A Model for Circadian Rhythms in *Drosophila* Incorporating the Formation of a Complex between the PER and TIM Proteins

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Abstract The authors present a model for circadian oscillations of the Period (PER) and Timeless (TIM) proteins in *Drosophila*. The model for the circadian clock is based on multiple phosphorylation of PER and TIM and on the negative feedback exerted by a nuclear PER-TIM complex on the transcription of the *per* and *tim* genes. Periodic behavior occurs in a large domain of parameter space in the form of limit cycle oscillations. These sustained oscillations occur in conditions corresponding to continuous darkness or to entrainment by light-dark cycles and are in good agreement with experimental observations on the temporal variations of PER and TIM and of *per* and *tim* mRNAs. Birhythmicity (coexistence of two periodic regimes) and aperiodic oscillations (chaos) occur in a restricted range of parameter values. The results are compared to the predictions of a model based on the sole regulation by PER. Both the formation of a complex between PER and TIM and protein phosphorylation are found to favor oscillatory behavior. Determining how the period depends on several key parameters allows us to test possible molecular explanations proposed for the altered period in the per^l and per^s mutants. The extended model further allows the construction of phase-response curves based on the light-induced triggering of TIM degradation. These curves, established as a function of both the duration and magnitude of the effect of a light pulse, match the phase-response curves obtained experimentally in the wild type and per^s mutant of *Drosophila*.

Key words circadian rhythms, biochemical oscillations, phase-response curve, *Drosophila*, PER, TIM, model

INTRODUCTION

Genetic and biochemical studies of the *period* (*per*) locus in *Drosophila* have led to important advances in unraveling the molecular bases of circadian rhythms (Konopka and Benzer, 1971; Hall and Rosbash, 1988; Baylies et al., 1993; Hall, 1995; Rosbash, 1995; Dunlap, 1996). An important clue to the mechanism by which *per* produces rhythmic behavior is that *per* mRNA itself varies in a circadian manner (Hardin et al., 1990). The fact that PER also varies periodically but follows the mRNA rhythm by several hours suggested that the mechanism of circadian oscillations involves a negative feedback exerted by PER on the transcription of the *per* gene (Hardin et al., 1990). Posttranslational regulation appears to be involved in the oscillatory

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mechanism, as PER is phosphorylated in a circadian manner (Edery et al., 1994b). Based on multiple phosphorylation of PER and on repression of *per* transcription by a phosphorylated form of the protein, a theoretical model has been proposed for circadian oscillations in the *Drosophila* PER protein and its mRNA (Goldbeter, 1995, 1996).

Recent experimental observations indicate that besides PER, a second protein, TIM (product of the *tim* gene), also plays a crucial role in the generation of circadian rhythmicity in *Drosophila* (Sehgal et al., 1994; Vosshall et al., 1994; Gekakis et al., 1995; Rutila et al., 1996). The PER and TIM proteins, both of which can be phosphorylated (Edery et al., 1994b; Zeng et al., 1996), form a complex that migrates into the nucleus and represses the expression of the *per* and *tim* genes. Moreover, light induces the rapid degradation of TIM and thereby controls the phase of the circadian rhythm (Hunter-Ensor et al., 1996; Lee et al., 1996; Myers et al., 1996; Zeng et al., 1996). Here, we extend the model based on PER alone to take into account the formation of the PER-TIM complex. We show that this extended model can account for the generation of circadian oscillations in *Drosophila*. We study the behavior of the model both in constant conditions (i.e., continuous darkness) and in the presence of light-dark (LD) cycles. The time course of the two proteins and of the *per* and *tim* transcripts in conditions of entrainment by a cycle of 12 h of light and 12 h of dark agrees with experimental observations. Besides simple periodic behavior, which is the most prevalent mode of oscillations, the model shows the possible occurrence of birhythmicity (coexistence between two stable rhythms) as well as aperiodic (i.e., chaotic) oscillations in a restricted range of parameter values.

We determine how the occurrence of sustained oscillations is affected by various parameters such as the rates of protein synthesis, protein and mRNA degradation, and formation or dissociation of the PER-TIM complex. These results allow us to test possible molecular explanations proposed for the change in period seen in the per^l and per^s mutants. A comparison with the model based on PER alone indicates that the formation of a complex between PER and TIM favors oscillatory behavior because the domain in parameter space in which sustained oscillations occur is then much larger. The model further shows that PER and TIM phosphorylation also favors the occurrence of sustained oscillations, although periodic behavior can in principle be obtained in the absence of such covalent modification.

The model based on repression by a PER-TIM complex allows the construction of phase-response curves (PRCs) as a function of the duration and magnitude of the effect of a light pulse when assuming that light triggers the onset of TIM degradation. The theoretically predicted PRCs match those obtained experimentally for both the wild type and per^s mutant of *Drosophila*.

MODEL FOR *DROSOPHILA* CIRCADIAN RHYTHMS INVOLVING THE FORMATION OF A COMPLEX BETWEEN THE PER AND TIM PROTEINS

Model Hypotheses

The model, schematized in Fig. 1, relies on the following assumptions (see Fig. 1 and its legend for a definition of the parameters): *per* mRNA, whose cytosolic concentration is denoted by M_P, is synthesized in the nucleus and transferred into the cytosol, where it is degraded; the rate of synthesis of PER is proportional to M_P. As in the previous model based on PER alone (Goldbeter, 1995, 1996), to take into account the fact that PER is multiply phosphorylated (Edery et al., 1994b) and to keep the model as simple as possible, only three states of the protein are considered: unphosphorylated (P_0), monophosphorylated (P_1), and bisphosphorylated (P_2). We determined that including a larger number of phosphorylated residues would unnecessarily complicate the model without altering significantly its dynamic behavior.

To take into account the role played by the formation of a complex between the PER and TIM proteins (Hunter-Ensor et al., 1996; Lee et al., 1996; Myers et al., 1996; Zeng et al., 1996), we consider a sequence of steps for TIM similar to the one already outlined for PER. Thus, we assume that *tim* mRNA, whose cytosolic concentration is denoted by M_T, is synthesized in the nucleus and brought into the cytosol, where it is degraded; the rate of TIM synthesis is proportional to M_T. For reasons of symmetry with PER, we further assume that the unphosphorylated form of TIM (T_0) is phosphorylated in a reversible manner into the forms T_1 and T_2; such covalent modifications have been observed recently (Zeng et al., 1996).

The role of PER and TIM phosphorylation still is unclear. Here, we assume that the fully phosphorylated forms (P_2 and T_2) are marked both for degradation and for the reversible formation of a PER-TIM

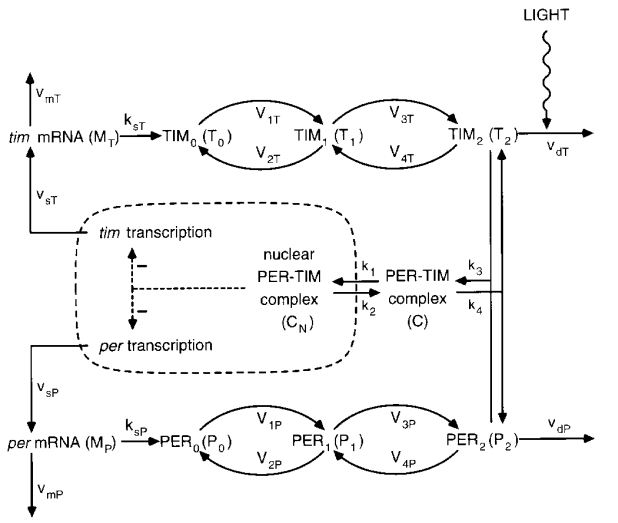

Figure 1. Scheme of the model for circadian oscillations in *Drosophila* involving negative regulation of gene expression by PER and TIM. *per* (M_P) and *tim* (M_T) mRNAs are synthesized in the nucleus and transferred into the cytosol, where they accumulate at the maximum rates v_{sP} and v_{sT}, respectively. There they are degraded enzymatically at the maximum rates, v_{mP} and v_{mT}, with the Michaelis constants, K_{mP} and K_{mT}. The rates of synthesis of the PER and TIM proteins, respectively proportional to M_P and M_T, are characterized by the apparent first-order rate constants k_{sP} and k_{sT}. Parameters V_{iP} (V_{iT}) and K_{iP} (K_{iT}) (i = 1, . . . 4) denote the maximum rate and Michaelis constant of the kinase(s) and phosphatase(s) involved in the reversible phosphorylation of P_0 (T_0) into P_1 (T_1) and P_1 (T_1) into P_2 (T_2), respectively. The fully phosphorylated forms (P_2 and T_2) are degraded by enzymes of maximum rate v_{dP} and v_{dT} and of Michaelis constants K_{dP} and K_{dT} and reversibly form a complex C (association and dissociation are characterized by the rate constants k_3 and k_4), which is transported into the nucleus at a rate characterized by the apparent first-order rate constant k_1. Transport of the nuclear form of the PER-TIM complex (C_N) into the cytosol is characterized by the apparent first-order rate constant k_2. The negative feedback exerted by the nuclear PER-TIM complex on *per* and *tim* transcription is described by an equation of the Hill type (see first terms in Equations 1a and 1e) in which n denotes the degree of cooperativity and K_{IP} and K_{IT} are the threshold constants for repression.

complex (C); the effect of phosphorylation of PER and TIM on the occurrence of oscillations is considered later. The PER-TIM complex is transported into the nucleus; the latter assumption holds with the observation that the nuclear localization of PER and TIM requires the association of the two proteins, although the formation of a complex also may occur in the absence of PER and TIM phosphorylation (Saez and Young, 1996). An alternative mechanism considers that PER and TIM form a complex and then are phosphorylated before entering the nucleus (Rutila et al., 1996). The results expected from such a mechanism should be similar to those obtained in the present model.

