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REVIEW

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# Clock mechanisms in zebrafish

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**Abstract** Recent research on the circadian system of the zebrafish is reviewed. This teleost has become an attractive model system because of its advantages for genetic analyses. Circadian rhythms of zebrafish behavior, visual system function, and pineal melatonin synthesis have been described, and behavioral and pineal rhythms are being used to identify and characterize clock mutants. Zebrafish heart, kidney, and embryonic cell lines contain circadian oscillators and phototransduction mechanisms for entrainment, suggesting that circadian pacemaking functions may be distributed throughout the animal. Studies of circadian system development in zebrafish have found that a molecular circadian oscillation in unfertilized oocytes persists through embryonic development with its phase intact, but that the pacemakers that drive rhythms of melatonin synthesis and behavior require environmental entraining signals late in development for initial synchronization. Zebrafish homologs of several of the core clock genes identified in other animals have been cloned. Transcripts for most of these are rhythmically expressed in multiple tissues. The interactions of clock gene products are for the most part similar to their interactions in mammals, although there are some potentially interesting differences.

**Keywords** Circadian rhythm · Clock gene · Development · Peripheral oscillator · Teleost

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## Introduction

Clock mechanisms in zebrafish are of interest both from a comparative standpoint and as a model system with unique experimental advantages. Teleost circadian systems have received relatively little attention in the past, with the exception of numerous studies of physiological rhythms of the pineal and retina, and a few studies demonstrating circadian control of behavior (cf. Cahill 2001). Whereas the zebrafish cannot really represent all of this large and diverse class of vertebrates, it does serve as one example for comparative analyses. Comparative issues aside, a major reason for the recent increase in attention to the zebrafish circadian system is its potential as a model system for the genetic analysis of clock mechanisms. Genetic approaches have been tremendously successful in elucidating circadian clock mechanisms in other model systems (cf. Allada et al. 2001; Loros and Dunlap 2001; Young and Kay 2001; Stanewsky 2002; Okamura et al. 2002). The zebrafish has become a major model system for mutational analysis of embryonic development, and this has led to development of the genetic and genomic technologies and resources required to identify mutant genes and determine their functions. These include methods for mutagenesis and transgenesis and the accumulation of rapidly growing genomic information. Point mutations can be induced easily and efficiently in the zebrafish germ line with chemical mutagens (Mullins et al. 1994; Solnica-Krezel et al. 1994), and insertional mutagenesis has also been successful (Amsterdam et al. 1999). A high-resolution meiotic map of the genome, based on simple sequence length polymorphisms (SSLPs), has been generated, enabling linkage mapping of mutations (Shimoda et al. 1999). Two zebrafish-mammalian radiation hybrid panels have been produced (Kwok et al. 1999; Chevrette et al. 2000) and used to generate gene maps that are anchored with SSLPs from the meiotic map (Geisler et al. 1999; Hukreide et al. 2001). These radiation hybrid maps, together with meiotic maps of zebrafish genes (Woods et al. 2000), facilitate cloning of mutant genes by suggest-

ing candidate genes that map to the same location. Together, these resources now make it possible positionally to clone genes identified by mutations; this will become easier with the completion of the zebrafish genome sequence, an effort that is currently under way. There have also been major advances in technology for the production of transgenic zebrafish, with the achievement of high rates of germ line transmission and tissue-specific transgene expression, and this will be instrumental in the analysis of gene function (Higashijima et al. 1997; Jessen et al. 1998).

Published studies of the zebrafish circadian system to date have focused on the characterization of behavioral and physiological rhythms, on the tissue distribution of circadian oscillators and photoreceptors, on the development of the circadian system, and on the expression patterns and functions of zebrafish homologs of known clock genes. These studies, reviewed here, not only provide information that will be critical for the genetic analysis of clock mechanisms, but have also revealed some important new principles about vertebrate circadian system organization.

