A fungus among us: the *Neurospora crassa* circadian system

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Neurospora crassa is the only molecular genetic model system for circadian rhythms research in the fungi. Its strengths as a model organism lie in its relative simplicity—compared to photosynthesizing and vertebrate organisms, it is a stripped-down version of life. It forms syncytial hyphae, propagates and reproduces, and the circadian clock is manifest in numerous processes therein. As with other model circadian systems, *Neurospora* features a transcription/translation feedback loop that is fundamental to an intact circadian system. The molecular components of this loop converge with those of blue light photoreception, thus bringing the clock and one of its input pathways together.

Key words: circadian rhythm / frequency / *Neurospora crassa*

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A circadian system in a fungus

Poor Brandt! He was the first to observe irregularities in *Neurospora crassa* growth patterns¹ but it was the circadian pioneer, Colin Pittendrigh, who demonstrated that these development patterns are a manifestation of a circadian clock.² In doing so, he set the stage for development of a fungal model organism for molecular genetic studies on circadian rhythms.

The fungi are a richly diverse collection of sessile poikilotherms. They extract what they can from their immediate environment, and are subject to the extreme conditions of nature. Thus, there is an obvious selective advantage for possessing an endogenous

timing system with which to anticipate the regular, daily changes in temperature, humidity and light. Circadian rhythms are thought to arise from exactly this sort of timing system, one which essentially prepares an organism for the coming dawn. Basic characteristics of these rhythms include self-sustained rhythmicity in constant conditions, a period of about 24 h, stability of the period of the oscillation in different temperatures and nutritional conditions (temperature or nutritional compensation) and entrainment of the oscillation to the environment.³ None of these characteristic properties are completely understood at the molecular level in any model system. Indeed, it is not at all clear why a self-sustained rhythm is necessary, given that environmental signals (especially light) are dependable and that a rapidly damping oscillation would serve equally well.⁴

Investigations into rhythmicity in the fungi have most often utilized spore formation, a discrete developmental event which is easily measured. The earliest of these studies were performed with *Pilobolus*, which conveniently shoots its spores with great force (up to a full 2 m!).⁵ Inconveniently, however, *Pilobolus* prefers dung for satisfying its particular nutritional requirements. Although a substitute media was described, circadian rhythms research moved on to a less demanding fungus, the ascomycete *Neurospora crassa*. *N. crassa* is a filamentous fungus which prefers warm, moist climates. Collections from nature are most successful between the latitudes 30° north or 10° south, though there are other *Neurospora* species that are found over an even wider range.⁶ *Neurospora* grows well on defined media, and it became an ideal organism, historically, for numerous genetic endeavors. As a result, the genome is extensively mapped by classical means⁷ and this information was recently supplemented by US and German genome sequencing projects (<http://www-genome.wi.mit.edu/annotation/fungi/neurospora/>, <http://www.mips.biochem.mpg.de/proj/neurospora/>).

One of the most versatile tools for screening *N. crassa* mutants is the race tube, first described in 1943.⁸ The race tube is a glass tube into which solid

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media is introduced. Inoculation of the fungus at one end initiates its growth along the length of the tube, at an approximately linear rate. Thus, mutations which reduce fitness by hindering growth rate are easily identified by visual inspection.

Another feature of *Neurospora* that was utilized for early genetic studies is its abundant production of conidia or asexual spores (See Figure 1). *Neurospora* growth and development includes asexual propagation, as well as sexual reproduction. The propagation state is characterized by linear growth of vegetative hyphae and conidia formation. The accumulation of orange, carotenoid-rich conidia is impressive under many conditions and its alteration or absence is, like linear growth rate, an easy visual screen. Using race tubes to study conidia formation has been the key for circadian research in *Neurospora* [Figure 1(b)]. Specifically, growth from one end of a race tube to the other takes days or weeks and about once per 22 h in constant darkness the circadian system dictates the switch from vegetative growth to asexual development, resulting in discrete accumulations of conidia (bands). The period of band formation does not change significantly between 20°C and 30°C and, therefore, is called temperature compensated. Rhythmicity persists without damping in constant darkness, though not in constant light, and is readily entrained by blue light. Thus, *Neurospora* satisfies the criteria for experimental research on circadian rhythms.

