Zebrafish arylalkylamine-*N*-acetyltransferase genes – targets for regulation of the circadian clock

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Abstract

Daily rhythms of melatonin production are controlled by changes in the activity of arylalkylamine-*N*-acetyltransferase (AANAT). Zebrafish possess two *aanat*s, *aanat1* and *aanat2*; the former is expressed only in the retina and the latter is expressed in both the retina and the pineal gland. Here, their differential expression and regulation were studied using transcript quantification and transient and stable *in vivo* and *in vitro* transfection assays. In the pineal gland, the *aanat2* promoter exhibited circadian clock-controlled activity, as indicated by circadian rhythms of Enhanced green fluorescent protein (EGFP) mRNA in AANAT2:EGFP transgenic fish. *In vivo* transient expression analyses of the *aanat2* promoter indicated that E-box and photoreceptor conserved elements (PCE) are required for expression in the pineal gland. In the retina, the expression of both genes was characterized by a robust circadian rhythm of their transcript levels. In constant darkness, the rhythmic expression of retinal *aanat2* persisted while the *aanat1* rhythm disappeared; indicating that the former is controlled by a circadian clock and the latter is also light driven. In the light-entrainable clock-containing PAC-2 zebrafish cell line, both stably transfected *aanat1* and *aanat2* promoters exhibited a clock-controlled circadian rhythm, characteristic for an E-box-driven expression. Transient co-transfection experiments in NIH-3T3 cells revealed that the two, E-box- and PCE-containing, promoters are driven by the synergistic action of BMAL/CLOCK and orthehodenticle homeobox 5. This study has revealed a shared mechanism for the regulation of two related genes, yet describes their differential phases and photic responses which may be driven by other gene-specific regulatory mechanisms and tissue-specific transcription factor profiles.

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Introduction

Life on earth has evolved under rhythmic day/night changes of environmental conditions. In order to adapt to and anticipate these changes, organisms developed endogenous circadian clocks which drive daily rhythms of physiological and behavioral processes (Pittendrigh 1993). In vertebrates, central circadian clocks are located within the hypothalamic suprachiasmatic nucleus (Reppert & Weaver 2001), the retina and the pineal gland (Klein *et al.* 1997). The core mechanism of the circadian clock in these organs involves intracellular autoregulatory transcriptional/translational feedback loops, which include positive and negative transcription factors. The positive clock proteins, BMAL and CLOCK, activate transcription by binding to E-box elements. Negative feedback involves rhythmic inhibition by clock proteins, PERIOD and CRYPTOCHROME, which suppress the activity of the positive regulators (Gekakis *et al.* 1998, Kume *et al.* 1999, Reppert & Weaver 2001). This mechanism in turn generates rhythmic expression of genes, collectively named clock-controlled genes (Jin *et al.* 1999, Ripperger *et al.* 2000, Cheng *et al.* 2002), which are believed to ultimately lead to diverse behavioral and physiological rhythms.

Melatonin is an important component of the endogenous circadian clock system in vertebrates. This hormone is typically produced during the night at two major sites: the pineal gland and the retinal photoreceptor cells (Klein *et al.* 1997). Pineal gland melatonin plays an endocrine role in the regulation of a variety of daily and annual physiological rhythms (Arendt 1995, Ekstrom & Meissl 1997). Retinal melatonin plays a paracrine role related to photoreceptor adaptation to darkness; it does not contribute to the large rhythms of circulating melatonin (Cahill & Hasegawa 1997).

Melatonin rhythms are generated by changes in the activity of serotonin-*N*-acetyltransferase (arylalkylamine-*N*-acetyltransferase; AANAT), a pineal gland- and

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retinal-specific enzyme (Klein *et al.* 1997). The increased production of melatonin during the night reflects increased AANAT activity and, in many species studied, increased *aanat* transcription (Ganguly *et al.* 2002, Falcon *et al.* 2003). Rhythmic *aanat* expression, AANAT activity, and hence melatonin production, are driven by an internal circadian clock and by external light signals (Foulkes *et al.* 1997, Klein *et al.* 1997, Li *et al.* 1998, Ganguly *et al.* 2002, Tosini & Fukuhara 2003). Moreover, E-box elements present in regulatory regions of *aanats* were shown to bind and mediate the activity of BMAL:CLOCK heterodimer (Chen & Baler 2000, Chong *et al.* 2000, Appelbaum *et al.* 2005). Consequently, *aanat* is considered a clock-controlled gene that serves as a link between the core molecular oscillator and the hormonal output signal, which in turn affects the whole organism.

In mammals, only a single *aanat* gene has been identified and it is expressed in both the pineal gland and retina. Like many other photoreceptor-specific genes, enhanced expression of *aanat* in these tissues is mediated by cone rode homeobox proteins (CRXs). A study with the *crx* knockout mouse revealed that CRX is essential for the expression of *aanat* in the pineal gland (Furukawa *et al.* 1999). Exclusively among vertebrates, teleost fishes have two *aanats*, *aanat1* and *aanat2* (Coon *et al.* 1999, Falcon *et al.* 2003, Zilberman-Peled *et al.* 2004). In zebrafish, *aanat1* is expressed only in the retina while *aanat2* is expressed in both the pineal gland and, at lower levels, also in the retina (Falcon *et al.* 2003).

In the current study, *in vivo* and *in vitro* analyses of *aanat1* and *aanat2* regulation by light, the circadian clock and its components were performed. The results indicate differential regulation of expression of these two related genes, highlighting that a combination of mechanisms fine-tune circadian clock-regulated outputs.

