ABSTRACT

One of the fundamental properties of cells is temporal regulation by the circadian clock. Molecular clock components have been identified in animals, plants, fungi and prokaryotes, revealing transcriptional negative feedback loops. These components must necessarily be tightly linked with input pathways (e.g., transducing light signals) to communicate information about environmental time-of-day. *Neurospora crassa* is a classic model system for research on the circadian clock and its molecular mechanisms. Practically all of the work on the *Neurospora* clock utilized a mutant (*bd*) which was discovered for its clear daily growth patterns. The location of *bd* mutant has recently been identified by Belden et al. to lie in the *ras-1* gene. Because the mutation is also known to modify light regulated gene expression, it could potentially alter the circadian phenotype. Belden et al. determined that it has no discernable effect on circadian oscillations in constant darkness – despite decreasing growth rate and increasing sporulation.
Circadian clocks evolved such that they regulate cellular metabolism to function optimally within the day and to anticipate daily changes in environmental qualities (e.g., light, temperatures or nutrients). A post hoc analytical study recently showed the scale of this regulation in proposing that the expression of practically all genes is modulated by the circadian clock in eukaryotic cells (Ptitsyn et al. 2007), as had already been shown for the prokaryote, *Synechococcus* (Liu et al. 1995). Whereas an intact clock confers an adaptive advantage (Yan et al. 1998), defective clocks may lead, for example, to increased incidences of certain cancers (Fu et al. 2002). Living against the clock, by experimental induction of chronic jetlag in mice (Davidson et al. 2006) or in humans, when social schedules are incompatible with individual circadian time (Wittmann et al. 2006), may challenge longevity and health. Chronobiologists are, thus, racing to understand how the circadian clock – a fundamental characteristic of all cells - is working on the molecular level and how it is regulating metabolism.

The *Neurospora* circadian clock

Research into circadian mechanisms – like in other fields – utilizes preferred model systems for both historical and methodological reasons. Mammals have long been represented by hamsters, due to their remarkably precise wheel-running rhythms (Davis 1980). In the genetic era of clock research, mice have won the competition over hamsters (Vitaterna et al. 1994). As with other genetic and behavioral breakthroughs, extraordinary insights concerning clocks have come from *Drosophila* (Konopka and Benzer 1971; Hardin et al. 1990). Circadian rhythms in plants were initially studied in many different species, ranging from beans (Bünning and Moser 1973) to Madagascan shrubs (Engelmann et al. 1961), but are now predominantly investigated in *Arabidopsis* (Park et al. 1999). Much of our early knowledge about cellular clocks stems from studying photosynthesizing unicellular algae, such as *Gonyaulax* (Hastings and Sweeney 1959), *Euglena* (Bruce and Pittendrigh 1958), or *Chlamydomonas* (Bruce 1972) but the eukaryotic unicells have now been overshadowed by the remarkable work on the prokaryote *Synechococcus* (Kondo et al. 1994; Nakajima et al. 2005).

The fungus *Neurospora crassa* was introduced to circadian research because of its conspicuous daily growth patterns (Sargent et al. 1956; Pittendrigh et al. 1959). *Neurospora* has many requisite advantages for an ideal model system, including rapid growth and a small, haploid genome, tractability for genetic studies and it is completely safe to work with (non-pathogenic and no teeth). It has no brain to confuse pacemaker and organ-specific clocks, and defies the confusion of sex and development; although it technically does develop and can
have sex, the former results in a terminal tissue left behind a leading growth front, and the latter is a relatively rare condition. This fungus is an excellent system combining genetic and biochemical approaches, and new tools are continuously being developed (a recent functional genomics project aims to make knockouts of every annotated ORF, a task that will promote *Neurospora* to the upper echelon of model systems beyond circadian research (www.dartmouth.edu/~neurosporagenome/)).

*Neurospora* has been used to establish many of the principles of molecular mechanisms of cellular clocks (Lakin-Thomas et al. 1990; Lakin-Thomas and Brody 2004; Brunner and Schafmeier 2006). Its circadian clock is measured by following outputs including the production of asexual spores (the conspicuous daily growth patterns) or gene expression, protein levels or enzyme activity. As in all organisms – from *Synechococcus* to humans, *Neurospora*’s circadian system and its outputs are synchronized (entrained) to precisely 24 h by environmental cycles (e.g., light or temperature). The clock was shaped through evolution by light-dark cycles, which are the most reliable environmental signal for synchronizing daily rhythms. It is the clock – in combination with environmental signals – that makes (most of) us diurnal and most rodents nocturnal. Although entrainment is the natural state of circadian clocks, they are often investigated experimentally using the common, yet remarkable phenotype of continuing their rhythmicity in constant conditions (with no decrease in amplitude). These free-running rhythms often deviate from 24 h. In the case of *Neurospora*, rhythms such as spore production occur once per 22 h in constant darkness.

