ORGANIZATION OF THE CIRCADIAN CLOCK AND CONTROL OF RHYTHMICITY IN FUNGI

A Dissertation

by

ANDREW VANDERFORD GREENE

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2005

Major Subject: Microbiology

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ABSTRACT

Organization of the Circadian Clock and Control of Rhythmicity in Fungi.

(August 2005)

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Chair of Advisory Committee: Dr. Deborah Bell-Pedersen

Circadian rhythms in biological processes occur in a wide range of organisms and are generated by endogenous oscillators. In *Neurospora crassa*, the FRQ-oscillator (comprised of FRQ, WC-1 and WC-2) is essential for rhythms in asexual sporulation and gene expression. How this oscillator signals to the cell to control rhythmicity is unknown. Furthermore, under certain growth conditions, rhythms are observed in FRQ-null strains, indicating the presence of one or more FRQ-less oscillators (FLOs). Interestingly, while circadian rhythms are observed in the related *Aspergillus spp.*, they lack the *frq* gene, leading to the hypothesis that a FLO is responsible for rhythms in *Aspergillus*. Thus, *Aspergillus* provides a useful organism to investigate the components of the FLO.

To investigate how an oscillator controls circadian output, we characterized the role of *N. crassa* NRC-2. The *nrc-2* gene is under control of the clock and encodes a putative serine-threonine protein kinase. In a NRC-2-null strain cultured in low glucose conditions, FRQ-oscillator-dependent outputs are arrhythmic, but are rhythmic in high glucose. Our data suggests a model whereby NRC-2 relays metabolic information to the

FRQ-oscillator to control rhythmic output. To understand the role of FLO(s) in the *N. crassa* circadian system, we examined regulation of the *ccg-16* gene. We show that *ccg-16* transcript rhythmicity is FRQ-independent, but WC-1-dependent. Furthermore, in contrast to current models for the FRQ-oscillator, we observed that rhythms in WC-1 protein accumulation persist in the absence of FRQ. These data support a new model involving two oscillators that are coupled through the WC-1 protein and that regulate different outputs. One approach to identify components of the FLO involved characterizing circadian rhythms in *Aspergillus spp*, which lacks FRQ. We find that *A. flavus* and *A. nidulans*, display circadian rhythms in sporulation and gene expression, respectively. Together, these findings provide a foundation for the identification of FLO components in both *Aspergillus* and *N. crassa*, that will ultimately lead to an understanding of how a multi-oscillator system can generate and coordinate circadian rhythmicity.

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NOMENCLATURE

CAMK-1	Calcium/calmodulin dependent protein kinase I
ccg	*clock-controlled gene*
CHX	Cycloheximide
CKI/II	Casein Kinase I/II
CM	*Aspergillus* Complete Medium
CT	Circadian Time
DD	Constant Darkness
FAD	Flavin Adenine Dinucleotide
FLO	FRQ-Less Oscillator
FRP	Free-Running Period
LD	Light:Dark cycle
LL	Constant Light
LOV	Light, Oxygen and Voltage domain
NLS	Nuclear Localization Sequence
PAS	PER, ARNT and SIM domain
PKC	Protein Kinase C
RHT	Retinohypothalamic Tract
SCN	Suprachiasmatic Nucleus
WCC	White-Collar Complex
ZT	Zeitgeber Time

CHAPTER I

INTRODUCTION

Circadian rhythms are a ubiquitous occurrence in the natural world. Circadian rhythms have been documented in many diverse organisms, and control a wide range of processes from cell division and nitrogen fixation in unicellular cyanobacetria (Ditty *et al.*, 2003), to asexual spore formation in fungi (Bell-Pedersen, 2000), to bioluminescence and buoyancy in the dinoflagellate *Gonyaulax* (Roenneberg and Merrow, 2002), to behavior in insects and mammals, as well as controlling rhythmic transcription and translation of genes in many species (Dunlap, 1999). The circadian clock governs all of these processes, and many others, in nature.

Circadian rhythms were first described in 1729 by the French scientist Jacques de Marian, who noted that the leaves of the mimosa plant moved up and down very slowly, with a period of about a day in constant environmental conditions (Sweeney, 1987). This observation highlights one of the cardinal properties of circadian rhythms: they persist even in the absence of environmental cues; thus, the mechanism required for the generation of these rhythms must reside within the organism itself. Over the next three centuries, and especially the past fifteen years, much has been learned regarding the processes regulated by, and the mechanisms controlling, circadian rhythms.

The circadian clock provides organisms with the ability to anticipate daily changes in the environment, such as changes in lighting, temperature, and humidity. This

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This dissertation follows the style of *Molcecular Microbiology*.

helps the organism to prepare for predictable environmental changes by modulating its physiology to take advantage of the periodic characteristics of the environment. The fruit fly *Drosophila*, for example, will eclose from its pupal case at dawn, a time of the day at which the temperature is low and humidity is high, to prevent desiccation of the young flies (Pittendrigh, 1954). This circadian behavior persists even in the absence of temporal cues, thus it is not simply a response to changing environmental conditions. The circadian clock of cyanobacteria has been shown to enhance the fitness of the organism; strains with an intrinsic period close to that of the environmental cycle have a reproductive advantage over strains whose intrinsic period differs substantially from that of the environment (Ouyang *et al.*, 1998). The filamentous fungus *Neurospora crassa* forms asexual and sexual spores with greater efficiency in light:dark (LD) cycles that mimic environmental cycles; exposure to too much or too little light results in decreased spore production, an effect which is dependent on a functional circadian clock (Tan *et al.*, 2004). The circadian clock, therefore, provides diverse organisms with an adaptive advantage.

All circadian clocks possess several formal characteristics. Foremost among these is the expression of the rhythm under constant conditions with a period length of roughly 24 hours. The period of these rhythms in constant conditions is called the free-running period (FRP). Circadian rhythms are also synchronized by external environmental stimuli, such that the period of the rhythm matches that of the environmental cycle, a process termed entrainment. Entrainment is dependent on the perception of external time cues, or *zeitgebers* (German: time-giver), by the molecular

clock components, and results in the shifting of the circadian clock to an appropriate phase. This phase shifting can be observed in organisms under constant conditions; one-time exposure to a *zeitgeber* can shift the phase of the free-running rhythm in a predictable manner, and this is dependent on both the intensity and time of day of exposure to the *zeitgeber*. Finally, the FRP of a circadian rhythm is essentially constant in different temperatures and nutritional conditions, properties called temperature and nutritional compensation, respectively. Although most biochemical reactions have a Q_{10} (the ratio of the reaction rate at a given temperature to the rate 10°C lower) of 2-3, the Q_{10} of circadian rhythms is close to 1, within a species-specific range (Pittendrigh, 1993). This enables the organism to keep a meaningful phase with respect to the environment over a range of ambient temperatures. Nutritional compensation refers to the ability of the rhythm to remain essentially constant over a range of nutritional conditions, which can substantially affect growth rate. The filamentous fungus *N. crassa*, for example, exhibits circadian rhythms in asexual spore development on media containing a variety of carbon and nitrogen sources with a FRP of about 22 hours, even though the rate of growth varies under different media conditions (Loros and Feldman, 1986).

The use of genetically tractable model organisms in circadian studies has dramatically improved our understanding of circadian rhythms, and the model organisms examined span many phylogenetic levels. The unicellular cyanobacterium *Synechococcus elongatus* is an extensively studied prokaryotic circadian model organism, and eukaryotic clock models include the filamentous fungus *N. crassa*, the

fruit fly *Drosophila melanogaster*, the plant *Arabidopsis thaliana*, the zebrafish *Danio rerio*, and the mammals Golden hamster (*Mesocricetus auratus*) and mouse (*Mus musculus*). The data compiled at the genetic, physiological and molecular levels in these species has led to a simplified description of the circadian clock as consisting of three major components: 1) one or more circadian oscillators, composed of "clock proteins", responsible for timekeeping and rhythm generation, 2) input pathways responsible for the perception and transmission of environmental information to the oscillator, and 3) output pathways originating from the circadian oscillator functioning to control overt rhythmicity (Eskin, 1979). These parts of the clock are diagrammed in Figure 1. This simple view of the circadian system has provided a valuable working model; however, in each model system added complexity exists. For example, some input components also serve as oscillator components, such as the WC-1 photoreceptor in *N. crassa* (Froehlich *et al.*, 2002). Output components may also feed back on the clock and modulate FRP, such as the *dbp* (D-site binding protein) gene in mammals (Lopez-Molina *et al.*, 1997).

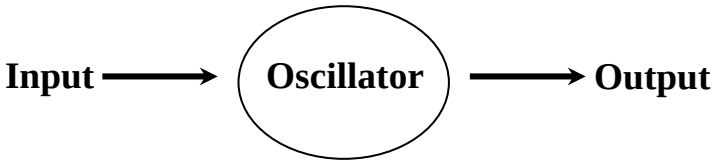

Figure 1. A simplistic view of a circadian clock. Daily environmental signals, such as light and temperature, are transduced to an oscillator, which can be reset by these stimuli but also runs in constant conditions. The oscillator controls outputs such as behavior and gene expression in a time of day specific manner through a signal transduction pathway(s).

Studies of circadian rhythms in diverse organisms have led to an appreciation of the similarities and differences that exist between the organization of clocks in these organisms. The clocks of unicellular systems, such as *S. elongatus* and *N. crassa*, appear to be composed of multiple oscillators located within a single cell (Bell-Pedersen *et al.*, 2005). In this system, individual oscillators have been shown to regulate different outputs, and coupling of the oscillators can increase the stability and precision of the clock mechanism (Lewis *et al.*, manuscript in preparation; Pittendrigh and Bruce, 1959).

The organization of the clock in multicellular eukaryotes is different. In multicellular eukaryotes, there is currently no evidence suggesting that multiple coupled oscillators are present in single cells. Instead, a variety of different cell types contain molecular oscillators that are composed of the same molecular machinery, but that regulate tissue-specific rhythms. In vertebrates, rhythms in non-brain (peripheral) tissues are coordinated by a light-entrainable pacemaker(s). Mammalian circadian rhythms are dependent upon the suprachiasmatic nucleus (SCN), which can generate self-sustained circadian oscillations and functions as a light-entrainable pacemaker (Klein *et al.*, 1991; Quintero *et al.*, 2003; Welsch *et al.*, 1995). Removal of the SCN results in a loss of circadian rhythms in behavioral processes (Turek, 1985), and transplantation of SCN tissue to SCN lesioned rats results in a restoration of behavioral circadian rhythmicity (Ralph *et al.*, 1990). The SCN, in contrast to the peripheral circadian oscillators, receives direct photic input from the retina via the retinohypothalamic tract (RHT) (Johnson *et al.*, 1988); this structure is required for entrainment to LD cycles. The current model of the mammalian circadian oscillator posits that the SCN serves as an essential bridge

between the environment and the organism's endogenous timekeeping machinery. Rhythms in clock genes are entrained in the SCN more rapidly by LD cycles than they are in peripheral tissues (Yamazaki *et al.*, 2000), and, in addition, peripheral rhythms are phase-delayed relative to the SCN by 4-12 hours, implying that it takes time for signals to travel from the SCN pacemaker to peripheral tissues (Zylka *et al.*, 1998). Transplantation of rhythmic, non-SCN cell types are unable to restore behavioral rhythmicity to SCN-lesioned animals (Earnest *et al.*, 1999). Rhythms in peripheral tissues persist in culture and in SCN-lesioned animals, suggesting that the SCN acts as a central light-entrainable pacemaker that acts to coordinate, rather than drive, rhythms in peripheral tissues (Yoo *et al.*, 2004).

The situation in the fruit fly, *D. melanogaster*, is distinct from that in vertebrates or unicellular organisms. Many tissues in *D. melanogaster* display free-running rhythms in expression of clock genes, and thus contain circadian oscillators (Hall, 2003). Oscillators in these tissues are directly entrainable to cyclic environmental cues, although they are not known to be responsible for the control of any circadian output. The known circadian outputs in *Drosophila* include locomotor activity, pupal eclosion and olfactory responses. Locomotor rhythms are controlled by the small ventral lateral neurons in the brain (Frisch *et al.*, 1994; Grima *et al.*, 2004; Renn *et al.*, 1999), while olfactory rhythms are under the control of odorant receptor neurons in the antenna (Tanoue *et al.*, 2004). These oscillators, however, do not appear to control other oscillators, and thus do not appear to have pacemaker function, unlike the mammalian SCN. Oscillators in isolated *Drosophila* peripheral tissues are able to free-run and are

light-entrainable (Plautz *et al.*, 1997), further strengthening the idea that there is no centralized pacemaker in *Drosophila*. However, recent evidence suggests that the oscillators are able to communicate with each other; pigment dispersing factor may facilitate communication between oscillator neurons in the brain, resulting in the coordinated control of locomotor rhythms (Grima *et al.*, 2004; Peng *et al.*, 2003; Stoleru *et al.*, 2004).

Despite the significant differences in the organization of the circadian clock in organisms ranging in complexity from single-celled eukaryotes to insects and mammals, a common thread exists between these organisms, and that is the observation that circadian rhythms are generated by coordinated multioscillatory systems, whether those oscillators are located within the same cell, or among different tissues. *N. crassa* is a useful model organism for addressing the question of the composition and coordination of multioscillatory systems, and this issue is addressed in Chapter III.

N. crassa: a model system for chronobiology

For over sixty years, the filamentous fungus *N. crassa* has been a model eukaryote in genetic and biochemical analyses. *Neurospora* is often one of the first organisms observed following a forest fire, colonizing burned vegetation in tropical and temperate regions (Jacobson *et al.*, 2004a; Jacobson *et al.*, 2004b; Perkins and Turner, 1988; Turner *et al.*, 2001). The “one gene, one enzyme” concept was first described using *N. crassa* in the 1940’s (Beadle and Tatum, 1941), and circadian studies have been performed with this organism since the late 1950’s (Pittendrigh *et al.*, 1959). The early

studies using *N. crassa* established that a free-running rhythm in asexual spore formation, or conidiation, exists and persists in constant darkness (DD) and constant temperature with a period of approximately 22 hours at 25°C. A major advantage to the use of *N. crassa* as a model for chronobiology is the ease with which the conidiation rhythm is monitored. Long (30-40 cm) glass tubes, called race tubes, are used to monitor the conidiation rhythm (Figure 2). Race tubes are bent upwards at both ends so that an agar medium can be placed inside. A small amount of inoculum is placed in one end of the tube, and the fungus grows linearly down the tube at a constant rate of about 3.5 cm/day at 25°C. Following inoculation, the tubes are held in constant light (LL) for one day. The growth front is marked and the tubes are transferred into DD, a treatment that sets the clock to dusk. The growth front is subsequently marked every 24 hours under a red safety light, which has no entraining effect on the clock (Sargent *et al.*, 1966). *N. crassa* conidiates at regular 22 hour intervals in DD, and, following removal from DD, a "fossil record" of the rhythm is visible on the tubes in the form of bands of orange conidia, facilitating growth rate and FRP calculation. The center of the band is commonly used as the phase reference point. Circadian studies with *N. crassa* make use of the *band* mutant, which does not affect the clock, but renders the organism insensitive to CO_2 buildup, which inhibits conidiation, and allows the organism to form discrete bands of conidia in closed race tubes (Sargent *et al.*, 1966).

The *N. crassa* conidiation rhythm is a true circadian rhythm; it persists under constant environmental conditions, is entrainable by LD and temperature cycles, and the FRP is temperature and nutritionally compensated (Francis and Sargent, 1979; Gardner

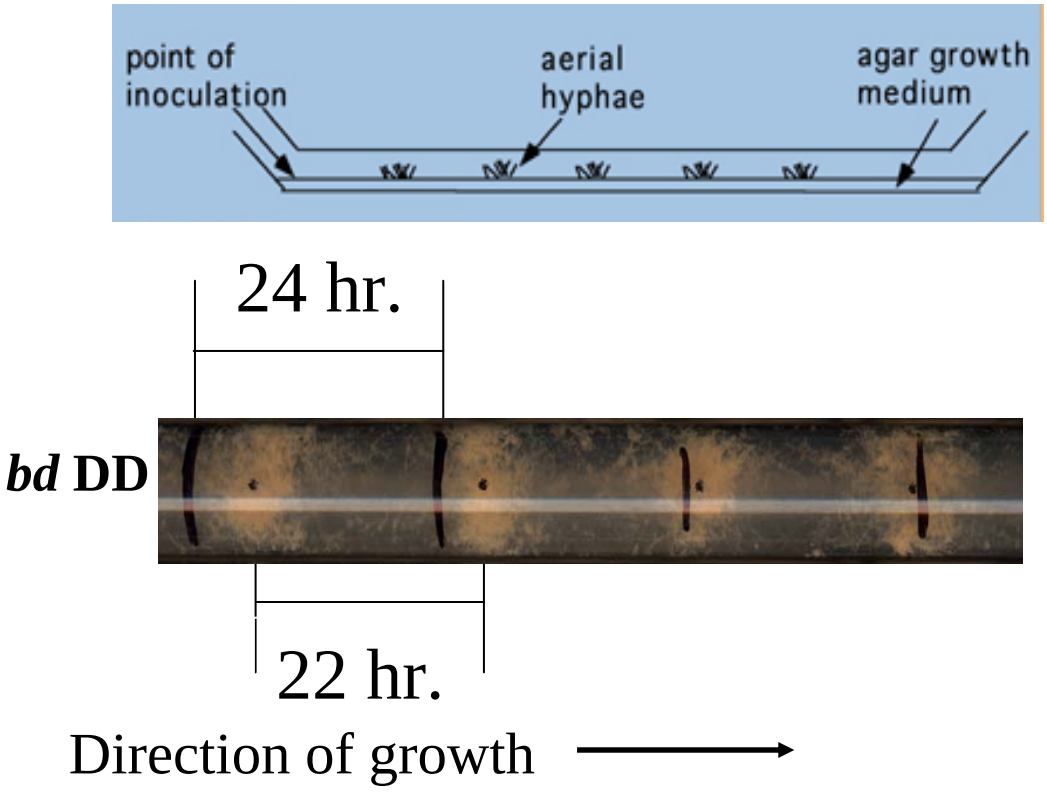

Figure 2. The race tube assay. A. Race tubes are long glass tubes bent upwards at each end and filled with solid growth medium. The tubes are inoculated on one end and the fungus is allowed to grow linearly down the tube. The fungus forms aerial hyphae bearing asexual conidia at regular intervals in DD. **B.** The *band* strain of *N. crassa* displays a free running conidiation rhythm in DD. Race tubes are marked daily (black lines) under a red safe light; the center of the conidiation band is marked with a black dot.

and Feldman, 1981; Gooch *et al.*, 1994; Nakashima and Feldman, 1980; Sargent *et al.*, 1966). Due to the ease of genetic and biochemical manipulation of this organism, the public availability of a complete genome sequence (www-genome.wi.mit.edu/annotation/fungi/neurospora), the ease of assay of the conidiation rhythm, and the mechanistic similarities between the molecular oscillator of *N. crassa* and those of higher eukaryotes, *N. crassa* serves to provide important information regarding basic clock mechanisms.

The FRQ oscillator

The most extensively characterized circadian oscillator in *N. crassa* is the FRQ-based oscillator. It consists of a transcription/translation-based feedback loop containing proteins that act as activators (WHITE-COLLAR 1 (WC-1) and WC-2) and inhibitors (FREQUENCY (FRQ)). The activators serve to facilitate transcription of the inhibitors, which then feed back to inhibit the activity of the activators. The inhibitors also function to positively regulate the levels of the activators, forming two interlocked feedback loops (Dunlap and Loros, 2004). This oscillator shares mechanistic similarity with other eukaryotic oscillators, such as those found in *Drosophila* and mammals (Harmer *et al.*, 2001).

The initial identification of *Neurospora* clock components was carried out using chemical and UV mutagenesis (Feldman and Hoyle, 1973; Feldman and Atkinson, 1978). The conidiation rhythms of the mutagenized survivors were assayed, and strains exhibiting altered FRPs and temperature compensation were selected for further study.

These initial studies identified over 20 mutations that affected these circadian properties, but one locus, the *frequency* locus, was represented in multiple strains from this screen, and was chosen for intensive study. The *frq* mutants exhibited FRPs ranging from 16 to 29 hours, and strains with long FRPs also displayed defects in temperature compensation. The *frq* alleles appeared to be clock-specific, as they do not affect any other cellular functions. These initial observations implied that *frq* encodes an important clock component (Feldman and Hoyle, 1973; Loros *et al.*, 1986). Subsequent studies included the cloning, sequencing, and knock-out of *frq*, as well as the generation of a strain containing *frq* under the control of an inducible promoter (Aronson *et al.*, 1994a; Aronson *et al.*, 1994b). These studies demonstrated that the loss of *frq* renders the organism arrhythmic, as does constituitive expression of *frq*. Rhythmic *frq* RNA is essential for circadian rhythmicity, and the phase of the developmental rhythm can be reset in a predictable manner by reductions in the amount of *frq* RNA. Overexpression of *frq* leads to a reduction in the amount of transcript originating from the native copy of this gene, suggesting that FRQ forms an autoregulatory feedback loop and inhibits its own expression (Aronson *et al.*, 1994b).

The FRQ protein contains several putative motifs, including a nuclear localization signal (NLS), two PEST domains, a helix-turn-helix motif, and conserved acidic and basic regions (McClung *et al.*, 1989). While orthologs of the FRQ protein are present only in fungi closely related to *Neurospora* (Lewis and Feldman, 1996; Merrow and Dunlap, 1994), sequence comparisons suggest that the most conserved region of the protein is an N-terminal coiled-coil motif (Lewis *et al.*, 1997). FRQ interacts with both

itself and the WC-1 and WC-2 proteins *in vivo*, and the coiled-coil region is essential for these interactions (Cheng *et al.*, 2001a). Interactions with the activator proteins WC-1 and WC-2 are necessary for normal clock function, as point mutations in FRQ that abolish these interactions render the organism arrhythmic (Cheng *et al.*, 2001a). The NLS is also essential for circadian function, removal of this sequence abolishes nuclear localization of FRQ, and renders the organism arrhythmic (Luo *et al.*, 1998). The PEST domains have been shown to be phosphorylated *in vitro* by casein kinase I (CKI) and deletion of these regions results in increased FRQ stability and lengthened FRP (Gorl *et al.*, 2001). There also exist two forms of the FRQ protein, arising from alternative translational start sites; however, while the choice of start site is regulated by temperature, no distinct activities have yet been attributed to these two forms (Garceau *et al.*, 1997).

The WC-1 and WC-2 proteins are also important components of the FRQ-oscillator. Both proteins contain zinc-finger DNA binding domains, transcriptional activation domains, and PAS (Per-Arnt-Sim) domains, as well as a LOV (Light-Oxygen-Voltage) domain in the case of WC-1 (Ballario *et al.*, 1998; Talora *et al.*, 1999). The PAS domains, which are protein:protein interaction domains that are common in clock proteins that are activators in circadian oscillators of other eukaryotic organisms, are required for homo- and heterodimerization of the WC proteins (Ballario *et al.*, 1996; Ballario *et al.*, 1998; Cheng *et al.*, 2002). The LOV domain binds flavin adenine dinucleotide (FAD) as a cofactor, and the WC-1 protein functions as a blue-light photoreceptor involved in the transduction of light information to the clock (Froehlich *et*

al., 2002; He *et al.*, 2002). Null strains of either *wc-1* or *wc-2* are arrhythmic (Russo, 1988), and both proteins are phosphorylated in response to light. WC-1 interacts with protein kinase C (PKC) in response to light, and PKC phosphorylates WC-1 *in vitro*. Overexpression of PKC results in a strong reduction in WC-1 levels (Franchi *et al.*, 2005). The WC-1 protein is also phosphorylated in DD, and mutation of several phosphorylation sites results in short period, low amplitude, or arrhythmic conidiation (He *et al.*, 2005). Together, FRQ, WC-1 and WC-2 form the FRQ-oscillator, and a model based on their activities is shown in Figure 3.

The *frq* gene is expressed in a circadian fashion; the mRNA levels peak at about circadian time (CT) 4. Circadian time divides an organism's FRP into 24 equal parts, and is a formalism used to compare strains and organisms with different FRPs. By convention, CT0 is defined as subjective dawn and CT12 as subjective dusk. Thus, *frq* mRNA peaks in the subjective morning. Levels of the FRQ protein peak around CT8, implying a delay between transcription and translation of the mRNA (Aronson *et al.*, 1994b; Garceau *et al.*, 1997). FRQ then moves into the nucleus, and this localization is required for free running rhythmicity (Luo *et al.*, 1998). Transcription of *frq* is dependent on both WC-1 and WC-2, which heterodimerize to form the WC complex (WCC). The WCC has been shown to bind to the *frq* promoter *in vitro* and presumably activate *frq* transcription, and FRQ interacts with the complex to inhibit the activity of the WCC, and thus, its own expression (Aronson *et al.*, 1994b; Cheng *et al.*, 2002; Denault *et al.*, 2001; Froehlich *et al.*, 2002; Garceau *et al.*, 1997; Merrow *et al.*, 2001). As the FRQ protein levels increase, the protein is phosphorylated by at least three

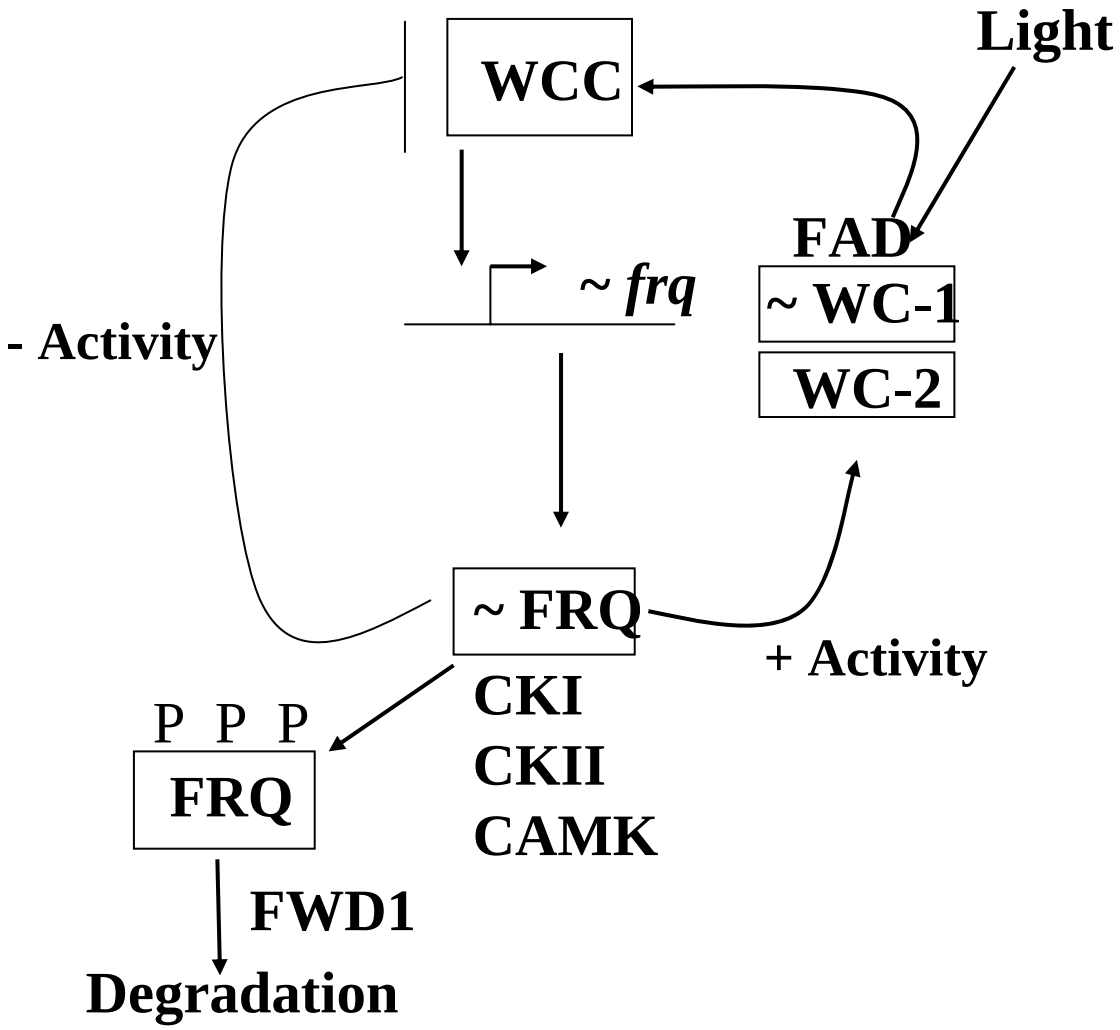

Figure 3. The current model of the FRQ oscillator. See text for details. P = phosphorylation, ~ = rhythmic accumulation.

kinases: a calcium/calmodulin-dependent kinase (CAMK-1), CKI and casein kinase II (CKII). This phosphorylation leads to the degradation of FRQ protein by a ubiquitin-mediated process, and is partially dependent on the F-box protein FWD-1 (Gorl *et al.*, 2001; He *et al.*, 2003; Yang *et al.*, 2001; Yang *et al.*, 2002). The build up of FRQ protein and its phosphorylation take about 14 hours, and this process appears to be a major contributor to the generation of a 22 hour FRP. FRQ also performs an activator role in this mechanism, and is thus also part of a positive feedback loop, serving to increase the levels of both WC-1 and WC-2 by an undescribed post-transcriptional mechanism. Levels of both WC-1 and WC-2 are low in a *frq*-null strain (Cheng *et al.*, 2001b; Lee *et al.*, 2000; Lee *et al.*, 2003). In the afternoon, when FRQ levels are high, WC-1 levels are low, but they begin to rise and peak antiphase to the peak in FRQ levels. While the WC-1 protein exhibits a low-amplitude circadian rhythm in abundance, both the *wc-1* and *wc-2* transcripts, as well as the WC-2 protein, are expressed at constant levels, thus, an uncharacterized post-transcriptional mechanism is thought to be responsible for WC-1 rhythmicity (Denault *et al.*, 2001; Lee *et al.*, 2000). By midnight, most FRQ protein has been degraded, and the WCC is now able to bind to the *frq* promoter and activate transcription. *frq* mRNA levels begin to rise and about 10 hours later they peak, thus completing the cycle (Figure 3).

A similar transcription/translation based circadian feedback loop also is present in organisms such as flies and mice, and, although the clock proteins are different, one common thread is that most clock proteins contain PAS domains. In these three organisms, the activators (WC-1 and WC-2 in *Neurospora*, CLOCK and CYCLE

(CYC) in *Drosophila*, and CLOCK and BMAL1 in mice) dimerize and activate expression of the inhibitors (FRQ in *Neurospora*, PERIOD (PER) and TIMELESS in *Drosophila*, and PER and CRYPTOCHROME in mice). The inhibitors in *Drosophila* and mice form heterodimers through their PAS domains and enter the nucleus to inhibit activation of their own transcription by the activators. The inhibitors are phosphorylated, leading to their degradation, as with FRQ, leading to reactivation of transcription by the activators. The circadian clocks of flies and mammals also contain a second feedback loop; rhythmic transcription of the activator proteins is directly controlled by VRILLE and PDP1ε in flies, and by REV-ERBα in mice. The genes encoding these proteins are, in turn, directly controlled by the activators CLK and CYC in flies, and CLK and BMAL1 in mice. The clocks of these organisms are thus composed of not one, but two interlocked feedback loops (Hardin, 2004).

Input to the FRQ-oscillator

The *N. crassa* clock is responsive to both light and temperature signals. Natural LD and temperature cycles serve to entrain the clock to local time, and this synchronization to the environment allows the fungus to coordinate and time physiological processes to the appropriate time of the day. Both light and temperature can reset the clock, and a light pulse given during the subjective night will delay the free running conidiation rhythm, while pulses during the subjective day do not significantly shift the phase of the rhythm (Crosthwaite *et al.*, 1995; Dharmananda, 1980). Changes in temperature can also reset the clock, and increases in temperature reset the clock to

dawn, while decreases in temperature set the clock to dusk (Liu *et al.*, 1998). Input of light and temperature information to the clock is beginning to be understood at the molecular level.

N. crassa is responsive to blue light, and several processes, including mycelial carotenoid biosynthesis, conidiation, orientation of perithecial beaks, and gene expression, are regulated by blue light. Both WC-1, the blue light photoreceptor, and WC-2 are integral to blue light perception in *N. crassa*. All blue light-regulated processes are insensitive to blue light in null mutants of *wc-1* or *wc-2* (Ballario and Macino, 1997; Degli-Innocenti and Russo, 1984; Harding and Turner, 1981). Blue light-responsive genes are directly bound by the WCC via consensus GATA promoter elements, and the *wc-1* and *wc-2* genes are also light inducible (Ballario *et al.*, 1996; Carattoli *et al.*, 1994; Linden and Macino, 1997). The WC-1 protein is rapidly and transiently phosphorylated in response to a light pulse, and no function has yet been attributed to this phosphorylation, though it does coincide with the transient induction of some light regulated genes (Schwerdtfeger and Linden, 2000).

Light resetting of the clock occurs by a rapid induction (within 5 min) of the *frq* transcript. This acts to set the clock to the morning, the time of day when *frq* levels peak. A light pulse given during the subjective night results in a rapid induction of *frq* mRNA, and resetting of the clock to the morning phase, while a light pulse given during the subjective day, when *frq* levels are already high, results in little or no phase shift (Crosthwaite *et al.*, 1995; Dharmananda, 1980) (Figure 4A). Light-mediated induction of

frq is dependent upon both the *wc-1* and *wc-2* genes (Collett *et al.*, 2002; Crosthwaite *et al.*, 1997).

The *N. crassa* FRQ oscillator can be entrained and reset by temperature cues. As mentioned above, two forms of the FRQ protein exist, a long form of 989 amino acids, and a short form of 890 amino acids. The long form is predominant at high temperatures, around 30°C, while the short form is the primary form present at low temperatures, around 20°C. Strains that make only the large form or only the small form are arrhythmic at low and high temperatures, respectively (Liu *et al.*, 1997). Temperature resetting is accomplished based on the total levels of FRQ in the cell. At high temperature (28°C), the total amount of FRQ is higher at the trough point of the cycle than the total amount at the peak point of the cycle at low temperature (21°C). A temperature decrease from 28°C to 21°C shifts the phase of the clock to the phase where FRQ is at its peak, which is the subjective evening, the time of day when the organism would be most likely to experience a temperature decrease in nature. A temperature increase from 21°C to 28°C shifts the phase of the clock to the phase where FRQ is at its trough, which occurs near the subjective morning. This results in a phase shift to the subjective morning, a phase where the organism would be most likely to experience an increase in temperature (Liu *et al.*, 1998) (Figure 4B).