Some experiments indicate (Edery et al., 1994b) that PER continues to be phosphorylated after its entry into the nucleus. We have verified that further phosphorylation of the nuclear form of the PER-TIM complex does not significantly influence the results reported subsequently. Thus, to avoid the introduction of additional variables, we have not considered any further phosphorylation of PER or TIM within the nucleus, although this could readily be done within the framework of the model.

Crucial to the mechanism of oscillations in the model is the negative feedback exerted by the nuclear form C_N of the PER-TIM complex on the synthesis of *per* and *tim* mRNAs. This negative feedback is described by an equation of the Hill type. To simplify the model, we consider that C_N behaves directly as a repressor; activation of a repressor on binding of C_N would not significantly alter the results. Finally, all equations contain a linear degradation term characterized by the rate constant k_d. (This constant is denoted by k_{dC} and k_{dN} for the cytoplasmic and nuclear forms of the PER-TIM complex, respectively.) This relatively small, nonspecific degradation term is not required for oscillations but ensures that a steady state exists even when specific degradation processes are inhibited.

Kinetic Equations

The time evolution of the 10-variable model is governed by the following kinetic equations in which all parameters and concentrations are defined with respect to the total cell volume:

$$\frac{dM_P}{dt} = v_{sP}\frac{K_{IP}{}^n}{K_{IP}{}^n + C_N{}^n} - v_{mP}\frac{M_P}{K_{mP} + M_P} - k_d M_P \quad (1a)$$

$$\frac{dP_0}{dt} = k_{sP}M_P - V_{1P}\frac{P_0}{K_{1P} + P_0} + V_{2P}\frac{P_1}{K_{2P} + P_1} - k_d P_0 \quad (1b)$$

$$\frac{dP_1}{dt} = V_{1P}\frac{P_0}{K_{1P} + P_0} - V_{2P}\frac{P_1}{K_{2P} + P_1} - V_{3P}\frac{P_1}{K_{3P} + P_1} + V_{4P}\frac{P_2}{K_{4P} + P_2} - k_d P_1 \quad (1c)$$

$$\frac{dP_2}{dt} = V_{3P}\frac{P_1}{K_{3P}+P_1} - V_{4P}\frac{P_2}{K_{4P}+P_2} - k_3P_2T_2 + k_4C - \nu_{dP}\frac{P_2}{K_{dP}+P_2} - k_dP_2 \quad (1d)$$

$$\frac{dM_T}{dt} = \nu_{sT}\frac{K_{IT}{}^n}{K_{IT}{}^n + C_N{}^n} - \nu_{mT}\frac{M_T}{K_{mT}+M_T} - k_dM_T \quad (1e)$$

$$\frac{dT_0}{dt} = k_{sT}M_T - V_{1T}\frac{T_0}{K_{1T}+T_0} + V_{2T}\frac{T_1}{K_{2T}+T_1} - k_dT_0 \quad (1f)$$

$$\frac{dT_1}{dt} = V_{1T}\frac{T_0}{K_{1T}+T_0} - V_{2T}\frac{T_1}{K_{2T}+T_1} - V_{3T}\frac{T_1}{K_{3T}+T_1} + V_{4T}\frac{T_2}{K_{4T}+T_2} - k_dT_1 \quad (1g)$$

$$\frac{dT_2}{dt} = V_{3T}\frac{T_1}{K_{3T}+T_1} - V_{4T}\frac{T_2}{K_{4T}+T_2} - k_3P_2T_2 + k_4C - \nu_{dT}\frac{T_2}{K_{dT}+T_2} - k_dT_2 \quad (1h)$$

$$\frac{dC}{dt} = k_3P_2T_2 - k_4C - k_1C + k_2C_N - k_{dC}C \quad (1i)$$

$$\frac{dC_N}{dt} = k_1C - k_2C_N - k_{dN}C_N. \quad (1j)$$

The total (nonconserved) quantities of PER and TIM proteins, P_t and T_t, are given by

$$P_t = P_0 + P_1 + P_2 + C + C_N \quad (2)$$

$$T_t = T_0 + T_1 + T_2 + C + C_N. \quad (3)$$

CIRCADIAN OSCILLATIONS IN PER AND TIM AND THEIR mRNAs

Circadian Oscillations

Numerical integration shows that in a large domain of parameter values, instead of evolving toward a stable steady state, the system governed by Equations 1a-j reaches a regime of sustained, periodic oscillations. Shown in Fig. 2A is the temporal variation in *per* and *tim* mRNAs in the symmetrical case in which identical rate constants are taken for corresponding steps involving PER and TIM. (The effect of asymmetrical conditions is considered later.) Also shown in Fig. 2A is the periodic variation in the nuclear PER-TIM complex and in the total amounts of PER and TIM. The corresponding oscillations in the various forms of PER and TIM and in the cytosolic and nuclear forms of the PER-TIM complex are shown in Fig. 2B, where the time scale has been enlarged to make clear the phase relationships among the different variables. As in the model based on PER alone (Goldbeter, 1995, 1996), sustained oscillations correspond to the evolution toward a limit cycle and away from the unstable steady state.

Parameter values in Fig. 2 have been chosen to yield a period close to 24 h in conditions corresponding to constant darkness. These values are somewhat arbitrary, and many other combinations of parameter values could yield a circadian period of oscillations. The values chosen in Fig. 2, however, are in a physiological range and yield an appropriate time course for the evolution of PER and TIM. The peak in *per* and *tim* mRNAs precedes the peak in the nuclear PER-TIM complex by about 5 h, whereas the shift between the mRNAs and the total amounts of PER and TIM is shorter, of the order of 3 h. Such a result roughly holds with the observation that the maximum in PER protein follows the peak in *per* mRNA by about 4 h (Zeng et al., 1994).

How the various parameters influence the period can be determined by numerical integration of Equation 1. Of particular interest is the dependence of the period on the rate of PER and TIM degradation. As shown in Fig. 3A, when increasing the linear rate of degradation of the nuclear PER-TIM complex, the period either decreases as k_{dN} increases (curve *a*) or passes through a minimum (curve *b*). The only difference between the conditions yielding curves *a* and *b* is that the latter is obtained for higher values of the maximum rates of dephosphorylation. The decrease in period seen in curve *a* as k_{dN} rises holds with the suggestion that the shortened period in the mutant per^s is due to enhanced degradation of the nuclear regulator of gene expression (Curtin et al., 1995).

Shown in Fig. 3B is the effect of a change in the rate of synthesis of PER and TIM. After passing through a maximum, the period is seen to decrease as the rate of protein synthesis increases. Moreover, sustained oscil-

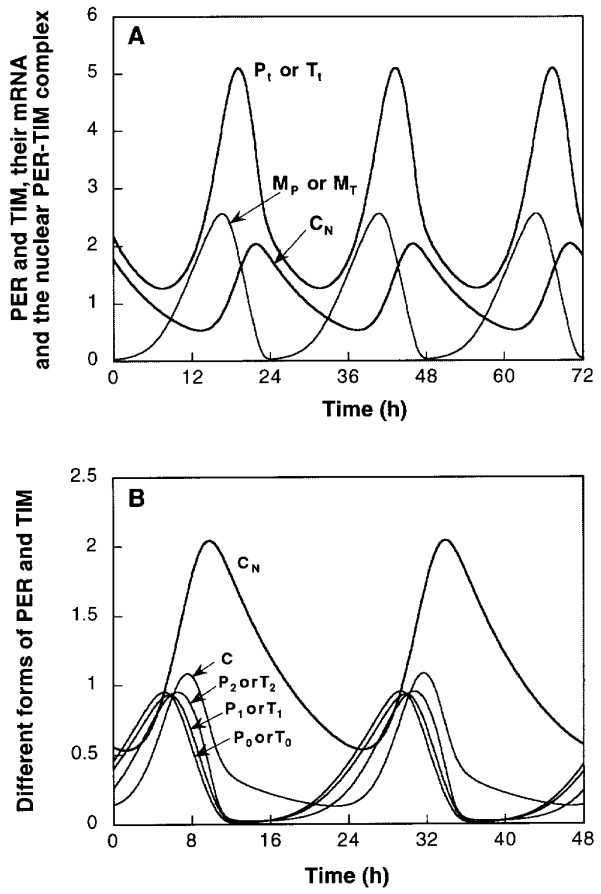

Figure 2. Sustained oscillations generated by the model based on negative regulation by a PER-TIM complex of *per* and *tim* expression in *Drosophila*. (A) Temporal variation in *per* and *tim* mRNAs (M_P and M_T) and in the total amount of PER and TIM proteins (P_t and T_t), together with the variation in nuclear PER-TIM complex (C_N). (B) Oscillations of the various forms of PER and TIM as well as the cytosolic and nuclear forms of the PER-TIM complex shown on an enlarged time scale. In this and subsequent figures, concentrations are tentatively expressed in nanomolars (nM). The curves are obtained in constant conditions (corresponding to continuous darkness) by numerical integration of Equations 1a-j. P_t and T_t are given by Equations 2 and 3. Parameter values are as follows: $v_{sP} = v_{sT} = 1$ nM h^{-1}, $v_{mP} = v_{mT} = 0.7$ nM h^{-1}, $K_{mP} = K_{mT} = 0.2$ nM, $k_{sP} = k_{sT} = 0.9$ h^{-1}, $v_{dP} = v_{dT} = 2$ nM h^{-1}, $k_1 = 0.6$ h^{-1}, $k_2 = 0.2$ h^{-1}, $k_3 = 1.2$ nM^{-1} h^{-1}, $k_4 = 0.6$ h^{-1}, $K_{IP} = K_{IT} = 1$ nM, $K_{dP} = K_{dT} = 0.2$ nM, $n = 4$, $K_{1P} = K_{1T} = K_{2P} = K_{2T} = K_{3P} = K_{3T} = K_{4P} = K_{4T} = 2$ nM, $k_d = k_{dC} = k_{dN} = 0.01$ h^{-1}, $V_{1P} = V_{1T} = 8$ nM h^{-1}, $V_{2P} = V_{2T} = 1$ nM h^{-1}, $V_{3P} = V_{3T} = 8$ nM h^{-1}, $V_{4P} = V_{4T} = 1$ nM h^{-1}.