Molecular dissection of the circadian system in *Neurospora*

A number of genes were identified in screens for clock mutants in *Neurospora*, one of which—*frequency* (*frq*)—was recovered multiple times.^{9,10} The *frq* mutants cover the range of phenotypes that one would expect of a defective central clock component: long and short free-running periods and arrhythmicity, loss of temperature and nutritional compensation and differences in entrainment properties to light.¹¹ Cloning and sequencing the gene gave little information as to how it achieves these feats¹² but a number of key experiments demonstrate some of its biochemical and genetic properties. FRQ protein moves between the nucleus and cytoplasm¹³ and is phosphorylated in a time-of-day specific manner.¹⁴ The phosphorylation regulates the half-life of the protein and also impacts the circadian period.¹⁵ Phosphorylation has been

Figure 1. (a) Asexual propagation by *Neurospora* involves elaboration of conidia on aerial hyphae. This micrograph shows formation of constrictions which segregate one or more nuclei into compartments. The final stage is complete separation of these units—the conidia—and their dispersal. Germination of conidia and growth of vegetative hyphae precede a subsequent round of conidial development (Reproduced with permission from Reference 60). (b) A race tube. The *bd A* strain (the generic wild type for circadian rhythms research) was inoculated at the left end. The filamentous fungus grows along the length of the race tube at an approximately linear rate. Areas of decreased pixel density are masses of aerial hyphae and conidia which accumulate about once per 22 h in constant darkness and punctuate the lawn of vegetative hyphae.

implicated in determining the activation state of many proteins, including transcription factors, in general, as well as the subcellular localization of proteins.^{16,17} Thus, it is possible that the multiple phosphorylation states of the FRQ protein could have numerous functions.

FRQ negatively regulates its RNA expression levels,¹⁸ and this feedback is believed to be the basis for circadian oscillations in RNA and protein levels that is characteristic for gene expression of many central clock components.^{14,19,20} Indeed, constitutive *frq* RNA expression in constant conditions results in loss of rhythmic conidiation.¹⁸ Although specific details between species differ, the *frq* regulatory (endogenous and exogenous) profile has features characteristic of a transcription/translation feedback loop, which is common to all circadian systems described genetically so far.

frq RNA was characterized for its behavior in response to light, a finding that should provide insights into entrainment of the circadian system by the environment. The RNA is induced rapidly by light, in a dose-dependent manner correlating with shifting the phase of the clock.²¹ *frq* can be light-

induced to equivalent levels at all times of day,^{21,22} consistent with the ability of light to induce large phase shifts at certain times of day (a type 0 phase response curve¹¹).

Light input meets the clock

The rapid light induction of *frq* RNA suggests that the gene is not far downstream of the light input pathway. How close is *frq* to the light input pathway? Experiments to address this question were facilitated by extensive studies from the past several decades that identified components—two single loci—which mediate the developmental and morphological responses of *Neurospora* to blue light. *Neurospora* is considered an ideal genetic system for the study of light signal transduction because there is a collection of distinct morphological outcomes following exposure to blue light, with no responses so far documented to red light. The *wc-1* and *wc-2* single mutants are impaired in almost all light-specific functions, showing a dark-grown phenotype in the presence of light.^{7,23–25} Sequence analysis, as well as DNA binding experiments, indicate that the WC proteins are zinc finger transcription factors.^{26,27} In addition, genetics and physiological studies suggest that WC-1 and WC-2 positively regulate the expression of the light-induced genes. The proteins also feature multifunctional PAS domains, which are protein-protein interaction domains that are commonly found both in proteins that sense environmental changes^{28,29} and in many clock-associated molecules.^{30,31} One of the WC-1 PAS domains was classified as a LOV (light oxygen voltage sensing) domain.³² The homologous region in the plant blue light photoreceptor NPH1 binds flavin mononucleotide, a chromophore that is likely to mediate light-dependent autophosphorylation.³³ According to classical photobiology studies, a flavin is also the predicted chromophore for the *Neurospora* photoreceptor.³⁴ Given that WC-1 and WC-2 are assembled in a white collar complex (WCC) in both the presence and absence of light,³⁵ it is possible that one of these proteins, or the complex itself, acts as a photoreceptor molecule.