Output from the FRQ oscillator

The circadian clock regulates a variety of processes in *N. crassa*; these are collectively called outputs. The conidiation rhythm is the most extensively characterized

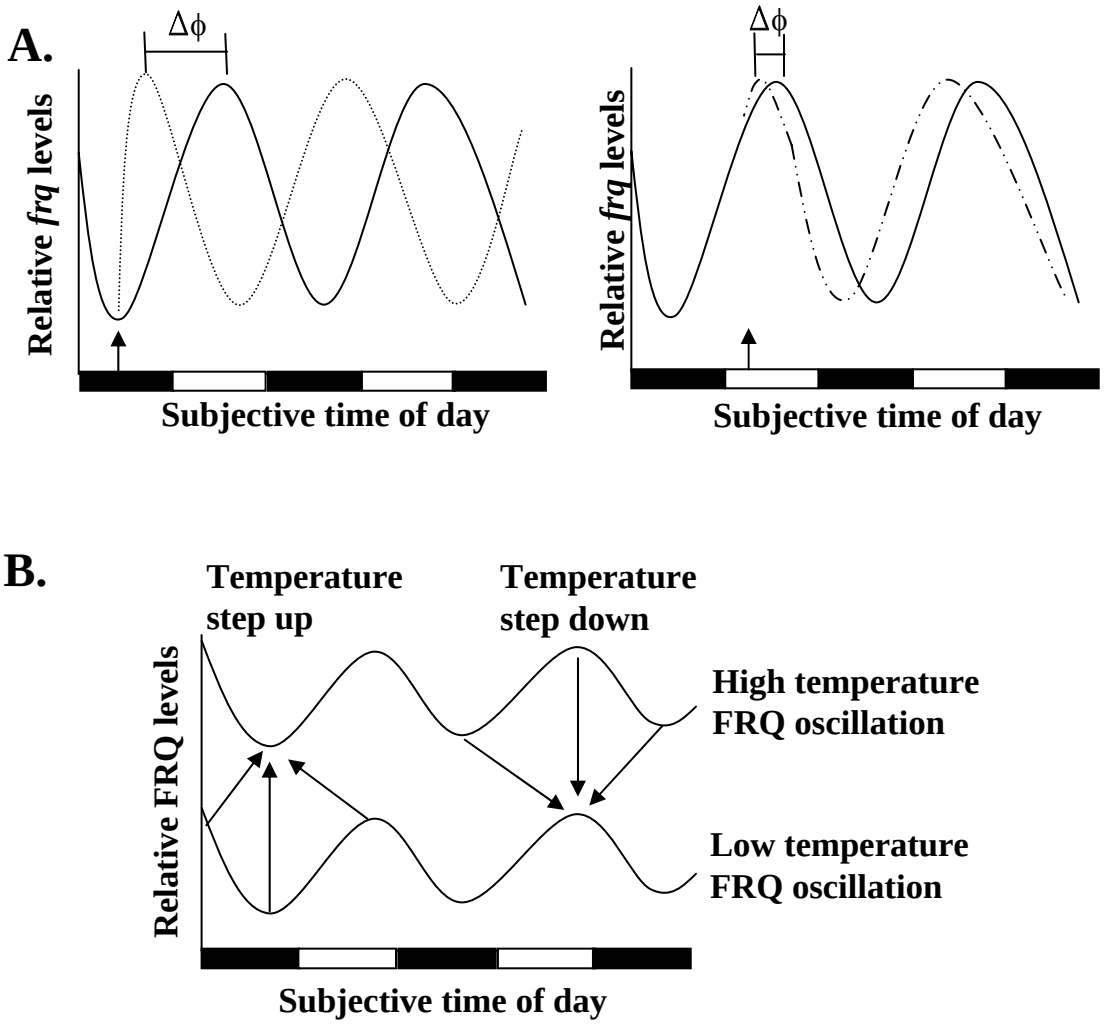

Figure 4. Light and temperature resetting of the FRQ oscillator in *N. crassa.* **A.** Light resets the FRQ oscillator through rapid induction of the *frq* transcript. A light pulse given during the subjective night resets the phase of the FRQ oscillator to morning (dotted lines), while a light pulse given during the subjective day has little or no effect on the phase (ϕ) of the oscillator. **B.** The overall levels of FRQ protein cycle at low levels in low temperature and higher levels at high temperature. When cultures are transferred from low to high temperature, the rhythm is reset to a phase corresponding to the low point in the circadian cycle (upward arrows). Transfer of cultures from high temperature to low temperature elicits the opposite effect, resetting the phase to the high point in the circadian cycle. Black bars = subjective night, white bars = subjective day, arrows = light/temperature shift. Figure adapted from Bell-Pedersen (2000).

output, but other outputs such as gene expression, enzymatic activity, CO_2 evolution and lipid metabolism have been described (Hochberg and Sargent, 1974; Loros *et al.*, 1989; Roeder *et al.*, 1982; Sargent and Kaltenborn, 1972; Shinohara *et al.*, 1998).

Recent research in *N. crassa* has revealed a number of genes controlled by the clock, however, the mechanisms controlling circadian gene expression in *N. crassa* remain a mystery. The initial experiments that demonstrated circadian control of gene expression were subtractive hybridization and differential screens (Bell-Pedersen *et al.*, 1996b; Loros *et al.*, 1989), which identified eight *clock-controlled genes* (*ccg*s). The *ccg*s are involved in a variety of cellular processes, including conidiation, glucose metabolism, mating and stress responses, highlighting the diversity of processes influenced by the clock. These *ccg*s all peak in expression in the late night to early morning, and mutations of these genes do not affect the normal functioning of the clock. Both *ccg-1* and *ccg-2* were shown by nuclear run-on assays to be controlled by the clock at the level of transcription (Loros and Dunlap, 1991).

More recent studies have utilized microarray technology to determine the extent of clock controlled gene expression in *N. crassa*. One study used microarrays representing roughly 1/7 of the genome, and found that transcript levels for 149 genes (20% of the genes on the array) were rhythmic in *N. crassa*. These genes encode proteins with diverse functions, and, importantly, were found to peak at all phases of the subjective day. Thus, the clock does not simply induce gene expression in the morning (Correa *et al.*, 2003). A separate study utilized a different microarray, representing about 1,100 genes. This study identified twenty genes expressed in a circadian fashion in DD,

all of which peak in transcript abundance during the late night to early morning (Nowrousian *et al.*, 2003). While neither study addressed the mechanisms controlling circadian gene expression, they supplied the circadian community with a number of potential candidate genes for control of rhythmic output. Despite the large number of *ccg*s identified to date, no output signaling component has yet been identified in *N. crassa*.

A complex clock: multiple oscillators in *N. crassa*

In the early days of circadian research, it was hypothesized that a circadian clock would consist of at least two mutually coupled oscillators, in order for an organism to maintain a stable phase relationship with its environment (Pittendrigh and Bruce, 1959). Recent years have witnessed the gradual buildup of evidence supporting the existence of multiple oscillators in *N. crassa*. A number of genes, such as the *period* genes, have been identified as mutations that affect the FRP or temperature compensation of the rhythm (Morgan *et al.*, 2001). The role played by these genes is unclear; they may be involved with the FRQ based oscillator, on an input or output pathway, or they may be components of a separate oscillator. Under certain growth conditions, *frq*-null strains are able to synchronize to temperature cycles, although this behavior is driven by the temperature cycle and does not show the characteristics of an entrained rhythm (Merrow *et al.*, 1999; Pregueiro *et al.*, 2005). Strains carrying null alleles of *frq* display conidiation rhythms under certain growth conditions. However, the FRP of these rhythms is variable, ranging form 12 to 35 hours, and is not temperature compensated.

The FRQ-null rhythm can be stabilized by the addition of farnesol or geraniol, and the rhythm can be entrained by temperature pulses (Granshaw *et al.*, 2003). Additionally, the *cel* and *chol* mutants, which have altered lipid metabolism, display rhythmic conidiation with variable FRPs, even in the absence of *frq* (Lakin-Thomas, 1998; Lakin-Thomas and Brody, 2000). These observations suggest that a second, temperature responsive oscillator that feeds into the conidiation pathway is present in the cell, and is activated or upregulated in response to farnesol and geraniol. This oscillator is referred to as the FRQ-less oscillator (FLO).

Other rhythms have also been observed in the absence of the FRQ oscillator. The *lm1* and *lm2* strains display rhythmic conidiation under conditions of constant light, and when combined with *frq* or *wc* null mutations, retain this rhythmicity (Suk-Seo, unpublished observations). Rhythms in nitrate reductase activity (Christensen *et al.*, 2004) and diacylglycerol levels (Ramsdale and Lakin-Thomas, 2000) persist in the absence of a functional FRQ oscillator (including loss of FRQ, WC-1 or WC-2), and the microarray experiments of Correa *et. al.* identified three genes that cycle with a ~24 hr. period in the *frq*-null strain (Correa *et al.*, 2003; Lewis *et al.*, manuscript in prep). These observations all indicate the existence of multiple FRQ-less oscillators (FLOs) that regulate different outputs in *N. crassa*. Some of the FLOs appear to require the FRQ oscillator for full circadian properties, such as stable rhythmicity with a ~24 hr. period, entrainment, and temperature compensation, whereas other FLOs appear to be more autonomous.

The evolution of fungal circadian clock genes

While our understanding of the molecular details of the circadian clock in *N. crassa* are rapidly advancing, relatively little is known regarding circadian rhythms in other fungal species. Circadian rhythms in spore discharge have been reported for the Zygomycete *Pilobolus sphaerosporus* (Bruce *et al.*, 1960), although no information concerning the molecular clock mechanism has arisen from studies of this organism. The degree to which circadian rhythms are found in the fungal kingdom and the diversity of process they control, is thus an intriguing question, and can help to elucidate the evolution of clocks in the fungi.

Homologs of the *N. crassa frq* gene have been identified in *Sordaria fimicola*, *Chromocrea spinulosa*, *Leptosphaeria australiensis* and *Podospora anserina* (Lewis and Feldman, 1996; Lewis *et al.*, 1997; Merrow and Dunlap, 1994). Two of these homologs partially rescue the phenotype of the *frq*-null strain of *N. crassa*, as chimeric *frq* genes containing both *N. crassa* and *S. fimicola* DNA can rescue circadian defects, while the full length *C. spinulosa frq* gene only rescues the pigment and conidiation phenotypes. The *L. australiensis frq* gene is unable to rescue any of the *frq*-null phenotypes in *N. crassa*. Thus, there is only a limited degree of functional conservation of the *frq* gene in these fungi.

To date, *frq* homologs have been identified only in the class Pyrenomycetes, members of the phylum Ascomycota. This group of fungi is distinguished by the presence of a perithecium, a fruiting body that can forcibly eject ascospores. The Pyrenomycetes include *Neurospora*, ergot and powdery mildews. The Pyrenomycetes

diverged from the other classes of the Ascomycota, about 240 million years ago (Berbee and Taylor, 2001). One of these other classes, the Plectomycetes, include medically and industrially important fungi such as *Aspergillus* and *Penicillium*, and are characterized by the presence of a cleistothecium fruiting body, which releases ascospores only on decay or disintegration. The absence of *frq* homologs from the Plectomycetes and other classes of fungi implies two possible situations regarding clock evolution in the fungi. First, the *frq* gene may have evolved in the Pyrenomycetes following their divergence from the Plectomycetes. Second, *frq* may have evolved in ancestral species prior to the divergence of the Plectomycetes and Pyrenomycetes, and was subsequently lost in the Plectomycetes or diverged significantly from the Pyrenomycete *frq* genes so as to be unrecognizable by sequence comparisons.

The Plectomycetes do contain homologs of both *wc-1* and *wc-2*, and deletions of both of the homologs *lreA* (*wc-1*) and *lreB* (*wc-2*) have been made in *Aspergillus nidulans*. Strains with deletions of either *lreA* or *lreB* have no apparent phenotype with respect to light regulated processes and development, thus, their function remains unclear, although no double deletion strain currently exists (H. Haas, unpublished data). The LREA and LREB proteins are both shorter than their *Neurospora* counterparts, being truncated at both the amino and carboxy-terminal ends. The LREA and LREB proteins contain conserved domains, including 3 PAS domains in LREA and one in LREB, as well as the GATA DNA-binding domains in both proteins. PAS domain proteins are also found in the clock proteins of many other species, including plants, flies and vertebrates, although multiple alignments of various PAS domains reveals low

similarity scores (Crosthwaite *et al.*, 1997). The LREA protein lacks the N- and C-terminal poly-Q domains, which are stretches of glutamine residues that have been implicated in transcriptional activation (Yuan *et al.*, 1991). Variations in the lengths of these poly-Q domains in field isolates of *N. crassa* have been demonstrated to affect the period length of the circadian rhythm (K. Lee *et al*., unpublished results). These data suggest that, if the first hypothesis regarding the evolution of FRQ is correct, in contrast to FRQ, the WC-1 and WC-2 proteins either arose prior to the divergence of the Plectomycetes and Pyrenomycetes, or that there has been less sequence divergence of these genes such that they can be recognized as orthologs. Additionally, the lack of poly-Q domains in the LREA and LREB proteins may indicate a clock-independent function for these proetins.

As fungal circadian rhythms are not unique to the Pyrenomycetes, a number of interesting questions arise, such as the identity of the components of these oscillators, as well as the mechanisms controlling circadian rhythmicity in other fungi. A tempting speculation is that the FRQ oscillator has evolved in the past 240 million years, and, given the evidence in *N. crassa* that other oscillators exist in the cell, analysis of circadian rhythms in other classes of fungi may elucidate the components of these FRQ-less oscillators. A candidate genus for these studies is *Aspergillus*. Genetic and biochemical research has been carried out in this organism for over fifty years, and much research has focused on asexual development, secondary metabolism and the cell cycle in this organism (Adams *et al.*, 1998; Osmani and Mirabito, 2004; Yu and Keller, 2005). *Aspergillus* is also, unlike *Neurospora*, agriculturally and industrially significant. While

Aspergillus does not form infection structures and is not considered a true plant pathogen, spores of *Aspergillus* will germinate and colonize crops such as corn and peanuts. Some species of *Aspergillus*, including *A. flavus* and *A. parasiticus*, make the toxic secondary metabolite aflatoxin. Presence of aflatoxin in crops at levels as low as 20 ppb can render corn crops unfit for animal consumption (USDA, 2002), and results in the loss of billions of dollars of crops annually.

Species such as *A. flavus* are difficult to work with in the laboratory setting, for instance, they have no known sexual state, and are thus unsuitable for traditional genetic analyses. Transformation of these species is difficult and few genetic markers exist in this species, however, the genome sequence of *A. flavus* has been completed recently and once it is available publicly, it will go a long way toward remedying the lack of genetic data for this organism. For these and other reasons, researchers have used *A. nidulans* as a model for *Aspergillus* biology. *A. nidulans* can be crossed readily in the lab, and many genetic and biochemical techniques have been devised for this organism over the past fifty years. *A. nidulans* has served as a model for aflatoxin biosynthesis, as it contains a cluster of genes required for aflatoxin biosynthesis. *A. nidulans*, however, lacks the genes encoding the final two enzymes in the aflatoxin pathway, and thus makes sterigmatocystin, the penultimate aflatoxin precursor, as a secondary metabolite (Brown *et al.*, 1996). Importantly, genetic evidence exists suggesting a connection between asexual development and sterigmatocystin biosynthesis in *A. nidulans* (Hicks *et al.*, 1997), and circadian rhythms in development in this species may imply circadian rhythms in toxin production, leading to novel targets for compounds designed to control

aflatoxin production. Investigations of circadian rhythms in *Aspergillus* and other fungal species will advance our understanding of the evolution of circadian rhythms in the fungi, and may lead to improved crop pathogen control strategies.

Objectives

Research in *Neurospora* and other systems has provided much information regarding the molecular mechanisms involved in the control of circadian rhythms. My work focuses on both mechanisms involved in control of circadian output in *N. crassa*, as well as the characterization of unique oscillators in both *N. crassa* and *Aspergillus*. Relatively little is known about circadian output pathways in *Neurospora*, and the question of clock evolution within the fungal kingdom remains to be addressed. My overall goal is to identify and characterize novel oscillators and output pathways in *Neurospora* and *Aspergillus*. In order to accomplish this goal, I have examined the following hypotheses:

1. The clock-controlled putative serine-threonine protein kinase *nrc-2* (*non-repressible conidiation gene-2*) is involved in the transduction of time of day information from the FRQ oscillator to circadian output processes in *N. crassa* (Chapter II). To test this hypothesis, I assayed expression of *ccg*s and FRQ oscillator components in an *nrc-2* insertional mutant strain. I found, in contrast to my hypothesis, that the *nrc-2* gene is involved in nutritional compensation of the circadian rhythm of *N. crassa*, and that circadian output is glucose-dependent in this strain. I also found that two forms of the WC-1 protein exist in wild type *N.*

crassa, and the *nrc-2* mutation alters the ratio of the two forms. These data suggest that *nrc-2* is involved in metabolic input to the FRQ oscillator at the level of choice of WC-1 translational start site.

2. A FRQ-less oscillator is involved in the control of rhythmic *ccg-16* expression (Chapter III). To test this hypothesis, I assayed rhythms in the WC-1 protein in a *frq*-null strain, and in LL, conditions under which the *ccg-16* is rhythmically expressed. I found that the WC-1 protein is rhythmic independent of FRQ protein; under conditions where FRQ is expressed at high levels (LL), WC-1 is rhythmic, and it is also rhythmic in a *frq*-null strain. As rhythmicity of the *ccg-16* gene is dependent on WC-1 but not on FRQ, these data indicate that WC-1 serves as a bridge between two oscillators in *N. crassa*.

3. *Aspergillus* can serve as an alternative model for fungal circadian clocks (Chapter IV). To test this hypothesis, I first established the presence of circadian rhythms of development in a field isolate of *A. flavus* and gene expression in a laboratory strain of *A. nidulans*. That these species display circadian rhythmicity is notable in that they harbor orthologs of the WC-1 and WC-2 genes (*lreA* and *lreB*, respectively), but no gene with significant similarity to FRQ exists in the genome sequence. The circadian rhythms in these fungi are light and temperature entrainable, but have very long FRPs ~33hr, and entrain to LD cycles in a unique manner. These data suggest that the circadian clock of *Aspergillus* is substantially different from that of *Neurospora.*

The results of this research are presented in the following chapters. Chapter II represents work that is in preparation for publication. Chapter III represents work that has been submitted for publication. Chapter IV is a published manuscript (Greene *et al.*, 2003). Each chapter contains an introduction and a discussion. Chapter V contains a summary of the work presented here, as well as conclusions and future research directions. Appendix A details efforts to establish a luciferase reporter system in *Aspergillus*. Appendix B describes the deletion of the *N. crassa velvet A* gene, and characterization of the circadian phenotype of the resulting deletion strain.

CHAPTER II

INACTIVATION OF THE PUTATIVE SERINE-THREONINE PROTEIN KINASE NRC-2 RESULTS IN A GLUCOSE-DEPENDENT LOSS OF CIRCADIAN RHYTHMICITY IN *NEUROSPORA CRASSA*

Summary

The putative serine-threonine protein kinase *nrc-2* was identified through microarray experiments as controlled by both circadian and developmental signals (Correa *et al.*, 2003). In this study, we investigate the role of this gene in circadian rhythms of *N. crassa*. Our initial data indicated that, under standard *ccg* assay conditions (0.03% glucose), output from the FRQ oscillator is disrupted in the *nrc-2^{pRAL-1}; bd* mutant strain. Free running rhythms in FRQ-dependent gene expression are abolished in the *nrc-2^{pRAL-1}; bd* mutant strain, and conidiation rhythms are undetectable. These defects are thought to arise downstream of the FRQ oscillator since light input to the oscillator and rhythms in the clock proteins FRQ and WC-1 are unaffected under high glucose (2.0%) conditions in the *nrc-2^{pRAL-1}; bd* mutant strain. Rhythmicity of the FRQ oscillator-independent gene *ccg-16* is unaffected in the *nrc-2^{pRAL-1}; bd* mutant strain. Thus, the role of *nrc-2* appeared to be specific to output from the FRQ oscillator. However, additional recent experiments call the role of *nrc-2* in circadian output into question because the *nrc-2^{pRAL-1}; bd* mutant strain was shown to have rhythmic

conidiation and gene expression on high glucose medium. We also establish the presence of two forms of the WC-1 protein, an integral clock component, in wild-type *N. crassa*. The *nrc-2*$^{pRAL-1}$*; bd* mutant strain makes primarily the larger of the two forms, suggesting that NRC-2 is required for proper choice of translational start site of the WC-1 protein. Three possible models for NRC-2 function are proposed; one suggests that NRC-2 is required for amplification of output signals from the FRQ oscillator in a glucose-dependent manner, such that under low glucose conditions, rhythmicity is abolished. A second model proposes that NRC-2 provides metabolic input to the FRQ oscillator at the level of choice of WC-1 translational start site. A third model posits that NRC-2 is required to maintain a certain level of FRQ and/or WC-1 expression under limited glucose conditions. Experiments to discern between these three models are also discussed.

Introduction

Microarray studies were performed by our group (Correa *et al.*, 2003), both in constant darkness (DD) and following developmental induction, with the objective of identifying clock-controlled signaling components that are also developmentally regulated, and ultimately elucidating the signaling pathway connecting the oscillator with rhythmic conidiation. Several candidate genes emerged from this study, one of them being *nrc-2* (*nonrepressible conidiation gene 2*), a putative serine-threonine protein kinase with strong similarity to the kinase domain of *Arabidopsis* NPH-1 (Huala *et al.*, 1997), as well as the putative serine-threonine kinases encoded by *kad5* from *S. pombe*

(Kothe and Free, 1998) and the *KIN82* gene from *S. cerevisiae* (Wilson *et al.*, 1992). The *nrc-2* transcript peaks in expression during the subjective night, prior to the time when *Neurospora* begins daily conidiation, and *nrc-2* also is induced 30 minutes to one hour following developmental induction. Based on these two observations from our microarray experiments (Correa *et al.*, 2003), combined with the strong similarity of the *nrc-2* gene product to other kinases, we reasoned that *nrc-2* was a good candidate for signaling time-of-day information from the FRQ oscillator to the conidiation output. An *nrc-2* mutant strain (*nrc-2*$^{pRAL-1}$) was available (Kothe and Free, 1998), and we chose to characterize its function in the *N. crassa* circadian system.

The *nrc-2* gene was identified in an insertional mutagenesis screen based on overexpression of a *ccg-1:tyrosinase* reporter on sorbose agar medium (Kothe and Free, 1998). The alteration in *ccg-1* expression in the *nrc-2* mutant provided additional stimulus for this study; misexpression of *ccgs* has previously been used by our group as a basis for the isolation of clock-affecting mutants (Vitalini *et al.*, 2004). The mutated *nrc-2* gene contains a plasmid insertion in the second intron of the coding sequence, resulting in inactivation of the gene. The *nrc-2*$^{pRAL-1}$ strain lacks normal vegetative hyphae and does not complete the conidiation process, displaying a conidial separation defect and a budding mode of growth on sorbose agar, suggesting that the mutant is unable to repress entry into the conidiation program (Kothe and Free, 1998). The *nrc-2*$^{pRAL-1}$ strain also grows slowly relative to wild-type *N. crassa*, a phenotype also observed in the *S. cerevisiae KIN82* mutant (http://www.yeastgenome.org).

This report describes the characterization of the effects of the *nrc-2* mutation on circadian rhythms of *N. crassa*. Specifically, we employ molecular techniques to examine rhythmicity of clock-controlled genes (*ccgs*) and FRQ oscillator proteins, as well as light and developmental regulation of clock components and *ccgs*. We confirm by northern blot analysis that the *nrc-2* gene is under clock control, with peak expression occurring during the subjective night. Our initial conclusions were that *nrc-2* is a circadian output from the FRQ oscillator, based on the lack of rhythms in FRQ-dependent *ccgs* and the robust rhythms in high glucose medium in both FRQ and WC-1 in the *nrc-2*$^{pRAL-1}$*; bd* strain. However, the role of *nrc-2* as an output from the FRQ oscillator has been modified based on the observation that the *nrc-2*$^{pRAL-1}$*; bd* mutant displays robust rhythms in conidiation and gene expression in non-standard high glucose medium. Mutation of *nrc-2* results in a glucose-dependent loss of circadian rhythmicity in conidiation, as well as FRQ oscillator-dependent genes. This medium-dependent loss of rhythmicity indicates a loss of nutritional compensation of circadian rhythms in the *nrc-2*$^{pRAL-1}$*; bd* mutant. The conidiation rhythm of wild-type *N. crassa* is nutritionally compensated (Loros and Feldman, 1986). On a variety of growth media containing different carbon and nitrogen sources, the free-running period of the rhythm remains the same, even though the rate of growth is variable. Mutations in other genes are also known to affect nutritional compensation, most notably in the *frq*^{9} allele, in which the free-running period is dependent on both temperature and the amount of carbon source present in the growth medium (Loros and Feldman, 1986). Finally, we observe that two forms of the clock component WC-1 exist in wild-type *N. crassa*, but only the larger of

the two forms is present in a *frq*-null strain, and the large form is the predominant form in the *nrc-2^{pRAL-1}; bd* mutant. We thus conclude that circadian rhythms in the *nrc-2^{pRAL-1}; bd* mutant are sensitive to carbon source levels, and *nrc-2* functions to influence the choice of WC-1 translational start site.

Materials and methods

Strains

The *nrc-2* mutant GTH16-T17 (*al-2; nrc-2^{pRAL-1}; aro-9; inv; qa-2; a; ccg1-tyrosinase*) (Kothe and Free, 1998) was kindly provided by Dr. Steve Free (SUNY Buffalo, NY). GTH16-T17 was crossed to strain 30-7 (*bd; A*), to obtain strain 167-403 (*al-2; nrc-2^{pRAL-1}; bd; a; ccg-1-tyrosinase*), which was used in all subsequent studies and is referred to as the *nrc-2^{pRAL-1}; bd* strain in this manuscript. The *band* (*bd*) mutation allows the circadian rhythm of conidiation to be easily monitored on closed race tubes, but does not affect the clock itself (Sargent *et al.*, 1966) and is considered the wild-type clock strain. The *nrc-2^{pRAL-1}; bd* strain is hygromycin resistant as the *ccg1-tyrosinase* construct contains the bacterial hygromycin resistance gene as a selectable marker. The *bd; A* strain FGSC 1858 (Fungal Genetics Stock Center, University of Kansas, Kansas City, KS), and *frq*-null strain 40-1 (*bd; frq^{10}; A*) were used in all subsequent studies.

Race tube cultures

Race tube assays were performed using either standard medium (Vogel's minimal medium with 0.3% glucose and 0.5% arginine plus 2% agar, pH 5.8) or high

glucose medium (Vogel's minimal medium containing 2% glucose and 2% agar, pH 5.8) as indicated. 14 mL of medium was used per race tube. Cultures were inoculated with dry conidia on one end, and allowed to germinate and grow in constant light (LL) for 24 hours at 30°C. Growth fronts were then marked and tubes were moved to constant darkness (DD) at 25°C for the remainder of the experiment. Growth fronts were marked every 24 hours to determine growth rate. Calculation of free-running period was performed as described (Dharmananda, 1980).

Timecourse experiments and northern blot analysis

Timecourse experiments were performed in liquid shake cultures (Nakashima *et al*., 1981; Loros *et al.*, 1989). Culture inoculum was obtained by culturing the *nrc-2*$^{pRAL-1}$*; bd* strain in stationary liquid cultures of high glucose Fries' medium (Fries' minimal medium containing 0.3% glucose, 0.5% arginine, 50 μg/ml biotin, pH 5.8) or 2% glucose Vogel's medium, pH 5.8 for 36-48 hours at 30°C. Mycelial disks were cut from the resulting mat using a size 7-9 cork borer and were transferred into liquid shake cultures. The *bd* and *bd; frq*^{10} strains were cultured in the same manner as above, except they were cultured for 20-30 hours prior to mycelial disk removal using a size 1 cork borer for 2% glucose medium, or a size 7-9 cork borer for low glucose Fries' medium. Liquid shake media were either low glucose Fries' (Fries minimal medium containing 0.03% glucose, 0.05% arginine, 50 μg/ml biotin**,** pH5.8) or 2% glucose Vogel's medium, pH 5.8, as indicated. Low glucose Fries' cultures were grown in 25 ml of medium in 50 ml Erlenmeyer flasks, while 2% glucose Vogel's cultures were grown in

125 ml of medium in 250 ml Erlenmeyer flasks. Cultures were allowed to grow for a minimum of one hour in constant light (550 lux) at 30°C, prior to transfer to constant darkness at 25°C, a treatment which sets the oscillator to dusk (Bell-Pedersen *et al.*, 1996b). Transfers were made at staggered intervals such that all samples are of similar age (within 8 h) at time of harvest, but represent different phases over two circadian cycles (Loros *et al.*, 1989). Cultures were harvested and RNA extracted as previously described (Yarden *et al.*, 1992) using RNA extraction buffer (0.1M sodium acetate, 1mM EDTA pH 8, 4% sodium dodecyl sulfate). Total RNA was quantified and 10μg of each sample was subjected to denaturing agarose gel electrophoresis (Lehrach *et al.*, 1977). Following electrophoresis, evenness of gel loading was determined by ethidium bromide stain. Gels were then washed and transferred to nitrocellulose membrane using 2X SSC. Efficient transfer was determined by observation of ethidium stained rRNA under short wave UV light, followed by UV crosslinking of the RNA to the membrane. Blots were subsequently prehybridized in 0.1% sodium pyrophosphate, 1 M sodium chloride, 50 mM Tris-HCL pH 7.5, 10X Denhardt's solution, 50% formamide, 100 μg/mL salmon sperm DNA for a minimum of 30 minutes, followed by addition of radiolabeled probe. For *ccg-16* blots, T3 and T7 primers were used to amplify the *ccg-16* coding sequence from plasmid pW06H2 (Nelson *et al.*, 1997). The PCR product was subsequently gel purified and used as a template for random primed DNA synthesis (DECAprime, Ambion, Inc. Austin, TX). RNA probes were used for all other genes examined in this study, and were generated using the plasmids pSP06F4 for *nrc-2* (Nelson *et al.*, 1997), pKL119 for *ccg-1* (Lindgren, 1994), pLW1K for *ccg-2* (Bell-

Pedersen *et al.*, 1992), pccg-7 for *ccg-7* (Shinohara *et al.*, 1998), and pKAJ106 for *frq* (Crosthwaite *et al.*, 1995)**,** and the Maxiscript Kit (Ambion, Austin, TX) labeled with 6000 Ci/mmol ^{32}P-UTP. T3 RNA polymerase was used for all riboprobes except for the *frq* probe, which was synthesized using T7 RNA polymerase. Probe hybridization proceeded overnight at 42°C for DNA probes or 58°C for riboprobes. Non-specific signal was washed from the membrane using two washes of 1X SSC + 0.1% SDS for 15 minutes at room temperature, followed by two washes in 0.2X SSC + 0.1% SDS at 58°C. Detection of probe was accomplished using film (Kodak X-OMAT Blue XB-1 film, Eastman Kodak Co., Rochester, NY). Densitometry was performed using NIH image 1.62.

Developmental induction

Developmental induction experiments were performed by growing stationary liquid starter cultures of the *nrc-2*$^{pRAL-1}$*; bd* strain for 36-40 hours in 2% glucose Vogel's medium, pH 5.8 and the *bd* strain for 20-24 hours in the same medium at 30°C. Mycelial mats were harvested and desiccated using a piece of Whatman paper in a Buchner funnel and application of a vacuum. Desiccated mycelia on filter paper were then laid on top of glass beads soaked in 2% glucose Vogel's medium, pH 5.8 at 25°C, such that the paper was damp, but the mycelia were not submerged in the medium. This treatment has been previously shown to induce developmentally regulated genes in *Neurospora* (Berlin and Yanofsky, 1985a). RNA was harvested and analyzed as above, although the experiment

was carried out in LL (950 lux) and the samples were collected for up to four hours following desiccation and air exposure.

Light induction

Stationary liquid starter cultures were grown in 2% glucose Vogel's medium, pH 5.8 for 36-40 hours (*nrc-2^{pRAL-1}; bd*) or 20-24 hours (*bd*) at 30°C. Liquid shake cultures in 2% glucose Vogel's medium (125 ml in 250ml Erlenmeyer flasks) were inoculated with mycelial disks cut using a size 7-9 cork borer (*nrc-2^{pRAL-1}; bd*) or a size 1 cork borer (*bd*). Cultures were then grown for 24 hours in LL (950 lux) at 25°C, followed by transfer to DD for 24 hours at 25°C. Light pulses were then administered by transferring cultures into the light (950 lux) at 25°C, followed by harvest 15 or 30 minutes later. RNA isolation and northern analysis were carried out as above.

Protein extraction and detection

All starter and liquid shake cultures were performed in 2% glucose Vogel's medium, pH 5.8. Starter cultures were stationary liquid cultures, and for liquid shake cultures 125 ml medium was used in 250 ml Erlenmeyer flasks. Timecourse assays were performed as stated above for RNA analysis. Harvesting of samples was performed as described above, with the exception that cultures were pressed between stacks of paper towels to dry the mycelia as much as possible prior to snap freezing in liquid nitrogen. Mycelia were ground to a powder using a mortar and pestle under liquid nitrogen, and total protein was extracted using 500µl protein extraction buffer (50 mM HEPES, 137

mM KCL, 10% glycerol, 1 mM EDTA, 10 μl/mL HALT protease inhibitor (Pierce Biotechnology Inc., Rockford, IL)). Total protein was quantified using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). 100 μg of total protein was subjected to SDS-PAGE in each experiment shown. For FRQ westerns, SDS-PAGE gels consisted of 8% acrylamide (29:1 acrylamide:bisacrylamide) resolving gels with 5% acrylamide (29:1) stacking gels. WC-1 westerns made use of SDS-PAGE gels with 5% acrylamide (74:1) resolving gels with 5% acrylamide (29:1) stacking gels. The remaining composition of these gels is as described in (Sambrook *et al.*, 1989). Gels were usually run overnight (16-20 hrs.) at 15-25 mA. This is especially important for WC-1 in order to discern the two forms of the protein. Following electrophoresis, protein was transferred to nitrocellulose membrane in 20% methanol, 0.2M glycine, 25mM Tris at 100 mA for 12-16 hours. Western blotting was accomplished using either mouse α-FRQ or mouse α-WC-1 antibodies (at 1/40 dilutions), both of which were kindly supplied by Dr. Martha Merrow (University of Munich, Munich, Germany). The α-FRQ antibody is specific to the large form of the FRQ protein. Secondary antibodies (goat anti-mouse HRP conjugate) were purchased from Bio-Rad Laboratories, Hercules, CA, and used at 1/5000 dilutions. Detection was accomplished using the SuperSignal West Femto Maximum Sensitivity Substrate (Pierce Biotechnology Inc., Rockford, IL) and Kodak film (Kodak X-OMAT Blue XB-1 film, Eastman Kodak Co., Rochester, NY).

