The *Neurospora* Circadian System

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Abstract The eukaryotic filamentous fungus *Neurospora crassa* has proven to be a durable and dependable model system for the analysis of the cellular and molecular bases of circadian rhythms. Pioneering genetic analyses identified clock genes, and beginning with the cloning of *frequency* (*frq*), work over the past 2 decades has revealed the molecular basis of a core circadian clock feedback loop that has illuminated our understanding of circadian oscillators in microbes, plants, and animals. In this transcription/translation–based feedback loop, a heterodimer of the White Collar–1 (WC-1) and WC-2 proteins acts both as the circadian photoreceptor and, in the dark, as a transcription factor that promotes the expression of the *frq* gene. FRQ dimerizes and feeds back to block the activity of its activators (making a negative feedback loop), as well as feeding forward to promote the synthesis of its activator, WC-1. Phosphorylation of FRQ by several kinases leads to its ubiquitination and turnover, releasing the WC-1/WC-2 dimer to reactivate *frq* expression and restart the circadian cycle. Light resetting of the clock can be understood through the rapid light induction of *frq* expression and temperature resetting through the influence of elevated temperatures in driving higher levels of FRQ. Several FRQ- and WC-independent, noncircadian FRQ-less oscillators (FLOs) have been described, each of which appears to regulate aspects of *Neurospora* growth or development. Overall, the FRQ/white collar complex feedback loop appears to coordinate the circadian system through its activity to regulate downstream-target clock-controlled genes, either directly or via regulation of driven FLOs.

Key words *frq*, *wc-1*, *wc-2*, FLO, circadian rhythm, evolutionary conservation, photoreceptor, feedback loop, light resetting, temperature responses

Neurospora crassa is a filamentous fungus, a model organism for the group of eukaryotes having the closest evolutionary relatedness to animals (Stechmann and Cavalier-Smith, 2003). *Neurospora*'s life cycle employs a handful of distinct cell types, but in its vegetative state, it grows as a syncytium, a growth habit more similar to that of muscle cells than that of fibroblasts. Because, unlike metazoans, there are not a lot of different cell types expressing rhythms at one time, or required to compose the whole adult organism, all the

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JOURNAL OF BIOLOGICAL RHYTHMS, Vol. 19 No. 5, October 2004 414-424
DOI: 10.1177/0748730404269116

power of genetics, genomics, and molecular biology can been focused on how the circadian system, from light and temperature input, to the core circadian oscillator, to various hierarchical slave oscillators and outputs, works at the level of the cell. The insights gained from this system have resulted from this ability to define and refine chronobiologically meaningful questions in a molecularly tractable way; in answering these questions, we have increased our insight into clocks in all organisms.

BIOLOGY AND GENETICS OF *NEUROSPORA* RHYTHMS

Fungi in general, including *Neurospora*, can express a variety of rhythms with various period lengths and characteristics (Bell-Pedersen, 2000; Ingold, 1971). These include rhythms in hyphal branching and/or in growth rate or morphology, the period lengths of which are a function of the temperature or nutritional conditions under which the fungus is growing. Such rhythms are undoubtedly important to the biology of the organisms but are not characterized as true circadian rhythms since they lack one or more of the characteristics defining rhythms as "circadian" (Bünning, 1973). One rhythm, however, meets the criteria: Work by Pittendrigh and colleagues in the 1950s showed that the developmental patterning of asexual conidiation seen when *N. crassa* grew over an agar surface was controlled by a circadian clock.

Neurospora is a haploid fungus that can grow and propagate asexually or via a sexual cycle. Both the asexual developmental cycle resulting in the production of macroconidiophores (conidia) and the sexual cycle that yields ascospores are circadianly regulated (Bobrowicz et al., 2002). In the former, a developmental switch leading to the production of conidia is activated in the subjective night. Interestingly, the clock is controlling the potential to develop rather than the developmental process itself, and once the switch is thrown, the actual process of development can take a long time or a short time, or even not take place at all, depending on the temperature and nutritional state of the organism. Also, growth conditions and genetic background can influence the phase when the process is initiated and terminated, although the peak and trough of conidiation are relatively constant for a given set of entraining conditions. Molecular and physiological rhythms can also be assayed using liquid (e.g., Loros et al., 1989) or solid (e.g., Brody and Harris, 1973) media, and real-time analysis of gene activity can also be followed in vivo using firefly luciferase as a reporter for gene expression (Mehra et al., 2002; Morgan et al., 2003).

The *Neurospora* system assumed real importance to rhythm studies when clock-mutant strains were identified (Feldman and Waser, 1971). Molecular analysis of the *Neurospora* clock was initiated shortly after that of *Drosophila* in the mid-1980s; it resulted in the cloning of the clock gene *frequency* (*frq*) and the completion of global screens for clock-controlled genes called ccgs (Loros et al., 1989). Molecular manipulations of *frq* expression in the 1990s proved *frq* to encode a central component of the core oscillator itself, and many additional clock genes, now more than 10, have since been characterized and cloned. With a genetic framework in place, steady progress has been made in understanding the molecular bases for sustainability of the rhythm, period length, resetting of the circadian system by light and temperature cues, and gating of input cues. Most recently, *Neurospora* has been suggested as a useful model for understanding photoperiodism (Tan et al., 2004b) and is proving to be a valuable system for examining the role of coupled feedback loops in clocks and for defining global features of circadian output.

