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Modeling circadian oscillations of the PER and TIM proteins in *Drosophila*

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The mechanism of circadian oscillations in the period (PER) and the timeless (TIM) proteins in *Drosophila* is investigated by means of a theoretical model. A first version of this model takes into account only the PER protein, while the second, extended version incorporates the formation of a PER-TIM complex. The model for the *Drosophila* circadian clock is based on multiple phosphorylation of PER (and TIM) and on the negative feedback exerted by the nuclear form of PER (or PER-TIM complex) on the transcription of the *per* (and *tim*) genes. Periodic behavior in the model occurs in the form of limit cycle oscillations. The size of the oscillatory domain in parameter space becomes larger as the degree of cooperativity of the negative feedback process increases, although sustained oscillations can occur in the absence of such cooperativity. The extended model accounts for the phase-shifts induced by light pulses, when incorporating the observation that light triggers TIM degradation.

1. INTRODUCTION

The molecular mechanism of circadian rhythmicity is being investigated in a variety of unicellular and multicellular organisms. Among these, *Drosophila* has yielded some of the most significant insights on how oscillations of 24 h period develop as a result of genetic regulatory mechanisms [1-4]. The initial experimental studies focused on the role of the *per* (*period*) gene in the generation of circadian rhythmic behavior in *Drosophila*. It was found that the *per* mRNA and the PER protein both display cyclical variations with a periodicity close to 24 h. The fact that the peak in protein follows the peak in mRNA suggested that the mechanism of oscillations rests on the negative feedback exerted by the PER protein on the expression of the *per* gene [5]. A similar mechanism involving a negative autoregulatory loop also underlies the generation of the circadian rhythm of the *frq* (*frequency*) gene product in *Neurospora* [6].

Recent experiments have pinpointed the role played in circadian rhythm generation in *Drosophila* by a second protein, TIM, encoded by the *tim* (*timeless*) gene [7], which forms a complex with PER [8-11]. The nuclear form of this complex appears to exert a negative feedback on the expression of the *per* and *tim* genes.

Based on these negative feedback processes, a theoretical model has been proposed for the generation of circadian rhythms in *Drosophila*. The model represents an extension of the original model proposed by Goodwin [12] who early on envisaged how the negative regulation

of a gene by its protein product can give rise to sustained oscillations. The first version of the model for circadian oscillations in *Drosophila* rests on the negative regulation exerted by PER alone [13,14]. This model has recently been extended to incorporate the formation of a complex between PER and TIM [15]. The two versions of the model as well as their main predictions are presented below.

2. MODELS FOR CIRCADIAN OSCILLATIONS OF PER AND TIM IN DROSOPHILA

2.1. Model based on PER (or TIM) alone

To generate circadian oscillations, a single, negative autoregulatory loop for the control of gene expression in principle suffices (as seems to be the case in *Neurospora* [6]), even if in *Drosophila* the formation of a complex between two proteins appears to be required [7-11]. This is confirmed by the analysis of a theoretical model based on the regulation exerted by PER alone (Figure 1). The model incorporates the multiple phosphorylation of PER which could play a role in gating the entry of the protein into the nucleus and/or protein degradation. Only two successive phosphorylations are considered; adding more phosphorylation steps would not alter significantly the dynamics of the system. The role of PER in the model schematized in Figure 1 could also be played by the TIM protein. In this model, what is required is that a single protein be capable of entering the nucleus and repressing directly or indirectly the expression of its gene.

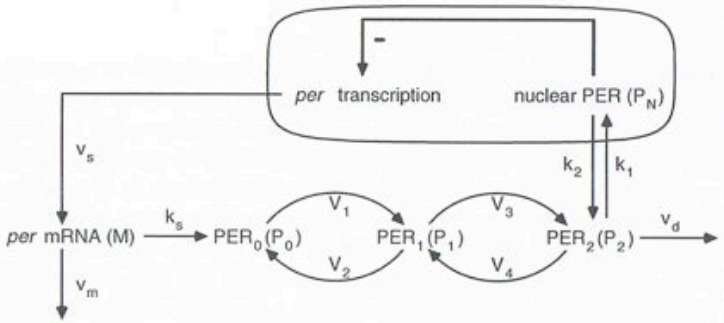

Figure 1. Scheme of the model for circadian oscillations in *Drosophila* based on the negative regulation exerted on gene expression by PER [13,14].