lations disappear when the rate of protein synthesis falls below a critical value. This is in agreement with the effect of inhibitors of protein synthesis, which are known to alter the period and to eventually suppress circadian rhythmicity in a variety of organisms (Karakashian and Hastings, 1963; Eskin et al., 1984; Taylor et al., 1982). The increase in period as the rate of protein synthesis progressively decreases down to a critical value beyond which circadian rhythms are suppressed has been observed in response to rising doses of anisomycin in the mollusk *Bulla* (Khalsa et al., 1992). It would be interesting to determine whether such a change in the period of circadian oscillations occurs on titrating the *Drosophila* circadian system with an inhibitor of protein synthesis.

The formation of the PER-TIM complex in the cytosol plays a key role in the present model. Accordingly, the rate constants k_3 and k_4 markedly affect the period of oscillations. The results of Fig. 3C indicate that the period decreases as the bimolecular association rate constant k_3 increases, whereas the opposite effect is seen as a function of the first-order rate constant k_4 for dissociation of the complex. Moreover, for both parameters, sustained oscillations occur only in a range bounded by two critical values, only one of which is shown for either k_3 or k_4 in Fig. 3C because the range extends over several orders of magnitude. The increase in period observed on decreasing the rate constant k_3 for the formation of the PER-TIM complex holds with the suggestion by Gekakis et al. (1995) that the formation of this heterodimeric complex is impaired in the long-period *per*^{l} mutant, possibly because of the competitive formation of PER homodimeric complexes (Huang et al., 1995).

Role of the Formation of a PER-TIM Complex and Effect of PER and TIM Phosphorylation

In the simpler model based on the regulation by PER alone (Goldbeter, 1995), sustained oscillations are obtained when repression is brought about by the nuclear form of the cytoplasmic phosphorylated protein, P_2. Regardless of whether phosphorylation of PER and/or TIM is actually required for producing oscillations, the present model allows us to assess the effect of phosphorylation as well as the role played by the formation of a complex between the two proteins in the generation of rhythmic behavior.

In Table 1, we summarize the results of a comparative study of the cases in which negative feedback on gene expression is exerted by the nuclear forms of P_0 or P_2 in the simple model based on PER alone (last two columns of table) or by the nuclear forms of the complexes P_2T_2, P_2T_0, or P_0T_0 in the extended model (first three columns of table). The latter three cases correspond, respectively, to the situations in which the complex between PER and TIM that exerts repression

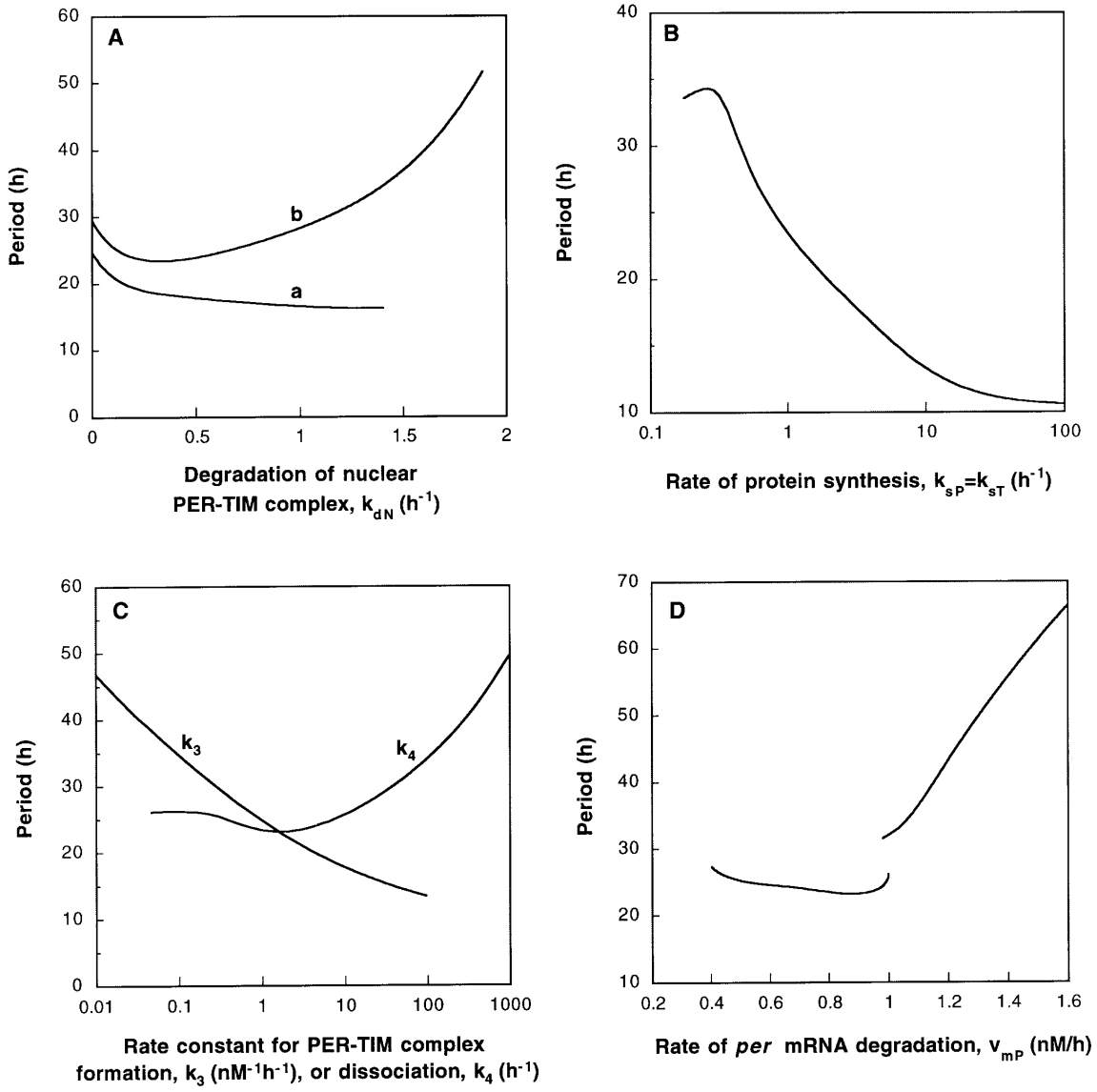

Figure 3. Dependence of the period on (A) the rate of degradation of the nuclear PER-TIM complex, (B) the rate of synthesis of the PER and TIM proteins, (C) the rate of formation and dissociation of the PER-TIM complex, and (D) the rate of *per* mRNA degradation. The curves are established under constant conditions corresponding to continuous darkness by analyzing Equations 1a-j by means of the program AUTO (Doedel, 1981), which allows the direct construction of bifurcation diagrams. Parameter values are the same as in Fig. 2. In panel A, curve *b*, the maximum dephosphorylation rates are $V_{2P} = V_{2T} = 5$ nM h^{-1}, $V_{4P} = V_{4T} = 5$ nM h^{-1}. In panel D, where v_{mP} varies and v_{mT} is fixed at the value given in Fig. 2, the coexistence between two stable rhythms (birhythmicity) is obtained in the narrow range $v_{mP} = 0.980$ to 0.998 nM h^{-1}.

forms when both proteins are fully phosphorylated, when only PER has to be fully phosphorylated while TIM is not phosphorylated, or when no phosphorylation occurs prior to the association of PER and TIM. In each case, we determined as a function of several key parameters of the model the occurrence of sustained oscillations and obtained the upper and lower critical values of the control parameter bounding the oscillatory domain. The magnitude of the oscillatory domain for the parameter is then measured by the ratio of these two critical values.

The results collected in Table 1 indicate that phosphorylation of PER and TIM prior to their association in a complex is not, in principle, required for producing sustained oscillations. This does not imply that oscillations should necessarily occur when protein

Table 1. Effect of PER and TIM phosphorylation on the occurrence of sustained oscillations in the model for circadian rhythms in *Drosophila*.

