Circadian rhythms: **Mop up the clock!**

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All circadian clock genes discovered in *Drosophila* have mammalian counterparts with extensive sequence homology. Similarities and differences have been identified between insect and mammalian oscillators. Recent studies have shed new light on two mammalian clock components: Mop3 and Per2.

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The physiology and behavior of most metazoan organisms show daily oscillations. These cycles are not simply consequences of light perception, but rather are generated by endogenous circadian clocks that can adapt the physiology of an organism to its needs in an anticipatory manner. Under constant conditions, circadian pacemakers produce rhythms with a period length of approximately, but not exactly, 24 hours. Hence, the phase of these oscillators must be reset every day by environmental light–dark cycles — the 'photoperiod' — in order for the organisms to stay in harmony with the outside world. Initially, such circadian clocks were believed to exist only in special brain structures: the suprachiasmatic nucleus (SCN) in mammals, the pineal gland in some birds and cold-blooded vertebrates, and the lateral pacemaker neurons in *Drosophila*.

Strikingly, during the past four years molecular oscillators have also been uncovered in most peripheral cell types of mammals, fish, and *Drosophila* (for review see [1]). In lower vertebrates and other metazoans, these peripheral oscillators are even sensitive to environmental stimuli such as light. In mammals, the currently held model posits that the central SCN pacemaker, whose phase is adjusted by daily light cycles, periodically synchronizes the phases of peripheral oscillators. According to two recent reports [2,3], this may be accomplished via an indirect route: namely, by regulating the time of feeding.

In 1971 Konopka and Benzer [4] provided the first compelling evidence that circadian clocks have a genetic basis. These authors identified mutations in a single locus, *period*, which changed or abolished the rhythmic eclosion of *Drosophila* embryos from their eggs. Since then, *period* and five additional *Drosophila* clock genes have been identified and isolated by molecular cloning. These include *timeless* (*tim*), *clock* (*clk*), *cycle* (*cyc*), *doubletime* (*dbt*) and *cryptochrome* (*cry*). The products of these clock genes can be assembled into a negative feedback loop that provides a plausible molecular mechanism for rhythm generation (for review, see [5]).

In the proposed circuitry, Clk and Cyc — transcription factors with PAS-helix-loop-helix domains — activate the transcription of the *per* and *tim* genes. Per and Tim proteins then block transcription of their own genes. As a consequence, Per and Tim levels decrease, and a new 24 hour wave of *per* and *tim* transcription can initiate. The robustness of this cycling may be enhanced by another interlocked feedback loop, in which Per positively regulates *clk* transcription [6], as well as by a number of post-transcriptional and post-translational mechanisms. For example, the stability of the Per protein is negatively regulated by phosphorylation by Dbt, a protein kinase related to the mammalian casein kinase 1ε [7]. Furthermore, the stability of the Tim protein may be independently regulated by cryptochrome, a blue light photoreceptor that appears to act as the major photoreceptor of the *Drosophila* circadian system [8,9].