The model of Figure 1 is described by a set of five kinetic equations describing the time evolution of the *per* mRNA (M) and of the unphosphorylated (P_0), mono- (P_1) and bisphosphorylated (P_2) forms of PER in the cytosol, as well as the nuclear form (P_N) of the protein (for further details, see refs. 13 and 14):

$$\frac{dM}{dt} = v_s \frac{K_1^n}{K_1^n + P_N^n} - v_m \frac{M}{K_m + M} - k_d M \tag{1a}$$

$$\frac{dP_0}{dt} = k_s M - V_1 \frac{P_0}{K_1 + P_0} + V_2 \frac{P_1}{K_2 + P_1} - k_d P_0 \tag{1b}$$

$$\frac{dP_1}{dt} = V_1 \frac{P_0}{K_1 + P_0} - V_2 \frac{P_1}{K_2 + P_1} - V_3 \frac{P_1}{K_3 + P_1} + V_4 \frac{P_2}{K_4 + P_2} - k_d P_1 \tag{1c}$$

$$\frac{dP_2}{dt} = V_3 \frac{P_1}{K_3 + P_1} - V_4 \frac{P_2}{K_4 + P_2} - k_1 P_2 + k_2 P_N - v_d \frac{P_2}{K_d + P_2} - k_d P_2 \tag{1d}$$

$$\frac{dP_N}{dt} = k_1 P_2 - k_2 P_N - k_d P_N \tag{1e}$$

The total (nonconserved) quantity of PER protein, P_t, is given by:

$$P_t = P_0 + P_1 + P_2 + P_N \tag{2}$$

Also incorporated in the above equations is a nonspecific degradation for each of the variables, at a low rate characterized by the apparent first-order rate constant k_d. The first term in the kinetic equation for the mRNA concentration (M) represents the nonlinear repression exerted by the nuclear form of PER on *per* transcription. This term is given by a function of the Hill type, characterized by the cooperativity degree, n. Phosphorylation-dephosphorylation kinetics is of the Michaelian type.

The numerical integration of Equations 1 shows that the PER control system can produce sustained oscillatory behavior. Many of the kinetic constants in these equations remain undetermined. When taking values in a plausible physiological range for the parameters, a period of the order of 24 h can be obtained. The peak in total PER protein then follows the peak in *per* mRNA by several hours (Figure 2), as observed in the experiments.

Sustained oscillations only occur in precise conditions, often in a range bounded by two critical values of a given parameter. This is illustrated by a stability diagram such as that established in Figure 3 as a function of two main control parameters, the rate of PER degradation, v_d, and the apparent first-order kinetic constant measuring the rate of PER entry into the nucleus, k_1. This diagram is obtained by subjecting the system of Equations 1 to linear stability analysis. This analysis indicates the domains in parameter space in which the PER control system evolves to a regime of sustained oscillations of the limit cycle type (I) or reaches a stable, nonoscillatory state (II). The diagram shows that for a given value of parameter k_1, there exists a window bounded by two critical values of parameter v_d in which sustained oscillations occur. The same situation arises for k_1 at a fixed value of v_d, although the range is then much wider.

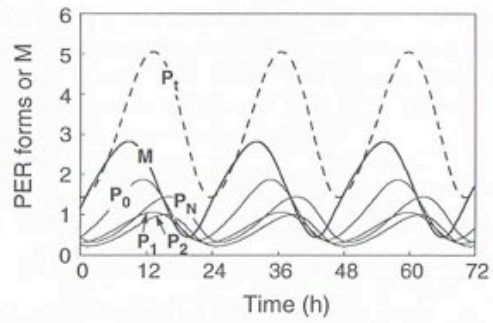

Figure 2. Circadian oscillations generated by the model based on PER alone. Parameter values are: v_s=0.76 nM h^{-1}, v_m=0.65 nM h^{-1}, v_d=0.95 nM h^{-1}, k_1=1.9 h^{-1}, k_2=1.3 h^{-1}, K_d=0.2 nM, n=4, V_1=3.2 nM h^{-1}, V_2=1.6 nM h^{-1}, V_3=5 nM h^{-1}, V_4=2.5 nM h^{-1}, K_1=K_2=K_3=K_4=2 nM, K_m=0.5 nM, K_I=1 nM, k_s=0.4 h^{-1}, k_d=0. In the absence of quantitative information, the concentration scale is tentatively expressed in nM.

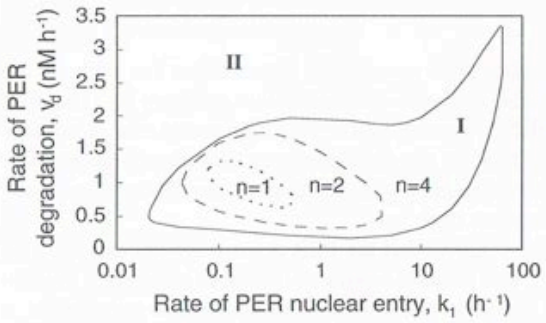

Figure 3. Stability diagram established as a function of the rate of PER nuclear entry and the rate of PER degradation for three values of the degree of cooperativity, n. (I) Evolution towards sustained, limit cycle oscillations; (II) evolution towards a stable nonoscillatory state. Oscillatory domains for n=1 or 2 are nested within the domain for n=4. Parameter values are the same as in Figure 2, except for K_1=K_2=K_3=K_4=20 nM and k_d=0.01 nM h^{-1}.

