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Introduction: Finding new clock components; past and future

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Abstract

The molecular mechanism of circadian clocks has been unraveled primarily by the use of phenotype-driven (forward) genetic analysis in a number of model systems. We are now in a position to consider what constitutes a clock component, whether we can establish criteria for clock components, and whether we have found most of the primary clock components? This perspective discusses clock genes and how genetics, molecular biology and biochemistry have been used to find clock genes in the past and the future.

Keywords

circadian clock components; clock genes; phenotype; forward genetics

The modern era of ‘clock genetics’ began in 1971 with the discovery by Ron Konopka and Seymour Benzer of the *period* (*per*) locus in *Drosophila* (Konopka and Benzer, 1971). At the time, even the existence of single-gene mutations affecting a process as complex as circadian rhythms was met with great skepticism. The eminent phage geneticist, Max Delbruck, is purported to have said, “No, no, no. It is impossible,” when told of Konopka’s results. Benzer’s reply: “But Max, you don’t understand. They’ve already been found” (Greenspan, 1990). Much has changed since the golden age of phage genetics, but much has also stayed the same. Thirty-three years later, we have dozens of examples of single genes that affect behavior (Kyriacou and Hall, 1994; Takahashi et al., 1994; Bucan and Abel, 2002). We have lived through decades when it was hard to clone a gene – now, we can manipulate genes at will. We have complete genome sequences for most of the important model organisms.

In the circadian field, we have identified cell autonomous circadian pathways that share a conserved transcriptional autoregulatory feedback mechanism across two domains of life (Dunlap, 1999; Young and Kay, 2001; Reppert and Weaver, 2002; Lowrey and Takahashi, 2004). And, we can already anticipate that circadian clock proteins will ultimately yield to structural biology (Uzumaki et al., 2004; Vakonakis et al., 2004; Ye et al., 2004). So where will this end, and what issues remain? In a sense, this is where much has stayed the same. We are upon the 50th anniversary of Pittendrigh’s analysis of temperature compensation of circadian oscillations in *Drosophila pseudoobscura* (Pittendrigh, 1954), yet we know very little more about this mechanism today. [Ironically, the *D. pseudoobscura* genome is being sequenced. Even Pittendrigh would not have expected this.] That is not to say that we haven’t learned much. To the contrary, we are approaching a complete catalog (‘parts list’) of circadian oscillator components in a number of organisms, and we even think we know how many of these parts work and interact with one another. But, a rigorous description of clock mechanism that includes an understanding of kinetics and stoichiometry still eludes us. Moreover, we need validation of such models *in vivo* (not in transfected cells!). Finally,

we have no deep understanding of how cell autonomous circadian oscillators interact in multicellular organisms ultimately to regulate behavior (Low-Zeddies and Takahashi, 2001).

In the beginning there was per

Benzer's bold foray into the genetics of brain and behavior in *Drosophila* remains one of the most remarkable achievements in biology - by unraveling complex systems using systematic screens focused on a judicious array of related phenotypes (Benzer, 1973). The *period* gene story remains the exemplar for genetic dissection of behavior (Hall, 2003); however, one should not forget the significance of the other Benzer mutants. The *Shaker* mutant defined the first potassium channel at the molecular level (Kaplan and Trout, 1969; Kamb et al., 1987; Papazian et al., 1987). The *dunce* mutant provided an entrée into the genetics of learning and memory (Dudai et al., 1976; Chen et al., 1986). The *sevenless* mutant defined a developmental pathway for photoreceptor determination (Banerjee et al., 1987; Hafen et al., 1987). And recent mutants such *bubblegum* and *methuselah* have uncovered genes in neurodegeneration and lifespan (Lin et al., 1998; Min and Benzer, 1999). So what is my point here? The discovery of circadian rhythm genes rests largely (indeed almost exclusively) upon the use of forward genetic screens to isolate circadian mutants, which in turn were used to identify the causative gene. Including *per* (Bargiello et al., 1984; Zehring et al., 1984), almost every important circadian locus has been defined by so called 'clock mutants.' This list includes *frequency* (*frq*), *timeless* (*tim*), *Clock*, *KaiABC*, and *double-time* which were first identified by positional cloning (McClung et al., 1989; Myers et al., 1995; Antoch et al., 1997; King et al., 1997a; Ishiura et al., 1998; Kloss et al., 1998). A second list includes *cycle* and *tau* which provided the primary phenotypic evidence that the underlying gene was a 'core component' (see below) (Rutila et al., 1998; Lowrey et al., 2000).

Phenotype, phenotype, phenotype

In retrospect, a compelling 'phenotypic profile' can be described to define what we now consider 'core clock genes.' First and foremost is an extreme phenotype: all of these key loci can be defined by mutations that alter period length by more than 15% (3–4 hours) or lead to a complete loss of circadian rhythms. When multiple alleles are available, period shortening and lengthening mutations are observed, as well as, loss-of-rhythm mutants. Ironically, *per* met all of these criteria from the beginning. Subsequently, *frq*, *tim* and *KaiABC* also produced this extreme range of phenotypes. In the absence of multiple alleles or in cases where null mutations are lethal, an extreme period phenotype appears to be sufficiently compelling to define a clock gene of interest. The mouse *Clock*, *Drosophila double-time* and hamster *tau* mutants fall into this category in which mutations caused 4-hour period changes and null mutations were not available.

