Circadian rhythms

new functions for old clock genes?

The mechanisms of circadian clocks, which time daily events, are being investigated by characterizing 'clock genes' that affect daily rhythms. The core of the clock mechanism in *Drosophila*, *Neurospora*, mammals and cyanobacteria is described by a transcription–translation feedback-loop model. However, problems with this model could indicate that it is time to look at the functions of these genes in a different light. Our *a priori* assumptions about the nature of circadian clocks might have restricted our search for new mutants in ways that prevent us from finding important clock genes.

Patricia L. Lakin-Thomas
pl106@mole.bio.cam.ac.uk

Department of Plant Sciences, University of Cambridge, Downing Street, Cambridge UK CB2 3EA.

How does a fungus, or a fruitfly, or a mouse, tell time? What are the molecular mechanisms of the circadian clocks (Boxes 1 and 2) that allow organisms to measure the passage of a day, and regulate their activities appropriately? There has recently been an explosion[1] of interest in this topic, with the isolation of new 'clock genes' (Box 2), the finding of homologous clock genes in *Drosophila melanogaster* and mammals, and the identification of a common sequence motif, the PAS domain (Box 2), in many of the gene products (see Table 1). According to many reviewers, the answer to my opening question is clear: '... there is a feedback loop ... that is centered on the transcription and translation of clock genes and clock proteins'[2]; 'Overwhelming evidence ... shows that core clock mechanisms involve 'clock genes', which participate in transcriptional–translational feedback loops'[1]; these are examples of many similar statements from recent reviews.

The orthodox view of clock mechanisms, as presented in the introductions to numerous papers in this field, is the transcription–translation feedback loop (Fig. 1a). In many organisms, one or more 'canonical clock genes' (Table 1) are rhythmically transcribed and translated into proteins that are rhythmically abundant and that feed back to rhythmically inhibit their own transcription. It might appear to the casual reader that this problem is essentially solved, and there is little left to do but fill in some species-specific details and tie up a few loose ends. But just how good is the evidence for this orthodox model? Those working inside this field are aware of the complexities and anomalies that are not explained by the simple model as it is often caricatured. In this perspective, I will play 'the devil's advocate' by focusing not on the successes of this model but on the remaining problems. In particular, new evidence from *Neurospora crassa* raises some basic questions: is the orthodox model adequate to explain the generation of circadian rhythmicity? If not, then what might be the functions of the canonical clock genes and what might be the rhythm-generating mechanisms?

Problems with the orthodox model in *Drosophila*

A transcription–translation feedback-loop model was first proposed for the circadian oscillator of *Drosophila*, and the current model is described in Fig. 1b. This model is built on a set of assumptions about the mechanism: the circadian oscillator is a causal chain, in which rhythmic transcription of dedicated clock genes begets rhythmic RNA levels, which beget rhythmic protein levels, which beget rhythmic DNA-binding activity, which begets rhythmic transcription, and so on; this sequence of events takes approximately 24 hours to complete one loop. The identity of the components might be different in different species, but the structure of this causal chain is assumed to be similar. Although this model has now been applied to *Neurospora*, mammals and cyanobacteria, it has never had the potential to be a universal mechanism. For example, in the giant alga *Acetabularia*, rhythmic photosynthesis can continue for many days in cells from which the nucleus has been removed and organellar RNA synthesis has been inhibited[3], so any mechanism depending on rhythmic gene transcription could not apply to this organism.

The first problem with this model is the time-delay question: all of the processes in this causal chain are normally carried out fairly rapidly, and some transcription factors can be transcribed, translated, transported into the nucleus and be acting on their nuclear targets within minutes. There must be time delays introduced at one or more points in the chain to make this loop take 24 hours to complete, but we do not yet have any kinetic data to indicate how these delays are accomplished.

BOX 1. Properties of circadian rhythms

Circadian rhythms are biological rhythms with periods of ~24 hours.
Rhythmicity is endogenous and self-sustaining, and continues under constant environmental conditions.
Period length is usually not exactly 24 hours under constant conditions but is genetically determined for that species.
Period length is usually temperature-compensated and varies little at different constant ambient temperatures.
Rhythmic environmental signals, mainly light and temperature changes, reset the phase to entrain the rhythm to exactly 24 hours.
Circadian rhythms appear to be 'ubiquitous in eukaryotes' and have also been demonstrated in the cyanobacteria.
Rhythmicity is found at the level of the single cell, and does not require complex tissue organization.
Uses for circadian rhythms include daylength measurement for photoperiodism, celestial navigation, timing of internal events to anticipate dawn or dusk, and timing of incompatible processes at different phases, but in many organisms the selective advantages of circadian rhythms have not yet been described.

0168-9525/00/$ – see front matter PII: S0168-9525(99)01945-9

BOX 2. Glossary

Circadian
Literally meaning 'about a day', refers to biological rhythmicity with a period of ~24 hours.

Clock
An entire circadian system, including the central oscillator and the input and output pathways (see Box 3). A clock can be entrained to the environmental day–night cycle and is used by an organism to track the passage of solar time. Alternatively (see 'clock gene'), refers only to the central oscillator mechanism.

Clock gene
Gene proposed as a component of a central oscillator. Alternatively[6] refers only to the negative elements of a transcription–translation feedback loop whose abundance is rhythmic.

Clock-affecting gene
Identified by a mutation that alters the period or persistence of rhythmicity. It might or might not be a component of a central oscillator.

Canonical clock gene
Gene proposed as a major component of a transcription–translation feedback loop. Also refers to similar genes identified in other organisms by sequence homology. Alternatively[25] refers to genes identified in screens for mutations that change the period or persistence of rhythmicity but have no known metabolic defects.

Non-canonical clock gene
Defined by mutations that change the period and either have known biochemical defects or affect the growth and viability of the organism. Also refers to clock-affecting genes not well characterized.

Entrainment
Process by which an endogenous circadian oscillator is forced to 'lock on' to an imposed environmental cycle (a zeitgeber) such as a light–dark cycle or temperature cycle so as to exactly match the imposed period·

Input
Pathway(s) through which a circadian oscillator receives information from the environment, such as light signals and temperature changes. This allows the oscillator to remain synchronized with the environmental day–night cycle.

