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Homeobox-Clock Protein Interaction in Zebrafish

A SHARED MECHANISM FOR PINEAL-SPECIFIC AND CIRCADIAN GENE EXPRESSION*

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In non-mammalian vertebrates, the pineal gland is photoreceptive and contains an intrinsic circadian oscillator that drives rhythmic production and secretion of melatonin. These features require an accurate spatio-temporal expression of an array of specific genes in the pineal gland. Among these is the arylalkylamine *N*-acetyltransferase, a key enzyme in the melatonin production pathway. In zebrafish, pineal specificity of *zfaanat2* is determined by a region designated the pineal-restrictive downstream module (PRDM), which contains three photoreceptor conserved elements (PCEs) and an E-box, elements that are generally associated with photoreceptor-specific and rhythmic expression, respectively. Here, by using *in vivo* and *in vitro* approaches, it was found that the PCEs and E-box of the PRDM mediate a synergistic effect of the photoreceptor-specific homeobox OTX5 and rhythmically expressed clock protein heterodimer, BMAL/CLOCK, on *zfaanat2* expression. Furthermore, the distance between the PCEs and the E-box was found to be critical for PRDM function, suggesting a possible physical feature of this synergistic interaction. OTX5-BMAL/CLOCK may act through this mechanism to simultaneously control pineal-specific and rhythmic expression of *zfaanat2* and possibly also other pineal and retinal genes.

The pineal gland (epiphysis) influences daily and annual physiological changes by transducing the 24-h rhythm in light and darkness into a rhythm in circulating melatonin; in all vertebrates, circulating melatonin is elevated at night (1). This rhythm is generated by large changes in the activity of arylalkylamine-*N*-acetyltransferase (AANAT),[1] the next-to-last enzyme in melatonin synthesis (2).

Aanat is expressed at high levels in the vertebrate pineal gland and to a variable degree in retinal photoreceptors, reflecting the common evolutionary origin of vertebrate photoreceptors (3, 4); expression in other tissues is undetectable. Fish have two *aanats*, *aanat1* and *aanat2*, which are preferentially expressed in the retina and pineal gland, respectively (5–7). In some but not all vertebrates, the large changes in pineal AANAT activity reflect a transcriptional regulation by a circadian clock (1, 5, 8, 9). In lower vertebrates, this clock is located within the pinealocytes (10). Consequently, these pineal glands exhibit rhythmic *aanat* expression in culture (11, 12).

Zebrafish *aanat2* (*zfaanat2*) displays two regulatory features, pineal/retinal photoreceptor specificity and daily rhythmicity (9, 13). Developmentally, the zebrafish pineal gland is formed prior to the retina; *zfaanat2* expression in the pineal gland begins as early as 22 h post-fertilization (hpf), whereas retinal *zfaanat2* expression begins 3 days post-fertilization (dpf) (9). Pineal specificity of *zfaanat2* is conferred by a downstream enhancer termed the pineal-restrictive downstream module (PRDM) (14). This sequence was shown to enhance the activity of *zfaanat2* and other promoters in the pineal gland and to silence expression of *zfaanat2* in other tissues. Thus, the PRDM can be viewed as an enhancer with a dual function. The 257-bp PRDM sequence contains two known regulatory elements, the PCE and the E-box; the former is present in triplicate and the latter as a single copy. Functional analysis has revealed that these elements are crucial for the PRDM-mediated pineal-specific expression (14).

Studies in rodents indicate that PCE recruits members of the cone rod homeobox (*crx*) gene family (15–17), that it is present in the promoter region of *aanat* (16), and that transcript levels of *aanat* in the pineal gland are greatly reduced in $Crx^{-/-}$ mice, providing evidence for the action of mammalian CRX on *aanat* expression (17). Evidence also exists for a role of the zebrafish CRX ortholog (18), orthodenticle homeobox 5 (OTX5), in *zfaanat2* regulation. Specifically, OTX5 knockdown has been shown to diminish *zfaanat2* expression in the pineal gland (13). The mechanism by which OTX5 enhances *zfaanat2* expression in the pineal gland has not been investigated; OTX5 may act through PCEs located in the *zfaanat2* promoter region and/or the PRDM (14, 19).

Although PCEs appear to be linked to pineal/retinal photoreceptor specificity, the second PRDM element of interest, the E-box, is generally associated with transcriptional regulation by the positive and negative components of the circadian oscillator in these tissues (20, 21). The positive element, a heterodimer of two basic helix-loop-helix (bHLH) proteins, BMAL and CLOCK, activates transcription through binding to an E-box element, and the negative components, PERIOD and CRYPTOCHROME, inhibit the activity of BMAL/CLOCK (22–24). Clock-controlled genes usually contain one or more E-boxes

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[1] The abbreviations used are: AANAT, arylalkylamine-*N*-acetyltransferase, serotonin *N*-acetyltransferase; PRDM, pineal-restrictive downstream module; OTX5, orthodenticle homeobox 5; BMAL, brain and muscle Arnt-like protein; CMV, cytomegalovirus; CRX, cone rod homeobox; DsRed, *Discosoma* red fluorescent protein; EGFP, enhanced green fluorescent protein; PCE, photoreceptor conserved element; MO, morpholino-modified antisense oligonucleotides; AVP, vasopressin; USF, upstream stimulatory factor; bHLH, basic helix-loop-helix; PCEs, photoreceptor conserved elements; WT, wild type; ANOVA, analysis of variance; RACE, rapid amplification of cDNA ends; dpf, days post-fertilization; hpf, h post-fertilization.

