REVIEW

Transcriptional Feedback Oscillators: Maybe, Maybe Not . . .

Patricia L. Lakin-Thomas[1]
York University, Toronto, Ontario, Canada

Abstract The molecular mechanism of circadian rhythmicity is usually modeled by a transcription/translation feedback oscillator in which clock proteins negatively feed back on their own transcription to produce rhythmic levels of clock protein mRNAs, which in turn cause the production of rhythmic levels of clock proteins. This mechanism has been applied to all model organisms for which molecular data are available. This review summarizes the increasing number of anomalous observations that do not fit the standard molecular mechanism for the model organisms *Acetabularia*, *Synechococcus*, *Drosophila*, *Neurospora*, and mouse. The anomalies fall into 2 classes: observations of rhythmicity in the organism when transcription of clock genes is held constant, and rhythmicity in the organism when clock gene function is missing in knockout mutants. It is concluded that the weight of anomalies is now so large that the standard transcription/translation mechanism is no longer an adequate model for circadian oscillators. Rhythmic transcription may have other functions in the circadian system, such as participating in input and output pathways and providing robustness to the oscillations. It may be most useful to think in terms of a circadian system that uses a noncircadian oscillator consisting of metabolic feedback loops, which acquires its circadian properties from additional regulatory molecules such as the products of canonical clock genes.

Key words circadian, rhythm, *Acetabularia*, *Synechococcus*, *Drosophila*, *Neurospora*, mouse

"All circadian oscillators are based on transcriptional feedback loops involving the canonical clock genes." This statement, or versions of it, can be found somewhere in the introduction to nearly every review on circadian rhythms and most primary data papers dealing with molecular mechanisms published in the past 10 years. (A survey of the references in this review found a similar statement in 8 out of 11 reviews and 16 out of 28 data papers, excluding my own.) It has sometimes been presented as an orthodoxy almost immune to criticism. As a congenital iconoclast, I am irresistibly attracted to the challenge of breaking down orthodoxies, and to that end I collect reports of anomalies that run counter to the predictions of this widely accepted model. Such anomalies have been increasing in frequency in recent years, to the point where this model is no longer universally accepted as a literal description of clock mechanisms. This review describes what I believe are the most salient examples of circadian systems that defy the predictions of the orthodox model.

Let me begin by defining the version of the orthodox model that I am considering (Fig. 1A). In this

1. To whom all correspondence should be addressed: Patricia L. Lakin-Thomas, Department of Biology, York University, 4700 Keele Street, Toronto, ON M3J 1P3, Canada; e-mail: plakin@yorku.ca.

