Circadian rhythms: molecular basis of the clock

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Much progress has been made during the past year in the molecular dissection of the circadian clock. Recently identified circadian genes in mouse, *Drosophila*, and cyanobacteria demonstrate the universal nature of negative feedback regulation as a circadian mechanism; furthermore, the mouse and *Drosophila* genes are structurally and functionally conserved. In addition, the discovery of brain-independent clocks promises to revolutionize the study of circadian biology.

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Abbreviations

bHLH	basic helix-loop-helix
Cry	*Cryptochrome*
CT	circadian time
DBP	D-binding protein
dbt	*double-time*
frq	*frequency*
PAS	PER ARNT SIM
per	*period*
SCN	suprachiasmatic nuclei
tim	*timeless*

Introduction

A major goal in the study of circadian biology is to elucidate the molecular mechanisms governing the circadian clock. To date, genetic approaches have yielded the most success in this endeavor: the *period* (*per*) and *timeless* (*tim*) genes in *Drosophila*, the *frequency* (*frq*) gene in *Neurospora*, and the *Clock* gene in the mouse were each cloned following mutagenesis screens for altered circadian phenotypes (Table 1) [1–3]. Studies in *Drosophila* on circadian mutants that either shorten (per^S), lengthen (per^L) or abolish (per^{01}, tim^{01}) rhythms in behavior and eclosion have provided a compelling molecular model of rhythmicity in that animal (reviewed in detail in [3] and [4] but only briefly below, as space restrictions prevent extensive description and referencing). The *per* and *tim* genes oscillate in mRNA expression, protein abundance, and protein localization [5–10]. The periods of these oscillations depend upon the *per* and *tim* alleles (i.e. the molecular period matches the behavioral period in a mutant *per* or *tim* genetic background). In addition, PER overexpression from an inducible promoter inhibits the endogenous *per* mRNA rhythm [11]. These observations indicate that the *per* and *tim* gene products regulate their own transcription. A ~6 hour delay between transcription and translation occurs in both gene products [6–10] and, furthermore, nuclear localization of PER and of TIM is blocked in tim^{01} and per^{01} flies, respectively [8,9]. PER physically interacts with TIM via its PAS domain [1] both *in vitro* [12] and in cell culture [13]. Finally, light degrades the TIM protein, which provides a mechanism for photic entrainment of the PER–TIM molecular cycle [8–10,14]. Integration of these observations results in the following model (Figure 1): the *per* and *tim* genes are transcribed during the subjective day (peak at circadian time [CT] 12–14) (Figure 1a); PER and TIM accumulate slowly until a threshold level of TIM is reached and stabilizes PER (Figure 1b,c). PER–TIM dimers enter the nucleus around CT 21 and inhibit transcription of their own genes (Figure 1d,e), but as the proteins turn over, inhibition is released and transcription begins again in the subjective morning (Figure 1f). Light-induced degradation of TIM during the early subjective night or the late subjective night decreases PER stability, which results in a phase delay or a phase advance in the cycle, respectively.

The current *Neurospora* model is analogous to that of *Drosophila* [15]. Similar to PER and TIM, FRQ appears to inhibit its own transcription [16]. In contrast to *Drosophila*, however, peak *frq* expression occurs during the day (CT 4–6), and light strongly induces *frq* transcription [16,17]. These differences indicate that while the negative feedback mechanism of circadian rhythmicity appears to be conserved, the required genes and regulatory pathways may differ from species to species.

In the past year, remarkable progress has been made in discerning the elements of the clock mechanism. Identification of positive elements (i.e. factors which activate rather than inhibit) allows the formal testing of the feedback loop model, and other new circadian genes provide additional information about the regulation of rhythmicity (Table 1). The discovery and functional analyses of these genes, comparison of circadian organization among divergent species, and new approaches in circadian biology are addressed in this review.

Closing in on closing the loop: new clock component genes and new aspects of circadian regulation

The mammalian clock

Molecular analysis of mammalian clock components began in earnest with the identification of the mouse *Clock* gene [18••,19••]. *Clock* was cloned by rescue of both the period length and period stability mutant phenotypes using a *Clock*-containing bacterial artificial chromosome transgene [18••]. Sequence analysis indicated that *Clock* encodes a putative bHLH-PAS domain transcription factor [19••]. This finding raised the intriguing possibility that CLOCK could act as a positive element within a transcription–translation negative

Table 1

Circadian clock gene expression, function and effects 1*.

Gene	Circadian phenotype	Circadian expression†	Acute light response	Reference
Mammals				
Clock	28 hour period, arrhythmicity	No	n.d.‡	[18••,19••,21••]
Per1	n.d.	Yes	↑mRNA	[21••,22••,26•]
Per2	n.d.	Yes	↑mRNA	[23•] [24•,25••]
Per3	n.d.	Yes	No	[25••]
Bmal1	n.d.	No	n.d.	[28•,29••,30]
Drosophila				
per	Many alleles, including 16 hour period, 19 hour period, 28 hour period, arrhythmicity	Yes	No	[3]
tim	Arrhythmicity	Yes	Protein degradation	[3]
dClock (Jrk)	Arrhythmicity; low *per*, *tim* expression	Yes (light–dark cycle)	n.d.	[36••,38••]
cycle (dbmal1)	Arrhythmicity; low *per*, *tim* expression	n.d.	n.d.	[37••,38••]
double-time	18 hour period, 27 hour period, arrhythmicity	No	No	[40••,41••]
Neurospora				
frq	Many alleles, including short period, long period, arrhythmicity	Yes	↑mRNA	[15]
white collar-1	Arrhythmicity, low *frq* expression	n.d.	n.d.§	[51•]
white collar-2	Arrhythmicity, low *frq* expression	n.d.	n.d.§	[51•]
Synechococcus				
kaiABC	Many alleles, including short period, long period, arrhythmicity	Yes	n.d.	[63]