Oscillator
Molecular mechanism that generates self-sustained rhythmicity, independently of the input and output pathways. Can also be called 'rhythm generating loop' or 'central clock mechanism'.

Output
Pathway(s) through which an oscillator influences cellular and organismal behaviour to produce observable rhythms in metabolism, activity, etc.

Parameter
A term used in mathematical modelling of dynamic systems, such as oscillators, referring to a constant quantity that does not change with time but that determines the rates at which other quantities (variables) change. For example, in a mathematical model of a biochemical oscillator, the K_m and V_{max} of an enzyme might be parameters.

PAS domain
Protein sequence motif found in some clock proteins and in many other proteins[18], predominantly associated with signalling pathways that transmit environmental information such as oxygen, redox state and light. Sometimes associated with protein–protein interactions.

Phase
Describes the time of occurrence of some landmark in a rhythm, relative to a control rhythm. For example, the time of occurrence of the peak of rhythmic activity in an animal relative to another animal, or relative to the dawn of a day–night cycle.

Phase shift
A single persistent change in the phase of a rhythm, caused perhaps by some outside perturbation such as exposure to a pulse of light.

Rhythm
Anything that can be seen to regularly oscillate in level or activity. Usually refers to the observable output of a central oscillator rather than the oscillator itself.

Semi-dominance (also incomplete, or partial, dominance)
Refers to a mutation that when heterozygous produces a phenotype that is neither that of the homozygous wild-type nor the homozygous mutant. For example, a mutation that produces a period of 20 h when homozygous in an organism with a wild-type period of 26 h might produce a period of 23 h in the heterozygote.

State variable
A term used in mathematical modelling of dynamic systems, such as oscillators, referring to a quantity that changes with time (a variable). The set of state variables is the minimum number of variables that are required to completely define the state of the system at one instant in time. For example, in a mathematical model of a biochemical oscillator, the concentrations of products and reactants might be state variables.

Temperature compensation
The property of maintaining approximately the same period at different constant temperatures.

Zeitgeber
Literally meaning 'time-giver', an externally imposed rhythmic signal, such as a light–dark cycle, that entrains an oscillator.

Zeitnehmer
Literally meaning 'time-taker' (by analogy with 'zeitgeber'), an input pathway that is itself rhythmically regulated by feedback from an oscillator. It will therefore create a rhythmic input to the oscillator even in constant environmental conditions.

(Note: The first definition given for each term is that used in this review. Alternative definitions used by other authors are given to aid the reader in understanding the literature.)

The second problem comes in attempting to establish the causal links between steps in the loop. Evidence from *Drosophila* has shown that the timing of the peaks of the RNA and protein products of the clock gene *period* (*per*) (Table 1), and the shapes of the abundance curves, cannot be explained by the simple 'rhythmic transcription begets rhythmic RNA begets rhythmic protein' sequence. The lag time between the *per* RNA peak and the PER protein peak requires the assumption both of rhythmic RNA stability and of rhythmic protein stability to account for the shape and amplitude of the rhythms[4]. Similarly, the lag time between protein accumulation in the cytoplasm and transcriptional repression requires rhythmic regulation of nuclear entry of the complex between PER and its partner TIM, encoded by the *timeless* (*tim*) gene (Table 1)[5]. Most critically, if the *per* gene is transcribed from a constitutive promoter, it can rescue rhythmicity in arrhythmic null mutant per^0 flies and the RNA and protein are found to be

TABLE 1. Canonical clock genes

Species	Gene	Clock mutant phenotype	Proposed function	Relationship to phototransduction	Refs
Drosophila melanogaster	*per*[a] (*period*)	Short-period, long-period, and arrhythmic alleles	Negative element		6, 29
	tim (*timeless*)	Short-period, long-period, and arrhythmic alleles	Negative element	TIM protein destabilized by light	6, 29
	clk (*jrk*)[a] (*clock, jerk*)	Arrhythmic	Positive element	Mutants blind for lights-on response	1, 6, 30
	cyc[a] (cycle; homologous to BMAL1 of mammals)	Arrhythmic	Positive element	Mutants entrain poorly to light–dark cycles	1, 6, 31
	cry (*cryptochrome*)		Photoreceptor?	Protein sequence homologous to photolyase, protein abundance light-regulated, altered light responses in mutants	32
Neurospora crassa	*frq* (*frequency*)	Short-period and long-period alleles; null mutants conditionally rhythmic, with poor temperature compensation	Negative element	RNA abundance increased by light, null mutants less sensitive to light	6, 12, 13, 16
	wc-1[a], *wc-2*[a] (*white-collar*)	Arrhythmic under standard conditions	Positive elements	Mutants blind to almost all light responses	6, 19
Mammals (mouse and human)	*per1*[a], *per2*[a], *per3*[a] (*period*)	Per2 knockout mice have short period, gradual loss of rhythmicity	Negative elements	RNA abundance of *per1* and *per2* increased by light, *per1* induction required for phase shifting	1, 6, 33, 34
	tim (*timeless*)	?	Negative element?		6
	clk[a] (*clock*)	Long period, gradual loss of rhythmicity	Positive element	Reduced light-induced gene expression in mutants	1, 6, 35
	BMAL1[a] (*MOP3*)[a] (brain and muscle Amt-like protein; member of the PAS superfamily)	?	Positive element		1, 6
	cry1, cry2 (*cryptochrome*)	Short or long periods; double mutant is arrhythmic	Negative element	Protein sequence homologous to photolyase, altered photoresponses in mutants	32, 36
Synecho-coccus sp. strain PCC 7942	*kaiA* (*cycle*)	Several long period alleles; null mutant arrhythmic	Positive element		6, 37
	kaiC (*cycle*)	Short and long period alleles; null mutant arrhythmic	Negative element		6, 37

[a]PAS-containing proteins (see Box 2).

rhythmically abundant, in spite of the constant rate of transcription[6]. Indeed, one set of authors[7] concluded that 'it is possible that transcriptional regulation is not necessary for PER cycling or even for circadian rhythms.' The same conclusion might apply to *tim*: it has recently been found that expression of a *tim* cDNA transgene can rescue rhythmicity in arrhythmic null mutant tim^0 flies, even though the *tim* RNA is not rhythmic in abundance (A. Sehgal, pers. commun.). According to the orthodox model (Fig. 1b), the level of the PER–TIM heterodimer in the nucleus is critical for transcriptional control, and the level of dimer would be rhythmic as long as one of the two proteins is rhythmically expressed. It appears that flies can be rhythmic if either *per* or *tim* is constitutively expressed; we don't yet know if flies are rhythmic with both genes constitutively expressed.