One could of course argue that this analysis is biased – we have only gone after the extreme mutations and so these are the ones that we know about. This is fair, but if we look more broadly at targeted mutations in which we begin with the gene and then ask what the phenotype might be, the 'extreme phenotypic profile' still appears to hold, especially if we allow the inclusion of double mutants for paralogous genes such as *Period* and *Cryptochrome* in mice. With the exception of the *Bmal1* (also known as *Mop3*) knockout, which has an extreme loss-of-rhythm phenotype by itself (Bunger et al., 2000), most gene-targeted null mutations have produced less extreme (more subtle) circadian phenotypes. The next best knockout is *Per2* which causes a 1.5 hour period shortening and eventual loss of rhythms in the majority of mice in constant darkness (Zheng et al., 1999). Knockouts of *Per1* and *Per3* fall into the subtle (0–1 hour period changes) category at best (Bae et al., 2001). Fortunately, the *Per1/Per2* double knockout has strong effects, causing a complete loss of rhythms in constant darkness (Bae et al., 2001; Zheng et al., 2001). The

Cryptochrome knockouts are also interesting. The *Cry1* null mutation causes a relatively subtle 1-hour shortening of period, while the *Cry2* null mutation causes a 1-hour lengthening of period (van der Horst et al., 1999; Vitaterna et al., 1999). Again, the *Cry1/Cry2* double knockout has a profound phenotype with a complete loss of circadian rhythms in constant darkness (van der Horst et al., 1999; Vitaterna et al., 1999).

In contrast to this strong class of mutant circadian phenotypes, there are also examples of circadian genes that are involved in the clock mechanism but are not essential for the generation of circadian rhythms. This second ‘nonessential category’ is exemplified by *Rev-erb* (Preitner et al., 2002). This gene is activated by the CLOCK-BMAL1 complex and is in turn regulated by PER/CRY-mediated repression. REV-ERB then feeds back on *Bmal1* (and *Clock*) to drive cycles of transcription that are out of phase with *Per* and *Cry* (Preitner et al., 2002). This pathway forms a second feedback loop in the circadian network that has been labeled as a “positive feedback” loop (which we now know is the result of two inhibitory steps). Null mutations of *Rev-erb* abolish the feedback effects of this pathway on *Bmal1* and transcription of *Bmal1* is constitutively elevated. However, circadian rhythm generation persists in the absence of REV-ERB, and there are only subtle effects on the period and stability of circadian rhythms in the behavior of knockout mice (Preitner et al., 2002). This specific example illustrates the quandry that exists when we define clock components: REV-ERB is clearly part of the native circadian oscillator network, yet it is not essential for the generation of circadian oscillations.

Additional examples of genes that would not be expected to produce extreme phenotypes include other elements of the circadian oscillator system such as those involved in input and output pathways. Again, it is difficult to make definitive statements here, but some generalizations apply. Inputs to the circadian oscillator can exert significant phase-shifting and period effects; however, loss-of-function mutations should not lead to arrhythmicity. Rather such mutations should lead to defects in entrainment as seen with the *cryptochrome^b* (*cry^b*) mutation in *Drosophila* (Stanewsky et al., 1998), or with compound mutations of the mammalian visual system involving melanopsin (Hattar et al., 2003; Panda et al., 2003). Output mutations can produce overt arrhythmicity, but strong effects on period or phase should not occur. Molecular readouts of core oscillator components (such as *Per*) can then be used to assess whether the circadian oscillator is intact. Good examples of the effects of loss-of-function mutations on output components are *Dbp*, a direct target of CLOCK-BMAL1 (Lopez-Molina et al., 1997; Ripperger et al., 2000), and *Pdf*, a peptide output signal in *Drosophila* (Renn et al., 1999). Caution is warranted, however, because feedback effects of output pathways such as locomotor activity can exert phase-shifting effects, subtle period changes, and modulation of clock gene expression (Van Reeth and Turek, 1989; Edgar et al., 1991; Maywood et al., 1999).

In spite of the complexities addressed above, we can define a set of criteria for primary (core or essential) clock components. The strongest case can be made when there are ‘strong’ (i.e., 3–4 hour) period shortening and period lengthening mutations, as well as, arrhythmicity for null mutations. *Drosophila per* and *tim* are the prototypical examples (Konopka and Benzer, 1971; Konopka et al., 1994; Sehgal et al., 1994; Rothenfluh et al., 2000). When homozygous null mutations are lethal, as in the case of *double-time* in *Drosophila*, the spectrum of extreme period mutations maybe compelling (Price et al., 1998). However, the lack of a loss-of-function null mutation tempers our ability to conclude that the gene is essential in the classic genetic sense. In mammals, where paralogous genes are common, compound null mutations are required to achieve a strong loss-of-rhythm phenotype (van der Horst et al., 1999; Vitaterna et al., 1999; Bae et al., 2001; Zheng et al., 2001). Finally, as seen for *Clock* and *tau*, substantially abnormal period mutants (caused by antimorphic mutations) may be sufficient to implicate the gene in circadian function in the absence of null mutations

(Vitaterna et al., 1994; King et al., 1997a; King et al., 1997b; Lowrey et al., 2000). In addition to period phenotypes, *Clock* and *Per2* mutant mice also become progressively arrhythmic in constant darkness, which are interesting examples of mutant alleles that have a combined period and loss-of-rhythm phenotype (Vitaterna et al., 1994; Zheng et al., 1999).

If an allelic series is not available, mutant phenotypes alone may not be sufficient to define core clock components. However, elucidating the specific biochemical functions of the proteins combined with a strong circadian phenotype can be ultimately compelling. For example in mammals, CLOCK was shown to interact with BMAL1 to activate transcriptionally the *Per* and *Cry* genes (Gekakis et al., 1998; Kume et al., 1999); and *Casein kinase 1 epsilon* (CK1 ϵ) was shown to phosphorylate the PERIOD, CRYPTOCHROME and BMAL1 proteins (Lowrey et al., 2000; Eide et al., 2002). Thus, a second set of criteria (beyond an extreme circadian phenotype in mutants) involves defining the biochemical function of putative clock components within circadian pathways.