Parameter	*n*	P_2T_2	P_2T_0	P_0T_0	*Parameter*	n	P_2	P_0
k_1 (h^{-1})	4	4216.9	2057.8	981.3	k_1 (h^{-1})	4	1065.3	208.7
	2	624.8	370.1	208.3		2	147.2	57.0
	1	110.5	67.7	40.3		1	33.7	16.1
k_2 (h^{-1})	4	730.4	254.0	72.1	k_2 (h^{-1})	4	131.46	4.9
	2	108.5	39.7	12.3		2	22.43	
	1	10.6				1		
$k_{sP} = k_{sT}$ (h^{-1})	4	11,181.1	407.4	77.0	k_s (h^{-1})	4	1394.6	
	2	40.4	16.6	7.6		2		
	1	2.5				1		
v_{dT} (nM / h)	4	232.2	175.0	95.4	v_d (nM / h)	4	8.4	
	2	115.2	91.3	69.0		2		
	1	26.7	16.5	11.8		1		

NOTE: Shown is the range of sustained oscillations given by the ratio of the maximum through the minimum value of the control parameter (k_1, k_2, $k_{sP} = k_{sT}$, or v_{dT}) yielding limit cycle oscillations. The data are obtained as a function of these five control parameters, for a degree n characterizing the cooperativity of repression equal to 1 (no cooperativity), 2, or 4. To test the effect of PER and TIM phosphorylation, the domain of sustained oscillations is determined when assuming that the PER-TIM complex exerting the negative control on gene expression is the form P_2T_2, P_2T_0, or P_0T_0 (first three columns). These results can be compared to those generated by the model based on regulation by PER (or, for reasons of symmetry of the model, TIM) alone (last two columns). The absence of any number denotes the absence of sustained oscillations for the particular set of parameter values considered. Other parameter values are as in Fig. 2. The range of sustained oscillations has been determined by means of the program AUTO (Doedel, 1981).

phosphorylation is blocked; the model indicates only that oscillations might in principle occur if a complex between P_0 and T_0 could form and migrate into the nucleus to repress the transcription of the *per* and *tim* genes. The data of Table 1 further indicate that a similar result (accompanied by the same caveat) holds for the model based on PER alone; parameter values can indeed be found for which oscillations are brought about by the negative feedback exerted by the nuclear form of the unphosphorylated species P_0.

The data of Table 1 show, however, that even if it is not in principle required for sustained oscillations, phosphorylation of PER and TIM nevertheless favors oscillatory behavior. Indeed, the size of the oscillatory domain for each parameter in Table 1 increases with the degree of phosphorylation of PER and TIM required to form the regulatory active complex (compare the first three columns of the table). Moreover, the formation of a complex between PER and TIM significantly favors the occurrence of sustained oscillations (compare the first and penultimate columns corresponding to the cases in which regulation is exerted by the nuclear forms of P_2T_2 or of P_2). Both in the extended model and in the simpler model based on PER alone, sustained oscillations can occur in the absence of cooperativity ($n = 1$) but clearly are favored by a large degree of cooperativity, as shown by the comparison of the size of the oscillatory domains when the Hill coefficient characterizing the repression function varies from $n = 4$ to $n = 2$ or 1.

Effect of Asymmetrical Conditions and of Entrainment by a Light-Dark Cycle

The results in Figs. 2 and 3A-C have been obtained in the fully symmetrical case in which identical values are taken for corresponding parameters in the steps involving PER and TIM in the scheme of Fig. 1. The participation of two distinct proteins in the formation of the active complex exerting repression raises the question of what might be the effect of differences in the parameters governing the evolution of these proteins and their mRNAs. It is highly probable that the corresponding parameters characterizing the synthesis and degradation of *per* and *tim* transcripts and of the two proteins do not possess the same values. That such an asymmetry occurs is indeed shown by the fact that the level of *tim* mRNA is larger by a factor of the order of 5 than that of *per* mRNA (Young et al., 1996). Moreover, in constant darkness, the level of PER in the tim^0 mutant is less than the level of TIM in per^0 flies (Myers et al., 1996; Zeng et al., 1996). Oscillations can readily be generated by the model in such asymmetrical conditions. Up to now, in Figs. 2 and 3A-C, we have studied the model in conditions symmetrical with respect to the time evolution of PER and TIM and their gene transcripts, in constant conditions corresponding to continuous darkness. We shall investigate the effect of asymmetries in the parameters corresponding to the two proteins and their mRNAs first in conditions of constant darkness and then in the presence of LD cycles.

In Figs. 4A-C are shown periodic oscillations in conditions of continuous darkness in a case in which the rate of synthesis of the *per* mRNA and its rate of degradation have been decreased and augmented, respectively, whereas the corresponding parameters for *tim* mRNA remain unchanged with respect to the symmetrical conditions of Fig. 2. In Figs. 4A-C, the rate of entry of the PER-TIM complex into the nucleus has been increased compared to Fig. 2 so as to retain a period close to 24 h. The oscillations of *per* and *tim* transcripts and of the total amounts of the two proteins (Fig. 4A) have been redrawn on different scales in Figs. 4B,C so as to allow better comparison between the curves related to PER and TIM (Fig. 4C) and to their mRNAs (Fig. 4B). Also indicated in Fig. 4B is the periodic variation of the nuclear form of the PER-TIM complex.

The curves in Figs. 4A-C show that slight differences in the rates of synthesis and degradation of the *per* and *tim* transcripts can account for the observed differences in their levels and also for differences in the levels of PER and TIM. Here, a threefold increase in the level of *tim* mRNA compared to *per* mRNA is associated with a fivefold increase in TIM levels as compared to PER levels. Further asymmetries in the parameters could involve a differential repression of *per* and *tim* genes by the nuclear PER-TIM complex as well as differences in the rates of synthesis, degradation, and phosphorylation or dephosphorylation of the PER and TIM proteins.

The results shown in Figs. 4A-C indicate that an asymmetry in the parameters characterizing the evolution of the two proteins or (as considered here) their mRNAs can introduce phase differences between the two transcripts and also between PER and TIM. Thus, Figs. 4B,C clearly indicate that the decrease in the levels of PER and *per* transcript relative to TIM and *tim* mRNA is accompanied by the appearance of phase differences between these variables. In the particular case considered in Figs. 4A-C, *per* mRNA (M_P) precedes *tim* mRNA (M_T) by close to 1.8 h, whereas the phase difference further increases up to 2.3 h when comparing total PER and TIM. The maximum in the nuclear form of the PER-TIM complex (C_N) is nearly concomitant with the trough in M_P and precedes by several hours the trough in M_T. Due to the change in waveform, indeed, the difference between the minima in M_P or M_T is larger than the difference between their peaks. Also noticeable is the effect of the parameter changes on the relative dynamics of the two proteins; in the case considered in Figs. 4A-C, the peak in PER precedes the peak in TIM, whereas the minimum in PER follows the trough in the second protein.

The effect of the same asymmetries in parameter values is illustrated in Figs. 4D-F in the presence of a cycle of 12 h of light and 12 h of dark (12:12 LD). These entrainment conditions are generally used in the experiments on circadian oscillations of PER and TIM in *Drosophila*. Therefore, the simulations shown in Figs. 4D-F allow a direct comparison with experimental observations. The existence of the LD cycle is reflected in the model by the periodic, square-wave variation of parameter v_{dT}, which measures the maximum rate of TIM degradation. To take into account the observation (Hunter-Ensor et al., 1996; Lee et al., 1996; Myers et al., 1996; Zeng et al., 1996) that light induces TIM degradation, we assume that in the dark phase, v_{dT} has the same basal value as in the case of continuous darkness (Figs. 4A-C) and is multiplied by a factor of 2 in the light phase (see Fig. 4D where the time evolution of v_{dT} is shown).

The difference between the cases of continuous darkness and entrainment by a LD cycle is not very significant. The most striking effect of the 12:12 LD cycle is that the drop in TIM is then steeper (cf. Figs. 4F and 4C). This drop is more precipitous than that of PER, which decreases over a more prolonged time course, in agreement with experimental observations (Zeng et al., 1996). The timing of the rise and fall of PER and TIM also agrees with experimental observations that are generally performed in flies entrained by a 12:12 LD cycle; as seen in Fig. 4F, the two proteins rise during the night phase, and their peak occurs during the second part of night, around Zeitgeber Time (ZT) 19, whereas the drop occurs during late night or early morning (Myers et al., 1996; Zeng et al., 1996). The data of Fig. 4E also agree with experimental observations that show (Hardin et al., 1990; Qiu and Hardin, 1996) that *per* mRNA reaches its maximum level about 4 h after the beginning of the dark phase (i.e., near ZT 16 in a 12:12 LD cycle), whereas the decrease in *per* mRNA abundance, which is steeper than the rising phase of the *per* transcript, occurs during the rest of the night hours.

The amplitude in the oscillations of the two proteins and of the *tim* and *per* mRNAs is larger in the conditions of entrainment by the LD cycle. This is due to the fact that the stronger, light-induced TIM degradation produces a more important drop in TIM. Due to the consequent reduction in negative feedback on