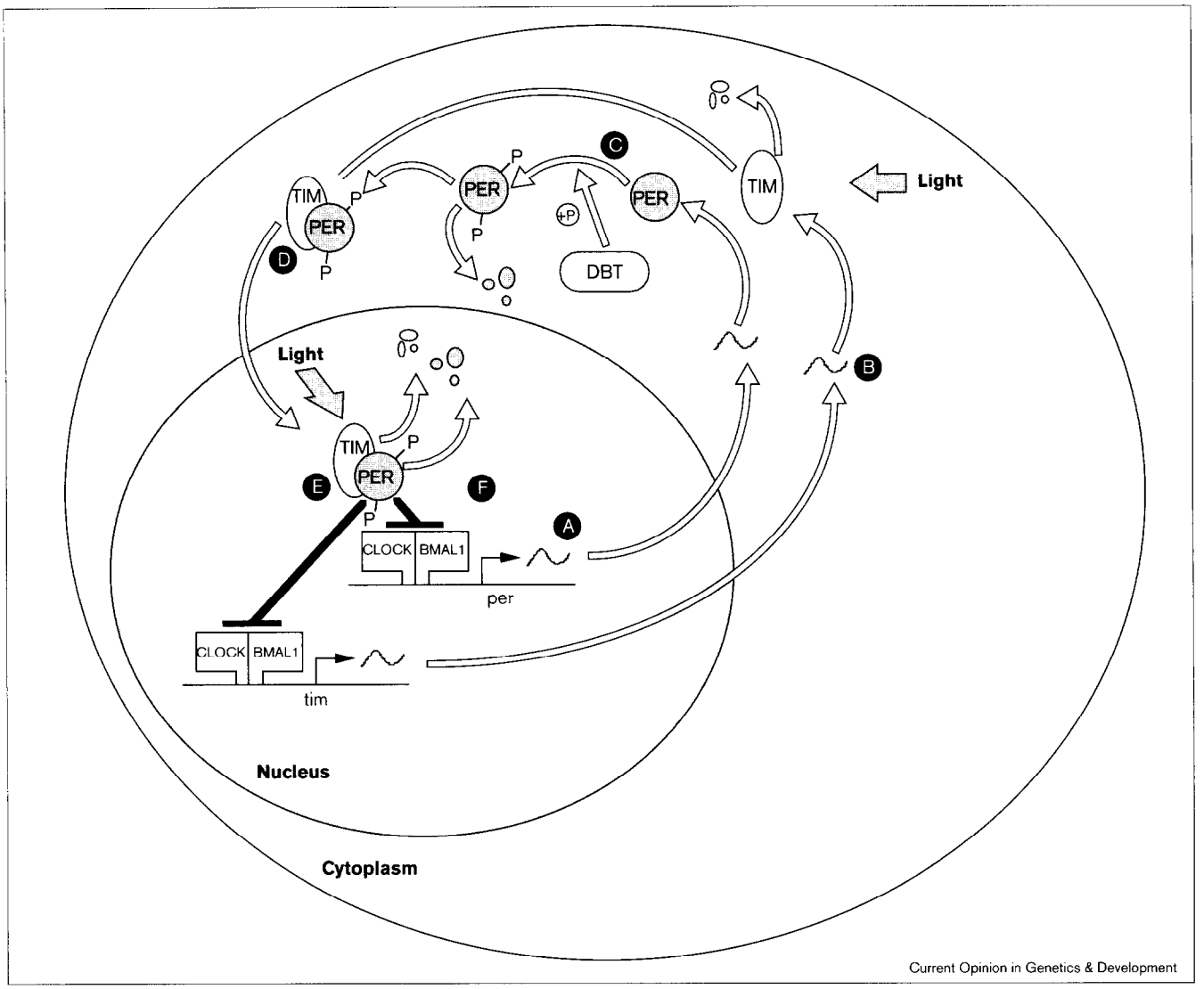
*'Genes' indicates known DNA and protein sequence. †Circadian rhythm of mRNA and/or protein expression. ‡n.d., not determined. §Mutations originally identified as a blue light blind phenotype.

feedback loop and could be inhibited by a mammalian version of PER via its PAS domain. Furthermore, the *Clock* mutation, an A→T transversion in the splice donor site of exon 19, results in a 51 amino acid deletion within the proposed activation domain and is consistent with the antimorphic nature of the *Clock* mutant phenotype [19••,20].

Following the cloning of *Clock*, the identification of *per* orthologs in human and in mouse by several independent laboratories underscored the possibility of a *Drosophila*-like feedback loop in mammals. These genes, prefixed human (h) or mouse (m) *Per1* [21••,22••], *Per2* [23•,24•], and *Per3* [25••], all encode proteins that contain a PAS domain. Circadian expression of m*Per1*, m*Per2*, and m*Per3* in the suprachiasmatic nuclei (SCN), the site of the mammalian clock, suggests that these genes could be circadian clock components [21••,22••,23•,24•,25••]. Confirmation of these genes as circadian components requires functional evidence: either a mutation within each gene or altered expression of each gene must result in an altered circadian phenotype in mice. In the SCN, m*Per1* expression peaks at CT 6 whereas m*Per2* peaks at CT 9; m*Per3* expression reaches its maximum at CT 6 and remains at that level until after CT 9. In tissues throughout the body, such as retina and skeletal muscle, expression of all m*Per* genes is delayed ~6 hours relative to their phase in the SCN [21••,22••,23•,24•,25••,26•]. Interestingly, a light pulse administered during the subjective night results in rapid, transient induction of m*Per1* expression (maximal induction within one hour) and delayed, transient induction of m*Per2* (maximal induction within 1.5 to 2 hours) [23•,24•,25••,26•]. In contrast, m*Per3* does not acutely respond to a light pulse [25••].