## Behavioral and physiological circadian rhythms in zebrafish

Circadian rhythms of locomotor activity, visual system function, and pineal melatonin synthesis have been described. My laboratory has examined circadian regulation of locomotor activity in adult and larval zebrafish (Hurd et al. 1998; Cahill et al. 1998). The activity of adults housed individually in small (50 ml) recording chambers was measured with infrared motion detectors (Hurd et al. 1998). There was considerable variability in the activity patterns measured in this way, and only about 70% of the fish showed strong activity rhythms, but some basic features of the circadian system were discernible. When maintained in a light:dark cycle, zebrafish were most active during the daytime. When the fish were kept in constant conditions, circadian rhythmicity in locomotor activity was observed for up to 10 days. The period of the freerunning locomotor rhythm was temperature-compensated, varying little over the range of 18–28.5°C, although it was longer in constant darkness (~25 h) than in constant dim light (~24.4 h). In some activity records, two rhythmic components with different circadian periods were observed, suggesting the regulation of activity by more than one circadian oscillator that can run independently.

The locomotor rhythms of larval zebrafish (5–18 days old), measured by a computerized video image analysis system, have proven to be much more robust and reliable (Cahill et al. 1998). The system that we use can simultaneously track the movements of up to 150 animals housed individually in 0.8-ml wells for a week in constant conditions. The activity records of over 95% of larval zebrafish display statistically significant circadian rhythmicity in this system, and satisfactory phase and

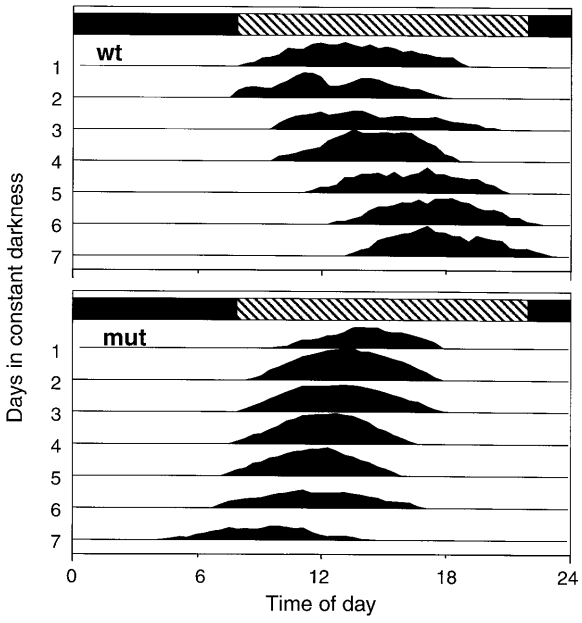


**Fig. 1** Locomotor activity rhythms of wild-type (*wt*) and circadian mutant (*mut*) larval zebrafish in constant conditons. Swimming of 10- to 17-day-old zebrafish under constant infrared light was monitored by a video image analysis system (Cahill et al. 1998). The data were smoothed with a 4-h running average, and activity levels greater than the daily mean were plotted. The prior LD cycle is indicated by the *hatched* (light) and *black* (dark) *bars* above the records. The two fish are homozygous *wt* and *mut* siblings from a cross of parents that were heterozygous for a chemically induced mutation that shortens the period of activity rhythms. The mutated gene has not as yet been identified

period estimates can typically be determined from 85%–90% of the animals. Larval zebrafish, like the adults, are most active during the subjective day, and the average freerunning period under constant infrared light is 25.5 h. We use these behavioral rhythms as an assay in a screen for chemically-induced semidominant mutations that alter the period of the rhythm. We have identified mutations that shorten or lengthen the period of the behavioral rhythm by 0.5–1.5 h in heterozygotes and 1.0–2.5 hours in homozygotes. Figure 1 shows actograms from a wild-type zebrafish with a typical 25 h period and of a mutant with a period shorter than 24 h. We are now in the process of mapping the mutations with the goal of cloning the mutated genes.