Responses that are impaired in the *wc* mutants include the light induction of *frq*,³⁶ suggesting that light input to the clock is mediated by the WC proteins. Genetic and biochemical evidence indicates how this transduction pathway functions. WC-1 and WC-2 are necessary for activation of *frq*

Figure 2. Molecular components of the *N. crassa* circadian system. A transcription/translation feedback loop is formed by the *frq*, *wc-1* and *wc-2* gene products. These components are necessary for a self-sustained oscillation in constant darkness (DD) as well as synchronization of the circadian output, conidiation, by light. In our model, these could function as the 'A oscillator' described by Pittendrigh.³ Additional oscillatory machinery exists independently of the transcription/translation loop, alternately called the FLO (FRQ-less oscillator⁶¹) or the 'B oscillator'³ or the rhythm generator.⁴ The *vvd* gene product regulates light input pathways.⁴³ *cgs* refers to downstream genes (clock controlled genes) that are regulated by the clock and thereby do its work. Wavy line indicates rhythmic expression in DD; straight line is non-rhythmic; sun is light-induced. See text for abbreviations. Adapted from Trends in Genetics, vol. 17, Merrow & Roenneberg, "The circadian cycle: is the whole greater than the sum of its parts?", pp. 4–7, copyright 2001, with permission from Elsevier Science.

transcription and for self-sustained rhythmicity.³⁶ Given that *frq* is weakly expressed in *wc-1* and *wc-2* mutant strains, the WC proteins may function as the transcriptional activators necessary for the positive aspect of the transcription/translation feedback loop (see Figure 2). In this model, the WCC activates *frq* transcription in constant conditions until the FRQ protein accumulates and acts to repress its own synthesis, perhaps by interacting with the WCC, thus providing a mechanism for self-sustained rhythmicity. Support for this configuration comes from interdependent regulation of *frq* and *wc-1* by their proteins, and a circadian regulation of the WC-1 protein product.^{22,37,38} Indeed, *frq* expression is not simply downstream of the WCC, but is necessary for the accumulation of *wc-1* RNA²² and protein product.^{22,37} It was also shown that FRQ complexes with the WC proteins,^{22,39} with WC-2 mediating the interaction of WC-1 and FRQ in a multimeric complex.³⁹

The role of FRQ in the regulation of the light response is further clarified by the finding that the presence of FRQ *per se* (i.e. constitutively expressed),

and not a FRQ/*frq* negative feedback loop, permits regulation of conidiation by light.²² In the absence of FRQ all light-induced conidial banding is absent.^{40–42} Other light-induced physiologies persist without FRQ, although there is a deficit in overall levels.⁴² Finally, the strength of light responsiveness, measured both for gene expression and carotenoid induction, depends on the circadian time, suggesting that the light input pathway is modulated by the clock.^{22,43} This is apparently a common feature of circadian systems, with circadian regulation of light input in *Drosophila*,⁴⁴ in the marine unicell *Gonyaulax*,^{45,46} and in plants.⁴⁷ Light input is not simply a linear transduction of signal to an oscillator, but rather an active mechanism which is itself circadianly regulated.

The problem that we are left with is how to characterize the circadian system on the molecular level: what is input, what is rhythm generator? The data concerning FRQ allow various hypotheses, including FRQ as rhythm generator or FRQ as input component to a downstream rhythm generator.⁴⁸ In *Arabidopsis thaliana* numerous photoreceptor molecules are period mutants: they have aberrant free running periods in constant conditions.⁴⁹ Further, the photoreceptors are transcription factors.⁵⁰ But in this plant model system, there are several candidates for components of a rhythm generator that are independent of the light input pathways.^{20,51,52} To date a similar component remains unknown in *Neurospora*, but physiological experiments suggest that an additional circadian machinery exists independent of the transcription/translation loop ('A' oscillator, see below) portrayed in Figure 2.⁴²

Unique insights from *Neurospora*

The original description of the 'arrhythmic' *frq*⁹ strain⁵³ in 1986 reports that under special conditions its conidiation is rhythmic, with a free-running period in the circadian range (see Figure 3, redrawn from Reference 54), demonstrating that oscillations in this time range can occur without functional FRQ protein. This important observation, which suggests FRQ-independent oscillatory behavior, has only recently been investigated further. Experiments using mutants in lipid metabolism pathways were characterized for their periodicity and shown to have extremely long periods in constant conditions (typically 35 to 100 h),⁴⁰ with or without FRQ. The possibility that this rhythmicity is related to the circadian clock mechanism is suggested by the

Figure 3. Determination of free running period in *frq* mutants (reanalysed based on data from Reference 54). The individual periods of conidial band formation are plotted according to their distribution and fitted for a Gaussian distribution. The period of *frq*⁺ (full triangle) is 20.6 $\pm$ 1.1 h, $r = 0.99$; *frq*⁹ (open square), 21.2 $\pm$ 3.6 h, $r = 0.88$; *frq*¹⁰ (open circle), 20.4 h $\pm$ 3.22, $r = 0.96$. Although on average the periods of the three strains are indistinguishable, clearly in the absence of FRQ stability and precision are lost. Also, the rhythmicity in the null mutants is seen only under special conditions, and it is neither temperature nor nutritionally compensated.^{10,54}

titration of appropriate supplements into the race tube media, which results in a systematic titration of the period to the circadian range. When the media is adequately supplemented to a point where growth rate is normal, the free running period reflects that of the *frq* allele.

