A Model of Molecular Circadian Clocks: Multiple Mechanisms for Phase Shifting and a Requirement for Strong Nonlinear Interactions

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A Model of Molecular Circadian Clocks: Multiple Mechanisms for Phase Shifting and a Requirement for Strong Nonlinear Interactions

Tjeerd Olde Scheper, Don Klinkenberg, Jaap van Pelt, and Cyriel Pennartz[1]
Graduate School Neurosciences Amsterdam, Netherlands Institute for Brain Research, Meibergdreef 33, 1105 AZ, Amsterdam, the Netherlands

Abstract A fundamental question in the field of circadian rhythms concerns the biochemical and molecular nature of the oscillator. There is strong evidence that circadian oscillators are cell autonomous and rely on periodic gene expression. In *Drosophila*, *Neurospora*, *Aplysia*, and vertebrates, circadian oscillators are thought to be based on molecular autoregulatory loops composed of transcription, translation, and negative feedback by proteins on nuclear transcription. By studying a mathematical model of molecular clocks based on this general concept, the authors sought to determine which features such clocks must have to generate robust and stable oscillations and to allow entrainment by external stimuli such as light. The model produced circadian oscillations as an emergent property even though a time delay in protein synthesis and rate constants of the feedback loop were much shorter than 24 h. Along with the delay in protein production, strong nonlinear interactions in macromolecular synthesis and nuclear feedback appeared to be required for the model to show well-behaved oscillatory behavior. Realistic phase-shifting patterns induced by external stimuli could be achieved by multiple mechanisms—namely, up- and downward perturbations of protein or mRNA synthesis or degradation rates. The model makes testable predictions about interactions between clock elements and mechanisms of entrainment and may help to understand the functions of the intricate molecular interactions governing circadian rhythmogenesis.

Key words circadian rhythm, *Drosophila*, entrainment, *Neurospora*, nonlinearity, phase shift, suprachiasmatic nucleus, transcriptional regulation

Circadian rhythms in behavioral and physiological functions are driven by an internal clock that can be entrained by environmental stimuli such as light or temperature. In the mammalian suprachiasmatic nucleus (SCN) and in mollusc basal retinal neurons, individual cells have been shown to act as autonomous circadian pacemakers (Michel et al., 1993; Welsh et al., 1995). Molecular mechanisms underlying circadian pacemakers are being elucidated at a rapid pace, both in some invertebrate species and in mice. In *Neurospora*, strong evidence implicates the products of the *frq* and *wc-2* genes in transcription- and translation-based autoregulatory feedback loops (Aronson et al., 1994; Dunlap et al., 1996; Crosthwaite et al., 1997). Specifically, FRQ protein appears to provide a negative feedback signal regulating the amount

1. To whom all correspondence should be addressed.

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of *frq* transcript, whereas WC-2 would act as a positive regulatory element by promoting expression of *frq*. In *Drosophila melanogaster*, the *per* and *tim* genes are thought to constitute essential components of the circadian clock. Synthesis of PER and TIM proteins follows an indistinguishable circadian rhythm that depends on a physical interaction between these proteins (Hardin et al., 1990; Sehgal et al., 1994, 1995; Vosshall et al., 1994; Gekakis et al., 1995; Myers et al., 1995; Young, 1996). PER-TIM association is believed to be required for cytoplasmic accumulation and nuclear translocation of PER, which in turn would act as a negative feedback signal on *per* and *tim* transcription. As concerns vertebrate biological clocks, the recent cloning of the *clock* gene in mice suggests that the CLOCK product may dimerize, bind to DNA, and act as a transcriptional activator (King et al., 1997). Three mammalian homologues of the *per* gene have thus far been identified: *mPer1*, *mPer2*, and *mPer3* (Albrecht et al., 1997; Tei et al., 1997; Shigeyoshi et al., 1997; Zylka et al., 1998b). Throughout these phylogenetically remote species, the PAS domain, which is important for protein dimerization, is emerging as a motif common to PER, WC-2, and CLOCK (Huang et al., 1993; Crosthwaite et al., 1997; King et al., 1997).