Protein stability assay

Starter cultures were grown in stationary liquid 2% glucose Vogel's medium for 36-40 hours (*nrc-2*$^{pRAL-1}$*; bd*) or 20-24 hours (*bd*) at 30°C. Disks were then cut from the resulting mycelial mats with a size 1 cork borer (*bd*) or a size 7-9 cork borer (*nrc-2*$^{pRAL-1}$*; bd*) and transferred into liquid shake culture consisting of 2% glucose Vogel's medium (125 ml medium in 250 ml Erlenmeyer flasks). Cultures were allowed to grow for 24 hours in LL (950 lux) at 25°C, then were transferred to DD for another 20 hours at 25°C, at which time 10 μg/mL cycloheximide was added. Harvests were performed immediately, 1 hour following cycloheximide treatment, and then every 2 hours following treatment for up to 9 hours total. Mycelia were harvested, extracted, and subjected to western analysis as above.

Phosphatase treatments

Starter cultures were grown in stationary liquid 2% glucose Vogel's medium for 36-40 hours (*nrc-2*$^{pRAL-1}$*; bd*) or 20-24 hours (*bd*) at 30°C. Disks were then cut from the resulting mycelial mats with a size 1 cork borer (*bd*) or a size 7-9 cork borer (*nrc-2*$^{pRAL-1}$*; bd*) and transferred into liquid shake culture consisting of 2% glucose Vogel's medium or 2% glucose Westergaard's medium (125 ml medium in 250 ml Erlenmeyer flasks). Cultures were harvested at 48 hours of total age, after 24 hours in DD, 24 hours of LL, or following a 30-minute light pulse (950 lux), all at 25°C, and total protein was extracted as above. 100 μg of total protein was treated with 400 U of λ protein phosphatase in the buffer provided (New England Biolabs, Beverly, MA) for 45 minutes

at 37°C. Untreated samples were mock-treated in phosphatase bufer and $MnCl_2$ for 45 minutes at 37°C. SDS-PAGE electrophoresis was subsequently performed as described above for WC-1 detection.

Results

The nrc-2 *transcript oscillates with a circadian period and peaks in the early subjective night*

In order to verify that *nrc-2* was indeed a night-specific clock-controlled gene (*ccg*), total RNA was isolated from cultures grown in DD for two circadian cycles (see Materials and Methods) and *nrc-2* transcript levels were determined by northern blot. In the clock wild-type *band* (*bd*) strain, the *nrc-2* transcript cycled with a 20-24 hour period with peak abundance at about CT 15-20, in agreement with previous microarray data (Correa *et al.,* 2003). Additionally, in the *frq*-null strain frq^{10}, the rhythm of *nrc-2* transcript abundance was abolished (Figure 5A and B). Together, these results indicated that *nrc-2* is a night-specific *ccg*, the rhythmic abundance of which requires a functional *frq* gene.

No nrc-2 *transcript is detectable in the* nrc-2^{pRAL-1} *strain*

In order to determine if the *nrc-*2^{pRAL-1} mutant was a true null allele, we performed northern blot analysis on dark grown liquid shake cultures of the *nrc-*2^{pRAL-1} mutant. No transcript was detected in the *nrc-*2^{pRAL-1}*; bd* strain (Figure 5C). Thus we conclude that this is indeed a null strain. Subsequent deletion of the entire *nrc-2* coding

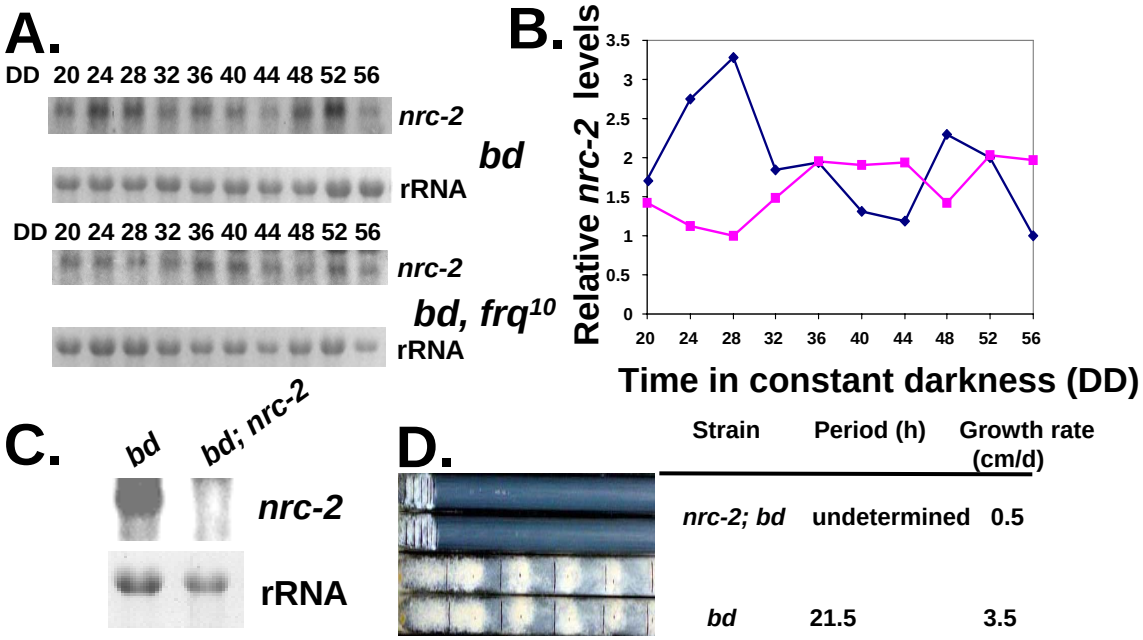

Figure 5. The *nrc-2* transcript is under clock control and a null mutation in this gene severely affects growth rate. A. The *nrc-2* transcript cycles in abundance with a circadian period, and this requires a functional FRQ oscillator. Northern blots of mRNA isolated from mycelia grown in 0.03% glucose + 0.05 % arginine Fries' medium every 4 hours for 2 consecutive days in DD were probed with an *nrc-2* specific riboprobe. Shown at top are representative results from three independent experiments obtained from FGSC 1858 (*bd; A*), results at bottom are from 40-1 (*bd; frq^{10}; A)*. Ethidium stained rRNA was used as a loading control. **B.** Densitometry of blots in (A). *nrc-2* transcript levels were normalized using ethidium stained rRNA as a loading control, and the lowest level of transcript for each strain was set to 1.0. Results for the *bd* strain are shown as blue diamonds, and results for the *bd, frq^{10}* strain are shown as pink squares. **C.** The *nrc-2$^{pRAL-1;}$ bd* strain contains no detectable *nrc-2* transcript. Cultures of FGSC 1858 (*bd*) and the *nrc-2^{pRAL-1}* strain were grown in 2% glucose Vogel's medium in constant darkenss for 38 hours prior to RNA harvest. Similar results were seen in 0.03% glucose + 0.05% arginine (not shown). Fries' medium. Ethidium stained rRNA is shown as a loading control. **D.** The *nrc-2^{pRAL-1}; bd* strain is arrhythmic. Race tube assays of the *nrc-2^{pRAL-1}* strain are shown (top 2 tubes) along with the wild-type *bd* strain (bottom 2 tubes). The period of conidiation is shown for the *bd* strain, and the growth rates (cm/day) are shown for both strains. Growth medium was Vogel's minimal medium with 0.3% glucose + 0.5% arginine.

region yielded a strain phenotypically indistinguishable from the *nrc-2^{pRAL-1}; bd* mutant (T. Lamb, unpublished observations).

Free-running conidiation rhythms are not detectable in the nrc-2 *mutant background*

To determine if *nrc-2* is required for circadian clock function and rhythmic development, the *nrc-2* strain GTH16-T17 (Kothe and Free, 1998) was crossed with the *bd* strain in order to obtain *nrc-2^{pRAL-1}; bd* progeny. Under conditions of DD and standard growth medium (1X Vogel's + 0.3%glucose + 0.5% arginine) at 25°C (Sargent and Kaltenborn, 1972), the *nrc-2^{pRAL-1}; bd* strain grew very slowly (0.5 cm/day) relative to the *bd* strain (3.5 cm/day), and no rhythm in conidiation was detectable in the *nrc-2^{pRAL-1}; bd* strain (Figure 5D). However, conidiation rhythms, if present, would be difficult to detect due to the extremely slow growth rate of the mutant. Therefore, this assay did not provide a useful measure of clock activity in the mutant strain.

The nrc-2 *mutation affects rhythms of a subset of* ccgs

In the absence of rhythmic conidiation on race tubes in the *nrc-2^{pRAL-1}; bd* mutant, we turned to molecular methods to assay the relationship between *nrc-2* and the circadian clock. If *nrc-2* is involved in the *Neurospora* clock, then this should be detectable by an alteration in clock output. The expression pattern of several *ccgs* was analyzed under DD in standard liquid shake medium (1X Fries + 0.03% glucose + 0.05% arginine) (Bell-Pedersen *et al.*, 1996b; Loros *et al.*, 1989). In the *nrc-2^{pRAL-1}; bd* mutant strain, levels of the morning-specific, *frq*-dependent *ccg-1* and *ccg-2* transcripts

were arrhythmic (Figure 6). Interestingly, the levels of these transcripts were reminiscent of a *frq*-null strain (Bell-Pedersen *et al.,* 1996a; Lindgren, 1994; Vitalini *et. al*, 2004), that is, *ccg-1* levels fluctuated around wild-type peak levels, while *ccg-2* levels were drastically reduced relative to wild-type. Long exposures of northern blots probed with *ccg-2* also revealed that the *ccg-2* transcript is arrhythmic. The high *ccg-1* levels in particular were not surprising, given that *nrc-2* was initially identified in a screen for mutations that resulted in *ccg-1* misexpression (Kothe and Free, 1998).

Both the *ccg-1* and *ccg-2* genes are induced during asexual development, as is *nrc-2* (Correa *et al.*, 2003; Lauter *et al.*, 1992; Lindgren, 1994). It is possible that *nrc-2* is necessary for proper expression of developmentally regulated genes. To ascertain whether *nrc-2* was only affecting the expression of developmentally regulated genes, we also monitored expression of *ccg-7,* which is not developmentally regulated (Bell-Pedersen *et al.*, 1996b) and encodes glyceraldehyde-3-phosphate dehydrogenase, a key glycolytic enzyme (Shinohara *et al.*, 1998). Similar to our results with *ccg-1* and *ccg-2*, *ccg-7* was arrhythmic in the *nrc-2*$^{pRAL-1}$*; bd* strain (Figure 6). Thus, *nrc-2* is not simply required for circadian rhythms of a subset of developmentally regulated *ccgs*.

The majority of *ccgs* peak in the late night to early morning; however, microarray experiments have recently shown that *ccgs* peak at varying phases throughout the subjective day (Correa *et al.*, 2003). One evening-specific gene, *ccg-16,* was shown to accumulate rhythmic mRNA in the absence of a functional FRQ oscillator. Consistent with a role for *nrc-2* in the FRQ oscillator or its outputs, *ccg-16* remained rhythmic with a similar phase and levels in the *nrc-2*$^{pRAL-1}$*; bd* mutant strain (Figure 6).

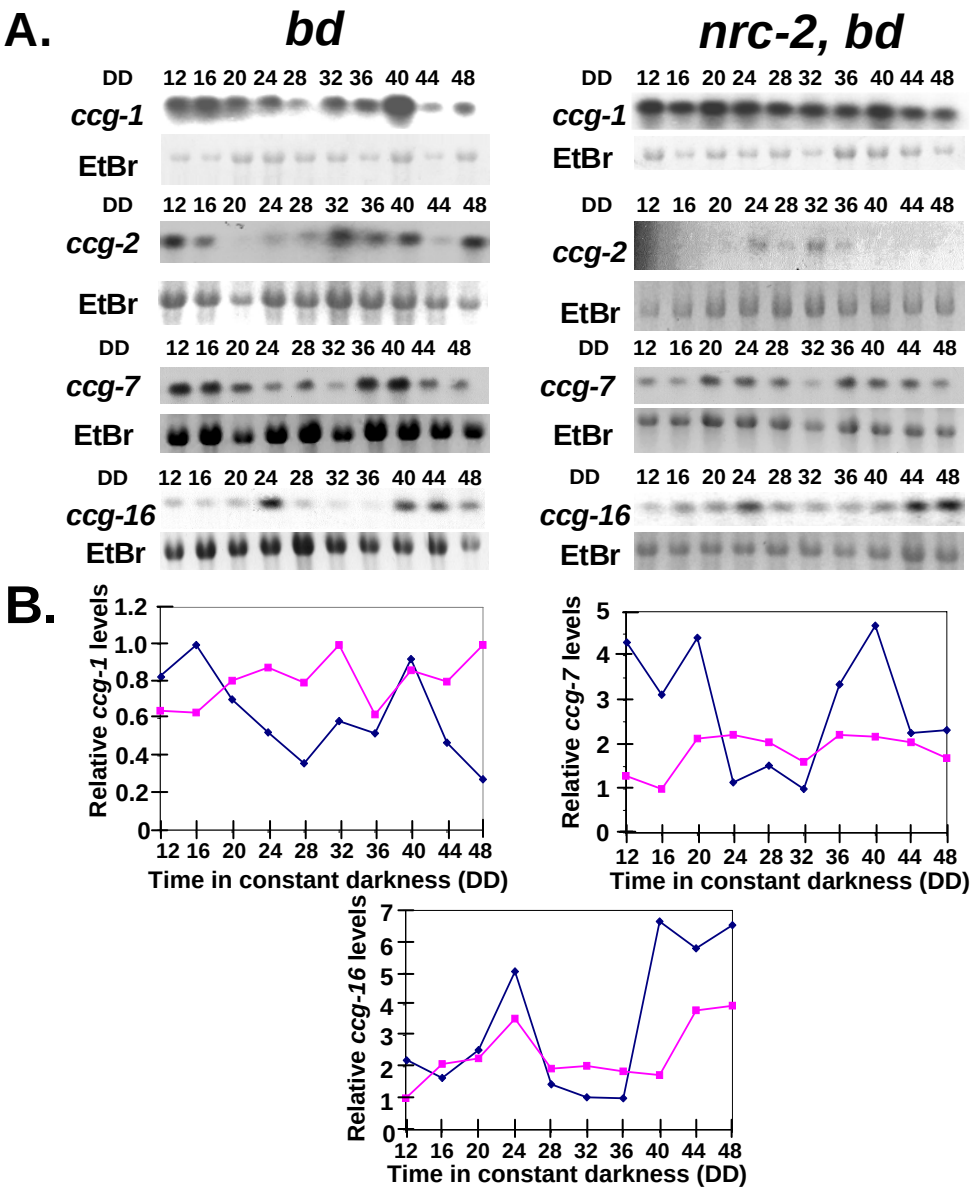

Figure 6. Rhythmicity of some clock-controlled genes is disrupted in the *nrc-2; bd* strain.
A. Northern blot analysis of four *ccgs* cultured in constant darkness was performed. Strains were cultured in Fries' minimal medium with 0.03% glucose and 0.05% arginine. Shown at left are results from strain FGSC 1858 (*bd, A*), at right are results from strain 167-403 (*al-2; nrc-2*$^{pRAL-1}$*; bd; a; ccg-1-tyrosinase*). Ethidium bromide stained rRNA is shown as a loading control. *ccg-1*, *-2*, and *-16* blots are representative of 5 independent experiments; *ccg-7* results are representative of 2 independent experiments. **B.** Densitometry of blots shown in (A). Due to the low levels of expression of *ccg-2* in the *nrc-2*$^{pRAL-1}$ strain, this gene was not normalized. Ethidium bromide stained rRNA was used to normalize transcript levels, and trough levels were set to 1.0 for each gene in each strain, except for the *ccg-1* blot, in which peak levels are set to 1.0. Results for the *bd* strain are shown as blue diamonds, results for the *nrc-2; bd* strain are shown as pink squares.

These data suggested that under standard *ccg* assay conditions (0.03% glucose, 0.05% arginine), *nrc-2* is required for rhythms in FRQ-dependent *ccgs*, while it is not involved in rhythmicity of FRQ-independent *ccgs*, such as *ccg-16*.

Developmentally regulated genes are inducible in the nrc-2^{pRAL-1} *strain*

The arrhythmic accumulation of *ccg-1*, *ccg-2*, and *ccg-7* in the *nrc-2^{pRAL-1}; bd* mutant strain suggested that *nrc-2* is required for FRQ-dependent rhythms; however, it is also possible that the effect on these genes is a general transcriptional effect, rather than a clock-specific effect. The promoter of the *ccg-2* gene has been extensively characterized and contains discrete elements responsible for transcriptional control by developmental, clock and light signals (Bell-Pedersen *et al.*, 1996a). To assay the clock-specificity of the *nrc-2^{pRAL-1}* mutation, we examined *ccg-1* and *ccg-2* mRNA levels after cultures were desiccated and exposed to air, a process known to induce conidiation in *N. crassa* (Berlin and Yanofsky, 1985b). Similar to previous reports, the levels of *ccg-1* and *ccg-2* transcripts increased within two hours of developmental induction in the *bd* strain, and peaked at four hours (Figure 7A and B) (Bell-Pedersen *et al.*, 1996a). In the *nrc-2^{pRAL-1}; bd* mutant strain, *ccg-1* levels rose by a similar fold as compared to the *bd* strain, although as expected the initial levels were elevated in the *nrc-2^{pRAL-1}; bd* mutant. The rate of increase of the *ccg-1* transcript was also altered in the *nrc-2^{pRAL-1}; bd* mutant, as the *ccg-1* transcript increased more rapidly than in the *bd* strain. Levels of *ccg-2* transcripts also increased in the *nrc-2* background, but the magnitude of this increase was difficult to quantify given the very low initial levels of this transcript in the *nrc-*

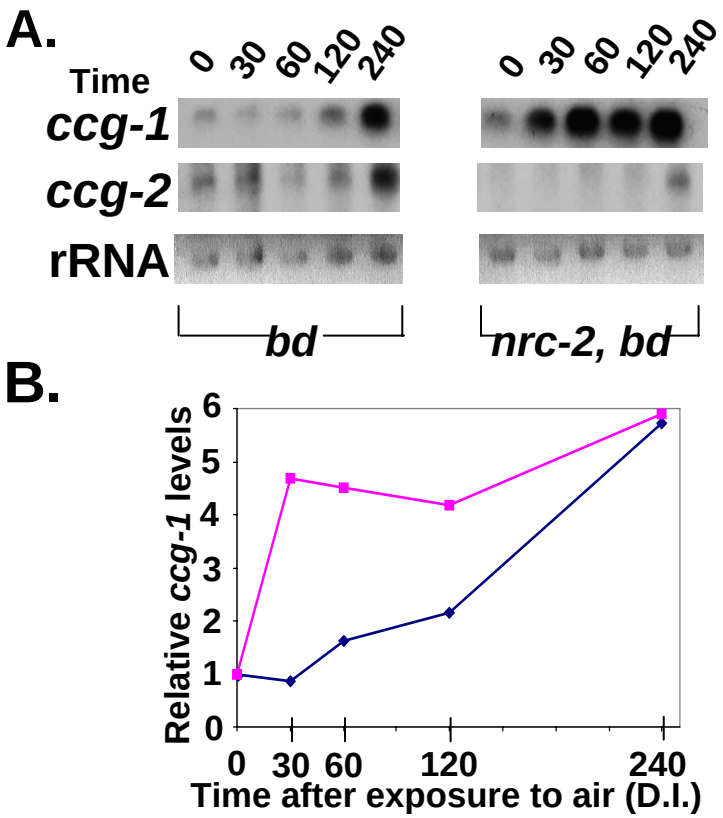

Figure 7. Developmental regulation of *ccg-1* and *ccg-2* in the *nrc-2^{pRAL-1}* mutant strain. A. Northern blot analysis of *ccg-1* and *ccg-2* expression following exposure to air. At time 0, standing liquid cultures grown in Vogel's minimal medium with 2% glucose were dried by vacuum and exposed to air in a sterile hood. Cultures were harvested and snap frozen at the time indicated (in minutes after exposure to air). Ethidium bromide stained rRNA is shown as a loading control. Strains used were FGSC 1858 (*bd, A*) and strain 167-403 (*al-2; nrc-2^{pRAL-1}; bd; a; ccg-1-tyrosinase*). Probes used were riboprobes specific to *ccg-1* or *ccg-2*, as indicated.

B. Densitometry of *ccg-1* results. Expression levels were normalized to ethidium bromide stained rRNA, and time 0 levels were set to 1.0. Data for FGSC 1858 (*bd, A*) are shown as blue diamonds, while data for strain 167-403 (*al-2; nrc-2^{pRAL-1}; bd; a; ccg-1-tyrosinase*) are graphed as pink squares. The *ccg-2* data are not normalized due to the low levels in the *nrc-2^{pRAL-1}* mutant at time 0.

2^{pRAL-1}; bd mutant (Figure 7A and B). These results indicate that the lack of rhythms in *ccg-1* and *ccg-2* does not stem from a general effect of the *nrc-2^{pRAL-1}* mutation on the regulation of these genes.

FRQ rhythms and stability are unaffected in the nrc-2^{pRAL-1} *strain*

The lack of rhythmicity of several FRQ-oscillator-dependent *ccgs* implied that *nrc-2* is required for FRQ-oscillator function. This requirement could conceivably be in an input pathway to the FRQ-oscillator, an output pathway from the FRQ-oscillator, or within the FRQ-oscillator itself. To determine whether *nrc-2* is part of an input or output pathway, or part of the molecular FRQ-oscillator, we assayed levels of known FRQ-oscillator components cultured in 2.0% glucose medium in DD. The *frq* gene is transcribed in a rhythmic manner with a peak in the early subjective morning (Aronson *et al.*, 1994b), while the FRQ protein peaks about four hours later and is progressively phosphorylated prior to its degradation (Garceau *et al.*, 1997; Liu *et al.*, 2000). Northern analysis indicated that although *frq* mRNA is rhythmic in the *nrc-2^{pRAL-1}; bd* mutant, the levels are drastically reduced relative to those in the *bd* strain (note the differing exposure times in Figure 8A). While *frq* levels are lower in the *nrc-2^{pRAL-1}; bd* strain, the phasing and period of the rhythm is comparable to that of the *bd* strain, indicating that the FRQ-oscillator is still functional in the *nrc-2^{pRAL-1}; bd* mutant (Figure 8A and B). Consistent with a functional FRQ-oscillator, western blot analysis of FRQ protein indicated that FRQ is rhythmic with a period and phase identical to that of the *bd* strain. In some replicate experiments, FRQ protein levels were slightly reduced in the *nrc-2^{pRAL-}*

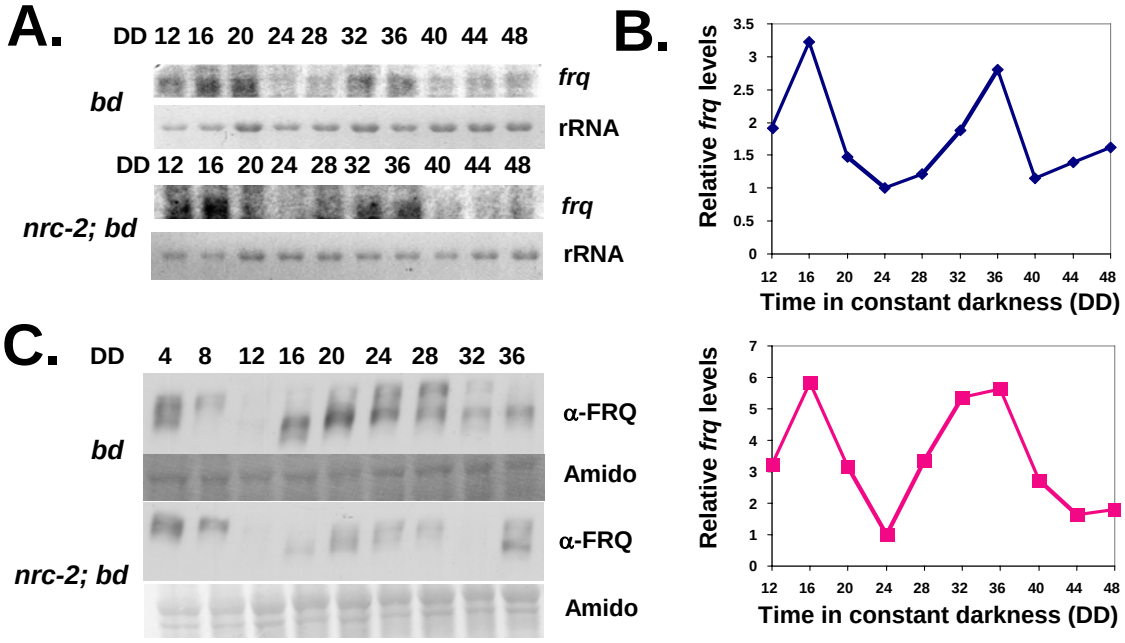

Figure 8. Rhythms in *frq* mRNA and FRQ protein persist in the *nrc-2; bd* strain.
A. Northern blot analysis of *frq* mRNA in DD. Total RNA was extracted from mycelia grown in 2% glucose Vogel's medium harvested every four hours in DD over the course of two days and was probed with a *frq*-specific riboprobe. Shown at top are results obtained from strain FGSC 1858 (*bd; A*) and at the bottom are results from strain 167-403 (*al-2; nrc-2^{pRAL-1}; bd; a; ccg-1-tyrosinase*). Ethidium bromide stained rRNA is shown as a loading control. The blot showing *frq* expression in *bd* was exposed for 48 hours; the *nrc-2* blot was exposed for 9 days.
B. Densitometry of the blots shown in (A). *frq* transcript levels were normalized to ethidium bromide stained rRNA and trough points for each strain were set to 1.0. Data for FGSC 1858 are plotted with blue diamonds; data for strain 167-403 are plotted using pink squares. **C.** Western blot analysis of FRQ protein levels. Total protein was extracted from mycelia grown in 2% glucose Vogel's medium harvested every four hours in constant darkness over the course of two days. Shown at top are results obtained from strain FGSC 1858 (*bd; A*) and at bottom are results from strain 167-403 (*al-2; nrc-2^{pRAL-1}; bd; a; ccg-1-tyrosinase*). Amido black stained protein is shown as a loading control.

[1]; bd strain (Figure 8C). Interestingly, while levels of *frq* mRNA were much lower in the *nrc-2[pRAL-1]; bd* mutant, FRQ protein levels were normal or only slightly reduced in this strain.

Stability of the FRQ protein is a critical feature of the *Neurospora* clock. FRQ protein begins to accumulate in the morning, and is progressively phosphorylated throughout the course of the day, leading up to peak levels in the afternoon. As the day progresses into the evening, FRQ is degraded and levels drop to trough levels during the night (Garceau *et al.*, 1997). Stability of the FRQ protein was examined in cultures grown in DD at the time of peak FRQ expression by treating the cultures with the translational inhibitor cycloheximide. Treatment with cycloheximide blocks *de novo* protein synthesis and allows for visualization of degradation of existing FRQ protein (Yang *et al.*, 2002). The degradation of FRQ in the *nrc-2[pRAL-1]; bd* mutant was not different from *bd* following cycloheximide treatment (Figure 9). Similar results were obtained following a light to dark transfer (data not shown), a treatment previously shown to destabilize FRQ (Yang *et al.*, 2002). Thus, these experiments indicate that *nrc-2* is not required for FRQ rhythmicity and that FRQ stability is unaffected in the *nrc-2[pRAL-1]; bd* strain.

Light input to the oscillator is unaffected in the nrc-2 *mutant strain*

The circadian clock is reset and synchronized in response to temporal cues, including light and temperature. In *N. crassa* light resetting requires WC-1 and WC-2 and is mediated by a rapid increase in *frq* mRNA and FRQ protein upon exposure of

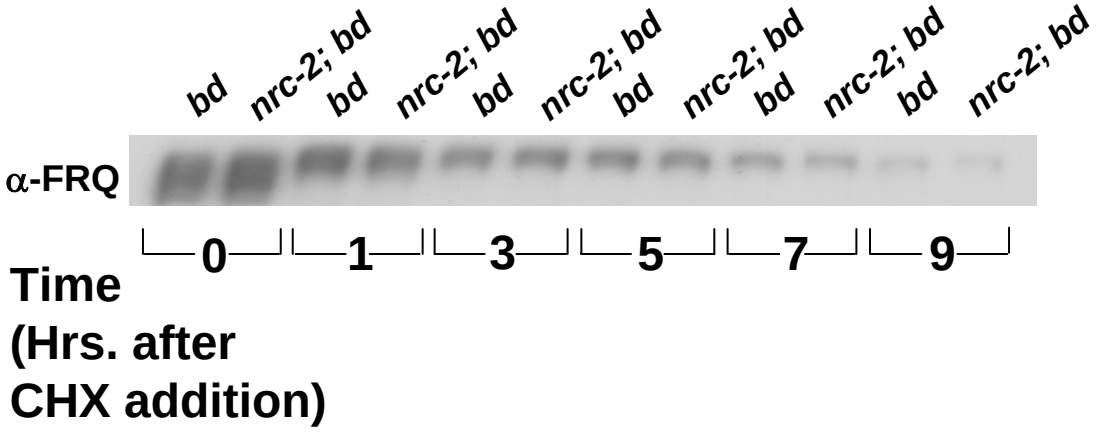

Figure 9. Stability of the FRQ protein is unaffected in the *nrc-2* mutant background. Western blot analysis was performed on total protein extracted from cultures grown in 2% glucose Vogel's minimal medium under DD. Prior to harvest, 10 mg/mL cycloheximide was added to the cultures. Cultures were harvested immediately (time 0) or up to nine hours following cycloheximide treatment. Strains used were FGSC 1858 (*bd; A*) or strain 167-403 (*al-2; nrc-2^{pRAL-1}; bd; a; ccg-1-tyrosinase*).

dark-grown cultures to light (Crosthwaite *et al.*, 1995). To address whether *nrc-2* is involved in light input to the circadian oscillator, dark-grown cultures of the *nrc-2^{pRAL-1}; bd* strain were exposed to a 15-minute light pulse (950 lux) prior to harvest. Total RNA was extracted and *frq* transcript levels were assayed by northern blot. In both the *bd* and *nrc-2^{pRAL-1}; bd* strains, *frq* levels increased rapidly relative to *frq* levels in DD (Figure 10). These results indicated that light input into the oscillator is unaffected in the *nrc-2^{pRAL-1}; bd* mutant.

The effects of the nrc-2^{pRAL-1} *mutation on WC-1*

WHITE COLLAR-1 (WC-1) is a central clock component that functions as both a blue light sensor and a transcription factor (Froehlich *et al.*, 2002). The WC-1 protein manifests a low amplitude circadian rhythm in DD, whereas *wc-1* mRNA accumulates without a circadian rhythm. WC-1 is subject to post-translational modifications, such as phosphorylation, over the course of the day. We show that WC-1 protein is rhythmic in the *nrc-2^{pRAL-1}; bd* mutant (Figure 11), however, the WC-1 protein migrated slightly further during gel electrophoresis at all times of the day in the *nrc-2^{pRAL-1}; bd* strain. These data suggested that *nrc-2* may play a role in post-translational modification of WC-1.

Protein Kinase C (PKC) has recently been shown to phosphorylate WC-1, though it is possible that other kinases also phosphorylate WC-1 (Franchi *et. al*, 2005). The *nrc-2* gene encodes a putative serine-threonine protein kinase (Kothe and Free, 1998), and may be involved in phosphorylation of WC-1. In order to determine if the observed

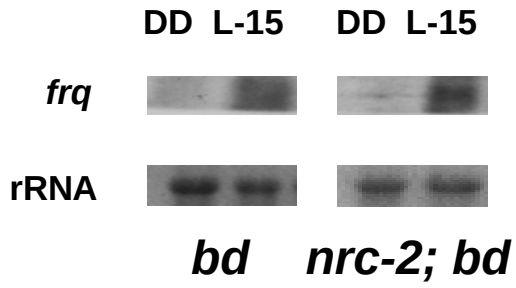

Figure 10. The *frq* transcript is light inducible in the *nrc-2* background. Liquid shake cultures of FGSC 1858 (*bd; A*); left, and strain 167-403 (*al-2; nrc-2^{pRAL-1}; bd; a; ccg-1-tyrosinase*); right, were grown in 2% glucose Vogel's minimal medium in DD for 24 hours. Cultures were subjected to a 15 minute light pulse (L-15) prior to harvest. *frq* transcript was detected using a specific riboprobe and ethidium bromide stained rRNA is shown as a loading control.

