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STOPPING TIME: The Genetics of Fly and Mouse Circadian Clocks

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■ **Abstract** Forward genetic analyses in flies and mice have uncovered conserved transcriptional feedback loops at the heart of circadian pacemakers. Conserved mechanisms of posttranslational regulation, most notably phosphorylation, appear to be important for timing feedback. Transcript analyses have indicated that circadian clocks are not restricted to neurons but are found in several tissues. Comparisons between flies and mice highlight important differences in molecular circuitry and circadian organization. Future studies of pacemaker mechanisms and their control of physiology and behavior will likely continue to rely on forward genetics.

INTRODUCTION

With the completion of the human genome sequence, the era of simple gene cloning and identification is nearing an end. With the advent of high throughput technologies such as DNA microarrays, the temporal and spatial expression patterns of thousands of genes will be known. The challenge for neuroscience will be to functionally link genes, including their complex expression patterns, to the output of the nervous system: behavior. Genetics will play a crucial role in navigating through this genomic jungle.

Forward genetics, the process of identifying mutant phenotypes to isolate genes, is best applied to a problem lacking molecular description. Forward genetics used to be "genetics," but the advent of recombinant DNA and reverse genetics necessitates a more useful term. Genetic screens require no prior hypothesis about the mechanism by which a system functions. The only requirements are an efficient means of mutagenesis and phenotypic screening. If there is an abundance of correlative molecular or cellular data, genetic analysis can also be helpful in

determining which variables are critical. Distinguishing between observations that are simply correlated with an output and those that are causal can be difficult. By randomly mutagenizing and assaying the consequence on a particular behavioral or physiologic parameter, one can identify key regulatory components.

Nowhere in neuroscience has the power of forward genetics been felt more acutely than in circadian rhythms. Genetics has propelled rhythms research to appear twice recently among *Science* magazine's breakthroughs of the year (1997, 1998). In this review, we discuss the key discoveries and how they were made possible by the confluence of genetics and genomics. We compare the molecular mechanisms and functions of different animal circadian systems, focusing on the fruit fly, *Drosophila melanogaster*, and the mouse. For the sake of brevity and to avoid redundancy with other recent reviews (Dunlap 1999, Ishida et al 1999, Edery 2000, Hall 2000, King & Takahashi 2000, Scully & Kay 2000, Young 2000a,b), we focus our efforts on circadian pacemakers and their systemic organization and outputs.

BACKGROUND

Animals temporally organize their behavior and metabolism to adapt to and anticipate the progression through the 24-hour solar cycle. The earth's 24-hour rotation has apparently dictated that all manner of life evolve to maximize fitness in this environment. Endogenous clocks serve to anticipate daily changes in their environment. The overt manifestations of these clocks are circadian rhythms. They are defined by periodicity in the absence of exogenous cues. Circadian rhythms are therefore not simply driven by the environment but arise from internal biological clocks. Circadian clocks only closely approximate but are not exactly 24 hours. Importantly, the timing of the clock is sensitive to light, among other environmental cues. The daily cycle of light and darkness resets internal clocks to maintain synchrony between the external solar day and internal biological clocks. Across a wide range of physiologic temperatures, the periodicity of rhythms is stable. This temperature compensation mechanism is particularly relevant in poikilotherms to prevent perturbations of the clock due to changes in the ambient temperature. However, even neural pacemaker tissues such as the mammalian suprachiasmatic nucleus (SCN) when maintained in vitro also defend against changes in temperature (Ruby et al 1999). Temperature compensation is thought to reflect an underlying feature of the clock mechanism. Clocks have been typically associated with regions of the nervous system, such as the SCN (Moore & Eichler 1972, Stephan & Zucker 1972). However, they are found in cells outside the nervous system and in organisms that do not have nervous systems. Even unicellular prokaryotes (e.g. photosynthetic cyanobacteria) exhibit robust circadian rhythms (Johnson & Golden 1999). Determining the molecular nature of these clocks awaited a forward genetic approach, first in *Drosophila* and other organisms (Konopka & Benzer 1971, Bruce 1972, Feldman & Hoyle 1973) and later in the mouse (Vitaterna et al 1994).

BEHAVIOR, GENETICS, AND THE FRUIT FLY

In the modern era the study of the genetic basis of behavior was pioneered by Seymour Benzer and colleagues, using the fruit fly, *Drosophila melanogaster.* Benzer's laboratory initiated genetic studies of many aspects of fly behavior, including circadian rhythms (Benzer 1971). The fruit fly had previously been a workhorse of geneticists, aiding in the discovery of many of the core principles of inheritance. *Drosophila* has a generation time of only 10 days, and its long history in the field of genetics has led to the development of many useful tools. Benzer's laboratory hoped to apply the genetic techniques optimized in this model system to questions related to behavior. Even then, much was known about circadian rhythms in *Drosophila* at the behavioral level. Fruit flies proceed through a series of characteristic developmental stages, beginning with the embryonic through a series of three larval stages to a pupal stage from which adults emerge or eclose. The fruit fly prefers to eclose at a particular time of day, reflecting gating by a circadian clock (Pittendrigh 1954). In fact, the word *Drosophila* means dew-loving, referring to its tendency to eclose in the morning. Ron Konopka, a graduate student in Benzer's laboratory, designed a screen of mutagenized populations, looking for mutant flies that eclosed with a different circadian phase (Konopka & Benzer 1971). In now classic studies, Konopka identified three such strains. When studying the individual strains under constant conditions, he saw that one had a long period rhythm of 29 hours, the second had a short period rhythm of 19 hours, and the third had no detectable rhythm. Remarkably, all three mutant phenotypes mapped to the X-chromosome to what appeared to be a single locus, which they called *period* (*per*).

The identification of these behavioral mutants suggested that one could study genes as a means to understanding behavior in general and circadian rhythms in particular. In part, the success of this screen reflected the unusual precision of this particular behavioral assay. Few behavioral assays can detect small differences on the order of 20% or less that distinguish their short and long period mutants. The ability to identify adult circadian rhythm mutants suggested that the mutated genes may not be vital to functions in development. If genes involved in circadian rhythms were also required for vital functions, then null mutations of such genes would never live to eclosion. The ability to identify mutants suggested that the function of the *period* gene might be relatively circumscribed. As a result, we now speak of "circadian rhythm genes" or "circadian clock genes."

TRANSCRIPTION AS NEURAL CODE: *Drosophila Period*

The work of Konopka and Benzer raised the possibility that forward genetics might more generally illuminate the circadian clockworks. However, the mutants and their intriguing phenotypes shed little light on the actual clock mechanism. This level of insight awaited the cloning of the *period* gene and more precisely, a

careful analysis of its temporal expression pattern. The cloning of PER was initially misleading, assigned to a group of cell surface proteins known as proteoglycans (Jackson et al 1986, Reddy et al 1986). This observation fit intercellular models of circadian clocks. However, more careful analysis of *per* sequence, expression, and function changed this view dramatically. Both PER protein and RNA are rhythmically expressed (Siwicki et al 1988, Hardin et al 1990, Zerr et al 1990). Importantly, the timing of the RNA fluctuations was sensitive to point mutations in the *period* protein (Hardin et al 1990). This set of observations placed these molecular fluctuations at the heart of the circadian pacemaker and led to the formulation of a model in which PER feeds back on its own transcription.

A clue to the mechanism of PER feedback came from sequence homologies with the basic helix-loop-helix (bHLH) transcription factors, *single-minded* (*sim*), and the aryl hydrocarbon receptor nuclear translocator (ARNT) (Crews et al 1988). However, *per* does not contain a canonical bHLH or other DNA-binding domain. The conserved domain, termed PAS (for ***p****er*-***A****RNT*-***s****im*), mediates dimeric PAS-PAS interactions (Huang et al 1993). In fact, it was shown that PER could inhibit transcriptional activation by ARNT and its bHLH-PAS partner, the aryl hydrocarbon receptor (Ahr) (Lindebro et al 1995). It was proposed that PER might feed back by inhibiting the activity of such bHLH-PAS transcription factor(s). Genetics would be crucial in determining which of these factors is the relevant in vivo target (see below).

For a decade, *period* was the only known circadian gene in animals. The intensive study of *period* led to the elucidation of several principles concerning clock genes. First, the gold standard for defining a clock gene is genetic: If one disrupts a gene, does it affect circadian rhythms? Second, genes that satisfy this first criterion are often (but not always) rhythmically transcribed. Third, clock proteins feed back and regulate their own transcription. Finally, there must be delays between activation and inhibition in order to generate free-running oscillations. In the absence of such delays, the system will damp to steady state.

TRANSCRIPTIONAL FEEDBACK

Fly Transcriptional Activators: CLOCK and CYCLE

Genes on the positive arm of the cycle appear to be well conserved. In *Drosophila* a pair of bHLH-PAS–containing transcription factors, CLOCK (CLK) and CYCLE (CYC), play roles as activators of clock genes. Arrhythmic mutant alleles of each gene abolish the rhythm of *period* and *timeless* (*tim*) transcription and RNA abundance and peg their levels near the trough of a dynamic cycle (Allada et al 1998, Rutila et al 1998). Interestingly, *Clk*, but not *cyc*, RNA and protein levels cycle over a 24-hour period (Bae et al 1998, 2000; Darlington et al 1998; Lee et al 1998). A circadian enhancer from the *per* promoter has been identified that is necessary and sufficient to confer cycling to a reporter gene (Hao et al 1997). Of note, a known binding site for bHLH-PAS transcription factors, an E-box

(CACGTG), is required for this activity (Hao et al 1997). Similar sequences have been identified in the *timeless* promoter (Darlington et al 1998). Transfection of CLK into a CYC-expressing *Drosophila* cell line results in activation from cotransfected *per* and *timeless* enhancers in an E-box-dependent fashion (Darlington et al 1998). CLK and CYC coimmunoprecipitate from fly head extracts and interact in yeast two-hybrid assays and as in vitro translated proteins (Darlington et al 1998, Lee et al 1999, Bae et al 2000). Thus, CLK and CYC appear to work together as a canonical heterodimeric transcription factor complex.

Mouse Transcriptional Activators: mCLOCK and BMAL1

For years following the cloning of *per*, many interpreted the failure to identify mammalian homologs as a sign that the two systems are not well conserved, at least at the molecular level. The recent expansion in both fly genes and their mammalian orthologs proved these observers wrong. In the mouse, the activators work similarly but perhaps on targets somewhat distinct from those in *Drosophila*. Genetic evidence is currently available from the mouse *Clock* mutant (*mClock*). *mClock* was originally identified in a behavioral forward genetic screen for mutants with circadian rhythm phenotypes (Vitaterna et al 1994). Homozygous mutants exhibit long period (28 hr) rhythms, which damp to arrhythmicity (Vitaterna et al 1994, Antoch et al 1997, King et al 1997). In these *Clock* mutant mice, several cycling genes appear to be downregulated, including *mouse Period1-3*(*mPer1-3*), *mouse Cryptochrome1-2*(*mCry1-2*), and *Bmal1*, implying a possible master regulatory role as a transcriptional activator (Zylka et al 1998b, Kume et al 1999, Shearman et al 2000b). Unlike *Drosophila Clock*, *mClock* does not cycle at the RNA level in the SCN (Shearman et al 2000b). However, *Bmal1* RNA levels appear to cycle in the mouse and rat suprachiasmatic nucleus, though with a low amplitude (Abe et al 1998, Oishi et al 1998a, Shearman et al 2000b). CLOCK and BMAL1 interact in two-hybrid assays and cooperatively activate from E-box elements in the *mPer1* promoter in transfection experiments (Gekakis et al 1998, Hogenesch et al 1998). It is tempting to equate mouse CLOCK and BMAL1 with fly CLOCK and CYCLE. Recent genetic evidence supports a role for BMAL1 (Bunger et al 2000). Although the 5′ promoter region of *mPer1* has been shown to confer cycling in vivo, it is not known if the E-boxes located there are necessary or sufficient for this cycling (Kuhlman et al 2000, Yamaguchi et al 2000, Yamazaki et al 2000). Complicating the picture is the presence of other homologous bHLH-PAS transcription factors, such as MOP9. MOP9 appears to exceed BMAL1's ability to activate with mCLOCK in transfection experiments (Hogenesch et al 2000). A MOP9 knockout will be required to distinguish the role of this gene.