What are clock components anyway?

Traditionally, circadian expression patterns or circadian oscillations in ‘activity’ of a putative clock component have been thought to be critical criteria for inclusion (Aronson et al., 1994). However, real examples have clearly shown that this assumption only applies to a subset of core clock components. Theory has defined state variables of limit cycle oscillators as clock components, but under this definition, only the feedback elements of the oscillator would fulfill this criterion. (Two examples of mathematical models of the circadian oscillator incorporating recent molecular information have appeared recently (Forger and Peskin, 2003; Leloup and Goldbeter, 2003).) While state variables obviously oscillate; and clock components such as PER, TIM, and CRY match this definition, other critical elements as defined above do not necessarily oscillate in an obvious manner. *Clock* and *tau* are two examples in which circadian expression may not be necessary for their function. Although for these specific cases, one could enter into a semantic discussion concerning whether the ‘activity’ of CLOCK or CK1 ϵ may be circadian. In any case, we know that circadian expression patterns are neither restrictive nor compelling since literally hundreds of downstream target genes exhibit circadian patterns (Akhtar et al., 2002; Panda et al., 2002; Storch et al., 2002; Ueda et al., 2002) and many components of input pathways to the circadian system can have circadian patterns (Kornhauser et al., 1990; Ginty et al., 1993; Emery et al., 1998; Heintzen et al., 2001; Mellow et al., 2001).

Perturbation analysis has been considered the gold standard for the identification of clock components. The classical view has been that if a putative component is part of the oscillator mechanism, then perturbation of the expression level or activity of the component must change the steady-state phase of the oscillator for pulse treatments, or may cause period changes for continuous treatments. This criterion is valid for distinguishing output elements, but has never been able to discriminate input pathways from effects on the oscillator itself. We now have clear examples of input components that oscillate and could fulfill the perturbation test, but do not appear to be clock components (Emery et al., 1998; Heintzen et al., 2001). In addition, we have examples of output pathways such as locomotor activity that can feedback and influence the period and phase of circadian systems (albeit with relatively weak phenotypic effects). So unfortunately the complexity of real circadian systems has compromised our desire to have simple criteria to define oscillator components.

So how do we define core clock components? Is this a valid concept, or does the complexity of circadian networks as in the case of genetic regulatory networks allow too many degrees of freedom to exist (Greenspan, 2001; Roenneberg and Mellow, 2003)? I would argue that the situation is not hopeless. We can distinguish among different levels of importance of clock components. As discussed above, it appears that only a limited number of genes can

be mutated to cause strong (3–4 hour) period changes for missense mutations and loss-of-rhythm phenotypes for null mutations. This allelic series definition incorporates both the classic genetic loss-of-function criterion for necessity, as well as, the perturbation criterion when applied to strong period changes. Defining the position of a putative component in a regulatory network (classically known as defining a pathway) describes how the component interacts with other elements. Moreover, describing the biochemical function of the putative element can further define its role within a circadian network. Some elements of the network may be essential – their removal (loss-of-function mutations) will lead to a loss-of-rhythm phenotype. Other elements of the network may not be essential either because there are alternate paths for the network to function or there may be true cases of redundancy or overlapping function. In these cases, the loss-of-function phenotypes will be subtle and compound mutations may be required to produce strong phenotypes. If a strong phenotype cannot be produced by compound loss-of-function mutations for a paralogous gene family, then these elements would be considered nonessential.

Ultimately, we would want to apply another level of perturbation tests for core clock components. This would require conditional expression of clock components. We would want to apply this test for a number of reasons. First, it is important to demonstrate that the phenotypic effects seen in classical mutants are due to the ‘ongoing’ or cycle-to-cycle effects of the mutation rather than some developmental effect (since classical mutants develop in this context). Second, conditional experiments can test whether rhythmic or constitutive expression is required for the component. Third, conditional expression in a wild-type background can be used to apply perturbation tests (phase shifts or period changes). Finally, conditional experiments can be used in a tissue-specific manner to test for cell autonomy, hierarchical regulation and other types of interactions. To date, only a few clock components have been tested in this way. Aronson et al. applied this test early on in the field (Aronson et al., 1994). In retrospect, the only deficit in this analysis was definitive measurement of the expression levels of the conditionally expressed genes which was performed later (Morrow et al., 1997). Similar analyses have been accomplished in *Cyanobacteria* (Ishiura et al., 1998). Tissue-specific and constitutive expression patterns have also been tested in *Drosophila* (Yang and Sehgal, 2001; Zhao et al., 2003). Thus far, no critical tests of this type have been performed in mammals particularly in the context of the intact animal. We are still a long way from critical testing of known clock components especially in mammals.

Are there more new clock components?