Recently, CLOCK was shown to dimerize with a protein named BMAL1 (MOP3), and the dimer is able to activate transcription by binding to an E-box element (Gekakis et al., 1998; Hogenesch et al., 1998). This dimer drives transcription of at least one mammalian homologue of *per*—namely, *mPer1*. In *Drosophila*, a similar mechanism for driving the transcription of *per* and *tim* appears to be at work, relying on the *Drosophila* clock gene *dClock* and *cyc*, a homologue of BMAL1 (Hao et al., 1997; Allada et al., 1998; Rutila et al., 1998; Darlington et al., 1998; Lee et al., 1998). Here, strong evidence for a negative feedback role of PER and TIM was obtained by showing that PER and TIM reduced dCLOCK-mediated induction of a reporter gene (Darlington et al., 1998).

When reconsidering the temporally overlapping expression patterns of PER and TIM in *Drosophila*, one may ask why two distinct proteins should be needed where, on intuitive grounds, only one would suffice to provide negative feedback to the nucleus. Is PER-TIM mediated nuclear feedback a specific and perhaps even accidental feature of the *Drosophila* clock (cf. Sauman and Reppert, 1996), or should similar interactions also be expected in other species? The recent discovery of a mammalian TIM ortholog is beginning to shed light on this matter, although the results of two studies (Zylka et al., 1998a; Sangoram et al., 1998) disagree on the essential point whether physical PER-TIM interactions occur in mammals.

Phase shifting by photic inputs in *Drosophila* may be mediated by rapid degradation of TIM protein (Lee et al., 1996; Myers et al., 1996; Zeng et al., 1996). Should enhanced degradation of clock protein be expected to constitute a universal mechanism, or can phase response curves of a wide range of species be explained by light-induced modulation of different clock parameters?

MATERIAL, METHODS, AND RESULTS

These and related questions were addressed in a general mathematical model that is structured as follows:

$$\frac{dM}{dt} = \frac{r_M}{k + P^n} - q_M M \tag{1}$$

$$\frac{dP}{dt} = r_P M(t-\delta)^m - q_P P \tag{2}$$

with M the relative mRNA concentration; P the relative concentration of protein that effectively provides negative feedback on transcription; r_M the rate of transcriptional activation; r_P the protein synthesis rate constant; q_M and q_P the mRNA and protein breakdown rate constants, respectively; n the Hill coefficient specifying the cooperativity of protein in nuclear feedback; δ the delay between translation onset and completed delivery of the effective protein product; m an exponent imposing nonlinearity on the production of effective protein; and k a scaling constant (Fig. 1). Due to the delay term δ, protein production is computed from the mRNA concentration M at time $(t - \delta)$. The conclusions drawn from this model remain the same when the negative feedback, specified by P^n, acts at the posttranscriptional instead of the transcriptional level (as in equations (1) and (2)) or when the delay δ includes not only translation (as in equation (2)) but also transcription (cf. Gumowski, 1981). Since P represents the protein that effectuates nuclear feedback, the delay may also include multiple phosphorylation steps (Edery et al., 1994; Garceau et al., 1997; Kloss et al., 1998; Price et al., 1998), nuclear translocation of protein as in the case of PER (Curtin et al., 1995), or processes mediating very slow derepression (Merrow

et al., 1997). The equations are essentially similar to the seminal model of Goodwin (1965) but differ on the important points that a delay (δ) and a nonlinearity (*m*) in protein production are taken into account. In consequence, the functional implications derived from the present simulations could not have been inferred from the Goodwin model. A related paper (Olde Scheper et al., 1999) focuses in more detail on the mathematical aspects of the model.

The current model reflects a generalized approach to molecular clocks since the equations must be understood as lumping together several reaction steps such as protein synthesis, phosphorylation, di- or multimerization, and nuclear translocation. This approach was chosen because it may help to define which minimal requirements should be fulfilled by biochemical feedback systems to display circadian oscillations. We preferred this approach over detailed simulations of reaction cascades (cf. Goldbeter, 1996; Leloup and Goldbeter, 1998) because it may facilitate definition of a general framework of constraints under which molecular clocks operate rather than illuminate a single case. In addition, the precise reaction steps and their kinetics are still unknown in species considered thus far.