Behavioral, physiological and molecular studies have revealed that several functional aspects of the visual system are regulated by the circadian system in zebrafish. Using a behavioral assay, Li and Dowling (1998) have shown that the threshold light intensity for detection of a visual stimulus is ~ 2 log units higher during the night than during the day (i.e. the visual system is most sensitive during the day). This is true for the dark-adapted responses of both rods and cones. The sensitivity rhythm persists for a few days in constant conditions, indicating that it is under the control of a circadian clock. The rhythm damps to a state of high sensitivity in prolonged constant conditions, a finding that has led to the

suggestion that, when this clock functions, it acts in some way to decrease visual sensitivity during the night. Electroretinograms (ERGs) recorded at different times of day showed that some, but not all, of the rhythm in visual sensitivity could be accounted for by changes in the outer retina, suggesting that more central parts of the visual system may also be regulated by the clock (Li and Dowling 1998). Neurochemical lesions of dopaminergic interplexiform cells in the retina abolish the rhythm in behavioral threshold and the rhythms in the ERG, indicating that these cells have an important role in visual system rhythmicity (Li and Dowling 2000b). Surprisingly, the behavioral threshold is high (in the nighttime state) at all times of day after these lesions, whereas ERG thresholds remain low (in the daytime state) at all times. Together, these data suggest that circadian clocks regulate the visual system at multiple levels, and that retinal dopamine plays multiple roles in visual rhythmicity. Further indications of the complexity in circadian regulation of the visual system come from a genetic mutation (*night blindness b*, *nbb*) that causes reductions in the dopaminergic interplexiform cells of the retina and in the efferent innervation of these cells by neurons of the terminal nerve in the olfactory bulb (Li and Dowling 2000a). In this mutant, the behavioral threshold after prolonged dark adaptation remains high throughout the day, whereas ERG responses are unaffected. This behavioral phenotype is mimicked by ablation of the olfactory epithelium and olfactory bulb. This supports the conclusion that olfactoretinal efferents have an important role in the regulation of visual sensitivity at the level of the inner retina. The data so far indicate that dopaminergic interplexiform cells and the olfactoretinal efferents that innervate them are necessary for the expression of several aspects of visual system rhythmicity. It is not yet clear whether they play a direct role in the generation of rhythms or are permissive for the expression of the rhythms generated in the retina.

Other retinal rhythms that have been studied in zebrafish include the synthesis of the hormone melatonin (Cahill 1996) and the expression of mRNA for interphotoreceptor retinoid binding protein (IRBP; Rajendran et al. 1996) and of core clock genes (described below). In the retinas of other vertebrates, melatonin has been shown to regulate rhythms of photoreceptor metabolism and of dopamine release by inner retinal neurons (cf. Cahill and Besharse 1995). As in other vertebrates, melatonin synthesis in zebrafish retina is elevated at night and low during the day. This rhythm persists for a few cycles in constant darkness in retinal organ culture, indicating that melatonin synthesis is regulated by a damped circadian oscillator located within the retina (Cahill 1996). IRBP is synthesized and secreted by photoreceptors and is believed to participate in the transfer of retinoids between photoreceptors and retinal pigment epithelium during the visual cycle. Expression of *Irbp* mRNA in zebrafish retina is higher during the day than during the night, and the rhythm persists for a few days in constant conditions in vivo (Rajendran et al. 1996). This mRNA

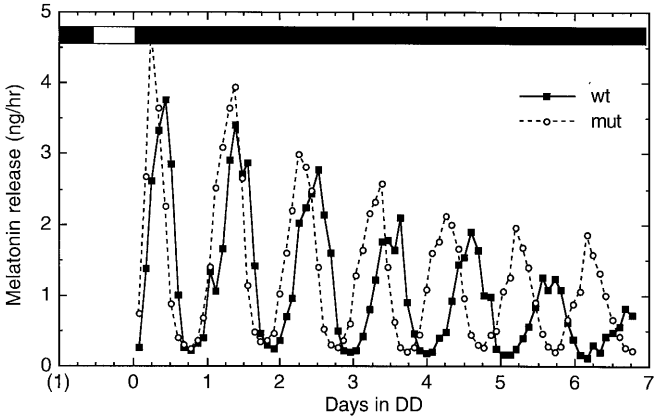


**Fig. 2** Circadian rhythms of melatonin release from cultured pineal glands of adult zebrafish. Pineals were maintained in constant darkness (DD) in a flow-through culture system, and melatonin release into the medium was measured by radioimmunoassay (Cahill 1996). The prior LD cycle is indicated on the *bar above* the graph. Records are from single pineal glands from a wild-type zebrafish (*filled squares*) and a homozygous mutant (*open circles*), from a line recovered in a behavioral screen