Materials and methods

Fish maintenance and examination of embryos

Zebrafish were raised in a light- and temperature-controlled recirculating water system under a 12 h light:12 h darkness (LD) cycle. To produce wild-type embryos, male and female zebrafish were paired in the evening, and spawning occurred within 1 h of lights on. Heterozygous TG(AANAT2:EGFP)Y8 transgenic embryos expressing enhanced green fluorescent protein (EGFP) in the pineal gland (Gothilf *et al.* 2002) were obtained from an outcross of adult heterozygous TG(AANAT:EGFP)Y8 and wild-type fish. Injected and un-injected wild-type and transgenic embryos were kept in a light-controlled refrigerated incubator at 28 °C as described (Appelbaum *et al.* 2004) and were collected at different time-points for *in situ* hybridization (ISH) analysis or for evaluation of EGFP expression.

Whole mount ISH

Embryos/larvae were fixed overnight in 4% paraformaldehyde and stored in 100% methanol. Whole mount ISH was performed with digoxyginin-labeled EGFP probe at a concentration of 1 ng/μl. Detection and quantification of EGFP mRNA was as described for *aanat2* (Ziv *et al.* 2005). Differences in the signal intensity between sampling times were determined using two-way ANOVA analysis.

In vivo transient expression assays of *aanat2* promoter constructs

In vivo transient expression assays of promoter–EGFP constructs were performed by microinjection of zebrafish embryos as described (Appelbaum *et al.* 2004). Each construct (see below and Fig. 3A) contained the *aanat2* promoter with various deletions and mutations, the EGFP reporter gene and a 257 bp enhancer termed pineal restrictive downstream module (PRDM) (Appelbaum *et al.* 2004). The PRDM was included because it was shown to be required for the pineal-specific expression of *aanat2* (Appelbaum *et al.* 2004). Each construct was tested in more than a hundred embryos and the expression patterns, pineal specific, ectopic or both, as illustrated in previous studies (Appelbaum *et al.* 2004, 2005), were monitored. Statistical differences in the expression pattern were determined by χ^2 analysis.

AANAT2-EGFP constructs for *in vivo* analysis

–1320 AANAT2-EGFP-PRDM

A 1320 bp fragment was PCR amplified using AANAT2-EGFP-PRDM (Appelbaum *et al.* 2004; Fig. 3A) as a template and sets of primers containing *SalI* and *BamHI* restriction sites. The PCR product was double digested with *SalI* and *BamHI* and ligated into *SalI*/*BamHI*-cut AANAT2-EGFP-PRDM, in place of the original promoter (GenBank accession number AF494081), yielding a 1320 bp promoter with a 343 bp 5′ deletion.

–502 AANAT2-EGFP-PRDM

A construct with 502 bp of *aanat2* promoter, i.e. a 1161 bp 5′ deletion, was generated as described above.

–254 AANAT2-EGFP-PRDM

1409 bp of the 5′ region of the *aanat2* promoter were deleted, yielding a 254 bp promoter. AANAT2-EGFP-PRDM was digested with *PstI* to produce two fragments, 1415 and 4742 bp; the latter was re-ligated, yielding the desired construct.

AANAT2(Δ-1237/-300)-EGFP-PRDM

A fragment from the center of the 1663 bp *aanat2* promoter was removed, yielding a 726 bp promoter. AANAT2-EGFP-PRDM was digested with *SpeI* to produce two fragments, 937 and 5220 bp; the latter was re-ligated, yielding the desired construct.

AANAT2(Δ-1237/-300)-EGFP-PRDM and E-box mutation

The two E-boxes within the 726 bp promoter, located at positions 1472–1477 and 84–89 upstream to the transcription start site, were each mutated. For each E-box, two complementary primers containing the desired mutation were utilized to introduce the mutations (CACGTG to CTCGAG) into AANAT2(Δ-1237/-300)-EGFP-PRDM, using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) as instructed by the manufacturer. Colonies with the desired mutation were selected on the basis of a *PmlI* and *XhoI* digestion pattern and sequencing. The resulting constructs were named AANAT2(Δ-1237/-300)-ME1-EGFP-PRDM and AANAT2(Δ-1237/-300)-ME2-EGFP-PRDM (Fig. 3A).

AANAT2(Δ-1237/-300)-MP-EGFP-PRDM

The three photoreceptor conserved elements (PCE) (TAATT) within the 726 bp promoter, originally located at positions −282 (PCE3), −1591 (PCE2) and −1605 (PCE1), were mutated. For each PCE, two complementary primers containing the desired mutation were sequentially utilized to introduce the mutations (TAATT was changed to TCGAG, GATCT and GGGCC for PCE1, PCE2 and PCE3 respectively) into AANAT2(Δ-1237/-300)-EGFP-PRDM, as described above, to obtain a triple PCE mutation construct. Colonies with the desired mutation were selected on the basis of *XhoI*, *BglII* and *ApaI* digestion pattern for PCE1, PCE2 and PCE3 respectively and sequencing.