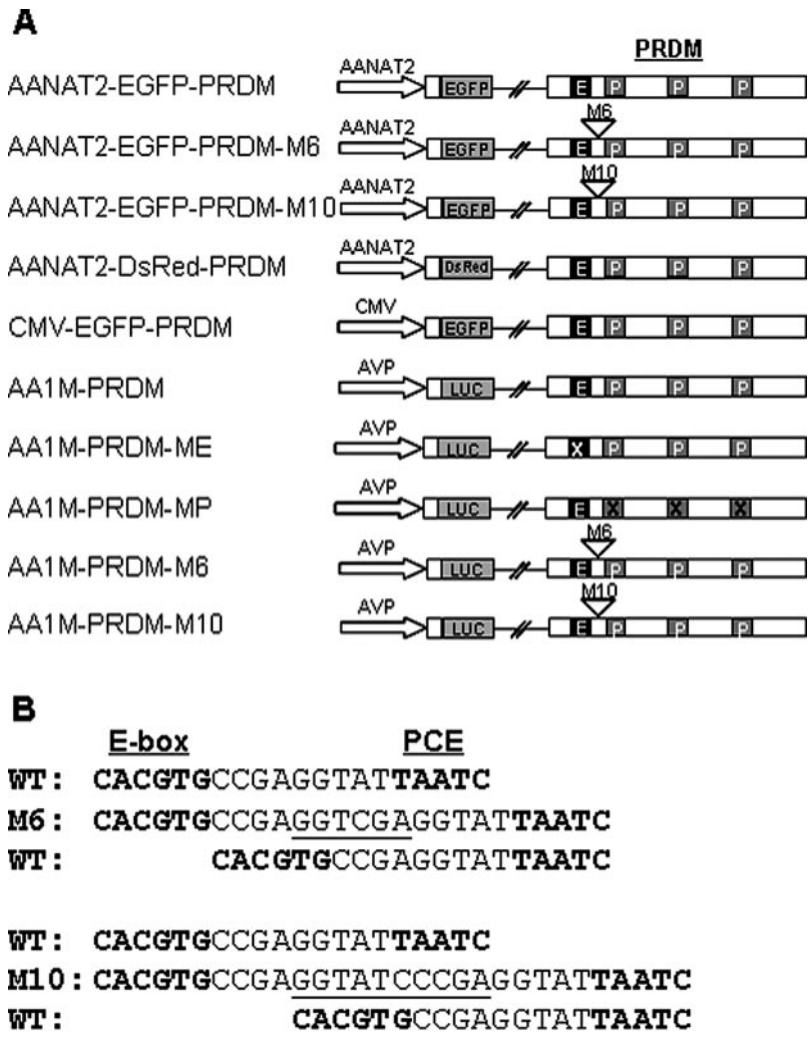

FIG. 1. **Promoter-reporter constructs.** *A,* names (*left*) and schematic illustrations (*right*) of the promoter-reporter DNA constructs containing AANAT2, CMV, or E-box-mutated AVP promoters (*open arrow*), EGFP, DsRed, or luciferase reporter genes and wild type or modified PRDM sequence. PRDM, enlarged in the illustration, contain E-box (*E* in *black box*) and three PCEs (*P* in *gray box*). Mutation of these elements is denoted with *X*. The location of insertions containing 6 (M6) or 10 (M10) nucleotides is marked with *triangles. B,* nucleotide sequence of the PRDM-E-box and adjacent PCE, and modifications resulting from the insertion (*underlined*) of 6 and 10 nucleotides. To illustrate that the adjacent flanking regions of each element were not disrupted by the insertions, E-box plus downstream sequences and PCE plus upstream sequences are compared with wild type sequence placed above and below the modified sequences, respectively.

in their promoter regions that mediate this action (25, 26). Rhythmic expression of *aanat* in the chicken pineal gland and the rat retina is thought to reflect this mechanism (27, 28). Likewise, fish *aanats* typically contain E-boxes in their proximal promoter (19); in the case of *zfaanat2*, an E-box occurs also in the PRDM (14). The functional role of these E-boxes in mediating circadian transcription, however, has not been determined.

To examine the regulatory mechanisms that are mediated by the PCEs within the PRDM and E-box and the relationship between these elements/mechanisms, *in vivo* gene knockdown analyses in combination with stable and transient expression, and *in vitro* transfection analyses of modified PRDM constructs in combinations with BMAL/CLOCK and OTX5 expression vectors have been done. In addition, *in vivo* and *in vitro* experiments were performed to examine whether the precise physical relationship of the PCEs and the E-box is critical for the PRDM-mediated mechanisms to take place. The results of these studies are presented here.

EXPERIMENTAL PROCEDURES

DNA Constructs (Fig. 1A)

AANAT2-EGFP-PRDM and Insertions—The distance between PRDM E-box and PCE1 was increased from 9 to 15 and 19 nucleotides. Initially six nucleotides were inserted between the two elements. Two complementary primers were utilized to introduce a six-nucleotide insertion (Fig. 1*B*) into the AANAT2-EGFP-PRDM construct (14) by using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) as instructed by the manufacturer, yielding AANAT2-EGFP-PRDM-M6. Next, a four-nucleotide insertion (Fig. 1*B*) was introduced into AANAT2-EGFP-PRDM-M6 yielding the construct AANAT2-EGFP-PRDM-M10. These insertions increased the distance between the E-box and the PCEs without changing the immediately adjacent 7–9 bases that could affect recognition and binding affinity (Fig. 1*B*).

AANAT2-DsRed-PRDM and CMV-EGFP-PRDM—These constructs were prepared as described previously (14).

AA1M-PRDM—PRDM (GenBank™ accession number AY380805) was placed in a luciferase reporter vector that contained an E-box-mutated AVP promoter (AA1M; see Ref. 29). This extensively characterized promoter was completely insensitive to BMAL/CLOCK transactivation (29). The PRDM was PCR-amplified by using AANAT2-EGFP-PRDM as a template and sets of primers containing NheI and KpnI restriction sites. The PCR product was double-digested with NheI and KpnI and ligated into NheI/KpnI-cut AA1M, yielding construct AA1M-PRDM.

AA1M-PRDM-ME—PRDM with a mutated E-box (PRDM-ME, CACGTG to CTCGAG) was PCR-amplified by using AANAT2-EGFP-PRDM-ME (14) as a template and subcloned into AA1M, upstream of the AVP promoter, as described above, yielding the construct AA1M-PRDM-ME.

AA1M-PRDM and PCE Mutation—The three PCEs within the PRDM were mutated. For each PCE, two complementary primers containing the desired mutation (TAATC (PCE1) to AGATC, GATTA (PCE2) to GTATA, and TAATC (PCE3) to ATATC) were utilized to introduce the mutations into AA1M-PRDM as described above. Consequently, novel restriction sites, BglII, EcoRV, and BsTZ17I, were introduced into the sequence in place of PCE1, PCE2, and PCE3, respectively. Mutated colonies were selected based on their digestion pattern with the above enzymes, and the presence of mutation was confirmed by sequencing. These constructs were named AA1M-PRDM-MP1, -MP2, and -MP3. Next, AA1M-PRDM-MP1 was sequentially mutated at PCE2 and PCE3 to get a triple PCE mutation construct AA1M-PRDM-MP.

AA1M-PRDM and Insertion—Six and 10 nucleotides were inserted in AA1M-PRDM between the E-box and PCE1 using a similar procedure and similar sequences as used for AANAT2-EGFP-PRDM insertions, yielding constructs AA1M-PRDM-M6 and AA1M-PRDM-M10.

Cytomegalovirus (CMV) promoter-driven mouse CLOCK (mCLOCK) and human BMAL1 (hBMAL1) expression vectors were generously provided by Drs. N. Gekakis and C. Weitz, Harvard University. Mammalian expression vectors for human USF-1 (hUSF-1) and mouse USF-2 (mUSF-2, SV40 promoter-driven) were gifts from Dr. M. Sawadogo, University of Texas. Zebrafish OTX5 expression vector (CMV-driven) was generously provided by Dr. Jennifer Liang, Case Western Reserve University.