JOURNAL OF BIOLOGICAL RHYTHMS, Vol. 21 No. 2, April 2006 83-92
DOI: 10.1177/0748730405286102

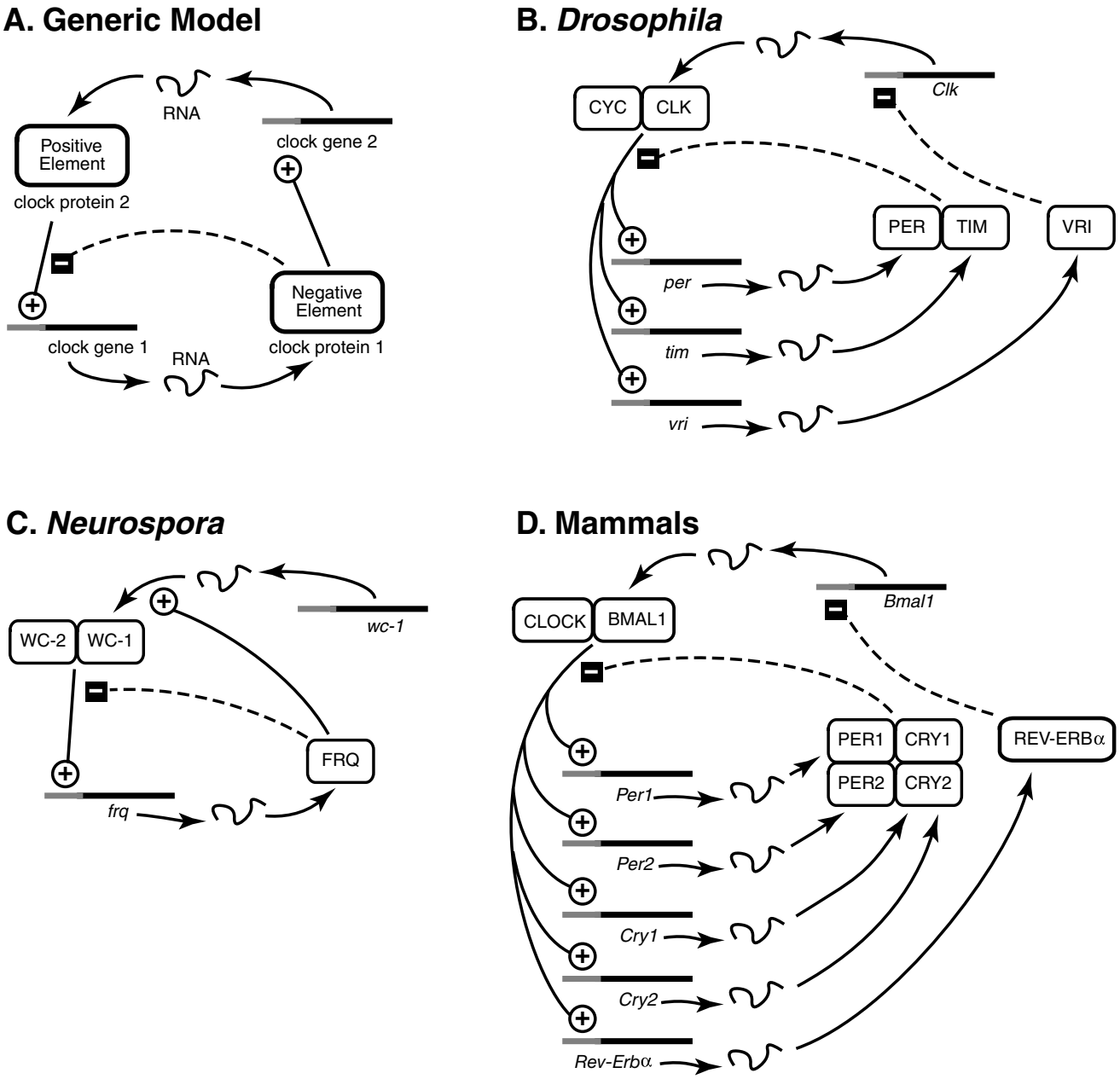

Figure 1. Transcription/translation oscillator (TTO) models for circadian clocks. (A) Generic TTO model with 2 interlocked loops. Clock gene 1 is transcribed into RNA and translated into protein. Clock protein 2 positively regulates transcription of clock gene 1. Clock protein 1 negatively regulates its own transcription by interfering with the positive effect of clock protein 2. Clock protein 1 also positively regulates production of clock protein 2, via either transcription or translation. Black bar represents gene coding sequence, gray bar represents transcription control regions. Biosynthetic pathways are shown as solid lines with arrowheads. Positive influence is shown as a solid line with circle head. Negative influence is shown as a dashed line with square head. Nuclear/cytoplasmic compartmentation, phosphorylation, degradation pathways, environmental inputs, and outputs to clock-controlled genes and observed rhythms have been omitted. (B) TTO model for *Drosophila*. A complex of CLK and CYC proteins activates transcription of *per*, *tim*, and *vri* genes. (*Pdp1*ε has been omitted for simplicity.) A complex of PER and TIM proteins inhibits the positive effects of CLK/CYC. VRI protein represses transcription of *Clk*. PER and TIM inhibit their own transcription, while simultaneously activating *clk* transcription by inhibiting expression of *vri*, and therefore *per* and *tim* RNA cycle in antiphase to *clk* RNA. CYC protein levels are not rhythmic. Derived from Hardin (2004). (C) TTO model for *Neurospora*. A complex of WC-1 and WC-2 proteins activates transcription of the *frq* gene. FRQ protein inhibits the positive effect of WC-1/WC-2 and also activates synthesis of WC-1 protein. Positive effects of FRQ on WC-2 expression are omitted; WC-2 protein levels are not rhythmic. Derived from Dunlap and Loros (2004). (D) TTO model for mammals. A complex of CLOCK and BMAL1 proteins activates transcription of *Per1*, *Per2*, *Cry1*, *Cry2*, and *Rev-Erb*α genes. A complex of PER1, PER2, CRY1, and CRY2 proteins inhibits the positive effects of CLOCK/BMAL1. REV-ERBα protein represses transcription of *Bmal1*. CLOCK RNA and protein levels may oscillate with a low amplitude. Derived from Hardin (2004) and Hastings and Herzog (2004).

model, there is a small set of identified "canonical clock genes" whose transcription is rhythmic and whose protein products feed back (directly or indirectly) to negatively regulate their own transcription. Rhythmic transcription causes rhythmic levels of RNA, rhythmic RNA causes rhythmic levels of proteins, and rhythmic levels of protein cause rhythmic transcription. Other factors, such as posttranslational modifications of proteins, nuclear import, interlocked loops, and so forth, may affect the time lags in the system and hence the period, and may affect the amplitude and hence the sustainability of the rhythms, but the driving engine for rhythmicity of the organism is rhythmic transcription of clock genes. I will use the abbreviation TTO (transcription/translation oscillator) as shorthand for this model. In the context of this review, TTO refers only to the feedback loops proposed for the identified canonical clock genes and not to the general idea of transcriptional/translational feedback loops. There may be such loops participating in rhythm generation that have not yet been identified.

The orthodox model makes many predictions about the behavior of organisms and genes, but 2 predictions are, I think, particularly critical. If the standard model for a clock mechanism driven by rhythmic transcription of clock genes is correct, then it follows that 1) holding transcription of clock genes constant should lead to arrhythmicity of the organism, and 2) knocking out the canonical clock genes should also lead to arrhythmicity of the organism. If these predictions fail, then we must conclude that rhythmic transcription of canonical clock genes is not required for rhythmicity of the organism. (In my view, it is not necessary for rhythmicity in knockout mutants to be fully normal in its circadian characteristics in order to falsify the TTO hypothesis.) It is my contention that in every system that has been tested, one or the other of these predictions has failed. I would like to review 5 model systems in which these predictions have been tested with varying degrees of rigor: *Acetabularia*, *Synechococcus*, *Drosophila*, *Neurospora*, and mammals. Most of these systems have been extensively and repeatedly reviewed in the literature. I have chosen to reference primarily the authoritative reviews published in the October 2004 special issue of the *Journal of Biological Rhythms* (volume 19, issue 5, pp 339-458).

REMEMBER *ACETABULARIA*!

Acetabularia was historically one of the pioneer organisms in circadian research, but it has fallen out of favor recently. An excellent summary of the work on this organism up to 1988 can be found in the invaluable monograph by Edmunds (1988). A review by the major investigators of *Acetabularia* rhythms was published in 1986 by Schweiger and colleagues. A summary of *Acetabularia* as an experimental system is also available (Mandoli, 1998). *Acetabularia* is a single-celled marine alga found in shallow waters near coral reefs. In the vegetative phase, it consists of a foot, or rhizoid, with which it attaches to the seabed, and a long stalk, up to 5 cm long (yes, cm, not mm!). At sexual maturity, it produces a cap in which nucleated gametes are formed. For most of its life, however, this giant cell gets along with a single nucleus in the rhizoid. What makes this organism useful for our purposes is that it can survive for many days after the nucleus-containing rhizoid is removed. These enucleated cells, and even parts of cells, can continue to photosynthesize with circadian rhythmicity for up to 7 weeks. Rhythms in chloroplast movement have also been demonstrated in enucleated cells (Woolum, 1991). Inhibitors of both nuclear and organellar RNA synthesis do not abolish photosynthetic rhythmicity in anucleate cells, but inhibitors of protein synthesis on 80S ribosomes will shift the phase and inhibit the rhythm of photosynthesis. These results demonstrate that daily transcription, from either the nuclear or organellar genomes, is not required to maintain rhythmicity, although protein synthesis is required. This does not necessarily implicate rhythmic protein synthesis; it merely indicates that some proteins may be short lived and require replacement. These cells are capable of storing long-lived RNAs in the cytoplasm, which accounts for their longevity in the absence of a nucleus. The conclusion from the work with *Acetabularia* is that rhythmic transcription of any gene is not required for rhythmicity of the organism, and so our 1st prediction of the TTO model is falsified. But is *Acetabularia* a special case? Is this bizarre giant single cell so unique in its biology that this anomaly can be safely ignored as a challenge to the TTO?

