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Temporal phosphorylation of the *Drosophila* period protein

(circadian rhythms/epitope tag/*per*/post-transcriptional regulation)

ISAAC EDERY*, LAURENCE J. ZWIEBEL†, MARIE E. DEMBINSKA, AND MICHAEL ROSBASH‡

Howard Hughes Medical Institute, Department of Biology, Brandeis University, Waltham, MA 02254

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ABSTRACT **The period gene (*per*) is required for *Drosophila melanogaster* to manifest circadian (≈24 hr) rhythms. We report here that *per* protein (PER) undergoes daily oscillations in apparent molecular mass as well as abundance. The mobility changes are largely or exclusively due to multiple phosphorylation events. The temporal profile of the classic short-period form of PER (PERS) is altered in a manner consistent with the mutant strain's behavioral phenotype. As changes in abundance and phosphorylation persist under constant environmental conditions, they reflect or contribute to a free-running rhythm. We suggest that the phosphorylation status of PER is an important determinant in the *Drosophila* clock's time-keeping mechanism.**

Daily fluctuations in behavioral and physiological phenomena are governed by an endogenous circadian (≈24 hr) pacemaker or clock. Although biological clocks are a pervasive feature of eukaryotic temporal organization and have been intensely studied, very little is known about the biochemical activities that underlie a circadian time-keeping mechanism. Some key insights have resulted from the use of genetic approaches, which have led to identification of putative clock components in several species (recent reviews, see refs. 1–4). Among the best candidates at present is the *Drosophila* period gene (*per*), required for the proper manifestation of circadian rhythms in locomotor activity and eclosion. The original three *per* alleles shorten (per^s; ≈19 hr), lengthen (per^l; ≈29 hr), or essentially abolish (per^{01}) the periodicity of both rhythms (5).

Since *per* is essential for the manifestation of circadian rhythms, identification of its biochemical function and characterization of its regulation should provide significant insight into the mechanisms that underlie biological clocks. Indeed, recent evidence has led us to suggest that *per* gene activity involves the circadian regulation of transcription (6–8). Although the biochemical function of PER has not been directly established, this proposal is supported by the demonstration that *per* transcription is influenced by PER protein activity (6, 7). A more direct effect on transcription is indicated by the observation that PER is a predominantly nuclear protein in the fly head (9), where the circadian pacemaker is located (10–12). Moreover, a recent study showed that PER contains a dimerization motif (termed PAS) shared by several transcription factors (13).

Consistent with a central role in clock function, PER expression itself is subject to circadian regulation. Immunohistochemical studies suggested that PER levels undergo circadian oscillations in abundance (14, 15), and these changes are very likely influenced by daily fluctuations in *per* transcription (7) and *per* mRNA levels (6). There is also indirect evidence for an as yet undefined post-transcriptional mechanism contributing to PER fluctuations (16).

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Biochemical analysis might reveal the nature of the post-transcriptional regulation that influences PER dynamics. It should also allow a direct visualization of PER, which has not been previously reported. To this end, we have characterized PER on Western blots and demonstrated that it undergoes dramatic daily changes in phosphorylation as well as abundance.

MATERIALS AND METHODS

Antibodies. Overexpression of PER was achieved by using Sf9 moth cells infected with a recombinant *per*-baculovirus (ref. 9; J. Rutila, personal communication). Homogenates containing PER were used to immunize female Sprague–Dawley rats (Charles River Breeding Laboratories) as described (9). Sera were affinity purified on a protein G-silica gel column (Genex) according to manufacturer's instructions.