Morpholinos Preparation

Gene knockdown experiments were performed using morpholino-modified antisense oligonucleotides (MO) (30, 31). MOs were obtained from Gene Tools, LLC (Philomath, OR). All MOs were designed according to the manufacturer's recommendations in order to bind to the sequences flanking and including the translation start codon (AUG). Sequences were as follows (sequence complementary to the start codon is underlined): OTX5 MO, CATGACTAAACTCTCTCTCTCTCTC (a gift from Dr. Reiko Toyama, NICHD, National Institutes of Health); BMAL1/3 MO, ATATCCATTCTTTGGTCTGCCATTA; BMAL2 MO, CAGATTTCATTTCCAGGTTGTCCAT; CLOCK1 MO, AGATACTGCTGTCATCCCGGTCTAT; CLOCK2 MO, TGTCCATGCTCACTCCTTCTCCCAT; CLOCK3 MO, ATGATAACTCGGTCTCATGGATCAG; and EGFP MO, ACAGCTCCTCGCCCTTGCTCACCAT. To test for morpholino specificity, BLAST analysis was performed using zebrafish genome sequence assembly from the Sanger Institute. None of the MOs are predicted to bind to gene sequences other than those targeted.

Pineal cDNA Library

Adult TG(AANAT2:EGFP)Y8 transgenic zebrafish (19), which express EGFP in the pineal gland under the control of the *zfaanat2* regulatory regions, were used. They were anesthetized (at ZT 1 and ZT 13) in 1.5 mM Tricane (Sigma) and sacrificed by decapitation; the pineal glands were isolated and removed under a fluorescent dissecting microscope. The use of fluorescent pineal glands facilitated the selective removal of pineal tissue. Total RNA was extracted using TriPure RNA isolation reagent (Roche Diagnostics) and was used as a template for synthesis of single strand 5′-RACE and 3′-RACE cDNA libraries using the SMART™ RACE cDNA amplification kit (Clontech) according to the manufacturer's protocols.

In Vitro Transient Transfection Assays

NIH-3T3 cells (ATCC, CRL-1658) were plated at a density of 3×10^4 cells per well in a 24-well plate (Costar, Cambridge, MA) and transfected 24 h later with a mixture containing Lipofectamine Plus (1.25/2.5 μl) reagents (Invitrogen), 10 ng of luciferase reporter vector driven by the AA1M-PRDM promoter (wild type (WT) and mutated versions), and 0.75 μg of a 1:1:1 expression vector mix (hBMAL1/mCLOCK/zOTX5 or hUSF1/mUSF2/zOTX5) or empty vector pcDNA (Invitrogen) in 50 μl of Vitacell Dulbecco's modified Eagle's medium (ATCC, Manassas, VA) without fetal bovine serum. On the following day, 0.5 ml of culture medium (Vitacell Dulbecco's modified Eagle's medium, supplemented 10% fetal bovine serum) was added to each well; plates were harvested 24 h later. Differences in transfection efficiency were taken into account by measuring the enzyme activity generated by a co-transfected thymidine kinase promoter-driven *Renilla* luciferase plasmid (0.5 ng). Firefly and *Renilla* luciferase enzyme activities were measured using the Stop and Glo kit (Promega, Madison, WI) following the manufacturer's instructions, and relative luciferase activity was determined for each well. To determine the effect of the mutations and of BMAL/CLOCK, OTX5, and their interaction, data were subjected to three-way ANOVA analysis. Results are the mean of at least three independent experiments each performed in triplicate.

In Vivo Transient Expression and Gene Knockdown Assays

In vivo transient expression assays of the EGFP-containing constructs were performed by microinjection of zebrafish embryos. Purified DNA constructs were diluted to 100 ng/μl in injection solution (0.1 M KCl, 0.05% phenol red). In co-injection experiments, constructs were dissolved together to a final concentration of 100 ng/μl each. *In vivo* knockdown assays were performed by microinjection of MOs at 0.8–4 μg/μl injection solution. Approximately 2 nl were injected into the cytoplasm of one-cell stage embryos using a micromanipulator and PV830 Pneumatic Pico Pump (World Precision Instruments, Sarasota, FL). In some experiments plasmids and MOs were injected together. One hundred to 400 embryos were injected using 3–4 different needles in each experiment and were incubated in 10-cm plastic dishes at 28 °C.

To calibrate and determine the dosage required for protein knockdown in the pineal gland, different doses of EGFP MO (0.16, 0.83, 4.1, 8.3, and 16.6 ng) were microinjected into one-cell stage TG(AANAT2:EGFP)Y8 embryos, and EGFP expression levels were monitored daily until 14 dpf. EGFP MO injection delayed the appearance of EGFP expression in a dose-dependent manner. Most embryos that were microinjected with 0.83–8.3 ng developed normally, and pineal EGFP expression was delayed until 12 dpf. The highest dose, 16.6 ng, caused developmental abnormalities, and the lowest dose, 0.16 ng, was ineffective (data not shown). Thus, injection of MO at 0.8–8 ng offers an efficient method for prolonged gene knockdown in the pineal gland.

Fish Maintenance and Examination of Embryos

Adult zebrafish were raised in a light and temperature-controlled recirculation water system under a 12-h light/12-h dark cycle at 28 °C and fed twice a day. To produce embryos, male and female zebrafish were paired in the evening, and spawning occurred within 1 h of the light cycle. Heterozygous TG(AANAT2:EGFP)Y8 transgenic embryos were obtained from an outcross of adult heterozygous TG(AANAT:EGFP)Y8 and WT fish. Injected embryos were placed in 10-cm Petri dishes with egg water containing methylene blue (0.3 ppm) and raised in a light-controlled refrigerated incubator at 28 °C. To prevent pigmentation, egg water was supplemented with 0.2 mM phenylthiocarbamide. Fluorescent protein expression in live embryos/larvae was determined under a stereo dissecting microscope (SZX12, Olympus) equipped with appropriate set of filters for excitation (460–490 nm for EGFP and 460–560 nm for DsRed) and emission (510–550 nm for EGFP and 550–590 nm for DsRed). In transient expression assays, embryos were sorted on the bases of their expression pattern, pineal-specific, ectopic, or both (14), on days 2–5 of development, and results were subjected to χ^2 analysis. In OTX5 knockdown assays, embryos/larvae were examined daily for pineal gland EGFP expression until 14 dpf. Embryos/larvae that were subjected to whole mount *in situ* hybridization were fixed overnight in 4% paraformaldehyde and stored in 100% methanol. *In situ* hybridization for the detection and quantification of *zfaanat2* mRNA was as described (9). The difference in the mean signal intensity between day and night and between treatments was determined using two-way ANOVA analysis.