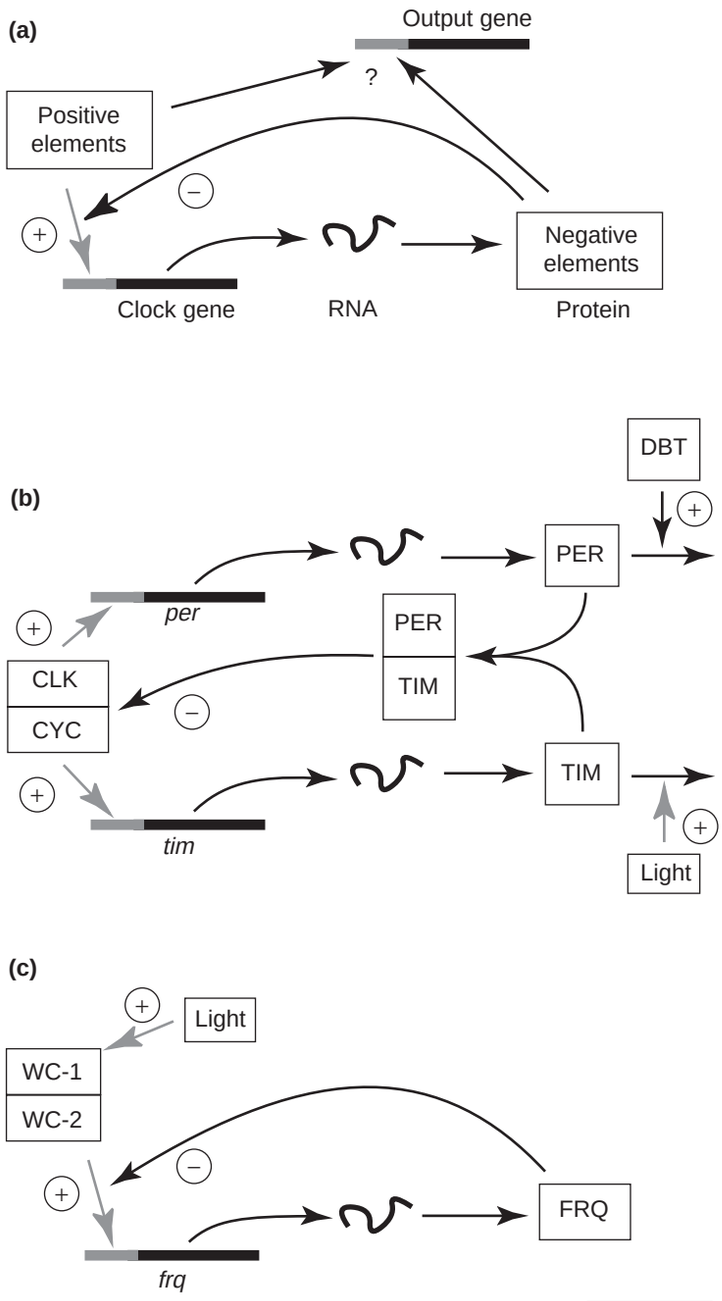
The third problem is the assumption that components of the loop are 'clock genes', dedicated to the proposed timekeeping function of the feedback loop[8]. The *Drosophila per* gene clearly plays an important role in circadian rhythmicity, but its regulation and functions are not dictated by, or restricted to, the orthodox feedback loop. The PER protein is nuclear and its levels are rhythmic in the lateral neurons (the brain cells that control circadian rhythmicity), but PER is expressed widely in other fly tissues, and in the ovaries the protein is neither nuclear nor rhythmic[8]. In the brains of silkmoth, PER appears to be functional in circadian rhythmicity but the protein, although rhythmic, is never nuclear[8], even though TIM protein is present and should allow nuclear transport according to the orthodox model[6] (see Fig. 1b). The *per* gene affects the period of the courtship-song rhythm in *Drosophila*, even though this rhythm has a period of ~1 minute, which is clearly too rapid to depend on a transcription–translation feedback loop[8]. Canonical clock genes must have other functions that are not explained by the model, and this raises the possibility that these proteins might have a primary function that is as yet unknown, and that their effects on circadian clocks might be secondary[8].

Problems with the orthodox model in *Neurospora*

The current model for the *Neurospora* transcription–translation feedback loop is described in Fig. 1c. The first problem in applying the orthodox model to *Neurospora* is that several of the basic assumptions behind the model have not yet been tested: although the abundance of the RNA product of the clock gene *frequency* (*frq*) (Table 1) is rhythmic[6], it has not yet been shown whether this gene is rhythmically transcribed or whether downregulation of *frq* RNA by FRQ protein occurs at the level of the promoter. For example, in a thorough analysis of the rhythmically expressed RNA-binding protein *At*GRP7 in *Arabidopsis*[9], it was shown that although the RNA levels are rhythmic, and the protein feeds back to control the level of its own RNA, this control is not at the level of transcription.

A second set of problems is similar to those in *Drosophila* concerning time delays and causal sequences.