A number of rhythm-affecting alleles have been identified (Dunlap et al., 2004a) whose review is beyond the scope of this article. This collection of clock mutants includes genes that affect the period length, such as frq^1 and frq^2, while other mutations (e.g., *chr*, *prd-1*, *prd-2*, *prd-3*, *prd-4*, *prd-6*, *wc-2*, and other *frq* alleles) affect both the period of the rhythm and temperature compensation. Several loci have been identified that can influence circadian or noncircadian timing, apparently by acting pleiotropically in a nutrition-dependent manner. The mechanisms through which they affect timing are not clear and have been reviewed (Dunlap et al., 2004a) but are not further considered here. The *frq* gene has been identified at least 8 separate times, with period variants ranging from 16 to 35 h, as well as mutants lacking circadian rhythmicity (although they do retain noncircadian rhythms). More recently, mutations in several FRQ turnover regulators, including CAMK-1, casein kinase II (CKII), protein phosphatases PP1 and PP2A (reviewed in Liu, 2003; Yang et al., 2004), and the E3 ubiquitin ligase FWD-1 (He et al., 2003), have been found to abolish rhythmicity or to affect period.

MOLECULAR ELEMENTS OF THE *NEUROSPORA* CLOCK

Fungal, insect, and mammalian clocks are all built around feedback loops in which heterodimeric PAS domain–containing transcription factors act as positive elements to drive expression of proteins that block the activity of those positive elements. Also, most eukaryotic circadian systems described to date actually operate using multiple, interlocked positive and negative regulatory connections (reviewed in Dunlap, 2004b; Sehgal, 2003). In addition to conservation seen in the general layout of these interconnected loops, the protein sequences of several components (e.g., White Collar–1 [WC-1], the kinases that phosphorylate FRQ, and the cullin-based ubiquitin ligase that mediates its turnover) all show significant similarities to components active in animal clocks (reviewed in Dunlap, 1999, 2004b; He et al., 2003; Lee et al., 2000; Liu, 2003).

Central components of the core *Neurospora* circadian feedback loop include both *frq* mRNA and FRQ and the transcription factors WC-1 and WC-2 that act together as the white collar complex (WCC; reviewed in Dunlap, 1999, 2004b; Liu, 2003; see Fig. 1). The WCC drives expression of the *frq* gene whose transcripts are heavily processed in a regulated manner (see below). Two distinct FRQ proteins can be translated from *frq* mRNAs (Garceau et al., 1997), either of which can act to dampen expression of its own transcript through its interactions with the WCC (Aronson et al., 1994a; Froehlich et al., 2003). As a result of the negative feedback of FRQ on *frq* expression, both *frq* mRNA and FRQ protein are rhythmically expressed in a daily manner. This cyclical expression is essential for clock function. Loss-of-function mutations in *frq* (Aronson et al., 1994b), *wc-1* (Crosthwaite et al., 1997), or *wc-2* (Collett et al., 2001) result in loss of circadian rhythmicity, although noncircadian oscillations can still be detected in some null strains and are discussed in detail below. Partial loss-of-function mutations in *wc-2* (Collett et al., 2001) or *frq* (Aronson et al., 1994b) can result in significant period-length changes (periods from 16 to 35 h) and to partial loss of temperature and nutritional compensation of the clock.

Dynamics of FRQ

Figure 1 serves as a guide for following the events within the *Neurospora* free-running clock cycle starting from late subjective night. At this point, most of the FRQ protein in the cell has recently been degraded,