SYNECHOCOCCUS: A CLOCK IN A TEST TUBE

The most spectacular demonstration possible of circadian rhythmicity in the absence of rhythmic transcription of clock genes has been published recently by Kondo's group (Nakajima et al., 2005). The properties of circadian rhythmicity in the cyanobacterium *Synechococcus* have been reviewed repeatedly (Bell-Pedersen et al., 2005; Ditty et al., 2003;

Iwasaki and Kondo, 2004; Johnson, 2004; Mittag, 2001), and I will focus only on those aspects that are relevant to the TTO model. Three genes, *kaiA*, *kaiB*, and *kaiC*, are required for rhythmicity; deletion of any 1 of them abolishes rhythmicity of the organism, and mutations in these genes can change the period. KaiB and KaiC proteins are rhythmic in amount, and rhythmic transcription of the *kaiBC* operon is inhibited by KaiC protein overexpression in an apparent feedback loop. KaiC autophosphorylates and is rhythmic in its phosphorylation state. A pulse of KaiC overexpression can reset the phase of the clock. The bacterial circadian system therefore began to resemble the eukaryotic model systems, and a transcription/translation feedback oscillator model was proposed for *Synechococcus* (Ishiura et al., 1998) based on analogy with *Drosophila* and *Neurospora*.

The evidence for a TTO operating in this bacterium was weakened by the observation that feedback inhibition of *kai* gene transcription does not require *kai*-specific promoters (Ditty et al., 2003; Xu et al., 2003; Nakahira et al., 2004), and the kinetics of the oscillator seemed to rely on the autophosphorylation of KaiC more than transcriptional control. Tomita et al. (2005) directly tested the TTO model by determining whether rhythmic expression of *kaiBC* is required for rhythmicity of the organism. *Synechococcus* is a photosynthetic organism and requires light to maintain metabolic activity; in constant darkness, transcription and translation are suppressed. These authors found that in DD, in spite of nearly undetectable levels of *kaiBC* RNA, and even in the presence of inhibitors of transcription and translation, the rhythmic phosphorylation of KaiC protein continued. The robust cycling of KaiC phosphorylation state was circadian in period and temperature compensated.

The coup de grace for the TTO model in *Synechococcus* was delivered by Nakajima et al. (2005) in an article that I believe is the most significant publication in chronobiology in many years and is destined to be a classic. KaiC protein had been shown previously to have both autophosphorylation and autodephosphorylation activities. KaiA enhances autophosphorylation of KaiC, while KaiB inhibits the effect of KaiA, suggesting that interactions between these 3 proteins could generate the oscillation of KaiC phosphorylation. These authors mixed purified recombinant KaiA, KaiB, and KaiC proteins in vitro in ratios similar to in vivo ratios, added some adenosine triphosphate, and assayed KaiC phosphorylation. The result was stunning: a 24-h, self-sustained, temperature-compensated rhythm of phosphorylation; a clock in a test tube. As an extra bonus, Nakajima et al. assayed the in vitro rhythms of KaiC proteins with mutations that alter the in vivo periods and found similar effects on the in vitro periods, clinching the demonstration that "KaiC phosphorylation is the molecular timer for the circadian rhythm of *Synechococcus*" (Nakajima et al., 2005, p 415).

Note that all the apparatus of a TTO is apparently present in *Synechococcus*: clock genes essential for rhythmicity, rhythmic transcription of those genes, rhythmic protein levels, and apparent feedback of those proteins onto their own transcription. And yet, none of that apparatus is required to generate circadian rhythmicity. But is this in vitro rhythm of protein phosphorylation just a fluke? Does "real" circadian rhythmicity, with output that drives rhythmic photosynthesis, etc., require the whole TTO? And do prokaryotes really matter when what many people are ultimately interested in is the mechanism of rhythmicity in animals like us? I believe the significance of these results with *Synechococcus* and *Acetabularia* is to warn us that we should approach the TTO models in other organisms with skepticism and demand rigorous tests of our predictions. We should not be too quick to apply the TTO model to any organism with feedback regulation of putative clock genes (Salomé and McClung, 2004).

DROSOPHILA: IS THE GLASS HALF FULL OR HALF EMPTY?

The TTO was first applied to data from *Drosophila* 15 years ago, in a landmark paper proposing negative feedback regulation by the PER protein onto its own transcription (Hardin et al., 1990). Very briefly, the current model (Hardin, 2004) is as follows (Fig. 1B): A complex of CLK and CYC proteins activates transcription of *per* and *tim* genes. A complex of PER and TIM proteins interacts with CLK-CYC to inhibit transcriptional activity. The *per* and *tim* genes are thereby rhythmically transcribed, driving rhythmic PER and TIM protein levels. The VRI protein represses *Clk* transcription, and *Clk* cycles in antiphase to *per* and *tim* due to the inhibition by PER-TIM of *vri* transcription (Fig. 1B). In this model, posttranslational processes are considered to be important for period and amplitude of the rhythm, but transcriptional control is the core of the oscillator. This model makes several strong predictions, including the following: 1) Antiphase cycling of *Clk* and *per* transcription is essential to the feedback loop, and 2) rhythmic

transcription of *per* and *tim* is required for sustained rhythmicity.