FIGURE 1. The orthodox transcription–translation feedback loop



(a) The transcription–translation feedback loop that is proposed to be common to many circadian oscillators. The rhythmic transcription of one or more clock genes produces rhythmic levels of clock RNA(s) that, in turn, produce rhythmic levels of clock protein(s). The clock protein(s) are negative elements that inhibit the transcription of their own genes, possibly by interfering with positive elements required to activate transcription of clock genes. When clock RNA and subsequently protein levels fall, transcription is activated and the cycle repeats. Rhythmic output could be controlled by either the negative or positive elements acting on output gene expression. (Adapted from Ref. 6.) (b) Proposed model of the *Drosophila* transcription–translation feedback loop. Two clock genes, *per* (*period*) and *tim* (*timeless*), are rhythmically transcribed and translated into their proteins, PER and TIM. The PER protein contains a PAS domain (see Box 2), but TIM does not. The proteins dimerize and the heterodimer is transported into the nucleus where it inhibits transcription of the *per* and *tim* genes. Two PAS-protein transcription factors, CLK (clock) and CYC (cycle), are required for activation of transcription of *per* and *tim*. The PER–TIM heterodimer inhibits transcriptional activation by the heterodimer CLK–CYC. The DBT (double-time) kinase phosphorylates and destablizes PER in the absence of TIM, keeping PER levels low until TIM levels rise sufficiently to allow heterodimerization and nuclear transport. Light affects this system by enhancing the breakdown of TIM. (Adapted from Ref. 6. See Refs 1, 2, 6 and 29 for evidence supporting this model.) (c) Proposed model of the *Neurospora* transcription–translation feedback loop. The clock gene *frq* (*frequency*) is rhythmically transcribed and translated into its protein, FRQ. The protein is transported into the nucleus where it inhibits transcription of the *frq* gene. Two PAS-protein transcription factors, WC-1 (white-collar) and WC-2, are required for activation of transcription of *frq*. The FRQ protein inhibits transcriptional activation by the heterodimer WC-1–WC-2. Light affects this system by activating transcription of *frq*, acting through WC-1 and WC-2. (Adapted from Ref. 6. See Ref. 6 for evidence supporting this model.)

There appears to be no time delay in the *frq* loop to account for the circadian period: FRQ protein appears in the nucleus very rapidly after activation of *frq* transcription[10]. *frq* RNA and nuclear FRQ protein levels both drop to a low level and remain there for most of the circadian cycle, and after a long lag, *frq* transcription begins again without a further drop in nuclear FRQ levels[10]. The causal role of *frq* gene products in determining the state of the oscillator is also uncertain: if the current model of the *frq* feedback loop[6] is correct, then the levels of *frq* RNA and FRQ protein should determine the phase of the clock. Yet, in a series of experiments to assay levels before and after abrupt changes in temperature that reset the phase[11], there was no correlation between the levels of *frq* gene products and the phase of the rhythm of conidiation (spore-formation) that is assayed as the output of the *Neurospora* clock. These results cannot be explained without assuming that there are additional components outside the *frq* loop that control the time delays and interpret the level of FRQ correctly.

The third and most critical argument against a *frq*-based feedback loop being responsible for generating rhythmicity in *Neurospora* is the abundance of evidence that rhythmicity can be found in null mutants with no functional *frq* gene product. This was evident from very early on in the history of the *frq* gene, when the *frq*^{9} strain was characterized[12]. Cultures carrying this allele of *frq* are usually arrhythmic early during growth, but rhythmic conidiation often appears after several days of growth. Unlike the wild type, the period of the mutant is sensitive to the carbon source in the growth medium and has poor temperature compensation. An identical phenotype was found for a true null allele, *frq*^{10}, created by gene deletion[13].

Null mutants of *frq* and the putative clock genes *white-collar* (*wc-1* and *wc-2*, Table 1) have recently been shown to be rhythmic in another type of assay[14]. Unlike the wild type, these strains are not rhythmic when grown in light–dark cycles, indicating they are insensitive to light: indeed, the *wc* mutants are 'blind' mutants, defective in all light responses[15]. However, they do show rhythmic conidiation in temperature cycles. Changing the period of the temperature cycle changes the phase between the conidiation rhythm and the imposed cycle, which is a characteristic of an oscillator entrained by an external stimulus, and is unlike the fixed-phase relationship expected from a rhythm that is not produced by an independent oscillator but is merely driven by the rhythmic stimulus. This kind of

TABLE 2. Some non-canonical clock genes[a]

Species	Gene	Defect	Clock phenotype	Refs
Hamster	*tau* (*period*)	Altered growth rate and metabolic rate (primary defect unknown)	Short period, instability of rhythmicity	38
Mouse	*Ncam* [(encoding a neural cell adhesion molecule(NCAM)]	Deletion of NCAM carrying polysialic acid in brain	Short period, instability of rhythmicity	39
Drosophila melanogaster	*dbt* (*double-time*)	Defective protein kinase	Short-period, long-period and arrhythmic alleles	1, 6, 29
Neurospora crassa	*cel* (*fas*) (*chain elongation, fatty acid synthesis*)	Fatty acid synthetase defective, requires long-chain saturated fatty acids for normal growth	Long period (30–40 h) when supplemented with unsaturated or short-chain fatty acids, poor temperature compensation	40, 41
	chol-1 (*choline*)	Defective in synthesis of phosphatidylcholine, requires choline for normal growth	Long period when choline-deficient (up to 60 h, depending on choline concentration) poor temperature compensation.	42, 43
	oli^{r} (*oligomycin resistant*), [*Mi*-3] (*mitochondrial mutant*), *cya-5* (*cytochrome aa*$_3$), and others	Defective in mitochondrial energy transduction	Short periods	41, 44
	arg-13 (*arginine*), *cys-4* (*cysteine*), *cys-9, cys-12*	Require amino acid supplementation	Short periods	6, 41
	glp-3 (*ff-1*) (*glycerol phosphate, female fertility*)	Enhanced glycerol utilization, female sterile	Short period	41
	phe-1 (*phenylalanine*)	Requires phenylalanine supplementation	Short period	41
	prd-6 (*period*), *rhy-1* (*rhythm*)	Unknown	Temperature-sensitive rhythms	23, 24
	prd-4 (*period*)	Unknown	Short period	41
	chr (*chrono*), *cla-1* (*clock-affecting*), *prd-1* (*period*), *prd-2, prd-3*	Unknown	Long periods	41

[a]See also Refs 6, 26 and 41 for additional clock-affecting genes in these and other organisms.

entrainment protocol can reveal a cryptic oscillator that is not producing a visible output (such as the conidiation rhythm) under constant conditions, but which can produce such an output with the additional stimulus of the entraining cycle.