**Molecular clock mechanisms – from the slant of Neurospora**

A transcriptional negative feedback loop is responsible for a daily increase and decrease in clock regulated gene expression. The components of this network have been determined with the toolbox of the modern geneticist, showing the products of the *frequency* (*frq*) and the *white collar* genes (*wc-1* and *wc-2*) serving repression and activating roles, respectively (Dunlap and Loros 2004). Notably, in addition to it’s role as a transcription factor, WC-1 is a blue light photoreceptor (He et al. 2002). Thus, both light and clock information appear to go via WC-1 on their way to gene regulation. To complicate matters further, the negative element, FRQ, is also necessary for the clock to “see” light (Merrow et al. 1999) and, in addition, maintains levels of its own activator, WC-1. Such complications are typical of clock and similar networks and other circadian systems have similarly constructed feedback loops, but animals, plants, fungi and prokaryotes possess unique sets of clock genes (Young and Kay 2001).
Another characteristic of most circadian systems is that they appear to be constructed from multiple oscillators – even those in simple unicells (Roenneberg and Morse 1993). This network quality was established for Neurospora in several ways. (i) Residual oscillations in the circadian range – as well as with longer periods – can be recorded in clock gene mutants (Loros and Feldman 1986; Lakin-Thomas and Brody 2000; Dragovic et al. 2002). (ii) Mutants of characterized clock genes (e.g., frq) can still be entrained (e.g., to temperature cycles) with circadian characteristics (Roenneberg et al. 2005). (iii) Circadian oscillations of isolated RNAs can be measured in apparently arrhythmic clock mutants (Correa et al. 2003).

The \textit{bandit} mutation

\textit{Neurospora} is an ideal 'simple' system for studying the complex genetic trait of circadian rhythmicity. The molecular mechanisms responsible for circadian rhythm generation have – at least in part – been worked out, and we have a glimpse of how multiple oscillators come together to form a molecular clock-network. Yet, a shadow of doubt hangs over the system because virtually all circadian experiments have been performed in a mutant background, called \textit{band} (\textit{bd}, referring to the enhanced banding in spore formation relative to the wild type strain(s); Fig. 8.1). Although model systems for genetic research are often stunted versions of their wild type cousins, the \textit{bd} mutation has been a particular concern because it has major effects on the cells' biology, leading – in addition to enhanced sporial banding – to substantial decreases in growth rate (Sargent et al. 1966). Furthermore, the \textit{bd} mutation enhances transcription of some genes in response to light (Arpaia et al. 1993). This was especially important due to the inseparable roles of clock and light input in \textit{Neurospora}. In order to lift the shadow, the gene responsible for the \textit{bd} mutation had to be identified and the mechanisms underlying its effects on banding and light expression elucidated.

The post-genomic era has now facilitated identification of the \textit{bd} mutation, showing that it lies in the \textit{ras-1} gene (Belden et al. 2007). The RAS protein is well characterized in other contexts and thus allows for experimentation to determine how \textit{bd} (now \textit{ras-1bd}) effects \textit{Neurospora}'s circadian system. RAS was first identified as an oncogene (it was identified in viral isolates from rat sarcoma tumors) (Diaz-Flores and Shannon 2007). Mutations in RAS are responsible for a daunting variety of cancers, making it an attractive target for developing anticancer therapies. RAS is bound to the cell membrane and catalyzes the conversion of GTP to GDP – it is a G protein. GTPbinding converts the protein to its active state which signals to downstream targets via hydrolysis to GDP. GTP
binding is effected by a family of facilitating proteins, suggesting a network of regulatory mechanisms. In theory, any one of these components (the G protein, the facilitators or the GTP, the acceptor of the signal) is regulated independently, thus lending specificity – also in the temporal domain – to a potent signal transduction mechanism. The location of RAS, in the cell membrane, suggests their role in sensing the extra-cellular environment (Neurospora tissue is a syncitium readily allowing inter-cellular signaling). Circadian clocks can be synchronized to regular changes of temperature (Merrow et al. 1999; Brown et al. 2002) or nutrition (Roenneberg and Rehman 1996), using these cues to reliably predict the time of the environmental day.

RAS-1 is constitutively expressed in Neurospora under constant conditions, both in wildtype and in the ras-1bd mutant (Belden et al. 2007), unlike so many other cellular components (e.g., (Correa et al. 2003)). Although this is a first crucial test, RAS-1 could still have circadian impact. Several key clock components, including WC-2 and casein kinase Ia, are expressed at constant levels (Crosthwaite et al. 1997; Görl et al. 2001). Alternatively, RAS-1 could use a clock regulated facilitator protein and become temporally regulated.

