



A CLOCK-less clock

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Mammalian physiology is governed by a complex circadian timing system that involves interacting positive and negative transcriptional feedback loops. A key role in this feedback loop was attributed to the PAS domain helix–loop–helix protein CLOCK, on the basis of a dominant-negative mutation in this transcription factor. However, recent experiments by Reppert and coworkers with *Clock* knockout mice suggest that CLOCK is dispensable for rhythmic gene expression and behavior, presumably because other proteins can substitute for CLOCK in these animals.

Introduction

The mammalian circadian clock is composed of a central pacemaker in the suprachiasmatic nucleus (SCN) in the brain that coordinates subsidiary oscillators in most peripheral tissues. The circadian timing system can measure time only approximately and therefore must be readjusted every day by external time cues (Zeitgebers), such as dark–light cycles for the SCN pacemaker and feeding cycles for peripheral clocks (for a review, see Ref. [1]). The rhythm-generating molecular circuitry is thought to rely on the opposing effects of transcriptional activators and repressors in generating a negative feedback loop. This feedback loop is responsible for ~24 h cycles of gene expression, physiology and behavior [2,3]. According to this model, the PAS domain helix–loop–helix proteins CLOCK and BMAL1 bind as heterodimers to E-box motifs present in the *Cry1*, *Cry2*, *Per1* and *Per2* genes (which encode proteins of the cryptochrome and period families) and thereby stimulate the transcription of these genes. Once the CRY and PER proteins reach critical concentrations, they form non-productive complexes with BMAL1–CLOCK heterodimers and thereby auto-repress their own transcription. In addition, BMAL1–CLOCK, PER and CRY proteins also regulate the expression of the orphan nuclear receptor REV-ERB α , which in turn represses transcription of *Bmal1*. This mechanism interconnects the positive and negative limbs of the circadian clockwork circuitry [4]. In turn, this molecular clockwork circuitry regulates the expression of many output genes, including metabolic enzymes, which manifests itself in overt rhythms in physiology and behavior.

The mouse *Clock* gene was originally identified and isolated by Joseph Takahashi and his coworkers through a heroic forward genetic screen for altered locomotor activity [5]. The CLOCK ^{Δ 19} mutant protein can still bind to regulatory elements on its target genes together

with BMAL1, but it fails to activate transcription [6]. Homozygous *Clock* ^{Δ 19}/*Clock* ^{Δ 19} mutant mice have exceedingly long period lengths and become arrhythmic when kept in constant darkness [5]. Moreover, circadian expression of many circadian genes is dramatically reduced in these mutant mice. Evidence from fruit fly genetics also supports a pivotal role for CLK, the *Drosophila* ortholog of CLOCK, in circadian rhythm generation: fruit flies homozygous for a strongly hypomorphic *clk* allele are behaviorally arrhythmic [7], although residual, low-amplitude rhythms in circadian gene expression can still be detected in these mutant flies.

Recently, DeBruyne *et al.* have generated and analyzed mice homozygous for a *Clock* null allele. Surprisingly, these animals still showed circadian locomotor activity rhythms in constant darkness, although the period length was somewhat shorter than that measured for wild-type mice. At the molecular level these mice continued to exhibit circadian rhythmicity in clock gene expression, albeit with somewhat reduced amplitude both in the SCN and in the liver [8].

Does this mean that CLOCK has no function in circadian rhythm generation? Clearly, genetic loss-of-function experiments do not address the question of whether a gene – or rather its product – participates in a biochemical process. They only provide an answer to the question of whether this gene product is essential for the process of interest. More often than not, mammalian genes exist as multiple isoforms with partially overlapping functions, and *Bmal1* is still the only known clock gene whose inactivation results in immediate arrhythmicity in the absence of Zeitgeber cues. Therefore, the most straightforward way to explain the results obtained by DeBruyne *et al.* [8] for *Clock* null mice is that *Clock* has one or more paralogs, as is the case for other clock components, such as period genes (*Per1*, *Per2* and *Per3*), cryptochrome genes (*Cry1* and *Cry2*), genes encoding nuclear receptors with circadian functions (*Rev-erba*, *Rev-erbb*, *Rora*, *Rorb* and *Rory*) and casein kinase genes (*CK1 δ* and *CK1 ϵ*) [3,9]. The possibility that the function of CLOCK is redundant would also be in line with the observation that the CLOCK ^{Δ 19} mutant protein functions in a dominant-negative fashion, possibly interfering with the binding of other CLOCK-related proteins.

So what could the functional paralogs of CLOCK be? Based on amino acid sequence similarity, Neuronal PAS Domain Protein 2 (NPAS2) is by far the closest cousin of CLOCK [10]. NPAS2 is a transcription factor expressed primarily in the mammalian forebrain that can bind E-box motifs as heterodimers with BMAL1 and transactivate components of the circadian regulatory apparatus [11,12].