RESULTS

Bmal and Clock Transcripts Are Expressed in the Zebrafish Pineal Gland—In zebrafish, three *bmal* and three *clock* genes have been identified (32–34) of which *zfclock1*, *zfbmal1,* and *zfbmal2* transcripts have been shown to be expressed in the pineal gland (32, 33). The presence of all six transcripts in the pineal gland was determined by PCR using adult TG(AANT2:EGFP)Y8 pineal-exclusive cDNA as a template. Fragments of the cDNAs encoding for zCLOCK1–3 and zBMAL1–3 were successfully amplified (data not shown), indicating that all six transcripts are present in this tissue.

BMAL/CLOCK Enhances zfaanat2 Expression in Vivo—In zebrafish, pineal *zfaanat2* expression begins at 22 hpf and a circadian clock-controlled rhythm of its transcript begins 2 dpf (9). To test *in vivo* the effect of BMAL/CLOCK heterodimers on *zfaanat2* expression, knockdown experiments were performed. Embryos were kept under a 12-h light/12-h dark cycle during the first 2 days of development, after which they were transferred to constant darkness. Embryos were collected at subjective mid-day and mid-night of the 3rd day of development, and *zfaanat2* mRNA levels were monitored by whole mount *in situ* hybridization. Non-injected embryos exhibited significant ($p < 0.001$) day/night differences in *zfaanat2* expression (Fig. 2), as documented previously (9, 13). A similar expression pattern was observed in embryos injected with 8 ng of control EGFP MO (Fig. 2), indicating that incompatible MO has no effect on *zfaanat2* expression. In contrast, microinjection of the mixture (1.6 ng each) of the BMAL/CLOCK MO resulted in a significant ($p < 0.001$) reduction in the level of *zfaanat2* mRNA and a reduction in the day/night differences (Fig. 2); injection of each MO separately was ineffective, probably reflecting functional

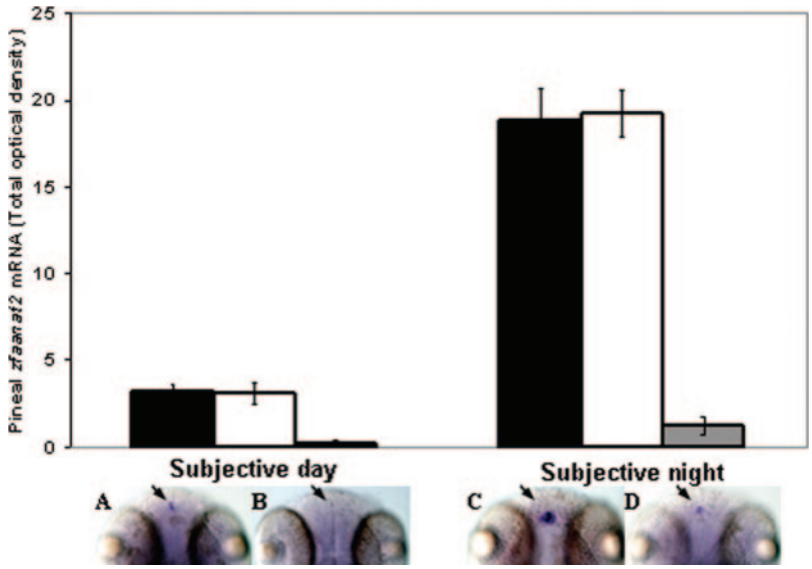

FIG. 2. **BMAL/CLOCK heterodimer enhances pineal *zfaanat2* expression *in vivo*; knockdown analysis.** Knockdown analysis was performed by microinjection of a mixture of BMAL/CLOCK morpholinos (*gray bars*) and a control EGFP MO (*white bars*) into one-cell stage embryos. Morpholino-injected and non-injected (*black bars*) zebrafish embryos were kept under 12-h light/12-h dark cycles during the first 2 days of development. On the 3rd day of development, embryos were placed under constant darkness and collected at mid-subjective day (CT 6; 54 hpf) and mid-subjective night (CT 18; 66 hpf). Zebrafish *aanat2* mRNA levels were monitored by whole mount *in situ* hybridization analyses. Each value represents the mean OD of pineal signals ($n = 12$) ± S.E. Values of *zfaanat2* mRNA were significantly higher at night in the non-injected and the control, EGFP MO-injected, embryos ($p < 0.001$, by ANOVA and Tukey's test). *zfaanat2* mRNA levels were significantly lower in BMAL/CLOCK MOs-injected embryos ($p < 0.001$, by ANOVA). Signal intensity was significantly affected by interaction between circadian time and treatment ($p < 0.001$, by 2-way ANOVA); there were no significant day/night differences in BMAL/CLOCK MOs-injected embryos. Photographs of representative EGFP MO (*A* and *C*) and CLOCK/BMAL MO mixture (*B* and *D*) of injected embryos sampled at mid-subjective day (*A* and *B*) and mid-subjective night (*C* and *D*) are shown below.

redundancy of these proteins (35). In accordance, the use of MOs directed toward negative components of the circadian oscillator resulted in a significant increase in *zfaanat2* expression.[2] The reduction in *zfaanat2* mRNA in response to BMAL/CLOCK knockdown may reflect a general disruption of the circadian clock. Because, as mentioned above, BMAL/CLOCK heterodimer enhances the expression of rat and chicken *aanat* through E-box elements, it is reasonable to suggest that this heterodimer may directly enhance the expression of *zfaanat2* in the pineal gland.

BMAL/CLOCK Dimers Activate Expression through PRDM E-box—Previous *in vivo* mutational analyses indicated that the perfect E-box located within the PRDM is required for enhancing the expression of *zfaanat2* in the pineal gland (14). To test whether BMAL/CLOCK heterodimers activate *zfaanat2* transcription through this E-box, AA1M-PRDM was co-transfected into NIH-3T3 cells with either an empty vector (pcDNA) or a 1:1 mixture of mBMAL and hCLOCK expression vectors. Reporter gene expression driven by PRDM was significantly ($p < 0.001$) enhanced 3-fold in the presence of hBMAL/mCLOCK (Fig. 3*A*). Moreover, when the E-box was mutagenized (AA1M-PRDM-ME construct), the heterodimer failed to transactivate the chimeric promoter (Fig. 3*A*). In contrast, E-box mutation did not alter the stimulatory action of USF, a bHLH protein known to bind E-boxes (36, Fig. 3*B*). Taken together, these results suggest that the BMAL/CLOCK heterodimer binds to the PRDM-E-box, resulting in increased transcription.