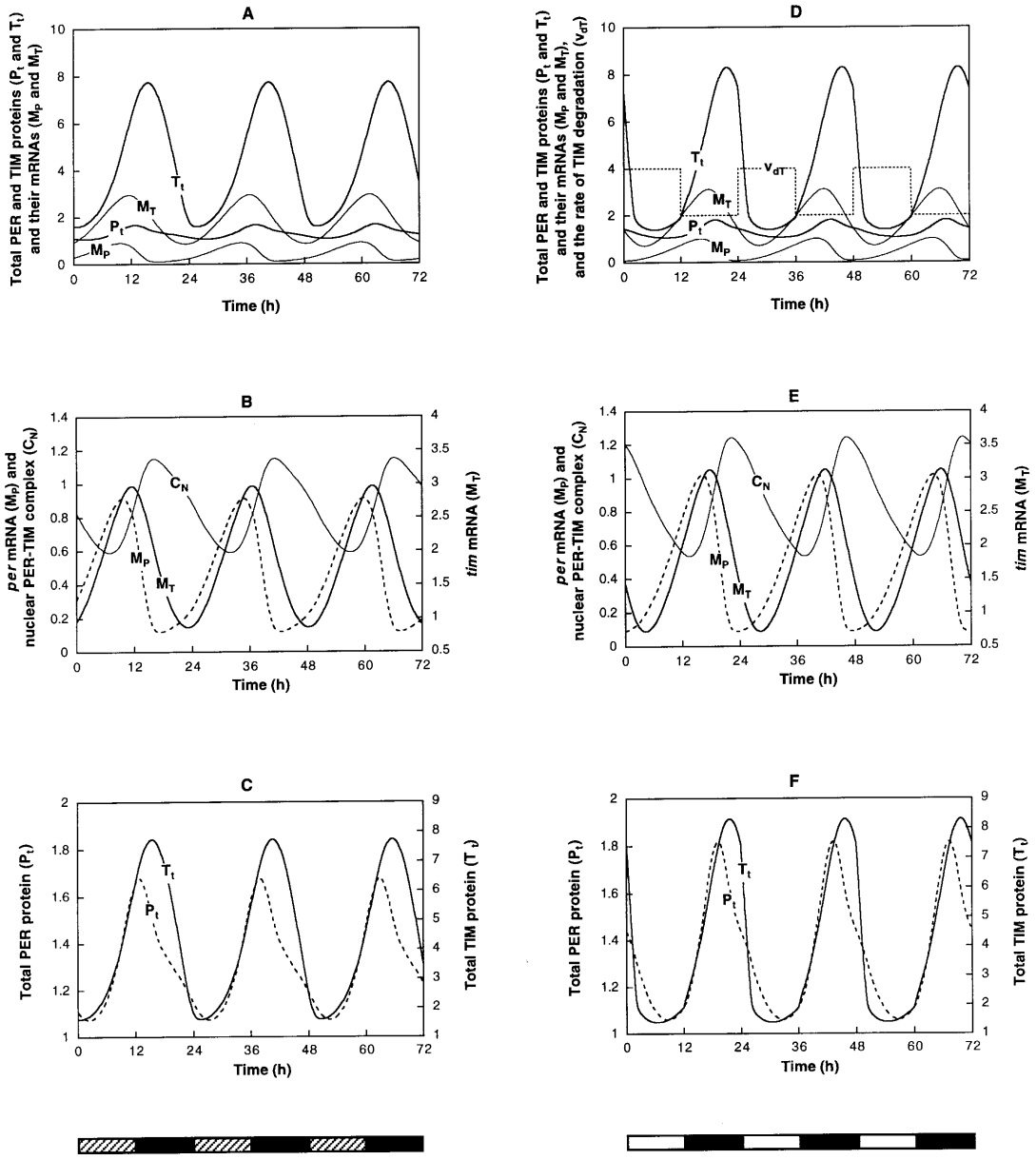

Figure 4. Effect of asymmetrical conditions and of entrainment by a light-dark (LD) cycle. Panels A-C on the left refer to the case of continuous darkness, whereas panels D-F on the right pertain to the entrainment by a 12:12 LD cycle. The curves are generated by numerical integration of Equations 1a-j. In panels A-C, the maximum rates of TIM degradation, v_{dT}, remain constant and equal to 2 nM/h. In panels D-F, to take into account light-induced TIM degradation, v_{dT} = 2 nM/h during the dark phase and is multiplied by 2 during the light phase. Other parameter values are identical for panels A-C and D-F. Shown in panels A and D are the time variations of the total PER (P_T) and TIM (T_T) proteins and of the *per* (M_P) and *tim* (M_T) mRNAs. These variations are enlarged for M_P and M_T in panels B and E, in which the variation of the nuclear form of the PER-TIM complex (C_N) also is shown. Panels C and F present enlargements of the variations in total PER and TIM proteins. With respect to the symmetrical conditions of Fig. 2, an asymmetry between the rates of synthesis (v_{sP} and v_{sT}) and degradation (v_{mP} and v_{mT}) of M_P and M_T has been introduced; here $v_{sP} = 0.8$ nM $h^{-1} < v_{sT} = 1.0$ nM h^{-1} and $v_{mP} = 0.8$ nM $h^{-1} > v_{mT} = 0.7$ nM h^{-1}. Moreover, parameter k_1 has been increased from 0.6 to 1.2 h^{-1} so as to keep a period close to 24 h. Other parameter values are as in Fig. 2.

gene expression, this leads to a larger rise in *per* and *tim* transcripts and, hence, to a larger increase in the two proteins.

The comparisons between Figs. 4B and 4E and between Figs. 4C and 4F further indicate that in the asymmetrical conditions considered in Fig. 4, the

phase differences between PER and TIM and between their transcripts are attenuated in the presence of an LD cycle compared to continuous darkness. While we find that PER (*per* mRNA) reaches its maximum slightly before TIM (*tim* mRNA), some experiments (Marrus et al., 1996; So and Rosbash, 1997) indicate that the reverse situation is encountered, although the peak in *per* transcript appears to slightly precede the peak in *tim* mRNA in some other experiments (Young et al., 1996). Further simulations of the model are needed to address this question. Moreover, recent experiments suggest (So and Rosbash, 1997) that post-transcriptional regulation of *per* (and possible *tim*) mRNA, exerted directly or indirectly by PER or the PER-TIM complex, might contribute to the circadian oscillatory mechanism. Such a regulation, not considered here, could also affect the phase relationship between PER and TIM.

Simple versus Complex Oscillatory Behavior

Only simple periodic oscillations are obtained in symmetrical conditions (Fig. 2). In the asymmetrical case, simple periodic oscillations remain the most common mode of oscillatory behavior, as illustrated in Fig. 4. However, the model shows that complex phenomena can arise from an asymmetry in the parameters governing the time evolution of PER and TIM and/or their mRNAs. Thus, two separated domains of instability of the steady state accompanied by sustained oscillations are obtained in Fig. 3D, where the period is plotted as a function of the maximum rate of specific degradation of *per* mRNA, v_{mP}, at a given value of v_{mT}. In a small range of v_{mP} values, the two domains of oscillations may overlap so that two stable rhythms coexist; the two regimes of stable oscillations are characterized by two distinct periods equal to 24.97 and 31.81 h for $v_{mP} = 0.99$ nM/h in the case of Fig. 3D. Such a phenomenon is known as *birhythmicity* (Decroly and Goldbeter, 1982; Goldbeter, 1996). The evolution toward either one of the two stable oscillatory regimes depends on the initial conditions.

We also have observed aperiodic oscillations (i.e., chaos) in the PER-TIM model. Shown in Fig. 5 is an example of such a chaotic behavior illustrated by the time evolution of *per* and *tim* mRNAs. Chaos and birhythmicity occur in relatively small ranges of parameter values and will be analyzed in more detail in a forthcoming publication.

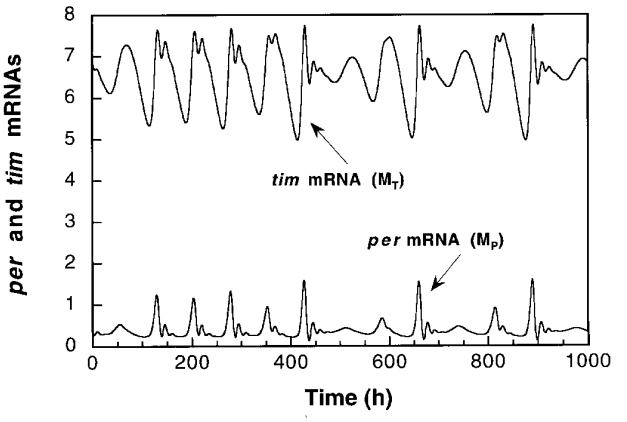

Figure 5. Aperiodic (i.e., chaotic) oscillations. Shown is the time evolution of *per* mRNA (M_P) and *tim* mRNA (M_T). The curves are obtained by numerical integration of Equations 1a-j for the parameter values of Fig. 2, with $v_{mT} = 0.4$ nM h^{-1} and $v_{dT} = 5.9$ nM h^{-1}.

PHASE-RESPONSE CURVES PREDICTED BY THE MODEL

A common method of characterizing circadian rhythms is to determine the phase shifts induced by chemical stimuli (e.g., inhibitors of protein synthesis) or pulses of light or temperature. PRCs yielding the new phase of the oscillations as a function of the phase of the unperturbed rhythm at which the stimulus is applied have been determined experimentally for circadian rhythms in a large number of organisms (Edmunds, 1988) including *Drosophila* (Hall and Rosbash, 1987; Edery et al., 1994a; Saunders et al., 1994). PRCs also have been determined theoretically for circadian oscillations (Drescher et al., 1982) in the early model proposed by Goodwin (1965) for oscillations in protein synthesis due to negative control of gene expression. The model for circadian rhythms in *Drosophila* can be tested to determine whether it can account for the PRC obtained experimentally in this organism.

