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Transcriptional Feedback and Definition of the Circadian Pacemaker in *Drosophila* and Animals

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The modern era of *Drosophila* circadian rhythms began with the landmark Benzer and Konopka paper and its definition of the *period* gene. The recombinant DNA revolution then led to the cloning and sequencing of this gene. This work did not result in a coherent view of circadian rhythm biochemistry, but experiments eventually gave rise to a transcription-centric view of circadian rhythm generation. Although these circadian transcription-translation feedback loops are still important, their contribution to core timekeeping is under challenge. Indeed, kinases and posttranslational regulation may be more important, based in part on recent in vitro work from cyanobacteria. In addition, kinase mutants or suspected kinase substrate mutants have unusually large period effects in *Drosophila*. This chapter discusses our recent experiments, which indicate that circadian transcription does indeed contribute to period determination in this system. We propose that cyanobacteria and animal clocks reflect two independent origins of circadian rhythms.

INTRODUCTION

Ron Konopka and Seymour Benzer are justifiably credited with initiating genetic studies of circadian rhythms in *Drosophila melanogaster* in 1971 (Konopka and Benzer 1971). They described three alleles of a single gene they called *period* (*per*), with fast clock properties (per^S), slow clock properties (per^L), and no apparent clock at all (per^0). These compelling phenotypes, coupled with the remarkable and fortuitous unfolding of the recombinant DNA revolution during that same decade, inspired a collaboration between my lab and Jeff Hall's to identify the *period* (*per*) gene and its function. Pranitha Reddy, a graduate student in my laboratory, led the effort to clone *per* DNA, and Will Zhering, a postdoc of Jeff's, led the effort to rescue the arrhythmic *per0* phenotype. This was the first rescue of a behavioral gene in any organism and arguably the first gene rescue of consequence that provided bona fide gene identification (Reddy et al. 1984; Zehring et al. 1984). An independent, parallel effort in Mike Young's laboratory at Rockefeller achieved these two goals at the same time (Bargiello and Young 1984; Bargiello et al. 1984).

In those early days of gene identification, the *period* gene product (PER) was a pioneer protein, meaning that its sequence did not provide strong clues about its function. This statement does not do justice to what is a more complicated tale, as it is more accurate to say that the few clues present in the sequence led us and the Young laboratory in a wrong direction, toward proteoglycans (Shin et al. 1985; Jackson et al. 1986; Reddy et al. 1986). However, a finding made principally by Steve Crews, then a postdoc in the Goodman lab at Stanford, raised a different possibility to me and my colleagues at Brandeis. Steve cloned and sequenced the transcription factor *single-minded* (*sim*), which turned out to have homology with PER (Crews et al. 1988). Although the region in question was small and had no known function or contribution to transcription (the domain was subsequently called PAS after the three founding members of the family: SIM, ARNT, and PER; Reyes et al. 1992), it suggested that PER might function to modulate gene expression at the transcriptional level, a very different role from that of proteoglycans.

We spent the next few years examining both hypotheses: proteoglycan function and the regulation of gene expression. The signature motif in PER that connected it with proteoglycans was a GT (glycine-threonine) repeat region. We discovered, however, that this motif was not well-conserved among *Drosophila* species, in contrast with other highly conserved regions of PER (Colot et al. 1988). More importantly, the motif was not necessary for PER function: Transgenic flies carrying a *period* gene with a deletion of this region were perfectly rhythmic (Yu et al. 1987).

Because these results made us doubt the proteoglycan hypothesis, a coherent gene expression picture began to emerge with the discovery that *period* mRNA levels undergo circadian oscillations under constant darkness (DD) as well as normal (LD) conditions. My postdoc Paul Hardin also showed that the phase and period of the *per* mRNA cycling are sensitive to the missense mutations in the *period*-encoded protein that advance, delay (speed up, slow down), or eliminate behavioral rhythms (Hardin et al. 1990). This *per* mRNA regulation was shown to be predominantly transcriptional (Hardin et al. 1992) and led to the proposal that PER inhibits its own gene expression and that this negative feedback loop is central to circadian timing. In a study designed to distinguish between the proteoglycan and the gene expression hypotheses, we collaborated with the Benzer laboratory to assay PER subcellular localization in fly brains by immunoelectron microscopy. The data indicated that PER was predomi-

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nantly nuclear (Liu et al. 1992), a result consistent with a direct role for PER in the transcriptional regulation. Indeed, the PAS domain of PER was shown to be a protein–protein interaction motif (Huang et al. 1993), and we imagined that a PAS-containing transcription factor was directly contacted and inhibited by PER.