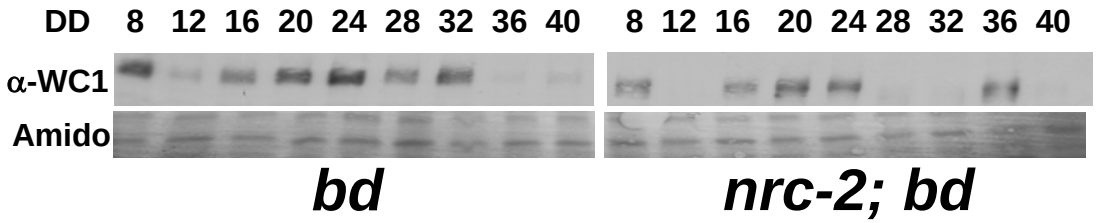

Figure 11. The WC-1 protein is rhythmic in the *nrc-2* background. Western blot analysis was performed on total protein extracts from cultures grown in 2% glucose Vogel's minimal medium, harvested every four hours for two consecutive days in DD. Shown at the top are results from strain FGSC 1858 (*bd; A*); shown at the bottom are results from strain 167-403 (*al-2; nrc-2^{pRAL-1}; bd; a; ccg-1-tyrosinase*). Also shown is amido black stained protein as a loading control.

increase in electrophoretic mobility of WC-1 in the *nrc-2*$^{pRAL-1}$*; bd* mutant was the result of a phosphorylation defect, total cell extracts were treated with λ protein phosphatase prior to SDS-PAGE. Samples harvested in LL showed a slower mobility band corresponding to the phosphorylated form of WC-1 in both the wild-type and *nrc-2*$^{pRAL-1}$*; bd* strains, indicating that *nrc-2* is not required for phosphorylation of WC-1. Surprisingly, phosphatase treated samples of wild-type *Neurospora* yielded 2 distinct forms of WC-1, a large form of approximately 135 Kda, and a small form of approximately 127 Kda (Figure 12)**.** Neither form was apparent in a WC-1 deletion strain (data not shown). The larger form was predominant in the *nrc-2*$^{pRAL-1}$*; bd* mutant strain (Figure 12A); in some replicates a small amount of the small form was present. The ratio of these two forms did not vary in a circadian manner throughout the day, although abundance of total WC-1 protein was rhythmic (Chapter III). Interestingly, the *frq*-null strain *frq*^{10} makes exclusively the large form of WC-1 (Figure 12C); the small form was never observed in this strain. The two forms of WC-1 do not appear to be regulated by light, as both forms of WC-1 were observed in the *bd* strain regardless of the lighting condition at the time of harvest (Figure 12A). Levels of the two forms of WC-1 were independent of the nitrogen content of the medium, as both forms were observed in cultures grown in low nitrogen (10 mM KNO_3) containing Westergaard's medium and in Vogel's medium (25 mM NH_4NO_3) (Figure 12B). These results demonstrated that both the *nrc-2*$^{pRAL-1}$ and *frq*^{10} mutations alter the ratio of the two forms of WC-1 such that the smaller form is absent or present in greatly reduced quantities. We hypothesize that the smaller form of WC-1 observed in the *bd* strain arises from an in-

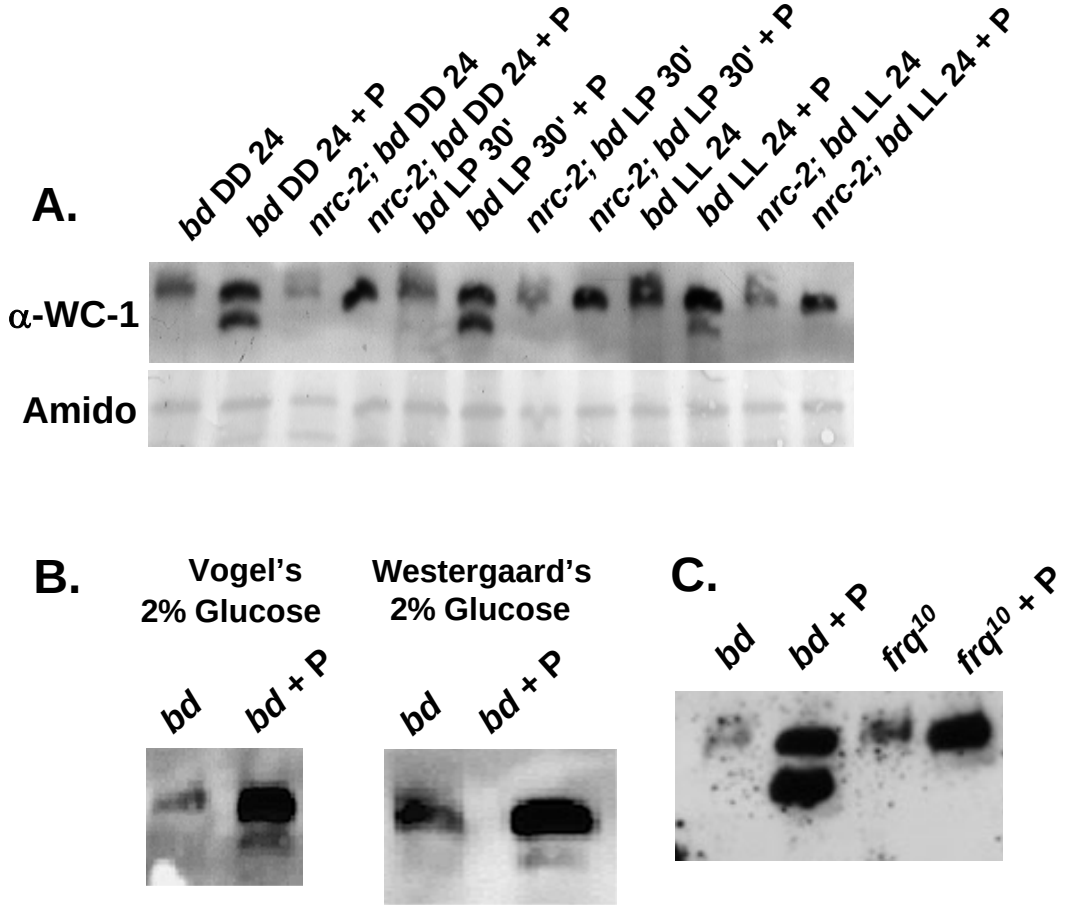

Figure 12. Two forms of WC-1 are detectable in wild-type; the *nrc-2*$^{pRAL-1}$ mutation affects the ratio of these forms. A. Two forms of the WC-1 protein are present in wild-type; primarily the larger form is present in the *nrc-2* background. Western blot analysis was performed on total protein extracts harvested after 24 hours in constant darkness (DD 24), 24 hours of constant light (LL 24), or a 30 minute light pulse (LP 30'). Mycelia were grown in 2% glucose Vogel's minimal medium. Total protein was treated with 400 units of λ protein phosphatase for 45 minutes at 37°C prior to SDS-PAGE (lanes with +P) or were mock treated with phosphatase buffer and $MnCl_2$ for 45 minutes at 37°C prior to SDS-PAGE. Strains used were FGSC 1858 (*bd; A*) or strain 167-403 (*al-2; nrc-2*$^{pRAL-1}$*; bd; a; ccg-1-tyrosinase*). Amido black stained protein is shown as a loading control. **B.** The two forms of WC-1 are not regulated by nitrogen levels. Mycelia were treated as above, and were grown in either 2% glucose Vogel's minimal medium or 2% glucose Westergaard's minimal medium as noted. **C.** Only the large form of the WC-1 protein is present in the FRQ-null strain. Mycelia were grown and treated as above, and the *frq*-null strain used was 40-1 (*bd; frq*^{10}*;A*).

frame translational start site located 87 codons downstream from the 5' AUG site (Figure 13). This putative start site contains a favorable Kozak consensus sequence (Kozak, 1986), and would give rise to a form of WC-1 approximately 10 KDa smaller than the full-length protein.

Glucose levels affect circadian rhythms in the nrc-2^{pRAL-1}; bd *strain*

The *Neurospora* circadian community uses a variety of media to assay different clock processes, depending on the process being measured. Rhythms in conidiation are commonly measured on race tubes containing solid Vogel's medium with 0.1-0.3% glucose, 0.17-0.5% arginine (Sargent and Kaltenborn, 1972). Expression of the *ccgs* is normally assayed in Fries medium with 0.03% glucose and 0.05% arginine (Bell-Pedersen *et al.*, 1996b; Loros *et al.*, 1989), concentrations low enough to suppress conidial development, which can mask clock regulation of the *ccgs.* Protein rhythms and rhythms in *frq* mRNA are generally performed using 2% glucose Vogel's medium (Aronson *et al.*, 1994b) (Garceau *et al.*, 1997); both *frq* and FRQ are expressed at nearly undetectable levels in low glucose (<0.05%) media (data not shown). Thus, many studies draw conclusions about clock function from experiments that are not standardized for media conditions.

In an attempt to increase the growth rate of an *nrc-2* deletion mutant, growth on non-standard race tubes containing 2% glucose Vogel's medium was examined. The growth rate of an *nrc-2* deletion strain was not only increased from 0.5 cm/day to 1.3 cm/day, but rhythms in conidiation were also observable (T. Lamb, unpublished data).

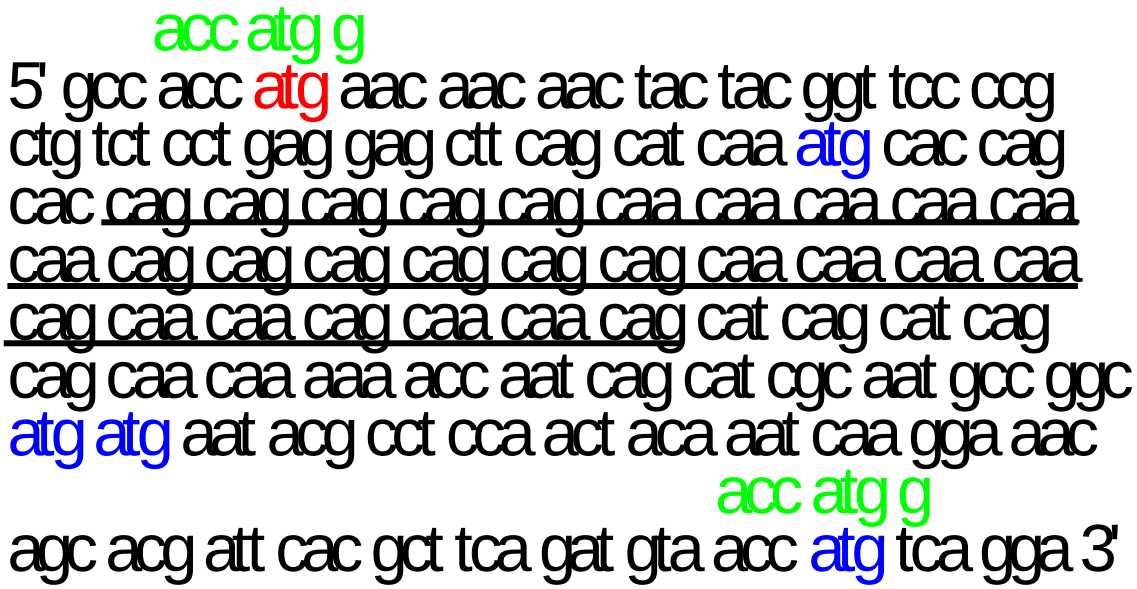

Figure 13. The 5' end of the WC-1 coding sequence contains four putative in frame translational start sites. Shown are about 250 nucleotides from the 5' end of the WC-1 coding region. The full-length protein uses the ATG start codon shown in red, the four putative downstream ATGs are shown in blue. Consensus Kozak initiation sequences (Kozak, 1986) are shown in green above the two closest matching sequences. Translation starting from the fourth ATG would yield a protein truncated by 87 amino acids, lacking the enitre poly-Q domain of the protein (underlined).

Similar results were obtained with the *nrc-2^{pRAL-1}; bd* strain (Figure 14 and data not shown). This conidiation persists with a near wild-type period of 21.2 hours. The *bd* strain also formed rhythmic bands of dense conidia on this medium; however, these were hard to discern given that this strain conidiated all throughout the circadian cycle, as compared to standard conditions. The growth rate of the *bd* strain was also variable on this medium (Figure 14). The observed rhythms in the *nrc-2^{pRAL-1}; bd* strain on high glucose medium suggested that the mutation renders the FRQ oscillator or FRQ-dependent outputs sensitive to the carbon levels in the growth medium, such that increased glucose levels restored rhythmicity to the cell. In 2% glucose Vogel's medium, rhythms of *ccg-1*, *ccg-2* and *ccg-7* mRNA accumulation were observed in both the *bd* and *nrc-2^{pRAL-1}; bd* strains with similar periodicity and phasing in both strains (Figure 15 and data not shown). Thus, mutation of the *nrc-2* gene disrupts FRQ-oscillator-dependent circadian rhythms in a glucose-dependent manner.

Discussion

This study focuses on the role of the putative serine-threonine protein kinase *nrc-2* in the circadian clock of *N. crassa*. Under standard assay conditions (0.03% glucose, 0.05% arginine Fries' medium) FRQ-dependent gene expression and conidiation are arrhythmic in the *nrc-2^{pRAL-1}; bd* mutant, however, under higher glucose concentrations (2% glucose Vogel's medium), circadian rhythms are observed in both conidiation and *ccg* transcription. Two forms of WC-1 are present in wild-type cells, while in the *nrc-2^{pRAL-1}; bd* mutant strain, WC-1 exists almost exclusively as the large form. We conclude

Strain	Period (h)	Growth rate (cm/d)
nrc-2; bd	**21.2**	**1.3**
bd	**22.3**	**3.0**

Figure 14. Circadian conidiation rhythms in the *nrc-2* mutant background are restored in high glucose medium. Strains were grown on 2% glucose Vogel's minimal medium in constant darkness. Shown are two representative tubes of the *nrc-2*$^{pRAL-1}$*; bd* strain 167-403 (top) and two tubes of the wild-type *bd* strain FGSC 1858 (bottom). The free-running period is shown for both strains, as well as the growth rate (in cm/day) for each strain.

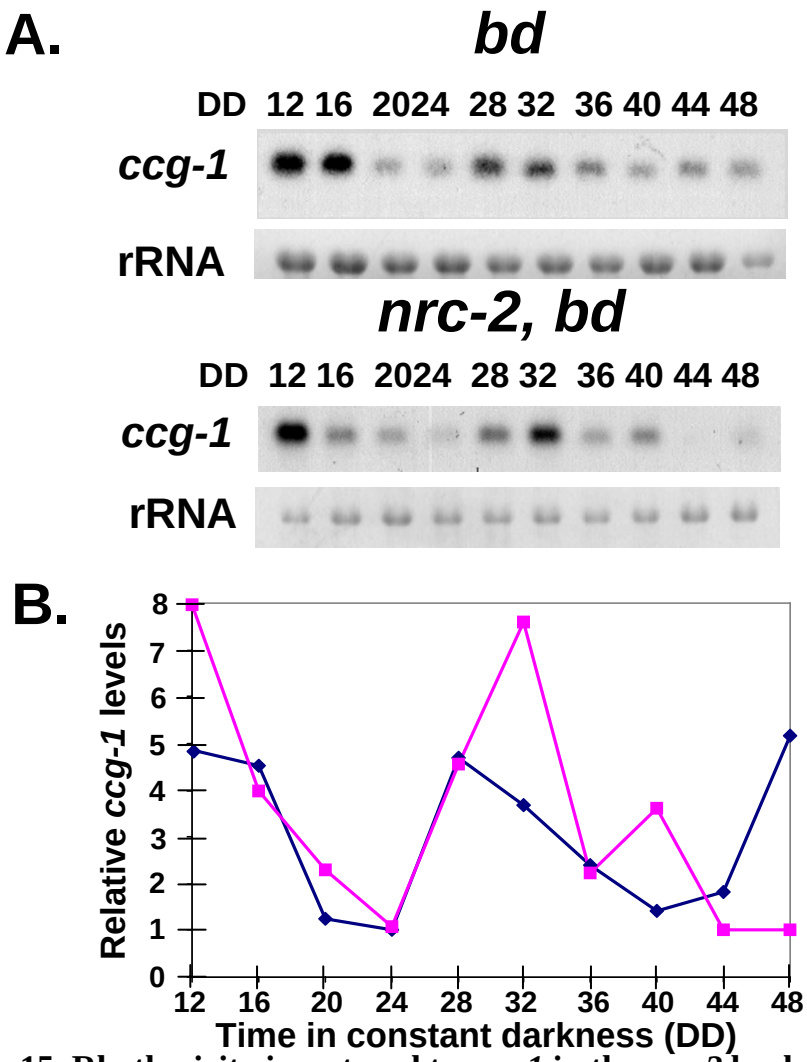

Figure 15. Rhythmicity is restored to *ccg-1* in the *nrc-2* background when cultured in high glucose medium. A. Northern blot analysis of *ccg-1* cultured in DD was performed. Strains were grown in 2% glucose Vogel's minimal medium and harvested every four hours for two consecutive days. Shown at the left are results from FGSC 1858 (*bd; A*), at the right are results from strain 167-403 (*al-2; nrc-2*$^{pRAL-1}$*; bd; a; ccg-1-tyrosinase*). Ethidium bromide stained rRNA is shown as a loading control. **B.** Densitometry of the blots shown in (A). Ethidium bromide stained rRNA was used to normalize transcript levels, and trough levels were set to 1.0 in each strain. Results for the *bd* strain are shown as blue diamonds, results for the *nrc-2; bd* strain are shown as pink squares.

that the FRQ oscillator is sensitive to glucose levels in the *nrc-2*$^{pRAL-1}$*; bd* mutant strain, and *nrc-2* is involved in the choice of translational start site of the WC-1 protein.

Circadian experiments involving *Neurospora* are typically carried out on various media. Conidiation rhythms are typically assayed on Vogel's minimal medium containing 0.1-0.3% glucose and 0.17-0.5% arginine (Sargent and Kaltenborn, 1972). Under lower glucose concentrations, *Neurospora* does not conidiate well, while higher glucose levels lead to masking of the circadian rhythm, resulting in conidiation at all phases of the circadian cycle. Rhythms in *frq* mRNA, FRQ and the WC proteins are best observed in 2% glucose Vogel's medium, under lower glucose concentrations these components are difficult to detect (Garceau *et al.*, 1997). The *ccgs* are best assayed under starvation conditions, in Fries' medium containing 0.03% glucose and 0.05% arginine, to prevent conidiation and masking of rhythms in developmentally regulated genes (Loros *et al.*, 1989). Using these media conditions, we arrived at the conclusion that *nrc-2* was a circadian output component, involved in signaling information from the FRQ oscillator to transcriptional control of the *ccgs*. However, cultures of the *nrc-2*$^{pRAL-1}$*; bd* strain on 2% glucose Vogel's race tubes revealed a rhythm in conidiation (Figure 14), and the *ccgs* proved to be rhythmic in the same medium (Figure 15), resulting in a major revision in our understanding of the role of *nrc-2* in the clock. The results of this study indicate that 2% glucose Vogel's medium is suitable for use in *ccg* expression studies as both developmentally regulated genes *ccg-1* and *ccg-2* are robustly rhythmic in this medium (Figure 15 and data not shown), as are the clock components FRQ and WC-1.

Although the growth rate of the *nrc-2*$^{pRAL-1}$*; bd* mutant is severely reduced (Figure 5), suggesting that the *nrc-2*$^{pRAL-1}$ mutation has other effects on the organism, we see a FRQ-oscillator-specific effect of the *nrc-2*$^{pRAL-1}$ mutation on *ccg* expression. Expression of the FRQ oscillator-independent gene *ccg-16* is rhythmic in the *nrc-2*$^{pRAL-1}$*; bd* strain under standard conditions (0.03% glucose), in which the FRQ-dependent *ccg* expression is arrhythmic (Figure 6). The clock-specific effect of this mutation is further supported by the proper developmental induction of the *ccgs* in the *nrc-2*$^{pRAL-1}$*; bd* strain. Clock, light, and developmental signals regulate the *ccg-1* and *ccg-2* genes, and in the case of *ccg-2*, discrete elements in the promoter have been shown to contain regulatory sequences responsive to each of these inputs (Bell-Pedersen *et al.*, 1996a). Although the levels of *ccg-1* and *ccg-2* prior to developmental induction are increased and reduced, respectively, in the *nrc-2*$^{pRAL-1}$*; bd* strain, the magnitude of induction of these genes is similar to that of the *bd* strain, indicating that induction of these genes by developmental signals is unaffected by the *nrc-2*$^{pRAL-1}$ mutation.

Circadian rhythms are nutritionally compensated in *N. crassa*. Similar free-running periods of the conidiation rhythm are observed on a wide range of media, which result in varying rates of growth (Loros and Feldman, 1986). Other mutations affecting media compensation have been isolated, such as the *frq*^{9} mutation. This strain harbors a premature stop codon in the *frq* coding sequence, and behaves as a clock null strain under most conditions. Rhythmic condiation is observable under certain conditions, and this rhythmicity is neither temperature nor nutritionally compensated (Loros and Feldman, 1986), providing further support to the hypothesis that temperature

compensation is merely one facet of a mechanism that functions to maintain constant period length in a variety of environmental conditions (Nakashima and Caldarola, 1973). The glucose dependent conidiation and gene expression rhythms indicate that media compensation is affected in the *nrc-2*$^{pRAl-1}$*; bd* strain, and though we did not test temperature compensation of the conidiation rhythms in the *nrc-2*$^{pRAl-1}$*; bd* strain, one would expect an alteration in temperature compensation if Nakashima and Caldarola's hypothesis is correct. Future experiments should assay temperature compensation in the *nrc-2*$^{pRAl-1}$*; bd* strain, as well as the extent of nutritional compensation in the *nrc-2*$^{pRAl-1}$*; bd* strain, by assay of conidiation rhythms on various levels and types of carbon sources.

Phosphorylation of WC-1 is not dependent on *nrc-2* (Figure 12). However, during the course of our studies we noticed that there are two forms of WC-1 present in wild type cells, differing in mobility by approximately 8 KDa. This is the first report of multiple forms of WC-1, though multiple forms of both FRQ and WC-2 have been described (Garceau *et al.*, 1997; Liu *et al.*, 1997). Given the large size of WC-1 (135 Kda), and the small size difference between the two forms, it is not surprising that this has been overlooked in the past. Special gel conditions are essential for resolving these two forms, such as a high acrylamide to bisacrylamide ratio (74:1) (K. Lee, personal communication) and long electrophoresis runs on large SDS-PAGE gels. We propose that these two forms of WC-1 arise from alternative translational start sites, with the short form arising from an in-frame AUG located 87 residues from the 5' start site (Figure 13). This downstream start site is in a favorable translational consensus sequence, and would give rise to a protein lacking the entire N-terminal poly-glutamine

domain, which has been implicated in DNA binding (Yuan *et al.,* 1991). It is also possible that the short form observed is a WC-1 degradation product, or that the protein undergoes a posttranscriptional modification such as alternative splicing, and purification of both forms of WC-1 followed by N-terminal sequencing will help to clarify this situation.

Other groups have reported truncated forms of WC-1 in several mutants. The *rhy-2* strain contains a plasmid inserted into the 5' coding region of the *wc-1* locus, resulting in a truncated WC-1 missing the 5' polyglutamine region. This strain is weakly responisve to light and is arrhythmic on standard medium (Toyota *et al.*, 2002). In a *wc-1*^{RIP} strain, FRQ is light-inducible (Dragovic *et al.*, 2002), indicating that this strain retains partial WC-1 function, with translation likely initiating downstream of a 5' stop codon. The *wc-1*^{MK1} mutant expresses FRQ in response to light but is defective in FRQ expression in DD, a process that is WC-1 dependent, and this strain produces primarily the N-terminal poly-Q domain of WC-1, but also low levels of the C-terminal portion of the protein, resulting from downstream translational initiation (Lee *et al.*, 2003). Here we report multiple forms of WC-1 in a wild-type strain, and other groups have seen this also (M. Brunner, personal communication), but the significance of these two forms remains unclear. Mutations in both *frq* and *nrc-2* affect media compensation, and given the similar effects of *nrc-2* and *frq* on *ccg* transcript levels (see below) and the forms of the WC-1 protein, it is tempting to speculate that metabolic information is transduced to the clock at the level of WC-1 translation start site choice through NRC-2 and FRQ. Efforts to address this question with regards to carbon levels have been unsuccessful,

however, as WC-1 is undetectable in low (0.03%) glucose medium (data not shown). The expression of *nrc-2* mRNA is weakly repressed under carbon starvation conditions (Xie *et al.*, 2004); thus, one might expect an alteration in the choice of translational start sites of WC-1 in a low glucose medium. Additionally, WC-1 is insensitive to nitrogen levels, as both forms are present at similar levels in the nitrogen limited Westergaard's medium as well as in the nitrogen rich Vogel's medium (Figure 12).

Environmental factors such as temperature have been shown to affect the choice of translational start site of the clock component FRQ. At lower temperatures, the smaller of the two forms of FRQ is predominant, while at higher temperatures, the larger form is more abundant. The absolute levels of FRQ also vary with temperature, providing a mechanism by which the cell can be reset by temperature cues (Liu *et al.*, 1998). A similar mechanism of translational regulation may explain the lack of rhythmicity of the *nrc-2* strain under low glucose conditions, the smaller form of WC-1 may be necessary for rhythmicity in limited carbon conditions.

Levels of both *ccg-1* and *ccg-2* mRNA are altered in the *nrc-2*$^{pRAL-1}$*; bd* background. *ccg-1* mRNA is elevated relative to wild-type, while the *ccg-2* transcript exhibits markedly reduced levels. This mirrors the effects seen on the levels of these transcripts in a *frq*-null background (Vitalini *et al.*, 2004). Interestingly, the *frq*-null strain contains only the larger of the WC-1 forms, and no small form is detectable (Figure 12). Thus, the altered levels and the arrhythmicity of the *ccgs* observed in a *frq*-null background may arise from the lack of the small form of WC-1, and this form may be important in controlling circadian output. A small amount of small WC-1 is

detectable in the *nrc-2*$^{pRAL-1}$*; bd* mutant, while none is visible in the *frq*-null strain in high glucose medium. If small WC-1 is required for *ccg* rhythmicity, then this can explain the *ccg* transcript rhythms observed in high glucose in the *nrc-2*$^{pRAL-1}$*; bd* strain. Further experiments using strains able to synthesize only the large or the small form of WC-1 will be needed to adequately address this issue.

The *nrc-2*$^{pRAL-1}$*; bd* mutation also results in drastic reductions in *frq* mRNA levels (Figure 8). However, this decrease does not correlate with FRQ protein levels, as levels are identical or slightly reduced in the *nrc-2*$^{pRAL-1}$*; bd* mutant (Figure 8 and 9). This observation suggests that a post-transcriptional mechanism exists to maintain FRQ protein levels independently of *frq* mRNA levels in *Neurospora*. Further, light induction of *frq* mRNA is normal in the *nrc-2*$^{pRAL-1}$*; bd* strain, an indication that the effects of *nrc-2* on *frq* levels are specific to DD conditions.

Taken together, these data establish the requirement of *nrc-2* for free-running rhythms in limited glucose media. Our data suggest several possible models for the function of *nrc-2* in *Neurospora* circadian rhythms. First, NRC-2 may interact with the output portion of the circadian system. This model proposes that the *ccg*s are affected by metabolic signals in an *nrc-2*-dependent manner such that under low glucose conditions, rhythms in these genes are abolished. Increases in the glucose content of the medium can override the requirement for *nrc-2*, implying an *nrc-2*-independent effect of glucose levels on circadian rhythms in these genes (Figure 16A). While this model reflects our initial hypothesis regarding NRC-2 function, we consider this an unlikely model to explain NRC-2 function because it does not explain our results with WC-1. A second

model suggests that NRC-2 serves to provide metabolic input to the FRQ oscillator, at the level of choice of WC-1 translational start site. This model implies that the short form of WC-1 has a FRQ-oscillator specific circadian function, as the *ccg-16* gene, which is dependent on WC-1 but not FRQ for rhythmcity (Chapter III), remains rhythmic in the *nrc-2*$^{pRAL-1}$ background. Some of the small form of WC-1 is made in the *nrc-2*$^{pRAL-1}$ strain in 2% glucose Vogel's medium, presumably enough to support rhythmicity in FRQ-dependent gene expression. As the *nrc-2*$^{pRAL-1}$ and the *frq*^{10} strains both show primarily the large form of WC-1 and display similar levels of the *ccg* transcripts, our model suggests that NRC-2 is responsible for signaling metabolic information to the FRQ-oscillator (Figure 16B). One experiment to address this model would be to assay WC-1 levels in the *frq*^{9} mutant, which displays rhythmicity in conidiation that is not nutritionally compensated. Our model would predict that the *frq*^{9} strain would make primarily the large form of WC-1. A third model posits that *nrc-2* is required to maintain a certain level of FRQ and/or WC-1 under low glucose conditions. While *frq* mRNA is reduced in the *nrc-2*$^{pRAL-1}$ background in 2% glucose Vogel's, levels of the FRQ protein are normal or only slightly reduced. We have not examined the levels of FRQ and WC-1 under low glucose conditions where the *ccg*s are arrhythmic, thus it is possible that *nrc-2* is required for maintenance of FRQ and/or WC-1 levels in low glucose (Figure 16C). Testing of Model 2 and 3 requires the assay of FRQ and WC-1 in low glucose medium. All three models are supported by the observation that the levels of FRQ-dependent *ccg*s are similar to those seen in a FRQ-null strain, indicating a loss of signal from the FRQ oscillator at some point in the pathway. Further experiments,

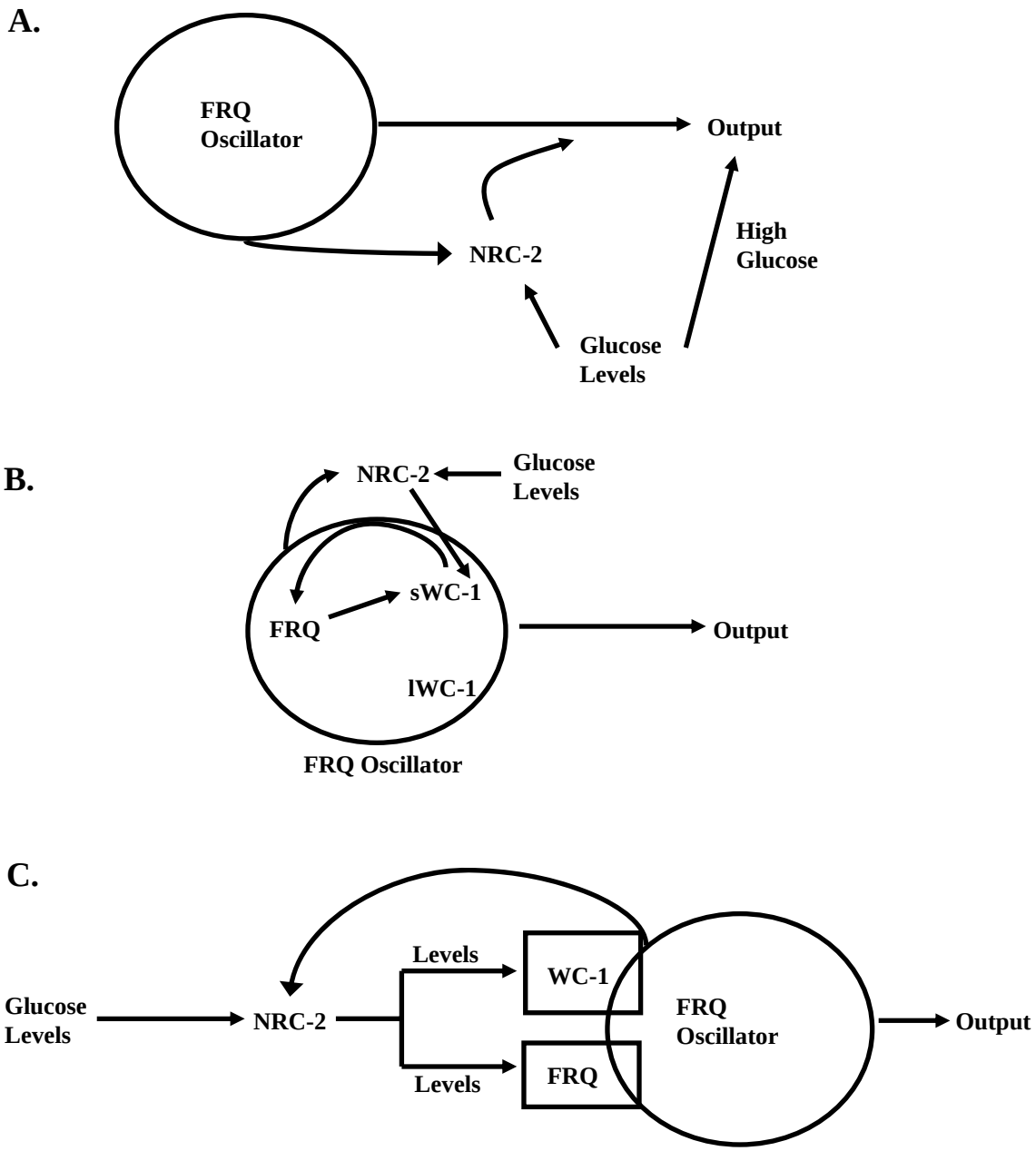

Figure 16. Models for the role of NRC-2 in the circadian clock of *N. crassa*. See text for details.

including the construction of strains able to make only the short or only the long form of WC-1 and the generation of antibodies to NRC-2 to determine the expression, localization, and proteins that interact with NRC-2, will be needed to clarify the role of *nrc-2* and the two forms of WC-1 in the *Neurospora* clock.

CHAPTER III

MULTIPLE OSCILLATORS COUPLED WITH THE WHITE COLLAR-1 PROTEIN REGULATE GENE EXPRESSION IN *NEUROSPORA CRASSA*

Summary

In *Neurospora crassa*, the FREQUENCY (FRQ), WHITE-COLLAR-1 (WC-1) and WC-2 proteins comprise the core circadian FRQ oscillator, which is directly responsive to light and controls daily rhythms in asexual development and gene expression (Aronson *et al.*, 1994a; Crosthwaite *et al.*, 1997). However, physiological and biochemical data have demonstrated the existence of additional oscillators in *N. crassa* that function in the absence of FRQ (called FRQ-less oscillators, or FLOs) (Christensen *et al.*, 2004; Correa *et al.*, 2003; Granshaw *et al.*, 2003; Lakin-Thomas and Brody, 2000; Loros and Feldman, 1986; Merrow *et al.*, 1999). A previous study identified a gene, W06H2, which peaks in mRNA abundance in the subjective evening and is robustly rhythmic in the absence of FRQ (Correa *et al.*, 2003). In this chapter, the regulation of this gene, now called *clock-controlled gene 16* (*ccg-16*) is examined in detail. We show that while the FLO responsible for *ccg-16* rhythms can function in the absence of the FRQ oscillator, coupling of this FLO to the FRQ oscillator through the WC-1 protein stabilizes the phase of the rhythm. Furthermore, the WC-1 protein accumulates in a rhythmic manner in the absence of FRQ and in constant light (LL), a condition where

FRQ protein levels are elevated and arrhythmic, suggesting that components of this FLO are involved in rhythmic accumulation of WC-1. Together, our data support the hypothesis that the *N. crassa* circadian system is composed of multiple oscillators, and that coupling of these oscillators functions to increase the stability and accuracy of the circaidan clock.

Introduction

Organisms ranging in complexity from single-celled cyanobacteria to humans depend upon their circadian clock to regulate 24-hour biological cycles such as metabolism, hormone production and sleep patterns. The endogenous circadian clock is synchronized by daily temporal cues such as light:dark (LD) and temperature cycles, and allows organisms to predict and anticipate changes in their environment. Despite the enormous differences in the phylogenetic origin and complexity of organisms that possess a circadian clock, a remarkably similar molecular mechanism is used to generate circadian rhythms. At the core of circadian clocks are cell autonomous oscillators composed of activators and inhibitors that form autoregulatory feedback loops (Dunlap *et al.*, 1999; Young and Kay, 2001). Components of these oscillators respond to environmental input, entrain the clock to external time and transfer time-of-day information through output pathways to regulate rhythmic processes, including gene expression.

In animals, a hierarchical system exists in which a master circadian oscillator, or pacemaker, located in the brain coordinates the activity of peripheral oscillators that

control rhythms in tissue-specific outputs (Bell-Pedersen *et al.*, 2005; Lowrey and Takahashi, 2004). Conversely, the clock in unicellular organisms must have the innate capacity to perform all functions required for the generation of circadian rhythmicity. Recent physiological evidence indicates that the circadian clocks of microbes are composed of multiple oscillators (Lakin-Thomas and Brody, 2004; Loros and Dunlap, 2001; Morse *et al.*, 1994; Nair *et al.*, 2002), and leads to the hypothesis that unicellular organisms harbor multiple oscillators which regulate diverse outputs in a manner analogous to circadian organization in metazoans. While multiple circadian oscillators have long been proposed to account for the precision, stability, and complexities of circadian rhythms, the nature and interactions of these oscillators remain elusive (Pittendrigh, 1960).

