In Vivo Disruption of *Xenopus* CLOCK in the Retinal Photoreceptor Cells Abolishes Circadian Melatonin Rhythmicity without Affecting Its Production Levels

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Xenopus laevis retinas, like retinas from all vertebrate classes, have endogenous circadian clocks that control many aspects of normal retinal physiology occurring in cells throughout all layers of the retina. The localization of the clock(s) that controls these various rhythms remains unclear. One of the best studied rhythmic events is the nocturnal release of melatonin. Photoreceptor layers can synthesize rhythmic melatonin when these cells are in isolation. However, within the intact retina, melatonin is controlled in a complex way, indicating that signals from many parts of the retina may contribute to the production of melatonin rhythmicity. To test this hypothesis, we generated transgenic tadpoles that express different levels of a dominant negative *Xenopus* CLOCK specifically in the retinal photoreceptors. Eyes from these tadpoles continued to produce melatonin at normal levels, but with greatly disrupted rhythmicity, the severity of which correlated with the transgene expression level. These results demonstrate that although many things contribute to melatonin production *in vivo*, the circadian clock localized in the retinal photoreceptors is necessary for its rhythmicity. Furthermore, these data show that the control of the level of melatonin synthesis is separable from the control of its rhythmicity and may be controlled by different molecular machinery. This type of specific "molecular lesion" allows perturbation of the clock in intact tissues and is valuable for dissection of clock control of tissue-level processes in this and other complex systems.

Key words: dominant negative CLOCK; transgenic Xenopus*; circadian clock; retinal photoreceptor; melatonin rhythm; arylalkylamine* N-*acetyltransferase (AANAT)*

Circadian oscillators exist in many tissues and control timing of local events (Damiola et al., 2000; Stokkan et al., 2001). This type of local circadian control is well established in the vertebrate retina, where many aspects of retinal physiology are regulated by an endogenous retinal circadian clock(s) (for review, see Besharse, 1988; Cahill and Besharse, 1995; Anderson and Green, 2000). This temporal regulation is a fundamental part of the normal function and maintenance of the retina, and disruption of these controls may contribute to retinal dysfunction and degeneration (Fain and Lisman, 1993).

Although retinal rhythms have been demonstrated in vertebrates of all classes, the *Xenopus laevis* retina has been a particularly valuable model system for these studies because of its large cells and its survivability in culture. In the *Xenopus* retina, several rhythmic phenomena are under the control of the circadian clock, including release of neuromodulators, gene expression, cone elongation, rod disc shedding (for review, see Besharse, 1988; Anderson and Green, 2000), and visual sensitivity (Manglapus et al., 1999).

Perhaps the most extensively studied rhythm in the retina is melatonin synthesis and release, which peaks during the night (Besharse, 1988; Cahill et al., 1991). Melatonin synthesis is under complex control, including regulation by the circadian clock, acute suppression by light, and suppression by dopamine, which is released from inner retinal cells (for review, see Cahill et al., 1991).

As in all clock systems, very little is known about how these various individual cellular rhythms are organized within the context of the intact tissue. It is unclear how many retinal cell types contain clocks or what the relationships of the various cellular clocks are. The few attempts that have been made to examine this issue have relied primarily on gross lesioning techniques. For example, destruction of the entire inner layers of the retina result in an enriched photoreceptor preparation that still maintains rhythmicity of melatonin production (Cahill and Besharse, 1993). However, within the intact eye, it is likely that other rhythmic processes may also contribute to the generation of the high amplitude melatonin rhythms. For example, dopamine from inner retinal neurons can both acutely suppress melatonin and affect the phase of the melatonin rhythm (Cahill et al., 1991).

The general molecular makeup of circadian clocks appears to be primarily conserved among all animals (for review, see Dunlap, 1999). One well conserved aspect of these clocks is the positive regulation of *period* gene expression by the transcription factor heterodimer composed of CLOCK and BMAL1/CYCLE (Bae et al., 1998; Darlington et al., 1998; Lee et al., 1998; Glossop et al., 1999), which is necessary for clock function in all cases where it has been tested (Vitaterna et al., 1994; Antoch et al., 1997; King et al., 1997; Allada et al., 1998; Darlington et al., 1998; Rutila et al., 1998; Bunger et al., 2000).

Here we have used a transgenic approach to introduce a mutant form of the *Clock* gene specifically into photoreceptor cells of the *Xenopus* retina. We demonstrate that expression of this mutant gene results in a very specific "molecular lesion" that ablates circadian clock function only in rods and cones in the context of the intact retina and results in arrhythmic melatonin production. Surprisingly, although melatonin is arrhythmic in these retinas, overall melatonin levels are not altered, demonstrating that the circadian regulation is separable from the general regulation of melatonin synthesis.

MATERIALS AND METHODS

DNA constructs. The transgene IRBP-XCLΔQ-GFP was constructed as follows. *Xenopus laevis Clock* cDNA (Zhu et al., 2000) (GenBank accession number AF227985) was truncated by PCR at bp 1629 using the following primers: 5′-CCTACAGAAGCATAAAGAAATCAGTGC-3′ and 5′-GA<u>AGATCT</u>T<u>ACCGG</u>TTTTGTTGGCGTTGATGATGGG-TC-3′ (this primer has introduced *Age*I and *Bgl*II sites at the end, underlined). The truncated *Clock* cDNA was then digested with *Eco*RI and *Bgl*II and inserted into the *Eco*RI- and *Bgl*II-digested pBluescript II vector (pBS-XCLΔQ). The mouse interphotoreceptor retinoid-binding protein (IRBP) promoter (−155/+101) (Boatright et al., 1997) was digested with *Bam*HI and inserted into the *Bam*HI site of the pBS-XCLΔQ (pIRBP-XCLΔQ). Then pIRBP-XCLΔQ was digested with *Age*I and *Sac*II, and the excised fragment was ligated with a pEGFP-1 vector (Clontech, Palo Alto, CA) that was linearized with the same enzymes to produce IRBP-XCLΔQ-green fluorescent protein (GFP).