Real-time PCR quantification of *aanat* mRNAs in the eyes

Expression of zebrafish, *aanat1*, *aanat2* and glyceraldehyde 3-phosphate dehydrogenase (*g3pdh*) in the eyes throughout the 24-h cycle was determined at the transcript level using quantitative PCR assays. Five adult fishes were killed every 4 h. Fish were anesthetized in tricane, decapitated, the eyes removed and total RNA was extracted using TriPure RNA isolation reagent (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. The mRNA (200 ng) was reverse transcribed using Oligo(dT) primer and MMLV reverse transcriptase (Promega, Madison, WI, USA). Transcript levels were determined by real-time PCR using the GeneAmp 5700 PCR thermocycler (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. Triplicate first-strand cDNA aliquots (2μl) from each sample served as templates in PCR using master mix, SYBR Green I fluorescent dye (Applied Biosystems) and 200 nM gene-specific primers. Amplification reactions were carried out under the following conditions: incubation at 50 °C for 2 min, then 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The copy number in unknown samples was determined by comparing threshold cycles values with those of recombinant plasmid standards (0·1–100 pmol) containing the cDNA inserts and normalized to the amount of *g3pdh* mRNA in each sample. Data were log transformed to achieve normality for statistical analysis. The significance of the effects of the photoperiodic condition (LD and constant darkness (DD), the time of day (ZT) and interactions of these variables on the levels of each mRNA was determined by ANOVA with repeated measures. Specific comparisons were performed using Tukey's post hoc tests.

aanat–luciferase constructs and expression vectors for *in vitro* analyses

pGL3–498 AANAT2

The proximal *aanat2* promoter was placed upstream of a luciferase reporter gene in the pGL3 vector (Promega). A fragment containing 498 bp of 5′-flanking region and the 122 bp 5′ UTR of the *aanat2* promoter was PCR amplified using AANAT2-EGFP-PRDM as a template and sets of primers containing *NheI* and *BglII* restriction sites. The PCR product was double digested with *NheI* and *BglII* and ligated into *NheI*/*BglII*-cut pGL3.

pGL3–784 NAT1

A fragment containing 784 bp of 5′-flanking region and the 92 bp 5′ UTR of the *aanat1* promoter was placed upstream of a luciferase reporter gene, as described for pGL3–498 AANAT2.

Cytomegalovirus (CMV) promoter-driven mouse CLOCK (mCLOCK) and hamster BMAL1 (hBMAL1) expression vectors were generously provided by Drs N Gekakis and C Weitz, Harvard University, MA, USA. Zebrafish orthodentical homeobox 5 (OTX5) expression vector (CMV driven) was generously provided by Dr Jennifer Liang, Case Western Reserve University, Cleveland, OH, USA.

Establishment of stable PAC-2 cell lines

PAC-2 cells were cultivated as previously described (Whitmore *et al.* 2000, Vallone *et al.* 2004). Cells were

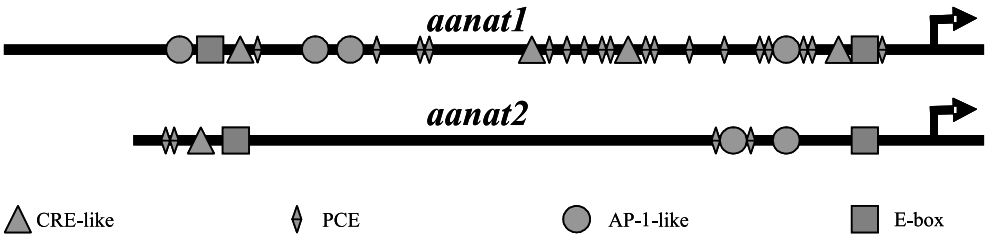


Figure 1 Transcription factor binding site distribution in the promoters of *aanat1* and *aanat2*. The relative location of E-boxes, PCE, AP-1-like and CRE-like elements in the promoters of *aanat1* (1930 bp) and *aanat2* (1663 bp) is shown. An arrow marks the start site of transcription.

transfected with linearized plasmids; the *aanat*–luciferase constructs (see above) and a neomycin resistance plasmid (pcDNA3,1 His-Myc(A); Invitrogen) at a molar ratio of 7:1. Electroporation was performed at 0·29 kV, 960 μF, using a Gene Pulser apparatus (Bio-Rad). Three days later, G-418 (GIBCO/BRL) was added at a final concentration of 800 μg/ml. During 1 month of selection, the concentration was gradually reduced to 250 μg/ml and 100–200 resistant colonies per transfection were obtained. Colonies were trypsinized and propagated as a single pool.

Luciferase assay in stable PAC-2 cell lines

In total, 3×10^4 cells per well were seeded into a 96-well Fluoplate (Nalge Nunc, Rochester, NY, USA). Alternate wells were left empty to minimize interference from bioluminescence crosstalk (estimated to be 2–3% in adjacent wells). After 12 h, 0·5 mM beetle luciferin, potassium salt (Promega) was added. The bioluminescence was assayed with a Topcount NXT counter (two-detector model; Packard). At least six independent stable transfections were made for each construct. All assays were performed at least three times. Each well was counted for 3 s at intervals of ~30 min. Plates were counted in an uninterrupted cycle. Between counting, plates were illuminated with a tungsten light source (20μW/cm^2). To ensure uniform illumination, transparent plates were intercalated between the sample plates. The counter was located in a thermostatically controlled dark room. Data were imported into CHRONO (T Roenneberg, University of Munich, Munich, Germany) and EXCEL (Microsoft) using the 'Import and Analysis' macro (S Kay, Scripps Research Institute, CA, USA). Period estimates were made by linear regression after peak finder analysis with CHRONO, measured under DD.

In vitro transient transfection assays in NIH-3T3 cells

NIH-3T3 cells (ATCC, CRL-1658) were plated and transfected with 10 ng luciferase reporter vector driven by *aanat1* (pGL3–784 AANAT1) or *aanat2* (pGL3–498 AANAT2) promoters and 0·75 μg of a 1:1:1 expression vector mix (hBMAL1/mCLOCK/OTX5) or empty vector pcDNA (as described in Appelbaum *et al.* 2005). 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) following the manufacturer's instructions. Data were subjected to two-way ANOVA analysis. Results are the means of at least three independent experiments each performed in triplicate.