The answer is a definite “yes.” There are many reasons for this opinion. (1) Even though we have complete genome sequences in humans, mice and rats (Lindblad-Toh, 2004), we are still far from understanding the function of a large fraction (~40%) of genes. We would be naively egotistical to think that we have already found all the core circadian clock genes just on the basis of our ignorance of gene annotation and function. (2) There are a significant number of candidate clock genes that have been proposed to be components of the circadian clock mechanism that require genetic evidence of strong circadian phenotypes before they can be admitted to the ‘inner circle.’ An example is the recent description of the *Dec1*(*Stra13*) and *Dec2* genes which have circadian expression patterns in the SCN and have strong inhibitory effects on CLOCK-BMAL1 activation in cell transfection/reporter gene assays (Honma et al., 2002; Grechez-Cassiau et al., 2004). What is still missing, however, is *in vivo* evidence that mutations can produce strong circadian phenotypes (loss-of-function or strong period mutants). (3) Quantitative genetic experiments analyzing circadian phenotypes in different inbred strains of mice reveal that the majority of quantitative trait loci (QTLs) do not map to known circadian genes (Shimomura et al., 2001). These allelic variants contribute to striking differences in circadian behavior in different inbred strains of mice and

suggest that a large number of circadian modifier genes remain to be identified. (4) Very few forward genetic screens for circadian mutations have reached saturation. In mice, we have barely scratched the surface. Indeed in a recent pilot screen in which two circadian mutants were recovered, one mutant was a new allele of a known locus and a second mutant defines a new circadian gene because no known circadian genes coincide with its map location. New large-scale recessive mutagenesis screens in mice promise to deliver many new circadian mutants (Moldin et al., 2001). Furthermore, because saturation mutagenesis can only be assessed empirically by recovery of multiple, new alleles only at previously known loci, it is likely that this level of screening has only been accomplished in *Cyanobacteria* (Kondo et al., 1994; Ishiura et al., 1998). In addition, even saturation mutagenesis is ultimately limiting because it may not be possible to recover circadian mutants for 'all' genes. We can already anticipate this result in the case of paralogous genes such as *Per* where compound mutations may be required to produce strong circadian phenotypes. (5) Microarray experiments have uncovered literally hundreds of cycling genes (Akhtar et al., 2002; Duffield et al., 2002; Panda et al., 2002; Storch et al., 2002; Ueda et al., 2002). In mice, about 10% of all detectable transcripts in any tissue express circadian profiles. A significant set of genes appear to cycle in the majority of tissues and most of these genes have not been previously assigned a function in the circadian network. It seems likely that at least a few of these cycling genes could define additional circadian clock genes. In addition, more comprehensive microarrays interrogating the entire transcriptome are now available so additional cycling genes will undoubtedly be uncovered (Su et al., 2004). (6) Biochemical interaction screens have identified a large number of proteins that can interact with known circadian clock proteins. For example, using chromatin immunoprecipitation (ChIP) assays on the *Dbp* promoter, Ripperger et al. found many proteins associated in a complex with CLOCK, most of which have not been identified (Ripperger et al., 2000). Yeast two hybrid screens have also yielded about 20 bonafide CLOCK-interacting proteins which remain to be explored (Gekakis et al., 1998). Such protein interaction screens have only begun to be pursued in earnest and so much remains to be explored in this arena using both traditional biochemistry as well as modern proteomics approaches. (7) Finally, many large-scale approaches such as genome-wide siRNA screens or overexpression screens with genome-wide cDNA collections are underway and are likely to reveal additional circadian clock genes. Thus, the future is bright. A daunting array of tools are being applied to circadian gene discovery and each of these is likely yield new insight into the circadian mechanism.

The Paradigm Shift: What have we lost and what have we gained?

The application of genetics, biochemistry, molecular biology and genomics to the study of circadian biology in the last decade has certainly led to a paradigm shift in the field. We have moved from analysis of circadian rhythms primarily at the formal level to a much more concrete mechanistic level. Much like the transition from classical to molecular genetics, the field of circadian rhythms has flourished while still maintaining a common language and set of problems. Perhaps it is somewhat ironic that genetics on the one hand and circadian rhythms on the other hand are so complementary. Circadian rhythms stands as one of the great success stories of classical genetic approaches to the study of behavior; while genetics and molecular biology have been the key to unraveling the network of genes that compose the circadian oscillator system.

So where are we today? As we stated at the beginning of this piece, we are well on our way to a complete description of circadian clock components in a number of model systems. And, we think we know how the core autoregulatory transcriptional feedback loop operates. However, we are still far from a quantitative understanding of any circadian oscillator with respect to kinetics, stoichiometry and cell biology in the context of real cells and organisms.

We still have little knowledge concerning how this molecular oscillator regulates the large number of pathways in the cell that are under circadian control – much less, how these cells communicate circadian information more broadly. A clear view has emerged that virtually every cell in the body has the capability of expressing circadian oscillations (Balsalobre et al., 1998; Yamazaki et al., 2000; Yamaguchi et al., 2003; Yoo et al., 2004). Now we need to know why. Are circadian rhythms so fundamental to metabolic efficiency that we cannot do without them (Rutter et al., 2002)? Or, are cellular circadian rhythms a vestige of a unicellular past where metabolism and environmental insult are more proximal to survival (Ouyang et al., 1998; Johnson and Golden, 1999)? We can only speculate. Recent links between circadian rhythms and cancer (Fu and Lee, 2003) and the cell cycle (Matsuo et al., 2003) in mice suggest that the relationship is more than vestigial.

How has this molecular view of circadian rhythms changed us? For a while, some of us may have lost some of our focus on systems and integrative questions (while we engaged in sprints to find new genes). But, with new tools such as *circadian promoter::reporter gene* technology and the knowledge that we are composed of populations of circadian oscillators (Millar et al., 1992; Plautz et al., 1997; Balsalobre et al., 1998; Yamazaki et al., 2000; Yamaguchi et al., 2003; Yoo et al., 2004), a renaissance in circadian systems biology is on the horizon. Lost forever is that black box that once represented *the* circadian clock.