rhythm is reflected in IRBP synthesis, but not in total retinal content of the protein, which remains constant throughout the day-night cycle (Cunningham et al. 2000). Turnover of IRBP is higher during the day than during the night, and it appears that the rhythms in mRNA and protein synthesis of IRBP compensate for this increased turnover to maintain constant levels of the protein.

The zebrafish pineal gland contains a light-sensitive circadian oscillator that drives robust rhythms of melatonin synthesis and release in vitro (Cahill 1996). This has also been shown for the pineals of several other teleosts (Falcón et al. 1989; Kezuka et al. 1989; Zachmann et al. 1992; Bolliet et al. 1996). Circadian rhythms of melatonin production by cultured zebrafish pineals persist with precise timing for a week or more, and we are using them to examine the effects of circadian mutations at the cellular level. Figure 2 shows the melatonin release rhythms of cultured pineals from a wild-type zebrafish and a short-period mutant that was identified in the behavioral screen described above (J. DeBruyne and G.M. Cahill, unpublished). The circadian rhythm in melatonin synthesis results, at least in part, from clock control of the mRNA for arylalkylamine-*N*-acetyltransferase (AANAT), the penultimate enzyme in melatonin synthesis (Bégay et al. 1998; Gamse et al. 2001). *Aanat* mRNA levels vary with a rhythm that parallels that of melatonin synthesis and persists in constant conditions. As in light-sensitive pineals of other species, light has two distinct effects on zebrafish pineal melatonin rhythms. It resets the clock in a phase-dependent manner, and it suppresses melatonin synthesis regardless of circadian phase (Cahill 1996). The acute suppression of melatonin synthesis by light appears to be mediated by a post-transcriptional mechanism, because light does not acutely affect *Aanat* mRNA (Bégay et al. 1998). In other species, light expo-

sure results in the proteosomal degradation of AANAT protein (Falcón et al. 2001); this is also likely to be true in zebrafish, although it has not been tested directly. In the pineal organs of other vertebrate classes, melatonin synthesis is regulated by sympathetic efferents via norepinephrine, but zebrafish melatonin rhythms are not affected by catecholamines (Cahill 1997). To date, no mechanisms for efferent regulation of the zebrafish pineal have been described.

## Light-sensitive peripheral oscillators and circadian system organization

Among the most striking recent discoveries about the zebrafish circadian system is that heart and kidney, in addition to cell lines derived from zebrafish embryos, contain both circadian oscillators and photoreceptive mechanisms sufficient for entrainment by LD cycles (Whitmore et al. 1998, 2000; Pando et al. 2001). This has been demonstrated by monitoring rhythms of *Clock* mRNA accumulation in cultured organs and cells. In vivo, *Clock* mRNA levels are rhythmic in heart, kidney, and spleen and in the brain, pineal, and eye (Whitmore et al. 1998). In all of these organs, *Clock* mRNA levels rise during the last part of the subjective day and peak in the early subjective night. When heart and kidney are removed to organ culture, these rhythms persist for two or more cycles in constant conditions, indicating that these organs contain circadian oscillators. When heart and kidney are exposed to LD cycles in vitro, the damping of *Clock* mRNA rhythms is prevented, and reversal of the LD cycle in vitro re-entrains the rhythm to the opposite phase (Whitmore et al. 2000). These data show that these organs contain phototransduction mechanisms sufficient for entrainment, in addition to circadian oscillators. Similar results have been obtained with cell lines derived from zebrafish embryos (Whitmore et al. 2000; Pando et al. 2001), suggesting that many cell types in zebrafish contain photoentrainable circadian oscillators. Light-sensitive circadian oscillators have previously been demonstrated in peripheral tissues of insects (Giebultowicz et al. 1989; Plautz et al. 1997), but these are the first demonstrations of such systems in peripheral organs of a vertebrate. It will be very interesting to see whether other vertebrates also have light-sensitive circadian oscillators in peripheral tissues. This will almost certainly not be true in mammals, which rely on their eyes for entrainment by LD cycles, but may be true in vertebrates of other classes, which are known to use extraretinal photoreceptors for entrainment.