Results

Putative regulatory elements in the two *aanat* promoters

Manual and computer analysis of the 5′ regulatory regions of the two genes revealed several putative transcription factor binding sites, some of which are shared by both promoters (Fig. 1). Two perfect E-box elements (CACGTG) were found in both promoters at approximately the same position upstream of the transcription start sites; nucleotides 84–89 and 1472–1477 in *aanat2* (Gothilf *et al.* 2002) and nucleotides 120–125 and 1447–1452 in *aanat1* (Fig. 1). Multiple copies of the PCE (TAATT/C) were present in both promoters; four PCEs in the *aanat2* and 18 PCEs in the *aanat1* promoter (Fig. 1). This element is known to mediate photoreceptor-specific expression by recruiting CRX and OTX5 in mice and zebrafish respectively (Chen *et al.* 1997, Li *et al.* 1998, Furukawa *et al.* 1999, Gamse *et al.* 2002, Appelbaum *et al.* 2005).

Other interesting elements are four cAMP-response elements-like (CRE) in the proximal promoter of *aanat1* and one in *aanat2* (Fig. 1). CRE has been shown to mediate the norepinephrine/cAMP-induced *aanat* expression in the rat pineal gland (Baler *et al.* 1997). In addition, an activator protein-1 (AP-1)-like binding site (Angel *et al.* 1987), a key element implicated in cell proliferation and speciation (Angel & Karin 1991), was centered at position −271 in *aanat1* and −224 and −307 in *aanat2*.

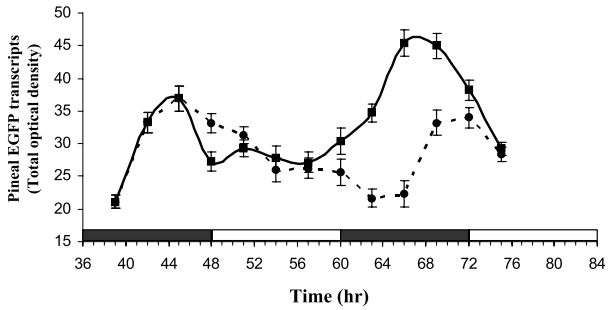


Figure 2 Rhythmic expression of EGFP mRNA driven by the *aanat2* regulatory region in the pineal gland. Zebrafish TG(AANAT2:EGFP)Y8 embryos were kept under LD during the first 2 days of development. On the third day of development, half were placed under LD (■) and half remained under DD conditions (●) and were collected every 3 h for 36 h. Zebrafish EGFP mRNA levels were monitored by whole mount ISH analyses. Each value represents the mean±S.E. optical density of pineal signals (n=12).

Clock-controlled regulation of the pineal *aanat2* promoter

To determine, *in vivo*, whether the clock-controlled *aanat2* mRNA rhythm reflects rhythmic promoter activity, EGFP mRNA levels in the pineal gland of TG(AANAT2:EGFP)Y8 transgenic zebrafish embryos (Gothilf *et al.* 2002) were monitored throughout the 24-h cycle. TG(AANAT2:EGFP)Y8 heterozygous embryos were kept under LD during the first 2 days of development. Under these photoperiodic conditions, the oscillator that drives rhythmic expression of pineal *aanat2* is functional (Gothilf *et al.* 1999, Ziv *et al.* 2005). On the third day of development, embryos were transferred to DD or kept under normal LD, collected at 3-h intervals for 36 h (39–75 hours post fertilization), and subjected to whole mount ISH analysis for EGFP mRNA. Under both LD and DD, embryos exhibited significant (P<0·001) day/night differences in EGFP mRNA expression (Fig. 2), indicative of rhythmic activity of the *aanat2* promoter.

Functional characterization of the *aanat2* promoter *in vivo*

The *aanat2* promoter was analyzed *in vivo* by microinjection of promoter–EGFP constructs into zebrafish zygotes followed by monitoring of fluorescence. Similar *in vivo* analysis of the *aanat1* promoter resulted in occasional ectopic EGFP expression but with no retinal expression, either because of late differentiation of the retina or the absence of elements in the cloned promoter that are essential for retinal expression.