Evidence is mounting for the existence of multiple oscillators in *N. crassa* (Aronson *et al.*, 1994a; Christensen *et al.*, 2004; Correa *et al.*, 2003; Granshaw *et al.*, 2003; Lakin-Thomas and Brody, 2000; Loros and Feldman, 1986; Merrow *et al.*, 1999). For example, in cultures grown in medium containing nitrate as the sole nitrogen source, daily rhythms in nitrate reductase activity are observed in constant darkness (DD) and LL, in wild-type strains and in strains that lack a functional FRQ oscillator (Christensen *et al.*, 2004). These data indicate that rhythms in nitrate reductase activity are controlled by a FLO. Strains lacking a functional FRQ oscillator display robust conidiation rhythms on media supplemented with farnesol or geraniol, intermediates in the sterol biosynthesis pathway, indicating that these compounds upregulate activity of a FLO (Granshaw *et al.*, 2003). However, molecular details of oscillator mechanisms in *N. crassa* are known for

only one oscillator, the FRQ oscillator. At subjective dawn, both *frq* mRNA and FRQ protein levels are low (Loros and Dunlap, 2001). WC-1 and WC-2, through PAS domain-mediated interactions, dimerize and form a WHITE-COLLAR Complex, or WCC, which directly activates *frq* transcription (Froehlich *et al.*, 2002; He *et al.*, 2002). About 4 h later, *frq* mRNA levels reach their peak, and FRQ protein begins to accumulate and enter the nucleus. Negative feedback occurs by the interaction of FRQ with the WCC, which interferes with the ability of the WCC to activate transcription of *frq*. For the rest of the day and into the evening, FRQ remains at sufficient levels in the nucleus to inhibit transcription of *frq*. FRQ also positively regulates the levels of WC-1 and WC-2 through two different proposed mechanisms: FRQ is hypothesized to positively regulate WC-1 levels posttranscriptionally leading to its rhythmic expression, and to increase the levels of *wc-2* mRNA (Cheng *et al.*, 2001b; Lee *et al.*, 2000; Merrow *et al.*, 2001). Completion of the feedback loop is achieved by the removal of FRQ from the nucleus, which ultimately results in activation of *frq* transcription by the WCC.

This chapter describes the characterization of the control of the FRQ oscillator-independent gene *ccg-16*. Specifically, we employ molecular techniques to determine the expression pattern of *ccg-16* in LL and DD in wild-type, *frq*- and *wc-1*-null strains. We find that rhythms in *ccg-16* mRNA persist in both LL and DD, in wild-type and *frq*-null backgrounds, although the phasing of *ccg-16* mRNA expression is variable in a *frq*-null strain. Rhythms in *ccg-16* mRNA are abolished in a *wc-1* null strain, indicating a requirement of this protein for *ccg-16* transcript rhythms. Assay of the WC-1 protein in LL and DD in the wild-type and *frq*-null strains revealed that rhythms in this protein are

FRQ-independent; they persist in LL, a condition in which FRQ levels are elevated and arrhythmic, and in the absence of FRQ. These data indicate that WC-1 serves to couple the FRQ oscillator to a FLO, and is required for rhythmic output from both oscillators.

Materials and methods

N. crassa *strains and growth conditions*

Growth media (Vogel's minimal media), vegetative growth conditions and sexual crosses were performed as described previously (Davis and De Serres, 1970). Race tube medium contains 1X Vogel's, 0.1% glucose, 0.17% arginine, 50 ng/ml biotin, and 1.5% agar. Race tube assays were performed in controlled environmental chambers (Percival Scientific Inc.). When relevant, light intensities were ~700 lux (14 µMol of photons/m^2/s) using broad range fluorescent lights (Phillips F20T12Cw 20W). All strains used in this study contain the *band* (*bd*) mutation, which allows visualization of the conidiation rhythm in race tube cultures (Sargent *et al.*, 1966). The ΔFRQ (*bd; frq*^{10}) and ΔWC-1 (*bd; wc-1*^{KO}) strains were produced by gene replacement resulting in null alleles and were described previously (Aronson *et al.*, 1994a; Lee *et al.*, 2003). The *bd; a* (40-9) and the *bd; frq*^{10}*; a* (40-2) strains were obtained from a backcross of *bd; a* (FGSC #1859, Fungal Genetics Stock Center, Kansas City, MO) with *bd; frq*^{10}*; A* (FGSC# 7490). The *bd; wc-1*^{KO}*; A* (418-3) strain was obtained by crossing *bd; wc-1*^{KO}*; his-3; a* (308-1), graciously provided by Jay Dunlap and Jennifer Loros (Dartmouth University, New Hampshire), with *bd; A* (FGSC #1858).

For RNA analysis, mycelial mat cultures were grown in liquid shake culture (100 rpm) in 25 ml of Fries' minimal medium (1X Fries Salts, 0.03% glucose, 0.05% arginine) in 50 mL Erlenmeyer flasks. For DD experiments, cultures were synchronized by transfer from LL 25°C to DD 25°C, from LL 30°C to DD 25°C or from DD 30°C to DD 25°C as indicated in the text and figure legends. For LL experiments, cultures were synchronized by transfer from DD 25°C to LL 25°C or from DD 30°C to LL 25°C. Transfer of cultures was performed so that cultures were approximately the same age (within 8 hours) at the time of harvest, but the circadian time of the cultures varied (Correa *et al.*, 2003; Loros *et al.*, 1989). For protein analysis, cultures were grown as above except mycelial mats were grown in 100 ml of Vogel's minimal medium (1X Vogel's salts, 2% Glucose).

Nucleic acid isolation and hybridization

Total RNA isolation and northern blotting was performed as previously described (Bell-Pedersen *et al.*, 1996a). For northern analysis of *ccg-1*, radioactive riboprobes were synthesized from pLK119 (Bell-Pedersen *et al.*, 1996a) by *in vitro* transcription (Maxiscript, Ambion Inc., Austin, TX) in the presence of [α^{32}P] UTP [3000 Ci mmol^{-1}] (Perkin Elmer Inc., Boston, MA) For northern analysis of *ccg-16*, T3 and T7 primers were used to amplify the *ccg-16* coding sequence by PCR from pW06H2 (Nelson *et al.*, 1997). The PCR product was gel purified and used as a template for random primed DNA synthesis (DECAprime, Ambion Inc., Austin, TX) in the presence of [α^{32}P] dCTP [6000 Ci mmol^{-1}] (Perkin Elmer Inc., Boston, MA).

Protein Isolation and Analysis

Total protein isolation was performed as described previously (Garceau *et al.*, 1997) with addition of HALT protease inhibitor cocktail per manufacturer's instructions (Pierce Biotechnology, Rockford, Il.). For WC-1 analysis, total protein extracts were treated with 200U λ protein phosphatase (New England Biolabs, Beverly, MA) at 37°C for 45 minutes prior to SDS-PAGE. Antibodies to FRQ (polyclonal) and WC-1 (monoclonal) were generously provided by Drs. Martha Merrow and Till Ronneberg (Institute for Medical Psychology, University of Munich).

Construction of a ccg-16 *knockout strain*

A *ccg-16*^{KO} strain was constructed by first generating a *ccg-16* knockout plasmid pZAL1 in which the CCG-16 coding sequence was replaced with the *Streptomyces hygroscopicus hygromycin phosphotransferase* (*hph)* gene. PCR was used to produce split marker fragments from the knockout plasmid for transformation into *N. crassa*. To create pZAL1, approximately 3 kb of 5' and 3' flanking sequence were amplified from genomic DNA using primers that contain sequences that overlap with the *hph* gene and the yeast pRS416 shuttle vector (New England Biolabs Inc.) (5' primers 5F-GTAACGCCAGGGTTTTCCCAGTCACGACGGAGGATGTGGTCAGTAACAG 5R-ACCGGGATCCACTTAACGTTACTGAAATCCTGCGAAACACTGGTGATGG 3' primers 3F-CGTTCTATAGTGTCACCTAAATCGTATGTGCACTGATGTCCAAACGCTC

3R-

GCGGATAACAATTTCACACAGGAAACAGCAGTGGCAGATGGTTGTCAGG). The *hph* gene was amplified from pCSN44 (Staben *et al.*, 1989) (hphF-ACATACGATTTAGGTGACACTATAGAACGCCCGTCGACAGAAGATGATATTG AAGGAGC hphR-AGCTGACATCGACACCAACG) and pRS416 was digested with *Xba*I and *Xho*I. The three PCR fragments were transformed into yeast (strain YF2) with the digested pRS416 shuttle vector to produce pZAL1 by recombination-mediated plasmid construction (Oldenburg *et al.*, 1997). Split marker fragments for *ccg-16* replacement were generated by amplification of 5' and 3' fragments by PCR using pZAL1 as a template (primers 5F and HSM-F AAAAAGCCTGAACTCACCGCGACG were used for the 5' fragment and primers 3R and HSM-R TCGCCTCGCTCCAGTCAATGACC were used for the 3' fragment). Each split marker fragment was gel purified and 300 ng was used to transform *N. crassa* (Margolin *et al.*, 1997). Transformants were plated on Vogel's minimal medium supplemented with 200 mg/ml hygromycin (Sigma). Southern blots were performed to identify strains that contained the *ccg-16* deletion and did not harbor additional copies of the *hph* gene (data not shown). Homokaryon deletion strains were obtained following a cross to *bd;* A (FGSC #1858) and *bd; frq*^{9}*; his-3* (94-1).

Results

Rhythmicity of the evening-specific ccg-16 *gene is independent of FRQ*

In DD and constant temperature, the wild-type clock strain *bd* (hereafter referred to as wild-type) displayed 22-h rhythms in conidiation, as viewed on race tubes (Figure 17A). However, under the same conditions, the FRQ-null strain, *bd; frq*^{10}*; a*, was arrhythmic. Similarly, the transcript of the morning-specific clock-controlled gene *ccg-1* accumulates in a rhythmic fashion in wild-type strains, peaking in the subjective morning (DD 12 and DD 36) in cultures that were synchronized by a light (30°) to dark (25°C) transition (Loros *et al.*, 1989), but was arrhythmic in the FRQ-null strain (Figure 17B, C). However, consistent with previous results (Correa *et al.*, 2003), a ~24 h rhythm in *ccg-16* mRNA accumulation was observed in both wild-type and FRQ-null strains, with peak mRNA levels occurring in the subjective evening in wild-type cells at DD 24 and 48 (Figure 17). In contrast to the wild-type, phase variability was observed in the peak of *ccg-16* expression between replicate experiments, suggesting that the phase of the oscillation in *ccg-16* mRNA levels is not as stable in strains lacking the FRQ oscillator as compared to wild-type (Figure 17 D).

The consistency and phase stability of the *ccg-16* oscillation observed in the FRQ-null strain is dependent on the exogenous cues used to synchronize the cultures. FRQ-null cultures synchronized by a temperature shift (30°C to 25°C) displayed a rhythm in *ccg-16* mRNA accumulation in all experiments. However, using a light to dark transition synchronized cultures in only half of the experiments and the phase of the rhythm was highly variable (data not shown). Wild-type strains, on the other hand,

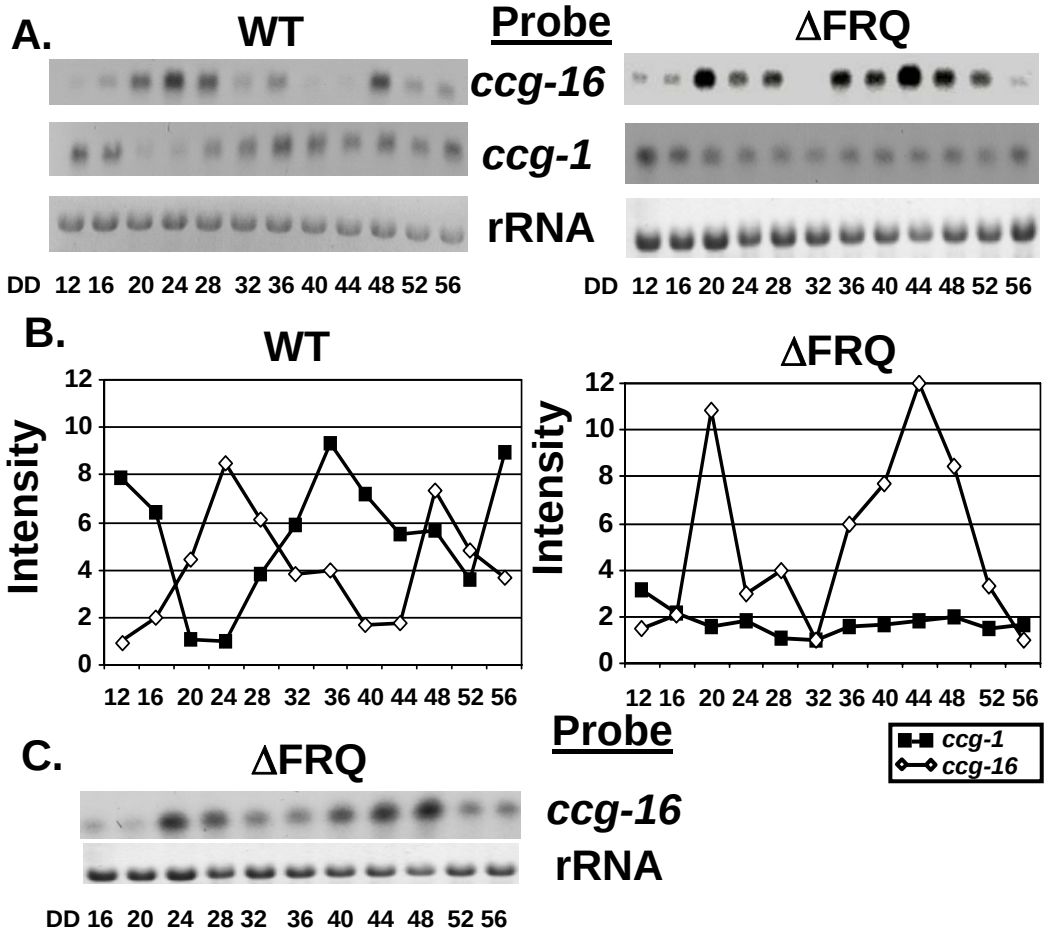

Figure 17. The *ccg-16* transcript is rhythmic in the absence of FRQ.
A. Representative northern blots of *ccg-16* and *ccg-1* in DD are shown. Cultures were harvested in DD 25°C at the indicated times (h) following transfer from LL 25°C to DD 25°C. Blots of RNA from WT (left) and ΔFRQ were probed with *ccg-1* and *ccg-16* probes as indicated. Ethidium stained rRNA was used as a loading control. **B.** Levels of mRNA (band intensity/rRNA) of *ccg-16* and *ccg-1* are plotted versus time. **C.** Northern blot of RNA from ΔFRQ cultures harvested at the indicated times in DD following a temperature shift from 30°C to 25°C. rRNA is shown as a control.

exhibited a robust rhythm in *ccg-16* levels with a stable phase when either light or temperature is used to synchronize the cultures. Together, these results indicate that a FLO is responsible for generating the *ccg-16* rhythm and that this oscillator responds better to temperature that light cues for synchronization.

We hypothesized that if *ccg-16* rhythms are independent of FRQ, then the rhythm should be maintained in cultures that overexpress FRQ. When *N. crassa* is grown in constant light, constitutive non-circadian conidiation is observed in race tube cultures (Figure 18A). Under these conditions, *frq* mRNA is expressed at high levels and does not accumulate in a rhythmic manner (Figure 18B). Consistent with FRQ-independent *ccg-16* rhythmicity, the levels of *ccg-16* mRNA oscillate with a ~24 h period in LL in both wild-type and FRQ-null strains (Figure 18C, D). In the FRQ-null strain, the period and phase of the *ccg-16* rhythm were variable, similar to the DD experiments when FRQ-null cultures were synchronized by light. In contrast, the FRQ oscillator-dependent *ccg-1* gene was not expressed in a rhythmic manner in LL in either strain. These data confirm that *ccg-16* is controlled by a FLO; *ccg-16* mRNA accumulation is rhythmic in the absence of the FRQ protein and in conditions where both the conidiation rhythm is abolished and FRQ is expressed at high levels in a non-circadian fashion.

Rhythms in ccg-16 *mRNA require the WC-1 protein*

We examined the possibility that WC-1 might be involved in the control of *ccg-16* rhythms. A WC-1 null strain, 418-3 (*bd; wc-1^{KO}; A*) grown in DD was used to

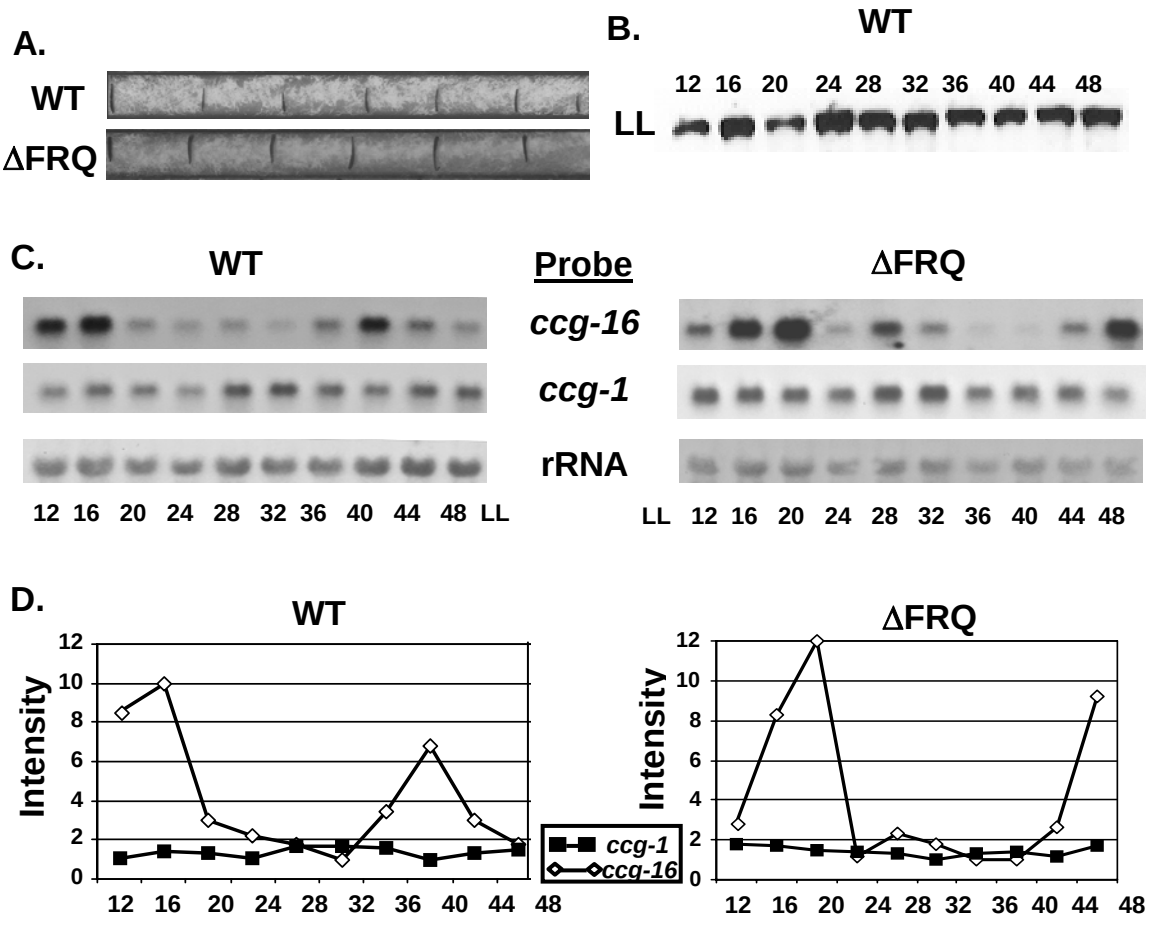

Figure 18. The *ccg-16* transcript is rhythmic in LL. A. Race tube cultures of wild-type (WT) (top) and ΔFRQ (bottom) strains were grown in DD for 24 h at 25°C prior to transfer to LL 25°C. The vertical lines represent 24 h of growth; the direction of growth is from left to right. **B.** Total protein isolated from WT cultures after transfer from DD 25°C to LL 25°C at the indicated times (h). Western blots were probed with polyclonal antibodies to FRQ.
C. Representative northern blots of *ccg-16* and *ccg-1* in LL are shown. Total RNA was isolated from WT and ΔFRQ strains after transfer from DD 25°C to LL 25°C at the indicated times (h). Northern blots were probed with *ccg-1* or *ccg-16* specific probes. rRNA was used as a loading control. **D.** mRNA levels (band intensity/rRNA) were determined for *ccg-1* and *ccg-16* and are plotted versus time.

examine *ccg-16* rhythmicity following temperature and light synchronization of cultures (30° C LL to 25° C DD). Although the levels of both *ccg-1* and *ccg-16* fluctuated slightly over the course of the day, the mRNA levels were arrythmic for both genes in 6/6 experiments (Figure 19A, B). Similarly, *ccg-16* and *ccg-1* mRNA accumulation in the WC-1 null strain in LL (synchronized by a 30°C DD to 25°C LL transfer) was arrhythmic. Together, these data indicate that WC-1 is required for rhythmic *ccg-16* mRNA accumulation.

WC-1 is rhythmic in the absence of FRQ and in LL

In wild-type *N. crassa*, *wc-1* transcript is present in the cell at constituitive levels in DD, whereas WC-1 protein accumulates with a low-amplitude circadian rhythm with a peak in the subjective morning, antiphase to the FRQ expression pattern (Lee *et al.*, 2000). Previous studies suggest that WC-1 rhythms require FRQ, as WC-1 cycles with a long period in the long period frq^7 strain in DD (Lee *et al.*, 2000). Current models propose that FRQ drives the rhythm in WC-1 accumulation via a posttranscriptional mechanism (Lee *et al.*, 2000). If WC-1 is required for rhythms in *ccg-16* accumulation in LL and in a FRQ-null strain, we hypothesized that WC-1 protein may also be rhythmic under these conditions.

We examined WC-1 protein levels in wild-type and FRQ-null strains in DD and LL following temperature and light synchronization (30°C LL to 25°C DD or 25°C DD to 30°C LL). Consistent with previous results (Chapter II and (Lee *et al.*, 2000), two forms of the WC-1 protein and a rhythm in WC-1 accumulation was observed in the

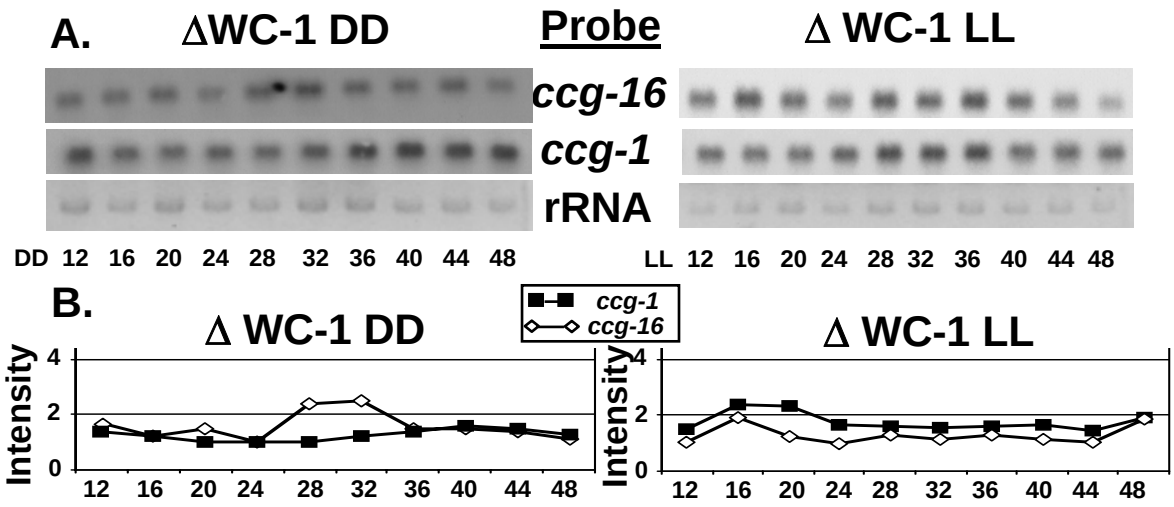

Figure 19. WC-1 is required for *ccg-16* rhythms. A. Total RNA was isolated from ΔWC-1 strains after transfer from LL 30°C to DD25 °C (left) and from DD 30°C to LL 25°C (right) at the indicated times (h). Northern blots were probed with *ccg-16* (top) and *ccg-1* (middle) probes. rRNA was used as a loading control (bottom). **B.** The mRNA levels (band intensity/rRNA) are plotted versus time.

wild-type strain in DD. In the FRQ-null strain, however, only the larger of the two forms and robust rhythms in WC-1 accumulation were observed in DD (Figure 20). Furthermore, in LL, rhythms in WC-1 accumulation were observed in both the wild-type and the FRQ-null strains, with the wild-type displaying two forms of the protein and the FRQ-null strain only showing the larger of the forms, and indication that oscillations in WC-1 protein levels do not require a functional FRQ oscillator (Figure 20). Consistent with previous results, overall levels of the WC-1 protein were significantly reduced in the FRQ-null strain, indicating that while FRQ is involved in the maintenance of overall WC-1 levels, it is not required for rhyhmicity of the WC-1 protein.

ccg-16 *is not required for WC-1 rhythms*

To determine if *ccg-16* is required for normal circadian rhythmicity and may be a possible component of a FLO, we created a deletion strain by replacing the predicted *ccg-16* coding sequence with the *S. hygroscopicus hygromycin phosphotransferase* (*hph*) gene in a wild-type strain. Normal circadian rhythms were observed in this strain, indicating that *ccg-16* is not required for circadian conidiation rhythms in DD (data not shown). In order to determine if CCG-16 is an output component, or if it feeds back on the oscillator, we assayed WC-1 levels in DD in the *ccg-16* knockout strain. Rhythms in WC-1 levels were observed in the wild-type and the *ccg-16* knockout (Figure 21), indicating that *ccg-16* is an output component and does not feed back on the circadian oscillator, at least at the level of WC-1 rhythmicity.

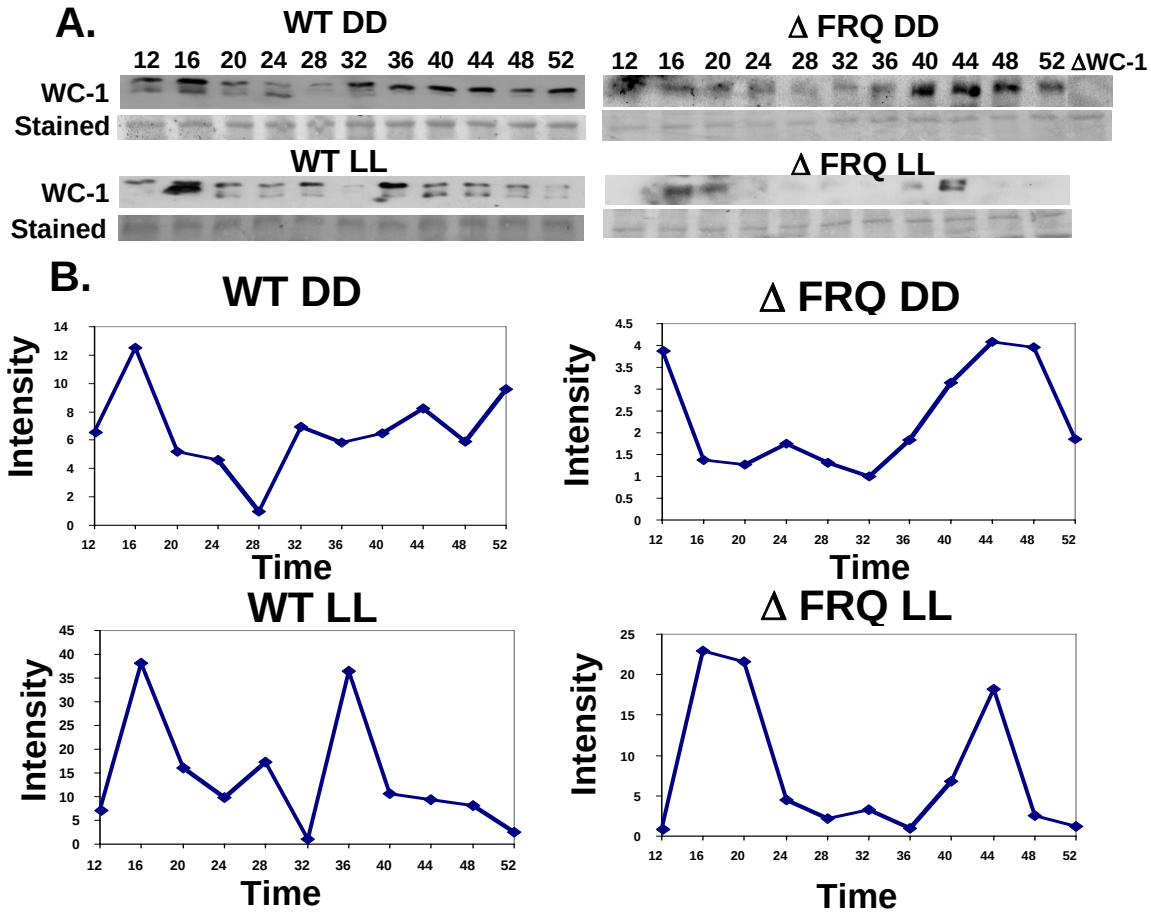

Figure 20. WC-1 protein accumulates in a rhythmic manner in LL and in the absence of FRQ. A. Total protein was isolated from WT (left) and ΔFRQ (right) strains harvested at the indicated times following transfer from LL 30°C to DD 25°C (top) or from 25°C DD to 30°C LL (bottom) and western blots were probed with WC-1 antibodies. Samples were treated with λ protein phosphatase prior to SDS-PAGE. Exposure times were 1 min. for WT and 3 min for ΔFRQ extracts. Extracts from a ΔWC-1 strain are shown in the ΔFRQ DD blot to demonstrate specificity of the antibody. Amido black stained membranes are shown below each blot and were used for loading normalization. **B.** Levels of WC-1 were normalized to amido black stained membranes; trough levels were set to 1. These results are representative of four independent experiments.

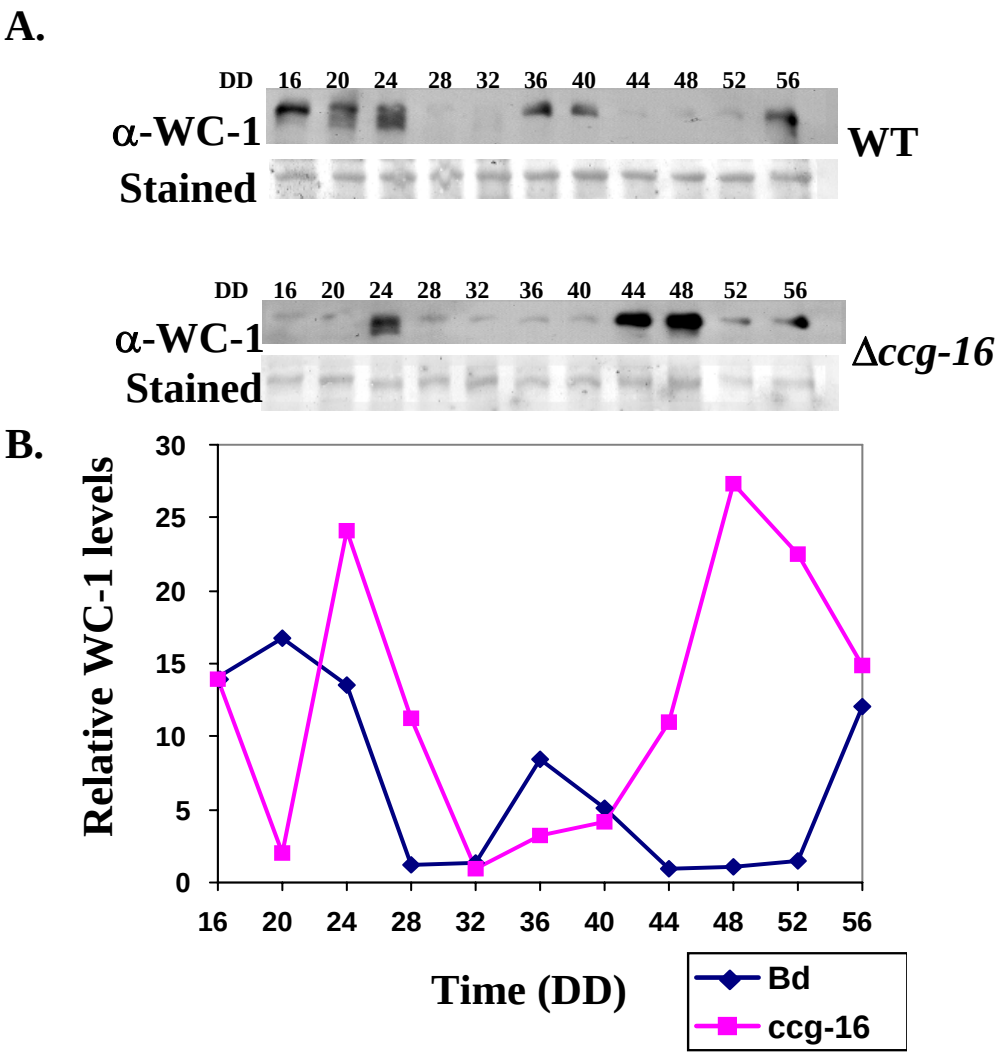

Figure 21. The WC-1 protein accumulates in a rhythmic manner in a Δ*ccg-16* strain. A. Total protein was isolated from WT (top) and Δ*ccg-16* (bottom) strains harvested at the indicated times following transfer from LL 30°C to DD 25°C, and western blots were probed with α-WC-1 antibodies. Amido black stained membranes are shown below each blot and were used for loading normalization. **B.** Relative WC-1 levels (band intensity/amido stained protein) are plotted versus time.

Discussion

The complexity of the circadian system of *N. crassa*, a relatively simple eukaryotic organism, is becoming increasing clear. It is now apparent that the traditional view of a linear pathway, leading from environmental cues to a circadian pacemaker to rhythmic output, can no longer sufficiently describe the circadian clock. While this view of the clock has been instrumental in the identification of the interlocked transcriptional and post-transcriptional feedback loops that compose the clock mechanism, future research will require an appreciation of the multioscillatory nature of *N. crassa* circadian rhythms.