This idea, as well as the role of the transcriptional feedback loop in circadian rhythms, was strengthened by the identification and cloning of the PAS-domain-containing *Drosophila* transcription factors CLOCK and CYCLE (Allada et al. 1998; Darlington et al. 1998; Rutila et al. 1998). This aspect of the fly story was preceded by the landmark identification and cloning of the mammalian circadian gene CLOCK (Antoch et al. 1997; King et al. 1997). The connections between flies and mammals were also made in that same year by the discovery of mammalian PERs (Sun et al. 1997; Tei et al. 1997). Taken together, the data indicated that a similar feedback system exists in *Drosophila* and mammals, and this situation is still largely true today.

Transcriptional feedback is also a feature of other circadian systems, much more distantly related to flies and mammals. Four years after the Hardin et al. (1990) feedback paper, a *Neurospora* study described mRNA oscillations of the key circadian gene *frequency* (*frq*) influenced by feedback from the FRQ protein (Aronson et al. 1994). In the two other major genetic systems with bona fide circadian rhythms, plants and cyanobacteria, feedback regulation at the level of transcription is prominent, and key transcription factor mutants affect circadian period in both organisms (Dunlap 1999).

The *Drosophila* transcriptional feedback loop is much more complex than this early work suggested. For example, the landmark identification and characterization of the *timeless* (*tim*) gene, the second clock gene identified in the *Drosophila* system, showed that *tim* mRNA also undergoes circadian oscillations and that TIM is a heterodimeric partner of PER and participates in transcriptional feedback regulation (Gekakis et al. 1995; Myers et al. 1995; Sehgal et al. 1995; Zeng et al. 1996). In addition, many kinases participate in modifying PER and TIM and contribute to circadian timing (Kloss et al. 1998; Price et al. 1998; Lin et al. 2002), a phenomenon recognized in the first cycling western blots of PER (Edery et al. 1994). A similar possibility exists in mammals as well, because kinases clearly have a key role in this system (Lowrey et al. 2000; Gallego et al. 2006).

The importance of phosphorylation to the circadian regulation of transcription has increased in prominence because of some breathtaking in vitro experiments in the cyanobacterial system. Recombinant versions of the three clock proteins KaiA, KaiB, and KaiC were incubated in vitro and shown to undergo circadian oscillations. Evident was an approximately 24-hour cycle of protein–protein associations as well as KaiC phosphorylation and dephosphorylation, which are even temperature-compensated (Nakajima et al. 2005; Tomita et al. 2005). The autokinase and autophosphatase activities of KaiC are integral features of this posttranslational timing system (Nishiwaki et al. 2007; Rust et al. 2007; Terauchi et al. 2007). So a current view is that the massive transcriptional regulation that this protein cycle directs in vivo, including transcriptional oscillations of the three Kai mRNAs, has a predominantly output function (downstream from the pacemaker) and/or its effects on timekeeping are relatively subtle compared to the phosphorylation/dephosphorylation cycle intrinsic to the three Kai proteins.

These stunning experiments force a serious consideration of the possibility that transcriptional regulation may be essential only for circadian output in flies and mammals. In other words, the transcriptional feedback loop may serve primarily to drive the oscillation of the hundreds or thousands of output mRNAs that are under clock control. In this view, the circadian transcription of *per* and *tim* also reflects this output feature and may have no more than a minor influence on the central timekeeping machinery. Is this true? Is the posttranscriptional regulation of PER, TIM (or CRY), CLK, and CYC (or BMAL) the sole key to circadian timing?

Although a definitive answer is lacking at present, there are other indications that the answer for the *Drosophila* system may be yes. A key experiment was reported several years ago by Sehgal and colleagues. Behavioral rhythmicity was reported in a strain in which both PER and TIM are generated from constitutive promoters, i.e., without the possibility of transcriptional feedback on these two key circadian genes (Yang and Sehgal 2001). Although behavioral rhythmicity of this strain was poor, it was clearly present. Moreover, PER oscillations could still be detected by antibody staining within brain circadian neurons (Yang and Sehgal 2001). The inescapable conclusion would appear to be that circadian function can take place without transcriptional feedback on *per* and *tim*, which is almost certainly dependent on the normal *per* and *tim* promoters. This focuses attention on the posttranslational regulation of PER and TIM, the interaction and regulation of the multiple kinases and phosphatases that modify these two key clock proteins. This view is congruent not only with the cyanobacterial system, but also with the fact that the strongest mutants in the fly system, those with the greatest period effects, are in the kinases or are suspected phosphorylation substrate mutants in PER and TIM (Kloss et al. 1998; Price et al. 1998; Lin et al. 2002). Nonetheless, recent work from our lab and others indicates that the core transcriptional feedback loop is important not only for overt behavioral rhythmicity (output) in *Drosophila*, but also for proper period determination as well as circadian amplitude.