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Moreover, *Npas2* knockout mice have mild phenotypes in circadian gene expression and behavior [13–15]. For example, *mPer2* expression in the forebrain is attenuated in these mice, and these animals also show sleep disturbances and a slightly shorter period length. However, there is one argument against NPAS2 – albeit not a decisive one. *In situ* hybridization experiments were unable to detect *Npas2* mRNA in the SCN of either wild-type or *Clock* knockout mice [8,16], and a functional SCN clock is required for circadian behavior and coordinated rhythmic gene expression in peripheral organs. Nevertheless, absence of evidence is not evidence for absence, and the final answer of whether CLOCK and NPAS2 are functionally redundant will have to come from the analysis of mice with loss-of-function mutations in both genes. The laboratories of Steven Reppert and Steven McKnight will probably soon provide definitive evidence for or against such a functional redundancy.

In the mean time, we would like to speculate on an alternative possibility. Recently, Doi *et al.* [17] have shown that CLOCK has an intrinsic histone acetyltransferase (HAT) activity. CLOCK specifically acetylates histones H3 and H4, and its HAT activity is enhanced in the presence of BMAL1 [17]. CLOCK versions carrying mutations within the acetyl coenzyme A binding motif are impaired in their HAT activity and appear to be defective in their ability to activate CLOCK–BMAL1-dependent expression of *mPer1* and the albumin D-element binding protein gene (*Dbp*) in a cell-based assay. Furthermore, the

work by Doi *et al.* suggests that the HAT activity of CLOCK is required to re-establish circadian gene expression in *Clock*^{Δ19} mutant cells [17]. These cells express a HAT-domain containing CLOCK version that, because of another peptide deletion, cannot activate target gene transcription. Interestingly, CLOCK has significant sequence homology with ACTR, a member of the steroid receptor coactivator (SRC) family of transcriptional coactivators, not only in its HAT domain but also within other domains (Figure 1). Thus, CLOCK and ACTR share the PAS domain – the basic helix–loop–helix region, the nuclear receptor interaction domain and the serine rich regions. This raises the possibility that ACTR (or another SRC family member) might have a role in the circadian clock feedback loop and substitute for CLOCK function in *Clock* null mice. The two alternative (but not mutually exclusive) scenarios for the functional substitution of CLOCK by NPAS2 or ACTR are schematically outlined in Figure 1a and 1b, respectively.

The model presented in Figure 1b posits that CLOCK acts as a coactivator rather than a DNA-binding partner of BMAL1. This obviously begs the question of how BMAL1 can recognize E-box motifs in the absence of CLOCK. In contrast to CLOCK, BMAL1 can bind E-box motifs as homodimers, although it has been speculated that these homodimers cannot activate the transcription of CLOCK–BMAL1 target genes [6]. One should bear in mind, however, that these conclusions are based on co-transfection experiments in which the massive overexpression of one protein (say BMAL1) results in efficient transcription

