Circadian rhythms: **PASsing time**
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Links are being discovered between the circadian clock mechanisms in different species. The *Neurospora* Frequency protein has a rhythm of abundance and phosphorylation similar to that of the *Drosophila* Period protein, and *Neurospora* and mouse clock components, like Period, have 'PAS' domains.

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The anniversary of the Greenwich meridian and the focus on chronometers in human navigation may leave biologists a little smug. Birds and bees had solved the time-keeping problem in navigation long ago, probably by adapting an existing, biological clock. These daily timers are present in all eukaryotes and in some prokaryotes. Their ubiquity has driven clock research in diverse organisms, and the diversity has recently paid off. New territory has been charted in the fungus *Neurospora*: one protein component of the clock is now accessible to investigation, and a second component has been genetically identified. An advance landing party has arrived at the molecular level in the mouse, with the cloning of the first gene, *Clock*, found to be involved in the mammalian clock. Despite the different starting points, these camps have come in sight of each other, and also of an earlier foray to the clock machinery of the fruit fly, *Drosophila*.

Cloning the *Clock* gene
Mice, humans and most other animals have an obvious cycle of activity and rest. The activity/rest rhythm persists for months in mice kept under constant darkness, with a period — the time between two peaks — of 23.4 hours. This circadian rhythm — from '*circa dies*', about a day — is no artefact of the Earth's rotation. The biological clock is in control. The biochemical mechanism of such clocks, however, has been an outstanding problem since they were described in plant leaf movements in the 18th century. Clock mutants have provided some of the most exciting insights in the 24 years since the identification of *period* (*per*) mutants in *Drosophila* and *frequency* (*frq*) mutant lines in *Neurospora* [1]. The nightly spinning of running wheels in the cages of several hundred mutant mice announced, in 1994, the first mouse screen for mutants with aberrant activity rhythms. The fruit of this screen was the *Clock* mouse, a long-period mutant that can

become arrhythmic in darkness [1]. *Clock* is clearly required for normal circadian rhythms — could this gene encode a gear of the mouse clockwork?

The molecular cloning of *Clock*, a critical step towards answering this question, has now been accomplished [2,3], a magnificent achievement of the Takahashi laboratory. Their map-based cloning approach first located the *Clock* gene to a 400 kilobase (kb) region of chromosome 5, cloned in yeast (YAC) and bacterial (BAC) artificial chromosome contigs. Shotgun sequencing revealed that *Clock* mutant mice carry a single base substitution at an intron splice site, which leads to mis-splicing of the RNA transcript [2]. Most compelling of all, the full sequence of the wild-type gene, in a 140 kb BAC, rescues the long-period phenotype of transgenic *Clock* mice, whereas chromosomal fragments that only partially overlap the gene do not [3].

The Clock protein sequence shows similarities to transcription factors, with a basic helix–loop–helix DNA-binding domain and a glutamine-rich transcription activation region [2]. This is known territory, but would not mark the Clock protein as a gear — it could fulfil other essential functions, either sustaining the clock mechanism or regulating the period of the clock, without participating in it directly. The most tantalising clue comes from two regions of homology between Clock and the 'PAS' protein–protein interaction domain. One of the prototypical PAS proteins is the protein encoded by none other than *Period* (*Per*), the *Drosophila* clock gene. The PAS domain in the Period protein interacts with Timeless, the product of a second clock gene: this interaction is a central feature of the fly's clockwork [1]. Clock gears have to turn as well as intermesh, however, and we do not yet know if the activity of Clock is rhythmically regulated. If it is, *Clock* may be the Plymouth Rock for a molecular exploration of the clock mechanism in mammals.

Frequency protein
The circadian clockwork, however constructed, must involve some unconventional biochemistry. For one thing, how can a series of biochemical reactions be stretched over a period as long as 24 hours? The system must incorporate some lengthy time delays. And how can the mechanism be insensitive to temperature over the physiological range? Any useful clock must be temperature-compensated, as are all circadian rhythms. The first studies of the Frequency protein (Frq) point to answers in *Neurospora*.

The *Neurospora frq* gene, like the mouse *Clock* gene, was identified by mutation. The *frq* sequence did not hint at

any biochemical function, but elegant studies by the Dunlap and Loros laboratories have shown that *frq* does participate directly in the fungal clockwork [1]. Thus, *frq* RNA accumulates in a circadian rhythm that peaks in the early morning (Figure 1), with a period that matches the period of the visible rhythm in sporulation. Moreover, the rhythmic expression of *frq* is actually required for the visible rhythm, and constant expression is no substitute. The *frq* gene exhibits the negative feedback required for any self-sustaining oscillation: *frq* overexpression represses transcription from the *frq* promoter.

The rhythm of *frq* RNA suggested that the Frq protein would also have a circadian rhythm, and this turns out to be true [4]. There is a 4 hour delay between the peak of *frq* RNA and the Frq protein peak. The complement of Frq proteins, however, is complex. The two main forms that are initially found on western blots give way to small groups of bands, reflecting the progressive phosphorylation of Frq during the late circadian day and early night [4]. The phosphorylation is reminiscent of similar modifications of Per protein, and may be involved in regulating Frq degradation.