Shown in Fig. 6A is the PRC obtained for the circadian rhythm of activity in the wild type and short-period mutant *per*^{s} of *Drosophila* using 1-min pulses of light. (The curves are redrawn from Fig. 2 of Hall and Rosbash [1987], which is based on data obtained by D. Orr and R. J. Konopka.) To account for these experimental results, we have taken into account the recent observations that light exerts its phase-shifting effects by inducing the rapid degradation of TIM (Hunter-Ensor et al., 1996; Lee et al., 1996; Myers et al., 1996; Zeng et al., 1996). Thus, as before, we assume that the

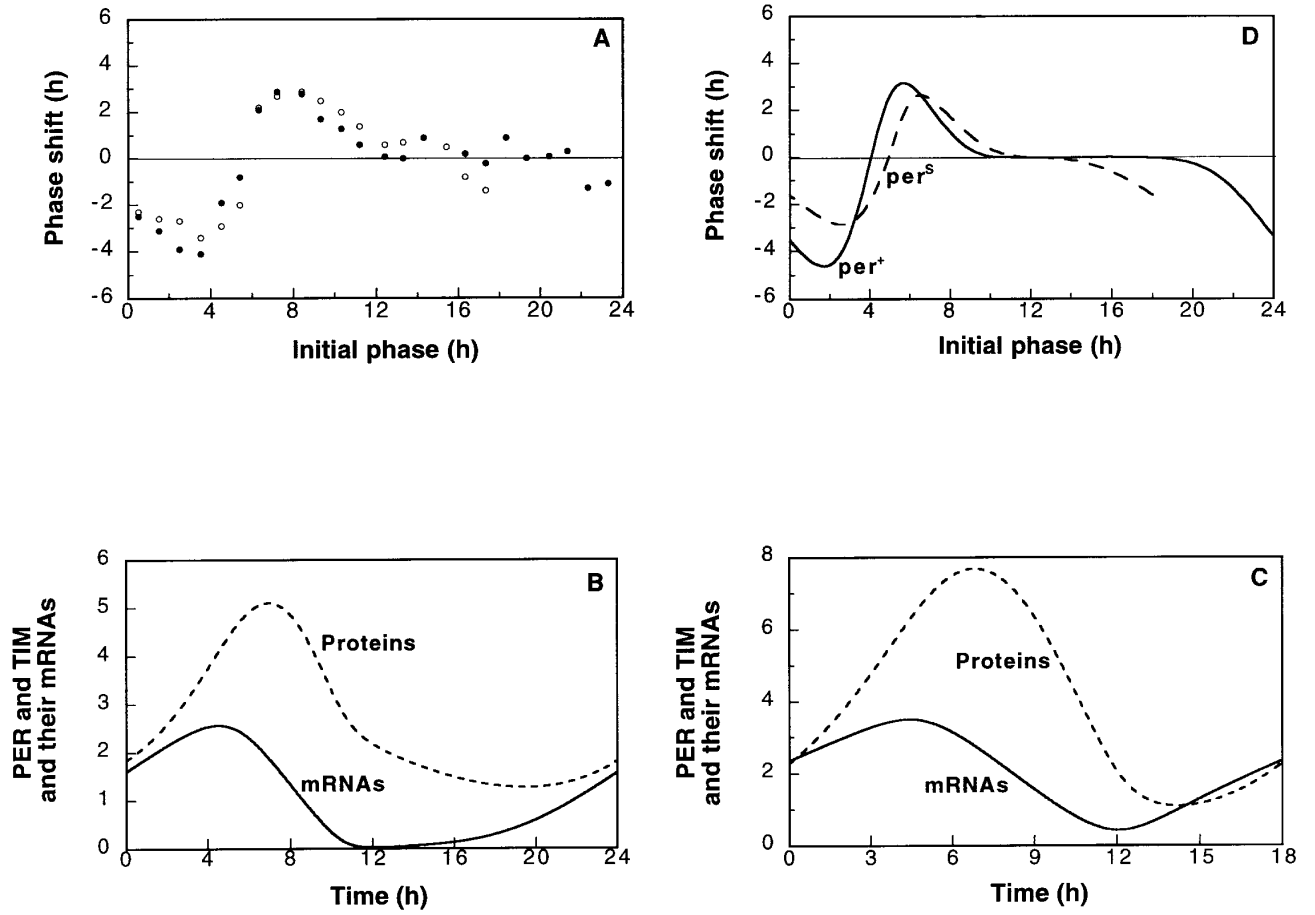

Figure 6. Phase-response curves (PRCs) obtained experimentally (A) and theoretically (D) for the wild type and *per*^{s} mutant in *Drosophila* following perturbation by light. The experimental curves in panel A, based on data obtained by R. J. Konopka and D. Orr using a 1-min light pulse, are redrawn from Fig. 2 of Hall and Rosbash (1987); black dots refer to the wild type, and unfilled circles pertain to the *per*^{s} mutant. The theoretical PRCs in panel D, pertaining to the wild type (solid line) and *per*^{s} mutant (dashed line), have been obtained by integration of Equations 1a-j, starting with the initial conditions corresponding to the particular phase of the unperturbed oscillations in panels B and C, after multiplying by a factor of 2 during 3 h parameter v_{dT} in Equation 1h, which measures the maximum rate of degradation of the fully phosphorylated form of TIM. Panels B and C show the unperturbed oscillations in PER and TIM and their mRNAs for the cases of the wild type and *per*^{s} mutant, respectively; in each case, phase zero is chosen, as in the experiments (Hall and Rosbash, 1987), so that the minimum in *per* mRNA occurs after 12 h. Phase zero corresponds to the beginning of the subjective night. Parameters in panel B and for the solid line in panel D (corresponding to the wild type) are as in Fig. 2; for panel C and the dashed curve in panel D (corresponding to the case of the *per*^{s} mutant), the value of k_{dN} measuring the degradation of the nuclear PER-TIM complex has been increased from 0.01 up to 0.45 h^{-1}. (See Fig. 3A, curve *a*, which shows how the period decreases when k_{dN} increases.) The values of the free-running periods in panels B and C are equal to 24.135 and 18.025 h, respectively.

effect of the light pulse is to increase, for a variable time, the maximum rate of TIM degradation, v_{dT}. Only the form T_2 is initially considered to be degraded specifically in the model, but the effect of degradation of all forms of the protein will be considered in Figs. 7E,F. The duration of the increase in TIM degradation can be much longer than the duration of the pulse itself because the perturbation might induce the synthesis of a TIM protease that could remain active for a certain time even after the light stimulus has been withdrawn.

To determine the effect of the perturbation, we must first fix the phase 0 of the unperturbed rhythm. In the wild type, the full period being equal to approximately 24 h, the phase spans the interval 0 to 24 h, whereas for the *per*^{s} mutant, the phase spans the interval 0 to 18 h. As in the experiments described by Hall and Rosbash (1987) (Fig. 6A), we specify in Fig. 6B (corresponding to the wild type) and Fig. 6C (corresponding to *per*^{s}) the initial phase (0 h) such that the minimum in *per* mRNA occurs at 12 h. In *Drosophila*,