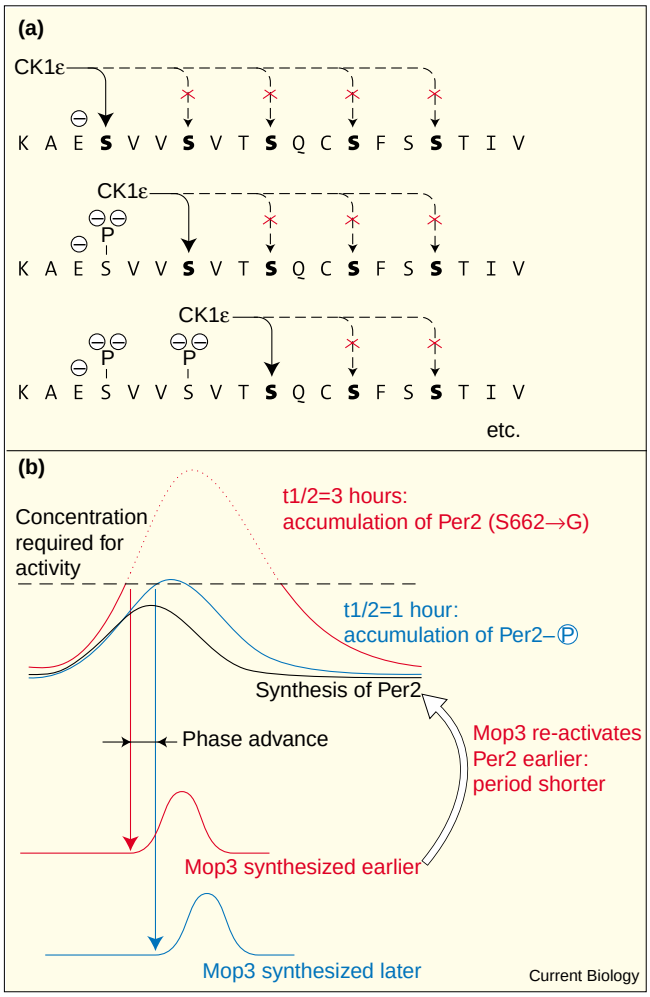
Within the past four years, mammalian counterparts to all of these *Drosophila* clock genes have been uncovered. This similarity was initially taken as evidence for the strict conservation of circadian timing mechanisms during animal evolution; however, the story has turned out to be somewhat more complicated. While the positive limb of the feedback loop does indeed involve the same effectors in insects and mammals (see below), the negative limb appears to be implemented by different players. Curiously, the two murine cryptochromes Cry1 and Cry2 are clearly essential repressors within the circadian feedback loop [10], but their role as circadian photoreceptors is still subject to debate (but see [11]). Although mPer2, one of the three murine period isoforms, is an essential clock component [12], there is as yet no genetic evidence supporting a role for this protein as a repressor. Rather, mPer2 appears to enhance the expression of the *Mop3* gene [13]. This review will focus on two recent papers that shed more light on the functions of Mop3 [14] and Per2 [15].

Mop3 and Clock

The PAS helix-loop-helix protein Mop3, also known as Bmal1, is the mammalian ortholog of *Drosophila* Cyc. It was initially identified as a dimerization partner of Clk in a yeast two-hybrid screen [16], but until recently it was not clear whether Mop3 is an essential component of the mammalian circadian pacemaker. Bunger *et al.* [14] have now provided an unambiguous answer to this question. They found that mice homozygous for a *Mop3* null allele

Figure 1

(a) A hypothetical Per2 phosphorylation cascade. Casein kinase 1ε (CK1ε) phosphorylates most readily serines in the vicinity of acidic amino acids or phosphorylated residues (which are also negatively charged). Serine 662 (S662) is an attractive substrate, and as a result of its phosphorylation S665 would become a more attractive site, and is itself phosphorylated. This phosphorylation would in turn render S668 attractive to casein kinase 1ε, and the same cascade can continue for S671 and S674. (b) A model for the effects of the Per2(S662-G) mutant – associated with the disorder FASPS (see text) – upon clock period. The lack of phosphorylation of Per2(S662-G) might render it more stable, causing it to accumulate more quickly (red curve) relative to its rate of synthesis (black curve). Because Per2(S662-G) accumulates more rapidly, Mop3 would be induced at an earlier time, and a new wave of *Per2* transcription would commence earlier. In dark–dark conditions, a shorter oscillator period would result; in dark–light conditions, this change would be manifested as a phase advance. The slower accumulation of phosphorylated Per2 (blue curve) would cause more time to pass before the threshold level of its activity is reached, and thereby delay the oscillator cycle. Although Per2(S662-G) would theoretically accumulate to a much higher level than phosphorylated Per2 in the absence of repression (dashed red line), the negative limb of the clock probably represses such higher accumulation. (Note that the half lives and accumulation profiles depicted in this figure are strictly hypothetical.)



display arrhythmic wheel-running activity in constant darkness. Moreover, their temporal recordings of m*Per1* and m*Per2* mRNA accumulation in the SCN, and of *Dbp* mRNA accumulation in the liver, indicate that circadian gene expression is abolished under these conditions.

These data clearly show that *Mop3* is a non-redundant gene required for circadian clock function. Interestingly, the *mop3*$^{-/-}$ mice under light–dark conditions are not simply active during darkness. Rather, they display variable light-anticipatory behavior that is difficult to explain by simple 'masking', the suppression of activity in night-active animals directly by light. It is possible that such anticipatory behavior might be generated by variations in clock gene transcription or protein levels. In subsequent analyses, it will be important to analyze the interplay between masking and clock function at the molecular level in these mice.