The 1st prediction concerns the interlocked loop of VRI and CLK that provides the positive factor for rhythmic transcription from E-boxes, including PER and TIM. *Clk* RNA cycles in antiphase to *per* and *tim* RNA, and these rhythms are assumed to be generated by the activity of the various activator and repressor proteins. Rhythmic RNA levels are in turn assumed to be at least partly responsible for generating rhythmic protein levels. The logic of the feedback loops requires some functional role for rhythmic RNA. A direct test of the functional role of rhythmic *Clk* RNA was carried out by expressing *Clk* from a *per* or *tim* promoter (Kim et al., 2002). This resulted in *Clk* RNA expressed at the same phase as *per* and *tim*, rather than antiphase, but no major effects were found on the cycling of CLK protein or the behavioral rhythmicity of the flies. In other words, the rhythmicity of *Clk* RNA appears to have no functional significance. This problem with the current model of the "2nd loop" is not a fatal flaw in the basic TTO model: The 2nd interlocked loop is not required to generate rhythmicity, and its role may be more concerned with stability, time delays, or output pathways (Hardin, 2004).

The 2nd prediction is more fundamental to the logic of the TTO model. Feedback of PER and TIM proteins on their own transcription, and subsequent generation of rhythmic PER and TIM levels by rhythmic RNA levels, is proposed to be the core of the rhythm generator. However, as others have pointed out before (Harms et al., 2004; Yang and Sehgal, 2001), several lines of evidence suggest that rhythmic levels of PER and TIM proteins are not dependent on rhythmic levels of mRNA. Expression of the *per* gene can be driven by other promoters that are not subject to PER/TIM feedback, producing constitutive levels of *per* RNA; yet the PER protein levels continue to cycle, and behavioral rhythmicity can be restored to arrhythmic *per* mutants in some cases (Cheng and Hardin, 1998; Frisch et al., 1994; Vosshall and Young, 1995). In these experiments, the cycling of PER protein might be attributed to rhythmic TIM levels because TIM is important for stabilizing PER protein. Constitutive expression of *tim* was also found to produce cycling TIM protein levels and behavioral rhythmicity in spite of the absence of *tim* RNA cycling (Yang and Sehgal, 2001). The crucial experiment was carried out by Yang and Sehgal (2001): What happens when both *per* and *tim* are constitutively expressed? The answer is that both proteins continue to cycle, and behavioral rhythmicity can be restored in null mutant flies.

One criticism of this work (Yang and Sehgal, 2001) is that rhythms in the double-constitutive flies are somewhat weak: About 42% of flies were rhythmic, and an additional 13% were classified as "weakly rhythmic." This can be interpreted as evidence that the normal feedback that produces RNA cycling is "important" for function (Hardin, 2004). This is the glass-half-empty attitude: only half the flies were rhythmic. I agree that rhythmicity in these flies is not normal, but I take the glass-half-full attitude: as many as half were rhythmic. My question is, What makes those 50% rhythmic? It isn't a transcriptional feedback oscillator centered on *per* and *tim*. As with *Clk*, RNA cycling of *per* and *tim* is clearly important for robustness, but it is not required for rhythmicity. However, all the evidence to date does suggest that rhythmic PER and TIM protein levels are required for behavioral rhythmicity.

NEUROSPORA: A PLETHORA OF FRQ-LESS OSCILLATIONS

The examples cited above for *Acetabularia*, *Synechococcus*, and *Drosophila* concern the question of whether rhythmic transcription of canonical clock genes is required for rhythmicity in the organism. *Acetabularia* does not require rhythmic transcription of any genes for rhythmic photosynthesis and chloroplast movement. In *Synechococcus*, Kai proteins may be able to produce rhythmic KaiC phosphorylation in vivo when the protein levels are constant. Currently, it appears that posttranscriptional and posttranslational mechanisms (Harms et al., 2004) may produce rhythmic levels of the PER and TIM proteins to sustain rhythms in *Drosophila*. The experiments described in the preceding sections have broken the link between rhythmic transcription of clock genes and output rhythms and falsify the 1st of my 2 critical predictions from the TTO model: Holding transcription of clock genes constant should lead to arrhythmicity of the organism.

My 2nd critical prediction is that knocking out the canonical clock genes should lead to arrhythmicity of the organism. In *Synechococcus*, the *kai* genes appear to be indispensable for 24-h rhythmicity, and deletion of any *kai* gene leaves the organism arrhythmic. In *Drosophila*, rhythmic PER and TIM proteins are generally thought to be required for rhythmicity. However, there are 3 reports suggesting the presence

of circadian oscillators in per^0 mutants: A 24-h photoperiodic clock operates normally in per^0 (Saunders, 1990); per^0 flies entrained to LD cycles show evidence of an entrained morning peak of activity (Helfrich-Förster, 2001); and introducing cry^b in a per^0 cry^b double-mutant restores the evening peak of activity in LD as well as other aspects of clock-like behavior (Collins et al., 2005).

The most compelling evidence for rhythmicity in the absence of canonical clock gene function comes from *Neurospora*. The TTO model for *Neurospora* (Dunlap and Loros, 2004) proposes that the central components of the feedback loop are the *frq*, *wc-1*, and *wc-2* genes (Fig. 1C). A complex of the 2 WC proteins (WCC) activates transcription of *frq*. The FRQ protein inhibits the activity of WCC to negatively regulate *frq* transcription. This results in rhythmic *frq* RNA and FRQ protein levels. Null mutations in any of these 3 genes should abolish rhythmicity, according to the TTO model. This prediction has been tested in *Neurospora*, and there are now multiple reports of rhythmicity in clock gene null mutants (Lakin-Thomas and Brody, 2004). These fall into 2 classes: rhythmicity of the conidiation rhythm, which is the standard output used to assay the state of the *Neurospora* clock, and rhythmicity at the molecular level.