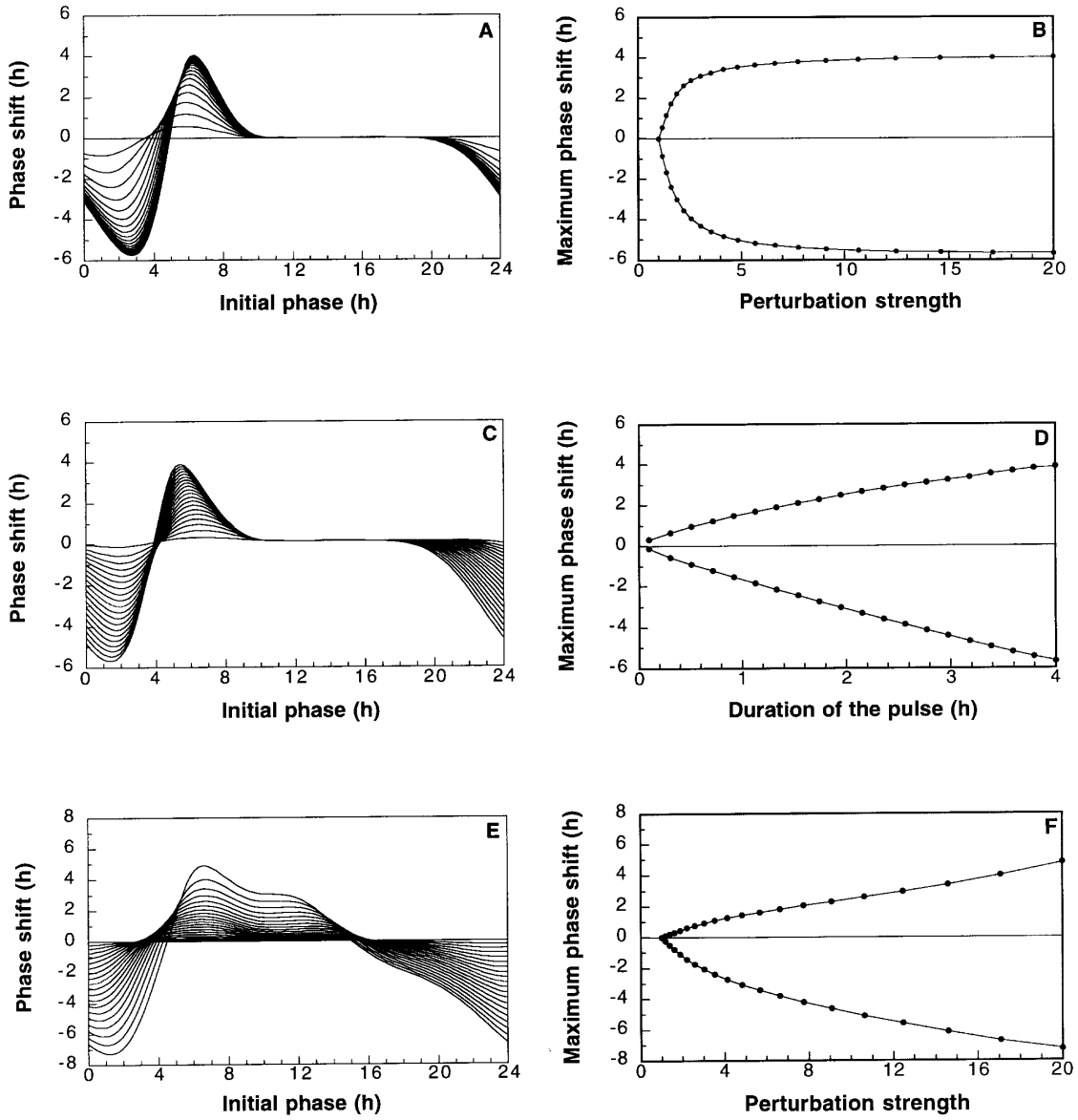

Figure 7. Influence of the magnitude and duration of the effect of a light pulse on the theoretically predicted phase-response curves (PRCs). In panel A, the PRC is obtained at a fixed value of the duration (2 h) as a function of increasing magnitude of the effect of the perturbation, referred to as perturbation strength. The latter is defined as the factor that multiplies the maximum rate v_{dT} of TIM (T_2) degradation in Equation 1h. The perturbation strength varies from 1 (no effect) to 20 (induction of maximum phase shift) by successive multiplications by a factor of 1.1708 so as to yield 20 logarithmically equidistant increments in v_{dT}. The maximum phase advance and phase delay obtained for each value of the perturbation strength are plotted in panel B. In panel C, the theoretical PRC is obtained at a fixed value of the perturbation strength (v_{dT} enhancement factor = 2) as a function of the duration of the effect of the light pulse. This duration varies from 6 min to 4 h by successive increments of 11.7 min. The maximum phase advance and phase delay for each curve are plotted in panel D. In panels E and F, the PRC and the maximum advance or delay are plotted as a function of the perturbation strength (varied as in panels A and B) at a fixed value of 30 min for the duration of the effect of the perturbation. In panels E and F, light is assumed to enhance by a similar factor the degradation of all free and complexed forms of the TIM protein (see text). Parameter values for panels A-D are as in Fig. 2. For panels E and F, the nonspecific linear degradation has been replaced by a specific Michaelian degradation term in the kinetic equations governing the time evolution of all forms of the TIM protein. In these conditions, parameter values for panels E and F have been chosen so as to yield a free-running period close to 24 h: $v_{sP} = v_{sT} = 0.8$ nM h^{-1}, $v_{mP} = v_{mT} = 0.6$ nM h^{-1}, $K_{mP} = K_{mT} = 0.4$ nM, $k_{sP} = k_{sT} = 0.9$ h^{-1}, $v_{dP} = v_{dT} = 2$ nM h^{-1}, $k_1 = 1.2$ h^{-1}, $k_2 = 0.6$ h^{-1}, $k_3 = 1.2$ nM^{-1} h^{-1}, $k_4 = 0.4$ h^{-1}, $K_{IP} = K_{IT} = 1$ nM, $K_{dP} = K_{dT} = 0.4$ nM, $n = 4$, $K_{1P} = K_{1T} = K_{2P} = K_{2T} = K_{3P} = K_{3T} = K_{4P} = K_{4T} = 1$ nM, $V_{1P} = V_{1T} = 4$ nM h^{-1}, $V_{2P} = V_{2T} = 1$ nM h^{-1}, $V_{3P} = V_{3T} = 4$ nM h^{-1}, $V_{4P} = V_{4T} = 1$ nM h^{-1}. Moreover, the Michaelis constant and maximum rate for degradation of the forms T_0, T_1 and C, C_N are given the values 0.5 nM and 0.1 nM h^{-1}, respectively. For symmetry reasons, we also consider a Michaelian rate of degradation for P_0 and P_1 characterized by the same parameter values.

this initial phase corresponds to the onset of the subjective night (Fig. 4E). The occurrence of a phase advance or phase delay is determined by comparing the position of the fourth minimum in *per* mRNA (M_P) after time zero in the perturbed and unperturbed systems.

To determine the phase shifts induced by a pulse of light, we must make assumptions for the effect of a 1-min pulse as to both the factor by which it increases the rate of TIM degradation and the duration of this enhancement. The effect of the magnitude and duration of the enhancement are considered in Fig. 7. Here, turning to the experimental observations of Fig. 6A, we find that these observations can well be accounted for by the model (Fig. 6D) when assuming that the degradation of T_2 is multiplied by a factor of 2 and that this enhancement in TIM degradation lasts 3 h. For the simulation of the wild type (solid line in Fig. 6D), a phase delay of up to 4 to 5 h is obtained between 0 and 4 h; this is followed by a phase advance of up to 3 h between 4 and 10 h. After that, between 10 and 19 h, no phase shift is obtained; a phase delay is observed thereafter, which merges with the delay occurring between 0 and 4 h. Going back to Fig. 6B, we see that a phase delay is obtained when the increase in TIM degradation triggered by light occurs during the rising phase of TIM (and PER); conversely, a phase advance is obtained when the increase in TIM degradation occurs when TIM is close to its maximum value. The absence of phase shift corresponds to the times at which TIM is near its minimum; the effect of an increase in TIM degradation should indeed be negligible at such phases of the oscillations.

To simulate the situation corresponding to the per^s mutant, we consider an enhanced rate of degradation of the nuclear PER-TIM complex according to the results of Curtin et al. (1995). The simulation (dashed line in Fig. 6D) yields good agreement with the experimental data redrawn in Fig. 6A; both in the experiments and in the model, what is most affected is the duration of the "dead zone" in which no significant phase shift is obtained. A comparison of Figs. 6B and 6C shows that the reduction of the dead zone originates from a shortened phase in which PER and TIM are near a minimum because both the decrease in the two proteins to their minimum and their subsequent rise are more rapid in the conditions of Fig. 6C.

The results of Fig. 6D indicate that a twofold increase in TIM degradation rate lasting at least 3 h can account for the experimental observations on phase shift by light. However, the magnitude and duration of the light-induced enhancement in TIM degradation remain unknown. Therefore, it is useful to investigate the effect of both quantities on the phase shifts predicted by the model. We have first obtained the theoretical PRC for an enhancement factor ranging from 1 to 20, increasing in 20 logarithmically equidistant steps (to this end, each value of the enhancement factor is multiplied by 1.1708), at a given duration (2 h) of the stimulus effect. In Fig. 7A are shown the 20 PRCs obtained for increasing the enhancement factor from 1 (no effect of light, i.e., zero phase shift) to 20 (maximum phase shift). In Fig. 7B are shown the maximum phase advance and phase delay obtained for each of the 20 values of the enhancement factor referred to as "perturbation strength." Each curve of Fig. 7A is thus represented by two points in Fig. 7B.

Fixing the perturbation strength by considering an enhancement factor of 2, we then determine the phase shifts produced when increasing the duration of the effect of the perturbation in 20 steps of 11.7 min each; the first and last values of the duration considered are equal to 6 min and 4 h. As in Fig. 7B, the maximum advance and delay corresponding to each curve of Fig. 7C are shown in Fig. 7D. The results of Figs. 7A-D indicate that the magnitude of the phase shifts increases with both the enhancement factor and the duration of the effect of perturbation by light; however, the increase with the perturbation strength (Fig. 7B) is more rapid and saturates, whereas the increase with the duration of the inhibitory effect is more progressive (Fig. 7D).

The results of Figs. 7A-D have been obtained assuming that only the degradation of the fully phosphorylated form T_2 is triggered by light. If we assume that light induces the degradation of all forms of the TIM protein (T_0, T_1, T_2, C, and C_N), then the model predicts that the PRC is significantly altered (Fig. 7E); the dead zone in which no phase shift occurs progressively shrinks as the perturbation strength increases in the manner described for Fig. 7A. Here the parameters of the model are chosen so as to yield a period close to 24 h when a specific Michaelian degradation term is included in the evolution equations for all forms of TIM. The duration of the effect of the perturbation is set to 30 min; too large phase shifts are indeed obtained when the duration is of the order of 2 h as in Figs. 7A-D.

Advances or delays of up to 12 h have been observed in *Drosophila* subjected to 6-h pulses of light, together with a transition of the PRC from Type 1 (weak perturbation) to Type 0 (strong perturbation) (Saunders et al., 1994). Such a transition occurs at

increased duration of degradation of all TIM forms in the conditions of Fig. 7E and also is observed in the model when further increasing the duration of the enhanced degradation of the form T_2 in the conditions of Fig. 7A or the perturbation strength in the conditions of Fig. 7C.

DISCUSSION

Recent experimental observations indicate that besides PER, a second protein, TIM (product of the *tim* gene), plays a crucial role in the generation of circadian rhythmicity in *Drosophila* (Sehgal et al., 1994; Vosshall et al., 1994; Gekakis et al., 1995; Rutila et al., 1996). The PER and TIM proteins form a complex that migrates into the nucleus and represses the expression of the *per* and *tim* genes; moreover, light induces the rapid degradation of TIM and thereby controls the phase of the circadian rhythm (Hunter-Ensor et al., 1996; Lee et al., 1996; Myers et al., 1996; Zeng et al., 1996). Taking into account these observations, we have extended the model for circadian oscillations based on regulation exerted by PER alone (Goldbeter, 1995, 1996) to incorporate the formation of the PER-TIM complex.

The characteristics of sustained oscillations in the extended 10-variable model are similar to those obtained in the minimal model considered previously. The comparison of the two models in the presence and absence of phosphorylation indicates that both the formation of the complex between PER and TIM and the phosphorylation of the two proteins (or one of them) favors sustained oscillations by introducing time delays in the negative feedback loop regulating gene expression. The favorable effect on oscillations, also observed for the degree of cooperativity of repression, is reflected in each case by an enlargement of the domain in parameter space in which sustained oscillations occur. The fact that circadian oscillations appear to be favored when the regulation of gene expression is exerted by a complex between the PER and TIM proteins rather than by a single protein is of particular interest because it suggests a possible evolutionary basis for such a heterodimeric mode of regulation.