This view is based in part on a characterization of a CYC-VP16 fusion protein in the *Drosophila* system (S. Kadener et al., in prep.). VP16 is a potent and well-studied viral transcriptional activator. It imparts to the CLK-CYC-VP16 complex enhanced (>5X) transcriptional activity relative to CLK-CYC, which derives most if not all of its transcription activator activity from CLK. This increase is manifest in tissue culture (S2) cells as well as in flies expressing CYC-VP16. These strains also have increased levels of CLK-CYC direct target gene mRNAs as well as a short period, implicating circadian transcription in period determination (S. Kadener et al., in prep.). The results therefore indicate that the level of transcriptional activation on natural promoters in vivo is sensitive to the

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nature and number of activation regions. The robust behavioral and molecular rhythms of CYC-VP16 flies more generally indicate that CLK-CYC-VP16 circadian function, including the mechanism(s) that temporally activate or repress transcription of this hyperactive complex, must be similar to those that regulate the activity of the wild-type CLK-CYC complex. Because the VP16 activation domain almost certainly functions differently from the CLK poly(Q) region, this indicates that the recruitment of specific activator and/or repressor proteins is unlikely to have a prominent role in the circadian regulation of transcription. A more likely mechanism involves the cyclical inhibition of CLK-CYC DNA binding. Importantly, this notion is consistent with recent chromatin immunoprecipitation results from the mammalian system as well as the fly system (Brown et al. 2005; Yu et al. 2006).

This correlation between increased transcriptional activity of the CLK-CYC complex and period shortening fits with several other pieces of data from the *Drosophila* system. An increase in *per* gene dose leads to flies with short periods. There is a decrease of approximately 0.5 hour for each additional gene copy up to three to four copies, which have an approximately 22–23-hour period (Smith and Konopka 1982; Baylies et al. 1987; Hamblen-Coyle et al. 1992). In addition, a hemizygous deletion that includes *clock* lengthens circadian period by about 0.5 hour (Allada et al. 1998). Although this deletion removes more DNA than just *clock* (including the adjacent clock gene *pdp1*), recent evidence is consistent with the idea that the amount of *clock* mRNA (and level of primary gene transcription) affects circadian period: A transgenic copy of *clock* shortens circadian period of otherwise wild-type flies by about 0.5 hour (J.S. Menet, pers. comm.). These observations are qualitatively similar to those showing the increase in transcription and period shortening caused by expression of CYC-VP16 in flies.

For the reasons described above, the strong effect of CYC-VP16 on period length might be due to a CYC-VP16-mediated change in the timing or level of *per* transcription. To test this possibility, we assayed the period of CYC-VP16-expressing flies in the context of UAS-*per*, i.e., a *period* gene that can be driven by Gal4 but not by CLK-CYC or CLK-CYC-VP16. Importantly, Sehgal and coworkers have previously shown that UAS-PER can rescue the arrhythmic per^{01} genotype with the pan-neuronal *elav*-Gal4 driver (Yang and Sehgal 2001), and we verified this finding (data not shown). Importantly, the *elav*-Gal4 driver in combination with UAS-CYC-VP16 (and a wild-type *per* gene) also manifests the approximately 2-hour period shortening. However, these two transgenes in combination with the UAS-PER and per^{01} fail to appreciably shorten period. This indicates that a major contributor to CLK-CYC-VP16 period shortening is indeed an increase in the levels and/or timing of *per* transcription. We also note the broad distribution of periods in individual flies from genotypes containing the UAS-PER: per^{01} combination compared to the tighter distribution in genotypes containing a *per* promoter; this is an additional indication that the strain without a *per* promoter is highly abnormal and that cyclic *per* transcription contributes to proper period determination.

An additional indication that period shortening is not simply due to an increase in *per* mRNA levels (and PER levels) by the more potent CYC-VP16 molecule is that an increase in PER dose with a UAS-PER transgene slightly increases rather than decreases period (data not shown). This is consistent with literature showing that overexpression of a UAS-*per* transgene does not shorten period (Kaneko et al. 2000; Yang and Sehgal 2001; Nawathean et al. 2007). The simplest interpretation is therefore that the shorter period of CYC-VP16 flies is due to an altered timing of *per* (and *tim*?) transcription. A steeper increase may reflect the enhanced potency of CLK-CYC-VP16, and a steeper decrease may reflect a faster accumulation of active PER repressor. The results more generally suggest that circadian transcription contributes to core circadian function in *Drosophila*.