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References

- Akhtar RA, Reddy AB, Maywood ES, Clayton JD, King VM, Smith AG, Gant TW, Hastings MH, Kyriacou CP. Circadian cycling of the mouse liver transcriptome, as revealed by cDNA microarray, is driven by the suprachiasmatic nucleus. *Curr Biol*. 2002; 12:540–550. [PubMed: 11937022]
- Antoch MP, Song EJ, Chang AM, 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. [PubMed: 9160756]
- 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. [PubMed: 8128244]
- Bae K, Jin X, Maywood ES, Hastings MH, Reppert SM, Weaver DR. Differential functions of mPer1, mPer2, and mPer3 in the SCN circadian clock. *Neuron*. 2001; 30:525–536. [PubMed: 11395012]
- Balsalobre A, Damiola F, Schibler U. A serum shock induces circadian gene expression in mammalian tissue culture cells. *Cell*. 1998; 93:929–937. [PubMed: 9635423]
- Banerjee U, Renfranz PJ, Pollock JA, Benzer S. Molecular characterization and expression of *sevenless*, a gene involved in neuronal pattern formation in the *Drosophila* eye. *Cell*. 1987; 49:281–291. [PubMed: 2882857]
- Bargiello TA, Jackson FR, Young MW. Restoration of circadian behavioural rhythms by gene transfer in *Drosophila*. *Nature*. 1984; 312:752–754. [PubMed: 6440029]
- Benzer S. Genetic dissection of behavior. *Sci Am*. 1973; 229:24–37. [PubMed: 4202065]
- Bucan M, Abel T. The mouse: Genetics meets behaviour. *Nature Reviews Genetics*. 2002; 3:114–123.
- Bunger MK, Wilsbacher LD, Moran SM, Clendenin C, Radcliffe LA, Hogenesch JB, Simon MC, Takahashi JS, Bradfield CA. Mop3 is an essential component of the master circadian pacemaker in mammals. *Cell*. 2000; 103:1009–1017. [PubMed: 11163178]
- Chen CN, Denome S, Davis RL. Molecular analysis of cDNA clones and the corresponding genomic coding sequences of the *Drosophila dunce+* gene, the structural gene for cAMP phosphodiesterase. *Proc Natl Acad Sci U S A*. 1986; 83:9313–9317. [PubMed: 3025834]
- Dudai Y, Jan YN, Byers D, Quinn WG, Benzer S. *dunce*, a mutant of *Drosophila* deficient in learning. *Proc Natl Acad Sci U S A*. 1976; 73:1684–1688. [PubMed: 818641]

- Duffield GE, Best JD, Meurers BH, Bittner A, Loros JJ, Dunlap JC. Circadian programs of transcriptional activation, signaling, and protein turnover revealed by microarray analysis of mammalian cells. *Curr Biol*. 2002; 12:551–557. [PubMed: 11937023]
- Dunlap JC. Molecular bases for circadian clocks. *Cell*. 1999; 96:271–290. [PubMed: 9988221]
- Edgar DM, Martin CE, Dement WC. Activity feedback to the mammalian circadian pacemaker: influence on observed measures of rhythm period length. *J Biol Rhythms*. 1991; 6:185–199. [PubMed: 1773091]
- Eide EJ, Vielhaber EL, Hinz WA, Virshup DM. The circadian regulatory proteins BMAL1 and cryptochromes are substrates of casein kinase Iepsilon. *J Biol Chem*. 2002; 277:17248–17254. [PubMed: 11875063]
- Emery P, So WV, Kaneko M, Hall JC, Rosbash M. CRY, a *Drosophila* clock and light-regulated cryptochrome, is a major contributor to circadian rhythm resetting and photosensitivity. *Cell*. 1998; 95:669–679. [PubMed: 9845369]
- Forger DB, Peskin CS. A detailed predictive model of the mammalian circadian clock. *Proc Natl Acad Sci U S A*. 2003; 100:14806–14811. [PubMed: 14657377]
- Fu L, Lee CC. The circadian clock: pacemaker and tumour suppressor. *Nat Rev Cancer*. 2003; 3:350–361. [PubMed: 12724733]
- 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. [PubMed: 9616112]
- Ginty DD, Kornhauser JM, Thompson MA, Bading H, Mayo KE, Takahashi JS, Greenberg ME. Regulation of CREB phosphorylation in the suprachiasmatic nucleus by light and a circadian clock. *Science*. 1993; 260:238–241. [PubMed: 8097062]
- Grechez-Cassiau A, Panda S, Lacoche S, Teboul M, Azmi S, Laudet V, Hogenesch JB, Taneja R, Delaunay F. The transcriptional repressor STRA13 regulates a subset of peripheral circadian outputs. *J Biol Chem*. 2004; 279:1141–1150. [PubMed: 14581485]
- Greenspan RJ. The emergence of neurogenetics. *Sem Neurosciences*. 1990; 2:145–157.
- Greenspan RJ. The flexible genome. *Nat Rev Genet*. 2001; 2:383–387. [PubMed: 11331904]
- Hafen E, Basler K, Edstroem JE, Rubin GM. Sevenless, a cell-specific homeotic gene of *Drosophila*, encodes a putative transmembrane receptor with a tyrosine kinase domain. *Science*. 1987; 236:55–63. [PubMed: 2882603]
- Hall JC. Genetics and molecular biology of rhythms in *Drosophila* and other insects. *Adv Genet*. 2003; 48:1–280. [PubMed: 12593455]
- Hattar S, Lucas RJ, Mrosovsky N, Thompson S, Douglas RH, Hankins MW, Lem J, Biel M, Hofmann F, Foster RG, Yau KW. Melanopsin and rod-cone photoreceptive systems account for all major accessory visual functions in mice. *Nature*. 2003; 424:76–81. [PubMed: 12808468]
- Heintzen C, Loros JJ, Dunlap JC. The PAS protein VIVID defines a clock-associated feedback loop that represses light input, modulates gating, and regulates clock resetting. *Cell*. 2001; 104:453–464. [PubMed: 11239402]
- Honma S, Kawamoto T, Takagi Y, Fujimoto K, Sato F, Noshiro M, Kato Y, Honma K. Dec1 and Dec2 are regulators of the mammalian molecular clock. *Nature*. 2002; 419:841–844. [PubMed: 12397359]
- 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. [PubMed: 9727980]
- Johnson CH, Golden SS. Circadian programs in cyanobacteria: adaptiveness and mechanism. *Annu Rev Microbiol*. 1999; 53:389–409. [PubMed: 10547696]
- Kamb A, Iverson LE, Tanouye MA. Molecular characterization of Shaker, a *Drosophila* gene that encodes a potassium channel. *Cell*. 1987; 50:405–413. [PubMed: 2440582]
- Kaplan WD, Trout WE 3rd. The behavior of four neurological mutants of *Drosophila*. *Genetics*. 1969; 61:399–409. [PubMed: 5807804]
- 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 W19H deletion, distal of Kit. *Genetics*. 1997a; 146:1049–1060. [PubMed: 9215907]