Fly Transcriptional Inhibitors: PERIOD and TIMELESS

The inhibitory complex in *Drosophila* seems to consist of PER and TIM. Null mutants of both *per* and *tim* are completely arrhythmic in constant darkness

(Konopka & Benzer 1971, Sehgal et al 1994). The levels of *per* and *tim* RNAs both oscillate daily (Hardin et al 1990, Sehgal et al 1995). In both arrhythmic mutants the levels of their own RNAs is middle to high in comparison with the dynamic range of a daily cycle (Hardin et al 1990, Sehgal et al 1994). These measurements of RNA levels have been largely confirmed by transcription run-on assays and in vivo measurements of promoter activity (Hardin et al 1992b, Brandes et al 1996, So & Rosbash 1997). Although TIM does not have a PAS domain, it coimmunoprecipitates with PER from fly head extracts (Zeng et al 1996). Furthermore, cotransfection of PER and TIM together represses CLK-mediated transcription from *per* and *tim* E-boxes (Darlington et al 1998, Rothenfluh et al 2000). PER and TIM coimmunoprecipitate with CLK and CYC from fly head extracts (Lee et al 1998, Bae et al 2000). This association appears to be specific to times of falling *per* and *tim* transcription. These four proteins have also been shown to associate as in vitro translated proteins (Lee et al 1999). Moreover, in vitro PER and TIM modestly reduce CLK-CYC binding to its target E-box (Lee et al 1999). Thus, there is strong evidence that PER and TIM behave as direct biochemical inhibitors of CLK/CYC-mediated transcriptional activation.

Is there functional specialization within this heterodimeric complex? TIM levels, but not PER levels, are suppressed within minutes of exposure of the organism to light (Hunter-Ensor et al 1996, Lee et al 1996, Myers et al 1996, Zeng et al 1996). TIM may therefore function to link transcriptional repression to external temporal cues. Furthermore, PER protein levels, but not *per* RNA levels, are low in tim^0 mutants, suggesting a positive role for TIM in PER stabilization (Price et al 1995). In addition to the presence of a PAS domain in PER, experimental evidence more strongly links PER than TIM to repression. Disappearance of PER but not TIM seems to correlate well with the turn-on of *per* and *tim* transcription (Marrus et al 1996, So & Rosbash 1997). Light pulses that degrade TIM, in a *tim* mutant (tim^{UL}), lead to rapid decreases in *per* RNA levels, perhaps by freeing PER monomer (Rothenfluh et al 2000). Furthermore, transfection of *per* mutants, which are constitutively nuclear in *Drosophila* S2 cells independently repress CLK/CYC transcriptional activity without TIM (Rothenfluh et al 2000). Therefore, PER may play the role of primary repressor, and TIM may transport and/or stabilize PER (Figure 1).

Mouse Transcriptional Inhibitors: mCRY and mPER

In the mouse the molecular nature of the circadian inhibitor appears to be distinct from that of *Drosophila*. The strongest candidates for components of an inhibitory complex are the cryptochromes. Cryptochromes are members of a blue-light sensitive family of proteins, which also includes UV-dependent DNA repair enzymes (photolyases) (Cashmore et al 1999). In flies strong evidence supports a role for cryptochrome in circadian photoreception (Emery et al 1998, 2000; Stanewsky et al 1998; Egan et al 1999; Ishikawa et al 1999). In mice genetic inactivation

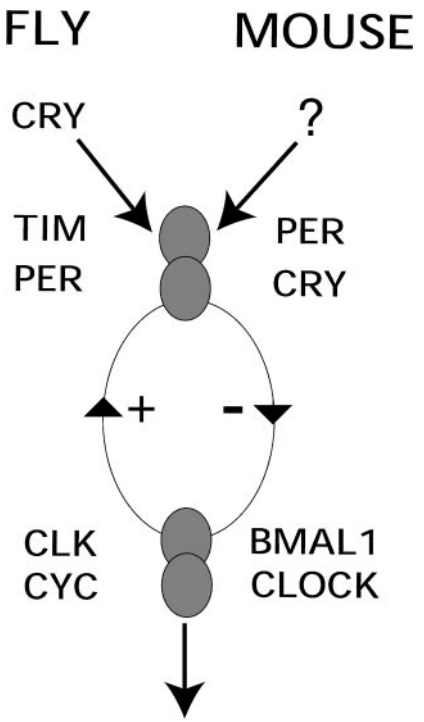

Figure 1 Schematic diagram of a circadian transcriptional feedback loop. CLK in Drosophila and BMAL1 in mammals are transcriptional activators that cycle in gene expression. CYC in Drosophila and mCLOCK in mice are transcriptional activators and heterodimeric partners of the above that do not cycle in gene expression. These complexes increase transcription of per and tim in Drosophila and mPer and mCry in mice. They are also thought to connect to output genes (arrow down). PER and mCRY play the role of primary transcriptional repressors. TIM and mPER transmit light information to the feedback loop and associate with these repressors. CRY behaves as a photoreceptor in flies. The mouse photoreceptor(s) is unknown.

of *mCry1* results in short period rhythms, whereas knockout of *mCry2* results in long period rhythms (Thresher et al 1998, van der Horst et al 1999, Vitaterna et al 1999). The double mutant displays no rhythm whatsoever, indicating a role for these genes in the central pacemaker (van der Horst et al 1999, Vitaterna et al 1999). *mCry1* and *mCry2* cycle at the RNA and protein levels in the SCN (Miyamoto & Sancar 1998, Kume et al 1999, Okamura et al 1999). In the double knockout of *mCry1* or *mCry2*, two of the CLOCK-BMAL1 target genes, *mPer1* and *mPer2*, are at high levels, consistent with a transcriptional suppressor role for the cryptochromes (Okamura et al 1999, Vitaterna et al 1999). These data are supported by transfection experiments, demonstrating potent transcriptional inhibition

by both *mCry1* or *mCry2* expression (Kume et al 1999). Moreover, mCRY1/2 interact with mCLOCK and BMAL1 in yeast two-hybrid assays (Griffin et al 1999). These data support a biochemically direct inhibitory role for the mCRYs, parallel to the role of PER-TIM or perhaps PER in the fly pacemaker mechanism (Figure 1).

One important question is whether mCRY1 and mCRY2 act alone or in concert with mammalian *Per* and/or *Tim*. Each CRY represses mCLOCK-BMAL1 activation in heterologous *Drosophila* S2 cells, which do not endogenously express dPER or dTIM (Saez & Young 1996, Shearman et al 2000b). The simplest model therefore is one in which mCRY1 and/or mCRY2 repress mCLOCK-BMAL1-mediated activation. A mystery remains as to why the *mCry1* and *mCry2* single knockouts have opposing period phenotypes, one shorter and one longer than 24 hours. Clearly, the transcriptional inhibitory assay of mCRY function in tissue culture cells, which does not significantly distinguish mCRY1 from mCRY2, does not explain the whole story.

If mCLOCK and BMAL1 are the activators and mCRY1/2 are repressors, what is the function of mPER? First, at least one of the mPERs satisfies the genetic gold standard. A deletion of the PAS domain in mPER2 results in shortened periods, which grade into arrhythmicity (Zheng et al 1999). All three mouse *period* genes cycle at the RNA and protein levels (Shearman et al 1997, Sun et al 1997, Tei et al 1997, Takumi et al 1998, Zylka et al 1998b, Hastings et al 1999, Field et al 2000). In these homozygous mutants, both *mPer2* and *mPer1* transcript levels are substantially reduced (Zheng et al 1999). This is in contrast to flies in which *per* transcript levels are middle to high in per^0 mutants (Hardin et al 1990). However, the nature of the mPER2 mutation complicates the interpretation of these results: A mutant protein might be produced and play a dominant-negative role. Indeed, transfection experiments show that this mutant allele is expressed and antagonizes wild-type mPER2 cellular localization (Shearman et al 2000b). Therefore, a true null mutation would be useful to determine the precise role of *mPer2* in the clock. Inactivation of the *mPer3* gene also has a slightly short period phenotype (Shearman et al 2000a). Despite being the otherwise most well studied of the *Period* genes, *mPer1*, when knocked out, has little effect on rhythms (Shearman et al 2000b). Functional redundancy of the *mPer* genes may be an obstacle to genetically deciphering their roles. Nonetheless, these data support a positive role for *mPer2* in promoting *mPer1* and *mPer2* RNA levels.

By analogy to *Drosophila*, one might expect mPER to be a transcriptional repressor of mCLOCK and BMAL1. The low RNA levels of *mPer1* and *mPer2* in the *mPer2* mutant animals are difficult to explain in this context. In fact, initial studies of mPER1 showed that it modestly repressed mCLOCK-BMAL1 activation (Sangoram et al 1998). However, the modest inhibition by mPER1 is dwarfed by that shown by either mCRY1 or mCRY2 alone (Kume et al 1999). In the absence of contradictory evidence, the simplest model is that mammalian *Period* genes do not behave like their repressing counterparts in flies.

How then does *mPer*(s) participate in the feedback loop, specifically *mPer2*, for which there is the strongest genetic evidence? Recently, it was proposed that

mPer2 participates positively in *Bmal1* RNA expression (Shearman et al 2000b). However,the peak values of *Bmal1* RNA are not dramatically affected in *mPer2* mutant animals (Shearman et al 2000b). Thus, there is no compelling evidence in favor of a specific function of mPER2. Interestingly, the mCRY and mPER proteins have been shown to engage in protein-protein contacts from SCN extracts (Field et al 2000). Furthermore, mPER2 protein levels are reduced in *mCry1-mCry2* double knockout mice (Shearman et al 2000b). It is therefore possible that in vivo the mPERs modulate the repressing activity of the mCRYs by direct physical association.

The mammalian *period* genes may also be involved in the response of the pacemaker to light. *mPer1* and *mPer2* RNAs as well as proteins are rapidly induced by light in the SCN (Albrecht et al 1997, Shearman et al 1997, Shigeyoshi et al 1997, Field et al 2000). Antisense oligonucleotides against *mPer1* reduce phase resetting, implicating mPER1 in this process (Akiyama et al 1999). However, the effect of *Period* gene knockout on light-induced *mPer* expression has yet to be determined. The in vivo role of the mammalian *Period* genes awaits true knockout of each of these genes as well as double and triple mutant combinations. The interactions with mCRY as well as light responsiveness implicate mPER in transducing light information, leading to a modulation of mCRY feedback, perhaps similar to the role of TIM in flies (Figure 1).