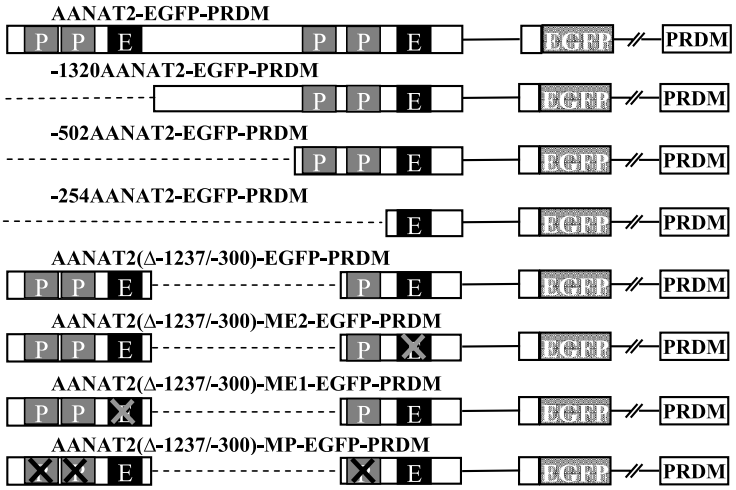
Injection of the AANAT2-EGFP-PRDM construct, which contained the entire promoter, resulted in EGFP expression in 39% of injected embryos. Among these EGFP-positive embryos, a signal in the pineal gland was observed in 87% and most exhibited a restricted signal. Ectopic expression of EGFP was observed in only 22% of EGFP-positive embryos (Fig. 3B), confirming previous results (Appelbaum *et al.* 2004, 2005). Constructs with 5′ promoter deletions, −1320AANAT2-EGFP-PRDM, −502 AANAT2-EGFP-PRDM and −254 AANAT2-EGFP-PRDM (Fig. 3A), generated significantly (P<0·01) lower pineal expression and higher ectopic expression (Fig. 3B). Injection of AANAT2(Δ-1237/-300)-EGFP-PRDM (Fig. 3A), which contains 726 bp of the proximal and distal promoter and lacks a region of repetitive DANA sequences (Izsvak *et al.* 1996), resulted in high levels of pineal EGFP expression (82%) and reduced ectopic expression (30%), similar to the wild-type construct (AANAT2-EGFP-PRDM; Fig. 3B). Since these promoter regions include E-box and PCE elements, the function of these elements was tested by injection of mutated constructs. Injection of E-box-mutated constructs (AANAT2(Δ-1237/-300)-ME1-EGFP-PRDM and AANAT2(Δ-1237/-300)-ME2-EGFP-PRDM; Fig. 3) significantly (P<0·001) reduced the pineal gland-specific expression and increased ectopic expression as compared with wild-type and the 726 bp promoters. Among the EGFP-expressing embryos, only 26% and 30% showed pineal expression and 97% and 98% showed ectopic expression for ME1 and ME2 respectively (Fig. 3B). The triple PCE mutation had an even stronger effect. Injection of AANAT2(Δ-1237/-300)-MP-EGFP-PRDM (Fig. 3A) resulted in ectopic expression in all EGFP-expressing embryos; 8% also demonstrated pineal expression (Fig. 3B). These results indicated that E-boxes and PCEs, distributed in the proximal and distal promoter, are essential for accurate transcription of *aanat2*.

Differential expression of *aanats* in the zebrafish retina

The daily expression pattern of *aanat1* and *aanat2* in the eyes was determined throughout the 24-h cycle. This analysis revealed a significant (P<0·01) effect of ZT on the mRNA levels of both *aanats* when fish were kept under normal LD cycles; both genes exhibited a daily rhythm (Fig. 4). Interestingly, under these conditions *aanat2* mRNA levels peaked at ZT10 and started to decline at ZT18, while *aanat1* mRNA levels peaked at ZT14 and started to decline at ZT22. These results suggested that under LD the two genes exhibit a rhythmic expression pattern with different phase.

To test whether these rhythms are driven by a circadian oscillator, fish were transferred from LD into DD and sampled throughout the first cycle as described above. The rhythmic expression of *aanat2* persisted in DD (Fig. 4), indicating that a retinal circadian oscillator controls expression of *aanat2* mRNA. In contrast, *aanat1*

A



B

Constructs	Pineal	Pineal & Ectopic	Ectopic	Positive/Injected	Sig.
AANAT2-EGFP-PRDM	53	6	9	68/173	
-1320AANAT2-EGFP-PRDM	4	28	65	97/128	*
-502AANAT2-EGFP-PRDM	3	33	20	56/107	*
-254AANAT2-EGFP-PRDM	18	37	37	92/200	*
AANAT2(Δ-1237/-300)-EGFP-PRDM	38	6	10	54/208	
AANAT2(Δ-1237/-300)-ME2-EGFP-PRDM	1	13	32	46/158	*
AANAT2(Δ-1237/-300)-ME1-EGFP-PRDM	2	16	51	69/142	*
AANAT2(Δ-1237/-300)-MP-EGFP-PRDM	0	9	101	110/330	*

Figure 3 (A) Promoter–reporter constructs. Names and schematic illustrations of the promoter–reporter DNA constructs containing wild-type, deleted or mutated *aanat2* promoter, EGFP reporter gene and the PRDM sequences. The promoters contain E-box (E in solid box) and PCEs (P in shaded box). Promoter deletions and mutations are symbolized by broken lines and X respectively. (B) *aanat2* promoter analysis, *in vivo*. Constructs shown in (A) were microinjected into zebrafish embryos. Deletions and mutations that resulted in a significant (Sig.) increase in the proportion of embryos exhibiting ectopic EGFP expression and a significant decrease in the proportion of embryos exhibiting pineal-specific EGFP expression ($P<0{\cdot}01$ by χ^2 analysis) as compared with the wild-type promoter (AANAT2-EGFP-PRDM) are marked with an asterisk. The appearance of pineal-specific and ectopic expression patterns is indistinguishable from those previously reported (Appelbaum *et al.* 2004, 2005).

mRNA levels were similar throughout the 24-h cycle, except for the last time-point, indicating that rhythmic *aanat1* expression was lost when fish were transferred into DD. These results suggested that different transcriptional regulatory mechanisms might drive expression of these two related genes within the same tissue.

Clock-controlled regulation of the two *aanat* promoters

The activities of *aanat1* and *aanat2* promoters were tested by stable transfection in the PAC-2 zebrafish cell line, which contains a light entrainable clock (Vallone *et al.* 2004). Cells were stably transfected with the constructs pGL3–784 AANAT1 and pGL3–498 AANAT2 in which luciferase is driven by promoters that contain one E-box. Expression was tested by exposure of the transfected cells to an LD cycle followed by DD and then reversed light:darkness cycles (DL).