For transient transfections, pCMV-XCL (wild-type *xClock*) and pCMV-XBM (wild-type *xBmal1*) were constructed as follows. pVAX1 (Invitrogen, Carlsbad, CA) was digested with either *Bam*HI and *Xho*I or *Eco*RI and *Xho*I, and *Bam*HI- and *Sal*I-digested *xClock* or *Eco*RI- and *Xho*I-digested *xBmal1*cDNA fragments (Anderson et al., 2001) were subcloned into the vector, respectively.

The luciferase reporter gene contains three tandem repeats of a fragment containing an E-box sequence reported from the mouse *Per1* gene promoter (Gekakis et al., 1998; Hida et al., 2000), which was cloned upstream of the rat prolactin basal promoter (Dr. Richard Day, University of Virginia; unpublished data) and firefly luciferase cDNA (pPer-Ebox).

Luciferase assay. Expression plasmids (0.1 μg each) and the luciferase reporter gene (0.1 μg) were cotransfected into COS-7 cells using Lipofectoamine following the manufacturer's instructions (Invitrogen, Gaithersburg, MD). After 24 hr incubation at 37°C, cells were lysed and examined for luciferase using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI). Luciferase activity was normalized by cotransfecting 25 ng of a control plasmid (pRL-TK, Promega) and measuring *Renilla* luciferase activity. Luciferase activity was measured using a luminometer (TD-20/20, Turner Designs, Sunnyvale, CA). ANOVA with Tukey-Kramer *post hoc* multiple comparison tests (InStat Software, Graphpad, San Diego, CA) were performed to determine whether luciferase activities are significantly different between wild-type and truncated CLOCK.

Transgenesis. *Xenopus laevis* adults were purchased from NASCO (Fort Atkinson, WI), and eggs and sperm were obtained from the adult frogs. Transgenic tadpoles expressing IRBP-XCLΔQ-GFP were produced using restriction enzyme-mediated integration (REMI) in which the transgene is stably inserted into the sperm genome, followed by fertilization of eggs with the sperm nuclei (Kroll and Amaya, 1996), with the following modifications. IRBP-XCLΔQ-GFP was linearized with *Spe*I and used as a transgene. The REMI reaction was prepared by mixing 1 μl of linearized plasmid (150 ng/μl) with 4 μl of sperm nuclei (~4 × 10^4 nuclei). After a 5 min incubation at room temperature, 1 μl 100 mM MgCl_2, 0.5 μl of a 1:10 dilution of *Spe*I (10 U/μl), 2 μl oocyte extract (preheated at 80°C for 10 min), and 9 μl sperm dilution buffer (250 mM sucrose, 75 mM KCl, 0.5 mM spermidine trihydrochloride, 0.2 mM spermidine tetrahydrochloride) were added and incubated for 10 min at room temperature. The reaction mix was diluted in MOH (10 mM KPO_4, 125 mM Kgluconate, 5 mM NaCl, 0.5 mM MgCl_2, 25 mM sucrose, 0.25 mM spermidine, 0.125 mM spermine) to a final concentration of ~3 sperm nuclei in 5 nl, and then injected into dejellied *Xenopus laevis* eggs at a rate of 5 nl/sec using a glass needle and a syringe pump (Model 1111, Harvard Apparatus, Holliston, MA). The concentration of the sperm nuclei was empirically determined to result in one functional sperm nucleus being delivered to the majority of eggs. Normally developing embryos were

maintained in 12 hr light/dark (LD 12:12) cycles until they reached the appropriate age for analysis (usually 2–3 weeks).

Genotyping. The tail tip was cut from each tadpole, and genomic DNA was isolated using the DNeasy Tissue Kit (Qiagen, Valencia, CA). PCR was then performed using AmpliTaq Gold (PE Applied Biosystems, Foster City, CA) and both GFP-specific and mouse IRBP promoter-specific primers. The sequences of the primers are as follows: GFP primers, 5′-CAAGCTGACCCTGAAGTTCATCTG-3′ and 5′-CG-GATCTTGAAGTTCACCTTGATG-3′; IRBP primers, 5′-ATCCCT-ACACAGACATGGCT-3′ and 5′-ATCCCAGAGCCTTGGCTCCT-3′. PCR conditions were as follows: 95°C for 10 min, 30 cycles of 94°C for 40 sec, 55°C for 1 min, 72°C for 1 min; and 72°C for 10 min.

Images. Eyes from transgenic tadpoles were dissected and fixed in phosphate-buffered 4% paraformaldehyde overnight, then infiltrated with 30% sucrose and embedded in O.C.T. compound (Ted Pella, Redding, CA). GFP and phase images were obtained from frozen sections (10 μm thickness) using an inverted fluorescence microscope (IX70, Olympus, Melville, NY).

Flow-through culture. Tadpoles were maintained under LD 12:12 cycles before the experiments. Eyes from entrained 2- to 3-week-old tadpoles were dissected shortly before normal dark onset, and the cornea and lens were removed. Eyecups from independent tadpoles were transferred to a well of a 96-well microtiter plate containing culture medium consisting of 80% defined balanced salts and amino acids (Cahill et al., 1991) and 20% Wolf and Quimby amphibian tissue culture medium (Invitrogen). The medium was supplemented with 100 μM 5-hydroxy-L-tryptophan to enhance melatonin production (Cahill and Besharse, 1990), and the pH of the culture medium was equilibrated with 5% CO_2/95% O_2 during flow-through culture. Eyecups were then cultured in flow-through chambers as described previously for 3–5 d in constant darkness (DD) (Cahill et al., 1991; Green et al., 1999). Temperature was maintained at 21°C. The culture plates were kept in light-tight chambers, and the medium was continuously delivered by a syringe pump (model 2000, Harvard Apparatus) to each well at a constant rate of 0.2 ml/hr. Superfusates were collected in a fraction collector over 4 hr intervals.