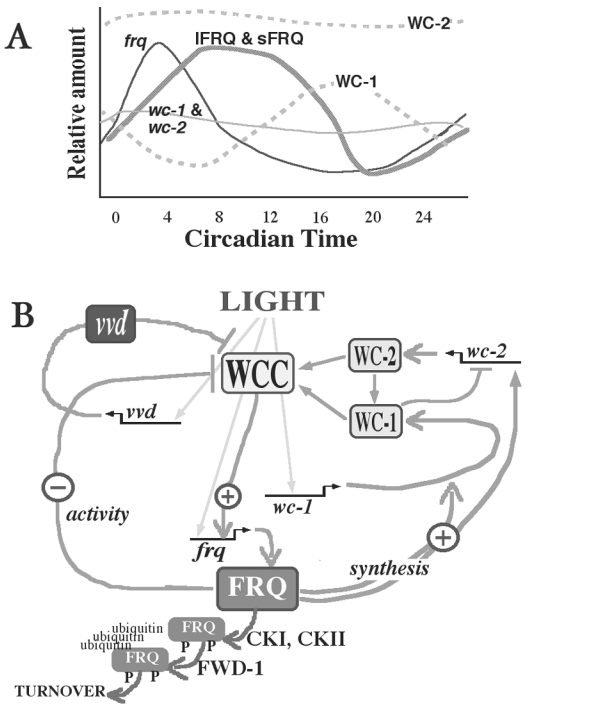

Figure 1. Known molecular components in the *Neurospora* circadian clock. (A) Temporal changes in the relative amounts of various RNAs and proteins having important roles in the clock cycle are shown. Lowercase italics denotes RNAs, and uppercase denotes proteins. lFRQ and sFRQ are the large and small proteins, respectively, arising from translation of *frq* mRNAs; see text for details. (B) Regulatory relationships between the genes and proteins with known roles in the clock are shown. Lines ending in bars connote negative regulation or repression, and arrows connote positive regulation. *P* on FRQ denotes phosphorylation. See text for details (modified from Dunlap, 1999, and Lee et al., 2000). WC = White Collar; WCC = white collar complex.

and *frq* RNA levels are low. WCC binds to the promoter of the *frq* gene and drives its transcription (Froehlich et al., 2003). *frq* transcript begins to appear by late subjective night, even before real or subjective dawn. By early morning, FRQ proteins appear, dimerize, and enter the nucleus, where they act to fulfill their role in the clock (reviewed in Dunlap, 1999, 2004b; Liu, 2003).

FRQ participates in several separate actions within the clock cycle, each essential to the daily rhythm. In the 1st and dominant role, specific interactions occur between FRQ and the WCC within the nucleus that block the activity of the WCC (Dunlap, 2004b; Froehlich et al., 2003; reviewed in Liu, 2003). So long as FRQ is present, WCC activity is diminished. As a result, by late in the subjective day, WCC activity has declined to its lowest level (Froehlich et al., 2003; Lee et al., 2000), and the loss of WCC activity results in

dampened expression of *frq* RNA. In a 2nd action, FRQ is phosphorylated by several kinases (CKI, CKII, CAMK1) that determine its stability (reviewed in Liu, 2003), with CKII playing the most important role. Phosphatases can further modulate this (Yang et al., 2004). FRQ phosphorylation may also influence FRQ-WCC interactions. Overall, *frq* mRNA levels peak in the midmorning (Aronson et al., 1994a; Crosthwaite et al., 1995), about 4 to 6 h before the peak of total FRQ in the afternoon (Garceau et al., 1997).

A 3rd role of FRQ in the cycle becomes apparent about now: FRQ promotes, through an unknown, posttranscriptional mechanism, the synthesis or accumulation of WC-1 from existing *wc-1* message. This results in a low-amplitude rhythm in WC-1 protein (Lee et al., 2000; reviewed in Liu, 2003). Because of this, levels of WC-1 begin to rise after FRQ appears, continuing even as phosphorylation-promoted degradation of FRQ begins. WC-1 rhythmicity is not essential for overt rhythmicity, but it promotes robustness of the rhythm. Although WC-1 and WC-2 influence each other's levels, and FRQ in some way promotes *wc-2* expression, WC-2 levels do not cycle but instead remain high and relatively constant (reviewed in Dunlap, 2004b ; Liu, 2003). WC-2 helps to mediate the interaction between FRQ and the WCC, and the stage of the circadian cycle can be gauged by the relative levels of FRQ or WC-1 with respect to the high and constant amount of WC-2. By late in the subjective day, FRQ is blocking activation of the *frq* promoter by the WCC while promoting WC-1 synthesis, increasing the level of the WCC. This creates a mass of WCC held inactive by FRQ (Froehlich et al., 2003; Lee et al., 2000).

Finally, the processive phosphorylation of FRQ promotes ubiquitination by the SCF-type ubiquitin ligase FWD-1 (He et al., 2003), triggering FRQ turnover (Garceau et al., 1997; Liu et al., 2000). As FRQ-promoted synthesis of WC-1 is balanced by WC-1 degradation, WC-1 levels peak at night, near to when FRQ levels reach a nadir (Lee et al., 2000). The block and then sudden release of WCC activation, combined with the wave of FRQ-promoted WC-1 synthesis, create a sharp transition in which high WCC activity initiates the next cycle to maintain a robust amplitude in the feedback loop (Froehlich et al., 2003).

The circa 24-h time constant for the *Neurospora* clock can be largely accounted for by the length of time required for FRQ synthesis (3-6 h) and recovery from FRQ repression via degradation (14-18 h; Merrow et al., 1997). Given this, and because many of the critical regulatory interrelationships between components of the *Neurospora* clock are becoming known, the *Neurospora* system is being actively modeled to gain insights into potential novel aspects of its regulation (Gonze et al., 2000; Leloup et al., 1999; Smolen et al., 2001). This promises to provide a basis for improved understanding of more cryptic clock properties such as temperature compensation (Ruoff et al., 1996). Modeling has also helped to underscore the similarities between fungal and animal circadian systems (Smolen et al., 2003).