The *aanat2* promoter drove a rhythm of expression under LD conditions with a peak of luciferase activity at ZT20·4 ± 1·47 as determined by peakfinder analysis (Fig. 5). This rhythmic expression was also maintained in DD with a free-running period of 25·19 ± 0·2 h and an accompanying rise in the basal expression levels (Fig. 5). When cells were then subjected to DL conditions, the phase of the rhythm gradually shifted to match the new light:darkness cycle but was still not completely entrained following three DL cycles. These results were

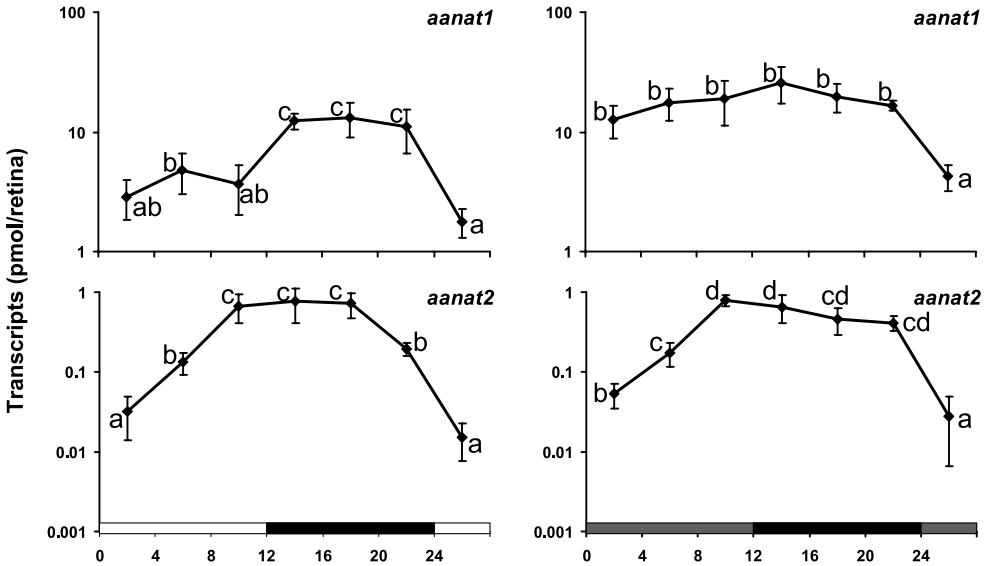


Figure 4 Daily expression pattern of retinal *aanat1* and *aanat2*. The amount of retinal *aanat1* and *aanat2* transcript in adult zebrafish was measured using real-time PCR and normalized relative to the levels of *g3 pdh* transcripts. One eye from each of five fish was sampled every 4 h, for 24 h, starting at ZT2, under LD (top and bottom left graphs, solid and open bars) and the first DD (top and bottom right graphs, solid and shaded bars). Data for normalized transcript levels of each *aanat* are presented as means±S.D. Values were compared by ANOVA ($P<0{\cdot}01$); different letters represent statistically different values within each graph ($P<0{\cdot}05$, Tukey's test).

consistent with previous observations on the entrainment of expression rhythms directed by E-box enhancer elements in PAC-2 cells (Vallone *et al.* 2004). The *aanat1* promoter showed a lower amplitude and less robust activity rhythm; however, in LD and DD, the timing of increases in *aanat1* expression resembled that of the *aanat2* peaks of expression. Thus, these stable luciferase reporter assays indicate that, in the context of PAC-2 cells, the *aanat2* and possibly the *aanat1* promoter is controlled by the endogenous, light-entrainable circadian oscillator.

The action of BMAL/CLOCK and OTX5 on *aanat* promoters

Previous results indicated that OTX5 and BMAL/CLOCK synergistically bind PCEs and E-box elements respectively, located within the PRDM region (Appelbaum *et al.* 2005). In order to test, *in vitro*, the action of BMAL/CLOCK and OTX5 on the PCE- and E-box-containing *aanat* promoters, the pGL3–784 AANAT1 and pGL3–498 AANAT2 promoter–reporter constructs were co-transfected with either an empty vector (pcDNA) or mixtures of mBMAL/hCLOCK and OTX5 expression vectors into NIH-3T3 cells. Co-transfection of the promoter–reporter constructs with OTX5 or BMAL/CLOCK alone did not increase reporter gene expression over control levels (Fig. 6). Nevertheless, co-transfection with both hBMAL/mCLOCK and OTX5 resulted in a >2-fold and >3·4-fold increase in luciferase activity, indicating that BMAL/CLOCK and OTX5, possibly through the E-box and PCE elements, cooperatively enhance the activity of *aanat* promoters.

Discussion

In the last two decades a great deal has been learned about the components and molecular organization of the circadian clock (Reppert & Weaver 2000, 2001). However, the pathways by which these clocks drive output rhythms are less well understood. It is currently believed that it is the transcriptional machinery of the core oscillator that directly regulates clock-controlled genes and these, in turn, serve as the 'hands of the clock' (Jin *et al.* 1999, Chen & Baler 2000, Chong *et al.* 2000, Ripperger *et al.* 2000, Cheng *et al.* 2002). The mechanisms that drive rhythmic and tissue-specific expression of *aanats*, key clock output relays, constitute important parts of this complex puzzle.

AANAT activity is regulated at the transcriptional, post-transcriptional and post-translational levels (Ganguly *et al.* 2002, Klein *et al.* 2002, Falcon *et al.* 2003, Kim *et al.* 2005). The current study focuses on the transcriptional regulation of this clock-controlled gene.