Preparation of Total Fly Head Extract. Approximately 3- to 7-day-old flies were placed in bottles that contained 5% sucrose and 2% agar. Flies were placed in incubators at 25°C, were subjected to 4 days of alternating cycles of 12-hr light followed by 12-hr dark conditions (LD), and on the fourth day were frozen at the indicated times (Figs. 1–3). For each time point ≈50 μl of heads isolated from frozen flies was placed in microfuge tubes and homogenized in 3 vol (relative to heads) of ice-cold HE solution (HE: 100 mM KCl/20 mM Hepes/5% glycerol/10 mM EDTA/0.1% Triton X-100/1 mM dithiothreitol/0.5 mM phenylmethylsulfonyl fluoride/10 μg of aprotinin per ml/5 μg of leupeptin per ml/1 μg of pepstatin A per ml, pH 7.5). The homogenates were centrifuged for 5 min and clarified supernatant was removed to new tubes. Protein concentration was determined using a Coomassie protein assay according to manufacturer's instructions (Pierce) and extracts were analyzed for PER by Western blotting.

Western Blotting. For each time point, equal amounts (μg) of protein (≈50 μg total) from total head extracts were mixed with 2× SDS sample buffer, boiled, and resolved by electrophoresis on 5.7% polyacrylamide (29.6:0.4, acrylamide:bisacrylamide ratio)/SDS gels. Size standards used for molecular mass determination were prestained SDS molecular mass markers from Sigma. Following electrophoresis, gels were electroblotted onto nitrocellulose for 23 min at 0.24 A using a semidry blotting apparatus according to manufacturer's specifications (Bio-Rad). The blot was incubated for 1 hr in blocking solution [5% skim milk (Bio-Rad) in TBST (10 mM Tris·HCl/150 mM NaCl/0.05% Tween 20, pH 7.5)] followed by 2 hr in the presence of B6PER anti-PER serum diluted 1:2000 in blocking solution. Subsequently, the blot was washed in TBST and incubated for 30 min in anti-rat IgG horseradish peroxidase-conjugated antibody (Amersham) di-

Abbreviations: LD, 12-hr light:12-hr dark; DD, total darkness; ZT, Zeitgeber time; PAP, potato acid phosphatase; HA, hemagglutinin.

*Present address: Department of Molecular Biology and Biochemistry, Center for Advanced Biotechnology and Medicine, Rutgers University, Piscataway, NJ 08854.
†Present address: Department of Cell and Developmental Biology, Harvard University, Cambridge, MA 02138.
‡To whom reprint requests should be addressed.

luted 1:3000 in blocking solution. After a further wash in TBST, visualization of PER was performed by chemiluminescence (Amersham) and autoradiography. Typically, exposures were for 10–30 min.

Immunoprecipitation. Head extracts were prepared as described above except that solution HP was used instead of HE [HP: HE containing 1% Triton X-100 (final concentration) and 0.1% SDS]. All subsequent steps were performed at 4°C. Nonspecific interactions were removed by incubating homogenates with 15 μl of a 1:1 slurry of Gammabind Sepharose (Pharmacia). Following a 15-min incubation with constant agitation, samples were centrifuged for 5 min and the clarified supernatant was removed to a fresh tube. Equal amounts (mg) of protein (≈1.5 mg total) were incubated with a 1:3000 dilution of anti-HA (12CA5, Babco, Emeryville, CA) monoclonal antibody. After 4 hr of incubation, 15 μl of a 1:1 slurry of Gammabind-plus Sepharose (Pharmacia) was added for an additional 1 hr. Beads were washed three times with 1 ml of HP, subsequently resuspended in 15 μl of 2× SDS sample buffer, boiled, and analyzed by Western blotting.

Bacterial and Heat-Inducible Expression of PER. Bacterial expression of PER (pET/PER) was done essentially as described (9), except that the entire PER coding region was used. Briefly, a plasmid encompassing the entire coding region of the major form of *per* mRNA (pCDA; ref. 17) was subjected to partial *Nco* I (5′ site; start of translation) and complete *Eco*RI (3′ site) digestion. The released 6.2-kb fragment was ligated directly into pET-11d (Novagen). The resulting plasmid, pET-11d*per*, was transformed into BL21-(DE3)pLysS recipient cells and induced by addition of isopropyl β-D-thiogalactopyranoside to a final concentration of 0.4 mM. Pelleted cells were resuspended in 2× SDS sample buffer and boiled. Heat-inducible expression of PER in flies (HspPER) was achieved by generating a new set of transgenic lines bearing a cDNA copy of *per* fused downstream of the heat shock 70 promoter (18). To induce HspPER, unentrained 3- to 7-day-old flies (we used the transgenic line designated per^{01};*hspc*-23a) were heat shocked for 1 hr at 37°C in a water bath and allowed to recover at 25°C for 1 hr. Head extracts were prepared, incubated with 2× SDS sample buffer, and boiled.