Circadian periodicity in mRNA and protein concentrations was observed under a broad range of parameter values. Figure 2 shows an example of a stable free-running rhythm with a period of 24.6 h. The time lag between peak concentrations of mRNA and protein was generally about 6 h, which is in the same range as found for PER in *Drosophila* (Zeng et al., 1994; Sauman and Reppert, 1996; Young, 1996) and *Neurospora* (Garceau et al., 1997). This time lag emerges from nonlinear interactions between clock components and cannot be directly derived from the value of δ, which was generally 4 h. Likewise, the overall period of the oscillation appeared to be an emergent property of the system. The ranges of *m* and *n* allowing for well-behaved circadian oscillations deserve special attention because they reflect strong nonlinear interactions in the production of effective protein and in negative feedback to the genome, respectively. With the parameter settings of Figure 2, we found that *m* was required to be larger than 1.81 and *n* larger than 1.27 to prevent a transition from oscillatory behavior (i.e., a limit cycle attractor) to steady-state concentrations (point attractor). Although the exact boundaries varied somewhat depending on the parameter settings, an extensive search through parameter space indicated that *m* or *n* was always required to be > 1. When

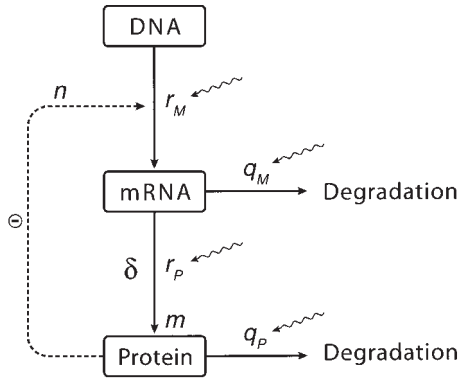

Figure 1. Schematic representation of the modeled molecular autoregulatory feedback loop generating a circadian rhythm in mRNA and protein abundance. mRNA is synthesized according to rate constant r_M and degraded at rate q_M. The rate constants for protein synthesis and degradation are represented by r_P and q_P, respectively. Production of the final protein product is subjected to a delay δ and dependent on mRNA abundance in a nonlinear fashion according to constant *m* (equation (2)). The protein exerts inhibitory control on mRNA synthesis, and the nonlinearity of this repressive action is represented by Hill coefficient *n* (equation (1)). The scaling constant *k*, which was kept at 1.0 throughout all simulations, has not been included in the scheme. The delay term δ can also be made to apply to both transcription and translation while preserving the functional architecture and oscillatory behavior of the model. Waving arrows indicate biochemical rate constants examined as targets of photic input.

m or *n* was set at 1, the other factor imposing nonlinearity (*n* or *m*, respectively) needed to be strongly enhanced (i.e., to about 4) to maintain stable oscillations. With "strong nonlinear interactions," we thus mean that at least one of the power constants operating in protein assembly (*m*) or transcriptional feedback (*n*) was considerably larger than unity. Even though no analytical proof is available at this point due to the presence of a delay term, these results suggest that in this model, strong nonlinearities, most likely implemented by macromolecular interactions, are required for stable oscillatory behavior at a biochemical level. In the absence of such interactions, mRNA and protein production accelerate too slowly to generate self-sustaining oscillations. Adding further posttranslational reactions to the feedback cycle would most probably not remove this need for strong nonlinearity because the net effect of such a reaction on the feedback cycle can be captured by enhancing δ and *m* or *n*. While equations (1) and (2) simulate mRNA-protein cycling with homomeric protein interactions, it can be inferred that robust oscillatory behavior also emerges when duplicating these equations for a second protein and implementing a hetero-