Products of the *frq* Gene

Early work on *frq* transcripts based on limited sequencing of available cDNA fragments (Aronson et al., 1994b) was largely consistent with a simple transcription unit, although inconsistencies in these data foreshadowed later identification of the *frq* antisense message (Kramer et al., 2003) and the elucidation of a much more complex transcription unit. *frq* transcripts arise from multiple promoters, and each *frq* transcript can be spliced in a complex manner (Colot et al., unpublished). Splicing determines the ratio of long to short FRQ proteins synthesized. In addition, a long antisense transcript arises from the region of *frq* beginning from a promoter 3′ to the sense *frq* coding region. Compared to sense *frq*, antisense *frq* transcript is expressed at low levels with a peak phase opposite to that of sense *frq*; it is also light induced and appears to play a role in ensuring precise entrainment to LD cues (Kramer et al., 2003).

The long form of FRQ contains 989 amino acids, with the shorter form lacking the first 100 amino acids (Garceau et al., 1997). Both forms appear needed for optimally robust rhythmicity, but form-specific requirements depend on ambient temperature. At temperatures in the low end of the physiological range (<~22 °C), short FRQ is required and less overall FRQ is needed, whereas at higher temperatures (>26 °C), the large form and higher overall levels are needed (Liu et al., 1997). Both forms can form homodimers and heterodimers and are transported to the nucleus, but presently no clues exist as to distinct molecular roles for either of the 2 forms.

Structure, Function, and Regulation of the WC Proteins

As noted above, fungal and animal clocks all use heterodimers of PAS domain–containing proteins as

transcriptional activators in core clock loops, but a novel aspect of the *Neurospora* system is that an additional domain of the PAS superfamily (a LOV domain) is present in WC-1. This allows the WCC heterodimer also to function as the principal photoreceptor in the cell (Froehlich et al., 2002; He et al., 2002). WC-1 is a functional and sequence homolog of mammalian BMAL1 (48% identical or similar to mouse BMAL1 over the entire length of BMAL1's 625 amino acids; Lee et al., 2000). When just the 611 amino acid central region of WC-1 showing overlap with BMAL1 is used to search all of GenBank (http://www.ebi.ac.uk/blast2/index.html?), human and mouse BMAL1 proteins are among the highest scoring animal proteins, all with scores better than 10^{-5}, denoting significant similarities (Lee et al., 2000). Given this, the general similarity in the logic of the circadian feedback loops, and the essentially identical role of BMAL1 in the analogous circadian feedback loop, it seems likely that the proteins and the clocks in which they play such an important part arose from a common clock-bearing ancestor and that they are undergoing rapid evolution (Crosthwaite et al., 1997).

WC-1 is regulated posttranscriptionally at the level of synthesis and through both phosphorylation and protein:protein interactions. WC-1 is needed for FRQ and WC-2 to interact and self-associates to some degree (reviewed in Liu, 2003). Under constant conditions in the dark, neither *wc-1* nor *wc-2* RNA levels vary substantially over time, but there is a clear rhythm in WC-1 protein content (Lee et al., 2000) that promotes the overt rhythm but is not essential for ongoing operation of the clock (reviewed in Dunlap, 2004b; Liu, 2003). The amount of WC-1 is similar to that of FRQ, but the rhythm in WC-1 protein content is out of phase with that seen in FRQ; it seems that it is the level of FRQ that ultimately controls the activity of the WCC and, therefore, transcription and the clock cycle. This interlocked loop, as well as loops formed through the positive action of FRQ on *wc-2* expression, the positive regulation of WC-1 by WC-2, and the repression of *wc-2* expression by WC-1 (reviewed in Liu, 2003), drives the clock (Fig. 1). Interlocked loops have also been described in flies and mammals (reviewed in Dunlap, 2004b; Sehgal, 2003), pointing to a similar functional organization underlying circadian oscillators in eukaryotes.

WC-2 is predominantly in the nucleus and does not appear to be highly regulated on a circadian time scale, although it is required for the interaction between WC-1 and FRQ (reviewed in Liu, 2003). Some WC-2 variants that fail to promote normal levels of light-induced expression of most light-regulated genes show little if any defects in the light induction of *frq* (Collett et al., 2002); this indicates that light-signal transduction cannot be as simple as WCC activation of all light-induced genes. A partial loss-of-function WC-2 displays a long circadian period length as well as a partial loss-of-temperature compensation (Collett et al., 2001), suggesting a role for the WCC both in compensation and in period-length determination.

RESETTING THE *NEUROSPORA* CIRCADIAN SYSTEM WITH ENVIRONMENTAL CUES

Entrainment resets the phase of the clock to coordinate with the phase of the external world. *Neurospora* has been instrumental for understanding the molecular mechanisms of entrainment by light (Crosthwaite et al., 1995), temperature (Liu et al., 1998), and gating (Heintzen et al., 2001), and core aspects of *Neurospora* light resetting are conserved in mammals (reviewed in Dunlap, 1999, 2004b; Liu, 2003).