Yet how are the strains missing the *per* and *tim* promoters rhythmic (Yang and Sehgal 2001)? Because other work in our lab during the past few years has highlighted the importance of brain circuitry to behavioral rhythmicity (Peng et al. 2003; Stoleru et al. 2004, 2005, 2007), we suggest that individual neurons from this per^{01}, *elav*-Gal4; UAS-PER strain might be even more impaired than indicated by the behavioral rhythms of this strain, i.e., circadian brain circuitry might help to compensate for poor core circadian function within individual cells. This is analogous to the superior circadian performance of behavioral rhythmicity and the suprachiasmatic nucleus (SCN) from mutant mouse strains compared to individual tissue culture cells (MEFs) derived from the same mutant strains (Liu et al. 2007). To put this into perspective, we are not certain to what extent individual circadian neurons even from a wild-type strain will manifest free-running rhythms in dissociated culture; to our knowledge, there is no report of such an experiment. On the basis of the importance of the *per* promoter in our recent CYC-VP16 experiments (S. Kadener et al., in prep.), a prediction is that individual dissociated neurons from the *per* promoterless strain will be substantially more impaired than wild-type neurons.

The conclusion that the core *Drosophila* transcriptional feedback loop is important not only for overt rhythmicity (output), but also for circadian amplitude was previously suggested and based on a different mutation in the core clock gene *clock*, Clk^{ar} (Allada et al. 2003). The mutation in this transcription factor gives rise to weak CLK activity, and homozygous Clk^{ar} flies have a reduced transcriptional amplitude of oscillating direct CLK-target mRNAs; this strain is also arrhythmic, even in LD (Allada et al. 2003).

The wider conclusion of the CYC-VP16 studies, the importance of the core transcriptional loop to period determination as well as circadian amplitude, is reinforced by our recent identification and characterization of a new clock gene, *clockwork orange* (*cwo*) (Kadener et al. 2007). It encodes a transcriptional repressor that synergizes with PER and inhibits CLK-mediated activation. Consistent with this function, the mRNA profiles of CLK–direct target genes manifest lower-amplitude oscillations in mutant flies, due predominantly to higher trough values. Because rhythmicity fails to persist in DD and there is little or no effect on average mRNA levels in

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the *cwo*-deficient strain, transcriptional oscillation amplitude appears to be linked to rhythmicity. The *cwo* mutant flies are long period before they become arrhythmic, consistent with delayed repression indicated by the RNA profiles. These findings and others suggest that CWO synergizes with PER to help terminate CLK-CYC-mediated transcription of direct target genes in the late night, a function that contributes to period determination as well as to rhythmicity. In this context of transcription and rhythmicity, it is interesting to note that all five of the validated CLK-CYC–direct target genes (*per, tim, vri, pdp1, cwo*) are known or suspected transcription factors.

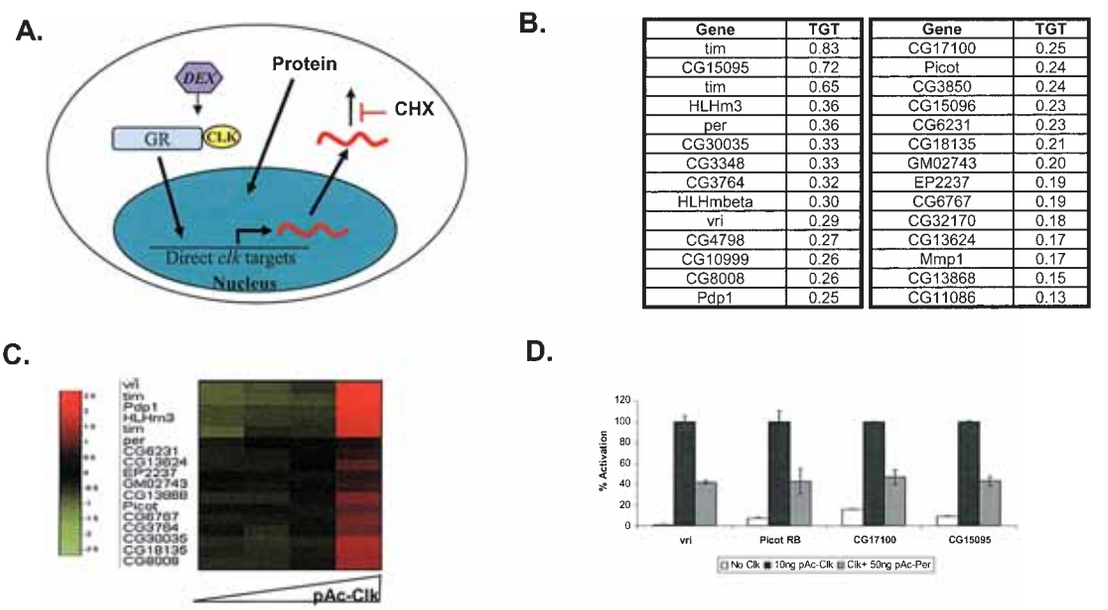
We note that *cwo* was identified and characterized independently in two other *Drosophila* studies (Lim et al. 2007; Matsumoto et al. 2007). Finally, the presence of two *cwo* orthologs in mammals (*Dec1* and *Dec2*) suggests a similar synergistic repression mechanism in this system, which would then be a collaboration between DEC and the CRY-PER repressor (Honma et al. 2002).