Transcriptional activation by bHLH factors such as CLOCK require dimerization and binding to a DNA promoter element called the E box (consensus 5′-CANNTG-3′) [27]. The CLOCK dimerization partner, *Bmal1* (for brain and muscle ARNT-like factor), was identified using a two-hybrid screen [28•,29••]; this gene of previously unknown function [30] also encodes a bHLH-PAS protein and is expressed in the SCN [29••]. Interestingly, an E box present in a 69 bp enhancer of the *Drosophila per* promoter is required for *per* mRNA cycling, which suggests a role for bHLH transcription factors in the circadian mechanism [31••]. Using a proximal fragment of the m*Per1* promoter which contains three E boxes, Gekakis *et al.* [29••] find that CLOCK and BMAL1 heterodimers bind the m*Per1* promoter and activate transcription. Furthermore, CLOCK–BMAL heterodimers can activate transcription from the three E boxes alone, whereas mutation of the E boxes abolishes DNA binding

Figure 1



Current molecular model of rhythm generation in *Drosophila*. The succession of events **(a–f)** occur over the course of ~24 hours. (a) CLOCK–BMAL heterodimers bind the *per* and *tim* promoters and activate mRNA expression from each locus; peak expression occurs ~CT 12. CLOCK–BMAL may also activate transcription other circadian-regulated genes (not shown). (b) *per* and *tim* mRNA are transported to the cytoplasm and translated into PER and TIM protein, respectively. (c) Regulation of protein levels occurs by two mechanisms: DBT protein phosphorylates and destabilizes PER, and light destroys TIM. Light during the early subjective night can phase-delay the clock. Small 'blobs' indicate degraded proteins. (d) PER and TIM levels slowly accumulate during the early subjective night; TIM stabilizes PER and promotes nuclear transport. Peak PER and TIM levels in the cytoplasm occur ~CT 19. (e) PER and TIM dimers enter the nucleus and inhibit CLOCK–BMAL-activated transcription; peak nuclear PER/TIM levels occur ~CT 21. (f) Protein turnover (combined with the lack of new PER and TIM synthesis) leads to derepression of *per* and *tim* mRNA expression; the cycle begins again (a) ~CT 2. Light during the late subjective night can phase-advance the clock.

and transcriptional activation [28•,29••]. Finally, the exon-19-deleted mutant form of CLOCK failed to activate transcription, a result fully consistent with the antimorphic *Clock* mutant allele [29••]. These results identify CLOCK and BMAL as positive elements in the transcription–translation loop; whether mPER1 inhibits CLOCK/BMAL activity awaits additional experimentation.

Further delineation of circadian output and input pathways in mammals has also been achieved recently. Expression of the basic leucine zipper transcription factor albumin site D-binding protein (DBP) oscillates under constant conditions in several tissues, including the SCN and the liver; interestingly, the phase of the rhythm in the SCN is advanced four hours relative to that in peripheral tissues [32•]. Animals homozygous for a targeted DBP-null mutation are less active than wild-type littermates but still display free-running rhythms, although period is ~30 minutes shorter than wild-type [32•]. In addition, the *dbp* gene does not require the DBP protein for its own expression [32•]. This

information suggests that *dbp* is an output gene rather than a part of the central oscillator mechanism. At the input level, targeted disruption of the melatonin 1a (Mel_{1a}) receptor showed that this receptor mediates the melatonin-induced inhibition of SCN neural activity, but that the phase-shifting effects of melatonin may be mediated by the Mel_{1b} receptor or by an unidentified receptor [33]. In addition, a putative input gene was identified in a cDNA subtraction screen for light-induced gene expression in the SCN; this gene, the zinc-finger transcription factor *egr-3*, is expressed in the ventral SCN and is gated by the circadian clock [34]. Finally, the mammalian blue-light photoreceptors cryptochrome 1 (*Cry1*) and cryptochrome 2 (*Cry2*) are expressed in the retina (*Cry1* and *Cry2*) and the SCN (*Cry1* only), thereby providing a new class of photopigments as candidates for the photic entrainment pathway in mammals [35•].

The *Drosophila* clock

Genetic and molecular approaches in *Drosophila* have led to recent discoveries that maintain this species as the best understood in the circadian field. Mutagenesis screening revealed the semidominant mutation *Jrk* and the gene dose-dependent mutation *cycle* (*cyc*), both of which either alter or abolish rhythms in behavior, *per* expression, and *tim* expression [36••,37••]. Concomitantly, d*clock* was isolated in a low-stringency screen with m*Clock*, and a *Drosophila*-expressed sequence tag clone with homology to h*Bmal1* was identified [38••]; d*clock* and d*bmal* were found to map to the *Jrk* and *cyc* loci, respectively. Darlington *et al.* [38••] have shown that dCLOCK (presumably with dBMAL) binds E box sequences to activate transcription of *per* and *tim*. Furthermore, co-expression of PER and TIM inhibits transcriptional activation by dCLOCK [38••]. These results thus appear to close the circadian loop: the positive elements dCLOCK and dBMAL activate transcription of the negative elements *per* and *tim*, the products of which eventually inhibit their own transcription via interaction with dCLOCK–dBMAL (Figure 1). The precise molecular interactions that mediate this inhibition are unknown; however, the action of PER–TIM on dCLOCK–dBMAL is relatively direct, as the E box element is necessary and sufficient for activation and inhibition of transcription [38••].