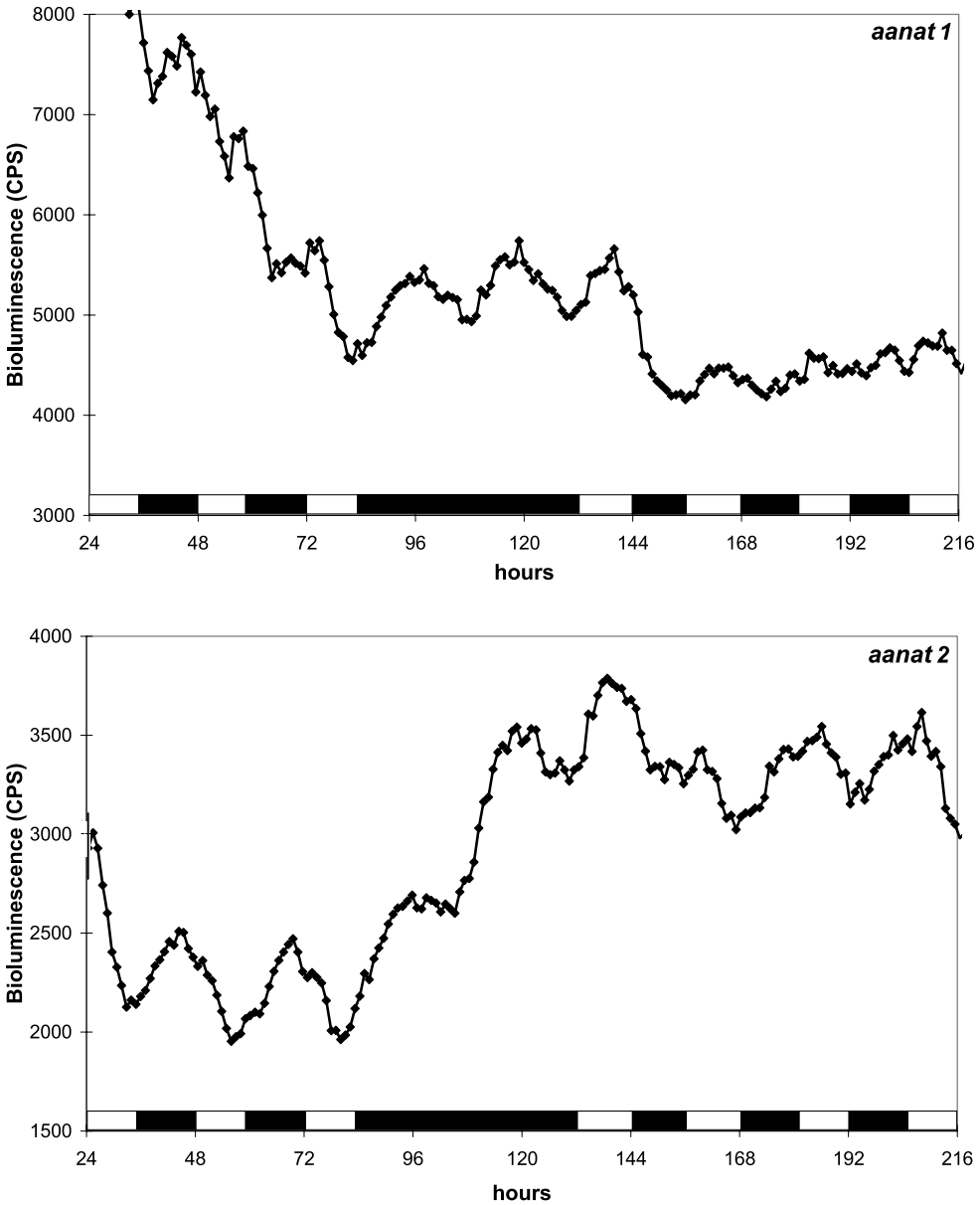


Figure 5 Temporal expression pattern of reporter gene driven by the promoters of *aanat1* and *aanat2*. Bioluminescence assay of pools of stably transfected *aanat* promoter–luciferase reporter PAC-2 zebrafish cells maintained for 8 days under various lighting conditions (open and solid bars show the light and dark periods respectively). Prior to the analysis, cells were exposed to an LD cycle for 5 days to ensure stable entrainment. Bioluminescence is plotted on the y axis (counts per second; CPS) and hours from the start of the assay on the x axis. Cells were maintained for 3 days in LD, then transferred to DD for 48 h, and finally subjected to DL for an additional 4 days. Representative data from single wells of cells are shown. Each well contains a pool of cells derived from 100–200 independent, stably transfected clones and so averages out inter-clone variability due to integration effects. The expression pattern of *aanat1* promoter-derived cells is presented in the upper chart and *aanat2* in the lower.

Strong support for the involvement of the core oscillator comes from the rhythmic activity of both zebrafish *aanat* promoters in the zebrafish PAC-2 cell line. Moreover, the rhythms generated by the *aanat2* promoter have similar characteristics to those generated by a heterologous promoter construct containing multimerized

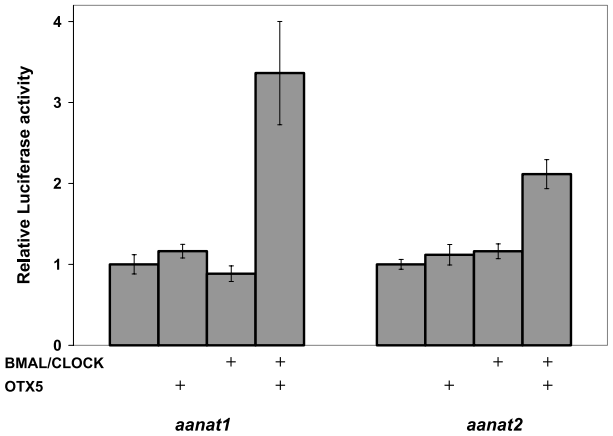


Figure 6 Co-operative activation by BMAL/CLOCK heterodimer and OTX5 via *aanat1* and *aanat2* promoters. NIH-3T3 cells were co-transfected with combinations of a luciferase reporter driven by *aanat1* or *aanat2* promoter constructs and expression vectors (indicated by +) of OTX5 and hBMAL/mCLOCK. Transcriptional activity is expressed as relative luciferase activity (means±S.E.). Values were normalized against the level of expression of empty vector (pcDNA). Statistical analysis was performed by two-way ANOVA.