A mammalian homolog of *Drosophila timeless* has also been identified. Inactivation of *mTimeless* (*mTim*) function in vivo results in early embryonic lethality (Gotter et al 2000). The role of *mTim* has been confused by divergent results. Some reports have shown no cycling of *mTim* RNA; others show induction by light (Koike et al 1998, Sangoram et al 1998, Zylka et al 1998a, Tischkau et al 1999). In *Drosophila*, *timeless* RNA and protein cycle in constant darkness conditions (Sehgal et al 1995, Hunter-Ensor et al 1996, Lee et al 1996, Myers et al 1996, Zeng et al 1996). Moreover, *Drosophila* TIM is degraded by light (Hunter-Ensor et al 1996, Lee et al 1996, Myers et al 1996, Zeng et al 1996). The only reports of mTIM protein show no cycling and no light responsiveness (Hastings et al 1999, Field et al 2000). In addition, a new *Drosophila* gene, *timeout*, has been identified, which more closely resembles mTIM (Benna et al 2000, Gotter et al 2000). It is unclear if *timeout* has any role in fly rhythms. These observations have led to the proposal that the mammalian *Timeless* may not be involved in circadian rhythms (Gotter et al 2000). However, mTIM coimmunoprecipitates from SCN with mCRY1 and mCRY2 (Field et al 2000). Because the *mTim* knockout is lethal in mice, circadian rhythms cannot be assayed in these animals. Therefore, the jury will remain out on the in vivo role of *mTim* until conditional knockouts are reported. The *dbt* gene in *Drosophila* is just one example of a gene with developmental lethality but a clear function in the pacemaker (Price et al 1998).

In summary (see Figure 1), the *Drosophila* bHLH-PAS DNA-binding proteins, CLK and CYC, activate transcription of the *period* and *timeless* genes. PER and TIM feed back and inhibit the activity of the CLK-CYC heterodimer. In the mouse,

Clock and *cycle* orthologs (mCLOCK and BMAL1) probably activate the *mPer* and *mCry* genes. However, both CRYPTOCHROMES (mCRY1 and mCRY2) feed back and inhibit the mCLOCK-BMAL1 heterodimer. mPERs may transduce light information to mCRYs. The role of mTIM remains unclear.

CODEPENDENT LOOPS?

One of the latest waves of circadian research deals with the role of positive and negative interdependent feedback loops. The principle was first laid out in examinations of *Drosophila Clock* RNA but has infected other organisms, including the mouse. Like *per* and *tim*, *Clock* is rhythmically expressed (Bae et al 1998, Darlington et al 1998). However, *Clock* RNA cycles antiphase to *per* and *tim*, peaking in the late night and early morning [Zeitgeber Time (ZT) 23, ZT 5; ZT 0 = lights-on ("sunrise"); ZT 12 = lights-off ("sunset")]. Consistent with this altered phase of cycling, *Clock* transcript levels respond differently in arrhythmic mutants and are low in the per^0 and tim^0 strains (Bae et al 1998); *per* and *tim* transcripts are at middle to peak levels in these mutants (Hardin et al 1990, Sehgal et al 1995). In cyc^0 and Clk^{Jrk} mutants, *Clk* transcripts are high, whereas *per* and *tim* transcripts are low (Glossop et al 1999). Finally, in per^0; cyc^0 and per^0; Clk^{Jrk}, levels are similar to single cyc^0 and Clk^{Jrk} mutants (Glossop et al 1999). These data led to a model in which there are two interconnected feedback loops: the classic one regulates *per* and *tim* transcription, with CLK/CYC acting positively and PER/TIM acting negatively; the other regulates *Clk* transcription, with PER/TIM acting positively and CLK/CYC acting negatively. Epistasis analysis in double mutants indicates that the effects of *per* and *tim* operate on *Clk* RNA through CLK/CYC. Analysis of various mouse knockout strains of circadian rhythm genes came to similar conclusions, although the names have changed. mPER2 is proposed to act as a positive regulator of *Bmal1* transcripts (Shearman et al 2000b), although these effects are modest (see above).On the other hand, mCRY1 and mCRY2 are thought to play the major negative role on *mPer* and *mCry* transcription (see above).

Interdependent loops even appear to be involved in the circadian rhythms of the fungus, *Neurospora crassa* (Lee et al 2000). In *Neurospora*, *frequency* (*frq*) was the first clock component identified (reviewed in Dunlap 1999). Like *per*, it is rhythmically expressed and feeds back negatively on its own transcription. *White collar-1* (*WC-1*) encodes a transcriptional activator that promotes expression of the *frq* gene. Unlike *frq*, *WC-1* RNA is not rhythmically expressed. However, its protein product (WC-1) does oscillate, consistent with circadian posttranscriptional regulation (Lee et al 2000). As expected, *frq* overexpression downregulates expression of the endogenous *frq* gene. On the other hand, this excess *frq* positively regulates WC-1 expression.

One problem with the interdependent feedback models, particularly from flies and mice, is the reliance on arrhythmic null mutants. Genetic studies of putative

rhythm genes are the gold standard in determining whether or not they are clock genes. However, analysis of mutant phenotypes can be difficult to interpret and even misleading about the specific role of the gene. Like setting off a row of dominoes, the absence of the gene(s) throughout development may result in consequences that may obscure true gene function in the adult. A second problem is the absence of corroborating biochemical data to support interdependent loops. As all rhythms we measure are abolished by per^0 and Clk^{Jrk}, it is not surprising that *per* rhythms (or any rhythm) depend on *Clk* or that *Clk* rhythms depend on *per*. Therefore, these analyses may lend little mechanistic insight into the exact nature of this regulation.

POSTRANSLATIONAL REGULATION

Gated Nuclear Entry?

Transcriptional feedback loops are ubiquitous in nature. A distinctive feature of circadian clocks is their timing. It has been proposed that it is the delay between synthesis and feedback that is necessary for circadian oscillations to occur. Furthermore, the magnitude of this delay may dictate the daily oscillatory frequency. Evidence is accumulating that posttranslational regulation of certain circadian rhythm proteins is crucial to setting up these delays and therefore to timing feedback.

Several features of circadian rhythm gene expression appear to be subject to posttranslational regulation. In the case of *Drosophila per*, the transport of PER from cytoplasm to nucleus seems to be circadianly gated, at least in the pacemaker lateral neurons (LNs) (Curtin et al 1995). As PER protein accumulates (ZT 8–17), immunohistochemical staining is detectable predominantly in the cytoplasm, appearing in a so-called doughnut pattern. After ZT 17, PER staining rapidly (over the next few hours) moves from predominantly cytoplasmic to predominantly nuclear in pacemaker neurons. These observations have been independently reproduced (Lee et al 1996, Matsumoto et al 1999). The importance of the gating of nuclear entry of PER is supported by genetic evidence. In per^L mutants the timing of nuclear entry is also delayed (Curtin et al 1995). These observations support a model of regulated nuclear entry of PER.

PER association with its heterodimeric partner TIME has been implicated in the temporal control of PER nuclear entry. TIME strongly associates with PER both in vitro and in vivo. The *time* gene was cloned in part by its ability to interact with PER in a yeast two-hybrid assay (Gekakis et al 1995). These in vitro findings have been confirmed by in vivo observations as well. In coimmunoprecipitation experiments with fly head extracts, PER and TIM are strongly associated (Zeng et al 1996). In tim^0 mutants, PER proteins levels are suppressed, even though RNA levels are relatively high, suggesting a role for TIM in PER stability (Price et al 1995). Furthermore, in tim^0 mutants, PER staining is constitutively cytoplasmic (Vosshall et al 1994). In cultured *Drosophila* S2 cells, nuclear

localization of transfected PER is completely dependent upon cotransfection of TIM (Saez & Young 1996). In *per*^{0} mutants, TIM protein is predominantly cytoplasmic (Hunter-Ensor et al 1996, Myers et al 1996). These observations led to a model in which association of PER with TIM is a prerequisite for nuclear co-entry of this heterodimer.

Though this model has been largely accepted, stray bits of evidence may ultimately undermine this nuclear entry model. Beyond the pacemaker lateral neurons, PER cycling is evident in several parts of the fly nervous system and in many different tissues throughout the fly. If gating is a necessary step to generate circadian macromolecular oscillations, then "doughnuts" should be observed in many other PER-expressing cells. In stark contrast to the current model, no cytoplasmic accumulation is observed prior to nuclear entry, despite robust PER cycling, as noted by Curtin et al 1995. PER has been previously shown to be a transcriptional repressor, and its entry to the nucleus has been proposed to be required for its activity. Gating of entry would be an elegant means of controlling the timing of repression and therefore the clock. However, the timing of nuclear entry does not coincide with the downturn of *per* transcription and in fact occurs several hours too late at about the time transcription has nearly reached trough levels (Curtin et al 1995, So & Rosbash 1997). Finally, gated nuclear entry has not yet been described for circadian systems of other organisms. If TIM is not gating PER nuclear entry, TIM's predominant function in *Drosophila* may therefore be to regulate PER stability. More careful studies in the ever-growing list of circadian model systems will be required to determine if gated entry is a ubiquitous feature of pacemakers.

Phosphorylation

Phosphorylation may also impose a delay on PER feedback. The phosphorylation state and hence the mobility of PER protein varies systematically over a 24-hour time course (Edery et al 1994). PER accumulates during the late day/early night (ZT 8–16). As night progresses, PER migrates more slowly, i.e. at larger apparent molecular weights. As PER disappears in the early morning, PER phosphorylation peaks, as measured by mobility. Immunoprecipitation of PER and in vitro phosphatase treatment returns PER to its baseline mobility, indicating a crucial role of phosphorylation in these mobility changes. Studies of TIM protein show similar progressive changes in mobility due to phosphorylation (Zeng et al 1996). In fact, TIM phosphorylation seems to occur in parallel with PER, implying similar regulation. The coincidence of the peak in phosphorylation with protein disappearance implicates phosphorylation as a key signal for protein degradation.

A major step forward in the understanding of the mechanistic basis of circadian phosphorylation awaited the identification of *doubletime* (*dbt*), a kinase involved in circadian rhythms. *dbt* was originally identified in genetic screens for mutants with altered circadian locomotor activity rhythms (Price et al 1998). Three *dbt* alleles were identified: one with short period rhythms (hence the mutant name; *dbt*^{S}), one with long period rhythms (27 h; *dbt*^{L}), and one homozygous lethal allele (*dbt*^{P}). As predicted, the long and short period alleles alter in parallel the

metabolism of PER and TIM expression, lending little mechanistic insight to the function of this gene (Price et al 1998). In this regard, the lethal allele turned out to be extremely important in establishing a model for *doubletime* function.