There is, however, an additional possibility for *cwo* function, as well as for the likely mammalian orthologs *Dec1* and *Dec2*. This is based on the many new candidate CLK-target genes in fly heads identified by the CLK-GR strategy (Fig. 1A,B). Intriguingly, a significant fraction of these genes are nonoscillating based on microarray studies of fly head RNA (Kadener et al. 2007). However, the S2 cell assays predict that most of them are bona fide CLK targets rather than a CLK-GR artifact (Fig. 1C,D); they may predominantly reflect *clk* function in noncircadian cell types. Indeed, a recent study reported that CLK expression is not restricted to circadian neurons in the fly brain (Houl et al. 2006).

This idea of noncircadian cell CLK function follows from the characterization of these new CLK–direct target genes (Kadener et al. 2007). For example, a clue to the difference between these new genes and canonical target genes is their response to the CWO repressor protein. In our hands, canonical clock genes respond to CWO addition but with only a modest reduction in CLK-CYC-mediated transcription (Kadener et al. 2007). This is exemplified by the *luc* reporter gene driven by the *tim* promoter (Fig. 2). (We suspect that the different results of Ueda and colleagues [Matsumoto et al. 2007], the apparent strong repression of CWO addition, is due to a different experimental protocol.) However, CWO works very effectively as a CLK-CYC inhibitor-competitor when it is added along with a small and limiting amount of PER (Fig. 2).

Our interpretation is that CWO is effective in collaborating with PER, because PER reduces the ability of CLK-CYC to bind to DNA (perhaps by promoting CLK phosphorylation; Yu et al. 2006). CWO can then compete effectively with the PER-weakened CLK-CYC complex for E-box access. In the absence of PER, CLK-CYC has a higher binding constant to canonical clock gene E boxes, so CWO does not compete well with CLK-CYC for access to canonical clock gene E boxes.

In contrast, the new direct target genes are better inhib-



Gene	TGT	Gene	TGT
tim	0.83	CG17100	0.25
CG15095	0.72	Picot	0.24
tim	0.65	CG3850	0.24
HLHm3	0.36	CG15096	0.23
per	0.36	CG6231	0.23
CG30035	0.33	CG18135	0.21
CG3348	0.33	GM02743	0.20
CG3764	0.32	EP2237	0.19
HLHmbeta	0.30	CG6767	0.19
vri	0.29	CG32170	0.18
CG4798	0.27	CG13624	0.17
CG10999	0.26	Mmp1	0.17
CG8008	0.26	CG13868	0.15
Pdp1	0.25	CG11086	0.13

Figure 1. Identification of direct CLK targets in *Drosophila* S2 cells and fly heads. (*A*) Diagram illustrating the approach for the identification of direct CLK targets from *Drosophila* S2 cells and fly heads. (Dex) Dexamethasone; (GR) ligand-binding domain of the glucocorticoid receptor; (CHX) cycloheximide. (*B*) Top 28 direct CLK targets identified by the approach described in *A*. (TGT) Targetness; this index was obtained by averaging the relative stimulation by dexamethasone from S2 cells and fly heads. (*C*) CLK protein expression activates most of the direct CLK-GR targets. Transient transfections were performed with varying amounts of pAc-*Clk* plasmid in S2 cells (0, 10, 30, and 100 ng). After 48 hours, cells were harvested and total RNA isolated. Microarray analysis was performed using the *Drosophila* 2.0 genomic Affymetrix chips. (*D*) Effect of CLK and PER expression on *vri*-Luc, *picot*-Luc, *CG15095*-Luc, and *CG17100*-Luc reporters on S2 cells. pAc-*Clk* and pAc-*per* refers to CLK- and PER-expressing plasmids, respectively. In all cases, cotransfection with pCopia-Renilla luciferase was performed to normalize for cell number, transfection efficiency, and general transcription effects.