OTX5 Enhances Pineal Expression in Vivo through PRDM—Zebrafish OTX5 augments *zfaanat2* expression in the pineal gland (13). The capability of OTX5 to enhance *zfaanat2* promoter activity in the pineal gland was tested *in vivo* by OTX5 knockdown. Transgenic embryos, TG(AANAT2:EGFP)Y8, were microinjected with OTX5 MO. The appearance of the EGFP signal in the pineal gland of OTX5 MO-injected embryos was delayed; expression of EGFP in injected embryos (88 individuals) could be detected only at 48 hpf, whereas the control embryos exhibited the typical 24-hpf EGFP-positive pineal gland. In addition, signal intensity was drastically reduced in injected embryos until the 6th day of development (Fig. 4). The fact that EGFP expression in OTX5 morphants recovered indicates that pineal development was not disrupted by OTX5 MO injection. The transgene in TG(AANAT2:EGFP)Y8 fish consists of the 1.8-kb 5′ and 3.5-kb 3′ regulatory region (19) that includes the PRDM (14). Accordingly, the above results indicate that OTX5 enhances *zfaanat*2 expression through one or both of these regions.

To test *in vivo* whether OTX5 action can be mediated through the PRDM, pCS2-CMV-EGFP-PRDM was injected into WT embryos along with OTX5 MO. Because the only *zfaanat2*-derived sequence in this construct is the PRDM, this co-injection restricted the tested activity of OTX5 to this region. Injection of pCS2-CMV-EGFP-PRDM alone resulted in a high percentage (96% of 135 individuals) of embryos expressing EGFP ectopically, of which 27% also displayed pineal expression, confirming previous results (14). In contrast, pineal expression did not occur in embryos that were co-injected with OTX5 MO; all EGFP-positive embryos (89% of 148 individuals) showed ectopic expression only (Fig. 5). Thus, knockdown of OTX5 completely blocks pineal expression of this PRDM-supplemented promoter, suggesting that OTX5 acts through the PRDM to enhance pineal expression of *zfaanat2*. Together with previous mutational analysis of PCEs within the PRDM (14), these results suggest that OTX5 action is mediated through PRDM-PCEs.

OTX5 Enhances BMAL/CLOCK-mediated Expression through PRDM-PCEs—To examine the capability of OTX5 to directly bind and activate *zfaanat2* through PRDM-PCEs, AA1M-PRDM reporter construct was co-transfected with zOTX5 expression vector into NIH-3T3 cells. OTX5 did not increase reporter gene expression over control levels; rather, most surprisingly, it reduced expression

[2] L. Ziv and Y. Gothilf, unpublished results.

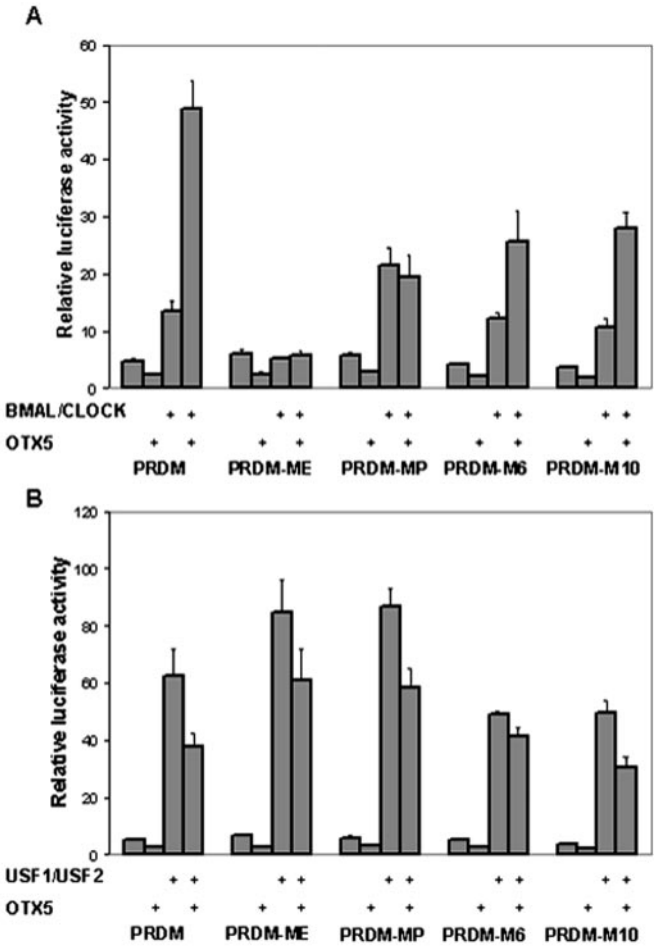

FIG. 3. **Cooperative activation by BMAL/CLOCK heterodimer and OTX5 via PRDM.** NIH-3T3 cells were transfected with a luciferase reporter driven by E-box-mutated AVP constructs (AA1M) (29) containing WT and mutated PRDM. Mutations include E-box mutation (*ME*), mutation of all PCEs (*MP*), and an insertion of 6 (*M6*) or 10 (*M10*) nucleotides between the E-box and PCEs. The cells were co-transfected with combinations of expression vectors (indicated by +) of zOTX5 and hBMAL/mCLOCK (*A*) or hUSF1/mUSF2 (*B*). Transcriptional activity is expressed as relative luciferase activity (mean ± S.E.). Results are the mean of three independent experiments performed in triplicate. Statistical analysis was performed by 3-way ANOVA.

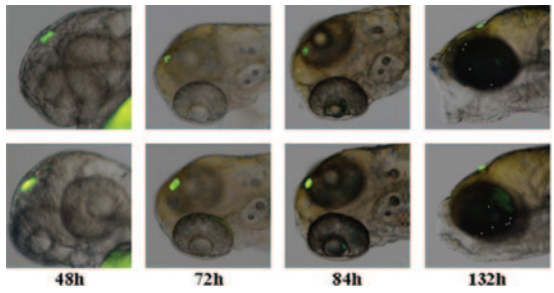

FIG. 4. **OTX5 enhances *zfaanat2* promoter activity; knockdown analysis in AANAT2:EGFP transgenic fish.** Knockdown analysis was performed by microinjection of OTX5 MO into one-cell stage TG(AANAT2:EGFP)Y8 embryos. Injected and non-injected live transgenic zebrafish embryos/larvae were examined daily for EGFP expression until 7 dpf. Shown are photographs of representative embryos/larvae (lateral view, anterior aspects to the *left*) with reduced (n = 88, *top panel*) and normal (*bottom panel*) levels of EGFP expression in the pineal gland of OTX5 MO-injected and non-injected transgenic embryos, respectively. Extra-pineal EGFP expression is absent. Expression of EGFP in OTX5 morphants was reduced until the 6th day of development, by which signal intensity was equal.