It has been believed that a clock operates in *Drosophila* from early in larval development (Brett 1955, Sehgal et al 1992). Subsequent studies have shown that PER and TIM are rhythmically expressed in the larval central nervous system, including the precursors of the adult pacemaker neurons (Kaneko et al 1997). These 16–20 neurons, called the ventral lateral neurons (LNVS), are located deep inside each hemisphere of the *Drosophila* brain and control circadian locomotor activity (see below). As the lethal *dbt* allele, dbt^P, did not induce lethality until after the larval stage, PER and TIM expression could be examined in homozygotes (Price et al 1998). In these homozygotes, PER and TIM cycling is abolished. PER specifically accumulates to very high levels in a hypophosphorylated form. The cloning of *doubletime* revealed that it encodes a kinase that is homologous to human casein kinase I epsilon (Kloss et al 1998). DBT coimmunoprecipitates with PER in cotransfection experiments (Kloss et al 1998). This model posits that DBT phosphorylation of PER is a key signal for PER degradation. However, no study has yet shown that DBT directly phosphorylates PER in *Drosophila*, although this is the likely working hypothesis.

Remarkably, genetic data also support a role for the mammalian homolog of DBT in rhythms. These data involved a spontaneously mutant golden hamster named *tau* (Ralph & Menaker 1988). *tau* is a semidominant mutant with shortened circadian periods. The *tau* locus was molecularly cloned and found to encode the hamster homolog of *Drosophila doubletime* or casein kinase I epsilon (Lowrey et al 2000). The shortened period of the *tau* hamster was attributed to the disrupted biochemical activity of the mutant TAU kinase (Lowrey et al 2000). It was found that this enzyme has a markedly reduced maximal velocity (V_{max}). The mutant protein is still able to associate with PER, although its ability to phosphorylate PER in vitro is reduced. Purified human casein kinase I epsilon (hCKI epsilon) also phosphorylates human PER1 in vitro (Keesler et al 2000). Moreover, in co-transfection experiments, hCKI epsilon associates with and significantly shifts the mobility of hPER1 owing to phosphorylation (Keesler et al 2000). Consistent with genetic evidence from *Drosophila*, hCKI epsilon also destabilizes hPER1 (Keesler et al 2000). Similar findings were made with mouse CKI (Vielhaber et al 2000). As in flies, casein kinase I epsilon appears to play a crucial role in the circadian rhythms in mammals, and the enzymes appear to play similar roles in both systems. Nonetheless, it remains unclear for both systems whether PER is the true target of DBT. In fact, preliminary mammalian studies do not find circadian regulation of electrophoretic mobility of mPERs in vivo as in *Drosophila* (Field et al 2000). Additional antibodies will help determine if mPERs undergo cyclic phosphorylation changes.

How might *doubletime* contribute to circadian timekeeping? Current models of DBT/CKI epsilon function focus on PER phosphorylation. One possibility is that PER, through its association with DBT, imposes circadian regulation on this kinase activity. Perhaps PER prevents DBT from autophosphorylation, which

has been shown to inactivate the enzyme (Rivers et al 1998, Gietzen & Virshup 1999). Thus, DBT activity rises with increasing PER levels, leading to an increase in PER phosphorylation. Phosphorylated PER may be a more avid transcriptional repressor, promoting or speeding up transcriptional feedback until PER is degraded and the cycle restarts. In mammals, CKI epsilon also increases PER phosphorylation, but this may lead to a reduction or slowing in mCRY negative feedback (Figure 1). These subtle differences may explain why reductions in kinase activity in mutants lead to opposing period changes: long in flies and short in hamsters (Lowrey et al 2000, Suri et al 2000).

CIRCADIAN ORGANIZATION

The Mammalian Pacemaker: The Suprachiasmatic Nucleus

It has been clear to physiologists that several parameters of biological function are subject to circadian influence. Moreover, studies of the suprachiasmatic nucleus indicated that its destruction resulted in a dramatic loss of much of this rhythmicity (Moore & Eichler 1972, Stephan & Zucker 1972). Moreover, SCN transplantation from another animal could rescue rhythmicity (Drucker-Colin et al 1984, Sawaki et al 1984, Lehman et al 1987). These studies led to an SCN-centric view of circadian systems. The SCN is a symmetrical paired group of approximately 20,000 neurons located just above the optic chiasm. Measurements of metabolic activity using 2-deoxyglucose uptake indicated that its metabolic activity was circadianly regulated (Schwartz & Gainer 1977). Furthermore, dissociated individual neurons showed clear circadian rhythms of spontaneous activity, indicating that rhythmicity was likely generated intracellularly (Welsh et al 1995). However, intercellular mechanisms cannot be excluded as important modifiers of such a rhythm. Key experiments to demonstrate the pivotal role of SCN as a pacemaker involved the short period *tau* hamster described above (Ralph & Menaker 1988). Lesion/transplantation experiments with *tau* animals demonstrated that the period of lesioned/transplanted animals was invariably controlled by the donor SCN (Ralph et al 1990).

Fly Circadian Organization

One of the unexpected dividends of the molecular genetic approach has been a deeper understanding of how circadian systems are organized. In *Drosophila*, the expression of *per* and other clock genes is not restricted to a handful of brain neurons but is widespread in diverse tissue types and organ systems (Liu et al 1988). These clock genes are not just widely expressed. In most cases, they continue to cycle, implying that their expression is relevant to various circadian functions (Hardin 1994).

Previous studies demonstrated that a diffusible signal from the *Drosophila* head confers rhythmicity. Transplantation of the brains of per^S animals into the

abdomens of arrhythmic per^0 animals rescued circadian activity rhythms (Handler & Konopka 1979). Based on these data, it might be expected that separating peripheral oscillators from the central pacemaker neurons in the *Drosophila* head would completely abolish cycling of gene expression. Consistent with this view, developmental mutants that significantly disrupt *per*-expressing LNVS result in largely behaviorally arrhythmic flies. For example, the *disconnected* (*disco*) mutation eliminates connections between photoreceptor cells in the *Drosophila* eye and targets in the optic lobe (Steller et al 1987). Furthermore, PER-positive lateral neurons are not detectable (Zerr et al 1990). As a result of this disruption in brain architecture, the flies are largely arrhythmic (Dushay et al 1989). However, peripheral cycling is robust in light-dark cycles and persists with some damping over time in constant darkness (Hardin et al 1992a). Consistent with these neuroanatomic mutants, decapitation does not abolish peripheral oscillations (Hege et al 1997). Impressively, when the Malpighian tubules (a *Drosophila* kidney analog) are transplanted from one fly to another, these tubules retain the circadian molecular cycling of the donor, even if out-of-phase with the recipient (Giebultowicz et al 2000). Therefore, although there are hormonal signals in flies, they do not strongly entrain peripheral molecular oscillators. Thus, the lateral neurons do not appear to be essential for peripheral cycling.

Surprisingly, these peripheral clocks respond and entrain to light. Transgenic flies containing the *per* promoter fused to firefly luciferase (*per-luc*) express this reporter gene in the spatial and temporal distribution of the *per* gene (Brandes et al 1996). Luciferase has been used previously as a method of continuous monitoring of gene expression in live organisms (Kay 1993). Because of the relatively rapid turnover of the luciferase enzyme, gene transcription changes are readily reflected in enzyme activity changes. Thus, transgenic *per-luc* flies exhibit cycling bioluminescence when fed on the substrate luciferin. Although clocks are light-sensitive, the degree of bioluminescence is not sufficient to affect behavioral rhythms. Such noninvasive monitoring systems have made the repeated sampling of RNA or protein values unnecessary and have allowed an "on-line" view of gene expression in individual animals.

To test whether these peripheral oscillators respond to light and cycle independently, investigators systematically removed various parts of the *Drosophila* body from a *per-luc* transgenic animal (Plautz et al 1997). They then placed legs,wings, antennae, and other parts into culture media containing luciferin. Once the parts were separated from the central pacemaker, investigators observed that rhythmic gene expression in these persisted but appeared to damp over time in constant darkness. The luminescence technique could not determine if the loss of rhythmicity was due to asynchronous oscillators between different cells or a loss of intrinsic oscillator amplitude within each cell. These separated tissues also demonstrated photoreceptive properties (Plautz et al 1997). Robust rhythmicity, lost after several days in constant darkness, could be reinitiated by reexposure to light cycles. Taken together, these studies reveal how molecular genetic studies can illuminate the organization of circadian systems.

Mammalian Circadian Organization

Studies of the organization of the *Drosophila* circadian system differ markedly with that found in mammals. As mentioned above, lesioning of the SCN abolishes activity rhythms, and transplants restore them to SCN-ablated animals (Ralph et al 1990). Transplantation of SCNs in semipermeable capsules also restores rhythmicity in hamsters (Silver et al 1996). Therefore, similar to *Drosophila* (Handler & Konopka 1979), diffusion of humoral molecules is sufficient to restore some rhythmicity in mammals. As in the case of fly rhythm genes, mammalian rhythm genes are not restricted to the suprachiasmatic nucleus. For example, *mClock* transcripts are found in several neural and nonneural tissues, such as the heart and lungs (King et al 1997). Examination of the temporal profile of *Per* expression in peripheral tissues (e.g. liver and skeletal muscle) finds robust molecular cycling as well as an interesting temporal lag when compared with the oscillations in the SCN (Balsalobre et al 1998, Zylka et al 1998b). Even lymphocytes in peripheral blood exhibit circadian gene cycling (Oishi et al 1998b).

Nevertheless, there are marked differences between flies and mammals in the control of circadian rhythmicity in peripheral oscillators. Unlike the case of the fly, lesioning of the central pacemaker, the SCN, abolishes most peripheral rhythmicity, at least as assayed at the tissue level (Sakamoto et al 1998). Again, loss of rhythmicity at the tissue level may be due either to asynchronous oscillations between different cells or to a loss of intrinsic oscillation amplitude within each cell. Although these observations, in general, differ substantially from *Drosophila*, autonomous light-sensitive peripheral oscillators also exist in mammals: Hamster retinal cells show robust and light-entrainable oscillation in culture (Tosini & Menaker 1996).

Studies using *per*-driven luciferase reporters also emphasize the heavy reliance of peripheral oscillators on central oscillators in mammals. Transgenic rats were constructed using the mouse *mPer1* promoter fused to a luciferase reporter (Yamazaki et al 2000). Various tissues and organs were removed from these animals and assayed for rhythmic bioluminescence in vitro. In culture, the SCNs from these animals exhibited robust rhythms for up to 32 days. Peripheral oscillators such as the liver, lung, and skeletal muscle were also rhythmic, but damped after a few cycles. This damping of peripheral oscillators is reminiscent of *Drosophila* peripheral oscillators. The authors then examined the response to rapid changes, i.e. advances or delays, in the environmental light cycle as might occur to someone experiencing jet lag. They observed that the SCN rhythm shifted rapidly to the new environmental light regime. However, the bioluminescence rhythm in the peripheral tissues was either lost for several days or lagged for many days before shifting to the new light cycle. The asynchrony between environmental light cycles and the SCN on the one hand and peripheral oscillators on the other hand may explain the symptomatology of jet lag.

Immortalized cell lines derived from rat SCN neurons exhibit circadian rhythms of metabolism and gene expression (Earnest et al 1999a,b). Remarkably,

transplantation of these SCN cell lines to the third ventricle restored circadian activity rhythm to SCN-lesioned animals (Earnest et al 1999b). Thus, these SCN cell lines may release appropriate phase-setting signals. Transformed cell lines, such as Rat-1 fibroblasts, exhibit circadian patterns of gene expression after a brief exposure of high concentrations of serum (Balsalobre et al 1998). It has been proposed that serum contains important circadian phase-setting factors. Cycling of genes in rat lymphocytes suggests that similar humoral factors may drive circadian clocks in these cells (Oishi et al 1998b).