The presence of light-sensitive circadian oscillators in multiple organs raises the issue of whether zebrafish have (or need) a central pacemaking system in the traditional sense. In other vertebrates, central pacemaking systems are made up of the eyes, the pineal gland, and the hypothalamic suprachiasmatic nuclei (SCN), with the relative influence of each on rhythm generation and entrainment varying among species. As described above, zebrafish retina and pineal both contain circadian oscillators that regulate melatonin synthesis, and circulating melatonin could act as a signal to drive or coordinate rhythms throughout the animal. However, in preliminary experiments, we have found that locomotor rhythms in larval zebrafish persisted and could be entrained by LD cycles after pinealectomy and ocular enucleation (M. W. Hurd and G. M. Cahill, unpublished). This indicates that neither of these organs is dominant in the circadian control of this behavior. In other teleosts, pinealectomy does alter the period and stability of behavioral rhythms (Kavaliers 1979, 1980), and subtle effects like these may have gone undetected in our experiments. In mammals, the SCN circadian pacemaker is entrained by LD cycles, and it in turn appears to entrain damped oscillators in peripheral tissues to coordinate rhythmicity (Yamazaki et al. 2000). This suggests the SCN as a candidate pacemaker for the regulation of zebrafish behavior, but the role of hypothalamic nuclei in the circadian system of zebrafish (or any other teleost) has not yet been addressed experimentally. Although there is as yet no direct evidence for a central pacemaking system in zebrafish, the issue has not been addressed sufficiently to rule it out. On the other hand, the presence of light-sensitive oscillators in peripheral tissues suggests the possibility that the zebrafish circadian system is made up largely of distributed pacemakers that are entrained independently by light. It should also be pointed out, however, that the molecular rhythms used to demonstrate peripheral oscillators have not yet been linked to any physiological rhythm.

## Development of the zebrafish circadian system

Zebrafish eggs are fertilized externally (usually at dawn), and embryos develop rapidly and synchronously from fertilized eggs to larvae that swim and eat in about 4 days, making them an excellent model for studies of embryogenesis. Ontogeny of the zebrafish circadian system has been studied at the molecular, physiological, and behavioral levels. The molecular studies suggest a primary role for maternal factors in the initiation and synchronization of circadian rhythmicity during development, whereas the physiological and behavioral studies indicate a crucial role for environmental entraining signals.

Delaunay et al. (2000) have used in situ hybridization and the reverse transcription/polymerase chain reaction technique to show that *Per3* mRNA is rhythmically expressed in unfertilized zebrafish oocytes, and that this rhythm persists through fertilization and embryogenesis. When embryos are raised in constant darkness, a rhythm with the same phase continues in the developing eyes and central nervous system until 5 days of age. These results indicate that LD cycles are not necessary for the initiation or synchronization of the *Per3* mRNA rhythm in embryos and larvae. Furthermore, when embryos were raised in constant light, the phase of the *Per3* mRNA

rhythm was not affected when eggs are fertilized at dusk instead of dawn, or when the temperature was lowered to slow development. These results indicate that the oscillator that controls this rhythm is not affected by the events of embryogenesis and lead to the conclusion that a circadian clock is inherited from the mother and is capable of freerunning through embryogenesis with its phase intact.