Melatonin measurement. Melatonin levels in the superfusate samples from flow-through culture were determined by radioimmunoassay (RIA) as described previously (Rollag and Niswender, 1976) and validated for measurement of melatonin (ruling out cross-reactivity to other related compounds) in our culture medium (Cahill and Besharse, 1990).

Period analysis. Circadian rhythmicity of melatonin release was evaluated using a fast Fourier transform–nonlinear least squares (FFT-NLLS) estimation method developed by Dr. Martin Straume (National Science Foundation, Center for Biological Timing, University of Virginia) (Plautz et al., 1997). Time series were classified as arrhythmic if no rhythms were returned by the algorithm, i.e., the cosine function could not be fit to the data at a 95% confidence level. Instead of applying any cutoff for the relative amplitude of period as reported in the previous paper (Plautz et al., 1997), we classified eyecups as rhythmic if the relative amplitude of the period was <1 (FFT-NLLS default setting).

Quantitative RT-PCR. After flow-through culture was complete, culture medium was removed, eyecups were homogenized, and total RNA was isolated using Trizol reagent (Invitrogen) following the manufacturer's instructions. RNA was then reverse transcribed into cDNA using Superscript II (Invitrogen) and used as a template for PCR. Real-time quantitative PCR was performed using GeneAmp 5700 Sequence Detection System and SYBR Green Master Mix that includes SYBR Green Dye and AmpliTaq Gold (PE Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. For the quantitation of the transgene expression, GFP-specific primers were used for the PCR reaction (5′-AGCAAAGACCCCAACGAGAA-3′, 5′-GGCGGCGGTCACGA-A-3′). For quantitation of endogenous arylalkylamine *N*-acetyltransferase (AANAT) expression, the following primers were used: 5′-GGA-AGGCTGGTGGCATTTATC-3′, 5′-CCGGTTTGTGAAAGGTGAG-TG-3′ (Drs. Steven Coon and David Klein, personal communication). Human 18S rRNA primers (PE Applied Biosystems) (5′-CGGCTA-CCACATCCAAGGAA-3′, 5′-GCTGGAATTACCGCGGCT-3′) were used as an endogenous control for the purpose of normalization. For each experiment, a standard curve was prepared for each primer set using as template dilution series of cDNA from transgenic eyes, where the most concentrated standard was assigned an arbitrary value of 10. The levels of GFP and 18S rRNA levels in each test sample were then determined on the basis of the standard curve. We then normalized the GFP expression levels to the 18S rRNA expression levels for each pair of
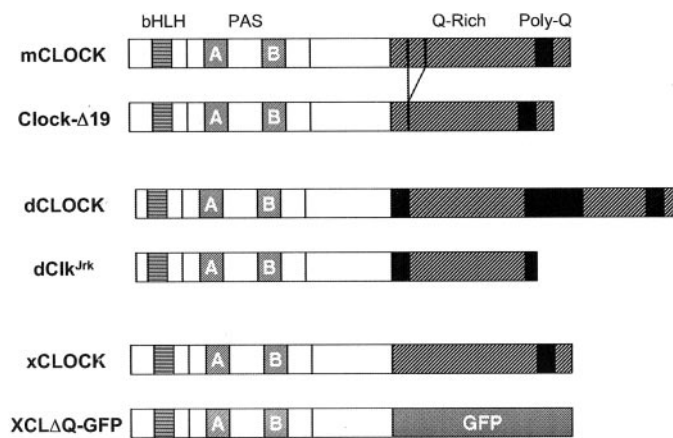
Figure 1. Construction of XCLΔQ-GFP, a dominant negative form of xCLOCK. Diagrammatic comparison of XCLΔQ-GFP with normal CLOCK proteins and their known mutant forms in mouse and *Drosophila*. *Hatched* and *filled regions* at the C terminus correspond to the glutamine-rich (Q-rich) and poly-glutamine putative transactivation domains, respectively. PAS-A and PAS-B domains are indicated by *A* and *B*. The mouse *Clock* mutation (*Clock-Δ19*) results in the deletion of 51 amino acids of the Q-rich transactivation domain. The *Drosophila dClk^Jrk* mutant has a nonsense mutation that results in truncation of part of the Q-rich domain. In this study, XCLΔQ-GFP was constructed by deleting the Q-rich region from xCLOCK and replacing it with an in-frame GFP cDNA.

eyes. The data shown are the averages of three or six independent measurements for each tadpole.

RESULTS

XCLΔQ acts in a dominant negative manner *in vitro*

We have previously characterized the *Xenopus* homolog of *Clock* (Zhu et al., 2000). Like its orthologs, the *Xenopus* CLOCK protein has a bHLH DNA-binding and protein interaction domain, a PAS protein–protein interaction domain near the N terminus, and a glutamine-rich (Q-rich) domain located at the C terminus that is thought to be a transcription activation domain (Zhu et al., 2000) (Fig. 1). From the structure of this protein, we predicted that an xCLOCK molecule lacking the activation domain would act as a dominant negative, because it would still bind DNA (the E-box element) and the BMAL1 binding partner but would not result in transcriptional activation (Gekakis et al., 1998). This prediction is supported by previous reports in which *Clock* mutants in both mouse (*Clock*) and *Drosophila* (*Clk^Jrk*) are semidominant in circadian phenotypes, and each mutation results in a partial deletion of the Q-rich domain at the C terminus (King et al., 1997; Allada et al., 1998) (Fig. 1).