Prominent among circadianly regulated hormones in humans is melatonin. However, peripheral rhythms persist in melatonin-deficient mice (Zylka et al 1998b). Another important class of circadian-controlled hormones is the glucocorticoids (Tronche et al 1998). Consistent with a possible role of glucocorticoids as output mediators, dexamethasone, a glucocorticoid analog, induces circadian rhythmicity in cultured Rat-1 fibroblasts (Balsalobre et al 2000). Moreover, dexamethasone transiently phase-shifts the rhythm of peripheral organs such as the liver. Finally, dexamethasone has no effect on the SCN rhythm, consistent with an absence of glucocorticoid receptor expression there. However, liver-specific knockout of the glucocorticoid receptor did not abolish circadian rhythmicity in the liver (Balsalobre et al 2000). Thus, glucocorticoids cannot be the sole entraining signals.

In zebrafish, rhythms of gene expression persist in peripheral organs such as the kidney and heart in vitro, i.e. separated from the brain (Whitmore et al 1998). Furthermore, as in the case of *Drosophila*, these peripheral oscillators appear to be directly light sensitive (Whitmore et al 1998). In fact, a zebrafish cell line has been shown to exhibit rhythmic gene expression in vitro (Whitmore et al 2000). Moreover, these rhythms are also entrainable by light. These data are more consistent with the more autonomous fly circadian organization.

Why these differences in organization? In small and partially transparent animals like *Drosophila* and zebrafish, light can easily penetrate into the body of the animal. Therefore, the presence of a photoreceptor (CRY in flies) allows a very simple way to synchronize the peripheral oscillators with the environment. On the other hand, larger and more opaque animals like mammals may need neurohormonal control of circadian oscillations in internal organs. Strikingly, the mammalian retina, which obviously receives light, has maintained light-sensitive cell-autonomous oscillation. The nature of the photoreceptor in this tissue or any mammalian circadian photoreceptor is still mysterious. In zebrafish, though, *zCRY4*, which is in sequence much closer to *Drosophila* CRY than to the mammalian CRYs, looks like an interesting candidate for study (Kobayashi et al 2000).

CIRCADIAN OUTPUT

Direct Output Targets

Although much progress has been made in identifying clock genes, little is known about how these genes connect to outputs. In many organisms, various fractions

of whole genomes appear to be under circadian control, from the vast majority in photosynthetic cyanobacteria to 1–5% in *Drosophila* (Liu et al 1995, Van Gelder et al 1995). Given the size of the *Drosophila* genome, hundreds of genes should cycle. These data implicate transcription in the control of various output genes and therefore in the control of behavioral and physiologic outputs. Most of these clock-controlled genes are probably indirectly controlled; many fewer are likely to be direct targets of clock genes. In our opinion, a direct interaction with a clock component has not yet been conclusively demonstrated for any output gene.

What then should be the criteria for a direct output target? As identification of cycling RNAs seems to be a straightforward strategy, it is likely that a large fraction of these will be controlled at the level of transcription. In this case, there should be direct binding of a circadian transcription factor, such as *Clock* to the promoter of the regulated gene. There are two main ways to show this convincingly. First, the construction of altered specificity mutants, i.e. mutants that alter DNA-binding specificity need to be coupled to mutants with correspondingly altered binding sites to prove direct binding in vivo. Second, chromatin immunoprecipitations could show that a factor is physically bound to its putative target in vivo. However, as these strategies are relatively difficult, studies of output gene regulation have heavily relied on transient transfection into tissue culture cells. Typically, DNAs expressing the relevant clock genes as well as the target promoters driving a reporter are transfected and allowed to express for 24–48 hours. The long temporal delay between expression and the downstream target gene allows for several intermediate steps to occur. Thus, the observed effects could easily be indirect. The relevance of the putative target site of the transcription factor needs to be assessed in vivo. Promoter elements need to confer cycling on reporter genes in transgenic animals. Mutation of the appropriate binding sites should abolish or reduce this cycling.

Most studies of output genes have tried to identify the targets of the CLK and CYC transcription complex, CACGTG. By simple randomness, this sequence will occur approximately once every four kilobases. The random probability that one will find such a sequence in the vicinity of a cycling gene is therefore relatively high. Moreover, expression is typically far higher in transfection experiments than what would be observed in vivo. The transfected target promoter DNA does not have the typical repressing chromatin structure of genomic DNA. Thus, under these highly artificial conditions, overexpressed transcription factors may find these vulnerable E-boxes, when they would not be accessible in vivo. Therefore, results based only on these types of experiments need to be interpreted very cautiously.

The neuropeptide gene, *arginine vasopressin*, is one putative output target in mammals. Besides many other functions, such as the physiologic control of water balance, this gene is also rhythmically expressed in the suprachiasmatic nucleus (Uhl & Reppert 1986). In *Clock* mutants the levels of *vasopressin* RNA are dramatically reduced and rhythmicity is abolished in the SCN (Jin et al 1999). In cell culture experiments, mCLOCK and its partner BMAL1 activate from E-boxes in the *vasopressin* promoter, providing a plausible explanation for the in vivo results and a model for how output might be generated (Jin et al 1999). However, it remains unclear whether the E-box sequence found in the *vasopressin* promoter is

required for its rhythmic expression in vivo. Moreover both the genetic evidence and the cell culture data leave open the possibility that the effect of mCLOCK-BMAL1 on *vasopressin* is indirect.

A more likely direct target output gene is the D-box binding element protein (DBP). DBP is rhythmically expressed in the liver and SCN among other tissues (Fonjallaz et al 1996). DBP activates the promoters of many enzymes involved in hepatic processes, including cholesterol metabolism (Lavery et al 1999). Mice lacking DBP exhibit a subtle circadian behavioral phenotype, implying that DBP may have a primary function in mediating output (Lopez-Molina et al 1997). Deletion of intragenic DBP regions substantially reduce promoter-driven expression (Ripperger et al 2000). Studies of in vivo promoter occupancy identified DNase I hypersensitive sites, suggesting that an exclusive focus on E-boxes is overly simplistic. Protein binding to DNA often renders the local DNA sensitive to DNase I. In the *Dbp* genomic region, as many as five different loci, some of which are intragenic, undergo circadian regulation of DNase I hypersensitivity. Control of output genes may therefore be much more complex than regulation by one or two factors. E-box motifs were identified and shown to bind mCLOCK in vitro. However, this binding activity does not cycle, raising questions about whether mCLOCK is the factor cyclically bound in vivo. Nonetheless, DBP regulates several enzymes circadianly expressed in the liver and is therefore an important link between the circadian pacemaker and the final outputs.

In combination with genetic approaches, recent work in *Drosophila* has focused on molecular methods to find clock-controlled genes. In a search for genes that are differentially expressed between wild-type and per^0 animals, the zinc finger transcription factor *vrille* (*vri*) was identified (Blau & Young 1999). Previous studies of the *vri* gene demonstrated that it was required for embryonic development (George & Terracol 1997). Like *per*, *vri* is rhythmically expressed in circadian pacemaker neurons (Blau & Young 1999). Furthermore, as in the case of *vasopressin*, the *Drosophila* orthologs of mCLOCK and BMAL1, CLOCK and CYCLE, activate a reporter gene fused to the *vri* promoter, which contains E-box target sites (Blau & Young 1999). Constitutive overexpression of *vri* lengthened behavioral rhythms and reduced expression of the *period* and *timeless* RNAs, indicating a potential role of this gene in the central pacemaker. This expression also downregulates the output molecule, pigment dispersing factor (PDF; see below). However, phenotypes due to misexpression or overexpression can be misleading, as they may induce functions that normal expression does not. Heterozygous deletions of the *vri* locus yield very subtle circadian phenotypes. Conditional rescue of the embryonic lethality and subsequent analysis of homozygous null mutant adults will be required to more fully specify the role of *vri* in the pacemaker.

Using a similar subtractive hybridization approach, another clock-regulated gene was identified called *takeout* (*to*) (So et al 2000). *takeout* was originally identified on the basis of its low expression in the cyc^0 mutant. Studies of a *takeout* mutant suggest that TAKEOUT protein is involved in the response to starvation (Sarov-Blat et al 2000). *takeout* expression is reduced in all circadian rhythm mutants tested, which distinguishes it from any other studied transcript. Although

takeout is also rhythmically expressed at the RNA level, the phase of this oscillation is distinct from other cycling RNAs from *Clk*, *per*, and *tim*. As in the case of *vri* and *vasopressin*, a search was undertaken to identify E-box target sites. Not only was an E-box identified, but there was also remarkable similarity in promoter regions flanking the E-box, suggesting that this region was functional. Surprisingly, this E-box was not sufficient to drive cycling when tested in vivo (which was not done for *vri*, *vasopressin*, or *Dbp* promoters). Alternatively, the lack of cycling could have been due to an artifact of the transgene. Nonetheless, the *takeout* mRNA cycling is not likely due to a direct effect of CLK and CYC. These studies highlight the importance of testing enhancer elements in vivo for circadian function.

Circadian Behavioral Output Molecules

Though the discovery of several new output genes and phenomena illustrates how pacemakers may generate output, they did not reveal any key mediator of behavioral rhythms. PDF is a neuropeptide that has been well studied in invertebrates and has been shown to phase-shift circadian clocks when injected into cockroaches (Petri & Stengl 1997). Initial studies with *Drosophila pdf* indicated that its expression was largely restricted to the head and absent in *disco* mutants (Park 1998). These mutants are largely arrhythmic and lack pacemaker lateral neurons (Dushay et al 1989, Zerr et al 1990). *pdf* mRNA and protein are specifically expressed in a ventral subset of these lateral neurons (LNvs) (Helfrich-Forster 1995, Park et al 2000). Furthermore, PDF is rhythmically expressed in the termini of these neurons (Park et al 2000). The role of *pdf* in these neurons was more clearly elucidated by the discovery of a *pdf* mutant, pdf^{01} (Renn et al 1999). Like *disco* mutants, these mutants display only weak or no rhythms. Because *disco* mutants exhibit far more neuroanatomical defects beyond just an absence of pacemaker lateral neurons, the *pdf* promoter was used to direct expression of the proapoptotic genes, *head-involution defective* (*hid*) and *reaper*, to the ventral lateral neurons. This expression resulted in the complete ablation of these neurons. The behavioral consequence was a circadian phenotype virtually identical to that of pdf^{01}, indicating that the prinicipal mediator of the circadian signal from these key pacemaker neurons is *pdf* (Renn et al 1999).

Is PDF synaptically released? To address the role of chemical synaptic transmission, the tetanus-toxin light chain (TeTxLC) was expressed in all pacemaker-containing cells (Kaneko et al 2000). TeTxLC blocks synaptic transmission by cleaving the synaptic protein, synaptobrevin. Although clock gene cycling was unaffected in these animals, rhythmic behavior was substantially reduced, indicating a role for synaptic transmission. Surprisingly, targeted expression of TeTxLC exclusively to the LN_{vs} did not affect circadian behavior,indicating that *pdf* release may operate through nonsynaptic mechanisms (Kaneko et al 2000). Furthermore, other cells may also be relevant to behavioral rhythms. In contrast to the SCN, which relies on the eyes for synchronizing its activity with the environment, the LNVS also contain a circadian photoreceptor and are therefore directly light

sensitive (Emery et al 2000). They are circadianly self-sufficient because they contain an input pathway, a molecular pacemaker, and an output. There does not appear to be a mammalian ortholog of PDF. It will be interesting to see what the mammalian version(s) of this output signal will be.