- King DP, Zhao Y, Sangoram AM, Wilsbacher LD, Tanaka M, Antoch MP, Steeves TD, Vitaterna MH, Kornhauser JM, Lowrey PL, Turek FW, Takahashi JS. Positional cloning of the mouse circadian clock gene. *Cell*. 1997b; 89:641–653. [PubMed: 9160755]
- Kloss B, Price JL, Saez L, Blau J, Rothenfluh A, Wesley CS, Young MW. The *Drosophila* clock gene double-time encodes a protein closely related to human casein kinase Iepsilon. *Cell*. 1998; 94:97–107. [PubMed: 9674431]
- Kondo T, Tsinoremas NF, Golden SS, Johnson CH, Kutsuna S, Ishiura M. Circadian clock mutants of cyanobacteria. *Science*. 1994; 266:1233–1236. [PubMed: 7973706]
- Konopka RJ, Benzer S. Clock mutants of *Drosophila melanogaster*. *Proc Natl Acad Sci U S A*. 1971; 68:2112–2116. [PubMed: 5002428]
- Konopka RJ, Hamblen-Coyle MJ, Jamison CF, Hall JC. An ultrashort clock mutation at the period locus of *Drosophila melanogaster* that reveals some new features of the fly's circadian system. *J Biol Rhythms*. 1994; 9:189–216. [PubMed: 7772790]
- Kornhauser JM, Nelson DE, Mayo KE, Takahashi JS. Photic and circadian regulation of c-fos gene expression in the hamster suprachiasmatic nucleus. *Neuron*. 1990; 5:127–134. [PubMed: 2116813]
- Kume K, Zylka MJ, Sriram S, Shearman LP, Weaver DR, Jin X, Maywood ES, Hastings MH, Reppert SM. mCRY1 and mCRY2 are essential components of the negative limb of the circadian clock feedback loop. *Cell*. 1999; 98:193–205. [PubMed: 10428031]
- Kyriacou CP, Hall JC. Genetic and molecular analysis of *Drosophila* behavior. *Adv Genet*. 1994; 31:139–186. [PubMed: 8036993]
- Leloup JC, Goldbeter A. Toward a detailed computational model for the mammalian circadian clock. *Proc Natl Acad Sci U S A*. 2003; 100:7051–7056. [PubMed: 12775757]
- Lin YJ, Seroude L, Benzer S. Extended life-span and stress resistance in the *Drosophila* mutant methuselah. *Science*. 1998; 282:943–946. [PubMed: 9794765]
- Lindblad-Toh K. Genome sequencing: three's company. *Nature*. 2004; 428:475–476. [PubMed: 15057812]
- 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 J*. 1997; 16:6762–6771. [PubMed: 9362490]
- Low-Zeddies SS, Takahashi JS. Chimera analysis of the Clock mutation in mice shows that complex cellular integration determines circadian behavior. *Cell*. 2001; 105:25–42. [PubMed: 11301000]
- Lowrey PL, Takahashi JS. Mammalian circadian biology: Elucidating genome-wide levels of temporal organization. *Annu Rev Genomics Hum Genet*. 2004; 5:407–441. [PubMed: 15485355]
- Lowrey PL, Shimomura K, Antoch MP, Yamazaki S, Zemenides PD, Ralph MR, Menaker M, Takahashi JS. Positional syntenic cloning and functional characterization of the mammalian circadian mutation tau. *Science*. 2000; 288:483–492. [PubMed: 10775102]
- Matsuo T, Yamaguchi S, Mitsui S, Emi A, Shimoda F, Okamura H. Control mechanism of the circadian clock for timing of cell division in vivo. *Science*. 2003; 302:255–259. [PubMed: 12934012]
- Maywood ES, Mrosovsky N, Field MD, Hastings MH. Rapid down-regulation of mammalian period genes during behavioral resetting of the circadian clock. *Proc Natl Acad Sci U S A*. 1999; 96:15211–15216. [PubMed: 10611364]
- McClung CR, Fox BA, Dunlap JC. The *Neurospora* clock gene frequency shares a sequence element with the *Drosophila* clock gene period. *Nature*. 1989; 339:558–562. [PubMed: 2525233]
- Morrow M, Franchi L, Dragovic Z, Gori M, Johnson J, Brunner M, Macino G, Roenneberg T. Circadian regulation of the light input pathway in *Neurospora crassa*. *Embo J*. 2001; 20:307–315. [PubMed: 11157738]
- Morrow MW, Garceau NY, Dunlap JC. Dissection of a circadian oscillation into discrete domains. *Proc Natl Acad Sci U S A*. 1997; 94:3877–3882. [PubMed: 9108072]
- Millar AJ, Short SR, Chua NH, Kay SA. A novel circadian phenotype based on firefly luciferase expression in transgenic plants. *Plant Cell*. 1992; 4:1075–1087. [PubMed: 1392609]
- Min KT, Benzer S. Preventing neurodegeneration in the *Drosophila* mutant bubblegum. *Science*. 1999; 284:1985–1988. [PubMed: 10373116]