We therefore constructed a truncated version of *xClock* in which a sequence encoding the Q-rich activation domain was removed and replaced with an in-frame GFP (Fig. 1, *XCLΔQ-GFP*). To test whether XCLΔQ-GFP has the predicted dominant negative effect *in vitro*, we performed transient transfection assays using a luciferase reporter gene. We cotransfected COS-7 cells with expression vectors encoding either wild-type xCLOCK (pCMV-XCL) or XCLΔQ-GFP (pCMV-XCLΔQ-GFP) along with xBMAL1 (pCMV-XBM) and a luciferase reporter gene under the control of a basal promoter and with a triple m*Per1* E-box cloned upstream (pPer-Ebox) (Gekakis et al., 1998). Although wild-type xCLOCK, along with xBMAL1, activated luciferase activity, no activation was observed when XCLΔQ-GFP and xBMAL1 were coexpressed (Fig. 2). In addition, when
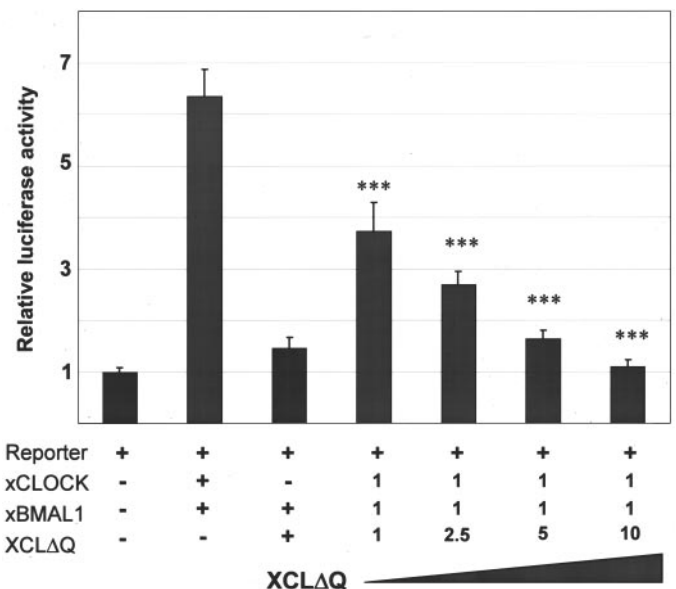

Figure 2. XCLΔQ acts as a dominant negative *in vitro*. Expression plasmids and the luciferase reporter gene (containing three repeats of *mPer1* E-box and a basal promoter) were cotransfected into COS-7 cells, and luciferase activities were measured from cell lysates. *xCLOCK* refers to a vector encoding wild-type xCLOCK (pCMV-XCL), *XCLΔQ* refers to a truncated xCLOCK (pCMV-XCLΔQ-GFP), and *xBMAL1* refers to wild-type xBMAL1 (pCMV-XBM). In the *right four columns*, pCMV-XCL and pCMV-XBM were cotransfected with increasing amounts of pCMV-XCLΔQ-GFP. The ratios of the transfected plasmids are indicated by *numbers* below each column. The data are averages from three independent transfections. ANOVAs with Tukey-Kramer multiple comparison tests were performed to determine significant difference in luciferase activity between wild-type and truncated xCLOCK (***$p < 0.001$).

XCLΔQ-GFP was cotransfected with wild-type xCLOCK and xBMAL1, it inhibited CLOCK/BMAL1-mediated activation of the luciferase activity in a dose-dependent manner (Fig. 2). Even at 1:1:1 ratio, significant inhibition of activation was observed, whereas complete inhibition was seen when 10-fold excess of pCMV-XCLΔQ-GFP was present ($p < 0.001$) (Fig. 2).

IRBP-XCLΔQ-GFP is specifically expressed in the retinal photoreceptor cells

A circadian oscillator located in the photoreceptor layer in *Xenopus* can regulate retinal circadian melatonin rhythms when this layer is cultured without the rest of the retina (Cahill and Besharse, 1993). To investigate the role of the photoreceptor clock in the regulation of melatonin synthesis *in vivo* (in the intact retina), transgenic studies were performed using the REMI method (Kroll and Amaya, 1996). We designed a transgene that expresses XCLΔQ-GFP in all photoreceptor cells, using the mouse IRBP gene promoter. This promoter drives reporter gene expression in rod and cone photoreceptors in retina and occasionally low level expression in the pineal gland in *Xenopus* (Fig. 3) (data not shown). We have never observed mouse IRBP-driven expression in any other body part or any nonphotoreceptor cells in the retina.