The fact that oscillations still may occur in the model in the absence of phosphorylation does not imply that the prevention of PER and/or TIM phosphorylation should necessarily preserve circadian oscillations. This should be the case only if the entry of the PER-TIM complex into the nucleus does not require phosphorylation of either PER or TIM or of both proteins and if the corresponding nuclear complex is capable of regulating the expression of the two genes.

Quantitative information on the values of most of the model parameters is not yet available from the experiments. Thus, parameter values were chosen somewhat arbitrarily, in a plausible physiological range, to obtain a period of oscillations close to 24 h as well as an appropriate time course for the different variables, as shown in Figs. 2 and 4. As experimental values for the parameters become available, these should be incorporated into the model to improve the comparison with experimental results. The present model might prove useful in helping to pinpoint the parameters for which a quantitative estimate is needed. When taking parameters that yield a period close to 24 h, we have shown that the model produces the appropriate relative time courses for PER and TIM and their mRNAs in conditions both of continuous darkness and of entrainment by a 12:12 LD cycle.

Simulations of the model have been performed in symmetrical conditions for PER and TIM (Fig. 2) and also in conditions where an asymmetry between the parameters governing the evolution of *per* and *tim* transcripts is taken into account (Fig. 4). Besides accounting for observed differences in the levels of the *per* and *tim* mRNAs, the simulations in the asymmetric case yield a result that may appear at first as counterintuitive; although the control of the expression of the two genes is governed by the same molecular species (i.e., the nuclear form of the PER-TIM complex), phase differences in the levels of *per* and *tim* mRNAs and also between the PER and TIM proteins nevertheless arise, owing to differences in the rates of synthesis or degradation of the two gene transcripts. A similar result would likely occur in the case of differences in the rates of synthesis or turnover of the two proteins. Recent reports of such phase differences (see, e.g., Marrus et al., 1996) suggest that asymmetries do exist between the parameters characterizing the two branches of the *Drosophila* circadian control system that converge with the formation of the PER-TIM complex.

A difference between the extended model involving PER and TIM and the simpler model based on PER alone pertains to the dependence of the period on the rate of protein degradation. In the model based on PER alone (Goldbeter, 1995, 1996), the period was shown to rise on increasing parameter v_d, denoted v_{dP} in Equation 1d. A similar result can be obtained in that model with respect to the nuclear degradation rate of PER. Here, by contrast, as shown in Fig. 3A, when increasing the linear rate of degradation of the nuclear

PER-TIM complex measured by the rate constant k_{dN}, the period either decreases as k_{dN} increases (curve *a*) or passes through a minimum (curve *b*) depending on the maximum rates of dephosphorylation. The decrease in period as a function of the rate of degradation of the nuclear PER-TIM complex in curve *a* could account for the shortening of the period in the mutant *per*^{s}, as suggested by Curtin et al. (1995). Thus, the model shows that it can be very difficult to predict, on intuitive grounds only, what will be the effect of a parameter such as the rate of degradation of the nuclear PER-TIM complex. This is because a given parameter may have antagonistic effects on the period, depending on the value of other parameters. Increasing the rate of degradation of the PER-TIM complex should, at first view, enlarge the period because it should delay the time at which this complex reaches the level at which it represses the expression of the *per* and *tim* genes. (Such an effect is seen in the right part of curve *b*.) Conversely, a larger level of the PER-TIM complex obtained with smaller values of k_{dN} should depress more considerably the transcription of the two genes and, therefore, will enlarge the time needed to build up PER and TIM to levels required for significant repression. This opposite effect of k_{dN} on the period can be seen in curve *a* and in the left part of curve *b*.

In the present model, PER and TIM possess only a negative regulatory effect on the expression of the *per* and *tim* genes. However, when nuclear localization of PER is blocked by mutation in *per* (Hardin et al., 1990) and *tim* (Vosshall et al., 1994) or by constant light (Qiu et al., 1996), the levels of *per* mRNA and of PER remain constant at a median level. By contrast, a large level of *per* transcript and of PER would be expected in the absence of PER entry into the nucleus and of the associated repression of gene expression. These observations suggest that some additional regulation is at work, perhaps involving other nuclear factors (Qiu et al., 1996). In the model, we observe, accordingly, a large increase in *per* and *tim* mRNAs and in PER and TIM when the formation of the PER-TIM complex is prevented; this is achieved by setting parameter k_3 equal to zero in Equations 1d, 1h, and 1i. We find that the levels of PER and TIM and of their gene transcripts can be brought down at a median level by assuming that the fully phosphorylated monomeric form of PER (P_2) is capable of entering the nucleus at a reduced rate and of exerting a mild inhibiting effect on *per* and *tim* expression compared to the larger rate of entry and stronger inhibition exerted by the PER-TIM complex.

The extended model based on the formation of a PER-TIM complex accounts well for the light-induced phase shifts observed experimentally in *Drosophila* when taking into account the effect of light on TIM degradation (Hunter-Ensor et al., 1996; Lee et al., 1996; Myers et al., 1996; Zeng et al., 1996). The model indicates that to obtain phase shifts of up to 4 h in response to a 1-min light pulse, as seen in the experiments, the effect of such a pulse on the degradation of TIM should be long lasting, of the order of 3 h for a twofold increase in the TIM degradation rate. We have used the model to show (Fig. 7) that the characteristics of the PRC, such as the maximum delay and maximum phase advance, are governed by both the duration of the effect of the light pulse and the enhancement of light-induced TIM degradation. In Figs. 6A,D, we compared the theoretically predicted PRCs to the experimental curves obtained in the wild type and *per*^{s} mutant of *Drosophila*. The model accounts for both curves and provides an explanation for the reduced dead zone observed in the PRC for the *per*^{s} mutant. Good agreement is obtained when considering that the light-induced enhancement of TIM degradation is twofold and that the effect of the light pulse lasts 3 h. As indicated by Figs. 7A-D, however, this choice certainly is not unique given that a variety of combinations of duration of the pulse effect and magnitude of degradation enhancement could produce similar PRCs.

The PRCs in Figs. 6D and 7A-D were obtained under the assumption that only the degradation of the free, fully phosphorylated form T_2 of TIM is triggered by light. When assuming that light induces the degradation of all free and complexed forms of TIM, the dead zone in which no phase shift occurs is progressively reduced (Fig. 7E) as the perturbation strength increases, whereas the magnitude of the phase shifts is larger at a given value of the duration and strength of the perturbation.

An intriguing result obtained in the extended model, in contrast to what is found in the simpler model based on regulation by PER alone, is that complex oscillatory phenomena can be observed when asymmetries are considered between the synthesis or degradation of PER and TIM and / or their mRNAs. In particular, the model shows that in a certain range of parameter values, two stable oscillatory regimes characterized by distinct periods may coexist in the same conditions, that is, for the same set of parameter values (Fig. 3D). Such a phenomenon, referred to as birhyth-

micity (Decroly and Goldbeter, 1982), has been observed in a number of theoretical models (Goldbeter, 1996) as well as in some chemical oscillatory systems (Alamgir and Epstein, 1983). For other parameter values, the extended model indicates that aperiodic oscillations (i.e., chaos) also may occur (Fig. 5). We plan to examine these complex oscillatory phenomena in more detail in a subsequent publication. It probably is too early to speculate on their possible physiological significance, particularly in view of the reduced range of parameter values in which they occur. Nevertheless, the intriguing question arises as to a possible link between birhythmicity and the phenomenon of rhythm splitting (Pittendrigh, 1960) in which a given rhythm splits into two different physiological rhythms characterized by distinct periods when an organism undergoes a change in environmental conditions. If so, rather than pointing to the existence of two distinct pacemakers, the phenomenon would originate from a single oscillator generating (perhaps in different tissues) either one of two stable rhythmic outputs. Likewise, the question arises as to a possible role for aperiodic oscillations in the emergence of arrhythmic behavior in certain *Drosophila* mutants (Konopka and Benzer, 1971). The latter behavior, however, is generally associated with the loss of function of the *per* or/and *tim* genes (Konopka and Benzer, 1971; Hall and Rosbash, 1988; Sehgal et al., 1994).

The phenomenon of temperature compensation represents one of the most conspicuous properties of circadian rhythms (Pittendrigh, 1954). The conditions for the occurrence of this phenomenon and for its loss in *per* mutant have been investigated theoretically in the simpler model based on regulation by PER (Leloup and Goldbeter, 1997; Hong and Tyson, 1997). It was found that different parameters have antagonistic effects on the period so that comparable changes in the values of these parameters as a function of temperature may compensate each other, resulting in a near constancy of the period. These conclusions on the control of the period of circadian oscillations by the various parameters hold in the present extended model, which takes into account the role of both PER and TIM in the generation of circadian rhythms in *Drosophila*.

The present results shed light on the conditions in which an autoregulatory feedback loop controlling gene expression may lead to circadian oscillations. Given that such a regulatory process has been invoked as a general mechanism for the origin of circadian rhythmicity (Takahashi, 1993), as further illustrated by the case of *Neurospora* (Aronson et al., 1994; Crosthwaite et al., 1997), the present theoretical model could be of value for describing the mechanism of circadian oscillations in various organisms besides *Drosophila*.

A recent report (So and Rosbash, 1997) points to a possible role for post-transcriptional regulation, for example, through modulation of *per* mRNA turnover, in the circadian oscillatory mechanism in *Drosophila*. That the feedback responsible for oscillations may operate (at least partly) outside the nucleus is also supported by observations in the silkmoth (Sauman and Reppert, 1996). It will be interesting to compare the predictions of the present model based on the control of gene expression with those of models involving different types of post-transcriptional regulation.

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