- Moldin SO, Farmer ME, Chin HR, Battey JF. Trans-NIH neuroscience initiatives on mouse phenotyping and mutagenesis. *Mammalian Genome*. 2001; 12:575–581. [PubMed: 11471049]
- Myers MP, Wager-Smith K, Wesley CS, Young MW, Sehgal A. Positional cloning and sequence analysis of the *Drosophila* clock gene, timeless. *Science*. 1995; 270:805–808. [PubMed: 7481771]
- Ouyang Y, Andersson CR, Kondo T, Golden SS, Johnson CH. Resonating circadian clocks enhance fitness in cyanobacteria. *Proc Natl Acad Sci U S A*. 1998; 95:8660–8664. [PubMed: 9671734]
- Panda S, Antoch MP, Miller BH, Su AI, Schook AB, Straume M, Schultz PG, Kay SA, Takahashi JS, Hogenesch JB. Coordinated transcription of key pathways in the mouse by the circadian clock. *Cell*. 2002; 109:307–320. [PubMed: 12015981]
- Panda S, Provencio I, Tu DC, Pires SS, Rollag MD, Castrucci AM, Pletcher MT, Sato TK, Wiltshire T, Andahazy M, Kay SA, Van Gelder RN, Hogenesch JB. Melanopsin is required for non-image-forming photic responses in blind mice. *Science*. 2003; 301:525–527. [PubMed: 12829787]
- Papazian DM, Schwarz TL, Tempel BL, Jan YN, Jan LY. Cloning of genomic and complementary DNA from Shaker, a putative potassium channel gene from *Drosophila*. *Science*. 1987; 237:749–753. [PubMed: 2441470]
- Pittendrigh CS. On temperature independence in the clock system controlling emergence in *Drosophila*. *Proc Natl Acad Sci USA*. 1954; 40:2697–2701.
- Plautz JD, Kaneko M, Hall JC, Kay SA. Independent photoreceptive circadian clocks throughout *Drosophila*. *Science*. 1997; 278:1632–1635. [PubMed: 9374465]
- Preitner N, Damiola F, Lopez-Molina L, Zakany J, Duboule D, Albrecht U, Schibler U. The orphan nuclear receptor REV-ERB α controls circadian transcription within the positive limb of the mammalian circadian oscillator. *Cell*. 2002; 110:251–260. [PubMed: 12150932]
- Price JL, Blau J, Rothenfluh A, Abodeely M, Kloss B, Young MW. double-time is a novel *Drosophila* clock gene that regulates PERIOD protein accumulation. *Cell*. 1998; 94:83–95. [PubMed: 9674430]
- Renn SC, Park JH, Rosbash M, Hall JC, Taghert PH. A pdf neuropeptide gene mutation and ablation of PDF neurons each cause severe abnormalities of behavioral circadian rhythms in *Drosophila*. *Cell*. 1999; 99:791–802. [PubMed: 10619432]
- Reppert SM, Weaver DR. Coordination of circadian timing in mammals. *Nature*. 2002; 418:935–941. [PubMed: 12198538]
- Ripperger JA, Shearman LP, Reppert SM, Schibler U. CLOCK, an essential pacemaker component, controls expression of the circadian transcription factor DBP. *Genes Dev*. 2000; 14:679–689. [PubMed: 10733528]
- Roenneberg T, Mrosovsky M. The network of time: understanding the molecular circadian system. *Curr Biol*. 2003; 13:R198–207. [PubMed: 12620213]
- Rothenfluh A, Abodeely M, Price JL, Young MW. Isolation and analysis of six timeless alleles that cause short- or long-period circadian rhythms in *Drosophila*. *Genetics*. 2000; 156:665–675. [PubMed: 11014814]
- 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. [PubMed: 9630224]
- Rutter J, Reick M, McKnight SL. Metabolism and the control of circadian rhythms. *Annu Rev Biochem*. 2002; 71:307–331. [PubMed: 12045099]
- Sehgal A, Price JL, Man B, Young MW. Loss of circadian behavioral rhythms and per RNA oscillations in the *Drosophila* mutant timeless. *Science*. 1994; 263:1603–1606. [PubMed: 8128246]
- Shimomura K, Low-Zeddies SS, King DP, Steeves TD, Whiteley A, Kushla J, Zemenides PD, Lin A, Vitaterna MH, Churchill GA, Takahashi JS. Genome-wide epistatic interaction analysis reveals complex genetic determinants of circadian behavior in mice. *Genome Res*. 2001; 11:959–980. [PubMed: 11381025]
- Stanewsky R, Kaneko M, Emery P, Beretta B, Wager-Smith K, Kay SA, Rosbash M, Hall JC. The cryb mutation identifies cryptochrome as a circadian photoreceptor in *Drosophila*. *Cell*. 1998; 95:681–692. [PubMed: 9845370]