Light responses in *Neurospora* are specific to blue light and require both *wc* genes. There are no known red or far-red responses, although the *Neurospora* genomic sequence revealed several putative photoreceptors including phytochromes and a cryptochrome (Borkovich et al., 2004), some of which are light regulated (Froehlich et al., unpublished; Liu, unpublished). A report of light effects in *wc* mutant strains (Dragovic et al., 2002) is controversial and was confounded by the use of partial loss-of-function *wc* strains rather than gene replacements (Cheng et al., 2003b; Lee et al., 2003; reviewed in Liu, 2003).

WC-1, the Circadian Blue-Light Photoreceptor, and Light-Induced Resetting

WC-1 uses FAD as a chromophore (Froehlich et al., 2002; He et al., 2002), with light inducing a change in DNA-bound WCC, promoting its activity. Light acts rapidly through the WCC to induce *frq* (reviewed in Dunlap, 1999, 2004b; Liu, 2003). This action is mediated by the WCC's binding to light-responsive elements (LREs) within the *frq* promoter (Froehlich et al., 2002, 2003). The proximal site controls clock phase and is required for full induction in response to light, while the LRE farthest from the promoter is required both for maximal light induction and for circadian rhythmicity in the dark. Globally, about 3% of the

genome is light induced, while about 7% is upregulated in the dark by overexpression of WC-1, consistent with light eliciting qualitative changes in WC-1 rather than simply increasing its levels (Lewis et al., 2002).

Complexities in the Light Response

Figure 2A describes a qualitative model for resetting (Crosthwaite et al., 1995). Although the initial response to light is transcriptional, the total response to light is beyond being merely transcriptional and beyond light simply yielding more FRQ. Both *frq* mRNA and FRQ responses depend on time of day and are further modulated acutely by light (reviewed in Liu, 2003). Phosphorylated FRQ accumulates in the light but may not be significantly degraded until transfer to dark (Collett et al., 2002; Tan et al., 2004a). Depending on the duration of light prior to darkness, the synthesis/decay kinetics of FRQ can vary substantially. The lag separating *frq* transcription and FRQ protein peaking can change by several hours. This is partly because FRQ is phosphorylated in a complex manner, some of it regulating turnover (e.g., He et al., 2003; Liu et al., 2000) and some doubtlessly influencing activity or interactions with other proteins by analogy to the case of PER (Nawathean and Rosbash, 2004). Thus, FRQ is qualitatively different depending on how long the organism has been in light, such that at transfer to darkness different phases may be assumed. In addition, antisense *frq* message is expressed out of phase with sense *frq* mRNA and is light induced. Loss of *frq* antisense results in much stronger light-phase-shifting, consistent with an important but still poorly understood role in clock light responses (Kramer et al., 2003). Last, circadian light responses are very different on the 1st versus later days in dark due to the action of the VVD repressor in gating (Heintzen et al., 2001).

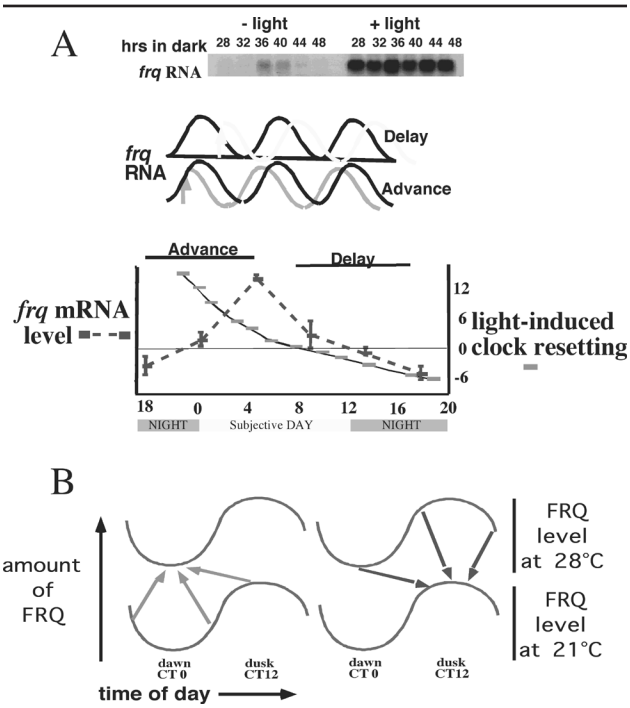

Figure 2. Environmental resetting of the *Neurospora* clock. (A) Light resetting—at the top is a Northern blot showing *frq* in mRNA collected at various times in the dark or light; the inherent rhythm in *frq* expression is seen at the left, and the high level of expression induced by 2 min of light is seen on the right. In the middle panel is a schematic model of how resetting works: Light always results in the induction of RNA. When this happens early in the cycle, before the normal peak in *frq* transcript levels, it yields an advance; if late in the cycle, it yields a delay. At the bottom are data showing the overlap of the daily rhythm in *frq* transcript with the daily response of the clock to light pulses. The data at the bottom conform qualitatively to the model in the middle panel (modified from Crosthwaite et al., 1995; Dunlap, 1999). (B) Resetting of the *Neurospora* clock with temperature treatments. The level of FRQ protein in cells is represented by the curved lines. At 21 °C, it cycles around a lower set point than at 28 °C. On the left, depicted schematically, is the result of a temperature step-up. Here, at any time of day, the level of FRQ in the cell at the time of the step-up is interpreted by the feedback relationships of the clock and seen to be low compared to the amount needed to effect repression of *frq* expression. Therefore, more *frq* and FRQ are made, an action normally occurring in the morning near dawn, to which a step-up sets the clock. On the right is a schematic describing the effects of a step-down in temperature. Here, no matter when the step occurs, there is enough FRQ in the cell to effect repression of *frq* expression (the phase corresponding to late in the day around dusk), so *frq* synthesis is stopped and the clock is reset to around dusk (modified from Dunlap, 1999).