Rhythmicity in null *frq* mutants and in *wc* mutants has also been shown in yet another context[16]. Two mutations that affect lipid metabolism in *Neurospora*, *chain elongation* (*cel*) and *choline* (*chol-1*) (Table 2), will lengthen the period of the conidiation rhythm under conditions that alter lipid composition. Under these conditions, the rhythm is sensitive to entrainment by light cycles but the temperature compensation is poor. In double-mutant strains carrying either *cel* or *chol-1* as well as a null *frq* allele or a *wc* mutation, the cultures are robustly rhythmic when lipid metabolism is abnormal, although they are arrhythmic when the lipid defects are repaired by the appropriate growth supplement. Under long-period conditions, these double mutants cannot be entrained to light–dark cycles, although the *chol-1* or *cel* parents can be entrained. This insensitivity to light in the null *frq* and *wc* mutants suggests a different role for these genes than the orthodox model indicates.

New functions for old clock genes?

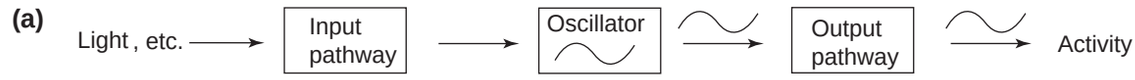
The evidence for a transcription–translation feedback loop involving canonical clock genes doesn't appear to be adequate for generating circadian rhythmicity in *Neurospora*. If so, then how might these genes affect rhythmicity and what might be their 'real' roles? We used to think we had a good set of criteria for recognizing components of a central circadian oscillator (see Box 3a). As more information became available about feedback from oscillators onto their input pathways, and feedback from output rhythms onto oscillators, it became more difficult to be confident about those criteria (Box 3b). A mathematical model has recently been published[17] demonstrating that, if the gene products are part of a rhythmically regulated input pathway, or 'zeitnehmer', they could fulfil nearly all the criteria of a central oscillator and yet not be part of a central 'rhythm-generating loop' (Box 3c).

A look at the column labelled 'Relationship to phototransduction' in Table 1 shows a remarkable similarity among canonical clock genes across species: many of them are intimately associated with light-input pathways. The clock proteins containing PAS motifs (Table 1) point to the same conclusion, as this protein motif is found in many proteins with signalling functions, sensing environmental information such as redox state, oxygen level or light[18]. This might indicate that circadian oscillators evolved from sensory input pathways[19], or it might mean that these genes are not components of central oscillator mechanisms but of input pathways, transducing environmental information to an oscillator. It is possible that all of the phenotypes of canonical clock mutants and details of molecular regulation of clock genes in all species examined so far could be explained by assuming that these transcription–translation loops are sensory input pathways that are rhythmically regulated by output from a central oscillator.

Limitations of clock-mutant screens

If canonical clock genes function in clocks as input components, then what are the central rhythm generators and why haven't they been identified in mutant screens for clock-affecting genes? Much excitement was generated by the finding of *per* and *tim* homologues in mammals, and the isolation of mutations in homologous *clk* genes that affect rhythmicity in *Drosophila* and mice. The same genes are continually being identified in mutant hunts, and the cross-species homologies are impressive. Does this prove the existence of a universal oscillator mechanism in the animal kingdom, or do the same clock genes keep arising because of limitations in the design of the screens for new mutants?

BOX 3. How do you recognize a component of an oscillator?



The traditional 'black box' model of a circadian system includes a single, non-redundant central oscillator that generates rhythmicity, an input pathway through which the oscillator receives information from the environment (such as light signals) to synchronize the oscillator with the environmental day–night cycle, and an output pathway through which the oscillator controls the observable rhythmic output (such as locomoter activity in animals or rhythmic conidiation in fungus). Information flow is one-way from left to right. The squiggle indicates a rhythmic signal.

The components of the oscillator can be identified as meeting some version of the following criteria. Putative components that meet these criteria are sometimes called 'state variables', although this term has little meaning in a biological context and should be reserved for mathematical models.

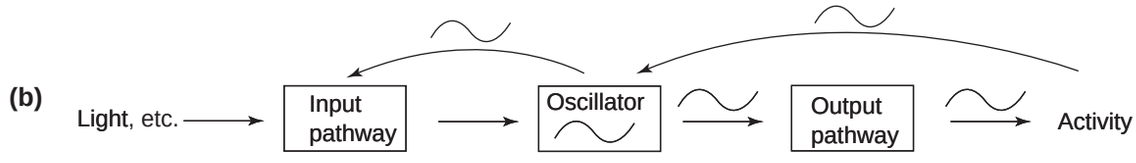
(1) Components of the oscillator (and output pathway) will be rhythmic in level or activity under constant environmental conditions. (In contrast, components of the input pathway are constant in level or activity in the absence of external signals.)

(2) The phase of the component's rhythm (and the phase of the rhythmic output) will be set by the environmental day–night cycle.

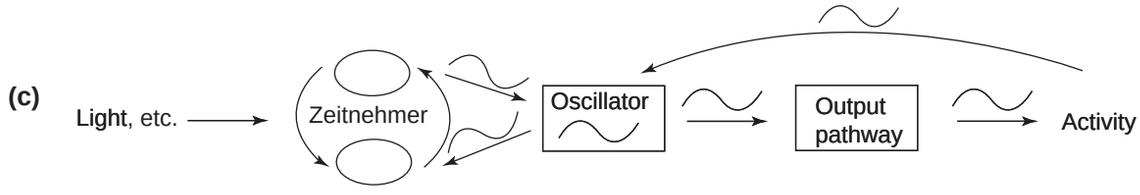
(3) A sudden induced change in the level or activity of an oscillator component (or an input component) will permanently change the phase of the output rhythm. (In contrast, a change in an output component will have no lasting effect on the phase of the rhythm.)

(4) Maintaining a rhythmic oscillator component at any constant level or constant activity will destroy rhythmicity of the output.