Regulation of the *Drosophila* circadian loop appears to occur at both the post-transcriptional and the post-translational levels. Using nuclear run-on experiments, So and Rosbash [39•] have demonstrated that *per* and *tim* are transcribed at high levels several hours before an RNase protection assay can detect their mRNA species. In addition, no rhythm in transcription rate was detected from a promoterless *per* gene that weakly restores rhythms of *per* mRNA accumulation to per^0 mutants. These results indicate that a post-transcriptional mechanism contributes to the observed cycle in *per* and *tim* mRNA expression [39•]. An important post-translational regulatory mechanism was also discovered recently. The mutation *double-time* (*dbt*) either shortens (dbt^S) or lengthens (dbt^L) period, and a *P* element-induced null or strongly hypomorphic mutation (dbt^P) results in pupal lethality [40••]. Remarkably, the *dbt* gene encodes a kinase with extensive homology to human casein kinase Iε [41••]. dbt^P homozygous mutant embryos express high levels of stable, unphosphorylated PER protein independently of circadian time whereas embryonic *tim* mRNA and protein rhythms are abolished [40••,42•]. These findings support a model in which DBT phosphorylates and destabilizes PER, thereby contributing to the translational delay of PER accumulation that is required for rhythmicity [40••,41••] (Figure 1). Finally, a post-translational modification other than phosphorylation within PER amino acids 637 and 848 appears to regulate cyclical PER degradation [43].

At the level of circadian output, analysis of the *lark* gene confirmed that it is under circadian clock control and therefore on the output pathway [44]. The *lark* gene product behaves like a repressor of eclosion, as the *lark* mutant allele results in early eclosion whereas additional copies of wild-type *lark* delay eclosion [45]. Despite the absence of a *lark* mRNA rhythm, LARK protein oscillates in abundance (peak and trough levels at CT 8 and CT 20, respectively) in the presence of a functional *per* gene [44]. Interestingly, *lark* is expressed both in lateral neurons, the proposed site of the *Drosophila* master clock, as well as in eclosion-regulating cells in the ventral nervous system [44]. These results suggest a specific output pathway for eclosion that is controlled by the central circadian oscillator.

The *Neurospora* clock

Continued analysis of the *Neurospora frq* gene in the past year has resulted in the identification of novel regulatory mechanisms. As in *Drosophila*, post-translational regulation plays a role in the *Neurospora* circadian clock: *frq* mRNA contains alternative translation initiation sites, the choice of which is mediated by environmental temperature [46••,47••]. At moderate temperatures (25°C), two forms are expressed, and each can support rhythmicity; however, at high temperatures (30°C) the short form of FRQ ($FRQ^{100-989}$) is unable to maintain rhythmicity, whereas at low temperatures (18°C) the full-length form cannot drive rhythms [47••]. In addition, temperature-shifting experiments in *Neurospora* have demonstrated that a shift from low temperature (21°C) to high temperature (28°C) strongly resets the clock to CT 0, whereas the opposite shift resets the clock to ~CT 12 [48••]. Finally, FRQ protein was shown to translocate to the nucleus and repress *frq* mRNA expression within 4 hours of the *frq* mRNA peak, with recovery from this repression taking the remainder of the circadian day [49•,50]. Thus, the details of circadian regulation between *Neurospora* and *Drosophila* continue to differ, as translational delay of PER and TIM in *Drosophila* versus long recovery period in *Neurospora* are used to maintain a 24-hour period.

The genes *white collar-1* (*wc-1*) and *white collar-2* (*wc-2*), which encode zinc-finger proteins, appear to be involved in circadian clock regulation [51•]. In particular, *wc-1* is necessary for light-induced *frq* expression, whereas *wc-2* may be

required for circadian *frq* expression [51•]. However, *wc-1* and *wc-2* have not been shown to bind the *frq* promoter or directly activate *frq* transcription. Sequence analysis indicates that FRQ itself may be a transcription factor, as FRQ shares moderate homology with known helix-turn-helix transcription factors [52]; but, again, no functional evidence in support of this hypothesis is available.

The cyanobacteria clock

The most recent circadian model system was established for cynobacterium *Synechococcus* strain PCC 7942, which displays circadian rhythms in bioluminescence despite a replication time of five to six hours [53•]. Using genetic complementation, a three-gene locus named *kaiABC* was shown to drive all circadian rhythms in this organism [54••]. These genes share no homology with any known genes. The KaiC product represses activity of the cluster, and overexpression of this gene can reset the phase of the clock. KaiA is required to drive *kaiC* expression. In effect, a single gene cluster in cyanobacteria appears to contain both negative and positive elements for a circadian negative feedback loop. These results demonstrate that circadian transcription–translation negative regulatory loops are conserved among living systems but the underlying genes differ among phyla.

Circadian organization: central pacemakers and peripheral oscillators

Conventional wisdom has it that the circadian clock resides in the brain in higher animal organisms. The lateral neurons in *Drosophila* appear to be important for circadian regulation [42•,55], and the suprachiasmatic nuclei are the site of the circadian clock in mammals [56]. In the past year, however, brain-independent circadian oscillators (cells capable of self-sustained rhythmic output) have been detected in many peripheral tissues of *Drosophila* and within cultured cell lines in mammals [57,58,59••,60••]. For example, the Malpighian tubules of both decapitated flies and non-decapitated control animals displayed identical circadian rhythms of PER-lacZ reporter expression and nuclear localization [57]. Kay and colleagues have extended this observation using a real-time luciferase reporter assay to show that the *Drosophila* body as a whole and in cultured segments sustains circadian rhythms in *per*-driven expression [58,59••]. Furthermore, every cultured tissue could be entrained by light, indicating that non-neural *Drosophila* cells are photoreceptive [59••].

Significant new evidence has been found for the existence of oscillators throughout the mammalian body. As discussed above, rhythms in m*Per1*, m*Per2*, and m*Per3* can be found in many non-neural body tissues [22••,23•,25••]. In cell culture, serum stimulation of rat-1 fibroblasts and H35 hepatoma cells elicits expression of several genes, including rat (r) *Per1*, r*Per2*, *dbp*, and *tef* (thyroid embryonic factor). Remarkably, the expression patterns of these genes then oscillate in a circadian manner in the presence of the cell cycle inhibitor cytosine β-D arabinofuranoside [60••]. This discovery provides definitive evidence of brain-independent mammalian clock cells. Furthermore, the relative phases of r*Per1* and r*Per2* expression in cell culture match those found in the liver *in vivo* [60••]. Finally, the r*Per1* and r*Per2* genes fulfill the criteria for immediate-early genes in that serum induction is rapid and independent of new protein synthesis [60••]. This finding is reminiscent of the immediate-early expression of c-*fos* and *jun-B* in the SCN in response to light and suggests that immediate-early signaling pathways may play a role in conveying photic information to the circadian clock in the SCN [61].