In another approach, the ras-1bd mutation was phenocopied using reducing agents and genetics showing that these manipulations also do not change the free running period of spore formation. Thus, concerning free running rhythmicity, there seems to be little if any effect on circadian timing. Despite this, when molecular components of the clock pathway were measured, most components (except for ras-1 itself) were found at unusual levels (see Table 8.1). For

Figure 8.1 Daily spore formation by the bd mutant (top) and 3 wild type strains, 74- OR23-1A, FGSC #8802 and FGSC #8860. We have adapted the race tube assay (omitting glucose from the media) for improved banding of wild type isolates. These strains were chosen to demonstrate the variety of circadian phenotypes that are recovered from natural isolates. One of them is adequate, one shows no regular banding (evaluated either by eye or digitally) and one of these strains (8860) looks as though it bands more robustly than the bd strain. Their growth rate relative to bd is indicated on the right. 74-OR23 was collected in the U.S., and 8802 and 8860 are from India. (Thanks to D. Jacobson for supplying the Indian strains.)
RNA (VVD is a light signaling modifier as well as yet another photoreceptor), levels are lower and later in the ras-1bd mutant. The RNAs of the clock genes frq and wc-1, and the output gene fluffy, all show higher levels. In light of the apparently normal free-running rhythms in darkness, there seems to be compensation on the level of RNA regulation within the circadian network. The results also may point to our ignorance on what role RNA regulation plays in circadian rhythm generation (Yang and Sehgal 2001).

Entrainment – the key property of the circadian clock

RAS-1bd apparently has no effects on free-running rhythms in Neurospora. But the circadian clock rarely, if ever, gets a chance to display its capacity to run free in nature – it is normally entrained which makes investigation of whether the mutation affects entrainment crucial, especially since this mutant leads to exceptionally high levels of light-induced gene expression (Arpaia et al. 1993). Although entrainment per se was not investigated by Belden et al., they did investigate how the clock and the expression of its output genes respond to light. The results produced puzzles which still remain to be solved. As in darkness, wc-1 RNA is expressed at elevated levels in the ras-1bd mutant compared to wild type in response to light, whereas the WC-1 protein is decreased. Given the normal circadian rhythmicity in constant darkness – despite altered clock gene RNA expression (see above) – these results clearly demonstrate that RNA levels are not a good gauge for function. But, even regulation of the protein does not tell

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Table 8.1 RNA and protein levels in the ras-1bd strain, relative to wild type. See Belden et al for methodological details; upward and downward arrows indicate increases and decreases, respectively, observed in the mutant strain relative to the wild type. Lack of rhythmicity is shown as a straight line, whereas circadian rhythms are indicated by the squiggle.

vvd RNA (VVD is a light signaling modifier as well as yet another photoreceptor), levels are lower and later in the ras-1bd mutant. The RNAs of the clock genes frq and wc-1, and the output gene fluffy, all show higher levels. In light of the apparently normal free-running rhythms in darkness, there seems to be compensation on the level of RNA regulation within the circadian network. The results also may point to our ignorance on what role RNA regulation plays in circadian rhythm generation (Yang and Sehgal 2001).
the full story, shifting the focus to post-translational modification as a critical factor in the circadian clock: as for many other transcription factors, WC-1 activity depends on phosphorylation state and sub-cellular localization (Schafmeier et al. 2006). Its de-phosphorylated nuclear form is most effective as a transcriptional activator, so if this is unaffected in ras-1bd, then it could – quantitatively - maintain its job in circadian regulation of frq transcription, even as overall amounts have fallen.

The fact that reduction of a key component in the so-called core clock of Neurospora does not have an effect on precise timing shows that we have to start thinking more about entire networks of molecular loops. Indeed, when wc-1 is expressed constitutively from an inducible promoter, all three so-called central clock proteins – WC-1, WC-2 and FRQ - are expressed at abnormally high levels, yet the free running period is perfectly normal (Cheng et al. 2001). Apparently, there is also compensation for clock protein levels among the central clock components.

Although Belden et al. investigated light induced gene expression in ras-1bd versus wild type, a remaining conundrum is what the mutation means for entrainment. The process of entrainment also refers to differences between individuals. In a population there is typically a normal distribution of entrained phases (chronotypes), ranging from extreme early to extreme late types with all the rest of the individuals somewhere in between. So if the bd mutation caused a shift within this chronotype distribution, then it would be a clock mutant – even when the free running period was identical to wild type. Circadian clock theory (based on both oscillator theory and wet experiments) associates phase of entrainment (chronotype) with individual differences in free running period. By this convention, the ras-1bd mutant would be expected to have a phase like any wild type strain with the same free running period. There are exceptions to this rule, reflecting the nature of the molecular clock as complex networks (e.g., see (Spoelstra et al. 2004; Merrow et al. 2005) for discussion). Thus without more elaborate experimentation on the effects of the bd mutation on the important state of the circadian system, entrainment, the verdict of whether or not it is a clock gene is still open. But even if it turns out that this mutant affects chronotype, the shadow of chasing a vigorously banding ghost can be swept aside. The new results show that the story of the Neurospora clock does not have to be re-written because all that remains is whether we have gained our current knowledge about this fascinating model system from a Neurospora lark or a Neurospora owl.

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References