(5) Mutations in oscillator components might change the period, alter temperature compensation, or abolish rhythmicity. (In contrast, mutations in output components cannot change the period or temperature compensation but might abolish rhythmicity. Mutations in input components would not affect temperature compensation but might change the period or abolish rhythmicity if, for example, the input pathway is constitutively activated and mimics constant light.)



The black box model has been modified to include the recent recognition that an oscillator can feed back on its input pathway(s) to rhythmically regulate their activity (as in the rhythmic levels of photoreceptor sensitivity in plants[26]), and the output pathway can feed back on the oscillator (as in the effect of forced running activity on the phase and period of the rodent oscillator[27]). These feedbacks make it more difficult to identify components of the oscillator by the set of criteria above. In the simplest case, the input pathway does not provide rhythmic input to the oscillator in constant environmental conditions, but its activity is 'gated' by the oscillator such that the response of the input pathway to external signals is rhythmic. Input pathways now satisfy criteria 1, 2 and 3, but would still not satisfy criteria 4 and 5.



The 'zeitnehmer' concept[17,28] modifies the black box further by recognizing that input pathways might include a feedback loop to downregulate the sensory transduction apparatus, and this loop can be regulated by the central oscillator. Even in constant environmental conditions, rhythmic input from the zeitnehmer loop stabilizes the central oscillator and maintains its oscillation at an appropriate amplitude and with an appropriately circadian period, and provides compensation by insulating the oscillator from temperature and metabolic fluctuations. The components of the zeitnehmer input loop can now fulfil criteria 1, 2, 3 and 5 listed in part (a) for the central oscillator, but possibly not criterion 4. The system might continue to oscillate in the absence of the zeitnehmer loop, but it might no longer be circadian in period or temperature compensated, and its amplitude could be either too high or too low to generate rhythmic output. It might be necessary to use special conditions, such as temperature cycles or a mutant background, to reveal the cryptic oscillation. The zeitnehmer concept raises several new questions: should the zeitnehmer loop now be defined as part of the oscillator, or as part of the input pathway? What is the relative importance of the components that generate self-sustained rhythmicity, and the components that stabilize and regulate that rhythmicity to produce a useful clock with a circadian period and compensation for changes in temperature and metabolism?

The original screens for clock mutants both in *Drosophila* and in *Neurospora* appear to have been based on a tacit *a priori* assumption about the mechanisms of circadian oscillators that might have prejudiced the screens in favour of a certain class of mutation (see Fig. 2a): it was assumed that clocks must be built out of special 'clock proteins' with no function other than timekeeping. Therefore, the most desirable mutations to study

would be those that affect only clock properties, such as the period, and have no other effects on growth or viability of the organism. The screens identified the *per* and *tim* genes in *Drosophila*, the *clock* gene in mice and the *frq* gene in *Neurospora* (Table 1). The mutations have all turned out to be semi-dominant, and this is now assumed to be a defining feature of clock gene mutations. These original screens have been very valuable in providing mutants that can be used as tools to manipulate rhythmicity without gross pleiotropic effects on other functions. They have demonstrated that the circadian system is indeed under genetic control, and that there are some gene products that are apparently dedicated to time-keeping functions.

An entirely different approach to identifying clock mutants was based on the assumption that rhythmicity is probably common in metabolic pathways with feedback regulation[20] (Fig. 2b), and a circadian clock mechanism is likely to incorporate housekeeping functions in the central oscillator. Therefore, the place to look for mutations affecting a central oscillator is among the metabolic mutants with known biochemical defects[21]. This approach identified several mutations in *Neurospora* affecting the period, most notably the *cel* mutant (Table 2). Since then, additional screens have identified several more clock-affecting genes among mutants with known biochemical defects (Table 2).