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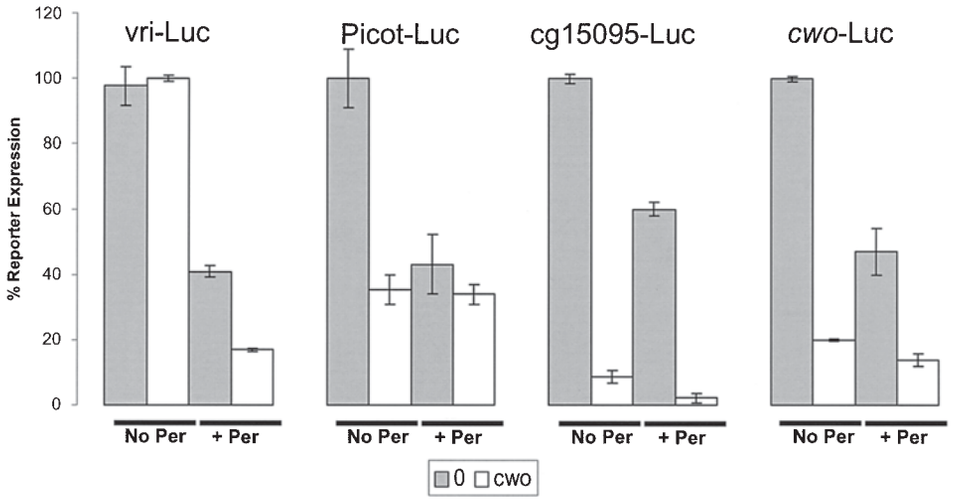

Figure 2. Effect of PER and CWO expression on CLK-mediated transcriptional activation. "No Per" indicates that 10 ng of pAc-*Clk* was cotransfected with 0 or 100 ng of pAc-*cwo* and the corresponding reporters. "+ Per" indicates that the transfection was performed as before, except that 50 ng of pAc-*per* was also cotransfected. A representative experiment is shown. (Duplicates for each condition were performed.)

ited by CWO alone under identical conditions (Fig. 2). Perhaps the E-box arrangements of these new CLK-target genes are different from those associated with canonical clock gene promoters. For example, CLK-CYC molecules may interact and therefore form very stable multimers on canonical clock gene E boxes (there appear to be closely spaced, multiple E boxes associated with the canonical clock gene promoters that are well-characterized), whereas they may interact less well with other E-box arrangements. At the risk of extending this speculation further, the argument suggests that CWO is of intermediate affinity, lower than that of CLK-CYC on optimally arranged E boxes, but higher than that of CLK-CYC on the other E-box arrangements. CWO would then serve to suppress transcription of the new direct target genes in clock cells.

To test this hypothesis, we manipulated the complex arrangement of E boxes and near-E boxes (*ter* boxes) within the *tim* promoter (Fig. 3). The deletion constructs with simpler arrangements of E boxes (fewer and/or fur-