(Fig. 3*A*). Nevertheless, co-transfection of AA1M-PRDM, hBMAL/mCLOCK, and zOTX5 resulted in a >10-fold increase in luciferase activity; this is significantly ($p < 0.001$) greater than the increase obtained with hBMAL/mCLOCK alone (Fig. 3*A*), indicating that zOTX5 enhanced the hBMAL/mCLOCK-mediated transcription. In contrast, OTX5 did not stimulate expression when co-transfected with hUSF-1/hUSF-2 (Fig. 3*B*); rather, there was a tendency to reduce the USF-stimulated expression, indicating that the additive effect of OTX5 is specific for BMAL/CLOCK activation. In support of the *in vivo* data described above, these results provide evidence that the action of OTX5 is mediated by the PRDM. To verify that OTX5 acts through the PCEs, co-transfection was done in the presence of PCEs-mutated construct, AA1M-PRDM-MP. Indeed, point mutations in the three PRDM-PCEs completely abolished the effect of OTX5 (Fig. 3*A*). These results indicate that there is a synergistic interaction between OTX5 and BMAL/CLOCK heterodimer and that OTX5 action is mediated by the PRDM PCEs.

The Distance between E-box and PCEs Is Crucial for PRDM Function in Vivo—The PRDM E-box and PCEs are central for the dual function of PRDM, *i.e.* enhancing pineal and blocking ectopic expression (14). To test *in vivo* the hypothesis of an interaction between these two elements, insertions were made between the E-box and the adjacent PCE, without changing the nucleotide sequences immediately flanking each element (Fig. 1*B*), and constructs were microinjected. With all constructs, nearly half (42–47%) of the injected embryos were EGFP-positive. Injection of AANAT2-EGFP-PRDM resulted in pineal EGFP expression in 87% of the EGFP-positive embryos, most of which were pineal-specific, whereas ectopic expression was found in 24% of EGFP-positive embryos (Fig. 6 and Table I), confirming previous results (14). In contrast, injection of AANAT2-EGFP-PRDM-M6, which contains six nucleotide insertion between the E-box and PCE1, resulted in significantly lower (45%; $p < 0.001$) pineal EGFP expression, of which only 21% exhibited pineal-specific signals. Ectopic expression on the other hand increased and was detected in 90% of the EGFP-positive embryos (Fig. 6; Table I). Extension of the distance between the E-box and PCE1 with AANAT2-EGFP-PRDM-M10 resulted in even less (27%) pineal EGFP expression, whereas ectopic expression was observed in 94% of the EGFP-positive embryos (Fig. 6; Table I). Thus, 6 and 10 nucleotide insertions between the E-box and the PCEs interrupted the dual function of PRDM. The effect of a 10-nucleotide insertion was confirmed by co-injection of AANAT2-EGFP-PRDM-M10 and AANAT2-DsRed-PRDM, in which the latter was used as the internal reference. Nearly half of the injected embryos (189 individuals) exhibited a fluorescent signal. Almost all positive embryos (93%) exhibited ectopic EGFP but not DsRed expression. Moreover, individual embryos that exhibited a pineal-specific EGFP and DsRed signal also exhibited ectopic EGFP but not DsRed expression (Fig. 6*E*). Overall, the above data indicate that the distance between the E-box and the PCE is crucial for PRDM function and points toward an interaction between the two elements.

Interaction between OTX5 and BMAL/CLOCK Heterodimer—To test further the effect of the distance between the E-box and PCEs on the synergistic interaction between the BMAL/CLOCK-E-box and OTX5-PCE complexes, constructs that contain insertions between these two elements, AA1M-PRDM-M6 and AA1M-PRDM-M10, were co-transfected with combinations of expression vectors into NIH-3T3 cells. In contrast to the effect of the E-box and PCEs mutations, the addition of 6 or 10 bases between these elements had no effect on the level of expression driven by hBMAL/mCLOCK or zOTX5 alone (Fig. 3*A*), indicating that the relevant binding sites were not disrupted. These insertions also did not affect the level of expression driven by the hUSF1/mUSF2/zOTX5 combination

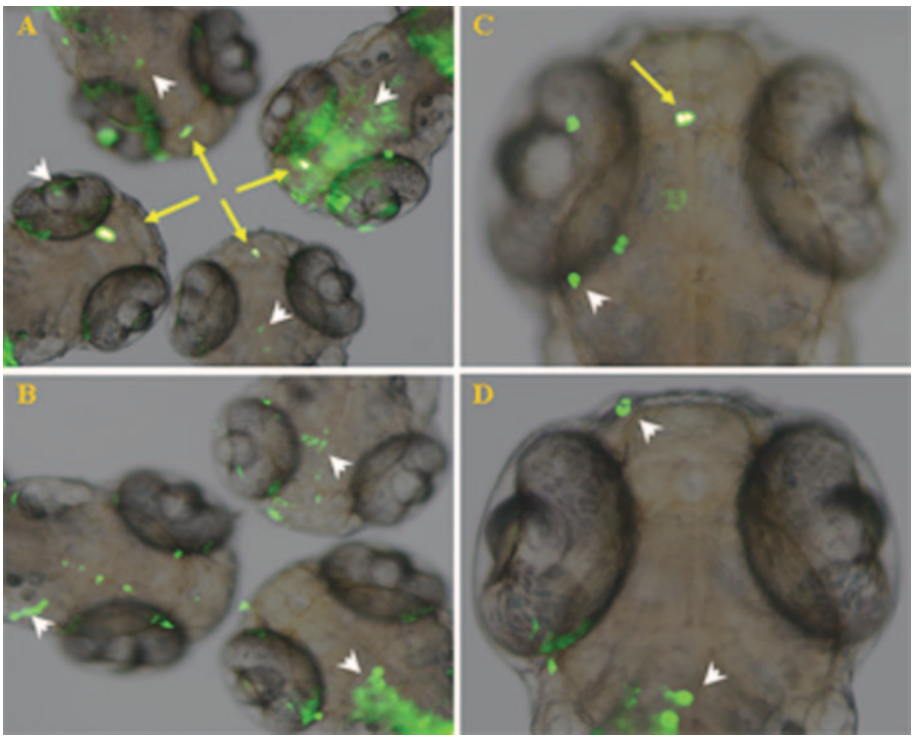

FIG. 5. **OTX5 action is mediated by PRDM; combined transient transfection and knockdown analysis.** CMV-EGFP-PRDM-injected embryos exhibit enhanced EGFP expression in the pineal gland ($n = 35$, *yellow arrows, A* and *C*) as well as ectopic EGFP expression (*white arrowheads*). In embryos that were co-injected with OTX5 MO, pineal expression is completely blocked, whereas ectopic expression is maintained ($n = 132$, *B* and *D*).