- Storch KF, Lipan O, Leykin I, Viswanathan N, Davis FC, Wong WH, Weitz CJ. Extensive and divergent circadian gene expression in liver and heart. *Nature*. 2002; 417:78–83. [PubMed: 11967526]
- Su AI, Wiltshire T, Batalov S, Lapp H, Ching KA, Block D, Zhang J, Soden R, Hayakawa M, Kreiman G, Cooke MP, Walker JR, Hogenesch JB. A gene atlas of the mouse and human protein-encoding transcriptomes. *Proc Natl Acad Sci U S A*. 2004; 101:6062–6067. [PubMed: 15075390]
- Takahashi JS, Pinto LH, Vitaterna MH. Forward and reverse genetic approaches to behavior in the mouse. *Science*. 1994; 264:1724–1733. [PubMed: 8209253]
- Ueda HR, Chen W, Adachi A, Wakamatsu H, Hayashi S, Takasugi T, Nagano M, Nakahama K, Suzuki Y, Sugano S, Iino M, Shigeyoshi Y, Hashimoto S. A transcription factor response element for gene expression during circadian night. *Nature*. 2002; 418:534–539. [PubMed: 12152080]
- Uzumaki T, Fujita M, Nakatsu T, Hayashi F, Shibata H, Itoh N, Kato H, Ishiura M. Crystal structure of the C-terminal clock-oscillator domain of the cyanobacterial KaiA protein. *Nat Struct Mol Biol*. 2004; 11:623–631. [PubMed: 15170179]
- Vakonakis I, Sun J, Wu T, Holzenburg A, Golden SS, LiWang AC. NMR structure of the KaiC-interacting C-terminal domain of KaiA, a circadian clock protein: implications for KaiA-KaiC interaction. *Proc Natl Acad Sci U S A*. 2004; 101:1479–1484. [PubMed: 14749515]
- van der Horst GTJ, Muijtjens M, Kobayashi K, Takano R, Kanno S, Takao M, de Wit J, Verkerk A, Eker APM, van Leenen D, Buijs R, Bootsma D, Hoeijmakers JHJ, Yasui A. Mammalian Cry1 and Cry2 are essential for maintenance of circadian rhythms. *Nature*. 1999; 398:627–630. [PubMed: 10217146]
- Van Reeth O, Turek FW. Stimulated activity mediates phase shifts in the hamster circadian clock induced by dark pulses or benzodiazepines. *Nature*. 1989; 339:49–51. [PubMed: 2654641]
- Vitaterna MH, King DP, Chang AM, Kornhauser JM, Lowrey PL, McDonald JD, Dove WF, Pinto LH, Turek FW, Takahashi JS. Mutagenesis and mapping of a mouse gene, Clock, essential for circadian behavior. *Science*. 1994; 264:719–725. [PubMed: 8171325]
- Vitaterna MH, Selby CP, Todo T, Niwa H, Thompson C, Fruechte EM, Hitomi K, Thresher RJ, Ishikawa T, Miyazaki J, Takahashi JS, Sancar A. Differential regulation of mammalian period genes and circadian rhythmicity by cryptochromes 1 and 2. *Proc Natl Acad Sci U S A*. 1999; 96:12114–12119. [PubMed: 10518585]
- Yamaguchi S, Isejima H, Matsuo T, Okura R, Yagita K, Kobayashi M, Okamura H. Synchronization of cellular clocks in the suprachiasmatic nucleus. *Science*. 2003; 302:1408–1412. [PubMed: 14631044]
- Yamazaki S, Numano R, Abe M, Hida A, Takahashi R, Ueda M, Block GD, Sakaki Y, Menaker M, Tei H. Resetting central and peripheral circadian oscillators in transgenic rats. *Science*. 2000; 288:682–685. [PubMed: 10784453]
- Yang Z, Sehgal A. Role of molecular oscillations in generating behavioral rhythms in *Drosophila*. *Neuron*. 2001; 29:453–467. [PubMed: 11239435]
- Ye S, Vakonakis I, Ioerger TR, LiWang AC, Sacchettini JC. Crystal structure of circadian clock protein KaiA from *Synechococcus elongatus*. *J Biol Chem*. 2004; 279:20511–20518. [PubMed: 15007067]
- Yoo SH, Yamazaki S, Lowrey PL, Shimomura K, Ko CH, Buhr ED, Slepka SM, Hong HK, Oh WJ, Yoo OJ, Menaker M, Takahashi JS. PERIOD2::LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues. *Proc Natl Acad Sci U S A*. 2004; 101:5339–5346. [PubMed: 14963227]
- Young MW, Kay SA. Time zones: a comparative genetics of circadian clocks. *Nat Rev Genet*. 2001; 2:702–715. [PubMed: 11533719]
- Zehring WA, Wheeler DA, Reddy P, Konopka RJ, Kyriacou CP, Rosbash M, Hall JC. P-element transformation with period locus DNA restores rhythmicity to mutant, arrhythmic *Drosophila melanogaster*. *Cell*. 1984; 39:369–376. [PubMed: 6094014]
- Zhao J, Kilman VL, Keegan KP, Peng Y, Emery P, Rosbash M, Allada R. *Drosophila* clock can generate ectopic circadian clocks. *Cell*. 2003; 113:755–766. [PubMed: 12809606]

- Zheng BH, Larkin DW, Albrecht L, Sun ZS, Sage M, Eichele G, Lee CC, Bradley A. The mPer2 gene encodes a functional component of the mammalian circadian clock. *Nature*. 1999; 400:169–173. [PubMed: 10408444]
- Zheng BH, Albrecht U, Kaasik K, Sage M, Lu WQ, Vaishnav S, Li Q, Sun ZS, Eichele G, Bradley A, Lee CC. Nonredundant roles of the mPer1 and mPer2 genes in the mammalian circadian clock. *Cell*. 2001; 105:683–694. [PubMed: 11389837]



Figure 1.
Photograph of Ron Konopka (left) and Seymour Benzer (right) taken in October 2000 in Pasadena, CA. Photo courtesy of Howard Hughes Medical Institute, Holiday Lectures on Science, December 2000, Clockwork Genes: Discoveries in Biological Time.