Multiple phosphorylation of PER also favors the occurrence of oscillatory behavior, although oscillations could in principle occur in the absence of such covalent modification of the protein. The model corroborates the view that PER phosphorylation may introduce time delays in the negative feedback loop, which strengthen its propensity to produce oscillations.

2.2. Model incorporating the formation of a complex between PER and TIM

The next step was to incorporate into the model the more recently characterized role of the TIM protein [8-11]. The formation of a complex between PER and TIM appears to be a necessary step in the transfer of these proteins into the nucleus where negative control of gene expression is exerted. The model incorporating the formation of a complex between the two proteins as well as multiple phosphorylation of PER and TIM is schematized in Figure 4.

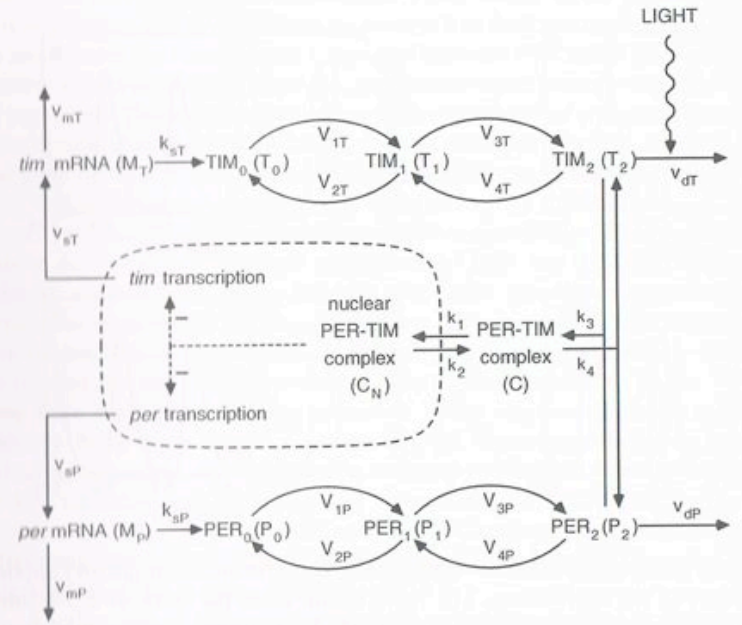

Figure 4. Scheme of the model for circadian oscillations in *Drosophila* incorporating the formation of a PER-TIM complex and the negative regulation exerted by the nuclear form of this complex on gene expression [15].

The model of Figure 4 can largely be viewed as a duplication of the model of Figure 1: it contains two symmetrical branches, leading to the synthesis and multiple phosphorylation of PER and TIM, respectively. The fully phosphorylated forms of PER and TIM are assumed to reversibly form a complex and to be marked for degradation. We have also obtained analogous results in the cases where the PER-TIM complex forms in the absence of prior phosphorylation of the two proteins and where all forms of PER and TIM are subjected to degradation.

The dynamic behavior of the regulated system is now described by a set of ten kinetic equations, of the form of Equations 1. The nonlinear negative feedback is exerted on the synthesis of *per* and *tim* mRNAs by the nuclear form of the PER-TIM complex. Numerical simulations indicate that sustained oscillations also occur in the extended model. The interest of this model is to allow a more direct comparison with experimental data. Thus, in contrast

to the predictions of the model based on PER alone, and in agreement with experimental observations, oscillations in PER and TIM persist in the extended model when the *per* mRNA is kept at a constant level (J.-C. Leloup and A. Goldbeter, manuscript in preparation). Moreover, the model can account for the effect of pulses of light which have been shown to produce phase shifts by triggering TIM degradation [8-11].