VIVID and the Gating of Light Responses

Gating refers to the ability of the circadian clock to modulate a response, in this case the sensitivity to resetting cues, as a function of time of day. The VVD protein is a member of the PAS protein superfamily (Heintzen et al., 2001), and its action comprises an autoregulatory negative feedback loop that closes outside of the core oscillator to effect gating (see Fig. 1). VVD is also a photoreceptor that binds a flavin chromophore (Cheng et al., 2003a; Schwerdtfeger and Linden, 2003). In response to light, VVD helps to modulate the organism's response to subsequent light signals, a process known as photoadaptation (reviewed in Liu, 2003). VVD expression is controlled in part by the clock. Importantly, significant expression of VVD is limited to the 1st day in constant darkness, and this probably accounts for the finding that light signals

have little clock-resetting effects when delivered on the 1st day after a light-to-dark transfer. Possibly related to the ability to gate light responses is a recent report proposing that both asexual reproduction and sexual reproduction in *Neurospora* may be seasonally regulated by night length and require the *frq* gene (Tan et al., 2004b).

Temperature Effects on the Clock

Ambient temperature affects rhythmicity in 3 ways. *Neurospora* will entrain to temperature steps and cycles, there are physiological temperature limits for operation of the clock, and the circadian period length is temperature compensated. Temperature effects are mediated in part through the amount and perhaps kind of FRQ protein (long vs. short) made. When either FRQ form is eliminated, the temperature range permissive for rhythmicity is reduced (Garceau et al., 1997; Liu et al., 1997). Clock resetting by temperature steps reflects posttranscriptional regulation. While *frq* transcript levels are influenced little by temperature, FRQ levels oscillate around a higher mean at higher temperatures, so that the "time of day" associated with a given number of molecules of FRQ is different at different temperatures. At peak at 25 °C, there are about 30 molecules of FRQ in the nucleus (Merrow et al., 1997), less than this number at 20 °C and more at 30 °C. Because at the lowest point in the curve (dawn) at 28 °C (the upper curves in Fig. 2B) there are more molecules of FRQ per nucleus than at the highest point in the curve (dusk) at 21 °C, a shift in temperature corresponds literally to a step to a different time and a shift in the state of the clock (Liu et al., 1998), although initially no synthesis or turnover of components occurs. Finally, temperature can be a stronger resetting cue than light (Liu et al., 1998).

OUTPUT FROM THE CLOCK

Efforts in *Neurospora* pioneered the study of ccgs and have contributed greatly to understanding how the clock delivers information to regulate downstream biological processes. The best-studied circadian phenotype in *Neurospora*, macroconidiation, can be triggered by environmental signals including blue light, desiccation, and nutrient starvation, as well as by the endogenous circadian clock. Conidiation involves a major morphological change that requires many novel gene products. Other physiological rhythms have been described, including the production of CO_2, a number of enzymatic activities, lipid and diacylglycerol metabolism, and even growth rate (Gooch et al., unpublished; reviewed in Loros et al., 2003). Fungi, in fact, can express a variety of rhythms in growth and spore formation, many of which are not by definition circadian (Bünning, 1973). A difficulty in the analysis of output pathways has been distinguishing circadian clock regulation from other metabolic or developmental regulations: Not every rhythm expressed by fungi is a circadian rhythm, nor is every rhythmic process controlling conidiation necessarily a part of the circadian clock.

An early assertion made in *Neurospora* (Loros et al., 1989) that has proven to be universally true in circadian systems is that daily clock control of gene expression would be a major aspect of output. Systematic screens for ccgs were initially executed in *Neurospora* (Loros et al., 1989), and more than 180 ccgs have since been identified through a combination of differential hybridization, cDNA sequencing, and cDNA microarrays (Correa et al., 2003; Nowrousian et al., 2003; reviewed in Bell-Pedersen, 2000; Loros et al., 2004).

The Spectrum of ccgs Reflects Growth Conditions

All aspects of the biology of the organism are under clock regulation (Table 1). In a microarray study (Nowrousian et al., 2003), the genes represented on the arrays were derived from RNAs expressed when cultures were slowly starving in the dark (reviewed in Bell-Pedersen, 2000; Loros et al., 2004). Exhaustive cDNA sequencing yielded a UniGene set of about 1100 genes, about 10% of the genome. Another study (Correa et al., 2003) sampled 1343 genes (~14% of the genome) derived largely from expressed sequence tag (EST) analysis of growing conidial, mycelial, and sexual tissues where many genes are expressed; these showed relatively little overlap with the 1st study. Most genes are expressed at the time of conidiation (late night to morning). Interestingly, only about 5% of the genes from starving cultures were clock regulated versus nearly 20% from the rapidly growing cultures.