(Fig. 3*B*). In contrast, when co-transfected with hBMAL/mCLOCK/zOTX5 combination, these insertions significantly ($p < 0.001$) reduced the expression from 10- to 6.2- and 7.5-fold, respectively (Fig. 3*A*). Thus, the insertions specifically affected the synergistic interaction of zOTX5 and hBMAL/mCLOCK.

The effect of the insertion mutations indicates that a productive synergistic effect between BMAL/CLOCK and OTX5 is dependent upon the presence of precise intervening elements, phasing, and/or distance between the E-box and PCEs that were rendered suboptimal after the insertion mutagenesis.

DISCUSSION

The molecular mechanism of the circadian clock has been well studied and characterized (22, 37, 38). In all organisms, it consists of clock proteins that form highly conserved positive and negative autoregulatory transcriptional-translational feedback loops that drive the rhythmic transcription of clock-controlled output genes. This study has revealed a new mechanism in which clock and homeobox proteins regulate expression of the clock control gene, *zfaanat2*, in the zebrafish pineal gland. This action is mediated by two transcription factor binding sites that are located within the PRDM, the E-box and PCE (14). The BMAL/CLOCK heterodimer, which is present in virtually all cell types (26, 32, 39), is recruited by the E-box, whereas the OTX5, which is photoreceptor-specific (13), is recruited by the PCEs. Jointly, OTX5-PCE and BMAL/CLOCK-E-box complexes enhance *zfaanat2* expression in the pineal gland.

The Role of the BMAL/CLOCK Heterodimer—The key proteins in the positive limb of the circadian clock, BMAL/CLOCK, activate clock-regulated expression of several genes, including chicken and rat *aanat*, through an E-box (22, 25, 27, 28, 37, 38, 40, 41). In the present study it was shown that BMAL/CLOCK enhances *zfaanat2* expression through the PRDM-E-box, suggesting that clock-controlled rhythmic expression of *zfaanat2* is mediated by this downstream element. Evidence in support of this hypothesis is the presence of all six positive limb clock genes, *i.e. zfclock1–3* and *zfbmal1–3* (current study) and the rhythmic expression of *zfclock1*, *zfbmal1*, and *zfbmal2* in the pineal gland (32, 33). In addition, all six genes exhibit rhythmic expression in the zebrafish eye (34).

The Role of OTX5—The positive clock proteins, BMAL/CLOCK, are ubiquitously expressed (26, 32, 39); therefore, their ability to enhance pineal-specific expression must be assisted by another pineal-specific transcription factor. Evidence obtained here indicates that this factor is the arrhythmic and photoreceptor-specific homeobox, OTX5, that associates with the rhythmic proteins BMAL/CLOCK to control *zfaanat2* expression. Furthermore, the *in vivo* knockdown and *in vitro* transient expression analyses indicate that the action of OTX5 is mediated by the PRDM-PCEs (Figs. 3 and 5). Gamse *et al.* (13) has stated that "OTX5 might be constitutively active but function in concert with a factor whose expression or activity cycles with a circadian periodicity in the pineal complex." This study indicates that, at least in the case of the *zfaanat2* gene, this factor is the BMAL/CLOCK heterodimer.

The Role of BMAL/CLOCK-OTX5 Interaction—The results of this study indicate that the combined activation by BMAL/CLOCK and OTX5 is required for maximal activity of a PRDM-containing promoter and that the distance between the E-box and PCEs is critical, indicating that *zfaanat2* expression is controlled by a synergistic interaction of the two complexes. This synergism is somewhat reminiscent of the activation of the rhodopsin promoter in retinal photoreceptor cells by CRX and the leucine zipper-containing factor, neural retina leucine zipper (15), which, most interestingly, physically interact with each other (42). Recently, a similar interaction involving OTX5 and neural retina leucine zipper has been demonstrated in the *Xenopus* retina (43). This study provides new evidence that OTX5 can synergistically interact with the bHLH clock protein, possibly adding another example of protein-protein interaction involving a photoreceptor-specific homeobox.

This combination of BMAL/CLOCK-E-box and OTX5-PCE complexes could enhance *aanat* expression in other vertebrates. For example, the chicken promoter contains eight PCEs and a functional E-box (27). It is possible that chicken CRX or a related protein binds these PCEs and interacts with BMAL/CLOCK-E-