Given the findings on *Mop3*$^{-/-}$ mice, the question arises of whether Clk, the dimerization partner of Mop3, is also an essential, non-redundant component of the molecular oscillator. The mouse *Clock* gene was originally isolated by Takahashi and coworkers [17] in a heroic forward genetic screen for altered locomotor activity. While the Clk mutant protein can still bind to the regulatory elements of its target genes, it is unable to activate transcription efficiently. Homozygous *Clock* mutant mice display rhythmic locomotor activity when kept under a 12 hour light–dark regimen, but show exceedingly long periods and then become arrhythmic when kept in constant darkness. Moreover, circadian expression of many circadian genes is dramatically attenuated in *Clock* mutants [13,18,19]. Nevertheless, given the potentially dominant-negative nature of the *Clock* mutation, it cannot yet be rigorously excluded that the function of Clk is redundant. The answer to this question will come from the examination of mice homozygous for *Clock* null alleles similar to those generated for *Mop3*.

Although transcription of the clock-controlled *Dbp* gene is strictly dependent upon Clk and Mop3 [14,19], *in vitro* binding studies suggest that Clk and Mop3 account for only a very small fraction of liver nuclear factors occupying E-boxes within Dbp enhancers [19]. These observations imply that Clk–Mop3 heterodimers cannot just find E-boxes of *Dbp* by its DNA binding specificity, but rather they must be guided to their target sites by specific cooperative interactions with other transcription factors bound to different *cis*-regulatory *Dbp* elements. Hence, we postulate that the positive limb of the circadian feedback loop involves additional transcriptional regulatory proteins that remain to be discovered.

Per2 and human rhythms

m*Per2* knockout mice become arrhythmic in constant darkness, suggesting that mPer2 is an essential component

of the mouse circadian clock [12]. A recent report by Toh *et al.* [15] demonstrates that Per2 also plays an important role in the human pacemaker. These researchers began by mapping a candidate gene for the inherited disorder known as 'familial advanced sleep phase syndrome' (FASPS), which causes affected individuals to awaken consistently at four a.m. The disease segregates in a highly penetrant autosomal-dominant manner, so linkage mapping in a large family with many FASPS-affected members allowed Toh *et al.* [15] to position a candidate gene to 2qter, a segment of chromosome 2 known to carry h*Per*, the human ortholog of m*Per2*.

Sequence comparisons of h*Per2* from FASPS affected and unaffected family members revealed a missense mutation in exon 17 — h*Per2* has 23 exons — that strongly correlated with the 'morning lark' phenotype. This nucleotide change results in the substitution of a serine by a glycine at position 662 (S662G). Why might this point mutation cause such a dramatic phenotype? Biochemical studies revealed that serine 662 is a phosphoacceptor site for casein kinase 1ε, a protein kinase previously identified as a clock component in *Drosophila* and hamster [7,20]. Casein kinase 1ε also phosphorylates hPer2 at other positions, but serine 662 appears to be the initial target site. The phosphorylation of serine 662 may facilitate similar modification of carboxy-terminal serines by successively converting poor casein kinase recognition sites into good ones (Figure 1a).

As phosphorylation by casein kinase 1ε may shorten the half-life of hPer, the underphosphorylated S662G mutant protein may accumulate more efficiently than the wild-type protein and thereby reach the threshold concentration required for its activity more rapidly. In turn, this may result in a faster buildup of Mop3 levels, and thereby result in a shortening of the period length (Figure 1b). Because under normal conditions the circadian clock is maintained to a 24 hour period by light, such a short period phenotype would manifest itself as an advanced-phase phenotype under light–dark conditions. Whether the molecular oscillator runs faster in FASPS-affected individuals has not yet been determined, but such experiments should be feasible with *in vitro* cultured skin fibroblasts or keratinocytes from affected and unaffected individuals. Although the circadian oscillator is dormant in cells kept in tissue culture for extended time periods, it can be reactivated by a short treatment with serum or chemicals that induce various signaling pathways [21,22].

Over the last four years, the repertoire of putative mammalian clock genes has expanded from zero to eight, and it is likely that these will be joined by additional ones. The discovery of mutations in human clock genes and the possibility to study such genes in tissue culture cells should render even the human circadian clock amenable to genetic and biochemical experimentation.

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