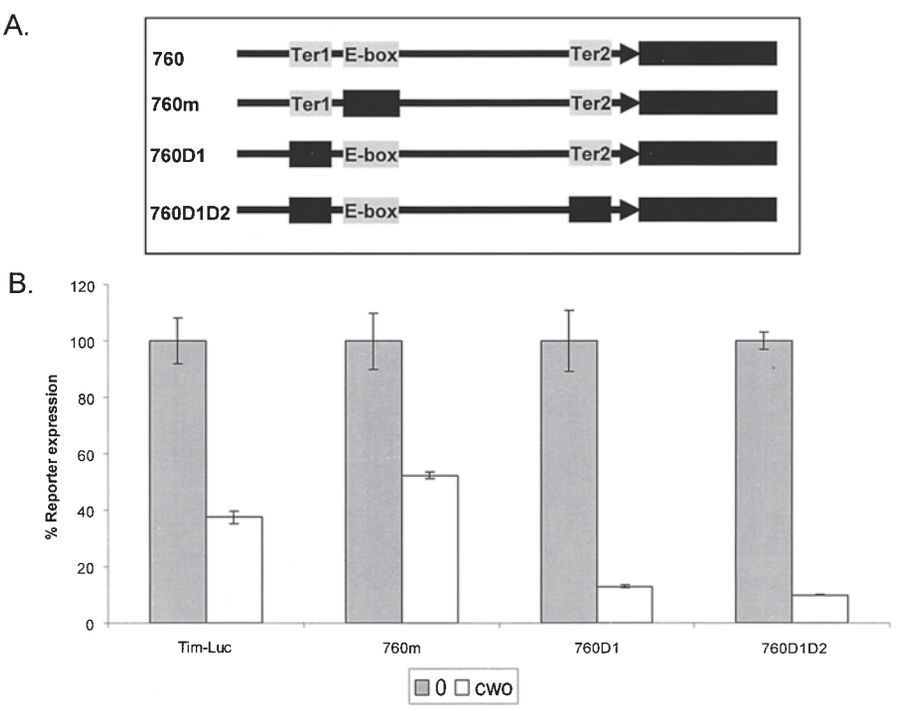

Figure 3. Different E-box arrangements alter the sensitivity of the *tim* promoter to *cwo* expression. (*A*) Schematic of the reporters used. (*B*) Effect of *cwo* expression (100 ng of pAc-*cwo*) on reporter expression. All experiments were performed in presence of 100 ng of pAc-*per*.

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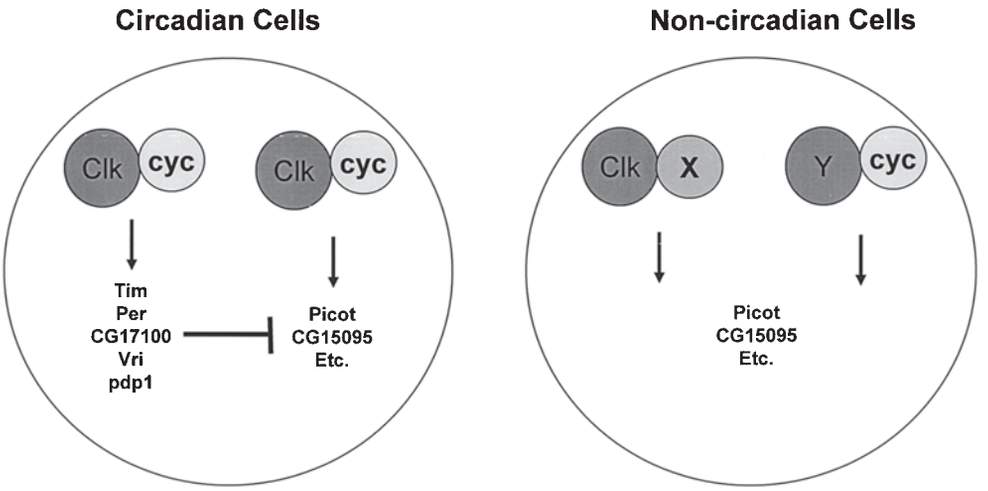

Figure 4. Proposed model of the role of CLK, CYC, and CWO in the specification of circadian cell types.

ther apart) are generally more sensitive to CWO. This suggests that it does indeed repress promoters that are not optimally designed to work with CLK-CYC. CWO therefore has a less restrictive E-box specificity and may therefore contribute to clock cell specification by preventing CLK from expressing inappropriate genes in bona fide clock cells (Fig. 4). We therefore suggest that the increased expression of the new direct target genes in the *cwo* mutant strain (Fig. 5) is due to significantly increased expression in circadian cells; the modest increase (Fig. 5) would then reflect unchanged levels in nonclock cells and dramatically increased clock cell levels. These are kept very low by CWO repression in wild-type strains.

These ideas fit with some of our previous work showing that ectopic CLK expression generates circadian clocks at additional locations within the brain (Zhao et al. 2003). The new insights into transcriptional circuitry presented here suggest that these additional locations are normal sites of CYC expression and that the induction of CLK in these cells creates clock neuron identity in part through the induction of CLK-CYC–direct target gene proteins including CWO and its repression of inappropriate transcription.