Conidiation rhythms have been observed in clock gene null mutants since the 1st reports of null mutations at the *frq* locus (Aronson et al., 1994; Loros and Feldman, 1986). Rhythmic conidiation can be seen in *frq* and *wc* null mutants simply by growing the strains in extra-long race tubes (Aronson et al., 1994; Dragovic et al., 2002; Loros and Feldman, 1986), but not all cultures are rhythmic, and it can take several days in culture before rhythms are produced. More reliable rhythmicity can be produced by adding farnesol or geraniol to the growth medium (Granshaw et al., 2003) or by creating double-mutant strains with the long-period lipid-deficient mutants *cel* or *chol-1* (Lakin-Thomas and Brody, 2000). Under conditions that do not promote reliable self-sustained conidiation rhythms, the application of temperature cycles or pulses will entrain conidiation rhythms that behave as expected for an endogenous oscillator when subjected to different entraining T-cycles (Lakin-Thomas, in press; Merrow et al., 1999; Pregueiro et al., 2005; Roenneberg et al., 2005). Two new mutations have been identified that permit reliable expression of rhythmic conidiation in *frq* and *wc* null mutants in LL and DD (Bell-Pedersen et al., 2005). The null mutants of the *frq* and *wc* genes should therefore be described not as arrhythmic but as "conditionally rhythmic": under appropriate conditions, rhythmicity can be restored to these mutants.

With so many instances of conidiation rhythmicity in clock gene null mutants, it is not surprising that molecular rhythms have been found in these mutants as well. Levels of the neutral lipid diacylglycerol (Ramsdale and Lakin-Thomas, 2000) and activity of the enzyme nitrate reductase (Christensen et al., 2004) are rhythmic in *frq* null mutants. Rhythmic mRNA levels have been found for several genes that continue to cycle in *frq* null mutants (Bell-Pedersen et al., 2005; Correa et al., 2003).

The existence of rhythmicity in null mutants leads to the conclusion that functional clock genes *frq*, *wc-1*, and *wc-2* are not required for rhythmicity and perhaps in some mutant strains not even for temperature compensation and light entrainability (Bell-Pedersen et al., 2005). The current debate revolves around the identity of the oscillator(s) driving these rhythms. In most cases, the rhythms found in *frq* and *wc* null mutants are missing 1 or more of the properties expected of "true circadian rhythms" (Dunlap and Loros, 2004): some are not robust, and/or are not temperature compensated, and/or are not light-entrainable, and/or have periods that vary with medium composition. The term "*frq*-less oscillator" (FLO) has been coined (Iwasaki and Dunlap, 2000) to describe the rhythms that are seen in *frq* null mutants. These rhythms are described as noncircadian, and it has been suggested that they represent additional slave oscillators, part of the circadian system but driven by the FRQ/WCC TTO (Dunlap and Loros, 2004). The rhythmicity seen in the 2 new mutations identified in the Bell-Pedersen laboratory has been described as an autonomous circadian oscillator with all the expected circadian properties (Bell-Pedersen et al., 2005). The *Neurospora* circadian system looks to some authors like a system of multiple oscillators with different inputs, with some FLOs acting as slaves to the TTO (Bell-Pedersen et al., 2005; Dunlap and Loros, 2004), or several interacting FLOs with the FRQ/WCC loop primarily acting as an input pathway (Lakin-Thomas and Brody, 2004).

FLOs seem to be proliferating like rabbits, and new FLOs are continuing to pop out of the shrubbery. Depending on how you count, there are at the moment about 10 different conditions that produce rhythmicity in *frq* and/or *wc* null mutants, and the properties of the rhythms are somewhat different in each condition. The term "*frq*-less oscillator" gives the impression that a separate oscillator mechanism is at work in each case, and I believe that is highly unlikely.

I propose that the acronym "FLO" should be expanded as "*frq*-less oscillaTION" instead of "oscillaTOR" to emphasize that what we are describing is an output rhythm (oscillation) and that we have no data on the underlying oscillator mechanism(s). We should consider the possibility that all of these oscillations are driven by the same core oscillator mechanism, which has yet to be identified. This core mechanism would not be "the circadian oscillator" in the sense that it would not have all the expected circadian properties in isolation but should instead be described as "the oscillator used by the circadian system." Only the genetically intact system would have all of the expected circadian properties, but various mutations and growth conditions could change some of the parameters of the system such that those properties are lost or altered.

MAMMALS: THE JURY IS STILL OUT

Tests of the TTO in mammals have not progressed as far as those in simpler organisms. The current model (Hastings and Herzog, 2004) was developed by analogy with the *Drosophila* system (Fig. 1D): The transcription of 2 *mPer* genes and 2 *mCry* genes is activated by the positive factor, a complex of CLOCK and BMAL1 proteins. Two PER and 2 CRY proteins form a complex and negatively regulate their own transcription. CLOCK/BMAL1 also activates expression of *Rev-Erbα*, and the protein product REV-ERBα represses *Bmal1* expression, creating a secondary loop to make BMAL1 rhythmic. Evidence in favor of this model is still incomplete, and, as suggested previously (Hastings and Herzog, 2004), we do not yet know whether rhythmic transcription is required for the clock mechanism.

Are these clock genes required for rhythmicity? Some evidence has been provided by mutations and gene knockouts, and the answer is similar to the evidence in *Neurospora*: It depends on the conditions (Hastings and Herzog, 2004). Experiments are ongoing to assay the effects of inactivating various combinations of clock genes in mice. Rhythmicity seems to depend on whether the animals are assayed in DD, LD, or LL, length of time in DD, age, and genetic background. In addition, different combinations of clock gene knockouts display different phenotypes.

Several laboratories have been looking at the phenotypes of knockouts of the negative factors in the TTO model, *Per*, and *Cry*. Single knockouts of *mCry1* or *mCry2* are rhythmic in DD (van der Horst et al., 1999). An $mPer2^{Brdm1}$ mutant, with a deletion in a PAS domain important for protein-protein interactions (Zheng et al., 1999), and knockouts of *mPer1* and *mPer2* (Bae et al., 2001; Zheng et al., 2001) are initially rhythmic in DD but may become arrhythmic in prolonged DD. The delayed arrhythmicity of $mPer2^{Brdm1}$ depends on genetic interactions and can be repaired in an *mCry2* mutant (Oster et al., 2002). Double mutants of *mPer1/mPer2* (Bae et al., 2001; Zheng et al., 2001), *mCry1/mCry2* (van der Horst et al., 1999), and $mPer2^{Brdm1}/mCry1$ (Oster et al., 2002) are immediately arrhythmic in DD. However, *mCry1/mCry2* double knockouts in LD cycles show pre-dark activity that cannot be accounted for by masking and that may be evidence for a damped endogenous oscillator (Mrosovsky, 2001). Double mutants *mPer1/mCry1* (Oster et al., 2003) and $mPer2^{Brdm1}/mCry2$ (Oster et al., 2002) are rhythmic in DD. The *mPer1/mCry2* mice are rhythmic when young, but rhythmicity decays with age (Oster et al., 2003). Can we make sense of the data? So far, it looks as if no single *Per* or *Cry* gene is indispensable for rhythmicity. Oster et al. (2002) have proposed a model in which the various combinations of PER and CRY proteins can form complexes with different transcriptional activities, and rhythmicity is supported only when that activity is within a particular range. The predictions of this model will continue to be tested with additional combinations of mutants. Definitive answers may be difficult to obtain if there are additional as-yet-unidentified genes that provide some backup functionality in clock gene knockouts.