Sleep in Flies?

One of the most controversial notions regarding circadian output in flies is that the behavioral cycles of flies mimic the mammalian sleep-wake cycle. Close observations of fly behavior during their 24 hour cycle demonstrate that flies can be immobile for long periods of time (over 2 hours in some cases) (Hendricks et al 2000, Shaw et al 2000). During this immobile state, flies exhibit an increased threshold to arousing, sensory stimuli. Importantly, this state is homeostatically regulated, like sleep. Rest-deprived flies will increase their rest subsequently. Similar stimulation during wake periods has no subsequent effect on rest behavior. Drugs that increase or decrease sleep in mammals, such as antihistamines and caffeine, increase and decrease rest in flies. As in the case of circadian rhythms, molecular and genetic studies of sleep (or this sleep-like state if you prefer) in flies may prove to be very influential in our understanding of the neurobiology of sleep.

CONCLUSION

The problem of circadian rhythms has been reduced to the molecular realm: a problem of understanding transcriptional feedback loops and the posttranslational regulation of their loop components. The past few years have seen a remarkable increase in the number of identified clock genes. There remains much to learn about how these genes regulate each other, especially in mammals, where we are truly at the dawn of the molecular era. In the next few years an important focus will be on posttranslational regulation and feedback. The identification of additional kinases and phosphatases as well as their regulatory features will be central to a better understanding of timing. In addition to understanding how the clock couples to output, the identification of key output molecules in mammals may have medical applications. Many of these discoveries will undoubtedly continue to capitalize on the power of forward genetics.

LITERATURE CITED

Abe H, Honma S, Namihira M, Tanahashi Y, Ikeda M, Honma K. 1998. Circadian rhythm and light responsiveness of BMAL1 expression, a partner of mammalian clock gene Clock, in the suprachiasmatic nucleus of rats. *Neurosci. Lett.* 258:93–96

Akiyama M, Kouzu Y, Takahashi S, Wakamatsu H, Moriya T, et al. 1999. Inhibition of

light- or glutamate-induced mPer1 expression represses the phase shifts into the mouse circadian locomotor and suprachiasmatic firing rhythms. *J. Neurosci.* 19:1115–21

Albrecht U, Sun ZS, Eichele G, Lee CC. 1997. A differential response of two putative mammalian circadian regulators, mper1 and mper2, to light. *Cell* 91:1055–64

Allada R, White NE, So WV, Hall JC, Rosbash M. 1998. A mutant Drosophila homolog of mammalian Clock disrupts circadian rhythms and transcription of period and timeless. *Cell* 93:791–804

Antoch MP, Song EJ, Chang AM, Vitaterna MH, Zhao Y, et al. 1997. Functional identification of the mouse circadian Clock gene by transgenic BAC rescue *Cell* 89:655–67

Bae K, Lee C, Hardin PE, Edery I. 2000. dCLOCK is present in limiting amounts and likely mediates daily interactions between the dCLOCK–CYC transcription factor and the PER-TIM complex. *J. Neurosci.* 20:1746–53

Bae K, Lee C, Sidote D, Chuang KY, Edery I. 1998. Circadian regulation of a Drosophila homolog of the mammalian Clock gene: PER and TIM function as positive regulators *Mol. Cell. Biol.* 18:6142–51

Balsalobre A, Brown SA, Marcacci L, Tronche F, Kellendonk C, et al. 2000. Resetting of circadian time in peripheral tissues by glucocorticoid signaling. *Science* 289:2344–47

Balsalobre A, Damiola F, Schibler U. 1998. A serum shock induces circadian gene expression in mammalian tissue culture cells. *Cell* 93:929–37

Benna C, Scannapieco P, Piccin A, Sandrelli F, Zordan M, et al. 2000. A second timeless gene in Drosophila shares greater sequence similarity with mammalian tim. *Curr. Biol.* 10:R512–13

Benzer S. 1971. From the gene to behavior. *JAMA* 218:1015–22

Blau J, Young MW. 1999. Cycling vrille expression is required for a functional Drosophila clock. *Cell* 99:661–712

Brandes C, Plautz JD, Stanewsky R, Jamison CF, Straume M, et al. 1996. Novel features of Drosophila period transcription revealed by real-time luciferase reporting. *Neuron* 16:687–92

Brett W. 1955. Persistent diurnal rhythmicity in Drosophila emergence. *Ann. Entomol. Soc. Am.* 48:119–31

1997. Breakthrough of the year. The runners-up. *Science* 278:2039–42

1998. Breakthrough of the year. The runners-up. *Science* 282:2157–61

Bruce VG. 1972. Mutants of the biological clock in Chlamydomonas reinhardi. *Genetics* 70:537–48

Bunger MK, Wilsbacher LD, Moran SM, Clendenin C, Radcliffe LA, et al. 2000. Mop3 is an essential component of the master circadian pacemaker in mammals. *Cell* 103:1009–17

Cashmore AR, Jarillo JA, Wu YJ, Liu D. 1999. Cryptochromes: blue light receptors for plants and animals. *Science* 284:760–65

Crews ST, Thomas JB, Goodman CS. 1988. The Drosophila single-minded gene encodes a nuclear protein with sequence similarity to the per gene product. *Cell* 52:143–51

Curtin KD, Huang ZJ, Rosbash M. 1995. Temporally regulated nuclear entry of the Drosophila period protein contributes to the circadian clock. *Neuron* 14:365–72

Darlington TK, Wager-Smith K, Ceriani MF, Staknis D, Gekakis N, et al. 1998. Closing the circadian loop: CLOCK–induced transcription of its own inhibitors per and tim. *Science* 280:1599–603

Drucker-Colin R, Aguilar-Roblero R, Garcia-Hernandez F, Fernandez-Cancino F, Bermudez Rattoni F. 1984. Fetal suprachiasmatic nucleus transplants: diurnal rhythm recovery of lesioned rats. *Brain Res.* 311:353–57

Dunlap JC. 1999. Molecular bases for circadian clocks. *Cell* 96:271–90

Dushay MS, Rosbash M, Hall JC. 1989. The disconnected visual system mutations in *Drosophila melanogaster* drastically disrupt circadian rhythms. *J. Biol. Rhythms* 4:1–27

Earnest DJ, Liang FQ, DiGiorgio S, Gallagher

M, Harvey B, et al. 1999a. Establishment and characterization of adenoviral E1A immortalized cell lines derived from the rat suprachiasmatic nucleus. *J. Neurobiol.* 39:1–13

Earnest DJ, Liang FQ, Ratcliff M, Cassone VM. 1999b. Immortal time: circadian clock properties of rat suprachiasmatic cell lines. *Science* 283:693–95

Edery I. 2000. Circadian rhythms in a nutshell. *Phys. Genomics* 3:59–74

Edery I, Zwiebel LJ, Dembinska ME, Rosbash M. 1994. Temporal phosphorylation of the Drosophila period protein. *Proc. Natl. Acad. Sci. USA* 91:2260–64

Egan ES, Franklin TM, Hilderbrand-Chae MJ, McNeil GP, Roberts MA, et al. 1999. An extraretinally expressed insect cryptochrome with similarity to the blue light photoreceptors of mammals and plants. *J. Neurosci.* 19:3665–73

Emery P, So WV, Kaneko M, Hall JC, Rosbash M. 1998. CRY, a Drosophila clock and light-regulated cryptochrome, is a major contributor to circadian rhythm resetting and photosensitivity. *Cell* 95:669–79

Emery P, Stanewsky R, Helfrich-Forster C, Emery-Le M, Hall JC, Rosbash M. 2000. Drosophila CRY is a deep brain circadian photoreceptor. *Neuron* 26:493–504

Feldman JF, Hoyle MN. 1973. Isolation of circadian clock mutants of Neurospora crassa. *Genetics* 75:605–13

Field MD, Maywood ES, O'Brien JA, Weaver DR, Reppert SM, Hastings MH. 2000. Analysis of clock proteins in mouse SCN demonstrates phylogenetic divergence of the circadian clockwork and resetting mechanisms. *Neuron* 25:437–47

Fonjallaz P, Ossipow V, Wanner G, Schibler U. 1996. The two PAR leucine zipper proteins, TEF and DBP, display similar circadian and tissue-specific expression, but have different target promoter preferences. *EMBO J.* 15:351–62

Gekakis N, Saez L, Delahaye-Brown AM, Myers MP, Sehgal A, et al. 1995. Isolation of timeless by PER protein interaction: defective interaction between timeless protein and long-period mutant PERL. *Science* 270:811–15

Gekakis N, Staknis D, Nguyen HB, Davis FC, Wilsbacher LD, et al. 1998. Role of the CLOCK protein in the mammalian circadian mechanism. *Science* 280:1564–69

George H, Terracol R. 1997. The vrille gene of Drosophila is a maternal enhancer of decapentaplegic and encodes a new member of the bZIP family of transcription factors. *Genetics* 146:1345–63

Giebultowicz JM, Stanewsky R, Hall JC, Hege DM. 2000. Transplanted Drosophila excretory tubules maintain circadian clock cycling out of phase with the host. *Curr. Biol.* 10:107–10

Gietzen KF, Virshup DM. 1999. Identification of inhibitory autophosphorylation sites in casein kinase I epsilon *J. Biol. Chem.* 274:32063–70

Glossop NR, Lyons LC, Hardin PE. 1999. Interlocked feedback loops within the Drosophila circadian oscillator. *Science* 286:766–68

Gotter AL, Manganaro T, Weaver DR, Kolakowski LF Jr, Possidente B, et al. 2000. A time-less function for mouse timeless. *Nat. Neurosci.* 3:755–56

Griffin EA Jr, Staknis D, Weitz CJ. 1999. Light–independent role of CRY1 and CRY2 in the mammalian circadian clock. *Science* 286:768–71

Hall JC. 2000. Cryptochromes: sensory reception, transduction, and clock functions subserving circadian systems. *Curr. Opin. Neurobiol.* 10:456–66

Handler AM, Konopka RJ. 1979. Transplantation of a circadian pacemaker in Drosophila. *Nature* 279:236–38

Hao H, Allen DL, Hardin PE. 1997. A circadian enhancer mediates PER–dependent mRNA cycling in *Drosophila melanogaster*. *Mol. Cell. Biol.* 17:3687–93

Hardin PE. 1994. Analysis of period mRNA cycling in Drosophila head and body tissues indicates that body oscillators behave

differently from head oscillators. *Mol. Cell. Biol.* 7211–18

Hardin PE, Hall JC, Rosbash M. 1990. Feedback of the Drosophila period gene product on circadian cycling of its messenger RNA levels. *Nature* 343:536–40

Hardin PE, Hall JC, Rosbash M. 1992a. Behavioral and molecular analyses suggest that circadian output is disrupted by disconnected mutants in *D. melanogaster. EMBO J.* 11:1–6

Hardin PE, Hall JC, Rosbash M. 1992b. Circadian oscillations in period gene mRNA levels are transcriptionally regulated. *Proc. Natl. Acad. Sci. USA* 89:11711–15