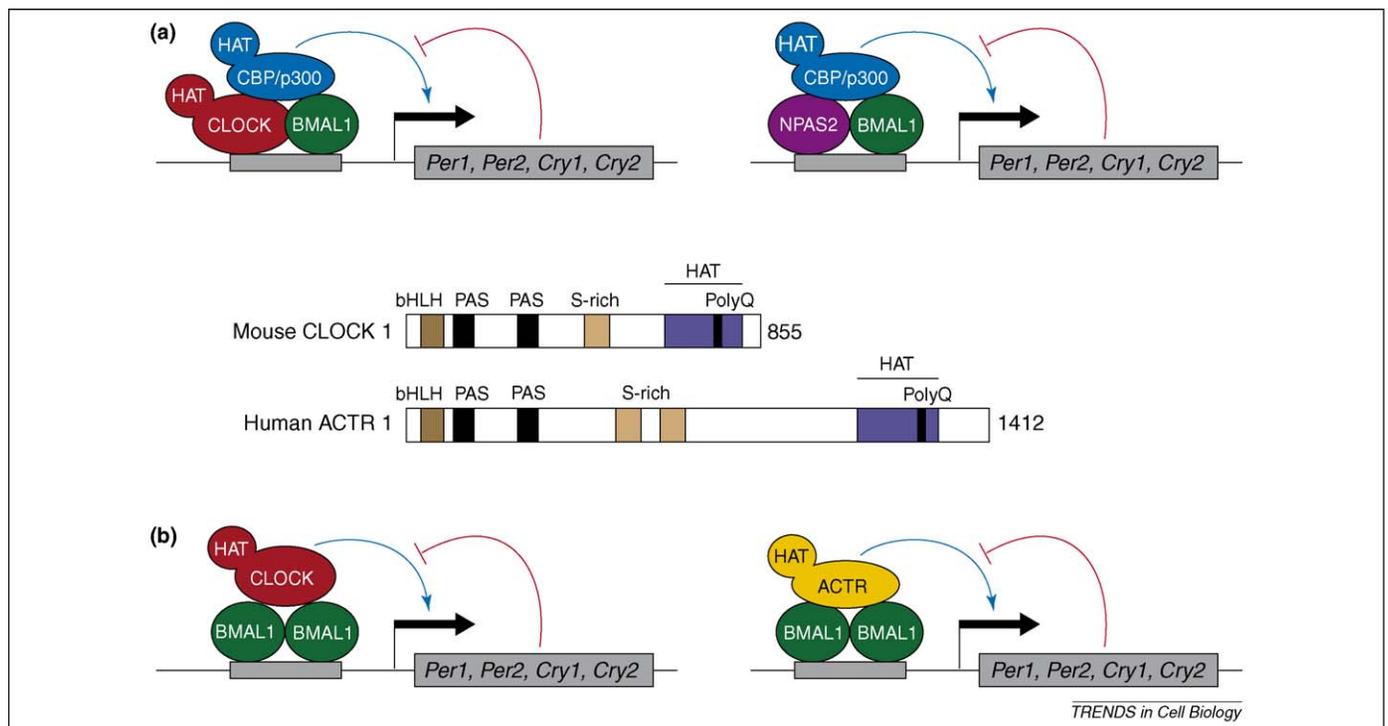


Figure 1. What are the functional paralogues of CLOCK? Simplified hypothetical models for negative feedback loops driven by the PAS helix–loop–helix transcriptional regulatory proteins BMAL1, CLOCK, NPAS2 and ACTR (see text). These proteins activate the expression of the genes encoding the repressors PER1, PER2, CRY1 and CRY2 (blue arrows), and multi-subunit protein complexes containing these repressors attenuate the transactivation potential of PAS HLH transcription (co)factors through unknown mechanisms. (a) Based on sequence similarity, NPAS2 (also called MOP4) is the most likely transcription factor substituting for CLOCK in *Clock* null mice. In this scenario, CLOCK–BMAL1 or NPAS2–BMAL1 heterodimers bind to E-box sequences of target genes (e.g. those encoding cryptochrome and period isoforms), recruit a coactivator complex (e.g. containing the CBP/p300 protein) and thereby stimulate transcription. Note that CLOCK contains a histone acetyl transferase (HAT) domain that is absent in NPAS [15]. (b) BMAL1 binds E-box motifs as homodimers (or heterodimers with unknown partners) and recruits CLOCK or ACTR as coactivators to the promoters of target genes. The structural homology of the histone acetyl transferases CLOCK and ACTR is depicted at the top. Adapted from Ref. [15]. Note that NPAS2 probably does not contain a histone acetyl transferase (HAT) domain and is thus unlikely to fulfill the function of a coactivator.

activation only if accompanied by the simultaneous overexpression of a partner that becomes rate-limiting under these conditions. In our opinion, such experiments cannot discriminate between the putative role of CLOCK as a DNA-binding partner or transcriptional coactivator. Although *in vitro* DNA binding assays with recombinant peptides encompassing the basic helix–loop–helix domains suggest that BMAL1 and CLOCK can bind DNA as heterodimers [11,12], they do not rule out the possibility that these two proteins also function as activator–coactivator pairs *in vivo*. Hence, if future experiments reveal that circadian rhythmicity is not abolished in mice deficient for both CLOCK and NPAS2 function, it will be important to examine the coactivator hypothesis in cells or mice deficient in the expression of various SRC coactivators.

Although the precise biochemical role of CLOCK in the core clockwork circuitry remains to be identified, the findings of DeBruyne *et al.* clearly suggest a function of this protein in the response of the circadian timing system to light [8]. In contrast to wild-type mice, *Clock*-deficient mice become active 2 h before turning the light off. Reppert and his coworkers also observed that *Clock* deficient mice had defects in resetting their clocks in response to light pulses. Specifically, these animals failed to set their clocks back after being exposed to light during the early night (dark phase), but advanced their clock much more dramatically than wild-type mice after an exposure to light during the late night. These observations with *Clock* knockout mice identified CLOCK as an important modulator of the synchronization pathway of the mammalian circadian timing system.

All in all, the findings by DeBruyne *et al.* [8] further illustrate how incomplete our understanding of the mammalian circadian clockwork still is. Indeed, there is little risk that chronobiologists will get bored within the next few years.

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