Phosphatase Treatment. Immunoprecipitation with anti-hemagglutinin (anti-HA) antibodies was as described above. Following the final wash in HP, the beads were equilibrated with 2× 1-ml washes in PAP solution (100 mM Mes/0.5 mM dithiothreitol/0.2 mM phenylmethylsulfonyl fluoride/20 μg of aprotinin per ml/10 μg of leupeptin per ml/10 μg of pepstatin A per ml, pH 6.0) and resuspended in 35 μl of PAP solution. Phosphatase treatment was performed by adding 2 units of potato acid phosphatase (PAP; Boehringer Mannheim; resuspended in 10 μl of PAP solution) directly to the slurry and further incubated for 30 min at 37°C. Subsequently, samples were placed on ice and washed with 2× 1 ml of ice-cold HP. Washed beads were resuspended in 30 μl of 2× SDS sample buffer, boiled, centrifuged, and the supernatant was directly analyzed by Western blotting using B6PER antibodies.

RESULTS

Temporal Changes in the Apparent Size of Wild-Type PER. Numerous past attempts to visualize wild-type PER by blotting have failed (I.E. and L.J.Z., unpublished data). A likely explanation is that PER levels are low and not biochemically detectable by any of the previously reported anti-PER antibodies. We therefore immunized rats with PER isolated from a baculovirus expressing system in the hope of generating better anti-PER antibodies. One such serum, termed B6PER, was found to be at least 10 times more sensitive than a previously reported anti-PER antibody (anti-EcoPER; ref. 9) when assayed by Western blotting (data not shown). Based on this initial result, we were encouraged to try the B6PER antibody to probe homogenates prepared from wild-type fly heads. Using the very sensitive chemiluminescent technique, we were able to detect a specific immunoreactive band migrating at ≈180 kDa (data not shown; see below).

Three criteria indicate that the ≈180-kDa band corresponds to the product of the *per* gene. (*i*) No signal was detected in extracts prepared from flies that carry the per^{01} mutation, which contains a stop codon within the PER open reading frame (refs. 19 and 20; data not shown; Fig. 1*A*, compare lanes 7 and 8). (*ii*) We assayed PER levels as a function of ZT (time in an alternating light:dark cycle; Fig. 1 *A* and *B*). Wild-type flies were entrained in the presence of alternating cycles of LD (where ZT12 is defined as onset of dark period and ZT0 as onset of light period), and head extracts were directly assayed by Western blotting with B6PER antibodies. As predicted from the earlier immunohistochemical results (14, 15), PER levels oscillate in a daily manner and reach maximal levels just prior to the onset of the light period (e.g., Fig. 1*A*, lane 6). Minimal levels occurred between ZT6 (lane 2) and ZT12 (lane 3), in excellent agreement with the histochemical results of Zerr *et al.* (15). (*iii*) As an independent indication that the observed band and fluctuations were due to PER, we assayed a transgenic strain bearing an epitope-tagged version of PER (HA/C; ref. 21). Previous studies showed that a hybrid protein containing a HA peptide at the carboxyl terminus of PER (HA/PER) had essentially indistinguishable biological activity from wild-type PER with respect to circadian rhythms (21). HA/PER was immunoprecipitated from head extracts with an anti-HA antibody (12CA5) and visualized by incubating Western blots in the presence of B6PER antibody (Fig. 1*C*). The fusion protein undergoes oscillations similar to those of wild-type PER (compare Fig. 1*C* to 1*A* and 1*B*), and no signal was