In contrast to the *Per3* mRNA rhythm, synchronous circadian rhythmicity has not been observed in melatonin production or locomotor behavior when zebrafish are raised in constant conditions. These rhythms are synchronized only if embryos are first exposed to environmental entraining signals relatively late in development (Kazimi and Cahill 1999; Hurd and Cahill 2002), suggesting that environmental cycles are required for the synchronization of rhythms and that some level of differentiation is required for entrainment to occur. Markers of pineal differentiation, including *Aanat* mRNA, are first detectable in the zebrafish pineal at 22–24 h postfertilization (hpf) near the end of the first night (Masai et al. 1997; Gothilf et al. 1999). When zebrafish are raised in a normal LD cycle, a diurnal rhythm in the melatonin content of pooled whole embryos is detectable by radioimmunoassay from the second night on (Kazimi and Cahill 1999). When embryos are transferred from constant light or an LD cycle to constant darkness at 26 hpf or any time later, a freerunning rhythm in melatonin content, phase-locked to the last dark onset is observed. However, when embryos are raised in constant darkness starting at either 14 or 20 hpf, no rhythm in melatonin content is detectable. These results suggest that the clock that controls melatonin synthesis is not synchronized by any timed maternal or developmental event, but by environmental entraining signals. Furthermore, the lack of entrainment by light during the early stages of embryogenesis suggests that some level of cellular differentiation is necessary before this clock becomes responsive to light. It is possible that melatonin rhythms do indeed develop in individuals raised in constant darkness, but with random phases, so that the population sampled in these experiments is arrhythmic.

In the case of locomotor activity, most individual zebrafish larvae are arrhythmic when maintained in constant darkness from 14 hpf on and tested at 4–9 days of age, and the weak rhythms that are observed in a minority of fish are not phase-locked to the maternal LD cycle (Hurd and Cahill 2002). In contrast, almost all larvae tested under similar conditions after exposure to an LD cycle through the first 3 or 4 days of life display robust, appropriately phased rhythms. These data argue against a role for maternal or developmental factors in the synchronization of behavioral rhythmicity and suggest that environmental LD cycles during the first few days of development not only entrain the clock, but also increase the amplitude of the behavioral rhythm. The effect on behavioral rhythm amplitude could reflect the light-induced initiation of a circadian oscillation, the synchronization of multiple oscillators, or conceivably, the light-induced coupling of a pacemaker to the behavioral output. The phase-setting effect of light for behavioral rhythms can be detected by the second day post-fertilization, but not on the first day post-fertilization. The stimulatory effect of LD cycles on rhythm amplitude increase progressively through the first 4 days of life, suggesting that the pacemaking system that drives locomotor rhythms continues to develop throughout this period.

The differences observed between development of the *Per3* mRNA rhythm and development of the melatonin and behavior rhythms could result from differences in experimental conditions. Alternatively, the clocks controlling the different rhythms may differ anatomically or in their molecular mechanisms. In other animals, results argue against either inheritance of maternal phase or a requirement for LD cycles to initiate behavioral rhythmicity. Both insects that are raised in constant conditions and rodents that are deprived of maternal timing cues develop normal behavioral rhythmicity, but with phases that are not synchronized (Page et al. 1990; Sehgal et al. 1992; Davis and Gorski 1988).

## Molecular clock mechanisms

The molecular mechanisms of circadian rhythm generation in zebrafish appear to have much in common with the more extensively studied mammalian system, although there are differences in detail that may be informative. The prevailing model for the core molecular mechanism of mammalian circadian rhythmicity is an autoregulatory feedback loop in which a transcription factor, formed by heterodimerization of CLOCK and BMAL1 proteins, activates transcription of three *Period* (*mPer1*, *mPer2* and *mPer3*) and two *Cryptochrome* (*mCry1* and *mCry2*) genes. The mPER and mCRY proteins form complexes that enter the nucleus, bind to the CLOCK:BMAL1 complex and inhibit transcription (cf. Allada et al. 2001; Young and Kay 2001; Okamura et al. 2002). This negative feedback loop produces circadian rhythms in expression of *mPer* and *mCry* transcripts. Mammalian *Bmal1* mRNA is also rhythmic, and this rhythmicity depends on mPER2 (Shearman et al. 2000). Zebrafish homologs of these mammalian clock genes have been cloned, and their expression patterns and the interactions of some of their products have been analyzed.