Early molecular studies in *N. crassa* circadian rhythms were based on the morning-specific circadian rhythms in conidiation and the morning-specific *ccg-1* and *ccg-2* genes (Aronson *et al.*, 1994b; Bell-Pedersen *et al.*, 1996b; Crosthwaite *et al.*, 1997). Although it was initially hypothesized that the circadian clock only controlled morning-specific processes, more recent data indicated that the clock regulates the mRNA levels of genes which peak in abundance at all phases of the circadian cycle (Correa *et al.*, 2003). In this study, the regulation of an evening-specific gene, *ccg-16*, is examined. We show that the *ccg-16* transcript cycles in the absence of FRQ and in LL, a condition in which FRQ protein levels are elevated and arrhythmic. These data indicate that the rhythms in *ccg-16* mRNA are controlled by a FLO which maintains rhythmicity independent of FRQ. Rhythms in *ccg-16* mRNA are dependent on WC-1, and WC-1 is rhythmic in the absence of FRQ and in LL, where FRQ levels are elevated and arrhythmic. This suggests that WC-1 is regulated by a FLO, and that this FLO provides

feedback on the FRQ oscillator through regulation of WC-1. A previous study examined the levels of the WC-1 protein in DD in the long-period *frq*^{7} strain, and found that the period of the rhythm in WC-1 abundance was lengthened accordingly (Lee *et al.*, 2000). These data indicate that while the FLO controlling WC-1 dependent *ccg-16* rhythmicity can oscillate in the absence of FRQ, FRQ oscillator rhythms can also affect the period of the WC-1 protein rhythms, indicating that both the FLO and the FRQ oscillator affect WC-1 rhythmicity. Our experiments, however, do not address whether WC-1 is a component of the FLO which controls *ccg-16* rhythms. In addition, our data suggest that the FLO requires a functional FRQ oscillator to maintain accurate phasing of circadian gene expression in DD, and that the FLO entrains more reliably to temperature cues than light cues. Finally, our data indicate that CCG-16 does not feed back on WC-1 rhythmicity, as rhythms in WC-1 and conidiation persist in a *ccg-16* null strain.

These data strongly indicate a need to revise the current model for *N. crassa* circadian rhythms. The previous model proposed that rhythms in WC-1 protein arose through regulation by FRQ at the post-transcriptional level (Lee *et al.*, 2000). Our data contradict this model and suggest that, although FRQ is required for maintenance of WC-1 levels and for synthesis of both the small and large forms of the protein, FRQ is not required for the post-transcriptional event leading to WC-1 rhythmicity. Thus, some other yet to be identified factor is involved in the generation of WC-1 rhythms, although our data do not indicate whether the FLO controlling *ccg-16* is involved in the generation of WC-1 rhythms.

This study extends our understanding of the *N. crassa* circadian system by providing evidence of a coupled, multioscillatory system that is composed of at least two oscillators, one the light responsive FRQ oscillator, the other a temperature responsive FLO. This parallels the 2-oscillator system proposed in 1959 by Pittendrigh and Bruce to explain differential responses of the *Drosophila* eclosion rhythm to light and temperature cues (Pittendrigh and Bruce, 1959). Their model proposes the existence of a light-entrainable "A" oscillator, which is coupled to a temperature entrainable "B" oscillator. Both oscillators are autonomous and can be directly entrained, but the B oscillator is dependent on the A oscillator for complete circadian properties and proper phasing. In *N. crassa*, the light-entrainable FRQ oscillator is analogous to the A oscillator, while the temperature-entrainable FLO controlling *ccg-16* rhythms corresponds to the B oscillator. The FLO would thus receive phase information form two sources, namely ambient temperature and the light-entrainable FRQ oscillator. The coupling of these two oscillators would serve to provide stability to the system, while simultaneously allowing each oscillator to be entrained to different inputs and control different outputs. In the absence of exogenous temporal cues, the stability of the circadian system would depend on this mutual coupling for maintenance of period and phasing of the oscillators. A similar situation likely exists in the network of oscillators present in the differentiated tissues of metazoans, in which coupling of the oscillators is thought to occur through diffusible signals from the master light-responsive pacemaker located in the brain. An understanding of how these oscillators communicate with one another is integral to understanding the circadian system.

In conclusion, these data illustrate the complexities of the *N. crassa* circadian system, and underscore the fact that much remains to be learned about the clock, even in a simple model system. Importantly, using rhythmic conidiation as an assay for clock function would not disclose mutations in components of the FLO, which are currently unknown, as is the extent of genes and processes regulated by the FLO. However, this study provides a foothold needed to solve the question of how multiple oscillators interact and generate precisely controlled circadian rhythms throughout the organism.

CHAPTER IV

A CIRCADIAN OSCILLATOR IN *ASPERGILLUS SPP.* REGULATES DAILY DEVELOPMENT AND GENE EXPRESSION.[1]

Summary

We have established the presence of a circadian clock in *Aspergillus flavus* and *Aspergillus nidulans* by morphological and molecular assays, respectively. In *A. flavus*, the clock regulates an easily assayable rhythm in the development of sclerotia, which are large survival structures produced by many fungi. This developmental rhythm exhibits all of the principal clock properties. The rhythm is maintained in constant environmental conditions with a period of 33 h at 30°C, it can be entrained by environmental signals, and it is temperature compensated. This endogenous 33-h period is one of the longest natural circadian rhythms reported for any organism, and this likely contributes to some unique responses of the clock to environmental signals. In *A. nidulans*, no obvious rhythms in development are apparent. However, a free running and entrainable rhythm in the accumulation of *gpdA* mRNA (encoding glyceraldehyde-3-phosphate dehydrogenase) is observed, suggesting the presence of a circadian clock in this species. We are unable to identify an *Aspergillus* ortholog of *frequency*, a gene required for

[1] Reprinted with permission from "A Circadian Oscillator in Aspergillus spp. Regulates Daily Development and Gene Expression" by Greene, A.V., Keller, N., Haas, H., and Bell-Pederson, D. (2003) *Eukaryotic Cell* **2:** 231-237. Copyright © 2003, American Society for Microbiology. All experiments were performed by A.V. Greene in the laboratories of D. Bell-Pedersen and N. Keller. H.Haas provided sequence information regarding the LREA and LREB proteins prior to publication. This chapter was written by A. V. Greene and D. Bell-Pedersen.

normal circadian rhythmicity in *Neurospora crassa*. Together, our data indicate the existence of an *Aspergillus* circadian clock, which has properties that differ from that of the well-described clock of *N. crassa*.

Introduction

Demonstrations of circadian rhythms are widespread. Among fungi, the ascomycete *Neurospora crassa* is the only model system in which the molecular mechanisms of circadian rhythms have been examined. Thus, we know essentially nothing about the mechanism of the clock in other fungal species. To compare the components of circadian clocks in related fungi, we have begun an investigation of circadian clocks in the medically and agriculturally important ascomycetes, *Aspergillus nidulans* and *Aspergillus flavus*.

Circadian clocks (composed of one or more oscillators) provide organisms with the ability to keep in synchrony with the external world (Edmunds, 1988). The clock generates a program with a duration of ca. 24 h, allowing anticipation of cyclic changes in the environment, particularly light and temperature changes. The formal properties defining circadian rhythms are nearly identical in all organisms studied thus far and provide the diagnostic criteria for identifying an endogenous circadian clock (Edmunds, 1988). By definition, a rhythm is circadian if it persists in the absence of temporal signals (e.g., constant temperature and darkness) with a period of ca. 24 h. A rhythm that persists under constant conditions is called a free-running rhythm, and its period is referred to as the free-running period (FRP).

A second fundamental characteristic of circadian rhythms is that they can be reset (a process termed entrainment) by external signals, such as the daily light and temperature cycles (Aschoff, 1981; Bruce, 1960). Entrainment results from perception of external time cues (*zeitgebers*) by one or more clock components and shifts the circadian clock to an appropriate phase. The magnitude and direction of the phase change is dependent on the intensity and duration of the *zeitgeber* and the phase of the internal circadian clock when the cue is given. Organisms can also entrain to environmental cycles of greater or less than 24 h within species-specific limits (Bruce, 1960). In this case, the circadian clock will systematically adopt a stable phase relationship to the imposed cycles and the rhythm will have the same period as the entraining cycle.

Another defining characteristic of circadian rhythms is that their FRPs are temperature compensated (Hastings and Sweeney, 1957). This means that over a physiologically defined range of temperatures, the FRP of a circadian rhythm remains relatively unchanged with a Q_{10} (the ratio of the rate at a given temperature to the rate at a temperature that is 10° lower) ranging from 0.8 to 1.4 (Hastings and Sweeney, 1957). This is in contrast to most biochemical reactions, which typically increase in rate as the temperature increases (Q_{10} ~2 to 3). Yet, because circadian rhythms can be entrained by temperature transitions (pulses or cycles), the system is not entirely temperature independent.

A simple model of a circadian clock includes three central components: a self-sustained oscillator(s), input pathways that convey environmental information and synchronize the oscillator(s) to the external world, and output pathways leading from the

oscillator(s) to the expressed rhythms. In *N. crassa*, the circadian clock directs the daily production of asexual spores (conidiospores) with a FRP of 22 h (Sargent *et al.*, 1966). Several proteins involved in rhythmicity have been identified in *N. crassa* (Loros and Dunlap, 2001). These include the FREQUENCY (FRQ), WHITE COLLAR-1 (WC-1), and WC-2 proteins. These clock components form a feedback loop in which the negative element FRQ feeds back to shut down its own expression presumably by repressing the activity of the positive-acting elements, WC-1 and WC-2 (Loros and Dunlap, 2001). There are functional similarities between the proteins of the *N. crassa* clock and those of other kingdoms. The *N. crassa* WC-1 and WC-2 proteins each contain PAS domains involved in protein-protein interactions, which are similar to those found in the mammalian clock proteins PERIOD, CLOCK, and BMAL1 (Dunlap *et al.*, 1999). WC-1 was recently shown to be a blue-light photoreceptor, linking the environment through an input pathway to the circadian oscillator (Froehlich *et al.*, 2002; He *et al.*, 2002). The output pathways are less well characterized in *N. crassa*, although several output clock-controlled genes are known that are involved in general stress responses, cellular metabolism, and development (Bell-Pedersen, 2000).

While *N. crassa* has greatly contributed to a molecular understanding of clock function, this has not been extended to other fungal genera. One genus of immense medical, industrial, and agricultural importance is *Aspergillus*. *A. flavus* is a toxigenic fungus that contaminates plants such as corn and peanuts with the carcinogenic metabolite aflatoxin and is responsible for billions of dollars of crop losses each year. Furthermore, infection by *Aspergillus spp*. (aspergillosis), including *A. flavus*, can lead

to allergic, superficial, saprophytic, or invasive disease, particularly in immunocompromised patients. This genus also includes the model eukaryote *A. nidulans*, which has been used to address several fundamental questions of biology (May and Adams, 1997). The asexual *Aspergillus spp*., such as *A. flavus*, produce two types of reproductive structures: conidiospores, which are small dispersal spores, and larger survival structures called sclerotia (Bennett *et al.*, 1979). Both conidiospore and sclerotium production are photoinducible (Calvo *et al.*, 1999), and recent studies have shown a link between sclerotium production and aflatoxin biosynthesis (Chang *et al.*, 2002). The sexual *Aspergillus spp*., including *A. nidulans*, generally do not produce sclerotia but produce asexual conidiospores and sexual ascospores (Adams *et al.*, 1998).

Several observations prompted our initial investigation of circadian rhythmicity in *Aspergillus*. First, we noticed that *A. flavus* exhibits a rhythm in sclerotial development; however, it was not known whether this rhythm is endogenously generated. Second, although both *A. flavus* and *A. nidulans* possess orthologs of the *N. crassa wc-1* and *wc-2* genes, no ortholog of *frq* appears to be present in either species. These data suggested that an *Aspergillus* clock might be constructed of some components that are different from those of *N. crassa* and indicated that investigation of the circadian system in *Aspergillus* could provide important information regarding clock mechanisms and their evolution.

Materials and methods

Strains and growth conditions

The strain of *A. flavus* used in the present study was 12S, a field isolate obtained from P. Cotty. Strains of *A. nidulans* include A4 (*ve+*) and *PW1 (argB2 methG1 biA1 veA1*) (Fungal Genetic Stock Center, Kansas City, Mo.). Unless otherwise indicated, strains were grown at 30°C on race tubes (Sargent *et al.*, 1966) containing 14 ml of *Aspergillus* complete medium (CM), comprised of 2% glucose, 1X *Aspergillus* salts plus trace elements, 0.2% peptone, 0.1% yeast extract, 0.1% Casamino Acids, and 1.5% agar at pH 6.5 (www.fgsc.net/methods/anidmed.html). Race tubes were 30-cm-long glass tubes bent up at both ends with an inner diameter of 1.5 cm. Conidia (~5 X 10^6) from 3- to 14-day-old plate cultures were suspended in 1 ml of water, and 2 μl of this spore suspension was used to inoculate the race tubes. Race tube cultures were incubated in a 24-h light-dark cycle (12:12 LD) for 3 to 7 days prior to transfer to constant darkness (DD) or constant light (LL) in controlled environmental chambers (Percival Scientific, Inc.) containing broad-spectrum fluorescent lights (Phillips F20T12 CW 20W). The light intensity was 45 μmol of photons/m^2/s (ca. 2,300 lx). Previous studies have shown that *Aspergillus* is responsive to both red and blue light but not to green light (Mooney and Yager, 1990b; Yager *et al.*, 1998). When cultures were grown in DD, a green safe light was used to observe and mark the growth front at the same time each day. The green safe light consisted of a green light bulb (Sylvania, 25 W) in a Kodak adjustable Safelight lamp covered with 20 layers of green cellophane. The emission spectrum of the green light bulb was measured by using a USB2000 spectroradiometer (Ocean Optics,

Inc.) in which a single peak was observed at 540 nm. In 12:12 LD cycles with the green light as the light source, the cultures were not entrained, and the growth rate of the cultures was not affected (data not shown). Digital photographs of race tubes were analyzed with the CHRONO program (Roenneberg and Taylor, 2000) generously provided by Till Roenneberg, and traces were generated by using NIH Image 1.61.

The ratio of the frequency of the average FRP at 40°C (28.4 h) to that at 30°C (33.4 h) was used to calculate the Q_{10} for the sclerotial rhythm between these temperatures. The estimated Q_{10} for the sclerotial rhythm between 28 and 30°C was calculated as the ratio of the frequency of the average FRP at 30°C (33.4 h) to that at 28°C (51.4 h) multiplied by 5.

Environmental entrainment

For LD entrainment assays, *A. flavus* 12S was grown on CM in a 10:10 LD cycle, a 12:12 LD cycle, or an 18:18 LD cycle at 30°C. For each cycle length, the race tubes were marked at the mycelial growth front at the dark-to-light transition for reference. For temperature entrainment assays, the cultures were inoculated onto CM race tubes and incubated in DD with 24-h cycles of 30°C (12 h) and 38°C (12 h). The race tubes were marked at the transition from 30 to 38°C.

To examine the phase response of *A. flavus* to external signals, strain 12S was cultured on race tubes and transferred to DD at 30°C after 3 days of growth in 12:12 LD at 30°C at the end of the last dark phase in the LD cycle. Cultures of *A. flavus* strain 12S were given a light pulse of 45 μmol of photons/m2/s (ca. 2,300 lx) for 1 h before transfer

back to DD 30°C. If we assume a 33-h FRP (see Results for details), the pulses were given starting at circadian time zero (CT0) of circadian day 3. Circadian time is a way to normalize biological time in strains or organisms with different endogenous period lengths to 24 circadian hours per cycle. By definition, CT0 is subjective dawn and CT12 is subjective dusk. Light pulses were given to a subset of race tubes at intervals of 4 circadian hours (every 5.5 local hours) over one circadian day (33 h). Thus, one set of tubes remained in the dark for the entire time, and other sets of tubes were pulsed at CT0, CT4, etc. After the pulse, the race tubes were returned to DD at 30°C for 4 days. The change in phase of the sclerotial rhythm after the light pulse was determined 3 days after the pulse compared to the nonpulsed control, and the data are plotted as a phase-response curve (PRC) (Pittendrigh, 1960).

Rhythmic RNA analysis

A. nidulans strain A4 was inoculated into petri dish cultures of minimal medium, comprised of 1 X *Aspergillus* salts plus 1% glucose and trace elements (www.fgsc.net/methods/anidmed.html). After 16 to 20 h of growth at 37°C in DD, sections of the resulting mycelial mat were cut with a 7-mm cork borer. Individual sections were transferred to liquid shaking cultures of low-glucose medium (0.1% glucose and 1X *Aspergillus* salts plus trace elements) to inhibit development (Loros *et al.*, 1989) and then incubated at 30°C in LL for 24 h. For free-running experiments, cultures were transferred from LL to DD at 4-h intervals so that, at the subsequent time of harvest, the cultures were all of the same age but represented different phases in the

circadian cycle. For entrainment experiments, cultures were transferred to alternating 12-h light at 38°C and 12-h dark at 30°C cycles and harvested after one full cycle every 4 h for 2 days. At harvest, the mycelia were frozen in liquid nitrogen. To extract RNA, frozen mycelia were ground with a mortar and pestle in RNA extraction buffer (0.1 M sodium acetate, 1 mM EDTA, and 4% sodium dodecyl sulfate [SDS] at pH 8). After four phenol extractions, total RNA was precipitated with ethanol. Northern analysis (10 μg of RNA/lane) was performed as described previously (Bell-Pedersen *et al.*, 1996b). Hybridization was to a probe made from a 500-bp exon of the *Aspergillus gpdA* gene, which was amplified from *A. nidulans* genomic DNA by using the upstream PCR primer 5'-GCCTTAAGCACCATTGAGACCTACGACGA-3' and the downstream primer 5'-GCCCTAGGTCAACTAGTTAGTCGAAGATG-3'. The upstream primer was designed to add an *Eco*RI restriction site (underlined), whereas the downstream primer was designed to add a *Bam*HI site (underlined) to facilitate cloning into a vector for sequence verification and maintenance. The PCR cycles were 94°C for 5 min, 50°C for 2 min, and 72°C for 3 min for a total of 30 cycles. The probe was labeled with [^{32}P]dCTP (6,000 Ci/mmol) by using the DECAprime II kit (Ambion, Austin, Tex.). Ethidium bromide-stained rRNA was used as a control for RNA loading.

Results

A. flavus *displays an endogenous rhythm in sclerotium production*

Preliminary experiments with petri dish cultures of *A. flavus* 12S indicated that the organism exhibits rings of sclerotia when a spore suspension is point inoculated and

allowed to grow in the absence of temporal cues. To investigate whether this developmental behavior was under control of a circadian clock, *A. flavus* 12S was grown on race tubes half-filled with CM (see Materials and Methods) in constant environmental conditions. We found that 12S cultures that were synchronized to 12:12 LD at 30°C and then transferred to DD consistently exhibited a free-running rhythm of sclerotium development with a period of 33.4 ± 0.2 h (mean ± the standard error of the mean [SEM]; n = 56) (Fig. 22A). The peak in sclerotium production occurred in the early evening, at ca. CT14. A stable 33-h free-running rhythm began immediately after transfer into DD; thus, there is no transient behavior. In addition, the rhythm persisted for up to 3 weeks in DD, until the time at which the cultures reached the end of the race tube (data not shown). When cultures were transferred from 12:12 LD cycles to LL at 30°C they formed sclerotia continuously for several days (Fig. 22B); however, we observed that ca. 20% cultures showed broad bands of sclerotia in LL after 5 days (data not shown).

In *N. crassa*, the period of the conidiation rhythm is essentially unchanged when cultures are grown on different media (Sargent and Kaltenborn, 1972). We examined *A. flavus* sclerotial rhythms when strain 12S was grown on potato dextrose agar (PDA), Champe's medium (2% glucose-0.5% yeast extract), and minimal medium (http://www.fgsc.net/methods/anidmed.html). *A. flavus* grew more slowly on these media (0.58 ± 0.04 cm/day [n = 11], 0.51± 0.03 cm/day [n = 12], and 0.33 ± 0.02 cm/day [n = 6] on PDA, Champe's medium, and minimal medium, respectively) compared to CM (0.93 ± 0.02 cm/day [n = 33]). Sclerotial rhythms were observed on minimal

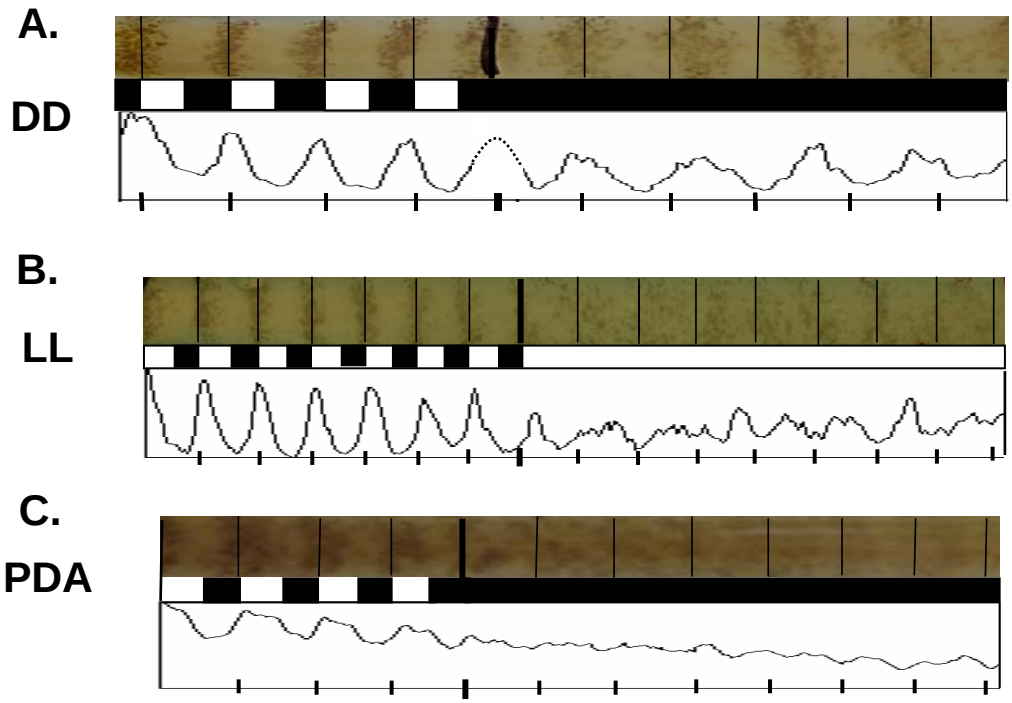

Figure 22. *A. flavus* forms bands of sclerotia in constant darkness. *A. flavus* strain 12S was inoculated onto race tubes containing CM and grown in 12:12 LD cycles for 5 days at 30°C before transfer into DD (A) or LL (B). The time of transfer is marked with a heavy black line. Bars below the race tubes represent the times that the cultures were in the light (white bars) or dark (black bars). Representative race tubes are shown. The direction of growth is from left to right. The mycelial growth front was marked at the same time each day (black lines) for reference. (C) *A. flavus* strain 12S was inoculated onto race tubes containing PDA medium and grown in 12:12 LD cycles for 7 days at 30°C before transfer into DD. In each panel, an intensity tracing of the race tubes is shown below, with the lines on the x axis representing the 24-h growth front marks.

medium in DD; however, no free-running rhythms in development were detected on PDA or Champe's medium (Fig. 22C and data not shown). These data indicated that the circadian rhythm in sclerotium development, unlike the *N. crassa* conidiation rhythm, is not well nutritionally compensated.

A. flavus *developmental rhythms are entrained by environmental cycles*

A defining property of circadian rhythms is that they are able to synchronize, or entrain, in response to cyclical cues in the environment (Aschoff, 1981). We examined *A. flavus* strain 12S to determine whether the sclerotial rhythm could be entrained by light or temperature cycles. Cultures were grown on race tubes in LD cycles that were 20, 24, or 36 h in duration. In each case, the period of the rhythm matched the entraining cycle (Fig. 23A). For example, the 12:12 LD cycle resulted in shortening the 33-h FRP to 24 h. Importantly, the phasing of the peak of sclerotium formation differed with cycle length (Fig. 23A). In the 10:10 LD cycle, the peaks of sclerotia occurred ca. 3 h (2.7 ± 0.1 h [n = 38]) prior to the dark-to-light transition. In the 12:12 LD cycle the peaks of sclerotia occurred slightly after the time of the dark-to-light transition (~0.75 ± 0.2 h [n = 23]), whereas in the 18:18 LD cycle the peaks of sclerotia occurred 3 h (~2.9 ± 1.64 h [n = 38]) after the dark-to-light transition. These observations demonstrated that the clock that controls the developmental rhythm is synchronized by the LD cycle and is not merely driven by the exogenous time cues since the phase of a driven rhythm would not change with respect to the length of the cycle.

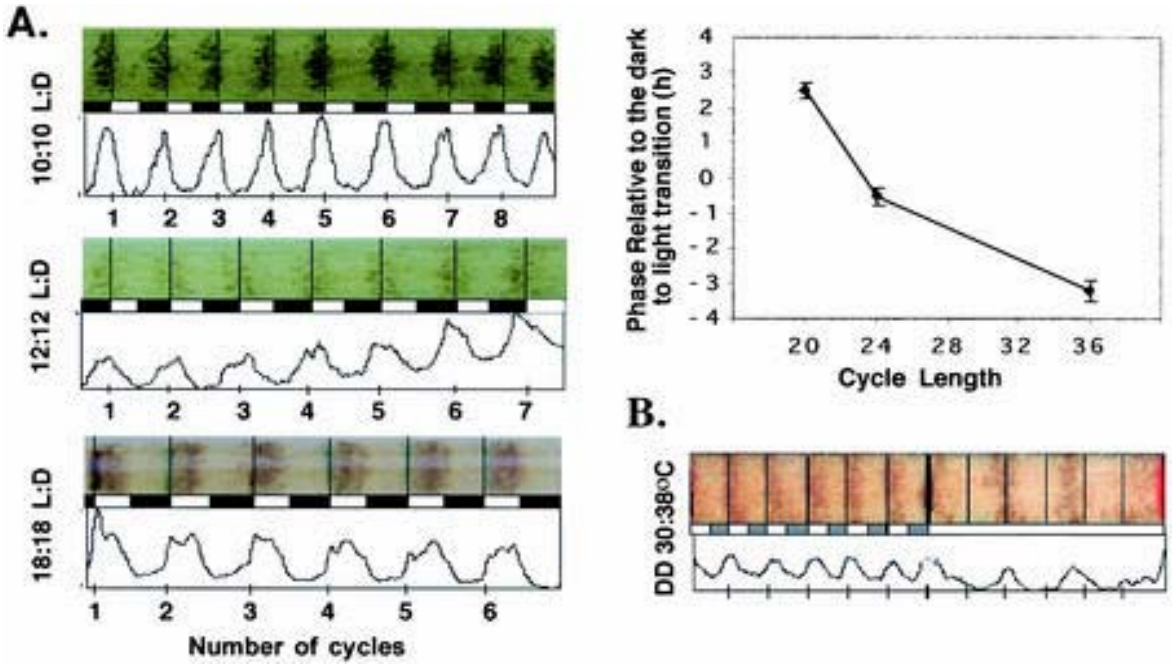

Figure 23. *A. flavus* 12S entrains to different light and temperature cycles. **(A)** *A. flavus* 12S was inoculated onto CM race tubes and allowed to grow for 3 days in LL at 30°C (not shown). Race tubes were then transferred into 20-, 24-, or 36-h LD cycles at 30°C. Representative race tubes are shown on the left, with bars and intensity tracings as in Fig. 22. The mycelial growth fronts were marked daily at the dark-to-light transition (black lines). The direction of growth is from left to right. The phase of the center of the sclerotial band relative to the dark-to-light transition was measured in hours and plotted (top right side of figure). Positive numbers indicate that the band occurred prior to the dark-to-light transition, and negative numbers indicate that the band occurred after the dark-to-light transition. Values are means ± the SEM (n = 23). **(B)** *A. flavus* strain 12S was inoculated onto CM race tubes and grown for 3 days in 12:12 LD at 30°C (not shown) and then transferred to DD with 24-h (12:12) cycles of 30 and 38°C. The mycelial growth front was marked at the transition from low to high temperature. The heavy line indicates when the cultures were transferred to constant 30°C. The bar below the race tubes indicates the time at 30°C (white) and 38°C (gray shaded). Tracings of the image are shown below the race tube.

The developmental rhythm was also entrained to a temperature cycle. In a 12:12 temperature cycle of 30 and 38°C, the period of the rhythm was 24 h (Fig. 23B). The sclerotial bands occurred ca. 3 h (~2.6 ± 0.1 h [n = 20]) after the transition from low to high temperature. When race tubes were placed in DD at 30°C after a few days in the temperature cycle, the cultures immediately returned to a 33-h period (Fig. 23B).

Light pulses reset the sclerotium developmental rhythm

A fundamental property of circadian oscillators is that short pulses of light and/or temperature can advance or delay the observed rhythm, depending on the phase of the oscillator at the time of the pulse. We examined the *A. flavus* sclerotial rhythm growing at 30°C in DD for phase shifting by using a 1-h light pulse given every 5.5 h for 33 h. The rhythm was responsive to the light pulses at all of the times tested and, as expected for a circadian rhythm, the magnitude of response varied over the course of the day. The data are plotted as a phase response curve (PRC) (Fig. 24). A light pulse given to cultures from mid-day to the early evening resulted in a phase advance of the rhythm compared to the nonpulsed control, with the largest advances occurring at subjective dusk (CT12). Phase delays were observed from subjective late night to dawn (CT20 to CT0), but only small changes in the phase were observed at these times. These data demonstrate that phase resetting occurs primarily by phase advances in *A. flavus*, since phase delays were very weak and infrequently observed.

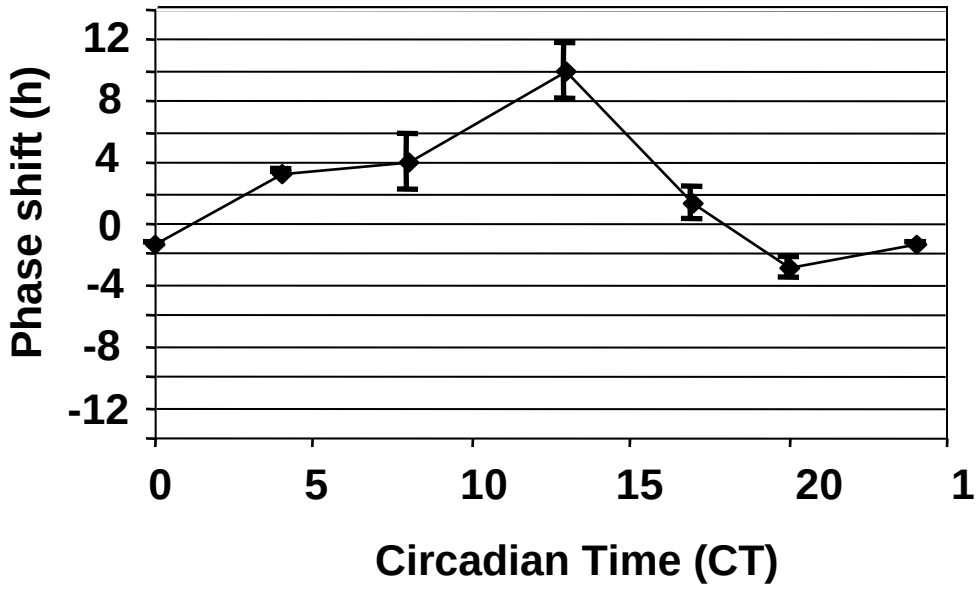

Figure 24. The sclerotial rhythm in *A. flavus* is reset by environmental signals. A PRC was generated as described in Materials and Methods. The phase response was calculated as the difference in position of the sclerotial band after light treatment compared to the untreated controls. The x axis indicates the circadian time (CT) as calculated from the 33-h FRP of *A. flavus* 12S on CM medium at 30°C; the y axis shows the phase shift measured in hours of advance (positive numbers) or delay (negative numbers). Values are means ± the SEM (n = 5).

The rhythms of sclerotium development are temperature compensated

Another defining feature of circadian rhythms, but one that is poorly understood, is that the FRP remains relatively constant (compensated) over a range of physiologically relevant temperatures; thus, we sought to determine whether the rhythm in sclerotium production is temperature compensated. The optimal growth temperature for *A. flavus* is 30 to 32°C, with a minimum of 10°C and maximum of 42°C (N. Keller, unpublished data). Temperatures below 28°C were not tested because *A. flavus* produces very few sclerotia below 28°C (data not shown). Cultures were grown on race tubes in DD at temperatures ranging from 28 to 40°C (Fig. 25A). Between 30 and 40°C the period and phase of the sclerotial rhythm remained essentially the same (Q_{10} 30 to 40°C = 1.2 [see Materials and Methods]), whereas the growth rate changed with culture temperature (Fig. 25B). At <30°C, the period of the sclerotial rhythm was longer than at higher temperatures (estimated Q_{10} at 28 to 30°C = 7.7), indicating that this is the lower limit of compensation.

A clock in A. nidulans *regulates gene expression*

In *A. nidulans*, red light is required for conidiation during a critical period of development. Wild-type strains will conidiate in the light, but they are aconidial in the dark. Strains harboring the *veA1* mutation conidiate in the absence of light (Mooney and Yager, 1990b). Therefore, we examined wild-type and *veA1* strains of *A. nidulans* on race tubes in LD cycles, in DD, and in LL at 30°C for rhythmic conidiation. Developmental rhythms were observed only in the wild-type A4 strain in the LD cycles;

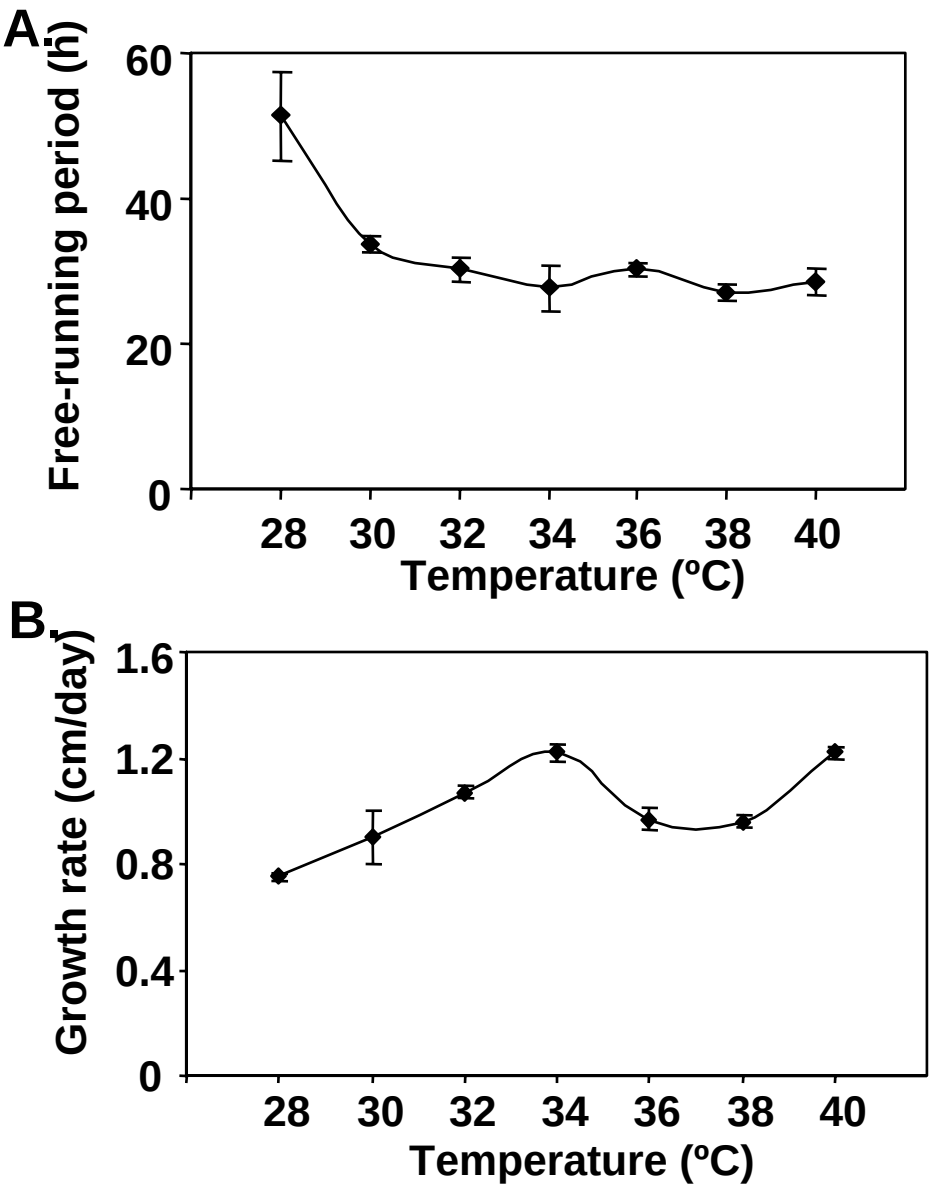

Figure 25. The sclerotial rhythm in *A. flavus* is temperature compensated. **(A)** *A. flavus* strain 12S was inoculated onto CM race tubes and allowed to grow at 30°C in 12:12 LD cycles for 3 days. Cultures were then transferred to the indicated temperatures in DD for 7 days. The data are plotted as the average FRP versus temperature. **(B)** The growth rate of *A. flavus* is plotted versus the temperature. In panels A and B, values are means ± the standard deviation (n = 14).

however, no developmental rhythms were observed under constant conditions. These data suggested that, unlike *A. flavus*, the observed *A. nidulans* developmental rhythm does not run free and is likely controlled directly by the environmental cycle and not by an endogenous circadian clock under these conditions.