E-boxes (Vallone *et al.* 2004). Rhythmic transcription of zebrafish *aanats* is regulated in part by the interaction of clock proteins BMAL/CLOCK and the homeobox protein OTX5. This has been shown *in vitro* using NIH-3T3 cells where *aanat* promoter activities were enhanced by co-transfection of BMAL/CLOCK:OTX5 (Fig. 6), and was further confirmed *in vivo* by demonstrating that their putative binding sites in the promoter, the E-box and PCE elements respectively, are important for enhanced pineal expression (Fig. 3).

The effect of BMAL/CLOCK/OTX5 on *aanat1* promoter activity was higher than for the *aanat2* promoter when tested in NIH-3T3 cells (Fig. 6). A reasonable explanation for these differences could be the larger number of PCE sites in the tested *aanat1* promoter. These promoter differences are also reflected in the retinal expression levels of the two genes (Fig. 4). An abundance of PCE sites occurs in other photoreceptor specific genes including chicken and rat *aanat*, rat pineal night-specific ATPase (*pina*) and zebrafish exorhodopsin (*exorh*) (Li *et al.* 1998, Chen & Baler 2000, Chong *et al.* 2000, Asaoka *et al.* 2002). In *aanat2*, additional PCEs are found in a downstream regulatory region, PRDM. This region, in conjunction with the promoter, has been shown to be important for enhanced pineal expression (Appelbaum *et al.* 2004). The current study confirms this observation and extends it by identifying PCE-containing regions and E-boxes as promoter elements that are important for this function. Although PRDM can drive enhanced pineal expression of the *aanat1* promoter (Appelbaum *et al.* 2004), it is as yet not known what drives retinal-specific expression of *aanat1*. Taken together, functional analyses of the *aanat* promoters in the current study link the transcriptional machinery of the core oscillator with the regulation of clock-controlled genes in photoreceptors.

In addition to the apparent common clock regulation, the results of the current study suggest that phase differences exist in the rhythmic expression of retinal *aanat1* and *aanat2*. A somewhat similar pattern was also found in pike; retinal *aanat1* mRNA peaks 6 h before that of pineal *aanat2* and this phase-lag persists under constant conditions, indicating differences between retinal and pineal clock regulation in the pike (Coon *et al.* 1999). Here, however, we show that these differences occur within the same tissue, the retina. Furthermore, *in vivo*, *aanat2* expression in pineal gland and retina is primarily, but not only, controlled by the circadian oscillator (Gothilf *et al.* 1999, Gamse *et al.* 2002, Ziv *et al.* 2005; Fig. 4) while *aanat1* is, at least in part, light regulated as was suggested for trout *aanat1* (Mizusawa *et al.* 2000, Besseau *et al.* 2005). These differences may indicate a gene-specific regulation within the retinal photoreceptors, or a different spatial expression pattern; the two *aanats* are expressed in different subsets of retinal cells. The apparent contradiction between the *in vivo* and *in vitro* expression pattern of *aanat1* may reflect regulatory differences between retinal photoreceptor cells and the PAC-2 cell line; although PAC-2 cells contain a light-entrainable circadian oscillator, the phototransduction mechanism may well be distinct from that present in the retina.

Gene duplication, commonly seen in teleost fish, is the result of a whole genome duplication that is predicted to have happened at the time when fish emerged from the vertebrate lineage. Functional subdivision may occur when two genes, formerly served by a single ancestral gene, are expressed in separate subsets of cells (Amores *et al.* 1998, Force *et al.* 1999). This may be the case with fish *aanat*; the ancestral gene might have been expressed in all photoreceptor cells, as seen in chickens and mammals, while subsequent mutations in the duplicated genes subdivided their function and profile of expression to the pineal gland (*aanat2*) and retina (*aanat1*), as seen in trout, pike and seabream (Coon *et al.* 1999, Benyassi *et al.* 2000, Zilberman-Peled *et al.* 2004). Since in zebrafish both *aanat* genes are co-expressed in the retina, it is likely that they are required for different retinal functions (Zilberman-Peled *et al.* 2006). This possibility is reflected in their differential tissue distribution and in their differential regulation by the circadian clock and light in the retina (Fig. 4).

Retinal *aanat*/melatonin is generally associated with visual adaptation to darkness (Cahill & Hasegawa 1997). However, in several fish species retinal melatonin levels were found to be high during the day (Falcon *et al.* 2003, Zilberman-Peled *et al.* 2006). In frog retina, a poor correlation between *aanat* activity and melatonin

production was found (Delgado *et al.* 1993). Moreover, in monkey and rat retina, *aanat* is expressed in the inner nuclear layer while there is no evidence in support of melatonin synthesis in this region (Coon *et al.* 2002, Liu *et al.* 2004). These cases in which *aanat* activity is not associated with darkness or with melatonin production suggest an additional role for *aanat* in the retina. Such a role may be the acetylation of arylalkylamines other than serotonin and/or synthesis of products other than melatonin (Falcon *et al.* 2003, Klein 2004, Zilberman-Peled *et al.* 2004, 2006).

Several experimental systems were used in the current study to explore and dissect the differential regulation of two related clock-controlled genes in photoreceptors. Future studies using these complementary approaches promise to provide important new insights into the links between the core molecular oscillator and rhythmic behavioral and physiological outputs.

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