Hastings MH, Field MD, Maywood ES, Weaver DR, Reppert SM. 1999. Differential regulation of mPER1 and mTIM proteins in the mouse suprachiasmatic nuclei: new insights into a core clock mechanism. *J. Neurosci.* 19:RC11

Hege DM, Stanewsky R, Hall JC, Giebultowicz JM. 1997. Rhythmic expression of a PER-reporter in the Malpighian tubules of decapitated Drosophila: evidence for a brain-independent circadian clock. *J. Biol. Rhythms* 12:300–8

Helfrich-Forster C. 1995. The period clock gene is expressed in central nervous system neurons which also produce a neuropeptide that reveals the projections of circadian pacemaker cells within the brain of *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. USA* 92:612–16

Hendricks JC, Finn SM, Panckeri KA, Chavkin J, Williams JA, et al. 2000. Rest in Drosophila is a sleep-like state. *Neuron* 25:129–38

Hogenesch JB, Gu YZ, Jain S, Bradfield CA. 1998. The basic-helix-loop-helix-PAS orphan MOP3 forms transcriptionally active complexes with circadian and hypoxia factors. *Proc. Natl. Acad. Sci. USA* 95:5474–79

Hogenesch JB, Gu YZ, Moran SM, Shimomura K, Radcliffe LA, et al. 2000. The basic helix-loop-helix-PAS protein MOP9 is a brain-specific heterodimeric partner of circadian and hypoxia factors. *J. Neurosci.* 20:RC83

Huang ZJ, Edery I, Rosbash M. 1993. PAS is a dimerization domain common to Drosophila period and several transcription factors. *Nature* 364:259–62

Hunter-Ensor M, Ousley A, Sehgal A. 1996. Regulation of the Drosophila protein timeless suggests a mechanism for resetting the circadian clock by light *Cell* 84:677–85

Ishida N, Kaneko M, Allada R. 1999. Biological clocks. *Proc. Natl. Acad. Sci. USA* 96:8819–20. Erratum. 2000. *Proc. Natl. Acad. Sci. USA* 97(16):9347

Ishikawa T, Matsumoto A, Kato T Jr, Togashi S, Ryo H, et al. 1999. DCRY is a Drosophila photoreceptor protein implicated in light entrainment of circadian rhythm. *Genes Cells* 4:57–65

Jackson FR, Bargiello TA, Yun SH, Young MW. 1986. Product of per locus of Drosophila shares homology with proteoglycans. *Nature* 320:185–88

Jin X, Shearman LP, Weaver DR, Zylka MJ, de Vries GJ, Reppert SM. 1999. A molecular mechanism regulating rhythmic output from the suprachiasmatic circadian clock. *Cell* 96:57–68

Johnson CH, Golden SS. 1999. Circadian programs in cyanobacteria adaptiveness and mechanism *Annu. Rev. Microbiol.* 53:389–409

Kaneko M, Helfrich-Forster C, Hall JC. 1997. Spatial and temporal expression of the period and timeless genes in the developing nervous system of Drosophila: newly identified pacemaker candidates and novel features of clock gene product cycling. *J. Neurosci.* 17:6745–60

Kaneko M, Park JH, Cheng Y, Hardin PE, Hall JC. 2000. Disruption of synaptic transmission or clock-gene-product oscillations in circadian pacemaker cells of Drosophila cause abnormal behavioral rhythms. *J. Neurobiol.* 43:207–33

Kay SA. 1993. Shedding light on clock controlled cab gene transcription in higher plants. *Semin. Cell Biol.* 4:81–86

Keesler GA, Camacho F, Guo Y, Virshup D,

Mondadori C, Yao Z. 2000. Phosphorylation and destabilization of human period I clock protein by human casein kinase I epsilon. *NeuroReport* 11:951–55

King DP, Takahashi JS. 2000. Molecular genetics of circadian rhythms in mammals. *Annu. Rev. Neurosci.* 23:713–42

King DP, Zhao Y, Sangoram AM, Wilsbacher LD, Tanaka M, et al. 1997. Positional cloning of the mouse circadian clock gene. *Cell* 89:641–53

Kloss B, Price JL, Saez L, Blau J, Rothenfluh A, et al. 1998. The Drosophila clock gene double-time encodes a protein closely-related to human casein kinase-I-epsilon. *Cell* 94:97–107

Kobayashi Y, Ishikawa T, Hirayama J, Daiyasu H, Kanai S, et al. 2000. Molecular analysis of zebrafish photolyase/cryptochrome family: two types of cryptochromes present in zebrafish. *Genes Cells* 5:725–38

Koike N, Hida A, Numano R, Hirose M, Sakaki Y, Tei H. 1998. Identification of the mammalian homologues of the Drosophila timeless gene, Timeless1. *FEBS Lett.* 441:427–31

Konopka RJ, Benzer S. 1971. Clock mutants of *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. USA* 68:2112–16

Kuhlman SJ, Quintero JE, McMahon DG. 2000. GFP fluorescence reports Period 1 circadian gene regulation in the mammalian biological clock. *NeuroReport* 11:1479–82

Kume K, Zylka MJ, Sriram S, Shearman LP, Weaver DR, et al. 1999. mCRY1 and mCRY2 are essential components of the negative limb of the circadian clock feedback loop. *Cell* 98:193–205

Lavery DJ, Lopez-Molina L, Margueron R, Fleury-Olela F, Conquet F, et al. 1999. Circadian expression of the steroid 15 alpha-hydroxylase (Cyp2a4) and coumarin 7-hydroxylase (Cyp2a5) genes in mouse liver is regulated by the PAR leucine zipper transcription factor DBP. *Mol. Cell. Biol.* 19: 6488–99

Lee C, Bae K, Edery I. 1998. The Drosophila CLOCK protein undergoes daily rhythms in abundance, phosphorylation, and interactions with the PER–TIM complex. *Neuron* 21:857–67

Lee C, Bae K, Edery I. 1999. PER and TIM inhibit the DNA binding activity of a Drosophila CLOCK-CYC/dBMAL1 heterodimer without disrupting formation of the heterodimer: a basis for circadian transcription. *Mol. Cell. Biol.* 19:5316–25

Lee C, Parikh V, Itsukaichi T, Bae K, Edery I. 1996. Resetting the Drosophila clock by photic regulation of PER and a PER-TIM complex. *Science* 271:1740–44

Lee K, Loros JJ, Dunlap JC. 2000. Interconnected feedback loops in the Neurospora circadian system. *Science* 289:107–10

Lehman MN, Silver R, Gladstone WR, Kahn RM, Gibson M, Bittman EL. 1987. Circadian rhythmicity restored by neural transplant. Immunocytochemical characterization of the graft and its integration with the host brain. *J. Neurosci.* 7:1626–38

Lindebro MC, Poellinger L, Whitelaw ML. 1995. Protein-protein interaction via PAS domains: role of the PAS domain in positive and negative regulation of the bHLH/PAS dioxin receptor-Arnt transcription factor complex. *EMBO J.* 14:3528–39

Liu X, Lorenz L, Yu QN, Hall JC, Rosbash M. 1988. Spatial and temporal expression of the period gene in *Drosophila melanogaster*. *Genes Dev.* 2:228–38

Liu Y, Tsinoremas NF, Johnson CH, Lebedeva NV, Golden SS, et al. 1995. Circadian orchestration of gene expression in cyanobacteria. *Genes Dev.* 9:1469–78

Lopez-Molina L, Conquet F, Dubois-Dauphin M, Schibler U. 1997. The DBP gene is expressed according to a circadian rhythm in the suprachiasmatic nucleus and influences circadian behavior. *EMBO J.* 16:6762–71

Lowrey PL, Shimomura K, Antoch MP, Yamazaki S, Zemenides PD, et al. 2000. Positional syntenic cloning and functional characterization of the mammalian circadian mutation tau. *Science* 288:483–92

Marrus SB, Zeng H, Rosbash M. 1996. Effect

of constant light and circadian entrainment of perS flies: evidence for light-mediated delay of the negative feedback loop in Drosophila. *EMBO J.* 15:6877–86

Matsumoto A, Tomioka K, Chiba Y, Tanimura T. 1999. timrit Lengthens circadian period in a temperature-dependent manner through suppression of PERIOD protein cycling and nuclear localization. *Mol. Cell. Biol.* 19:4343–54

Miyamoto Y, Sancar A. 1998. Vitamin B2-based blue-light photoreceptors in the retinohypothalamic tract as the photoactive pigments for setting the circadian clock in mammals. *Proc. Natl. Acad. Sci. USA* 95:6097–102

Moore RY, Eichler VB. 1972. Loss of a circadian adrenal corticosterone rhythm following suprachiasmatic lesions in the rat. *Brain Res.* 42:201–6

Myers MP, Wager-Smith K, Rothenfluh-Hilfiker A, Young MW. 1996. Light-induced degradation of TIMELESS and entrainment of the Drosophila circadian clock. *Science* 271:1736–40

Oishi K, Sakamoto K, Okada T, Nagase T, Ishida N. 1998a. Antiphase circadian expression between BMAL1 and period homologue mRNA in the suprachiasmatic nucleus and peripheral tissues of rats. *Biochem. Biophys. Res. Commun.* 253:199–203

Oishi K, Sakamoto K, Okada T, Nagase T, Ishida N. 1998b. Humoral signals mediate the circadian expression of rat period homologue (rPer2) mRNA in peripheral tissues. *Neurosci. Lett.* 256:117–19

Okamura H, Miyake S, Sumi Y, Yamaguchi S, Yasui A, et al. 1999. Photic induction of mPer1 and mPer2 in cry-deficient mice lacking a biological clock. *Science* 286:2531–34

Park JH, Hall JC. 1998. Isolation and chronobiological analysis of a neuropeptide pigment-dispersing factor gene in *Drosophila melanogaster*. *J. Biol. Rhythms* 13:219–28

Park JH, Helfrich-Forster C, Lee G, Liu L, Rosbash M, Hall JC. 2000. Differential regulation of circadian pacemaker output by separate clock genes in Drosophila. *Proc. Natl. Acad. Sci. USA* 97:3608–13

Petri B, Stengl M. 1997. Pigment-dispersing hormone shifts the phase of the circadian pacemaker of the cockroach Leucophaea maderae. *J. Neurosci.* 17:4087–93

Pittendrigh C. 1954. On temperature independence in the clock-system controlling emergence time in Drosophila. *Proc. Natl. Acad. Sci. USA* 40:1018–29

Plautz JD, Kaneko M, Hall JC, Kay SA. 1997. Independent photoreceptive circadian clocks throughout Drosophila. *Science* 278:1632–35

Price JL, Blau J, Rothenfluh A, Abodeely M, Kloss B, Young MW. 1998. double-time is a novel Drosophila clock gene that regulates PERIOD protein accumulation. *Cell* 94:83–95

Price JL, Dembinska ME, Young MW, Rosbash M. 1995. Suppression of PERIOD protein abundance and circadian cycling by the Drosophila clock mutation timeless. *EMBO J.* 14:4044–49

Ralph MR, Foster RG, Davis FC, Menaker M. 1990. Transplanted suprachiasmatic nucleus determines circadian period. *Science* 247:975–78

Ralph MR, Menaker M. 1988. A mutation of the circadian system in golden hamsters. *Science* 241:1225–27

Reddy P, Jacquier AC, Abovich N, Petersen G, Rosbash M. 1986. The period clock locus of *D. melanogaster* codes for a proteoglycan. *Cell* 46:53–61

Renn SC, Park JH, Rosbash M, Hall JC, Taghert PH. 1999. A pdf neuropeptide gene mutation and ablation of PDF neurons each cause severe abnormalities of behavioral circadian rhythms in Drosophila. *Cell* 99:791–802. Erratum. 2000. *Cell* 101(1): following 113

Ripperger JA, Shearman LP, Reppert SM, Schibler U. 2000. CLOCK, an essential pacemaker component, controls expression of the circadian transcription factor DBP. *Genes Dev.* 14:679–89.