Comparison of mammalian *per* expression data shows that the phase of these circadian genes is advanced between three and nine hours (depending on lighting conditions, species, and laboratory) in the SCN relative to the rest of the body [22••,23•,25••,32•,60••], suggesting that the SCN play a special role within the collection of cellular oscillators. Indeed, twenty-five years of physiological evidence has demonstrated that the SCN contains the required mammalian pacemaker — the oscillator that drives period and phase in other oscillating cells [62]. The method by which the SCN directs circadian rhythmicity throughout the body is unknown, but two general mechanisms are possible: the SCN could drive rhythms in passive, non-oscillating cells, or, conversely, the SCN could coordinate cell-autonomous oscillators. The discovery of peripheral oscillators strongly supports the latter model. Indeed, the physiological organization of circadian rhythmicity can be compared to the hierarchy of cardiac pacemakers: in the heart, the sinoatrial (SA) node controls the period of cardiac rate but, in the absence of the SA node, the atrioventricular (AV) node regulates rhythm. In the absence of either node, individual cardiac cells are capable of rhythmic contraction. This analogy could be applied to the circadian system, where some unknown factor(s) place the SCN at the top of the circadian hierarchy to coordinate cells in the body as a precisely functioning unit.

Conclusions

The tremendous progress towards the molecular dissection of the circadian clock places the circadian field in an exciting era. The identification of new circadian genes and the delineation of regulatory mechanisms in diverse model organisms have underscored the universal nature of the circadian clock yet also suggested phylogenetic differences in its assembly. Future studies will undoubtedly focus on each gene's functional role and interactions with other circadian genes within the organism. Finally, the discovery of brain-independent circadian clocks should allow the elucidation of the molecular circadian mechanism and provide a better understanding of the physiological circadian hierarchy.

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References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Crews ST, Thomas JB, Goodman CS: **The *Drosophila single-minded* gene encodes a nuclear protein with sequence similarity to the *per* gene product.** *Cell* 1988, **52**:143-151.

2. Takahashi JS: **Molecular neurobiology and genetics of circadian rhythms in mammals.** *Annu Rev Neurosci* 1995, **18**:531-553.

3. Rosato E, Piccin A, Kyriacou CP: **Circadian rhythms: from behavior to molecules.** *Bioessays* 1997, **19**:1075-1082.

4. Rosbash M: **Molecular control of circadian rhythms.** *Curr Opin Genet Dev* 1995, **5**:662-668.

5. Hardin PE, Hall JC, Rosbash M: **Feedback of the *Drosophila period* gene product on circadian cycling of its messenger RNA levels.** *Nature* 1990, **343**:536-540.

6. Edery I, Zwiebel LJ, Dembinska ME, Rosbash M: **Temporal phosphorylation of the *Drosophila* period protein.** *Proc Natl Acad Sci USA* 1994, **91**:2260-2264.

7. Sehgal A, Rothenfluh-Hilfiker M, Hunter-Ensor M, Chen Y, Myers MP, Young MW: **Rhythmic expression of *timeless*: a basis for promoting circadian cycles in *period* gene autoregulation.** *Science* 1995, **270**:808-810.

8. Hunter-Ensor M, Ousley A, Sehgal A: **Regulation of the *Drosophila* protein timeless suggests a mechanim for resetting the circadian clock by light.** *Cell* 1996, **84**:677-685.

9. Myers MP, Wager-Smith K, Rothenfluh-Hilfiker A, Young MW: **Light-induced degradation of TIMELESS and entrainment of the *Drosophila* circadian clock.** *Science* 1996, **271**:1736-1740.

10. Zeng H, Qian Z, Myers MP, Rosbash M: **A light-entrainment mechanism for the *Drosophila* circadian clock.** *Nature* 1996, **380**:129-135.

11. Zeng H, Hardin PE, Rosbash M: **Constitutive overexpression of the *Drosophila period* protein inhibits *period* mRNA cycling.** *EMBO J* 1994, **13**:3590-3598.

12. Gekakis N, Saez L, Delahaye-Brown A-M, Myers MP, Sehgal A, Young MW, Weitz CJ: **Isolation of *timeless* by PER protein interaction: defective interaction between *timeless* protein and long-period mutant PER^L.** *Science* 1995, **270**:811-815.

13. Saez L, Young M: **Regulation of nuclear entry of the *Drosophila* clock proteins period and timeless.** *Neuron* 1996, **17**:911-920.

14. Lee C, Parikh V, Itsukaichi T, Bac K, Edery I: **Resetting the *Drosophila* clock by photic regulation of PER and a PER-TIM complex.** *Science* 1996, **271**:1740-1744.

15. Dunlap JC: **Genetic and molecular analysis of circadian rhythms.** *Annu Rev Genet* 1996, **30**:579-601.

16. Aronson BD, Johnson KA, Loros JJ, Dunlap JC: **Negative feedback defining a circadian clock: autoregulation of the clock gene *frequency*.** *Science* 1994, **263**:1578-1584.

17. Crosthwaite SK, Loros JJ, Dunlap JC: **Light-induced resetting of a circadian clock is mediated by a rapid increase in *frequency* transcript.** *Cell* 1995, **81**:1003-1012.

18. •• Antoch MP, Song E-J, Chang A-M, Vitaterna MH, Zhao Y, Wilsbacher LD, Sangoram AM, King DP, Pinto LH, Takahashi JS: **Functional identification of the mouse circadian *Clock* gene by transgenic BAC rescue.** *Cell* 1997, **89**:655-667.