To further investigate possible circadian rhythmicity in *A. nidulans*, we isolated RNA from wild-type cultures grown in LL for 1 day and then transferred to DD and harvested at 4-h intervals over two consecutive days. Total RNA was probed with the *A. nidulans gpdA* gene encoding GAPDH (glyceraldehyde-3-phosphate dehydrogenase). Homologs of *gpdA* have been shown to be rhythmically expressed in several organisms (Fagan *et al.*, 1999; Temme *et al.*, 2000), including *N. crassa* (Shinohara *et al.*, 1998). We observed a rhythm in the accumulation of *gpdA* transcripts that has a period of 28 to 32 h (Fig. 26A). Similar to the variability observed between experiments in the time of peak message accumulation for *N. crassa* clock-controlled genes (Bell-Pedersen *et al.*, 1996b), the phase of the *A. nidulans gpdA* rhythm can vary up to 8 h in separate experiments (Fig. 26A). These data suggested the presence of a functioning circadian clock in *A. nidulans* with an FRP similar to that of *A. flavus*. To determine whether this rhythm could be entrained, we examined the *gpdA* rhythm in strains grown in cycles of 12 h of light at 38°C and 12 h of dark at 30°C (Fig. 26B). Simultaneous light and temperature cycles were used because neither light nor temperature alone was sufficient to entrain the rhythm. In the 24-h light and temperature cycle, the *gpdA* rhythm was entrained with a period of 24 h, with peaks of mRNA occurring at the end of the dark-cold phase.

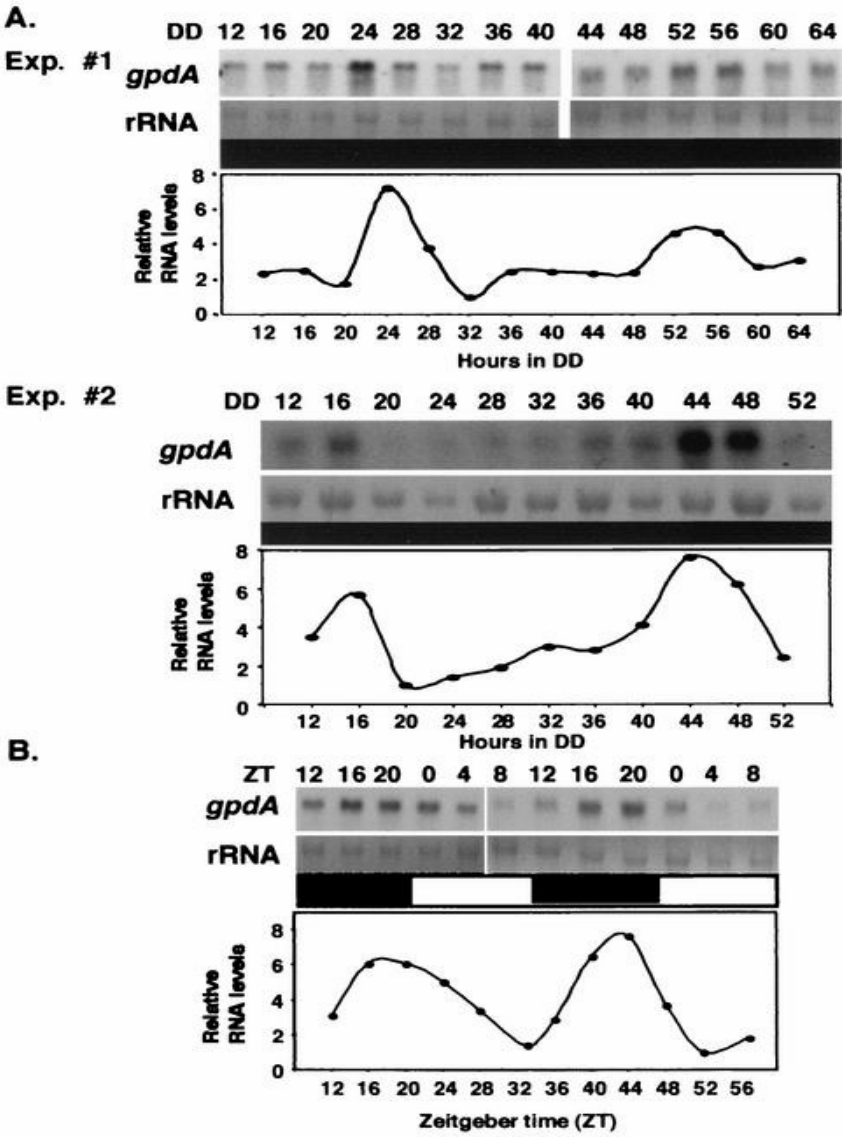

Figure 26. *gpdA* mRNA accumulates rhythmically in *A. nidulans* strain A4. (A) Total RNA was isolated from *A. nidulans* strain A4 grown in DD (black bar) and harvested every 4 h for 2 consecutive days. Two independent experiments are shown. The Northern blots were probed with a PCR product derived from the *A. nidulans gpdA* gene to identify the 1-kb *gpdA* mRNA. Ethidium bromide-stained 25S rRNA is shown as an RNA loading control. A plot of the relative band intensities from the Northern blot is shown below. This experiment was repeated four times with similar results; however, as revealed in a comparison of the two experiments shown, the phasing of the peak of expression varied over eight circadian hours. **(B)** Total RNA was isolated from *A. nidulans* strain A4 grown in cycles of 12 h of light at 38°C (white bar) and 12 h of dark at 30°C (black bar) and then harvested every 4 h for 48 h. Northern blots were probed as in A. The *zeitgeber* time (ZT) is the time in hours from the start of a 12:12 LD cycle. The lights were turned on at ZT0 and turned off at ZT12. The relative band intensities are plotted below.

Identification of clock gene orthologs in Aspergillus

To investigate the conservation of clock components in *Aspergillus* that are required for robust circadian rhythmicity in *N. crassa*, we cloned and sequenced *A. nidulans* orthologs of *N. crassa wc-1* and *wc-2* (M. Schoeser and H. Haas, unpublished data). LreA (accession no. 470313; 836 amino acids [aa]) is 37% identical and 55% similar over 758 aa to WC-1 (accession no. X9430; 1,168 aa), and LreB (accession no. 82072; 417 aa) is 35% identical and 51% similar over 333 aa to WC-2 (accession no. Y09119; 530 aa) (data not shown). In both cases, the overall similarity extends beyond the PAS domains of WC-1 and WC-2. No similar sequences were found in the current limited *A. flavus* database; however, in Southern analyses DNA fragments hybridizing to *lreA* and *lreB* were detected (data not shown).

Several different attempts were made in the present study and in other studies (Lewis *et al.*, 1997; Merrow and Dunlap, 1994) to identify a FRQ ortholog in *Aspergillus*. Based on sequence information, no putative ortholog was apparent in the *A. nidulans* genome sequence database (http://www-genome.wi.mit.edu), the *A. flavus* EST database, or in the newly released *A. fumigatus* database (www.tigr.org/tdb/e2k1/afu1/). Furthermore, efforts to amplify an FRQ-specific band by PCR were unsuccessful, as were attempts to identify a band by low-stringency Southern assays in both species. Based on these data, we conclude that *Aspergillus* lacks an obvious FRQ ortholog. This suggests that the *Aspergillus* circadian clock does not require a protein with strong similarity to FRQ.

Discussion

Our data demonstrate that the observed rhythm in sclerotium formation in *A. flavus* strain 12S is under the control of an endogenous circadian oscillator. The rhythm persists in constant conditions, is entrained and reset by environmental signals, and is temperature compensated. However, although the canonical clock properties are present in the *A. flavus* clock, several features of the rhythm differ from those of other eukaryotes.

The FRP of the *A. flavus* developmental rhythm at 30°C is 33 h and thus is significantly longer than a typical circadian rhythm, which is usually close to 24 h. The longest documented circadian rhythms to date are 29 h in the bean plant *Phaseolus* (Bunning, 1960) and ~30 h for the rhythm of promoter activity of the *Arabidopsis* chlorophyll a/b-binding protein (*cab2*) in DD (Millar *et al.*, 1995). It has generally been assumed that circadian clocks have FRPs close to 24 h in order to maintain a stable phase relationship to the earth's 24-h rotational cycle; the inherent cycle cannot be too far away from the environmentally driven cycle for optimal performance (Pittendrigh, 1960). However, our data suggest that this assumption may require reevaluation and strongly support the importance of investigating circadian rhythms in more organisms. Thus, the long FRP in *A. flavus* suggests that the clock in this organism operates differently than clocks in other well-studied organisms that run closer to 24 h, such that it runs more slowly when environmental cues are absent.

A circadian oscillation can be phased correctly to local time by resetting (entraining) the clock every day to precisely 24 h. Thus, despite the long FRP of *A. flavus*, the sclerotial rhythm is entrained by light and temperature cycles, as would be expected to occur in the natural environment. The steady-state phase relationship between an entrained circadian rhythm and the *zeitgeber* has been shown to be dependent on the FRP and the period of the *zeitgeber*, such that the phase relationship of the entrained rhythm to the *zeitgeber* will change as the period of the *zeitgeber* is shortened or lengthened (Aschoff, 1981; Moore-Ede *et al.*, 1982). In all other organisms examined, when the FRP is shorter than the *zeitgeber* period, the peak of the entrained rhythm occurs before the time cue (positive number), whereas when the FRP is longer than the entraining cycle, the peak of the entrained rhythm occurs after the time cue (negative number). This is the case for wild-type *N. crassa* strains grown in temperature cycles, whereby conidial development occurs after a warm to cold transition when the entraining cycle is shorter than the FRP and occurs before a warm-to-cold transition when the entraining cycle is longer than the FRP (Merrow *et al.*, 1999). However, clock-controlled sclerotium development in *A. flavus* occurs after the lights are turned on (negative value) when the LD cycle is greater than the FRP, and occurs before lights are turned on (positive value) when the cycle is shorter than the FRP. The difference between the phase of the rhythm during entrainment in *A. flavus* and all other organisms examined to date may be directly related to the extremely long FRP in *A. flavus*. Furthermore, the unusual steady-state phase relationship during entrainment in *A. flavus*

is close to what would be theoretically predicted based on the long FRP and the PRC to light (Pittendrigh and Minnis, 1964).

Pulses of light reset the *A. flavus* sclerotial rhythm and only large advances, but not large phase delays, were observed. This finding contrasts with the remarkable similarity in PRCs to light in other organisms, in which phase advances of the rhythm occur in the late subjective night and early subjective day and phase delays occur in the late subjective day and early subjective night (Pittendrigh, 1960). In fact, the PRC for light in *A. flavus* most closely resembles the phase response of organisms grown in the light to a short dark pulse (Johnson, 1992). Typically, species with FRPs longer than 24 h tend to show larger advances than delays, whereas the reverse is true for species and individuals with FRPs shorter than 24 h (Bruce, 1960; Pittendrigh *et al.*, 1991). For example, if an organism has an FRP of 26 h, a daily light exposure would need to produce a 2-h advance each cycle in order for the entrained rhythm to have a period of 24 h. The *A. flavus* rhythm may be an extreme example of this phenomenon, whereby the oscillator may be primarily phase advanced by light in order to keep pace with the environment due to its naturally long FRP.

Our data demonstrating an endogenous circadian clock are not as well developed for *A. nidulans* due to a lack of an easily observable rhythm in this species. However, our experiments demonstrating a free-running and entrainable rhythm in the accumulation of *gpdA* provide support for the existence of a circadian clock. The long period of the free-running rhythm also suggests that similarities may exist between the clocks of *A. nidulans* and *A. flavus*.

The Ascomycete subgroup Euascomycete radiated into several monophyletic groups (estimated at ca. 240 million years ago), including the Plectomycetes, which include *Aspergillus*, and the Pyrenomycetes, which include *Neurospora* (Berbee and Taylor, 2001). *frq* orthologs are present in several species of Pyrenomycetes (Lewis and Feldman, 1996; Lewis *et al.*, 1997; Merrow and Dunlap, 1994), but *frq* has not been found in members of the Plectomycetes. *Aspergillus spp*. lack a detectable *frq* gene, suggesting that the FRQ-based oscillator in *N. crassa* is not conserved among the ascomycetes. Orthologs of *N. crassa wc-1*, which encodes a blue-light photoreceptor (Froehlich *et al.*, 2002; He *et al.*, 2002), and *wc-2* are present in *A. nidulans* and *A. fumigatus* and are predicted in *A. flavus*. In preliminary data, we observed that *gpdA* was rhythmic in *A. nidulans* strains lacking the *wc-1* ortholog *lreA* (data not shown). *Aspergillus* displays blue-light responses (Champe *et al.*, 1994; Yager *et al.*, 1998). Together, these data imply that the primary evolutionary force for maintaining the *wc* genes in the Ascomycetes was to allow blue-light-sensing, rather than circadian rhythms.

The apparent absence of FRQ in *Aspergillus*, along with the unusual properties of the circadian clock in *A. flavus*, suggests that the *Aspergillus* clock differs from the well-described *N. crassa* clock. Furthermore, we have been unable to demonstrate rhythms in *gpdA* in *A. flavus*, suggesting that outputs from the clock differ in *A. nidulans* and *A. flavus*. This difference in output gene expression may reflect the different developmental programs and ecological niches of these two closely related fungal species. The difference in outputs from the clock may also be reflected in the lack of an observable developmental rhythm in *A. nidulans*. Alternatively, the conditions needed to observe

the developmental rhythm in *A. nidulans* may not have been met by our experiments. In either case, our data support the notion that the lack of developmental rhythms in different fungal species or isolates does not rule out the existence of a circadian clock and is consistent with the idea that circadian rhythmicity is a general phenomenon in *Aspergillus*. A well-described case in point is in *N. crassa*. Here, the conidiation rhythm is normally obscured in wild-type strains grown in closed culture tubes due to high CO_2 concentrations. However, strains harboring the *band* mutation display conidiation rhythms under the same culture conditions (Sargent *et al.*, 1966).

Evidence now exists for a FRQ-less oscillator (FLO) in *N. crassa* that can be entrained by temperature cycles (Merrow *et al.*, 1999). It is tempting to speculate that the *Aspergillus* clock and the FLO are related and that the FLO is ancestral to the *N. crassa* FRQ-based oscillator since both oscillators display canonical clock properties and yet have several unusual features, including poor nutritional compensation (Loros and Feldman, 1986; Merrow *et al.*, 1999). Ultimately, comparisons between the *N. crassa* and *Aspergillus* oscillators will allow investigation of whether circadian clocks have diverse evolutionary origins or whether molecular adornments have been added to a common ancestral mechanism.

CHAPTER V

SUMMARY AND CONCLUSIONS

The data presented in this dissertation represent efforts to understand the mechanisms involved in the control of circadian rhythmicity in *N. crassa* and *Aspergillus*. In an attempt to identify circadian output components in *N. crassa*, we examined the circadian phenotype of the *nrc-2*$^{pRAL-1}$ mutant. This study demonstrated the requirement of *nrc-2* for circadian rhythms in conidiation and gene expression in glucose-limited media; rhythms in these processes are observable in high glucose media. The effects of this mutation on the clock are FRQ oscillator specific, as circadian expression of the FRQ oscillator independent gene *ccg-16* is observed in the *nrc-2*$^{pRAL-1}$ mutant under conditions where FRQ dependent *ccg*s are arrhythmic. This study also establishes the presence of two forms of the WC-1 protein in a wild type clock strain, as well as a role for *nrc-2* in the regulation of these two forms, however, the significance of these two forms remains unknown.

Our data suggest three possible models for NRC-2 function in the circadian system of *N. crassa* (Figure 16). Model 1 involves a role for NRC-2 in circadian output from the FRQ oscillator; under low glucose conditions, NRC-2 is required for rhythmicity of FRQ dependent *ccg*s. However, increasing the glucose content of the medium can override the requirement for NRC-2 in *ccg* rhythms. One possible mode of action for NRC-2 in this model would be that it is required to amplify output signals from the FRQ oscillator under conditions of limited carbon source. For instance, NRC-2

may be required for post-translational modification of a transcription factor controlling circadian gene expression. Two other models are proposed that place NRC-2 on the input arm of the FRQ oscillator. Model 2 has NRC-2 involved sensing of glucose levels in the mdium, and signaling that information to the clock to control the choice of WC-1 translational start site. This model predicts a function of the short form of WC-1 in the generation of FRQ dependent output rhythms. This short form presumably has no role in the generation of FRQ-independent rhythms, such as the *ccg-16* rhythm, as the FRQ-null strain makes only the large form of WC-1. Model 3 assigns a role for NRC-2 in maintenance of FRQ and/or WC-1 protein levels, such that under conditions of low glucose, levels of these proteins are not sufficient to maintain rhythmicity. It must be noted that these models are not mutually exclusive of each other; for example NRC-2 may be required for normal levels of both oscillator and output proteins. Additionally, all three models involve a decrease or loss of signal from the FRQ oscillator, such that the levels of the *ccg*s in the *nrc-2*$^{pRAL-1}$ mutant mimic those of the FRQ-null strain.

We are currently unable to distinguish between these three models. One important experiment will be to assay both the levels and the rhythmicity of FRQ and WC-1 under low glucose conditions, although attempts to assay WC-1 in 0.03% glucose medium failed to yield any detectable protein in the wild-type. Thus, assay of WC-1 and FRQ in a range of glucose concentrations should yield valuable results. Model 1 and 2 predict that the levels and rhythmicity of FRQ and WC-1 will be normal in the *nrc-2*$^{pRAL-1}$ mutant, as only output components and/or the forms of the WC-1 protein are affected under these models. Model 3 predicts decreased levels and/or loss of rhythmicity of FRQ

and WC-1; thus assay of these proteins in low glucose medium is a critical experiment. Generation of strains that express only the long and only the short forms of WC-1 will also provide much information as to the nature of the circadian defects in the *nrc-2*$^{pRAL-1}$ strain. A loss of rhythmicity in FRQ dependent outputs in a strain expressing only the long form of WC-1 would strongly support Model 2. It will also be important to examine the forms of WC-1 in low glucose medium in the *nrc-2*$^{pRAL-1}$ strain, as small amounts of the short form of WC-1 are detectable in high glucose medium. If the short form is important in *ccg* rhythms, one would expect there to be no short WC-1 present when the strain is cultured in low glucose medium. Model 2 can also be addressed using NRC-2 and FRQ overexpression strains. If NRC-2 and FRQ are in a pathway that regulates the choice of translational start site of the WC-1 protein, we would expect that the small form of WC-1 would be the predominant form in both NRC-2 and FRQ overexpression strains. Additionally, assay of the forms of WC-1 in the *frq*^{9} strain may provide useful information. The *frq*^{9} strain displays conidiation rhythms of variable period length, and these rhythms are not medium compensated. Thus, if the choice of WC-1 translation site is regulated by the metabolic state of the cell, one would expect that mainly the large form of WC-1 would be present in the *frq*^{9} mutant strain. It also will be important to generate antibodies to the NRC-2 protein. If Model 1 is correct, we would expect that the NRC-2 protein may interact directly with circadian output components. Coimmunoprecipitation assays may be performed to identify NRC-2 interacting proteins, which, under this model, would be important in the control of FRQ dependent outputs. Alternatively, yeast two hybrid screens may be initiated to identify these

interacting proteins. NRC-2 specific antibodies will also prove useful in the examination of NRC-2 protein expression. Although the *nrc-2* transcript is rhythmic, we know nothing about the expression pattern of the NRC-2 protein. NRC-2 antibodies will allow us to examine parameters such as protein rhythms and subcellular localization, and will help us to understand the function of this protein.

Finally, our data indicate that the FRQ dependent *ccg*s *ccg-1*, *ccg-2* and *ccg-7* are arrhythmic in the *nrc-2*$^{pRAL-1}$ mutant. Microarray analysis has identified 150 *ccg*s in *N. crassa*, and it is possible that only a subset of FRQ dependent genes are altered in the *nrc-2*$^{pRAL-1}$ mutant in low glucose conditions, although *nrc-2* does not simply affect circadian expression of developmentally regulated genes. The extent of alteration in *ccg* expression in the *nrc-2*$^{pRAL-1}$ mutant can be examined using microarrays; if the *nrc-2*$^{pRAL-1}$ mutation only affects a subset of FRQ dependent genes, we would expect to see a number of rhythmic transcripts in a microarray experiment.

Our studies involving the *nrc-2* gene have highlighted the interplay between metabolism and the circadian clock. Analyses of the circadian system of *N. crassa* and other organisms have also indicated a relationship between the metabolic state of the cell and circadian rhythmicity. For instance, circadian rhythms of conidiation in *frq*-null strains of *N. crassa* are observable when the growth medium is supplemented with farnesol or geraniol (Granshaw *et al.*, 2003), and when the lipid synthesis mutations *cel* and *chol* are introduced in a *frq*-null genetic background (Lakin-Thomas and Brody, 2000). Experiments performed in mammals indicate that peripheral oscillators in the liver can be entrained and uncoupled from the SCN pacemaker by restricted feeding

schedules (Damiola *et al.*, 2000). Also, the DNA binding activity of the activators CLOCK and BMAL1 in mammals is regulated by the redox state of nicotinamide adenine dinucleotide (NAD) cofactors *in vitro*. The reduced forms of these cofactors enhance the DNA binding activity of CLOCK and BMAL1 heterodimers, while the oxidized forms inhibit DNA binding activity, providing a direct link between the metabolic state of the cell and regulation of oscillator function (Rutter *et. al*, 2001). These examples highlight both the importance of metabolism in circadian rhythms and the idea that multiple oscillators, either within a single cell or housed in separate tissues, are responsible for the generation of overt rhythmicity.

In an attempt to characterize other oscillators in *N. crassa*, two separate studies were performed. The first involved the characterization of the *ccg-16* gene. We find that rhythms in this gene persist in the absence of FRQ, but require WC-1. Assay of the WC-1 protein in the FRQ-null strain revealed that WC-1 levels are rhythmic in the absence of FRQ. The *ccg-16* transcript also accumulates rhythmically in LL, as does the WC-1 protein in both the wild-type and FRQ-null strains. These data contradict previous models that stipulate that rhythms in WC-1 require FRQ. Rhythms in the *ccg-16* transcript are reliably observed when cultures are synchronized by a temperature shift, rather than a light to dark transition, indicating that these rhythms are controlled by a FRQ-less oscillator (FLO) that is more sensitive to temperature signals than light signals. This FLO appears to be coupled to the FRQ-oscillator; although rhythms in WC-1 persist in the absence of FRQ, in the long period frq^7 strain, the WC-1 protein cycles with a long period (Lee *et. al*, 2000). This indicates that when the FRQ oscillator is

present, it can influence the period of the WC-1 rhythm. These data indicate a need for revision of the current model of the circadian clock of *N. crassa*, particularly the point involving the posttranscriptional regulation leading to WC-1 rhythmicity, which we show is FRQ-independent. However, FRQ is required for maintenance of WC-1 levels in DD; in our experiments WC-1 levels are drastically reduced in the FRQ-null strain. It is unclear, however, whether the FLO controlling *ccg-16* rhythms is responsible for WC-1 rhythmicity. This situation bears similarity to the two oscillator model proposed by Pittendrigh and Bruce to explain the eclosion rhythm in *Drosophila* (Pittendrigh and Bruce, 1959). Coupling of these two oscillators would lend stability to the system, and also provide a means for each oscillator to entrain to different inputs while controlling separate outputs. Further, these data suggest that it is the rhythms in FRQ, rather than WC-1, that are important for rhythmic conidiation. Components of this FLO remain to be identified, however.

This study represents an important step in our understanding of the oscillator system of *N. crassa*. The *ccg-16* gene represents a tool that can be used to identify components of a FRQ-less oscillator. For example, analysis of the promoter of this gene will identify motifs required for rhythmicity of this gene, and assays can then be employed to identify proteins that bind to these motifs. In this manner, output components of the FRQ-less oscillator may be identified. Furthermore, reporter screens making use of the *ccg-16* promoter will be useful in the identification of mutations that express *ccg-16* with an altered period, or are arrhythmic. Such mutations should lead to the identification of FRQ-less oscillator components. In addition, biochemical and

genetic approaches, such as immunoprecipitation experiments and yeast two hybrid screens to identify WC-1 interacting proteins, might also help us to identify FRQ-less oscillator components.

Our data indicate that the FRQ-less oscillator driving *ccg-16* expression is more sensitive to temperature input than light input. This aspect of our model must be examined in more detail. To address this point, the phase response curves (PRCs) for the *ccg-16* gene must be examined. If the FRQ-less oscillator is entrained by temperature, while the FRQ oscillator is entrained by light, we would expect distinct PRCs for FRQ dependent genes such as *ccg-1* and *ccg-2*, and FRQ-less oscillator dependent genes such as *ccg-16*. If, on the other hand, entrainment is mediated exclusively through the FRQ oscillator, or through the coupling of the two oscillators, then we would expect the PRCs for all *ccg*s to be similar. It must be noted that the determination of PRCs by northern blot will be difficult, if not impossible, given the temporal resolution of the assay. Experiments of this nature will require a reporter system such as the luciferase reporter, used in *Synechococcus*, *Arabidopsis*, and vertebrates.

Finally, we have not addressed the extent of *ccg* control by the FRQ-less oscillator. The observation that the evening-specific *ccg-16* gene is rhythmic in a FRQ-null strain suggests the possibility of a morning-specific FRQ oscillator and an evening-specific FRQ-less oscillator. The data presented here are insufficient to support or refute this hypothesis, but we now have the means to address this hypothesis. Microarray analysis of rhythmic gene expression in DD in the *frq* null strain will reveal the extent of *ccg* control by the FRQ-less oscillator.

In a second attempt to identify FRQ-less oscillators in *N. crassa*, we characterized circadian rhythms in two species of *Aspergillus*. We reasoned that as *Aspergillus* does not contain a *frq* homolog in its genome, circadian rhythms in this genus may be controlled by an oscillator ancestral to the FRQ oscillator. Identification of components of the *Aspergillus* oscillator, followed by deletion of their homologs in *N. crassa,* could provide a way to identify FRQ-less oscillator components, as well as address the evolution of circadian rhythmicity in the fungal kingdom. The circadian rhythms we observed in *Aspegillus* possess several unique properties. Our data indicate that *A. flavus* displays a temperature compensated circadian rhythm in sclerotium production, which free runs with a 33 h period. This period is longer than any known natural free running period, however, the organism is able to entrain to environmental cycles with a period of 24 h. This long free-running period likely contributes to the unique phase responses of the sclerotium rhythm to light pulses, as we observe primarily phase advances at all times of day. This observation is sensible in that a slow running clock would need to be advanced throughout the day in order to keep pace with daily environmental rhythms. The sclerotium development rhythm in *A. flavus* also is not nutritionally compensated; developmental rhythms were only observed on one of several media tested. However, we observed temperature compensation of the sclerotium rhythm between 30°C and 40°C. *A. nidulans* does not display any circadian rhythms in development; although free running and entrainable rhythms are observed for the *gpdA* transcript. The unique properties of the *Aspergillus* circadian rhythms, combined with

the absence of a FRQ homolog, make *Aspergillus* an interesting model in which to investigate fungal circadian rhythmicity.

Laboratory studies using *A. nidulans* over the past 50 years have resulted in the development of many sophisticated molecular, genetic and biochemical techniques, making *A. nidulans* the species of choice for investigation of circadian rhythms. Future studies should focus on the identification of clock components in *A. nidulans*. While we demonstrate circadian rhythmicity in gene expression in this species, screens for mutants expressing *gpdA* arrhythmically or with altered periods by northern blot would be hopelessly time consuming and expensive. Circadian studies using *A. nidulans* will require a more easily assayed reporter. We have demonstrated that firefly luciferase is functional in *A. nidulans* (Morgan *et al.*, 2003); however, attempts to establish free-running and entrained rhythms in luciferase expression driven by the *gpdA* promoter have not proven successful (Appendix A). Thus, further efforts with luciferase or another reporter, such as green flourescent protein or jellyfish aequorin, are necessary.

Should additional effort at developing an *Aspergillus* reporter system prove fruitless, other options exist for identification of clock components. The LREA and LREB proteins, homologs of *N. crassa* WC-1 and WC-2, are prime candidate clock components in *Aspergillus*. These proteins may interact with other clock components in *Aspergillus*; thus, approaches such as coimmunoprecipitation and yeast two hybrid experiments may result in the identification of novel clock components. Another approach is to isolate total protein from cultures grown in DD and subject the protein to 2D electrophoresis to identify proteins that oscillate over the course of the day.

Sequencing of the N-terminus of these proteins will result in the identification of putative clock components, and knockout of the corresponding genes can then be performed to assay their role in the *Aspergillus* circadian system. Additionally, whole genome microarrays are available for *A. nidulans*, and studies utilizing this technology will provide information regarding the extent of clock control of gene expression in this species, as well as provide candidate genes for further analysis of function with regards to circadian rhythms in *Aspergillus*. Orthologs of these putative clock components can then be knocked out in *N. crassa*, and the circadian phenotype may be assayed. These approaches using *Aspergillus* may ultimately prove valuable at identifying components of the FRQ-less oscillator of *N. crassa*.

In summary, these studies have revealed novel findings that help us to better understand the molecular clockworks of fungi, such as free running rhythms in the WC-1 protein in the absence of FRQ and in LL, as well as circadian rhythms in *Aspergillus*. Studies of the *nrc-2* gene in *N. crassa* highlight the interplay between metabolism and the circadian clock in this species. Together, this work has described the existence of another oscillator involved in circadian rhythmicity of *N. crassa*, and also described a testable model for metabolic sensing by the FRQ-oscillator (Figure 27). Identification and characterization of circadian rhythms in *Aspergillus* will provide the basis for future research into the nature of other fungal oscillators, as well as the evolution of the clock mechanism within the fungal kingdom.

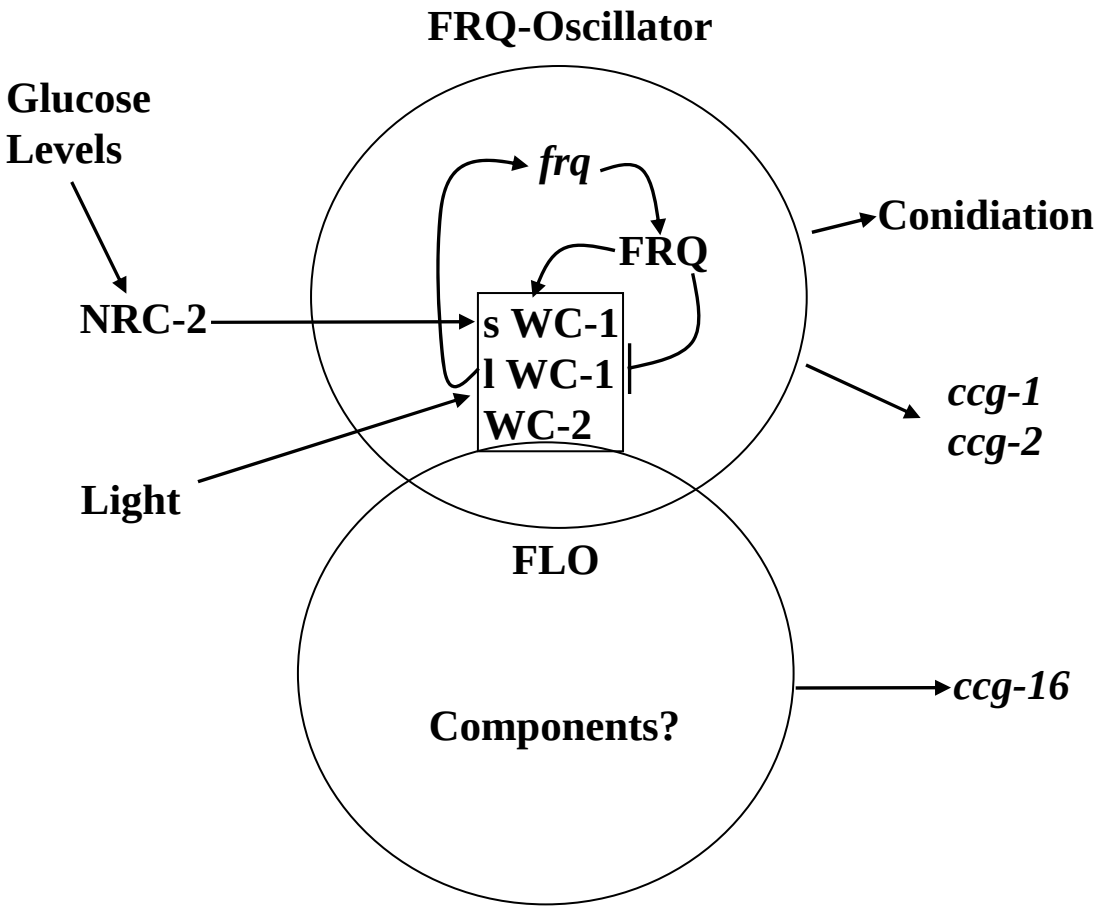

Figure 27. New insights into the *N. crassa* circadian system. The data presented here indicate that the circadian clock of *N. crassa* is composed of at least two distinct oscillators. One, the FRQ-oscillator, has been well-characterized, and is responsive to light signals through the WC-1 protein and the metabolic state of the cell through the NRC-2 protein. The FRQ-oscillator is responsible for the control of rhythmic conidiation and *ccg* expression, including *ccg-1* and *ccg-2*. Our data suggest the existence of a second oscillator, the components of which have yet to be described. This oscillator requires rhythms in the WC-1 protein, but not the FRQ protein, for rhythmic output, as assayed by rhythmicity of the *ccg-16* gene. This oscillator is proposed to lend stability and accuracy to the *N. crassa* circadian system.