The second line of evidence for the existence of circadian machinery independent of the *frq* transcription/translation loop comes from entrainment experiments with temperature and light. In the absence of FRQ, entrainment of conidiation by light is not apparent,^{40–42} consistent with the close interaction of FRQ with dedicated light input pathway components. However, temperature cycles (alternating between 22 °C and 27 °C) will entrain conidiation in these clock mutants.⁴² That the underlying oscillatory machinery is only capable of a weak oscillation is indicated by the inability to achieve a 'frequency demultiplication', a phenomenon that occurs when entraining cycles are half of the free-running period and the oscillation is robust enough to consolidate to a period that is

double the length of the entraining cycle (note: the term frequency as used here has no connection to the *frq* gene in *Neurospora*). The strains that are capable of a free-running rhythmicity under 'standard' conditions, e.g. *frq*⁺, *frq*¹ or *frq*⁷, will frequency demultiply. That *frq* deficient strains do not demultiply may reflect an oscillator with a low amplitude or one that is not self-sustained under these conditions.

What does it mean when oscillatory features such as entrainment and free-running rhythmicity are observed in the absence of a central clock component such as FRQ? One explanation is that the oscillations are simply non-circadian, and in the intact system, one would never see them, and they need not be considered further with regards to the clock mechanism. This view has been taken⁵⁵ based on a lack of temperature and nutritional compensation in the rhythmicity of the *frq* null strains.^{10,54} An alternative view places the FRQ transcription/translation feedback loop upstream of the actual rhythm generator, with the clock gene exerting its effect on the circadian system in constant conditions as an endogenously regulated sensory input pathway.^{22,42,48,56}

Yet another possibility recalls classical experiments performed with *Drosophila pseudoobscura* and its eclosion rhythm. Protocols using either a temperature or a light pulse resulted in distinctly different patterns of phase shifting.³ The interpretation of these experiments was that the circadian system is composed of two oscillators. One (the A oscillator) is light-entrained and is generally the pacemaker, and the other (the B oscillator) can be entrained by temperature and is normally difficult to distinguish individually. If this model applies to *Neurospora*, why are there no known components of the B oscillator? None of the mutant screens which have been used to date have attempted to distinguish A versus B oscillators, so, by default, they are biased to reveal genes that encode the major components of the A oscillator. On the other hand, the very nature of the B oscillator is problematic to characterize on almost any level, given that, in the intact system, it is normally a slave to the A oscillator, and in the absence of A it is not very robust, so reluctant that it requires entraining cycles to get something going.

The A and B oscillator model is an elegantly parsimonious explanation for complex and confounding physiologies. The molecular genetic era has streamlined circadian systems further, maybe even too far: in the case of *Neurospora*, all circadian

properties are in some way regulated by a single gene. FRQ confers entrainment by light, temperature and nutritional compensation, free-running periodicity, etc. That it is central to an intact and robust system is without question, however, it could be but one part of a complex network of loops that have, for example, been proposed to represent cellular pathways.⁵⁷ In this scenario, the transcription/translation feedback loop would be but one of a series of loops, which together produce circadian physiology. Further, if FRQ is part of a light input pathway, this would not exclude the existence of additional feedback loops that modify the light response in other ways. Heintzen *et al.*⁴³ argue that such interconnected loops would be redundant, but modelling tells us that each could serve a distinct purpose, namely facilitating stable, temperature compensated circadian rhythmicity.^{4,58} Clearly, the circadian oscillation must be insulated from transient variations, and FRQ seems to fulfill this function: in the absence of FRQ a circadian free-running period is seen, but it is less precise (See Figure 3, redrawn from Reference 54). Perhaps the next step is to determine how FRQ integrates various signals from metabolism: indications are that cellular redox state fluctuates with onset of conidia formation in *Neurospora*.⁵⁹ Why couldn't a flavin molecule, tuned to pick up photons, just as well accept electrons from programmed (circadian) biochemical processes in the cell?

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