Complete rescue of the *Clock* mutant phenotypes is achieved using bacterial artificial chromosome transgenes, which shows that functional rescue by transgenesis is a viable method of gene cloning in mammals. Another interesting observation: period length is shortened to values beyond wild-type in transgenic lines containing a high copy number of the transgene.

19. •• King DP, Zhao Y, Sangoram AM, Wilsbacher LD, Tanaka M, Antoch MP, Steeves TDL, Vitaterna MH, Kornhauser JM, Lowrey PL *et al.*: **Positional cloning of the mouse circadian *Clock* gene.** *Cell* 1997, **89**:641-653.

Genetic and physical mapping is combined with a three-tiered molecular approach to transcription unit analysis leading to the identification of the *Clock* gene sequence. Sequence analysis indicates that *Clock* encodes a bHLH-PAS transcription factor. The *Clock* mutation results in deletion of a portion of the putative activation domain, which is consistent with the antimorphic nature of the mutation.

20. King DP, Vitaterna MH, Chang AM, Dove WF, Pinto LH, Turek FW, Takahashi JS: **The mouse *Clock* mutation behaves as an antimorph and maps within the W^{19H} deletion, distal of *Kit*.** *Genetics* 1997, **146**:1049-1060.

21. •• Tei H, Okamura H, Shigeyoshi Y, Fukuhara C, Ozawa R, Hirose M, Sakaki Y: **Circadian oscillation of a mammalian homologue of the *Drosophila period* gene.** *Nature* 1997, **389**:512-516.

A novel PCR-based approach, which took advantage of relatively conserved sequence within the functionally significant PAS domain, is used here to identify a mammalian ortholog of the *Drosophila period* gene; significant sequence homology occurs throughout the entire gene in human, mouse, and *Drosophila*. Mouse (m) *Per* expression cycles in a circadian manner in the SCN.

22. •• Sun ZS, Albrecht U, Zhuchenko O, Bailey J, Eichele G, Lee CC: ***RIGUI*, a putative mammalian ortholog of the *Drosophilia period* gene.** *Cell* 1997, **90**:1003-1011.

This group cloned human *RIGUI* (now renamed h*per1*) in a chromosome 17 transcript mapping project. m*Per1* (called m*RIGUI* here) expression displays a circadian rhythm in the SCN as well as in the retina, the pars tuberalis, and the Purkinje neurons; interestingly, expression in the SCN is advanced 6–12 hours relative to the other tissues.

23. • Shearman LP, Zylka MJ, Weaver DR, Kolakowski LFJ, Reppert SM: **Two *period* homologs: circadian expression and photic regulation in the suprachiasmatic nuclei.** *Neuron* 1997, **19**:1261-1269.

See annotation [25••].

24. • Albrecht U, Sun ZS, Eichele G, Lee CC: **A differential response of two putative mammalian circadian regulators, *mper1* and *mper2*, to light.** *Cell* 1997, **91**:1055-1064.

See annotation [25••].

25. •• Zylka MJ, Shearman LP, Weaver DR, Reppert SM: **Three *period* homologs in mammals: differential light responses in the suprachiasmatic circadian clock and oscillating transcripts outside of brain.** *Neuron* 1998, **20**:1103-1110.

These papers [23•,24•,25••,26•] collectively illustrate several new, fundamentally important results in mammalian circadian biology. First, at least three mammalian *per* homologs exist. Second, all three genes respond differently to light administered during the subjective night: m*Per1* expression increases and decreases rapidly, much like the immediate-early gene response; m*Per2* expression increases in a delayed fashion, then decreases rapidly; and m*Per3* expression does not change in response to light. Finally, the phase of each m*Per* is advanced 3–9 hours in the SCN relative to other body tissues including the retina, skeletal muscle, and testis.

26. • Shigeyoshi Y, Taguchi K, Yamamoto S, Takekida S, Yan L, Tei H, Moriya T, Shibata S, Loros JJ, Dunlap JC, Okamura H: **Light-induced resetting of a mammalian circadian clock is associated with rapid induction of the *mPer1* transcript.** *Cell* 1997, **91**:1043-1053.

See annotation [25••].

27. Weintraub H, Davis R, Tapscott S, Thayer M, Krause M, Benezra R, Blackwell TK, Turner D, Rupp R, Hollenberg S: **The MyoD gene family: Nodal point during specification of the muscle cell lineage.** *Science* 1991, **251**:761-766.

28. • Hogenesch JB, Gu Y-Z, Jain S, Bradfield CA: **The basic helix-loop helix-PAS orphan MOP3 forms transcriptionally active complexes with circadian and hypoxia factors.** *Proc Natl Acad Sci USA* 1998, **95**:5474-5479.

CLOCK is shown to be a dimerization partner of MOP3 (BMAL1), and the E-box promoter element is shown to direct reporter expression in the presence of CLOCK/MOP3 (BMAL1). In addition, MOP3 forms transcriptionally functional dimers with MOP4 (NPAS2), HIF1α, and HIF2α.

29. •• Gekakis N, Staknis D, Nguyen HB, Davis FC, Wilsbacher LD, King DP, Takahashi JS, Weitz CJ: **Role of the CLOCK protein in the mammalian circadian mechanism.** *Science* 1998, **280**:1564-1569.

The authors of this paper describe the first definitive positive elements in the mammalian circadian clock mechanism: CLOCK and BMAL1 activate m*Per1* expression directly. The CLOCK/BMAL1 dimer can bind both the *Drosophila per* promoter E box and the m*Per1* promoter E boxes to drive expression of a reporter gene; furthermore, the mutant *Clock* product fails to transactivate the *mPer1* promoter.

30. Ikeda M, Nomura M: **cDNA cloning and tissue-specific expression of a novel basic helix-loop-helix/PAS protein (BMAL1) and identification of alternatively spliced variants with alternative translation initiation site usage.** *Biochem Biophys Res Commun* 1997, **233**:258-264.