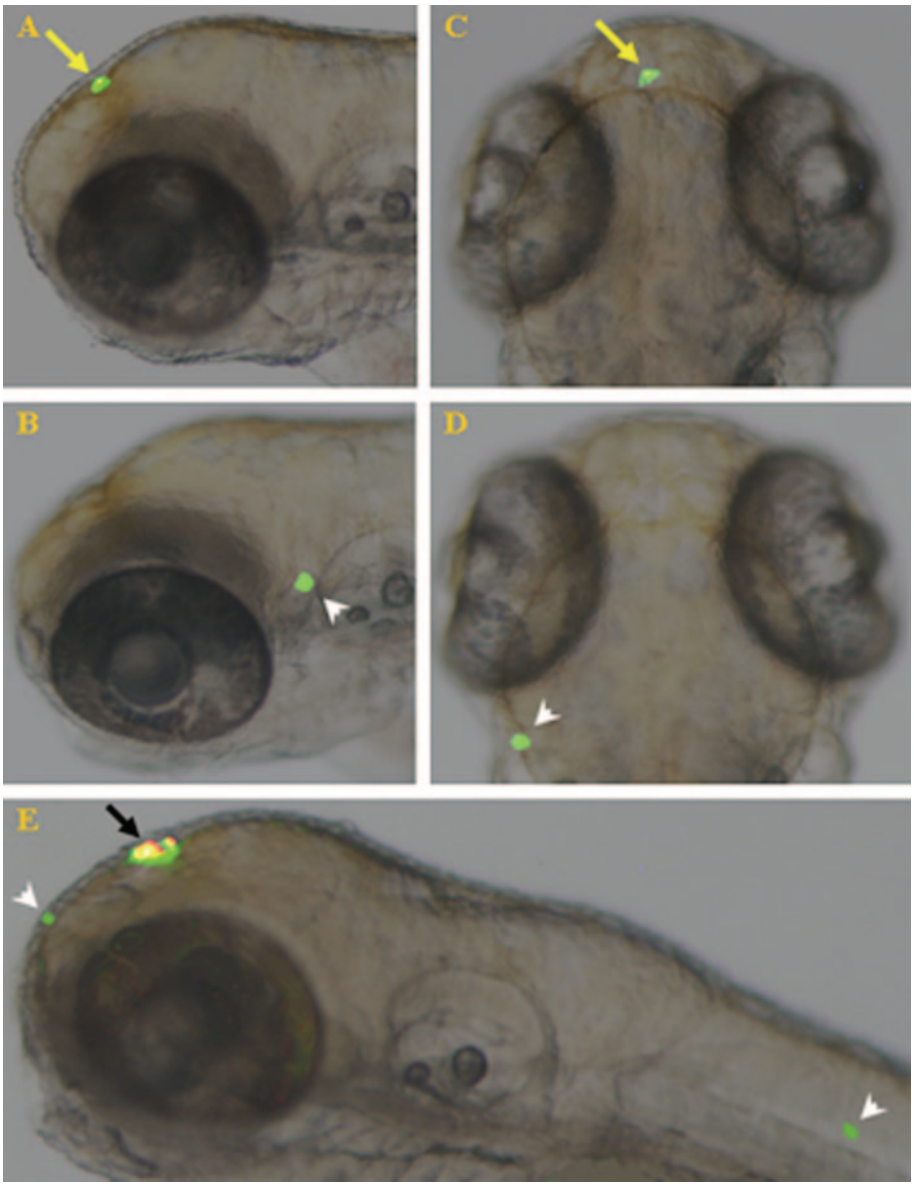

FIG. 6. **Transient expression patterns of fluorescent proteins.** Lateral (*A* and *B*) and dorsal (*C* and *D*) view of representative 3-day-old embryos exhibiting pineal-specific (n = 34, AANAT2-EGFP-PRDM-injected embryo, *A* and *C, yellow arrowheads*) and ectopic EGFP expression (n = 140, AANAT2-EGFP-PRDM-M10-injected embryo, *B* and *D, white arrowheads*). *E,* lateral view of an AANAT2-DsRed-PRDM and AANAT2-EGFP-PRDM-M10-injected, 4-day-old embryo, exhibiting pineal expression of both DsRed and EGFP (n = 16, *black arrow*) along with ectopic EGFP expression (*white arrowheads*).

TABLE I

The distance between E-box and PCEs is important for PRDM function: insertion mutations analysis

Constructs with WT or mutant PRDM were microinjected into zebrafish embryos. Extension of the distance between the E-box and PCEs by 6 (M6) or 10 (M10) nucleotides resulted in a significant increase in the proportion of embryos exhibiting ectopic EGFP expression and a significant decrease in the proportion of embryos exhibiting pineal-specific EGFP expression ($p < 0.001$ by χ^2 analysis). Representative photographs of pineal-specific and ectopic EGFP expression are given in Fig. 6.

Constructs	Pineal	Pineal and ectopic	Ectopic	Positive/injected
AANAT2-EGFP-PRDM	34	5	6	45/107
AANAT2-EGFP-PRDM-M6	13	48	74	135/284
AANAT2-EGFP-PRDM-M10	11	40	140	191/398

box to enhance chicken *aanat* transcription. The same strategy may work downstream of the Fugu *aanat2* gene (www.ensembl.org/Fugu_rubripes) and in the first intron of the rat *aanat* (44, 45), which also contain combinations of E-box and PCEs. Thus, the proposed mechanism may generally apply to *aanat*. In zebrafish, the pineal expression of additional rhythmic genes is influenced by OTX5 (13). It is therefore possible that the scenario of BMAL/CLOCK-E-box OTX5-PCE interaction is important for the expression of many additional genes that genetically define the pineal gland and are essential for pineal function. Moreover, in addition to other identified transcriptional mechanisms discussed above (15, 42, 43), this mechanism may regulate gene expression in retinal photoreceptors.

Transcriptional enhancement by homeobox-bHLH protein interactions is not restricted to the pineal gland. Several studies have shown that bHLH transcription factors can associate with homeobox proteins to form enhancer or promoter specific protein complexes, which result in a synergistic activation of the target gene in other tissues (46). A well studied example is the transcriptional activation of the insulin gene promoter, where the homeobox PDX1-PCE like (TAAT) and the bHLH heterodimer NeuroD1/E2A-E-box complexes synergistically cooperate to increase gene expression (47). A recent study (48) demonstrates that bHLH and LIM homeodomain factors physically interact and synergistically activate transcription which then promotes differentiation of motor neuron.

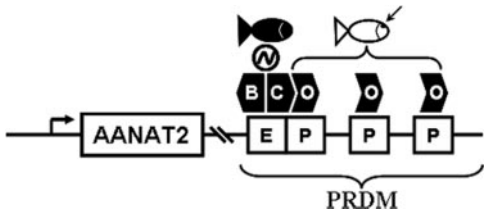

FIG. 7. **Proposed mechanism for rhythmic and specific expression of *zfaanat2* in the pineal gland.** Pineal expression of *zfaanat2* is enhanced by a synergistic interaction of OTX5-PCE and CLOCK/BMAL-E-box complexes. A functional E-box (*E*) and three PCEs (*P*) are located within a downstream regulatory region (PRDM). These elements recruit the rhythmic (sinus wave) and ubiquitously expressed (dark fish) transcriptional factors heterodimer BMAL/CLOCK (*B* and *C*) and the arrhythmic and pineal-specific expressed (white fish, arrow indicates pineal expression location) OTX5 (*O*), respectively. The interface contact between the protein shapes represent the possibility of physical interaction between BMAL/CLOCK and OTX5.

The above examples indicate that unique combinations of homeobox and bHLH proteins may lead to activation of cell-specific sets of target genes. Similarly, in the pineal gland, it is the synergistic binding of the homeobox, OTX5, and bHLH and BMAL/CLOCK that leads to activation of *zfaanat2* and possibly other pineal-specific genes. Because in the current model the bHLH proteins are rhythmic clock proteins, the homeobox-bHLH interaction may lead to the activation of pineal-specific sets of genes and to the temporal-specific, or rhythmic, activation of genes (Fig. 7). A similar mechanism may contribute to the low expression of *zfaanat2* in retinal photoreceptor cells. Indeed, the photoreceptor-specific zOTX5 augments the expression of rhythmic genes in the pineal gland but has no effect on the expression of non-rhythmic genes and has therefore been associated with circadian regulation (13). In a similar way, the current report provides evidence for the involvement of circadian genes BMAL/CLOCK, mediated by the PRDM-E-box, in determining tissue-specific expression.

The mechanism suggested in the current study can achieve exquisite control of gene expression. By simultaneously recruiting the temporal (BMAL/CLOCK) and spatial (OTX5) regulatory factors, the *zfaanat2* PRDM operates, in essence, as a four-dimensional coincidence transcriptional detector. Given the evidence for physical interaction between homeobox and bHLH proteins, and the effect of insertion mutations presented here, it is not improbable that the synergistic action of OTX5 and BMAL/CLOCK involves a physical interaction (Fig. 7). This mechanism sets the time and place of gene expression.

The involvement of additional pineal (49) and extra pineal bHLH and homeobox proteins in the regulation of *aanat* and other downstream rhythmic genes should be further investigated to elucidate the fine-tuning of pineal-specific and clock-controlled gene expression.

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