We first tested the IRBP-XCLΔQ-GFP transgene for general translation, stability, and cellular localization by producing transgenic *Xenopus* tadpoles and visualizing the fluorescent XCLΔQ-GFP in the retina. Sections through the retinas of these transgenic tadpoles verified that IRBP-XCLΔQ-GFP was stably expressed in both rod and cone cells and was localized in the cell bodies,
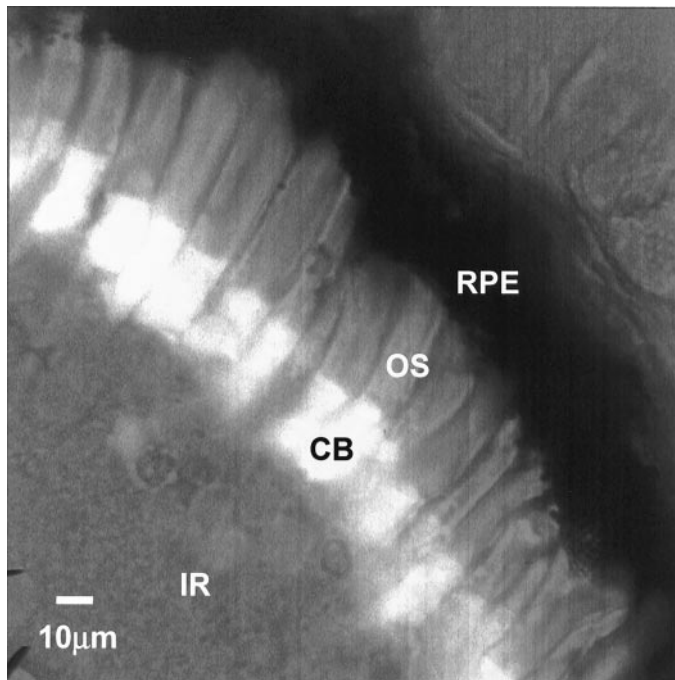
Figure 3. XCLΔQ-GFP accumulates in the photoreceptor cell bodies. An image of a section through a transgenic tadpole retina expressing XCLΔQ-GFP under the control of the IRBP promoter. XCLΔQ-GFP is present in the cell bodies and inner segments of all the photoreceptor cells. *CB*, Photoreceptor cell body; *OS*, photoreceptor outer segment; *IR*, inner retina; *RPE*, retinal pigment epithelium. Note that this is one of the strongest expressors of GFP among many transgenic eyes examined. This is a merged image of GFP signal and phase.

including nuclei (Fig. 3). No staining was observed in any other cell types. The cells expressing this transgene looked morphologically normal at the light microscopic level. It is of note that we observed different levels of GFP expression in eyes from different transgenic tadpoles, and in some cases GFP was not expressed at a detectable level, even when transgene was stably incorporated (data not shown).

Transgenic eyecups show abnormal rhythms of melatonin release but normal average melatonin levels

Circadian rhythms of melatonin release in *Xenopus* can be monitored over time when *Xenopus* retinas or eyecups are maintained in a flow-through culture system (Cahill et al., 1991). To investigate the involvement of xCLOCK in the regulation of circadian melatonin release, we performed eyecup culture for several days using transgenic tadpoles expressing XCLΔQ-GFP, with wild-type siblings as controls. Embryos were held in cyclic light (LD 12:12) until they reached 2–3 weeks of age, then eyecups were dissected and maintained in flow-through culture in DD for 3–5 d. The transgenic technique results in a mixture of developing embryos, some carrying the transgene and some nontransgenic (wild-type) siblings. The tail of each embryo was saved for PCR analysis to determine the genotype of each animal after the flow-through culture was complete. Therefore, each experiment contained wild-type nontransgenic tadpoles as controls that were obtained from the same set of microinjections and were maintained in exactly the same conditions as the transgenic animals.

Most of the wild-type eyecups showed circadian rhythms of melatonin synthesis (87%; average $\tau = 24.6 \pm 0.6$ hr) (Fig. 4*A*, Table 1), consistent with previous reports (Cahill and Besharse,
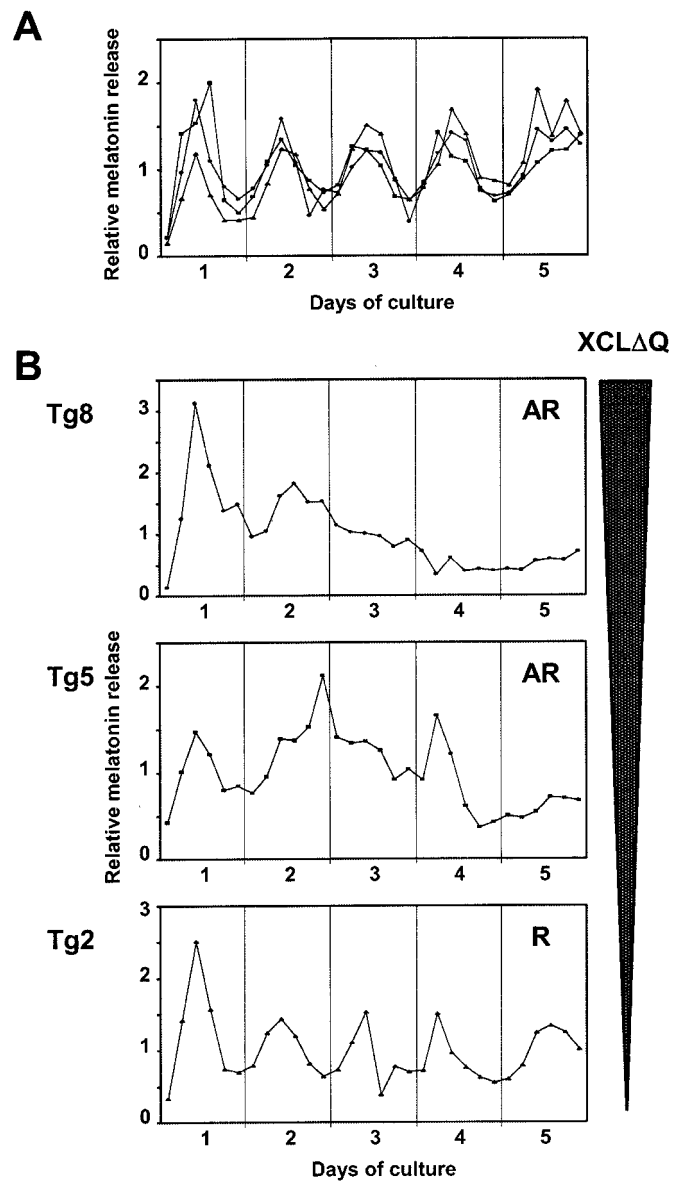