The positive factors in the mammal TTO model are also under scrutiny through mutational studies. Mice with homozygous mutations in *Clock* become arrhythmic after several days in DD, but rhythmicity can be restored by a light pulse (Vitaterna et al., 1994). Some *Clock* mutants continue to show rhythmic activity for 10 days or more in DD, and firing rhythms of SCN neurons in brain slices from these mutants can be rhythmic in vitro for more than 5 days (Nakamura et al., 2002). *Bmal1* knockout mutants are immediately arrhythmic in DD (Bunger et al., 2000). However, the *Rev-Erbα* knockout is rhythmic in DD and LL (Preitner et al., 2002), in spite of the loss of rhythmicity of *Clock*, *Bmal1*, and *Cry1* transcription in this mutant. *Cry2* transcription is also not rhythmic in wild-type or the *RevErbα* knockout (Preitner et al., 2002). These results may indicate that rhythmic transcriptional control is not required in the mouse circadian system.

A surprising finding is that constant light of appropriate brightness can restore rhythmicity to

some arrhythmic mutants. Single mutants *mPer2*^{Brdm1} (Steinlechner et al., 2002) and *Clock* (Spoelstra et al., 2002) remain rhythmic in LL, although they become arrhythmic in DD. The *Clock* mutation is not a deletion but produces a defective protein, and it is conceivable that some functionality is restored by elevating the level of this protein in LL. An unpublished study (Oster, personal communication) reported that rhythmicity can be restored to the double mutant *mPer2*^{Brdm1}/*mCry1* in bright LL, even though it is immediately arrhythmic in DD. We do not yet know how many other arrhythmic strains can be made rhythmic in appropriate genetic backgrounds or lighting conditions. A particularly interesting question is whether bright light can restore rhythmicity to the *Bmal1* knockout.

CONCLUSIONS

How should we deal with the increasing number of anomalies that challenge the orthodox TTO model? One approach is to ignore them, or try to bury the data, in the hope that they will eventually be explained away and the TTO will emerge triumphant. I believe that the number and credibility of the anomalies are too great to allow us to ignore them. A 2nd approach is to invoke additional noncircadian oscillators whenever rhythmicity is seen in the absence of a functional TTO. This is the approach often taken with *Neurospora*. However, I think the time might be right to try to unify multiple manifestations of rhythmicity into a single mechanism rather than continuing to split the system into multiple oscillators. A 3rd approach is to throw out the TTO altogether as irrelevant and look elsewhere for the oscillator that drives rhythmicity. This is too radical even for me; there is a lot of rhythmic transcription in cells, and I suspect some of it is there for a reason. That brings us to the 4th approach, which is to try to integrate rhythmic transcription into a larger circadian system.

If the TTO is no longer accepted as a literal description of how the oscillator works, it is still a useful description of the rhythmic behavior of clock RNAs and proteins in intact organisms under "normal" laboratory conditions. What is rhythmic transcriptional control of canonical clock genes for, if not to function as the core of a circadian oscillator? Some answers have been suggested by Roenneberg and Merrow (1998), Hardin (2004), and other authors. Transcriptional control may increase the amplitude of cycling proteins, contributing to the robustness of the oscillator and adding stability, allowing the circadian system to function in fluctuating environments outside of our well-controlled laboratories (Hardin, 2004). Transcriptional rhythms may also be driving rhythmic outputs and/or regulating the phase of output pathways. Another possibility is that the TTO may function as a rhythmic input pathway: The genes involved in the TTO feedback loop in *Neurospora* (*frq*, *wc-1*, and *wc-2*) are important for temperature compensation and light input. A rhythmic input pathway, or *zeitnehmer* (Roenneberg and Merrow, 1998, 2001), could be responsible for transmitting environmental information to the oscillator and for providing other circadian properties such as self-sustainability and temperature compensation.

Where should we look for "the oscillator used by the circadian system" if it's not the canonical TTO? Why haven't the components of this oscillator been identified yet? If there is a "rhythm generator" (Roenneberg and Merrow, 2001) still lurking in the undergrowth, it may be hiding among the "housekeeping functions" of the cell, utilizing essential genes that don't turn up in mutant screens because inactivation of these genes is lethal (Lakin-Thomas, 2000). Many genes that affect clock function other than the canonical clock genes have been identified in, for example, *Neurospora* (Lakin-Thomas and Brody, 2004) and mammals (Shimomura et al., 2001), and further analysis of these genes could lead us to some candidate oscillator components.

In conclusion, I would like to provide my vision of the circadian system. I don't see it as "the circadian oscillator," a small, defined set of clock genes that function in a transcriptional feedback loop with the sole function of providing rhythmic output that is robust, self-sustained, temperature compensated, and light sensitive. I see the circadian system as more flexible, with a rhythm generator ("the oscillator used by the circadian system") that is embedded in metabolic processes and that does not have all the circadian properties on its own but that interacts with gene products (including the canonical clock genes) that provide environmental input, output pathways, temperature compensation, stability, and robustness (Roenneberg and Merrow, 1998). These components may have greater or lesser influence on the entire system, depending on environment and genotype. The most basic message I would like to convey is simply that we should continue to be open minded about the functions of clock genes and the possibility of rhythm generators yet to be identified.