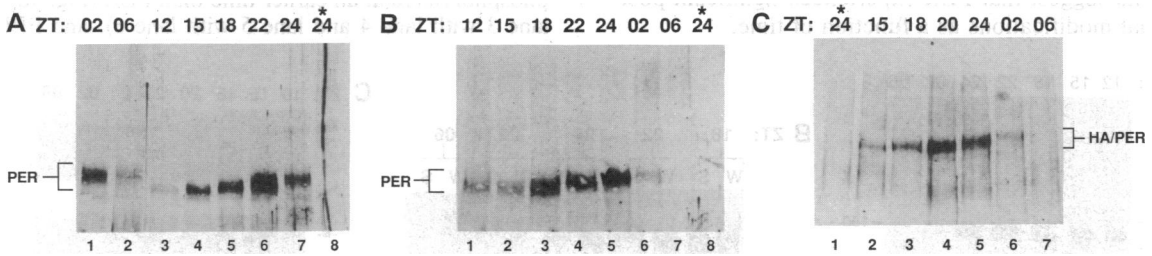

FIG. 1. Time-dependent changes in mobility and abundance of *per* protein (PER). (*A*) Total head extracts were prepared from either LD-entrained wild-type (lanes 1–7) or per^{01} (lane 8, *) flies, frozen at the indicated times, and analyzed for PER by Western blotting using anti-PER antibodies. (*B*) Identical to *A* except an independent experiment is shown. (*C*) Total head extracts were prepared from either HA/C (lanes 2–7) or wild-type (lane 1, *) flies frozen at the indicated times, immunoprecipitated with anti-hemagglutinin (anti-HA) antibodies, and subsequently analyzed by Western blotting in the presence of anti-PER antibodies. The Zeitgeber time (ZT, hours) of fly collection is shown at the top and the size range of PER (*A* and *B*) and HA/PER (*C*) is indicated to the left and right of each panel, respectively. Comparisons of head extracts prepared at the different time points (*A* and *B*) did not reveal any differences in total protein staining (I.E., unpublished data).

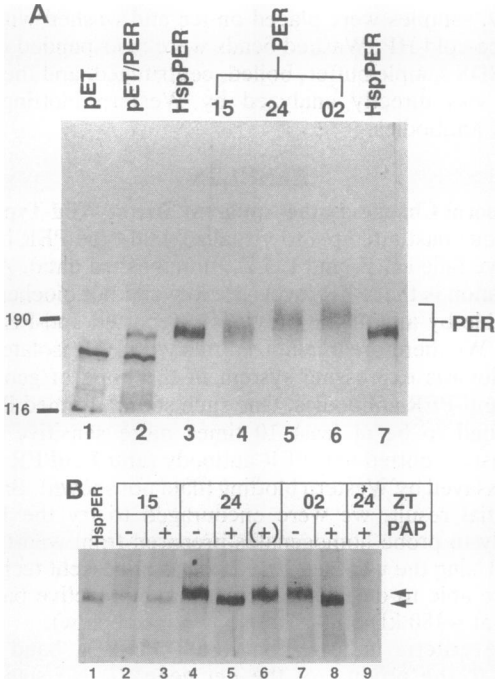

FIG. 2. Temporal mobility shifts of wild-type PER are due to phosphorylation. (*A*) Comparative Western blot of PER expressed in different systems. The different sources of PER loaded in each lane are shown at the top of the gel (lanes 1–7); extracts were prepared as described in the text. For wild-type PER (lanes 4–6) approximately equal immunoreactive PER material was loaded (total protein; ZT15, 200 μg; ZT24, 50 μg; ZT02, 80 μg), and the time (ZT, hours) of fly collection is indicated (top). The size ranges of the different sources of PER are indicated to the right. Migration of molecular mass markers (in kDa) is indicated to the left. (*B*) Total head extracts from either HA/C (lanes 2–8) or wild-type (lane 9, *) flies were immunoprecipitated with anti-HA antibodies. Treatment of washed immune complexes with PAP was performed as described in the text. ZT of fly collection, and either addition of PAP (+) or its absence (−) are indicated (top). Prior to addition of PAP, the phosphatase inhibitor sodium phosphate (pH 7.2) was added to a final concentration of 100 mM (lane 6). At these times of collection and treatment, HA/PER migrates with essentially two distinct sizes (indicated by arrows at right). For comparative purposes, a sample of HspPER (lane 1) was also included.