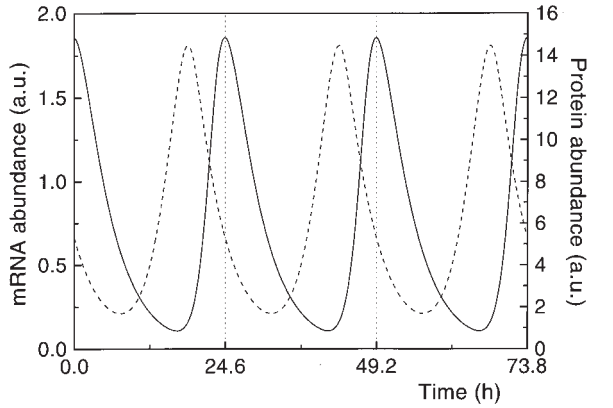

Figure 2. Example of circadian oscillations in mRNA (left-hand ordinate, dotted line) and protein abundance (right-hand ordinate, solid line; a.u. = arbitrary units). The oscillation period amounted to 24.6 h, and the phase lag between the peaks in mRNA and protein levels was 6 h. The initial concentrations were 0.67 and 14.8 a.u. for mRNA and protein, respectively. Parameter settings: $r_M = 1.0\ h^{-1}$; $q_M = 0.21\ h^{-1}$; $r_P = 1.0\ h^{-1}$; $q_P = 0.21\ h^{-1}$; $\delta = 4.0$ h; $m = 3.0$; $n = 2.0$; $k = 1.0$. Note that time marks relate to the peaks in protein concentration.

meric interaction, as in the case of PER and TIM in the *Drosophila* clock (given the PER-TIM dimer, n can be taken equal to 1). This was confirmed by simulation.

Entrainment was studied by determining phase shifts induced by single-pulse perturbations of the synthesis or degradation rates of mRNA or protein (r_M, r_P, q_M, or q_P; Fig. 1). This approach leads to acute changes in state variables, in line with Pittendrigh's (1974) concept. Figures 3A and 3B show phase response curves (PRCs) obtained by a 1-h enhancement of the protein degradation rate q_P and an inhibition of the protein synthesis rate r_P, respectively. Qualitatively similar results were obtained when mRNA degradation and synthesis rates were manipulated. PRCs based on enhanced or inhibited protein or mRNA degradation were usually characterized by two discrete zero crossings (Fig. 3A), similar to PRCs predominantly found in some invertebrate species, including *D. melanogaster* and *Aplysia* (Takahashi et al., 1993; Levine et al., 1994; Myers et al., 1996). Indeed, the curve of Figure 3A is similar to a type of PRC commonly found in *Drosophila*, in which phase shifting probably depends on light-induced degradation of TIM protein (Lee et al., 1996; Myers et al., 1996; Zeng et al., 1996). In contrast, PRCs obtained by enhanced or inhibited protein or mRNA synthesis generally displayed a "dead zone" (i.e., a time zone yielding zero or very small phase shifts), much like PRCs observed in many vertebrates (Pittendrigh, 1974; Takahashi et al., 1993; Zhang et al., 1996; Gillette, 1996). In this context,