ACKNOWLEDGMENTS

This review is an expanded version of a talk given in the Visions of Synthesis session of the 2005 Gordon Conference on Chronobiology, Newport, Rhode Island, and the author thanks Martha Gillette for organizing this meeting. Many thanks to Nicholas Mrosovsky for challenging the author to pursue this topic. The author's work is supported by NSERC Discovery Grant 250133-02.

REFERENCES

Aronson BD, Johnson KA, and Dunlap JC (1994) Circadian clock locus *Frequency*: protein encoded by a single open reading frame defines period length and temperature compensation. Proc Natl Acad Sci U S A 91:7683-7687.

Bae K, Jin X, Maywood ES, Hastings MH, Reppert SM, and Weaver DR (2001) Differential functions of *mPer1*, *mPer2*, and *mPer3* in the SCN circadian clock. Neuron 30:525-536.

Bell-Pedersen D, Cassone VM, Earnest DJ, Golden SS, Hardin PE, Thomas TL, and Zoran MJ (2005) Circadian rhythms from multiple oscillators: lessons from diverse organisms. Nat Rev Genet 6:544-556.

Bunger MK, Wilsbacher LD, Moran SM, Clendenin C, Radcliffe LA, Hogenesch JB, Simon MC, Takahashi JS, and Bradfield CA (2000) *Mop3* is an essential component of the master circadian pacemaker in mammals. Cell 103:1009-1017.

Cheng Y and Hardin PE (1998) *Drosophila* photoreceptors contain an autonomous circadian oscillator that can function without *period* mRNA cycling. J Neurosci 18:741-750.

Christensen MK, Falkeid G, Loros JJ, Dunlap JC, Lillo C, and Ruoff P (2004) A nitrate induced *frq*-less oscillator in *Neurospora crassa*. J Biol Rhythms 19:280-286.

Collins BH, Dissel S, Gaten E, Rosato E, and Kyriacou CP (2005) Disruption of Cryptochrome partially restores circadian rhythmicity to the arrhythmic *period* mutant of *Drosophila*. Proc Natl Acad Sci U S A 102:19021-19026.

Correa A, Lewis AZ, Greene AV, March IJ, Gomer RH, and Bell-Pedersen D (2003) Multiple oscillators regulate circadian gene expression in *Neurospora*. Proc Natl Acad Sci U S A 100:13597-13602.

Ditty JL, Williams SB, and Golden SS (2003) A cyanobacterial circadian timing mechanism. Annu Rev Genet 37:513-543.

Dragovic Z, Tan Y, Görl M, Roenneberg T, and Merrow M (2002) Light reception and circadian behavior in "blind" and "clock-less" mutants of *Neurospora crassa*. Embo J 21:3643-3651.

Dunlap JC and Loros JJ (2004) The *Neurospora* circadian system. J Biol Rhythms 19:414-424.

Edmunds LN (1988) *Cellular and Molecular Bases of Biological Clocks*. New York: Springer-Verlag.

Frisch B, Hardin PE, Hamblen-Coyle MJ, Rosbash M, and Hall JC (1994) A promoterless *period* gene mediates behavioral rhythmicity and cyclical *per* expression in a restricted subset of the *Drosophila* nervous system. Neuron 12:555-570.

Granshaw T, Tsukamoto M, and Brody S (2003) Circadian rhythms in *Neurospora crassa*: farnesol or geraniol allow expression of rhythmicity in the otherwise arrhythmic strains *frq*^{10}, *wc-1*, and *wc-2*. J Biol Rhythms 18:287-296.

Hardin PE (2004) Transcription regulation within the circadian clock: the E-box and beyond. J Biol Rhythms 19:348-360.

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

Harms E, Kivimae S, Young MW, and Saez L (2004) Posttranscriptional and posttranslational regulation of clock genes. J Biol Rhythms 19:361-373.

Hastings MH and Herzog ED (2004) Clock genes, oscillators, and cellular networks in the suprachiasmatic nuclei. J Biol Rhythms 19:400-413.

Helfrich-Förster C (2001) The locomotor activity rhythm of *Drosophila melanogaster* is controlled by a dual oscillator system. J Insect Physiol 47:877-887.

Ishiura M, Kutsuna S, Aoki S, Iwasaki H, Andersson CR, Tanabe A, Golden SS, Johnson CH, and Kondo T (1998) Expression of a gene cluster *kaiABC* as a circadian feedback process in cyanobacteria. Science 281:1519-1523.

Iwasaki H and Dunlap JC (2000) Microbial circadian oscillatory systems in *Neurospora* and *Synechococcus*: models for cellular clocks. Curr Opin Microbiol 3:189-196.

Iwasaki H and Kondo T (2004) Circadian timing mechanism in the prokaryotic clock system of cyanobacteria. J Biol Rhythms 19:436-444.

Johnson CH (2004) Precise circadian clocks in prokaryotic cyanobacteria. Curr Issues Mol Biol 6:103-110.

Kim EY, Bae K, Ng FS, Glossop NRJ, Hardin PE, and Edery I (2002) *Drosophila* CLOCK protein is under posttranscriptional control and influences light-induced activity. Neuron 34:69-81.

Lakin-Thomas PL (2000) Circadian rhythms: new functions for old clock genes? Trends Genet 16:135-142.

Lakin-Thomas PL (in press) Circadian clock genes frequency and white collar–1 are not essential for entrainment to temperature cycles in *Neurospora crassa*. Proc Natl Acad Sci U S A.

Lakin-Thomas PL and Brody S (2000) Circadian rhythms in *Neurospora crassa*: Lipid deficiencies restore robust rhythmicity to null *frequency* and *white-collar* mutants. Proc Natl Acad Sci U S A 97:256-261.

Lakin-Thomas PL and Brody S (2004) Circadian rhythms in microorganisms: new complexities. Annu Rev Microbiol 58:489-519.

Loros JJ and Feldman JF (1986) Loss of temperature compensation of circadian period length in the *frq-9* mutant of *Neurospora crassa*. J Biol Rhythms 1:187-198.

Mandoli DF (1998) What ever happened to *Acetabularia*? Bringing a once-classic model system into the age of molecular genetics. Int Rev Cytol 182:1-67.