detected in wild-type-derived extracts that were assayed in an identical manner (Fig. 1*C*, lane 1).

In addition to the oscillations in PER levels, the apparent size of PER dramatically increases between its initial appearance (ZT12; Fig. 1*A*, lane 3) and disappearance (ZT06; lane 2). This mobility shift was very reproducible, as only minor variations in pattern were observed in >10 independent experiments (compare Fig. 1*A* to 1*B*; data not shown). The observations suggest that PER experiences significant post-translational modifications as a function of time.

Apparent Size Changes Are Due to Phosphorylation. The magnitude of the mobility shifts is further indicated by comparing PER synthesized from various sources (Fig. 2*A*). Bacterially expressed PER (pET/PER; compare lanes 2 and 1) migrates with an apparent molecular mass of ≈150 kDa, somewhat larger than its predicted molecular mass of 127 kDa (17). HspPER, derived from flies bearing a heat-inducible copy of *per*, migrates marginally slower than pET/PER (compare lanes 3 and 7 to lane 2). To facilitate direct comparisons, we analyzed approximately equivalent amounts of immunoreactive wild-type PER from several key times (lanes 4–6). The results suggest that PER is less extensively modified at ZT15 (lane 4) and highly modified at ZT24 (lane 5) and ZT02 (lane 6), with apparent molecular masses from ≈155 kDa to ≈190 kDa. Moreover, it appears that particular size ranges of PER are restricted to specific times, or nearly so.

To investigate the possibility that the mobility changes involve phosphorylation, head extracts containing HA/PER were immunoprecipitated, treated with PAP, and subsequently assayed by Western blotting (Fig. 2*B*). For each time point analyzed, PAP treatment resulted in faster migrating HA/PER (compare lanes 2, 4, and 7 to lanes 3, 5, and 8, respectively). The mobilities of all PAP-treated samples were essentially indistinguishable from each other (compare lanes 3, 5, and 8) and from HspPER (lane 1), indicating that the phosphatase reaction went to completion and that dephosphorylated wild-type PER has an apparent molecular mass very similar to that of bacterially expressed PER. The PAP-induced mobility shift was not due to contaminating proteases, since a phosphatase inhibitor (sodium phosphate) completely blocked any noticeable size shift (compare lanes 5 and 6). The results indicate that most, and perhaps all, of the time-dependent size increase in wild-type PER is due to phosphorylation. As no higher molecular mass forms are detectable in the wild-type extracts, it is highly unlikely that PER is subject to other extensive post-translational modifications; hence, it is not a proteoglycan as suggested (22, 23).

Temporal Changes in Abundance and Phosphorylation Are Altered in *per* Mutants. To investigate further the extent to which the temporal changes in the abundance and phosphorylation of PER correlate with clock function, we assayed extracts derived from the classic short-period mutant (per^s). Under standard LD conditions, these mutant flies maintain 24-hr periods but manifest a substantial (≈3 hr) phase advance of their evening activity peak (24). Consistent with these behavioral observations, the general pattern of the mutant protein (PERS) is similar to that of the wild-type protein, but the abundance and phosphorylation changes of PERS occur earlier than those of the wild-type protein. For example, PERS has accumulated to substantial levels by ZT12, a time at which little wild-type protein has accumulated (e.g., compare Fig. 3*A*, lane 1, with Fig. 1*A*, lane 3). A greater proportion of PERS is extensively phosphorylated at an earlier time than PER (Fig. 3*B*, compare lane 3 with lane 4 and lane 5 with lane 6), and PERS levels