Figure 4. Melatonin release from wild-type and transgenic eyes. Each *line* represents melatonin release from a single pair of eyes in a flow-through culture for 5 d in constant darkness. Media fractions were collected every 4 hr and assayed for melatonin. Relative melatonin levels (melatonin content in each fraction divided by average) are shown to better allow comparisons between different animals. *A*, Wild-type eyes; *B*, patterns of melatonin release from three representative transgenic tadpole eyes are shown (*Tg8*, *Tg5*, and *Tg2* refer to the numbering used in Fig. 6). The expression level of XCLΔQ was highest in *Tg8*, intermediate in *Tg5*, and lowest in *Tg2* (see also Fig. 6). Of these tadpoles, only *Tg2* showed a significant circadian rhythm of melatonin release. *Tg5* showed some indication of rhythmic release with long period, although melatonin release was not significantly rhythmic by our criteria. Circadian rhythmicity of melatonin release was analyzed using FFT-NLLS. *AR*, Arrhythmic; *A*, rhythmic.

1990, 1993; Cahill et al., 1991; Green et al., 1999). Those few wild-type eyes that were judged arrhythmic showed weak rhythms that damped and were statistically nonrhythmic. In contrast, only 29% of the transgenic eyecups showed circadian expression of melatonin, with periods similar to those of wild-type siblings (Table 1). Of the transgenic tadpoles, rhythms were disrupted in 71%, including 8.5% that exhibited rhythms with extremely long

Table 1. Summary of the melatonin release rhythms

| | Number of animals | | | | Period (hr ± SEM) | % Abnormal | |
	Total	R	LP	AR	R + LP	AR	Total (AR + LP)
XCLΔQ-GFP	24	7	2	15***	26.4 ± 1.5	62.5	71.0
Wild type	23	20	1	2	24.6 ± 0.6	8.7	13.0

Circadian rhythmicity of melatonin release was analyzed using FFT-NLLS. Total % abnormal includes both arrhythmic and rhythmic eyes with long periods (>28 hr). R, rhythmic (period range between 20 and 28 hr); LP, long period (>28 hr); AR, arrhythmic (no significant rhythm at a 95% level of confidence). *** indicates significant difference between wild-type and transgenic eyes determined by Fisher's exact test ($p < 0.001$).
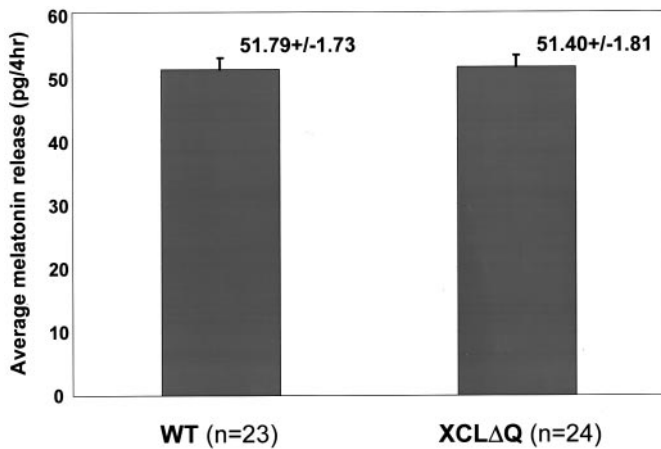

Figure 5. Total melatonin levels were not affected in transgenic eyes. Average melatonin release from eyes from transgenic and wild-type tadpoles was calculated from all fractions of all flow-through experiments. Values on the figure are average melatonin content (picograms per 4 hr) ± SEM.

periods (average $\tau = 33.9$ hr), and 62.5% showed no significant rhythmicity (Fig. 4*B*, Table 1). We also compared the average melatonin production levels (averaged over all fractions) of the transgenic and wild-type eyecups, but no significant difference was detected between those two distinct genotypes (Fig. 5). It is of note that even the arrhythmic transgenic eyes showed an initial peak of melatonin release during the first night after transfer from LD 12:12 to DD, which is comparable in level to the peak of the wild-type eyes. This peak is most likely the result of melatonin rebound after the previous light period, as has been observed previously in *Xenopus* retina and chick pineal cells (Zatz, 1991; Green et al., 1999). Although we do not know the mechanism driving this "rebound" phenomenon, this suggests a direct effect of light or light cycles on the amplitude of the melatonin rhythm, and this effect still exists in the transgenic eyes.

XCLΔQ alters the circadian expression of melatonin in a dose-dependent manner

As mentioned above, we observed variability in the phenotypes of retinal melatonin release among individual transgenic tadpoles. This incomplete penetrance is most likely caused by variability in transgene expression levels in individual tadpoles as observed when visualizing GFP fluorescence. To test this possibility, we compared transgene expression levels in individual eyes from some of our tadpoles by quantitative real-time RT-PCR. At the end of the flow-through experiments, total RNA was extracted from the cultured eyecups, and cDNA was synthesized from the RNA for use as a template in quantitative PCR. We averaged the relative expression levels of the transgene from six independent
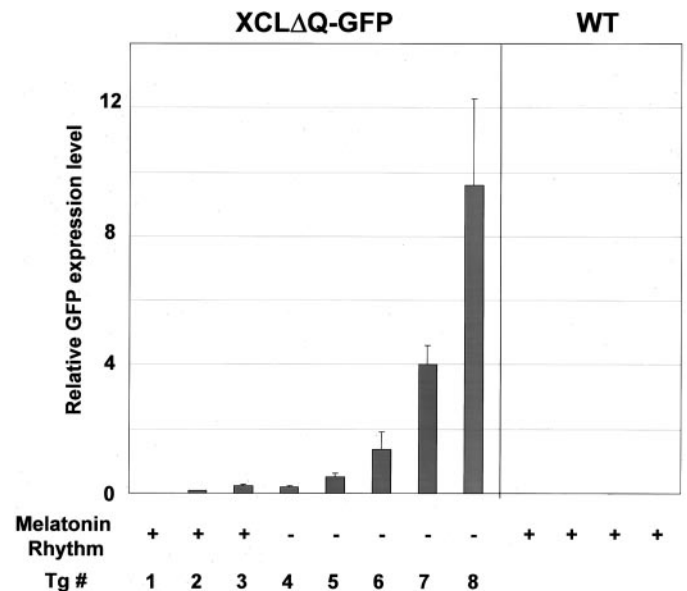