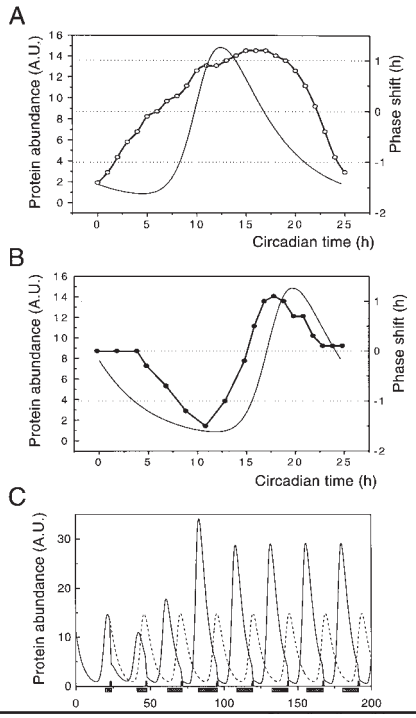

Figure 3. Phase shifting induced by single-pulse or repeated perturbations of protein synthesis and degradation. (A) Single-pulse (1-h) enhancement of protein degradation rate q_P from 0.21 to 0.42 h^{-1}. The phase response curve (PRC; thick line) is shown in relation to the cycle in protein abundance (thin line). Qualitatively similar to a type of PRC commonly found in *Drosophila melanogaster*, the simulated curve exhibited two zero crossings and no "dead zone." The temporal relationship between protein peak and PRC resembles that found for PER and TIM and the behaviorally assayed PRC in *Drosophila* (Myers et al., 1996; Zeng et al., 1996). A single-pulse inhibition of protein degradation resulted in a similar PRC, albeit that the protein peak coincided with maximal phase delays. (B) Single-pulse (1-h) inhibition of protein synthesis rate r_P from 1.0 to 0.0 h^{-1}. The peak in protein abundance (thin line) overlapped the time zone of phase advance (thick line). Note the clear presence of a dead zone at the beginning and end of the circadian cycle. Single-pulse enhancement of protein synthesis produced a similar curve, although the protein peak now overlapped the time zone of phase delay. (C) Stable periodic entrainment by enhancement of protein degradation at regular intervals. The free-running oscillation in protein abundance (shown by the dotted line for comparison) had a period of 24.6 h, while external stimuli (black vertical bars on abscissa) were applied at 24-h intervals. Each external stimulus produced a 1-h enhancement of q_P from 0.21 to 0.42 h^{-1}. The solid line represents the entrained protein oscillation. The gray horizontal bars below the abscissa denote time lags between protein peaks and external stimuli. These time lags are seen to vary in length for a few cycles after the onset of entraining stimuli (transient cycling), before settling into a pattern of constant periodic behavior. Parameter settings were as in Figure 2. Note that circadian times in (A) and (B) are arbitrary and cannot be aligned with zeitgebers or overt behavioral measures as used to define circadian times in experimental studies. Plots (A) and (B) were designed to facilitate comparison with experimental PRCs of *Drosophila* and vertebrate species.

it is interesting to note that *mPer1* RNA is rapidly induced by light exposure during the subjective night but not during the day (Albrecht et al., 1997; Shigeyoshi et al., 1997; Zylka et al., 1998b). Some parameter settings produced a PRC without a clear dead zone when protein synthesis was inhibited. Thus, qualitatively similar PRCs can be produced by dissimilar biochemical mechanisms, implying that the shape of a particular PRC does not allow firm conclusions about the underlying biochemical process targeted by photic input. However, these findings do warrant the conclusion that to explain the occurrence of a "dead zone," it is not necessary to invoke an additional clock-controlled gating mechanism determining the sensitivity to phase-shifting stimuli (cf. Gillette, 1996).

When applying perturbations of r_M, r_P, q_M, or q_P at regular intervals, stable periodic entrainment was achieved after a few transient cycles (Fig. 3C). Interestingly, the occurrence of these cycles shows that transient behavior observed in behavioral output should not be necessarily ascribed to the gradual motion of a clock-controlled output component toward a steady-state phase relation to the pacemaker (cf. Pittendrigh, 1974) but could as well reside within the pacemaker itself, even though resetting of the state variable is instantaneous.

DISCUSSION

The main conclusion to be drawn from our modeling work is that in a clock architecture with a time delay as outlined in Figure 1, strong nonlinear interactions in protein production or in negative transcriptional feedback appear to be essential properties for well-behaved oscillatory behavior. Including a delay and the nonlinear interactions in the negative feedback loop was not only necessary but also sufficient to reproduce the characteristic dynamic features of circadian clocks, indicating two crucial components of a basic, "minimal" model. The assumption of a delay can be biologically justified by strong evidence for biochemical processes underlying long time intervals between the transcription of clock genes and the final production of a clock product mediating negative feedback on transcription—especially slow phosphorylation of PER in *Drosophila* and other clock proteins (Edery et al., 1994; Curtin et al., 1995; Merrow et al., 1997; Garceau et al., 1997; Kloss et al., 1998; Price et al., 1998). It should be emphasized that the actual delay between mRNA and protein peak (about 6 h) was clearly different from the value of delay parameter δ (4 h). Thus, this simulated mRNA-protein peak delay was not "plugged into" the equations and is comparable to biologically measured delays (Zeng et al., 1994; Sauman and Reppert, 1996; Young, 1996; Garceau et al., 1997). The nonlinear interactions in protein production or negative feedback would be implemented biochemically by homo- or heteromeric protein-protein interactions and/or phosphorylation reactions. Protein-protein interactions may occur in the assembly of a protein product that eventually mediates transcriptional feedback ($m > 1$; e.g., PER-TIM or PER-PER dimerization) but may also be found at the level of transcriptional inhibition itself ($n > 1$; e.g., nonlinear repression of CLOCK-mediated gene induction by the PER-TIM complex). Unless the cooperativity in transcriptional feedback would be extremely high, the model predicts that protein di- or multimerization in the autoregulatory feedback loop will not be restricted to PER and TIM in *Drosophila* and will also be found in other species. Two studies on mTIM confirm this prediction in a preliminary sense, albeit that they disagree on several essential points (Sangoram et al., 1998; Zylka et al., 1998a). Whereas Sangoram et al. (1998) found human TIM (hTIM) to interact with *Drosophila* and mouse PER proteins and hTIM-mPER1 to inhibit CLOCK-BMAL1-induced transactivation of the *mPer1* promoter, Zylka et al. (1998a) failed to obtain evidence for mPER-mTIM interactions. However, Zylka et al. (1998a) did identify various interactions between mPER homologues. Thus, both studies argue in favor of physical association between clock proteins, but whether mPER-mTIM or mPER-mPER interactions are most vital to clock functioning remains to be determined.