Transcripts for most of the putative clock genes in zebrafish are expressed rhythmically in tissues throughout the animal. They are also rhythmically expressed in embryonic cell lines that can be induced to oscillate by LD cycles and provide a valuable model for further analysis of the molecular mechanisms of rhythm generation and entrainment (Whitmore et al. 2000; Pando et al. 2001). The approximate relative phases of these transcript rhythms are depicted in Fig. 3. As described above, zebrafish *Clock* mRNA is rhythmically expressed both in structures that are considered candidate central circadian pacemakers and in almost all peripheral tissues tested (Whitmore et al. 1998). In all tissues, *Clock*

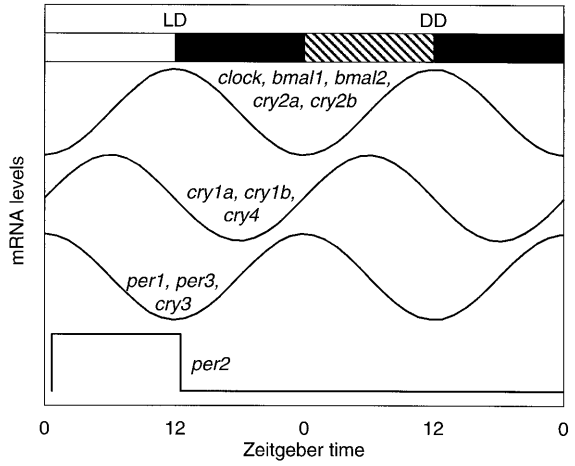


**Fig. 3** Rhythms in the expression of zebrafish clock genes. The approximate timing of mRNA rhythms in *LD* and *DD* is illustrated. The rhythm in *Per2* mRNA is driven by the LD cycle, whereas other rhythms persist in constant darkness (*LD* light dark cycle, *open bar* light phase, *black bar* dark phase, *DD* constant darkness, *hatched bar* subjective day, *black bar* subjective night)

mRNA rises during the late part of the day, peaks in the early night, and reaches a low point near dawn (Fig. 3). This rhythmicity in zebrafish *Clock* mRNA differs from the situation in the mammalian SCN, where *Clock* mRNA appears to be constitutively expressed (Shearman et al. 1999).

Zebrafish genes for two partners of Clock (*Bmal1* and *Bmal2*) were identified in yeast two-hybrid screens (Cermakian et al. 2000). Zebrafish *Bmal1* is clearly an ortholog of mammalian *Bmal1*, and *Bmal2* is probably orthologous to the more recently discovered mammalian *Mop9*/*Bmal2* genes (Hogenesch et al. 2000; Ikeda et al. 2000). Both of the zebrafish BMAL proteins can bind to CLOCK and have intrinsic transcriptional activity, and both genes are rhythmically expressed in many tissues, suggesting that both may be involved in circadian rhythm generation. There are differences in the activities and expression patterns of the two zebrafish BMAL proteins (Cermakian et al. 2000). BMAL1 appears to bind to CLOCK more tightly than BMAL2, whereas BMAL2 is a more potent transcriptional activator than BMAL1, indicating that the two proteins are not entirely redundant. Generally, the mRNA rhythms for the two *Bmals* parallel the *Clock* mRNA rhythm (Fig. 3), with peaks during the late day or early night (Cermakian et al. 2000). However, there are subtle differences in the timing of the two *Bmal* mRNA rhythms within some tissues, and subtle differences among tissues in the timing of each *Bmal* mRNA rhythm. In all cases, these rhythms differ from *Bmal1* mRNA rhythms in mammalian SCN, which peak later, in the middle to late night (Shearman et al. 2000). In the Z3 cell line, rhythms of *Clock*, *Bmal1*, and *Bmal2* transcripts peak during the middle of the night, somewhat later than in vivo (Pando et al. 2001). Surprisingly, transcripts of these genes that are upregulated during the night are also transiently stimulated by light in these cells, a phenomenon that has not been detected previously either in vivo or in organ cultures.