Figure 6. Relative expression levels of the XCLΔQ-GFP transgene in the eyes. Quantitative RT-PCR was performed using GFP primers on RNA from each pair of eyes after they were used for the flow-through culture. Relative expression levels of the GFP mRNA were determined and normalized to the endogenous control (18S rRNA). Each value is the average of six independent measurements. *Tg1–Tg8* indicate expression levels of the transgene from pairs of eyes from individual transgenic tadpoles. The presence (+) or absence (−) of melatonin rhythmicity in flow-through culture is noted for each tadpole. *Tg8*, *Tg5*, and *Tg2* correspond to the transgenic eyes that produced the melatonin profiles shown in Figure 4*B*.

measurements of each sample and found a large range of transgene expression levels among all eyes tested (Fig. 6). This variability in expression levels could be attributable to differences in copy numbers of the transgene or its integration sites, or both. These different expression levels correlate well with the melatonin phenotype: only the eyecups with low expression of the transgene had exhibited rhythmic melatonin release, whereas stronger expressors were arrhythmic (Figs. 4*B*, 6). As an example, among the three melatonin profiles from transgenic eyes shown in Figure 4*B*, the highest expressor (*Tg8*) was arrhythmic, whereas the lowest (*Tg2*) showed a statistically significant circadian rhythm of melatonin release. The eyecups that express the transgene at an intermediate level (*Tg5*) did not produce statistically rhythmic melatonin, although a few peaks can be observed (Figs. 4*B*, 6).

XCLΔQ expression does not affect endogenous AANAT mRNA levels

AANAT is one of the melatonin synthetic enzymes, and its activity peaks at night in all species examined (Klein et al., 1997).

Table 2. XCLΔQ alters melatonin rhythmicity, but not the endogenous AANAT mRNA levels

| Genotype | Relative mRNA level | | Melatonin rhythm |
	XCLΔQ-GFP	AANAT (% of WT)	
WT	ND	100.0	R
Tg	ND	74.2 ± 10.5	R
Tg	1.2 ± 0.2	72.3 ± 1.8	R
Tg	1.6 ± 0.1	86.5 ± 3.2	R
Tg	20.1 ± 3.0	128.9 ± 7.9	AR
Tg	91.1 ± 11.6	63.5 ± 6.6	AR

Quantitative RT-PCR was performed using GFP (for XCLΔQ-GFP mRNA) and *Xenopus* AANAT primers (for endogenous AANAT mRNA) on RNA from some pairs of eyes after they were analyzed for rhythmicity. Eyes were collected for RNA extraction at the end of the flow-through culture period (circadian time 13–14). Relative expression levels of the GFP and AANAT mRNAs were determined and normalized to the endogenous control (18S rRNA). Each value is the average of three independent measurements (± SEM). ND, Not detectable; R, rhythmic; AR, arrhythmic.

A recent report describes circadian transcriptional regulation of AANAT in chicken, where an E-box in the chicken AANAT 5′-flanking region was shown to be essential for its transcriptional activation and rhythmic expression (Chong et al., 2000). The CLOCK/BMAL1 heterodimer is capable of directly binding to this E-box and enhancing its transcription in transient transfection assays, suggesting that CLOCK controls melatonin production by directly regulating expression of this enzyme involved in its synthesis (Chong et al., 2000). To examine whether expression of XCLΔQ alters AANAT mRNA levels in our transgenic eyes, we performed quantitative real-time RT-PCR using AANAT-specific primers on some of the transgenic and wild-type eyes used previously for flow-through culture. These eyes had been collected from the flow-through culture in early subjective night (circadian time 13–14). Although we observed variation in the expression levels of XCLΔQ-GFP and a range of melatonin profiles among individuals, there were no significant differences in the AANAT mRNA levels between strong and weak expressors of the transgene, nor between wild-type and transgenic animals (Table 2).

DISCUSSION

In this study, we generated transgenic *Xenopus laevis* tadpoles that express a truncated xCLOCK (XCLΔQ-GFP) in the photoreceptor cells. This approach demonstrates that it is feasible to specifically manipulate a molecular clock component in a subset of cells within the intact retina in such a way that circadian clock function is compromised. Our data indicate that a circadian clock in the photoreceptors is not only sufficient but also necessary to generate circadian rhythms of melatonin release. However, a functional photoreceptor clock is not necessary to generate normal levels of melatonin.

Although most of the transgenic eyes did not show significant circadian rhythms of melatonin release, some were rhythmic. Because copy numbers and integration sites of the transgene can vary among tadpoles when the REMI method is used, we expected eyes from individual transgenic tadpoles to express XCLΔQ at different levels. In fact, our results from quantitative RT-PCR (Fig. 6) indicate extreme differences in the transgene expression levels. These experiments also demonstrate that those transgenic eyes that were capable of rhythmic melatonin release expressed XCLΔQ-GFP at low levels. In contrast, the arrhythmic

eyes expressed the transgene at higher levels (Figs. 4B, 6). These data suggest dose effects of the transgene expression, which is consistent with the dose-dependent effect observed in our transient transfection (Fig. 2).