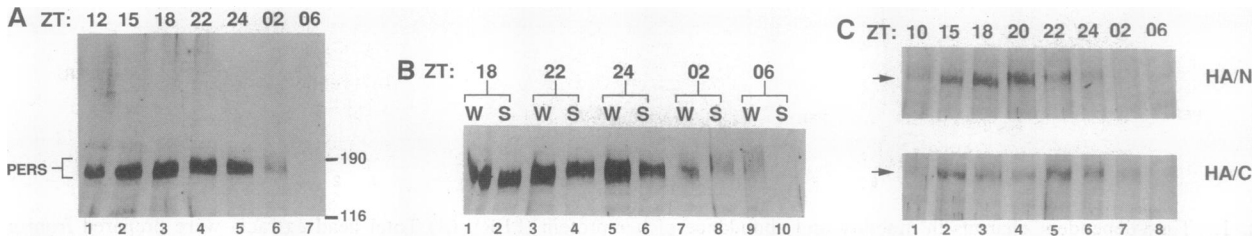

FIG. 3. Altered temporal changes in the abundance and phosphorylation of short-period mutants of PER. Total head extracts were prepared from flies maintained under LD conditions and analyzed by Western blotting. The time of collection (ZT, hours) is indicated at the top of each panel. (*A*) per^s flies were assayed and the size range of PERS is indicated to the left. (*B*) Separate bottles containing either wild-type (W) or per^s (S) flies, as indicated at the top, were incubated together during LD and assayed at the same time. (*C*) Either HA/N or HA/C flies, as indicated at the right, were maintained and assayed as in *B*. The HA-tagged versions of PER are indicated to the left by arrows.

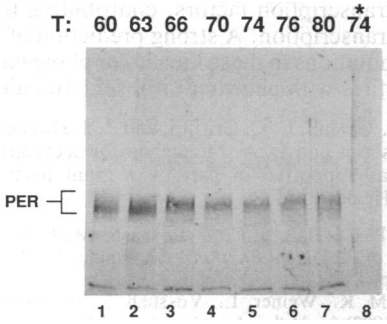

FIG. 4. Temporal phosphorylation of PER continues under constant environmental conditions. Upon completion of 4 days of LD, flies were maintained in DD for 2 days. On the third day of DD, flies were frozen at the indicated times (T, hours) starting 60 hr after completion of the fourth LD cycle. Head extracts were prepared from either wild-type (lanes 1–7) or per^{01} (lane 8, *) flies and analyzed by Western blotting. The size range of PER is indicated to the left. The faint discrete band just below PER was detectable in lane 8 on the autoradiogram but not on the photograph, due to underloading and smearing of this sample. It is due to a cross-reacting protein, as it is always detectable independent of PER.

decrease at earlier times—i.e., PERS levels are decreasing by ZT22, well before a decrease is observed for PER (Fig. 3*B*). The observed phase advance in the mutant protein's abundance fluctuations is in close agreement with previous histochemical results (15). It is also consistent with a previous study indicating that per^s mRNA cycling is phase advanced under identical LD conditions (6).

These observations were verified by analyzing an independent short-period strain, which expresses a hybrid version of PER modified at its amino terminus with a HA epitope tag (HA/N). For unknown reasons, these flies have circadian rhythms with ≈21-hr periods, compared to the essentially wild-type rhythms (≈24-hr periods) of the HA/C strain described above (21). A comparison of extracts from these two strains (Fig. 3*C*) indicates that the HA/N profile is phase advanced relative to the HA/C profile—e.g., peak levels of the HA/N protein are reached at ZT20 (lane 4), as compared to ZT22 for the HA/C version (lane 5). In sum, the biochemical data suggest that the protein fluctuations, including the phosphorylation changes, contribute to circadian clock function (see *Discussion*).