The dynamics of the Frq feedback loop have now been directly measured, following the induced expression of *frq* [5]. Down-regulation of endogenous *frq* RNA occurs rapidly, within 3–6 hours, but *frq* expression takes a surprising 14–18 hours to recover from repression. Frq phosphorylation presumably contributes to the second, slow process. Together, these two intervals could account for the full 22 hour period of *Neurospora* [5]. In other words, the levels of *frq* RNA and Frq protein (in its multiple states) alone may be sufficient to specify any phase in the *Neurospora* clock (Figure 1).
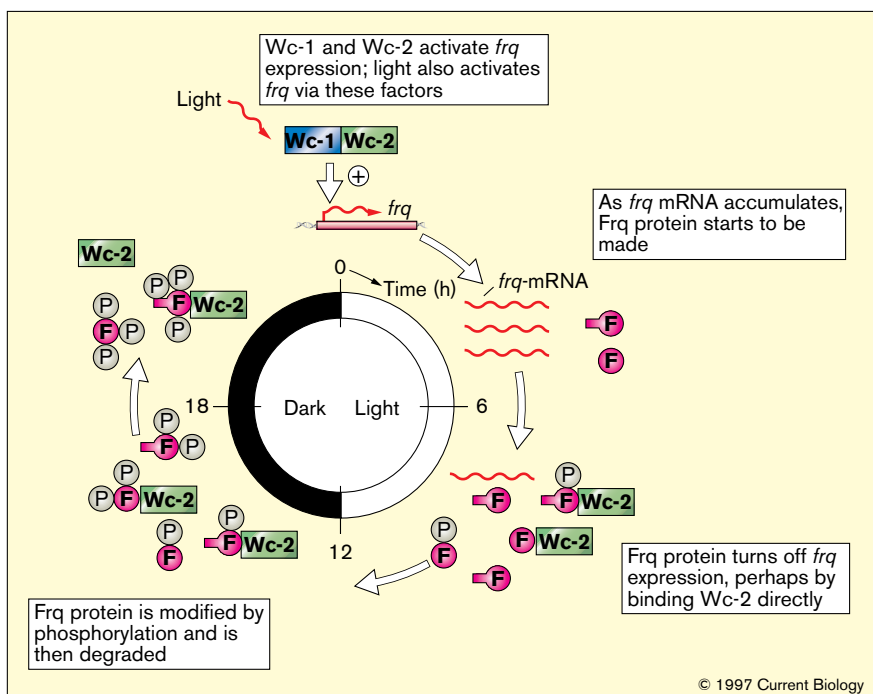
Part of the complexity of Frq proteins is due to translational control. The Frq proteins are synthesised from two ATG codons, 99 amino acids apart [6]. Both forms of Frq are present in the wild-type fungus, but mutants that lack one or the other ATG indicate that the Frq proteins are qualitatively different. The long Frq is sufficient to maintain rhythms at high temperature (30°C) but not at low temperature (18°C). The reverse is true of the short form. The wild-type fungus expresses more of the long Frq protein, compared to the short form, at high temperature, presumably compensating for the temperature-specificity of the Frq proteins [6]. The total amount of Frq also increases with temperature, strongly suggesting that the fungal clock requires substantial, dynamic changes in its components to operate over its full temperature range, in contrast to current models for *Drosophila* [1].

White collar proteins

The *frq*/Frq cycle includes negative feedback on *frq* expression. A self-sustaining oscillator, such as the circadian clock, also requires a positive input, in order to avoid damping out under the negative regulation alone. Even this intricate oscillator would not make a useful clock, though, for clocks must be synchronised with the local day/night cycle. Light performs this function in

Figure 1

A model of the *Neurospora* circadian clock. The *frq* gene is transcribed near subjective dawn (time 0), and *frq* RNA is translated to yield two forms of Frq protein (F). The Frq proteins rapidly repress *frq* expression, possibly through effects on the Wc-2 transcriptional activator (the direct binding depicted here is speculative). A prolonged phase of post-translational modification (including phosphorylation) follows, before Frq is degraded and transcription can increase once more. Light can activate *frq* expression independently, through Wc-1 and Wc-2.

Wc-1 and Wc-2 activate *frq* expression; light also activates *frq* via these factors

Light

As *frq* mRNA accumulates, Frq protein starts to be made

Frq protein turns off *frq* expression, perhaps by binding Wc-2 directly

Frq protein is modified by phosphorylation and is then degraded

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Neurospora by rapidly inducing *frq* expression [1]. The mechanism of *frq* induction is unknown, but saturating screens for 'blind' mutants that fail to respond to light have identified mutations in only two genes, *white collar 1* (*wc-1*) and *wc-2*, which are likely to encode components of the light-response machinery.

Sporulation is arrhythmic in the *wc* mutants, but this arrhythmia is not due to a lack of synchronisation, as was expected — *frq* expression is almost undetectable in the *wc* mutants [7]. The *wc* products activate *frq* expression, providing the necessary positive input to the *frq*/Frq cycle and transducing the light signal for resetting the clock. The clock in *wc-1* mutants lacks only Frq; priming the system with *frq* produces at least two subsequent cycles of *frq* expression. Priming with *frq* does not, however, initiate any cycling in *wc-2* mutants, indicating that more than Frq is required for rhythmicity in this strain. Most simply, the *wc-2* product may also be a central part of the clock mechanism (Figure 1), the positive factor that is antagonised daily by Frq [7].

The Macino laboratory has recently reported that the *wc* genes encode zinc-finger DNA-binding proteins, consistent with their role in activating gene expression [8,9]. Startlingly, both proteins also contain a PAS domain, which now links the circadian clocks of mice, flies and fungi. This landmark in the molecular map of circadian clocks cannot be followed blindly, for many proteins are known to contain PAS domains. Behind the PAS landmark, however, we may have found completely new territory — the list of PAS proteins includes both plant and bacterial photoreceptors (phytochromes and the photoactive yellow protein) [7,8]. Has an ancestral connection between photoreceptors and rhythms been retained, in the close dependence of present-day circadian clocks on light signalling? Clock researchers will be scanning the horizon for the return of this landmark, especially in the clock genes of plants and bacteria.

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