31. Hao H, Allen DL, Hardin PE: **A circadian enhancer mediates PER**
•• **dependent mRNA cycling in *Drosophila melanogaster*.** *Mol Cell Biol* 1997, **17**:3687-3693.
The first indication that a bHLH transcription factor may be involved in the circadian mechanism came from this work. A 69 bp enhancer in the *Drosophila Per* promoter that is necessary and sufficient for rhythmic expression of *Per* was isolated. This enhancer contains an E box, the DNA element that bHLH transcription factors bind. Mutation of the E box abolished rhythmic *per* expression, which suggested that bHLH factors are a part of the circadian loop.

32. Lopez-Molina L, Conquet F, Dubois-Dauphin M, Schibler U: **The DBP**
• **gene is expressed according to a circadian rhythm in the suprachiasmatic nucleus and influences circadian behavior.** *EMBO* 1997, **16**:6762-6771.
These authors made the first observation of a gene, *dbp* (*albumin site D binding protein*), whose phase of expression is advanced in the SCN relative to peripheral tissues. Targeted deletion of the *dbp* gene does not abolish circadian rhythms in behavior but period length decreases by ~30 minutes.

33. Liu C, Weaver DR, Jin X, Shearman LP, Pieschl RL, Gribkoff VK, Reppert SM: **Molecular dissection of two distinct actions of melatonin on the suprachiasmatic circadian clock.** *Neuron* 1997, **19**:91-102.

34. Morris ME, Viswanathan N, Kuhlman S, Davis FC, Weitz CJ: **A screen for genes induced in the suprachiasmatic nucleus by light.** *Science* 1998, **279**:1544-1547.

35. Miyamoto Y, Sancar A: **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* 1998, **95**:6087-6102.
Expression of new photopigments in the retina and SCN suggest the existence of an entrainment-specific photic response pathway.

36. Allada R, White NE, So WV, Hall JC, Rosbash M: **A mutant**
•• ***Drosophila* homolog of mammalian *Clock* disrupts circadian rhythms and transcription of *period* and *timeless*.** *Cell* 1998, **93**:791-804.
The circadian mutation *Jrk* severely disrupts rhythmicity in behavior, *per* expression and *tim* expression in the heterozygous state and abolishes these rhythms in the homozygous state. *Jrk* is the *Drosophila* ortholog of mammalian *Clock*; the *Jrk* mutation results in truncation of a large portion of the putative activation domain.

37. Rutila JE, Suri V, Le M, So WV, Rosbash M, Hall JC: **CYCLE is a**
•• **second bHLH-PAS clock protein essential for circadian rhythmicity and transcription of *Drosophila period* and *timeless*.** *Cell* 1998, **93**:805-814.
The circadian mutation *cycle* lengthens behavioral period in the heterozygous state and abolishes behavioral, *per* expression, and *tim* expression rhythms in the homozygous state. *cycle* is the *Drosophila* ortholog of *Bmal1*; a nonsense mutation just downstream of the PAS-B region corresponds well with the circadian phenotype.

38. Darlington TK, Wager-Smith K, Ceriani MF, Staknis D, Gekakis N,
•• Steeves TDL, Wietz CJ, Takahashi JS, Kay SA: **Closing the circadian loop: CLOCK-induced transcription of its own inhibitors *per* and *tim*.** *Science* 1998, **280**:1599-1603.
This group have independently identified the *Drosophila* orthologs of *Clock* and *bmal1*, then demonstrated the activities of the known circadian genes: first, dCLOCK activates expression of d*per* and d*tim* through the E box present in each gene's promoter; and second, PER and TIM directly inhibit dCLOCK to decrease their own expression.

39. So WV, Rosbash M: **Post-transcriptional regulation contributes to**
• ***Drosophila* clock gene mRNA cycling.** *EMBO* 1997, **16**:7146-7155.
Using the nuclear run-on assay, these authors confirm that *per* and *tim* mRNA transcription rates cycle in a circadian manner but that the amplitude and phase of these rhythms differ unexpectedly from amplitude and phase in their mRNA abundance rhythms. This finding suggests that post-transcriptional regulation, in addition to transcriptional activation, affects mRNA cycling of circadian genes.

40. Price JL, Blau J, Rothenfluh A, Abodeely M, Kloss B, Young MW:
•• ***double-time* is a new *Drosophila* clock gene that regulates PERIOD protein accumulation.** *Cell* 1998, **94**:83-95.
See annotation [41••].

41. Kloss B, Price JL, Saez L, Blau J, Rothenfluh A, Wesley C, Young MW:
•• **The *Drosophila* clock gene *double-time* encodes a protein closely related to human casein kinase I-epsilon.** *Cell* 1998, **94**:97-107.
The isolation of a new circadian mutant in *Drosophila* and the positional cloning of its gene introduce an important regulatory mechanism of the circadian clock. *double-time* encodes a protein with high homology to human casein kinase Iε; this homology combined with the lack of PER phosphorylation in *dbt*^{P} mutants suggests that the *dbt* product phosphorylates PER, which destabilizes PER and possibly contributes to its translational delay.

42. Kaneko M, Helfrich-Forster C, Hall JC: **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* 1997, **17**:6745-6760.
This elegant work demonstrates circadian expression of *per* and *tim* in *Drosophila* larvae. Expression of these genes was found in the lateral neurons and a subset of the larval dorsal neurons; surprisingly, two larval dorsal neurons express PER and TIM in antiphase to the other neurons.

43. Dembinska ME, Stanewsky R, Hall JC, Rosbash M: **Circadian cycling of a PERIOD-β-*galactosidase* fusion protein in *Drosophila*:evidence for cyclical degradation.** *Jour Biol Rhyth* 1997, **12**:157-172.