It is of note that average total melatonin secretion levels were not affected by dominant negative xCLOCK (Fig. 6). This indicates that xCLOCK is involved only in regulating melatonin rhythmicity and not absolute levels of melatonin. These data also demonstrate that photoreceptor cells of the transgenic retina are metabolically active and still produce melatonin, and the loss of circadian rhythmicity is not caused by a general toxic effect of XCLΔQ-GFP overexpression. Also, more importantly, these results suggest that there are other mechanisms that control melatonin synthesis and that melatonin synthesis and its rhythmicity are separable events.

Circadian control of melatonin rhythms may occur at several levels. It is known that the melatonin rhythm is regulated, at least in part, by dopamine, another neuromodulator that is expressed during the day. Dopamine acutely suppresses the activity of one of the melatonin synthetic enzymes, AANAT (Iuvone and Besharse, 1986; Boatright et al., 1994), and also causes phase-dependent phase shifts of the circadian clock controlling melatonin rhythms (Cahill et al., 1991). Because melatonin (presumably coming from the photoreceptors) also acutely suppresses dopamine, it has not been known previously which arm of this negative feedback loop (and which cell type) is most critical for driving melatonin rhythms. Our data indicate that the melatonin rhythms are driven by clocks in photoreceptor cells and not simply by rhythmic dopamine.

Other evidence for complex control of melatonin rhythmicity in *Xenopus* comes from studies in which the inner retina was lesioned with detergent treatment (Cahill and Besharse, 1993). Although these photoreceptor layers maintained rhythmic release of melatonin, the period was slightly lengthened. In addition, overall levels of melatonin released were reduced by ~90% when the retinal pigment epithelial layer was removed, suggesting that these cells also contribute to the regulation of melatonin production. Therefore, although the photoreceptor clocks are both necessary and sufficient for driving melatonin rhythms, these other cell types appear to influence melatonin levels and rhythmicity.

The CLOCK protein has been implicated in transcriptional control of both the core clock mechanism and other downstream rhythmic genes (Jin et al., 1999; Park et al., 2000). A previous report suggests that in chicken, AANAT transcription is regulated through an E-box in the AANAT 5′-flanking sequence (Chong et al., 2000). Transient transfection experiments demonstrated that this E-box could bind both CLOCK/BMAL1 and the closely related MOP4/BMAL1 heterodimers, resulting in activated transcription (Chong et al., 2000). Our present data suggest that in *Xenopus* retina, melatonin rhythmicity is regulated by CLOCK, but probably not through direct CLOCK control of AANAT transcription (Table 2). Because our measurements of AANAT mRNA levels could only be done at a single time point (the end of the flow-through experiment), we cannot rule out the possibility that the XCLΔQ-expressing eyes may have altered AANAT levels at other time points. However, our data that show no change in overall melatonin levels suggest that this is not the case (Fig. 5). In *Xenopus* retina, AANAT activity is under circadian control (Besharse and Iuvone, 1983), and one possibility is that CLOCK controls circadian NAT activity rhythms in *Xenopus* retina indirectly by regulating some other gene(s). In chicken and

Xenopus retina, another melatonin synthetic enzyme, tryptophan hydroxylase, is also expressed in a circadian manner, implying that the transcription of this gene may also be controlled by CLOCK or some other clock-controlled factor (Green et al., 1996; Chong et al., 1998). However, because our culture medium contains excess 5-hydroxy-L-tryptophan (the product of tryptophan hydroxylase) to enhance melatonin levels, any potential effect of this mutation on tryptophan hydroxylase would not be observed in these experiments.

Because our study demonstrated that total melatonin production is not altered in the transgenic eyes, we suggest that the role of CLOCK is not to simply turn on melatonin synthesis at night. If this were the case, then the transgenic animals that are arrhythmic (presumably with disrupted CLOCK-mediated transcriptional activation) would show constitutively low levels of melatonin. Although the reason for the normal levels of melatonin in these eyes is not known, one possibility is that the melatonin synthetic pathway is under both positive and negative transcriptional control, both of which require CLOCK. Alternatively, it is possible that melatonin synthesis is regulated through a negative feedback loop in which CLOCK directly or indirectly activates melatonin synthesis, which eventually feeds back to suppress melatonin output to trough levels. If this were the case, then the transgenic retinas that are missing the activation mechanism may never show the negative feedback and melatonin might remain at average levels. The present data cannot answer this question directly, but this method provides a means for more careful dissection of these control pathways in future studies.

Finally, this study raises the question of whether the circadian clock controlling melatonin release rhythms is localized either in rods or cones or in both photoreceptor cell types. In *Xenopus* eyes, all of the clock gene homologs identified thus far are expressed in both rod and cone photoreceptor cells (Zhu et al., 2000; Anderson et al., 2001; Zhu and Green, 2001), implying that both cell types have a circadian oscillator(s). Another interesting issue is whether the circadian clock that controls rhythmic melatonin expression regulates other outputs as well. As mentioned above, many physiological rhythms are under the control of the ocular clock(s), although it is not clear whether one circadian oscillator in the photoreceptor cells directs the many different rhythms in the retina, or whether more than one oscillator, located in different retinal cell types, controls different circadian processes. The localization of the clock controlling melatonin synthesis and other rhythmic events can be addressed by using distinct cell type-specific promoters to target XCLΔQ-GFP followed by the assessment of different rhythmic outputs.

In conclusion, we have succeeded in making transgenic *Xenopus* tadpoles in which the xCLOCK function is abolished or altered, specifically in photoreceptor cells in the intact animal. The expression of this dominant negative transgene in *Xenopus* is a valuable tool to selectively inactivate circadian clock function in specific cells and should allow careful dissection of the mechanism of circadian clock function within a complex tissue such as the retina.

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