Merrow M, Brunner M, and Roenneberg T (1999) Assignment of circadian function for the *Neurospora* clock gene *frequency*. Nature 399:584-586.

Mittag M (2001) Circadian rhythms in microalgae. Int Rev Cytol 206:213-247.

Mrosovsky N (2001) Further characterization of the phenotype of *mCry1/mCry2*-deficient mice. Chronobiol Int 18:613-625.

Nakahira Y, Katayama M, Miyashita H, Kutsuna S, Iwasaki H, Oyama T, and Kondo T (2004) Global gene repression by KaiC as a master process of prokaryotic circadian system. Proc Natl Acad Sci U S A 101:881-885.

Nakajima M, Imai K, Ito H, Nishiwaki T, Murayama Y, Iwasaki H, Oyarna T, and Kondo T (2005) Reconstitution of circadian oscillation of cyanobacterial KaiC phosphorylation in vitro. Science 308:414-415.

Nakamura W, Honma S, Shirakawa T, and Honma K (2002) *Clock* mutation lengthens the circadian period without damping rhythms in individual SCN neurons. Nat Neurosci 5:399-400.

Oster H, Baeriswyl S, van der Horst GT, and Albrecht U (2003) Loss of circadian rhythmicity in aging *mPer1*$^{-/-}$ *mCry2*$^{-/-}$ mutant mice. Genes Dev 17:1366-1379.

Oster H, Yasui A, van der Horst GT, and Albrecht U (2002) Disruption of *mCry2* restores circadian rhythmicity in *mPer2* mutant mice. Genes Dev 16:2633-2638.

Pregueiro AM, Price-Lloyd N, Bell-Pedersen D, Heintzen C, Loros J, and Dunlap J (2005) Assignment of an essential role for the *Neurospora frequency* gene in circadian entrainment to temperature cycles. Proc Natl Acad Sci U S A 102:2210-2215.

Preitner N, Damiola F, Molina LL, Zakany J, Duboule D, Albrecht U, and Schibler U (2002) The orphan nuclear receptor REV-ERB alpha controls circadian transcription within the positive limb of the mammalian circadian oscillator. Cell 110:251-260.

Ramsdale M and Lakin-Thomas PL (2000) *sn*-1,2-diacylglycerol levels in the fungus *Neurospora crassa* display circadian rhythmicity. J Biol Chem 275:27541-27550.

Roenneberg T, Dragovic Z, and Merrow M (2005) Demasking biological oscillators: properties and principles of entrainment exemplified by the *Neurospora* circadian clock. Proc Natl Acad Sci U S A 102:7742-7747.

Roenneberg T and Merrow M (1998) Molecular circadian oscillators: an alternative hypothesis. J Biol Rhythms 13:167-179.

Roenneberg T and Merrow M (2001) The role of feedbacks in circadian systems. In *Zeitgebers, Entrainment and Masking of the Circadian System*, Honma K and Honma S, eds, pp 113-130, Sapporo, Japan, Hokkaido University Press.

Salomé PA and McClung CR (2004) The *Arabidopsis thaliana* clock. J Biol Rhythms 19:425-435.

Saunders DS (1990) The circadian basis of ovarian diapause regulation in *Drosophila melanogaster*: is the period gene causally involved in photoperiodic time measurement? J Biol Rhythms 5:315-331.

Schweiger H-G, Hartwig R, and Schweiger M (1986) Cellular aspects of circadian rhythms. J Cell Sci 4(Suppl):181-200.

Shimomura K, Low-Zeddies SS, King DP, Steeves TD, Whiteley A, Kushla J, Zemenides PD, Lin A, Vitaterna MH, Churchill GA, and Takahashi JS (2001) Genome-wide epistatic interaction analysis reveals complex genetic determinants of circadian behavior in mice. Genome Res 11:959-980.

Spoelstra K, Oklejewicz M, and Daan S (2002) Restoration of self-sustained circadian rhythmicity by the mutant *Clock* allele in mice in constant illumination. J Biol Rhythms 17:520-525.

Steinlechner S, Jacobmeier B, Scherbarth F, Dernbach H, Kruse F, and Albrecht U (2002) Robust circadian rhythmicity of *Per1* and *Per2* mutant mice in constant light, and dynamics of *Per1* and *Per2* gene expression under long and short photoperiods. J Biol Rhythms 17:202-209.

Tomita J, Nakajima M, Kondo T, and Iwasaki H (2005) No transcription-translation feedback in circadian rhythm of KaiC phosphorylation. Science 307:251-254.

van der Horst GT, 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, and Yasui A (1999) Mammalian Cry1 and Cry2 are essential for maintenance of circadian rhythms. Nature 398:627-630.

Vitaterna MH, King DP, Chang AM, Kornhauser JM, Lowrey PL, McDonald JD, Dove WF, Pinto LH, Turek FW, and Takahashi JS (1994) Mutagenesis and mapping of a mouse gene *Clock*, essential for circadian behavior. Science 264:719-725.

Vosshall LB and Young MW (1995) Circadian rhythms in *Drosophila* can be driven by *period* expression in a restricted group of central brain cells. Neuron 15:345-360.

Woolum JC (1991) A re-examination of the role of the nucleus in generating the circadian rhythm in *Acetabularia*. J Biol Rhythms 6:129-136.

Xu Y, Mori T, and Johnson CH (2003) Cyanobacterial circadian clockwork: roles of KaiA, KaiB and the *kaiBC* promoter in regulating KaiC. Embo J 22:2117-2126.

Yang Z and Sehgal A (2001) Role of molecular oscillations in generating behavioral rhythms in *Drosophila*. Neuron 29:453-467.

Zheng B, Albrecht U, Kaasik K, Sage M, Lu WQ, Vaishnav S, Li Q, Sun ZS, Eichele G, Bradley A, and Lee CC (2001) Nonredundant roles of the *mPer1* and *mPer2* genes in the mammalian circadian clock. Cell 105:683-694.

Zheng B, Larkin DW, Albrecht U, Sun ZS, Sage M, Eichele G, Lee CC, and Bradley A (1999) The *mPer2* gene encodes a functional component of the mammalian circadian clock. Nature 400:169-173.