44. McNeil GP, Zhang X, Genova G, Jackson FR: **A molecular rhythm mediating circadian clock output in *Drosophila*.** *Neuron* 1998, **20**:297-303.

45. Newby LM, Jackson FR: **Regulation of a specific circadian clock output pathway by lark, a putative RNA-binding protein with repressor activity.** *J Neurobiol* 1996, **31**:117-128.

46. Garceau NY, Liu Y, Loros JJ, Dunlap JC: **Alternative initiation of**
•• **translation and time-specific phosphorylation yield multiple forms of the essential clock protein FREQUENCY.** *Cell* 1997, **89**:469-476.
These authors provide the first demonstration of FRQ protein oscillations in both abundance and mobility. In addition, alternative initiation events leading to two forms of FRQ are described.

47. Liu Y, Garceau NY, Loros JJ, Dunlap JC: **Thermally regulated**
•• **translational control of FRQ mediates aspects of temperature responses in the *Neurospora* icircadian clock.** *Cell* 1997, **89**:477-486.
This paper introduced a new regulatory mechanism in *Neurospora*: alternative translational initiation mediated by temperature. This work provides a molecular entry into the study of temperature compensation in *Neurospora*.

48. Liu Y, Merrow M, Joros JJ, Dunlap JC: **How temperature changes**
•• **reset a circadian oscillator.** *Science* 1998, **281**:825-829.
An elegant paper that details how temperature can reset a circadian oscillator by its effects on FRQ protein levels.

49. Merrow MW, Garceau NY, Dunlap JC: **Dissection of a circadian**
• **oscillation into discrete domains.** *Proc Natl Acad Sci USA* 1997, **94**:3877-3882.
Using an inducible *frq* construct in a *frq* loss-of-function genetic background, these authors demonstrate that FRQ protein rapidly inhibits *frq* transcription and that recovery from this inhibition requires the rest of the circadian day.

50. Luo C, Loros JJ, Dunlap JC: **Nuclear localization is required for function of the essential clock protein FRQ.** *EMBO* 1998, **17**:1228-1235.

51. Crosthwaite SK, Dunlap JC, Loros JJ: ***Neurospora wc-1* and *wc-2*:**
• **transcription, photoresponses, and the origins of circadian rhythmicity.** *Science* 1997, **276**:763-769.
Evidence of zinc-finger proteins as positive elements in the circadian loop is provided in this paper. The *wc-1* gene is required for response to light, and the *wc-2* gene appears to be essential in circadian rhythm generation.

52. Lewis MT, Morgan LW, Feldman JF: **Analysis of *frequency (frq)* clock gene homologs: evidence for a helix-turn-helix transcription factor.** *Mol Gen Genet* 1997, **253**:401-414.

53. Kondo T, Mori T, Lebedeva NV, Aoki S, Ishiura M, Golden SS:
• **Circadian rhythms in rapidly diving cyanobacteria.** *Science* 1997, **275**:224-227.
The unexpected presence of circadian rhythms in bioluminescence and mRNA expression in an organism with a generation time of five to six hours suggests the existence of a universal circadian mechanism, but the details of this mechanism in prokaryotes are expected to differ significantly from that in eukaryotes.

54. Ishiura M, Kutsuna S, Aoki S, Iwasaki H, Andersson CR, Tanabe A,
•• Golden SS, Johnson CH, Kondo T: **Expression of a gene cluster *kaiABC* as a circadian feedback process in cyanobacteria.** *Science* 1998, **281**:1519-1523.
The cloning and characterization of the genes responsible for all circadian phenotypes in cyanobacteria indicate that the negative regulatory loop is indeed conserved throughout evolution; however, the nature of the gene products differ among phyla.

55. Hall JC: **Tripping along the trail to the molecular mechanisms of biological clocks.** *Trends Neurosci* 1995, **18**:230-240.

56. Ralph MR, Foster RG, Davis FC, Menaker M: **Transplanted suprachiasmatic nucleus determines circadian period.** *Science* 1990, **247**:975-978.

57. Hege DM, Stanewsky R, Hall JC, Giebultowicz JM: **Rhythmic expression of a PER-reporter in the Malpighian tubules of decapitated *Drosophila*: evidence for a brain-independent circadian clock.** *J Biol Rhythms* 1997, **12**:300-308.

58. Plautz JD, Straume M, Stanewsky R, Jamison CF, Brandes C, Dowse HB, Hall JC, Kay SF: **Quantitative analysis of *Drosophila period* gene transcription in living animals.** *J Biol Rhythms* 1997, **12**:204-217.

59. •• Plautz JD, Kaneko M, Hall JC, Kay SA: **Independent photoreceptive circadian clocks throughout *Drosophila*.** *Science* 1997, **278**:1632-1635.
Both whole bodies and cultured tissue segments of *Drosophila* are shown to contain endogenous circadian oscillators. Remarkably, all *per*-expressing cells are photoreceptive, an observation that challenges the somewhat narrow view that only the brain can regulate rhythms in the body.

60. •• Balsalobre A, Damiola F, Schibler U: **A serum shock induces circadian gene expression in mammalian tissue culture cells.** *Cell* 1998, **93**:929-937.
This paper demonstrates the existence of functional circadian clocks in cell culture, a discovery that should allow the elucidation of the circadian mechanism sooner rather than later. In addition, the immediate-early kinetics of circadian gene induction suggest a role of immediate-early gene expression in the circadian pathway.

61. Kornhauser JM, Mayo KE, Takahashi JS: **Light, immediate-early genes, and circadian rhythms.** *Behav Genet* 1996, **26**:221-240.

62. Weaver DR: **The suprachiasmatic nucleus: a 25-year retrospective.** *J Biol Rhythms* 1998, **13**:100-112.

63. Kondo T, Tsinoremas NF, Golden SS, Johnson CH, Kutsuna S, Ishiura M: **Circadian clock mutants of cyanobacteria.** *Science* 1994, **266**:1233-1236.