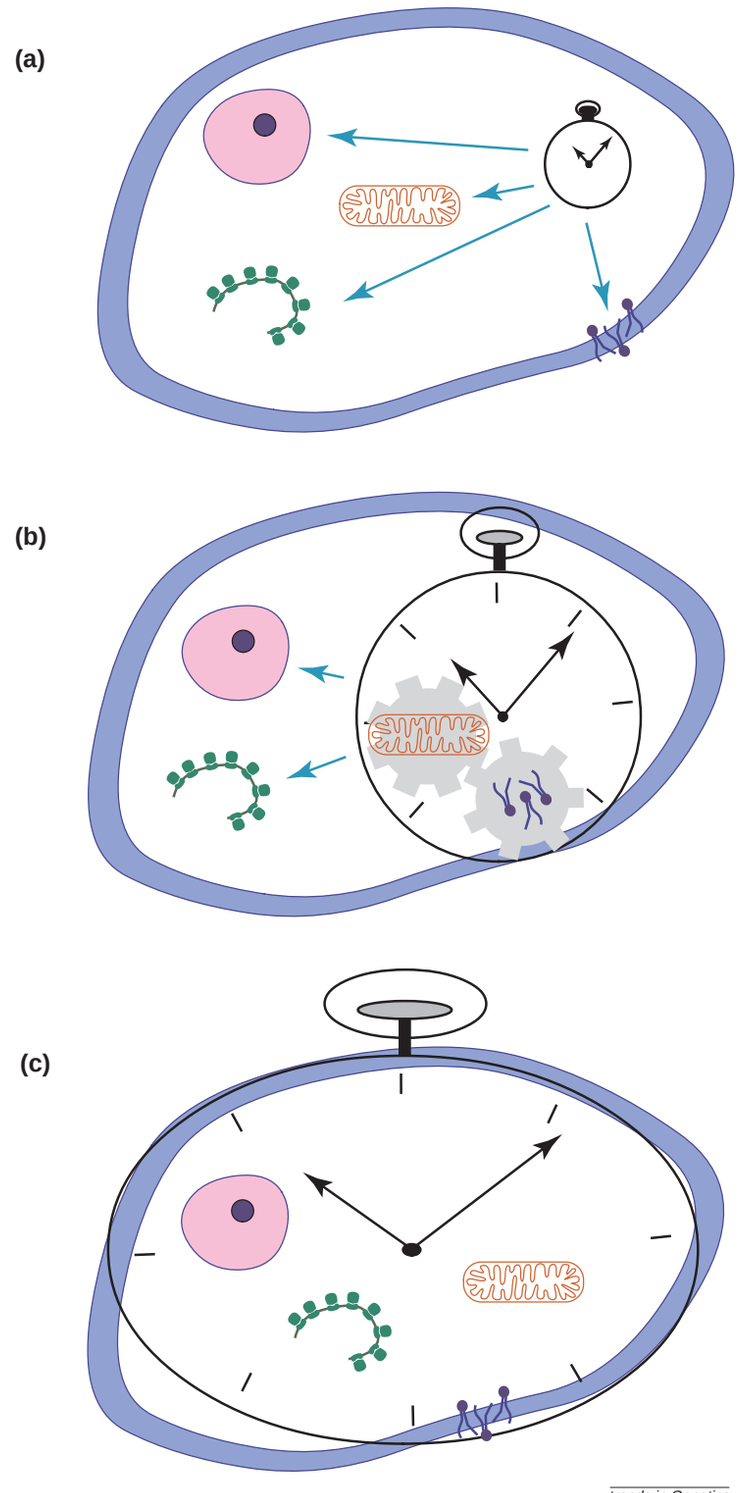
How to look for a central oscillator

All of the canonical clock genes are semi-dominant and are dispensable for growth and viability in homozygotes, and the design of the mutant hunts explains why this is so: only fully viable mutants were chosen for study, and only mutations with semi-dominant effects could be detected in the screens in diploid organisms. Therefore, the current set of canonical clock genes might represent only those clock-affecting genes that are 'luxury' genes, in particular, sensory input components that are dispensable in a controlled laboratory situation without impairing the organism's viability. If the core oscillator in a particular organism includes 'housekeeping' functions, it would be invisible to these screens.

Newer *Drosophila* screens have widened the field and can now detect mutations in genes that are fully viable in heterozygotes but impaired in homozygotes[22]. This approach has yielded the *double-time* (*dbt*) gene, which encodes what appears to be a protein kinase that is responsible for phosphorylating the *per* gene product. It is lethal at the pupal stage in homozygotes, so must have functions beyond its role in PER phosphorylation. This is an encouraging direction, but the search for new clock genes must be widened further, as fully recessive mutations would still not be picked up in the *Drosophila* screens.

Another approach to the problem of finding new classes of mutants is to search for temperature-sensitive mutations that display altered clock phenotypes only at restrictive temperatures. This approach has recently been successful in *Neurospora* and has produced two temperature-sensitive clock mutants so far [*period-6* (*prd-6*) and *rhythm-1* (*rhy-1*), see Table 2][23,24]. If the oscillators at the core of circadian systems are built out of housekeeping genes, then perhaps we should look to the lower organisms where biochemical genetics is well established if we want to work out the details of an oscillator mechanism. The non-canonical clock genes in *Neurospora* (Table 2) might provide a rich field for further investigation.

FIGURE 2. A tale of three clocks



Three models for the relationship between a circadian clock and a cell. A eukaryotic cell is shown with nucleus and representative metabolic functions: protein synthesis, mitochondria and lipid metabolism. Arrows represent clock output controlling cellular metabolism. (a) The clock is made out of special 'clock proteins' with no function other than time-keeping, like a disposable pocket-watch. Therefore, clock gene products can be destroyed by mutation, with no effect on the growth and viability of the organism. (b) The clock mechanism is built out of a few housekeeping functions, such as lipid metabolism or energy metabolism. Therefore, clock mutations affecting the central mechanism will have pleiotropic effects on growth and viability. (c) The entire cell is the clock mechanism, and all cellular functions contribute to maintaining rhythmicity and time-keeping. This 'holistic' approach might be correct, but would make it impossible to analyse the oscillator mechanism.

Conclusions

Although identifying new clock-affecting genes will be important in developing our understanding of how circadian clocks work, the next stage of this research has barely begun. Before we can really understand how rhythmicity is generated in a particular circadian system, we need not just qualitative identifications of components but quantitative information about the kinetics of the reactions in which those gene products participate and about the dynamic interactions between the components. To answer the questions raised at the end of Box 3, we will need a complete, quantitative description of the contribution to rhythmicity and input–output made by each clock-affecting gene product in a particular species. When we have this data, we might find that the contribution and relative importance of a component changes depending on the tissue, or the developmental or nutritional status of the organism. In this utopian future, the hard edges around the black boxes in Box 3 will dissolve and it will no longer make sense to debate whether or not a certain gene product is a component of a core oscillator or an input or output pathway, or whether it is a 'state variable' or a 'parameter'. A truly satisfying answer to our question 'how does a fungus tell time?' will come not from genetics, but from quantitative biochemistry, aided by mathematical modelling.

Acknowledgements

My apologies to those authors whose work has not been cited due to space limitations. Many thanks to S. Brody, M. Merrow, T. Roenneberg and A. Sehgal for permission to cite data in advance of publication, and to S. Brody, M. Hastings, M. Merrow, A.T. Winfree, and two anonymous reviewers for insightful comments and improvements to the manuscript.