Light-induced and temperature-shift-regulated genes have also been examined in clock-competent and in *frq*-null strains, as well as in strains overexpressing WC-1 in the dark (Correa et al., 2003; Nowrousian et al., 2003). As expected, light-induced genes do not require the clock but do require WC-1. Lewis et al. (2002) found about 7% of the genome to be

Table 1. Functions of Neurospora *ccg*s

Functional Category	*No. of* ccgs
Cell division	1
Signaling/communication	16
Cell structure/cytoskeleton	8
Cell defense	4
Development	11
Gene regulation	5
Metabolism	42
Protein processing	10
Protein synthesis	33
Unclassified	50

NOTE: ccg = clock-controlled gene. Data expanded from Correa et al. (2003) and Nowrousian et al. (2003).

upregulated in response to WC-1 overexpression, consistent with the dark-specific circadian-related role for WC-1. Surprisingly, among the array set corresponding to ESTs from starving, dark-grown cultures, the temperature response appeared to require a functional circadian clock (Nowrousian et al., 2003), suggestive of an unanticipated role of the circadian circuitry as an environmental temperature sensor. However, this study sampled only the small subset of the genome expressed in liquid culture, placing caveats on the conclusions. It is well known, for instance, that temperature cycles can drive rhythms in conidiation on solid media, even in *frq*-null strains (see below), rhythms that must entail changes in gene expression. That these were not seen suggests either that the driven rhythms seen on solid media are not seen in liquid and/or that the sample of genes on the arrays was too small or skewed. Consistent with this, Correa et al. (2003) recently described genes that cycle in an *frq*-null strain, albeit with a somewhat altered phase. This exciting observation, searched for but absent among the genes examined by Nowrousian et al. (2003), could provide an entrée to an FRQ-less oscillator (FLO).

Mechanisms of Circadian Control

If a gene is rhythmically expressed as measured by its mRNA levels, then either transcription and/or mRNA turnover must be clock regulated, and clock-controlled transcription seems to be the rule in the few cases carefully examined. *cis* analysis of the *eas* (*ccg-2*) promoter has separated sequence-specific regions that confer light, developmental, and circadian regulation. A 68-base pair sequence located close to the start of transcription has been identified, an activating circadian element (ACE), that is sufficient to confer clock regulation on this and other promoters (reviewed in Bell-Pedersen, 2000). The core ACE sequence, AACTTGGCCAAGTT, is distinct from the core LRE sequence, CGAT(N)CCGCT, bound by the WCC (Froehlich et al., 2002) that was shown to be necessary and sufficient for clock regulation of *frq* and potentially other clock-regulated genes (Froehlich et al., 2003). That both of these elements mediate clock control more generally is suggested by their appearance in some of the promoters of ccgs identified using microarrays (Correa et al., 2003). Some genes have neither element, suggesting additional clock-control elements and the presence of hierarchical control, in which the oscillator directly regulates oscillator-proximal controllers that in turn regulate more downstream genes. In general, individual genes are subject to combinatorial control by multiple environmental and internal processes, only one of which is the clock. Several laboratories have undertaken genetic screens aimed at identifying components within these pathways of control.

FLOs AND OTHER NONCIRCADIAN FEEDBACK LOOPS

Genetic and molecular analyses of the FRQ/WCC circadian feedback loop have been remarkably successful in explaining circadian phenomena. However, the circadian system may include other oscillators as well as simple clock-regulated genes and proteins. Strains bearing null *frq* alleles have long been known to occasionally express a rhythm in conidial banding, although one that has lost most circadian characteristic features (Aronson et al., 1994b; Loros and Feldman, 1986). The term *FLO* has been used to distinguish such noncircadian, possible slave oscillators from the FRQ/WCC circadian feedback loop (Iwasaki and Dunlap, 2000; Fig. 3). Several FLOs have been identified, including ones with at least partial temperature compensation (D. Bell-Pedersen, personal communication), but none have yet been shown either to be inherently circadian or to influence the operation of the FRQ/WCC feedback loop in the circadian clock. Identification of components of these FLOs represents one of the most active areas of research.