Rivers A, Gietzen KF, Vielhaber E, Virshup DM. 1998. Regulation of casein kinase I epsilon and casein kinase I delta by an in vivo futile phosphorylation cycle. *J. Biol. Chem.* 273:15980–84.

Rothenfluh A, Young MW, Saez L. 2000. A TIMELESS-independent function for PERIOD proteins in the Drosophila clock. *Neuron* 26:505–14

Ruby NF, Burns DE, Heller HC. 1999. Circadian rhythms in the suprachiasmatic nucleus are temperature-compensated and phase-shifted by heat pulses in vitro. *J. Neurosci.* 19:8630–36

Rutila JE, Suri V, Le M, So WV, Rosbash M, Hall JC. 1998. CYCLE is a second bHLH-PAS clock protein essential for circadian rhythmicity and transcription of Drosophila period and timeless. *Cell* 93:805–14

Saez L, Young MW. 1996. Regulation of nuclear entry of the Drosophila clock proteins period and timeless. *Neuron* 17:911–20

Sakamoto K, Nagase T, Fukui H, Horikawa K, Okada T, et al. 1998. Multitissue circadian expression of rat period homolog (rPer2) mRNA is governed by the mammalian circadian clock, the suprachiasmatic nucleus in the brain. *J. Biol. Chem.* 273:27039–42

Sangoram AM, Saez L, Antoch MP, Gekakis N, Staknis D, et al. 1998. Mammalian circadian autoregulatory loop: a timeless ortholog and mPer1 interact and negatively regulate CLOCK-BMAL1-induced transcription. *Neuron* 21:1101–13

Sarov-Blat L, So WV, Liu L, Rosbash M. 2000. The Drosophila takeout gene is a novel molecular link between circadian rhythms and feeding behavior. *Cell* 101:647–56

Sawaki Y, Nihonmatsu I, Kawamura H. 1984. Transplantation of the neonatal suprachiasmatic nuclei into rats with complete bilateral suprachiasmatic lesions. *Neurosci. Res.* 1:67–72

Schwartz WJ, Gainer H. 1977. Suprachiasmatic nucleus: use of 14C-labeled deoxyglucose uptake as a functional marker. *Science* 197:1089–91

Scully AL, Kay SA. 2000. Time flies for Drosophila. *Cell* 100:297–300

Sehgal A, Price J, Young MW. 1992. Ontogeny of a biological clock in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. USA* 89:1423–27

Sehgal A, Price JL, Man B, Young MW. 1994. Loss of circadian behavioral rhythms and per RNA oscillations in the Drosophila mutant timeless. *Science* 263:1603–6

Sehgal A, Rothenfluh-Hilfiker A, Hunter-Ensor M, Chen Y, Myers MP, Young MW. 1995. Rhythmic expression of timeless: a basis for promoting circadian cycles in period gene autoregulation. *Science* 270:808–10

Shaw PJ, Cirelli C, Greenspan RJ, Tononi G. 2000. Correlates of sleep and waking in *Drosophila melanogaster*. *Science* 287: 1834–37

Shearman LP, Jin X, Lee C, Reppert SM, Weaver DR. 2000a. Targeted disruption of the mPer3 gene: subtle effects on circadian clock function. *Mol. Cell. Biol.* 20:6269–75

Shearman LP, Sriram S, Weaver DR, Maywood ES, Chaves I, et al. 2000b. Interacting molecular loops in the mammalian circadian clock. *Science* 288:1013–19

Shearman LP, Zylka MJ, Weaver DR, Kolakowski LF Jr, Reppert SM. 1997. Two period homologs: circadian expression and photic regulation in the suprachiasmatic nuclei. *Neuron* 19:1261–69

Shigeyoshi Y, Taguchi K, Yamamoto S, Takekida S, Yan L, et al. 1997. Light-induced resetting of a mammalian circadian clock is associated with rapid induction of the mPer1 transcript. *Cell* 91:1043–53

Silver R, LeSauter J, Tresco PA, Lehman MN. 1996. A diffusible coupling signal from the transplanted suprachiasmatic nucleus controlling circadian locomotor rhythms. *Nature* 382:810–13

Siwicki KK, Eastman C, Petersen G, Rosbash M, Hall JC. 1988. Antibodies to the period gene product of Drosophila reveal diverse tissue distribution and rhythmic changes in the visual system. *Neuron* 1:141–50

So WV, Rosbash M. 1997. Post-transcriptional regulation contributes to Drosophila clock gene mRNA cycling. *EMBO J.* 16:7146–55

So WV, Sarov-Blat L, Kotarski CK, McDonald MJ, Allada R, Rosbash M. 2000. Takeout, a novel Drosophila gene under circadian clock transcriptional regulation. *Mol. Cell. Biol.* 20:6935–44

Stanewsky R, Kaneko M, Emery P, Beretta B, Wager-Smith K, et al. 1998. The cryb mutation identifies cryptochrome as a circadian photoreceptor in Drosophila. *Cell* 95:681–92

Steller H, Fischbach KF, Rubin GM. 1987. Disconnected: a locus required for neuronal pathway formation in the visual system of Drosophila. *Cell* 50:1139–53

Stephan FK, Zucker I. 1972. Circadian rhythms in drinking behavior and locomotor activity of rats are eliminated by hypothalamic lesions. *Proc. Natl. Acad. Sci. USA* 69:1583–86

Sun ZS, Albrecht U, Zhuchenko O, Bailey J, Eichele G, Lee CC. 1997. RIGUI, a putative mammalian ortholog of the Drosophila period gene. *Cell* 90:1003–11

Suri V, Hall JC, Rosbash M. 2000. Two novel doubletime mutants alter circadian properties and eliminate the delay between RNA and protein in Drosophila. *J. Neurosci.* 20:7547–55

Takumi T, Taguchi K, Miyake S, Sakakida Y, Takashima N, et al. 1998. A light-independent oscillatory gene mPer3 in mouse SCN and OVLT. *EMBO J.* 17:4753–59

Tei H, Okamura H, Shigeyoshi Y, Fukuhara C, Ozawa R, et al. 1997. Circadian oscillation of a mammalian homologue of the Drosophila period gene. *Nature* 389:512–16

Thresher RJ, Vitaterna MH, Miyamoto Y, Kazantsev A, Hsu DS, et al. 1998. Role of mouse cryptochrome blue-light photoreceptor in circadian photoresponses. *Science* 282:1490–94

Tischkau Y, Barnes JA, Lin FJ, Myers EM, Barnes JW, et al. 1999. Oscillation and light induction of timeless mRNA in the mammalian circadian clock. *J. Neurosci.* 19:RC15

Tosini G, Menaker M. 1996. Circadian rhythms in cultured mammalian retina. *Science* 272:419–21

Tronche F, Kellendonk C, Reichardt HM, Schutz G. 1998. Genetic dissection of glucocorticoid receptor function in mice. *Curr. Opin. Genet. Dev.* 8:532–38

Uhl GR, Reppert SM. 1986. Suprachiasmatic nucleus vasopressin messenger RNA: circadian variation in normal and Brattleboro rats. *Science* 232:390–93

van der Horst GT, Muijtjens M, Kobayashi K, Takano R, Kanno S, et al. 1999. Mammalian Cry1 and Cry2 are essential for maintenance of circadian rhythms. *Nature* 398:627–30

Van Gelder RN, Bae H, Palazzolo MJ, Krasnow MA. 1995. Extent and character of circadian gene expression in *Drosophila melanogaster*: identification of twenty oscillating mRNAs in the fly head. *Curr. Biol.* 5:1424–36

Vielhaber E, Eide E, Rivers A, Gao ZH, Virshup DM. 2000. Nuclear entry of the circadian regulator mPER1 is controlled by mammalian casein kinase I epsilon. *Mol. Cell. Biol.* 20:4888–99

Vitaterna MH, King DP, Chang AM, Kornhauser JM, Lowrey PL, et al. 1994. Mutagenesis and mapping of a mouse gene, Clock, essential for circadian behavior. *Science* 264:719–25

Vitaterna MH, Selby CP, Todo T, Niwa H, Thompson C, et al. 1999. Differential regulation of mammalian period genes and circadian rhythmicity by cryptochromes 1 and 2. *Proc. Natl. Acad. Sci. USA* 96:12114–19

Vosshall LB, Price JL, Sehgal A, Saez L, Young MW. 1994. Block in nuclear localization of period protein by a second clock mutation, timeless. *Science* 263:1606–9

Welsh DK, Logothetis DE, Meister M, Reppert SM. 1995. Individual neurons dissociated from rat suprachiasmatic nucleus express independently phased circadian firing rhythms. *Neuron* 14:697–706

Whitmore D, Foulkes NS, Sassone-Corsi P. 2000. Light acts directly on organs and cells in culture to set the vertebrate circadian clock. *Nature* 404:87–91

Whitmore D, Foulkes NS, Strahle U, Sassone-Corsi P. 1998. Zebrafish Clock rhythmic expression reveals independent peripheral circadian oscillators. *Nat. Neurosci.* 1:701–7

Yamaguchi S, Mitsui S, Miyake S, Yan L, Onishi H, et al. 2000. The 5′ upstream region of mPer1 gene contains two promoters and is responsible for circadian oscillation. *Curr. Biol.* 10:873–76

Yamazaki S, Numano R, Abe M, Hida A, Takahashi R, et al. 2000. Resetting central and peripheral circadian oscillators in transgenic rats. *Science* 288:682–85

Young MW. 2000a. Circadian rhythms. Marking time for a kingdom. *Science* 288:451–53

Young MW. 2000b. The tick-tock of the biological clock. *Sci. Am.* 282:64–71

Zeng H, Qian Z, Myers MP, Rosbash M. 1996. A light-entrainment mechanism for the Drosophila circadian clock. *Nature* 380:129–35

Zerr DM, Hall JC, Rosbash M, Siwicki KK. 1990. Circadian fluctuations of period protein immunoreactivity in the CNS and the visual system of Drosophila. *J. Neurosci.* 10:2749–62

Zheng B, Larkin DW, Albrecht U, Sun ZS, Sage M, et al. 1999. The mPer2 gene encodes a functional component of the mammalian circadian clock. *Nature* 400:169–73

Zylka MJ, Shearman LP, Levine JD, Jin X, Weaver DR, Reppert SM. 1998a. Molecular analysis of mammalian timeless. *Neuron* 21:1115–22

Zylka MJ, Shearman LP, Weaver DR, Reppert SM. 1998b. Three period homologs in mammals: differential light responses in the suprachiasmatic circadian clock and oscillating transcripts outside of brain. *Neuron* 20:1103–10