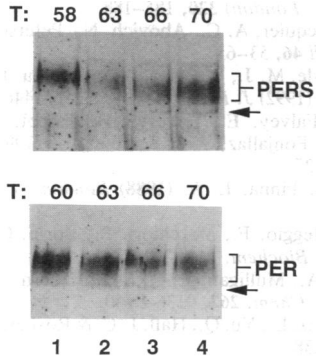

FIG. 5. The temporal phosphorylation changes in PERS are consistent with the mutant endogenous rhythm. Either per^s (*Upper*) or wild-type (*Lower*) flies were subjected to identical environmental conditions as described in the legend to Fig. 4 except that, in the case of per^s flies, flies were frozen starting 58 hr after completion of the fourth LD cycle. Total head extracts were analyzed by Western blotting. The time of collection (T, hours) is indicated at the top of each panel and the size ranges of PERS and PER are indicated to the right. To facilitate comparison of the mobility shifts, a background reference band is indicated (arrows).

Temporal Phosphorylation of PER Continues Under Constant Darkness. Importantly, the time-dependent changes in PER levels and phosphorylation status persist in constant conditions [total darkness (DD); Fig. 4]. As the changes in PER levels are less dramatic than in LD (Fig. 1), they appear to have been damped during the 3 days of DD. This is consistent with histochemical observations of PER fluctuations under free-running conditions (15). It is also consistent with an earlier report demonstrating that the amplitude of *per* mRNA cycling is damped in DD (6), suggesting that the RNA and protein fluctuations are correlated. The amplitude damping may result from desynchronization under constant conditions and/or a more direct, positive effect of the recurring light stimuli on the robustness of the molecular oscillations. In any case, the differential phosphorylation of PER persists under free-running conditions and is therefore likely subject to circadian regulation.

Since per^s flies have ≈19-hr rhythm periods in DD (5), it was of interest to determine if the PERS temporal changes would also parallel the mutant phenotype under these conditions. To optimize sensitivity, we incubated wild-type and mutant flies for 3 days in DD before taking time points for extract preparation; as the mutant strain "gains" 5 hr/day relative to the wild-type strain, they should be ≈180° out of phase after 3 days. The results indicate that PERS experiences a decrease in mobility at the same time that PER undergoes a mobility increase—i.e., the oscillations of the two populations are indeed ≈180° out of phase (Fig. 5). A similar strategy was previously applied to per^s mRNA cycling with similar results (6). We conclude that the temporal changes in PERS phosphorylation under constant conditions (DD) correlate well with the altered time-keeping mechanism that underlies the rhythmic behavior of this mutant strain.

DISCUSSION

A previous report from this laboratory indicated that a post-transcriptional regulatory mechanism is likely to contribute to *per*'s regulation (16). The observation that the abundance of PER is maximal ≈4–6 hr after the peak of *per* mRNA levels (i.e., ZT14-16; ref. 6) also suggests that a strictly transcriptional mechanism cannot be solely responsible for the regulation of PER [see Wuarin *et al.* (25) for a theoretical discussion of the general relationship between mRNA cycling and protein cycling]. The results in this report provide direct evidence that at least part of this post-transcriptional regulation is at the level of phosphorylation, which is likely to influence PER's metabolism or activity (Fig. 6). At present, it is impossible to estimate the relative contributions of this post-translational regulation and of transcriptional regulation to the observed fluctuations in PER levels.

The apparently continuous size increase of PER between ZT12 and ZT2 (Fig. 1) is possibly due to multiple, sequential phosphorylation events—i.e., the phosphorylation of certain sites may be dependent upon the prior phosphorylation of others. Similar interdependent phosphorylation pathways have been documented in other systems (26–28). The data also suggest that a defined hyperphosphorylated form(s) of PER might act as a signal for degradation. Although PER's complex spatial expression pattern (14, 15, 29–31) might introduce tissue heterogeneity into the biochemical observations, we observe very similar temporal changes in isolated eyes, where PER expression is restricted to photoreceptor cells (H. Zeng and M.R., unpublished data). Since a large number of different potential phosphorylation sites are present in PER (I.E., unpublished data), it is uncertain which of these are modified and how many different kinases participate in these phosphorylation events.