Use of Temperature Cycles to Probe FLO Dynamics

The *Neurospora* clock shows typical circadian behavior on temperature cycles of different period

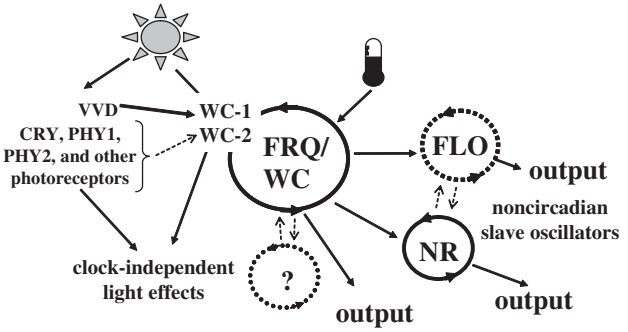

Figure 3. A model for oscillators and networks controlling and integrating light and temperature influences in the *Neurospora* circadian system. Solid loops, lines, and arrows represent known feedback loops or regulatory relationships, and dotted lines represent those predicted to exist or that could exist in the wild-type circadian system. Adapted from National Academies Keck Futures Initiative Signaling National Conference, http://63.251.167.36/nakfi/progressive/GeneticandMolecularDissectionofE/index.htm. WC = White Collar; FLO = FRQ-less oscillator.

lengths, for instance, phase leading when the free-running period length is greater than the entraining cycle period. Under temperature entrainment, the waveform of the rhythm changes with the period of the entraining cycle since it requires (as expected) half of the cycle to transit from trough to peak and vice versa. When normally arrhythmic *frq*-null strains are placed on a 5 °C temperature cycle, a banding rhythm is dependably elicited (Merrow et al., 1999; reviewed in Roenneberg and Merrow, 1999). Unlike the case for wild-type strains though, peaks always occur in the cold period and troughs always in the warm period: Typical circadian entrainment is lost, strongly suggesting that the rhythm is simply being driven. However, if instead of peaks or troughs a novel phase reference point on the rise of the curve from trough to peak is used, now the derived phase does vary with the period length of the cycle as if *frq*-null strains are not simply driven (Merrow et al., 1999); this has been taken as evidence that the FLO is the rhythm generator (reviewed in Roenneberg and Merrow, 1999). Clearly, though, the result depends on the phase reference point used, and since the waveform itself changes with period of the driving cycle and standard reference points of peak and trough do not show this change, the data seem most consistent with a model in which FLO is simply driven by temperature cycles but not entrained to them. Based on this, the simplest interpretation is that this FLO represents a slave oscillator.

Additional FLOs Are Known

Recently, Correa et al. (2003) have identified a 2nd FLO in a microarray study. Its phase is set by the LD transfer, making this FLO distinct from the original FLO described above. A 3rd class of FLO showing periods of up to 100 h is seen in strains bearing the choline-repairable morphological mutant *chol-1* (e.g., Lakin-Thomas and Brody, 2000). Unsupplemented *chol-1* strains show abnormal colony morphology and grow episodically so that the fastest rate of linear extension can be 8 times the slowest growth. The rhythm lacks temperature compensation but, interestingly, is still pH compensated (Ruoff and Slewa, 2002). It may be that the robust rhythms uncovered by choline starvation in the mutant reflect morphological cycles whose appearance masks, or perhaps is gated by, normal circadian regulation of banding. Potentially the best understood example of a FLO comes from study of a nitrate reductase rhythm; nitrate reductase is regulated in a circadian manner in clock wild-type strains, but the rhythm persists in *frq*- and *wc*-null mutants (e.g., Christensen et al., 2004). Since nitrate reductase expression is regulated by metabolites arising from assimilated nitrate, there is the basis for a feedback loop oscillator within nitrate metabolism that could constitute this FLO. A simple and plausible view unifying this observation, and all the FLOs, is that these are slave oscillators that can be coupled to the FRQ/WCC–associated circadian system (Fig. 3). Absent the FRQ/WCC loop, they can run on their own in a noncircadian manner.

SUMMARY

Steady advances deriving from the identification, cloning, and subsequent manipulation of putative clock genes and proteins have allowed us to improve our views of the clock over the past decades. Fifteen years ago, the oscillatory system was imagined simply as 3 discrete components, input, oscillator, and output, and to be based on a single feedback loop directly driving outputs. Although FRQ, WC-1, and WC-2, as well as their modifiers and regulators, are now known to be clock components, the observation that WC-1 is in addition the circadian blue-light photoreceptor has clearly united aspects of input and oscillator. Another known output gene product and independent photoreceptor, VVD, acts broadly to influence both phase and abundance of many of the other ccgs, as well as

the *frq* gene, and to influence input. Noncircadian oscillators such as the FLOs and the putative nitrate reductase oscillator can operate on their own or within a circadian system coordinated with the FRQ/WCC loop and, perhaps, yet other undescribed feedback loop oscillators. *Neurospora* has its own assets as a model system—its syncytial nature more closely reflecting the biology of a muscle cell than a uninucleate cell; its excellent genetics, biochemistry, and molecular biology; and the ease with which it can be cultured. There is every reason to expect work on *Neurospora* to continue to inform work on animal and plant as well as fungal clocks, in just the way a good model should.

ACKNOWLEDGMENTS

The authors wish to apologize for gaps in the citation of the primary literature that resulted from an editorial requirement to triage the reference list. We thank Yi Liu and D. Bell-Pedersen for comments on an earlier version of the article. This research was supported by grants MH44651 to JCD and JJL, NSF MCB-0084509 to JJL, and R37GM34985 to JCD.

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