This role of CLK in clock cell specification is analogous to the role of PAX6 in eye specification and begs the question: How did a gene involved in intracellular circadian circuitry acquire a role in cell-type specification? We suggest that the answer is also similar to PAX6, which influences the transcription of rhodopsin and has a role in eye specification. Presumably, an ancient PAX6 molecule had a role in the transcription of one or more rhodopsin progenitors in single-cell organisms before multicellularity arose some 600 million years ago. Added later to this initial function was a more complex tissue specification role with the recruitment of other direct target genes as well as regulatory loops (Pichaud et al. 2001). We imag-

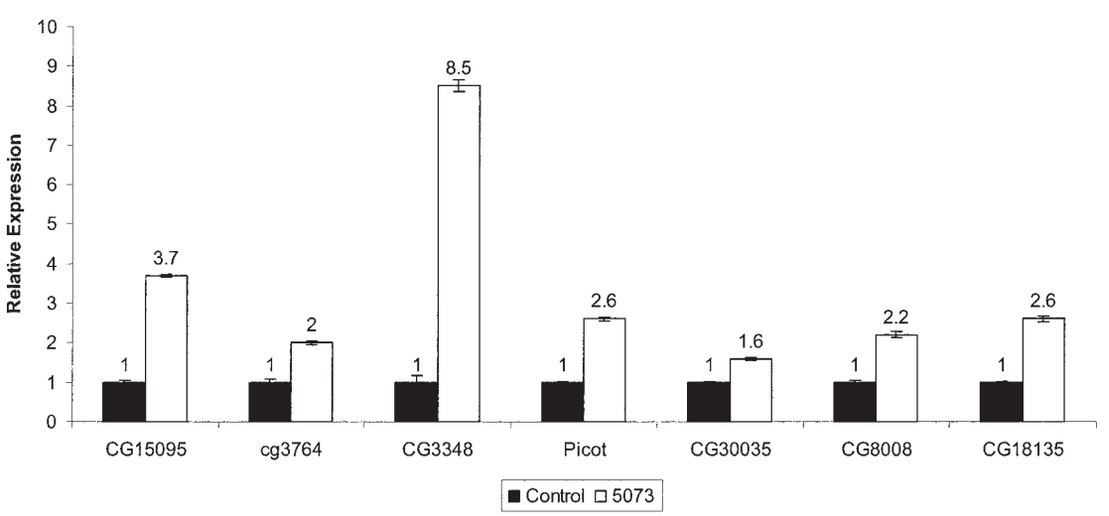

Figure 5. Noncircadian CLK–direct target levels are significally higher in *cwo*-deficient flies. The expression value for each gene was obtained by averaging the microarray-based expression level across six time points. The values were then normalized to the ones obtained in control flies.

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ine a similar early role in circadian rhythmicity for an ancient CLK-CYC heterodimer and its primary targets that we suspect were already present and functioning long ago in the clocks of our single-cell ancestors. More complex regulatory features, including those involving brain circuitry, were added more recently.

These evolutionary speculations suggest that circadian clocks have arisen at least twice in evolution: once in animal progenitors and once in cyanobacteria progenitors. (There are good arguments that circadian clocks have arisen more than twice, but it is best to keep things simple here.) Separate origins are supported by the total lack of sequence conservation between cyanobacterial and animal circadian clock proteins. It is also consistent with what appear to be different cellular properties of the two systems: Individual bacterial cells keep excellent circadian time, essentially indistinguishable from a bacterial culture (Mihalcescu et al. 2004), whereas individual eukaryotic cells (separated SCN cells, for example) show substantially more variation in period than an intact SCN or the organism (Welsh et al. 1995). Consistent with this view, animal clock properties appear to be more generally dependent on system or network properties (Liu et al. 2007). This difference in single-cell precision and intercellular communication echoes two additional differences between the systems: (1) rhythmic transcriptional oscillations are global in cyanobacteria, because most if not all genes are controlled by the same fundamental mechanism (Liu et al. 1995; Woelfle and Johnson 2006); in contrast, animal genes under circadian transcriptional regulation are regulated by different factors with only a minority apparently affected directly by the CLK-CYC heterodimer (McDonald and Rosbash 2001). (2) The KaiC kinase and phosphatase appear to be unique and of singular importance to timekeeping (McClung 2007; Terauchi et al. 2007). In contrast, animal rhythms have recruited several common enzymes involved in many other cellular processes (Kloss et al. 1998; Price et al. 1998; Lin et al. 2002). On the basis of the work summarized here as well, the circadian clockworks that govern intracellular animal rhythms may use a more equitable and complicated division of labor between gene expression and posttranslational regulatory mechanisms than the cyanobacterial clockworks. The intricacies of gene expression regulation may even explain why the strongest period mutations are in kinase genes, i.e., gene expression feedback loops may be more highly regulated (buffered) and therefore less easy to respond to single-gene mutations than mutations in kinases that affect clock protein turnover. We speculate that these differences between animal and cyanobacterial clocks reflect their independent evolutionary origins as well as the development of multicellularity.

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