A strikingly similar mechanism has been proposed for the regulation of the FAR 1 gene during the cell cycle of the budding yeast, *Saccharomyces cerevisiae*. The FAR 1 pro-

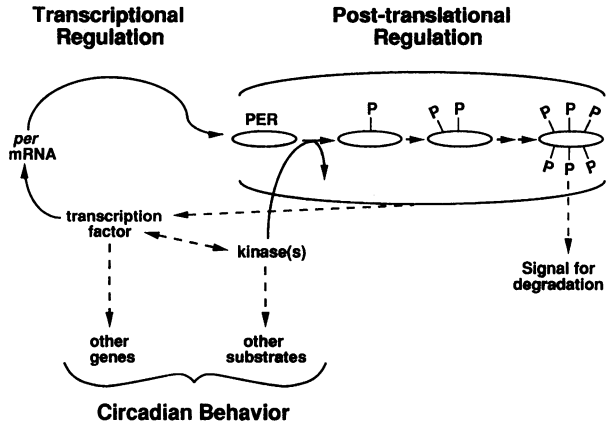

FIG. 6. Model for *per* function and regulation. At least two regulatory pathways are likely to contribute to fluctuations in *per* protein levels. One pathway is a PER-dependent transcriptional loop that results in daily oscillations in *per* mRNA. PER may directly modulate the activity of a transcription factor as described in the text. Another is a post-translational pathway that involves extensive, sequential phosphorylation of PER. A particular phosphorylated species might act as a signal for degradation. In this model PER might either directly or indirectly (for example, by influencing the activity of transcription factors or kinases) affect the output from the clock, resulting in circadian behavior. Solid lines indicate established pathways; dashed lines indicate possible pathways that have not been established.

tein is differentially phosphorylated, and its disappearance is governed largely independently of transcriptional mechanisms (32). More generally, protein phosphorylation and proteolysis are critical components of the feedback loops that are integral to the temporal and directional control of the cell cycle (33–35), implying that circadian clocks and the cell cycle may have similar mechanisms, if not components.

The biochemical profiles of the short-period strains correlate with their altered behavioral rhythms (Figs. 3 and 5). Western blots of extracts from the long-period per^{l} mutant also correlate with unique features of its rhythms and show substantial differences from those of the short-period or wild-type strains (M.E.D., unpublished data). These results are, however, somewhat more complicated than those from the short-period strains and, as a consequence, will be presented elsewhere.

In all cases, the observations on the mutant strains suggest that biochemical temporal profiles contribute to the clock mechanism. This implies the existence of feedback loops between other components of the time-keeping machinery and PER, which give rise to the observed oscillations in abundance and phosphorylation state. We imagine two classes of models by which PER might affect its own temporal profile. (*i*) The mutant proteins might have altered activity, resulting, for example, in the altered regulation of a kinase, which causes in turn an altered regulation of PER's phosphorylation. PER might contribute to the circadian regulation of some of the relevant modifying enzymes at the transcriptional level (cf. ref. 36). (*ii*) The activity of the mutant protein may be identical to that of wild-type PER but interact differently with the regulatory machinery—e.g., the short-period proteins may be more favorable substrates for the relevant kinase(s) (Fig. 6). Since the mutation is due to a Ser → Asn change (19, 20), it will be of interest to determine if this site itself is normally phosphorylated.

In either case, the results in this report raise the intriguing possibility that the phosphorylated status of PER is a key clock component: the presence or absence (or the relative ratios) of specific phosphorylated forms of PER could help define circadian time (Fig. 6). For example, PER's phosphorylation status could affect its ability to dimerize with PAS-containing transcription factors, contributing to temporally modulated transcription. A strong prediction of this hypothesis is that mutations in these kinases or phosphatases should give rise to flies with aberrant or absent circadian rhythms.

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