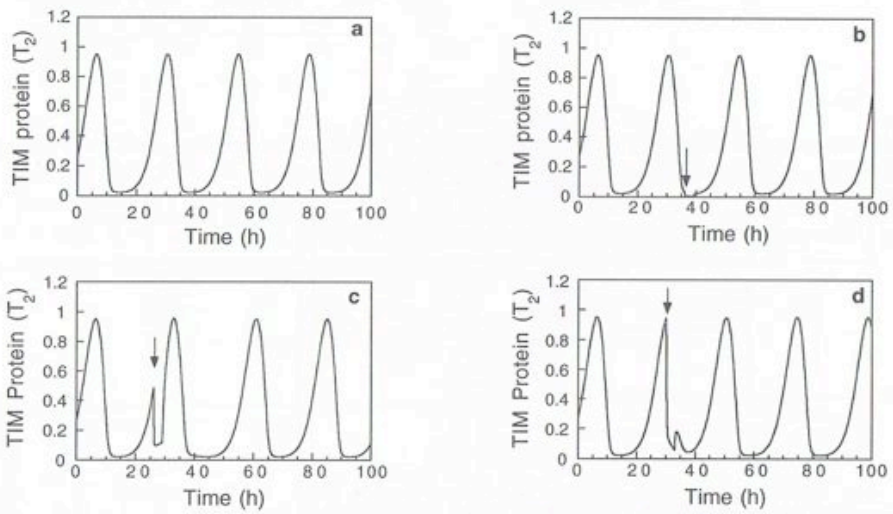

Figure 5. Light-induced phase-shifts obtained theoretically for a perturbation (arrow) applied at three different phases of the oscillations. The perturbation takes the form of a 3-h long, twofold increase in TIM degradation triggered by light. (a) Unperturbed oscillations (shown for comparison); (b) absence of significant phase shift; (c) phase delay; (d) phase advance.

The effect of a light pulse is introduced in the model by enhancing by a certain factor (e.g. twofold) for a given amount of time (e.g. 3 h) the maximum rate of TIM degradation. The direction of the phase shift depends on the phase of the oscillations (Figure 5a) at which the perturbation is applied. As observed in the experiments, phase advances are observed near the maximum of TIM (Figure 5d), phase delays occur during the rising phase of the protein (Figure 5c), while perturbations applied when TIM is at a minimum do not result in any significant phase shift (Figure 5b).

These results can be used to systematically construct phase response curves in which the new phase of the oscillations is plotted as a function of the phase at which the perturbation is applied. A family of phase response curves has been generated as a function of both the possible duration of the effect of the light pulse (which may exceed the duration of the pulse itself) and of the factor by which the TIM degradation rate is enhanced [15]. The results match the phase response curves obtained experimentally for the effect of light pulses in the wild type and in the per^s mutant of *Drosophila* [16].

3. DISCUSSION

The analysis of experimentally-based models for circadian oscillations of the PER and TIM proteins in *Drosophila* throws light on the conditions in which negative autoregulatory feedback loops in genetic control systems can give rise to sustained periodic behavior. The simplest model based on regulation by the PER (or TIM) protein alone already accounts for the occurrence of sustained oscillations in the levels of the protein and its mRNA, with a phase difference of several hours, as observed in the experiments. The extension of the model to incorporate the formation of a complex between PER and TIM leads to similar results, but further allows us to explain how the oscillations cease to occur if the formation of the complex is prevented. The extended model predicts that sustained periodic behavior of PER and TIM should subsist even when the level of *per* or *tim* mRNA is held constant. When incorporating the light-induced degradation of TIM, this model also accounts for the phase response curves obtained in *Drosophila* for the phase shifts elicited by light pulses.

Given the qualitative and semi-quantitative agreement with experimental observations, the model developed for circadian oscillations of PER and TIM in *Drosophila* can be used to address a number of issues and test alternative hypotheses regarding the occurrence and properties of circadian rhythms in this and other organisms such as *Neurospora*, in which a similar mechanism of circadian rhythmicity has been uncovered [6]. Thus the question arises as to what are the main determinants of circadian periodicity. Is the 24 h period due to the long time scale of several key processes in the negative feedback loop, and, if so, can we identify these slow steps? Alternatively, all steps might occur individually on a faster, ultradian time scale, and the circadian behavior could result from the fact that the accumulation of key variables such as the nuclear form of the PER-TIM complex or the mRNAs of the proteins is slowed down by the nearly equal counterpoise provided by the reverse reactions (dephosphorylation of PER and TIM, exit of their complex from the nucleus...) or by the degradation of the proteins and their mRNAs.

Another use of theoretical models for circadian rhythms in *Drosophila* pertains to the molecular bases of temperature compensation [17,18] and to the nature of the *per* mutations yielding the short- or long-period phenotypes, which are characterized by a period of 19 h or 28 h, respectively [19]. The simulations of the model [15] support the experimentally-based views that the increased nuclear degradation of the PER-TIM complex is responsible for the shortening of the period in per^s [20], whereas impaired formation of the PER-TIM complex in the cytosol likely underlies the increase in period in per^l [21,22]. Likewise, it will be interesting to investigate by means of the extended model for oscillations in PER and TIM the possible molecular bases underlying the changes in period observed in other mutants of *Drosophila* which are currently being characterized.

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