Our model assumes a single rate constant r_M for mRNA synthesis. Apparently, one factor mediating constant transcriptional activation is sufficient to generate well-behaved oscillatory behavior and phase shifting in the model. Experimentally, however, it recently has become clear that a dimerization of CLOCK and BMAL1, occurring both in *Drosophila* and mice, is important for driving transcription of *per* (Gekakis et al., 1998; Hogenesch et al., 1998; Darlington et al., 1998), pointing to an additional nonlinearity in the positive regulation of clock gene transcription. Furthermore, dCLOCK cycles in phase with PER and TIM (Lee et al., 1998). In an extension of the current model, it would be interesting to examine the consequences of this additional nonlinear process for oscil-

latory behavior and to test whether the model can reproduce the near-synchronous phasing of positive and negative regulatory elements as observed in *Drosophila*. Another possible extension relates to Cheng and Hardin's (1998) finding that circadian oscillations in PER can continue in *Drosophila* photoreceptors that constitutively express high levels of *per* mRNA. While a model with only homomeric protein interactions (equations (1) and (2)) would not sustain protein oscillations when negative feedback is abolished in this manner, it would be interesting to test whether a model with two interacting clock proteins (P1 and P2) would show oscillations in P1 in the absence of p1 mRNA cycling but in the presence of p2 mRNA cycling (cf. Cheng and Hardin, 1998). This is also relevant for evaluating a possible causal role of TIM in clock functioning, given the absence of mTim mRNA cycling in mice (Zylka et al., 1998a; Sangoram et al., 1998). On account of the current simulations, we predict that P1 levels would oscillate as long as the nonlinearities in P2 production and nuclear feedback would be sufficiently strong to sustain P2 and p2 mRNA cycling and P1-P2 dimerization would not be rate limiting.

With respect to entrainment, we showed that insensitivity of the clock to phase-shifting stimuli within circumscribed circadian time zones does not necessarily rely on gating mechanisms external to the biochemical oscillator (cf. Gillette, 1996) since this phenomenon can also result directly from time-varying patterns of mRNA and protein concentration. Furthermore, PRCs that are similar in shape can arise from light-induced modulation of different clock parameters. Even though PRCs based on accelerated protein breakdown turned out to be devoid of dead zones, analogous to TIM degredation in *Drosophila*, similar curves can be found on the inhibition of protein synthesis. Interestingly, perturbations of protein or mRNA synthesis generally gave rise to PRCs with dead zones, similar to PRCs in many vertebrates such as mice, in which light indeed enhanced *mPer1* mRNA synthesis during the subjective night (Albrecht et al., 1997; Shigeyoshi et al., 1997; Zylka et al., 1998b).

It should be borne in mind that these conclusions are based on the basic reaction kinetics of equations (1) and (2) and may not be generalized when specific biochemical reaction steps are added or other assumptions are made that complicate or expand this core model (cf. Griffith, 1968; Pavlides, 1973; Edmunds, 1988; Lewis et al., 1997; Roenneberg and Merrow, 1998). However, the kinetics and architecture of the model were designed on the basis of recently acquired biological data and can be considered biologically plausible. By avoiding highly specific assumptions about the structure of biochemical cascades, our conclusions on requirements for a delay and strong nonlinear interactions may well prove to be generalizable to more specific clock models. With the molecular tools that have become recently available in hand, testing the general validity of the model ought to be feasible in the near future.

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