References

1 Reppert, S.M. (1998) A clockwork explosion! *Neuron* 21, 1–4
2 Dunlap, J.C. (1998) An end in the beginning. *Science* 280, 1548–1549
3 Edmunds, L.N. (1988) *Cellular and Molecular Bases of Biological Clocks*, Springer-Verlag
4 Zeng, H. *et al.* (1994) Constitutive overexpression of the *Drosophila period* protein inhibits *period* mRNA cycling. *EMBO J.* 13, 3590–3598
5 Curtin, K.D. *et al.* (1995) Temporally regulated nuclear entry of the *Drosophila period* protein contributes to the circadian clock. *Neuron* 14, 365–372
6 Dunlap, J.C. (1999) Molecular bases for circadian clocks. *Cell* 96, 271–290
7 So, W.V. and Rosbash, M. (1997) Post-transcriptional regulation contributes to *Drosophila* clock gene mRNA cycling. *EMBO J.* 16, 7146–7155
8 Hall, J.C. (1996) Are cycling gene products as internal zeitgebers no longer the zeitgeist of chronobiology? *Neuron* 17, 799–802
9 Staiger, D. and Apel, K. (1999) Circadian clock-regulated expression of an RNA-binding protein in *Arabidopsis*: characterization of a minimal promoter element. *Mol. Gen. Genet.* 261, 811–819
10 Luo, C. *et al.* (1998) Nuclear localization is required for function of the essential clock protein FRQ. *EMBO J.* 17, 1228–1235
11 Liu, Y. *et al.* (1998) How temperature changes reset a circadian oscillator. *Science* 281, 825–829
12 Loros, J.J. and Feldman, J.F. (1986) Loss of temperature compensation of circadian period length in the *frq-9* mutant of *Neurospora crassa*. *J. Biol. Rhythms* 1, 187–198
13 Aronson, B.D. *et al.* (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
14 Merrow, M. *et al.* (1999) Assignment of circadian function for the *Neurospora* clock gene *frequency*. *Nature* 399, 584–586
15 Ballario, P. and Macino, G. (1997) White collar proteins: PASsing the light signal in *Neurospora crassa*. *Trends Microbiol.* 5, 458–462
16 Lakin-Thomas, P.L. 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
17 Roenneberg, T. and Merrow, M. (1998) Molecular circadian oscillators: an alternative hypothesis. *J. Biol. Rhythms* 13, 167–179
18 Taylor, B.L. and Zhulin, I.B. (1999) PAS domains: internal sensors of oxygen, redox potential and light. *Microbiol. Mol. Biol. Rev.* 63, 479–507
19 Crosthwaite, S.K. *et al.* (1997) *Neurospora wc-1* and *wc-2*: transcription, photoresponses, and the origins of circadian rhythmicity. *Science* 276, 763–769
20 Hastings, J.W. *et al.* (1976) Group Report 1: Basic Features. In *The Molecular Basis of Circadian Rhythms* (Hastings, J.W. and Schweiger, H-G., eds), pp. 49–62, Dahlem Konferenzen Life Science Research Reports
21 Brody, S. and Martins, S. (1973) Effects of morphological and auxotrophic mutations on the circadian rhythm of *Neurospora crassa*. *Genetics* (Suppl.) 74, s31
22 Price, J.L. *et al.* (1998) *double-time* is a novel *Drosophila* clock gene that regulates PERIOD protein accumulation. *Cell* 94, 83–95
23 Morgan, L.W. and Feldman, J.F. (1997) Isolation and characterization of a temperature-sensitive circadian clock mutant of *Neurospora crassa*. *Genetics* 146, 525–530
24 Chang, B. and Nakashima, H. (1998) Isolation of temperature-sensitive rhythm mutant in *Neurospora crassa*. *Genes Genet. Sys.* 73, 71–73
25 Dunlap, J.C. (1993) Genetic analysis of circadian clocks. *Annu. Rev. Physiol.* 55, 683–728
26 Millar, A.J. (1999) Biological clocks in *Arabidopsis thaliana*. *New Phytol.* 141, 175–197
27 Mrosovsky, N. (1996) Locomoter activity and non-photic influences on circadian clocks. *Biol. Rev.* 71, 343–372
28 Roenneberg, T. and Merrow, M. (1999) Circadian systems and metabolism. *J. Biol. Rhythms* 14, 449–459
29 Young, M.W. (1998) The molecular control of circadian behavioral rhythms and their entrainment in *Drosophila*. *Annu. Rev. Biochem.* 67, 135–152
30 Allada, R. *et al.* (1998) A mutant *Drosophila* homolog of mammalian *Clock* disrupts circadian rhythms and transcription of *period* and *timeless*. *Cell* 93, 791–804
31 Rutila, J.E. *et al.* (1998) CYCLE is a second bHLH-PAS clock protein essential for circadian rhythmicity and transcription of *Drosophila period* and *timeless*. *Cell* 93, 805–814
32 Lucas, R.J. and Foster, R.G. (1999) Circadian rhythms: Something to *cry* about? *Curr. Biol.* 9, 214–217
33 Akiyama, M. *et al.* (1999) Inhibition of light- or glutamate-induced *mPer1* expression represses the phase shifts into the mouse circadian locomoter and suprachiasmatic firing rhythms. *J. Neurosci.* 19, 1115–1121
34 Zheng, B. *et al.* (1999) The *mPer2* gene encodes a functional component of the mammalian circadian clock. *Nature* 400, 169–173
35 Shearman, L.P. and Weaver, D.R. (1999) Photic induction of *period* gene expression is reduced in *clock* mutant mice. *NeuroReport* 10, 613–618
36 Kume, K. *et al.* (1999) mCRY1 and mCRY2 are essential components of the negative limb of the circadian clock feedback loop. *Cell* 98, 193–205
37 Ishiura, M. *et al.* (1998) Expression of a gene cluster *kaiABC* as a circadian feedback process in cyanobacteria. *Science* 281, 1519–1523
38 Oklejewicz, M. *et al.* (1997) Metabolic rate changes proportionally to circadian frequency in *tau* mutant Syrian hamsters. *J. Biol. Rhythms* 12, 413–422
39 Shen, H. *et al.* (1997) Role of neural cell adhesion molecule and polysialic acid in mouse circadian clock function. *J. Neurosci.* 17, 5221–5229
40 Mattern, D.L. *et al.* (1982) Circadian rhythms in *Neurospora crassa*: a mutation affecting temperature compensation. *Proc. Natl. Acad. Sci. U. S. A.* 79, 825–829
41 Lakin-Thomas, P.L. *et al.* (1990) Circadian rhythms in *Neurospora crassa*: biochemistry and genetics. *Crit. Rev. Microbiol.* 17, 365–416
42 Lakin-Thomas, P.L. (1996) Effects of choline depletion on the circadian rhythm in *Neurospora crassa*. *Biol. Rhythm Res.* 27, 12–30
43 Lakin-Thomas, P.L. (1998) Choline depletion, *frq* mutations, and temperature compensation of the circadian rhythm in *Neurospora crassa*. *J. Biol. Rhythms* 13, 268–277
44 Brody, S. (1992) Circadian rhythms in *Neurospora crassa*: the role of mitochondria. *Chronobiol. Intl.* 9, 222–230