A zebrafish homolog has been identified for each of the three mammalian *Period* genes. Expression of the *Per3* gene is rhythmic in the eye and brain of embryonic and larval zebrafish, and in unfertilized oocytes and in the Z3 cell line (DeLaunay et al. 2000; Pando et al. 2001). These rhythms peak near dawn in an LD cycle and persist in constant conditions. To date, *Per1* and *Per2* expression patterns in vivo have not been described, but they have been examined in the Z3 cell line (Pando et al. 2001). A *Per1* mRNA rhythm parallels that of *Per3*, and both of these rhythms persist for a few cycles in constant conditions (Fig. 3). In contrast, *Per2* mRNA is stimulated in these cells by light, but it is not rhythmic in constant conditions. This differs from the situation in the mammalian SCN, where all three *Per* genes are rhythmically expressed in constant conditions, and *Per1* and *Per2* are both stimulated by light (Shearman et al. 1997). It more closley resembles *Period* gene regulation in *Xenopus* retina, where *Per1* is regulated by the clock and *Per2* by light (Zhuang et al. 2000). This has led to the suggestion that *Per2* may act in entrainment pathways in both of the nonmammalian systems (Zhuang et al. 2000; Pando et al. 2001).

A striking finding is that zebrafish have six rhythmically expressed *cryptochrome* genes (Kobayashi et al. 2000). Even though zebrafish are known to have two paralogs of many mammalian single-copy genes as a result of a whole-genome duplication in the teleost lineage (Postlethwait et al. 1998), this large number of *Cry* genes was unexpected. Phylogenetic analysis has shown that four of these (*Cry1a*, *Cry1b*, *Cry2a*, and *Cry2b*) are most similar to *mCry1*. Each of these four zebrafish CRY proteins can inhibit transcriptional activation by mammalian CLOCK:BMAL1 dimers, and all are rhythmically expressed in the eye, brain, and body. The mRNA rhythms of *zCry1a* and *zCry1b* peak during the daytime, whereas rhythms of *zCry2a* and *zCry2b* peak later, in the evening (Fig. 3), suggesting that they are not entirely redundant. A fifth gene, *Cry3*, also clusters with *mCrys* in phylogenetic analyses. However, the product of this gene does not inhibit CLOCK:BMAL1-mediated transcription, and Kobayashi et al. (2000) place it in a separate class. The *Cry3* gene is expressed rhythmically, with an mRNA peak in the morning. The sequence of the sixth zebrafish cryptochrome (*Cry4*) is most divergent from all other vertebrate *Crys*, and its product also does not inhibit CLOCK:BMAL1-mediated transcription. It is however rhythmically expressed, with an mRNA peak during the day (Fig. 3). Expression patterns for the *Cry* genes in Z3 cells have not as yet been reported. It will be interesting to learn whether their distinctive in vivo patterns of rhythmicity are preserved in the cell line. The diversity of zebrafish CRYs raises the question of whether any act as photoreceptors, as do the CRYs of *Drosophila* and plants. This seems a strong possibility, given that many tissues throughout the animal are photosensitive, but no direct evidence for CRY-mediated photoreception in ze-

brafish has been published to date. It is also possible that some of the zebrafish CRYs function mainly outside the circadian system.

The available data on molecular clock mechanisms in zebrafish described above are in general consistent with the mammalian model. Most of the putative clock genes are rhythmically expressed, and the interactions of their protein products in heterologous systems are similar to those of their mammalian homologs. Two differences from the mammalian system that may be important lie in the regulation of *Per2* and in the number of *Cry* genes. To date, we have no information on clock protein expression patterns in zebrafish in vivo, or on posttranscriptional and posttranslational mechanisms that are likely to play important roles in rhythm generation. Finally, we do not yet know how disrupting the products of these genes will affect physiological and behavioral rhythmicity. This can be approached in zebrafish by the analysis of clock mutants and transgenics.

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