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APPENDIX A

FIREFLY LUCIFERASE AS A CIRCADIAN REPORTER IN *ASPERGILLUS NIDULANS*

Summary

The data presented in this appendix represent efforts to establish firefly luciferase as a circadian reporter in *Aspergillus nidulans*. We used the clock regulated *A. nidulans gpdA* promoter to drive luciferase activity. Luciferase activity is easily monitored in 96-well plates using an automated luminometer; thus, establishment of luciferase as a circadian assay would greatly help in screening for clock mutants with altered periods or loss of circadian function. Our data indicated that, in conditions of constant darkness (DD), no rhythms in luciferase were observable, and most strains used showed one broad peak in luciferase activity at varying times in DD. When entrained by combinations of light:dark (LD) and temperature cycles, at most two peaks of luciferase activity were observable, and the peaks occured at varying phases, even when the same strain was cultured in separate wells on the same plate. These data indicate that, while expressed at high levels in *A. nidulans,* our *gpdA*:luciferase fusion does not act as a reliable reporter of circadian gene expression in *A. nidulans,* and is unsuitable as a reporter in large-scale mutagenesis screens.

Introduction

The characterization of circadian rhythms in *Aspergillus* has opened an avenue for the study of the evolution of circadian clocks within the fungal kingdom. While *A. flavus* displays free-running and entrainable rhythms in sclerotium development, this species of *Aspergillus* is rather difficult to work with under laboratory conditions, due to the lack of sexual state and the dearth of selectable markers, among other things. The related species *A. nidulans* provides a more tractable model for circadian studies, due to the ease of transformation and sexual crossing of this species. Unfortunately, wild-type *A. nidulans* does not manifest a free-running developmental rhythm, an assay that could be used in a large scale mutagenesis screen. *A. nidulans* does, however, display free-running and entrainable rhythms in the abundance of the *gpdA* transcript. Construction of a rhythmic reporter system making use of the rhythmic expression of *gpdA* would provide a convenient, non-invasive means of monitoring real-time clock-controlled gene expression in single cultures.

The luciferase reporter system, especially the *luciferase* (*luc*) gene from the firefly beetle *Photinus pyralis*, is desirable as a reporter for circadian gene expression in *Aspergillus* because the product of the *luc* gene does not require post-translational modification for enzymatic activity, and the enzyme has a short half-life, rendering it useful in assays measuring circadian gene expression. Indeed, use of the *luc* gene as a circadian reporter in several species has led to substantial advances in the identification of clock-associated genes (Brandes *et al.*, 1996; Kondo *et al.*, 1993; Kondo and Ishiura, 1994; Liu *et al.*, 1995; Millar *et al.*, 1992; Millar *et al.*, 1995). The LUC enzyme

catalyzes the ATP-dependent reaction of luciferin and oxygen and emits a photon of visible light. This reaction can easily be monitored in cultures grown in 96-well plates, making the luciferase assay of considerable value in large scale mutagenesis assays. We have previously shown that a modified version of firefly luciferase, which reflects the *N. crassa* codon bias, is expressed under control of the *N. crassa ccg-2* promoter in *A. nidulans* (Morgan *et al.*, 2003). In this appendix, we make use of the *gpdA* promoter to drive expression of the *luc* gene in DD and entraining cycles. The *gpdA* transcript displays free-running rhythms in accumulation in DD, and this rhythmicity is entrainable by combinations of LD and temperature cycles (Chapter IV). We find that, although luciferase activity is expressed at high levels by *A. nidulans*, no free running rhythmicity in DD is observed following one day in a 12:12 LD cycle. Furthermore, when luciferase activity is monitored in entraining cycles of 12 hours light, 34°C: 12 hours dark, 28°C, rhythms are observed, although on an inconsistent basis. These rhythms show variable period and phasing and in all cases do not persist for more than three cycles. Together, these data demonstrate that use of the gpdA:luciferase fusion as an assay for circadian gene expression in *A. nidulans* will not be useful. However, these experiments do not address if luciferase under control of a different promoter or a modified form of the *gpdA* promoter has the potential to be a reliable reporter of circadian rhythms in this species.

Materials and methods

Construction of the gpdA:luc *fusion*

Construction of the *gpdA:luc* fusion was achieved by digestion of pAVG3, which contains the *gpdA* promoter derived from pAN52.1-NOT (Punt *et al.*, 1991), a generous gift from Dr. Peter Punt, with the restriction enzyme *Bst*EII. This liberates a 1.8 kb *gpdA* promoter from pAVG3. This fragment was subsequently ligated into the pluc6ΔBS vector, which is identical to the pluc6 vector of Morgan *et al.*, 2003, except that the *Bst*EII site in the *his-3* gene has been removed by site directed mutagenesis. This ligation produced a fusion of the *gpdA* promoter, including the entire 5' untranslated sequence, to the *luc* coding sequence, preserving the *gpdA* AUG translational start site, while introducing no base changes to the *luc* coding sequence. Orientation of the promoter was confirmed by DNA sequencing. The resulting plasmid, named pgpdAmluc12, contained the *gpdA:luc* fusion, as well as the *ccg-2* promoter directly 5' to the *gpdA* promoter, and the *N. crassa his-3* gene. To re-locate the fusion construct to a plasmid without the *ccg-2* promoter and the *his-3* gene, PCR was performed on pgpdAmluc12 using the following primers: gpdxba: 5' GCT CTA GAA GCG TCC CTT TAA GG 3', and lucds: 5' ATG GTG GAG ATG GCA AGC 3'. These primers amplify the 3.5 kb *gpdA:luc* fusion, which was subsequently cloned into PCR-TOPOII (Invitrogen, Carlsbad, CA) to give pgpdmluc:TOPO1. The identity of this plasmid was confirmed by DNA sequencing. Plasmid pgpdmluc:TOPO1 was used in all subsequent *Aspergillus* transformations.

Strains used in this study

The strain used for all transformations with the *gpdA:luc* fusion was RAVG3.42 (*pyrG89; yA2; wA2; nicA2; veA*$^{+}$). This strain was constructed by crossing FGSC A767 (*pyrG89; nicA2; veA1*) with FGSC A230 (*yA2; wA2; argA1; veA*$^{+}$). These strains were obtained from the Fungal Genetics Stock Center, Kansas City, KS. Crosses were carried out by growing mycelial mats overnight on *Aspergillus* complete medium with 2% glucose plus appropriate supplements, followed by transfer of mycelial disks (cut with a size 7 cork borer) to *Aspergillus* minimal medium with 1% lactose as sole carbon source (http://www.fgsc.net/methods/anidmed.html). Cultures were grown in DD until mature cleistothecia were observed. All strains used in this study were maintained on *Aspergillus* minimal medium with 1% glucose as carbon source, with appropriate supplementation (http://www.fgsc.net/methods/anidmed.html).

Aspergillus *transformation*

The pgpdmluc:TOPO1 plasmid was co-transformed into RAVG3.42 with plasmid pNiaD3, a generous gift from Dr. Rosie Bradshaw, which carries a copy of the *N. crassa pyr4* gene, and can complement the *pyrG89* mutant of *A. nidulans* (Bird and Bradshaw, 1997) or pPyr4, which contains a genomic clone of the *N. crassa pyr4* gene. Transformation was achieved using standard protoplasting techniques, and yielded 11 transformants that expressed luciferase in a stable manner. These strains were named TAVG3.42-#, and have either NiaD (co-transformed with the pNiaD3 plasmid) or Pyr4

(co-transformed with pPyr4) after the strain number. All 11 strains were used in subsequent luciferase assays.

Luciferase assay

In vivo assays were performed in *Aspergillus* minimal medium containing either 1% or 0.1% glucose. Opaque 96-well microtiter plates were inoculated with 150 μl of medium and 10^6 conida and incubated for 12 hours in DD, followed by 12 hours in LL before assaying luciferase activity. Luciferin (Promega) was added to a final concentration of 10μM just prior to assay of the sample, and luciferase activity was detected immediately after the start of the assay (within 10 min.), suggesting that luciferin is readily taken up by *Aspergillus*. The luciferase assay was subsequently allowed to proceed for 5-7 days in either DD at 28°C, or in a cycle of 12 hours light at 34°C, followed by 12 hours dark at 28°C. Luciferase activity was measured *in vivo* using the Packard Top-Count Multiplate Luminescence and Scintillation Counter (Hewlett-Packard) and is presented as the number of photons/sec (cps). Data from the Top-Count Luminometer were collected and analyzed using the I-and-A program developed in the laboratory of Dr. Steve Kay (Scripps Research Institute, La Jolla, CA).

Results and discussion

Construction and expression of a gpdA:luc *fusion in* A. nidulans

A vector for expression of the firefly *luciferase* (*luc*) gene in *A. nidulans* was constructed in which *luc* was placed under control of the promoter of the clock-regulated

gpdA gene. 1.3 kb of the *gpdA* promoter has been analyzed for control sequences, and several elements involved in transcription activation and initiation have been identified in this sequence (Punt, 1990). Our fusion included 1.8 kb of the *gpdA* promoter, and thus all known transcriptional control sequences, including the *pgk* and *gpd* boxes, which function as transcriptional enhancers (Punt, 1990; Punt, 1995) and the *ct* box, which is required for initiation of transcription at the correct site (Punt, 1990) (Figure A-1). This fusion also contains the entire 5' untranslated region of the *gpdA* gene. Northern blot assays of endogenous *gpdA* mRNA levels measured over two circadian cycles showed rhythms in transcript abundance with an amplitude of ~5- to 8-fold (Chapter IV). Thus, our intent was to drive rhythmic expression of luciferase activity using the *gpdA* promoter. This construct was transformed into *A. nidulans* and was ectopically integrated into the genome. Transformants showed luciferase activity as assayed by both Top Count Luminometer detection and by the use of a CCD camera (LN/CCD Detector, Roper Scientific, Trenton, NJ) (Figure A-2 and A-3, and data not shown). To test circadian expression of the *gpdA:luc* construct in *A. nidulans*, co-transformants expressing luciferase were inoculated into 96-well plates and subjected to a 12 hour dark:12 hour light cycle prior to addition of beetle luciferin and transfer to the Top Count Luminometer in DD at 28°C. The peak levels of luciferase activity were about 1 x 10^6 cps; however, no circadian rhythmicity in luciferase expression was observed in any of the transformants (Figure A-2). Most wells displayed a single peak in luciferase activity ranging from 12 to 84 hours after the start of the assay; activity in several of the wells fluctuated, but did not show any circadian rhythmicity. Low levels of luciferase

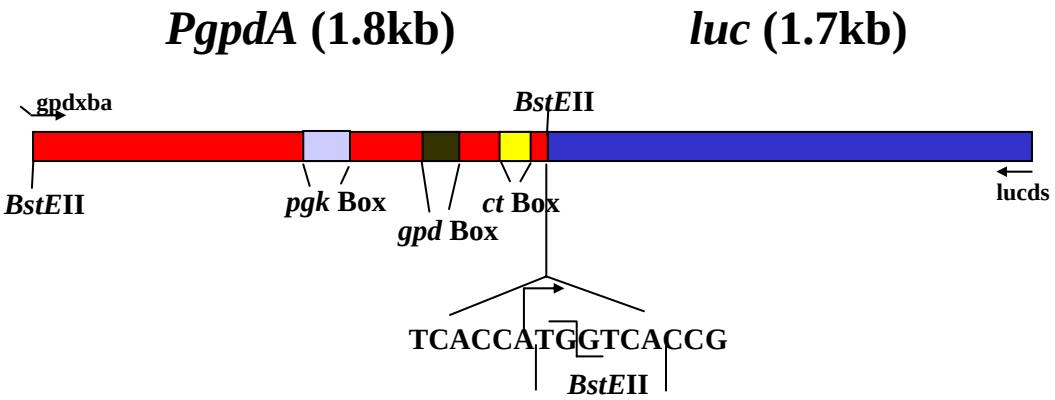

Figure A-1. The *gpdA:luc* fusion construct. A 1.8 kb fragment of the *gpdA* promoter was digested with *BstE*II and ligated to the *luc* coding sequence. This fragment contains the *pgk* and *gpd* box transcriptional enhancers, as well as the *ct* box, which is necessary for correct transcriptional initiation. The fusion construct contains the entire 5' untranslated sequence of the *gpdA* gene, and the site of fusion was confirmed by DNA sequencing. Arrow = start of translation, dividing line = site of fusion.

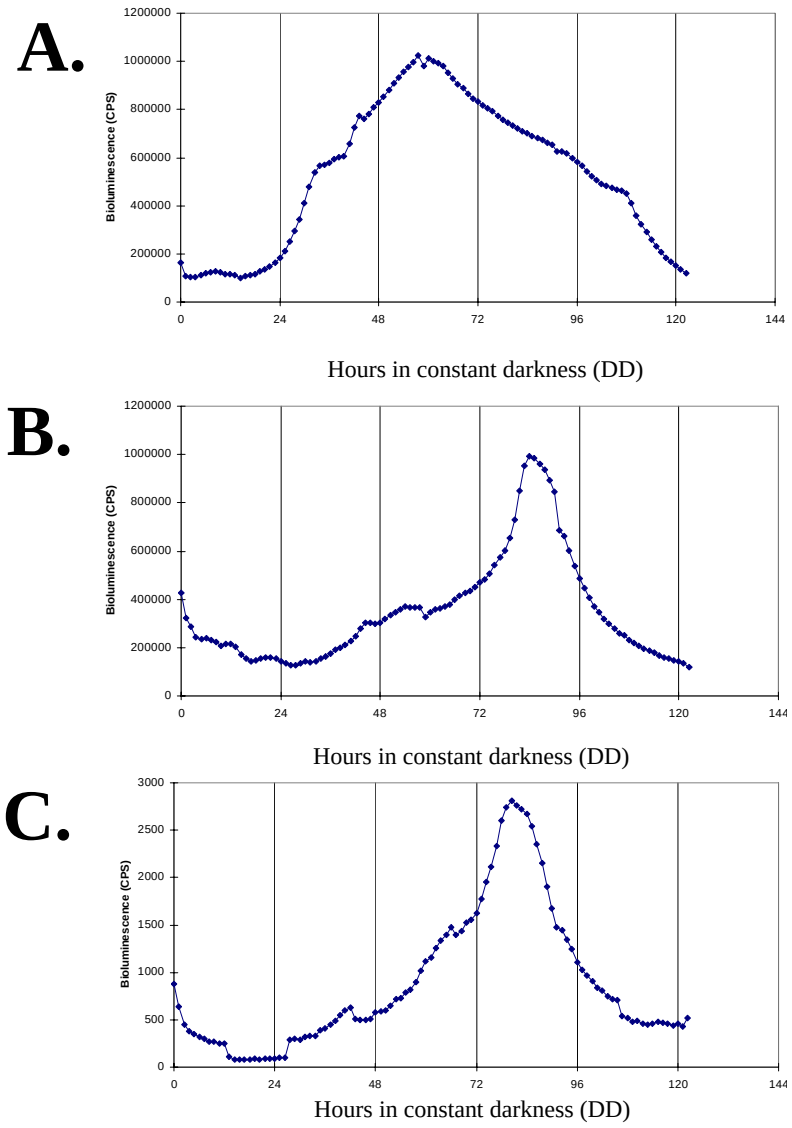

Figure A-2. Expression of *in vivo* firefly luciferase activity in *A. nidulans* transformants in DD. (A, B) Expression of *in vivo* luciferase activity in two separate wells containing the same transformant, TAVG3.42-7Pyr4. The data presented here are from the same 96-well plate. Cultures were grown in one cycle of 12 hr dark: 12 hr light prior to the start of the assay, which was performed in DD at 28°C. **(C)** Expression of *in vivo* luciferase activity from the untransformed strain RAVG 3.42. This data is from the same 96-well plate as in A and B. Note the low activity levels detected in this well.

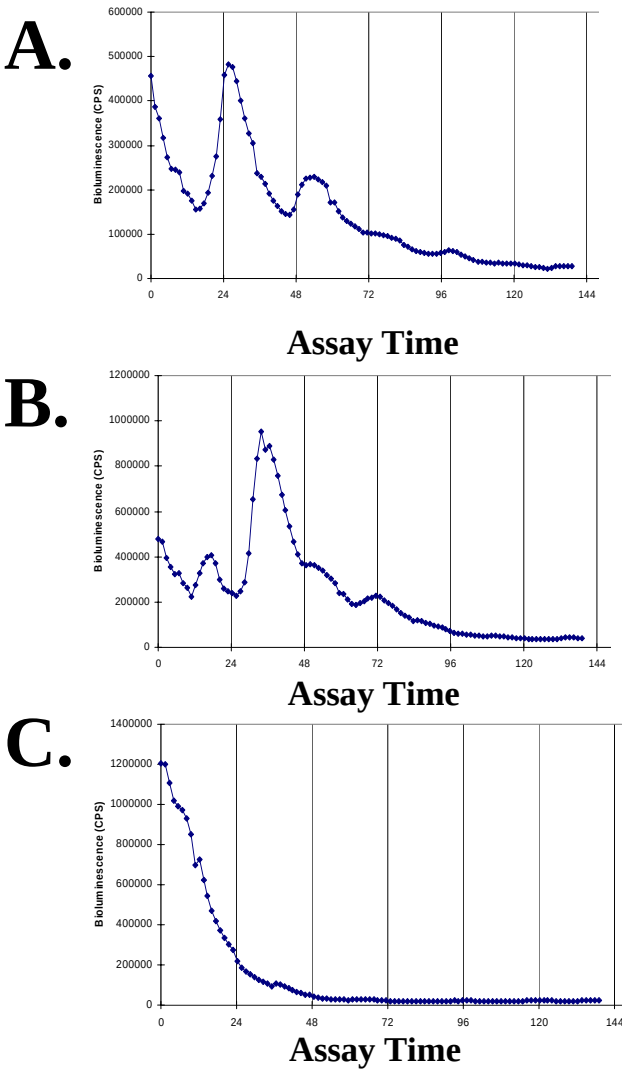

Figure A-3. Expression of *in vivo* firefly luciferase activity in *A. nidulans* transformants in entraining cycles. All cultures were grown in cycles of 12 hr light at 34°C, 12 hr dark at 28°C. Time 0, 24, 48, etc. represent the start of the light/warm portion of the cycle. **(A)** Expression of *in vivo* luciferase activity from TAVG3.42-7Pyr4. This graph is representative of an entrained culture, giving two peaks of luciferase activity 20-28 hr apart. This culture gave a period of 27 hr., and is representative of 17% of the behavior observed from 17% of the cultures in this assay. **(B)** Expression of *in vivo* luciferase activity from TAVG3.42-7Pyr4. This graph is representative of a rhythmic, but not entrained, culture (expression peaks are greater or less that 20-28 hr. apart). The peaks in this graph are 17 hr. apart, and is representative of 32% of the cultures in this assay. **(C)** Expression of *in vivo* luciferase activity from TAVG 3.42-7Pyr4. This graph represents arrhythmic luciferase expression, observed in 49% of cultures in this assay.

activity were detectable in the negative control wells, suggesting that either luciferase activity of the transformants was spilling over into adjacent wells, or that *Aspergillus* displays low levels of endogenous bioluminescence.

The lack of circadian rhythms in luciferase could be because the cultures were not entrained well. Although a simple light-to-dark transition is sufficient to entrain the *gpdA* rhythm in liquid shake cultures of *A. nidulans*, it is possible that in the luciferase assay the cultures were simply not synchronized. To test this possibility, cultures were assayed on the Top Count Luminometer while in cycles of 12 hours light at 34°C: 12 hours dark at 28°C. The resulting data indicate that, at best, two cycles of luciferase rhythms are observed even in an entraining cycle (Figure A-3). The phasing of this rhythmicity is inconsistent, however, as different wells containing the same strain show different phases. 17% of all wells (15/88) showed entrained rhythms (defined as two peaks 20-28 hr. apart) with variable phases, while 32% of all wells (28/88) showed non-entrained rhythms (period is longer or shorter than 24 hr.), with two or three activity peaks. The remaining 51% of wells were classified as arrhythmic. Representative traces obtained from one strain, TAVG3.42-7Pyr4 are shown in Figure A-3. This strain represents the most reliable luciferase expression in entraining cycles: 3/8 wells containing this strain were entrained with varying phases, 1/8 wells were rhythmic but non-entrained, the remaining 4/8 wells were arrhythmic.

The inconsistencies in this assay cast doubt regarding the utility of the *gpdA*:luciferase fusion as a reporter of circadian gene expression in *A. nidulans*. While the *gpdA*:luciferase fusion used in this study contained all known transcriptional control

sequences, it is possible that this promoter fragment is not sufficient to drive rhythmic expression of luciferase RNA. Additional promoter sequence may be necessary for this, or 3' untranslated sequence may be necessary for RNA rhythms. Northern blot assays should be performed to ascertain whether the *gpdA* promoter used is sufficient to drive rhythms in *luc* mRNA.

Several other options exist for the improvement of the luciferase system, including tests on different media, varying the amount of conidia in the innoculum, and varying the amount of luciferin used in the assay. Morgan (2003) determined an optimal concentration of luciferin for assays using *N. crassa*, perhaps the concentration used here is too high to give sufficient temporal resolution. The media used in our assays support rhythmic *gpdA* expression in liquid shake culture (Greene *et al.*, 2003); however, we have not investigated *gpdA* rhythms in stationary cultures such as the ones used in this study. In addition, although GAPDH activity has been shown to be rhythmic in *N. crassa* (Shinohara *et al.*, 1998), we currently have no information regarding GPDA protein rhythms in *A. nidulans*. It is possible that while the *gpdA* transcript cycles with a circadian period, translation of *gpdA* is constitutive. This could explain the lack of consistent rhythms in luciferase activity in our assays. However, the oscillation of luciferase activity in entraining cycles argues against this possibility. A final possible explanation for these inconsistent results is that the fungus conidiates in stationary media. Asexual development has been shown to regulate *ccg*s in *N. crassa*, and developmental regulation can override clock regulation. Thus it may be necessary to

develop assay conditions which delay or prevent the organism from conidiating in order to observe consistent rhythmicity from this promoter.

APPENDIX B

DELETION AND ANALYSIS OF THE CIRCADIAN PHENOTYPE OF THE *VELVET A* GENE IN *NEUROSPORA CRASSA*

Summary

During the course of our investigations of circadian rhythms in *Aspergillus niduans*, we noticed that the standard laboratory strain, the *veA1* mutant strain, did not display free running rhythms in *gpdA* mRNA, while the wild type *veA*$^{+}$ strain showed robust *gpdA* mRNA accumulation rhythms in constant conditions. The VEA protein is required for red light responses in *A. nidulans*, suggesting a possible role in circadian function in this species. VEA is also required for sexual development in *A. nidulans*; a deletion strain is unable to make cleistothecia. Sequencing of the *N. crassa* genome revealed a gene that is homologus to the *A. nidulans veA* gene. To investigate the role of the *veA* homolog, called *ncveA* in this appendix, in circadian rhythms in *N. crassa*, this gene was deleted by homologus gene replacement and its circadian phenotype was assayed on race tubes. We find that, while the Δ*ncveA; bd* strain showed increased conidiation on standard race tubes, it displayed free-running and light-entrainable conidiation rhythms similar to the wild-type clock strain. In addition, the Δ*ncveA; bd* strain was able to produce both perithecia and viable ascospores, indicating that the NCVEA protein is not required for sexual development in *N. crassa*.

Introduction

Asexual development of the filamentous ascomycete *Aspergillus nidulans* is red-light-dependent. Growing wild-type *A. nidulans* in the light results in primarily conidiation, while growth of the fungus in darkness results in an abundance of sexual cleistothecia. Red light stimulates conidiation in *A. nidulans*, and an immediate shift to far red light inhibits this inductive effect on conidiation (Mooney and Yager, 1990a). The effect of red light has been shown to be dependent on a functional *velvet A* (*veA*) gene; the inductive effect of red light is abolished in a *veA1* mutant strain and the organism conidiates regardless of the lighting condition (Mooney and Yager, 1990a). The *veA1* strain has been commonly used in research laboratories because it forms conidia in any lighting condition and in larger quantities than the wild-type, making it easy for researchers to acquire large numbers of conidia for experiments (Kafer, 1965).

Recent experiments with the *veA* gene in *A. nidulans* indicated that the *veA1* allele is not a complete loss of function allele. The *veA1* strain can produce cleistothecia and sterigmatocystin, an aflatoxin precursor; however, deletion of the *veA* gene resulted in a strain unable to make both cleistothecia and sterigmatocystin (Kato *et al.*, 2003; Kim *et al.*, 2002). Other secondary metabolic pathways are affected in the Δ*veA* strain, as this strain also produces no penicillin (Kato *et al.*, 2003). A similar phenotype has been observed in the closely related fungus *A. parasiticus*, which, when the *veA* gene was deleted, was unable to make sclerotia (a type of large survival spore) and aflatoxin (Calvo *et al.*, 2004), indicating that the function of this gene is conserved within the genus *Aspergillus*. Overexpression of *veA* from an inducible promoter in *A. nidulans*

resulted in overproduction of cleistothecia, even under conditions that repress sexual development, leading to the hypothesis that *veA* is a positive regulator of sexual development and a negative regulator of asexual development (Kim *et al.*, 2002). Sequencing of the *veA1* allele revealed a single nucleotide substitution in the start codon of the gene, resulting in a predicted protein arising from a downstream AUG with a 36 amino acid N-terminal truncation (Kim *et al.*, 2002). The VEA protein, while bearing no significant similarity to any protein with known function, contains a putative PEST domain (Kim *et. al*, 2002), a motif which has been implicated in regulation of protein stability, and is involved in FRQ protein stability in *Neurospora* (Gorl *et al*., 2001), as well as a putative Nuclear Localization Sequence (NLS) (Kim *et al.*, 2002). However, the role of these domains in VEA function remains to be tested.

During the course of our work with *A. nidulans* circadian rhythms, we noticed that a *veA1* strain was arrhythmic for *gpdA* expression, while the wild type *veA*$^{+}$ strain displayed robust rhythms in *gpdA* mRNA abundance. Our hypothesis regarding this observation is that *veA* is a clock component in *A. nidulans*, and is required for free-running rhythms in *gpdA* abundance. The presence of the PEST and NLS domains in the protein sequence support this hypothesis; both PEST-dependent protein degradation and NLS-mediated nuclear localization are essential for the circadian function of the FRQ protein of *N. crassa* (Gorl *et al.*, 2001; Luo *et al.*, 1998). In addition, mutation of clock components can affect light responses in *Neurospora*. For example, deletion of either the *wc-1* or *wc-2* gene results in a strain that is both blind to blue-light and is arrhythmic in constant darkness (DD) (Crosthwaite *et al.*, 1997; Russo, 1988).

The *N. crassa* genome contains a homolog of the *A. nidulans veA* gene. To investigate the role of this gene in *N. crassa* circadian rhythms, we generated a null mutation of the *ncveA* gene by homologous gene replacement. The resulting Δ*ncveA* strain displays several non-circadian phenotypes, including overconidiation and a slight pigment defect; however, the Δ*ncveA* strain conidiates rhythmically in DD and entrains to a light:dark (LD) cycle, and is able to cross and produce viable ascospores. Our data indicate that the NCVEA protein functions in a manner distinct from the *A. nidulans* VEA, and is not required for overt developmental rhythmicity.

Materials and methods

Strains, media conditions and sexual crosses

The *bd; A* strain FGSC 1858 was used for all *N. crassa* experiments. *A. nidulans* experiments made use of strain A4 (*veA*$^{+}$) and PW1 (*biA1; argB2; methG1; veA1*). *N. crassa* cultures were maintained on standard minimal media with appropriate supplements (Davis and De Serres, 1970), as were *A. nidulans* cultures (www.fgsc.net/methods/anidmed.html). Crosses involving *N. crassa* were performed on Westergaard's medium, and strains were co-inoculated, or the female strain was allowed to grow for ~7 days prior to addition of a conidial suspension of the male strain of opposite mating type. Ascospores were isolated and germinated as described (Davis and De Serres, 1970).

Timecouse assays

A. nidulans strain PW1 and A4 were inoculated into petri dish cultures of minimal medium, comprised of 1X *Aspergillus* salts plus 1% glucose and trace elements, arginine, methionine and biotin (www.fgsc.net/methods/anidmed.html). After 16 to 20 h of growth at 37°C in DD, sections of the resulting mycelial mat were cut with a 7-mm cork borer. Individual sections were transferred to liquid shaking cultures of low-glucose medium (0.1% glucose and 1X *Aspergillus* salts plus trace elements, arginine, methionine, and biotin) to inhibit development, and then incubated at 30°C in constant light (LL) for 24 hr. For free-running experiments, cultures were transferred from LL to DD at 4-h intervals so that, at the subsequent time of harvest, the cultures were all of the same age but represented different phases in the circadian cycle. At harvest, the mycelia were collected under a dim green safe light and frozen in liquid nitrogen (Greene *et al.*, 2003). To extract RNA, frozen mycelia were ground with a mortar and pestle in RNA extraction buffer (0.1 M sodium acetate, 1 mM EDTA, and 4% sodium dodecyl sulfate [SDS] at pH 8). After four phenol extractions, total RNA was precipitated with 95% ethanol and 3M sodium acetate, pH 5.8. Northern analysis (10 mg of RNA/lane) was performed as described previously (Lehrach *et al.*, 1977). The blot was probed with a 500-bp exon of the *A. nidulans gpdA* gene, which was amplified from *A. nidulans* genomic DNA using the upstream PCR primer 5'-GCCTTAAGCACCATTGAGACCTACGACGA-3' and the downstream primer 5'-GCCCTAGGTCAACTAGTTAGTCGAAGATG-3'. The probe was labeled with

[^{32}P]dCTP (6,000 Ci/mmol) by using the DECAprime II kit (Ambion, Austin, Tex.). Ethidium bromide-stained rRNA was used as a control for RNA loading.

Plasmid construction

To generate a construct for deletion of the *ncveA* gene, a 4.9 kb *ncveA* genomic fragment was amplified from *N. crassa* strain FGSC 1858 (*bd; A*) using the primers DeltaveAL: 5'-GGTTGTCGAGAGAAACAGCA-3' and DeltaVAR: 5'-TGGCTCTCTTTCCCGTATCA-3'. The PCR product was cloned into the pCRII-TOPO vector (Invitrogen, Carlsbad, CA) to give pΔveA26. For a selectable marker, the hygromycin resistance gene (*hph*) was chosen. A modified *hph* cassette was amplified using pCSN44 (Staben *et al.*, 1989) as a template and the primers HygavrL: 5'-ATCCTAGGGGCGAATTGGGTACCGGG-3' and HygavrRN: 5'-ATCCTAGGCAGGAATTCGATATCAAG-3'. These primers add 5' and 3' *Avr*II restriction sites to the *hph* cassette. The resulting 2.5 kb PCR product was cloned into pCRII-TOPO to give pavrhyg-TOPO2.

Both pavrhyg-TOPO2 and pΔveA26 were then digested with *Avr*II, and the 2.5 kb *hph* marker was ligated into the 5.5 kb pΔveA26 fragment, which completely lacks the *ncveA* gene. The resulting plasmid was named pveAKO2 and was used as a template in a PCR designed to amplify the *hph* cassette, flanked by 1kb of the *ncveA* genomic region on the 5' side, and 0.6 kb of the *ncveA* genomic region on the 3' side. The resulting 4.1 kb PCR product was transformed into *N. crassa* strain FGSC 1858 (*bd; A*)

by spheroplasting (Pratt and Aramayo, 2002). Twenty-one Hygromycin resistant transformants were isolated and characterized further.

Southern blot analysis

Genomic DNA was extracted from hygromycin resistant transformants using the CTAB method (Taylor and Natvig, 1987; Zolan and Pukkila, 1986), and digested with *Sex*AI and *Msc*I, both of which cut outside the *ncveA* region, and do not cut the *hph* cassette. Digested DNA was subjected to electrophoresis and Southern blotting as described (Sambrook *et al.*, 1989), and was hybridized to PCR product generated by the primers Velsou5: 5'-TCGAGTGCAAACCGTTGA-3' and Velsou3: 5'-CAAGGGTCCCAGGCACTA-3'. All DNA probes were labeled with [32P]dCTP (6,000 Ci/mmol) by using the DECAprime II kit (Ambion, Austin, TX).

Race tube cultures

For race tube assays, either standard medium (0.3% glucose, 0.5% arginine + 1X Vogel's) or a low glucose medium (0.05% glucose, 0.5% arginine + 1X Vogel's) were used. For free-running assays in DD, cultures were inoculated and allowed to sit in LL at 25°C for 24 hours, followed by transfer to DD at 25°C. Cultures were marked daily under a red safety light. For assays in entrainment cycles, cultures were inoculated onto race tubes and immediately placed into the light phase of a 12:12 hour LD cycle. Cultures were marked daily at the time of lights on.

Results and discussion

gpdA *expression is arrhythmic in the* A. nidulans veA1 *mutant strain PW1*

In three independent experiments, robust circadian rhythms in the accumulation of the *gpdA* mRNA, which encodes glyceraldehyde-3-phosphate dehydrogenase, were observed in the wild-type A4 strain (*veA*$^{+}$) of *A. nidulans*, but not in the PW1 strain, which carries the *veA1* mutation (Figure B-1). These data suggested that *veA* may function in the circadian clock mechanism in *A. nidulans*. To examine if *veA* is universally involved in fungal circadian rhythms, we were interested in determining if a *veA* homolog plays a role in the circadian clock of *N. crassa*. This homolog, referred to as the *ncveA* gene in this appendix, encodes a putative 554 aa protein that is 61% similar and 49% identical to the *A. nidulans* VEA protein, over an N-terminal 287 aa stretch. This region of strong similarity includes the NLS, but not the PEST domain, although the C-terminus of the two proteins, including the PEST domain, are weakly similar (Figure B-2).

Deletion of the N. crassa ncveA *gene*

In order to assay the function of the *ncveA* gene in circadian rhythms of *N. crassa*, we deleted the entire gene by homologus gene replacement. The *ncveA* ORF was replaced with the hygromycin resistance *hph* gene (Materials and Methods and Figure B-3). Homologus gene replacement was confirmed by Southern blot, and our data indicate that the initial transformant (called Δ14) was heterokaryotic (Figure B-4). The initial heterokaryotic deletion strain, when grown on Vogel's minimal medium, was

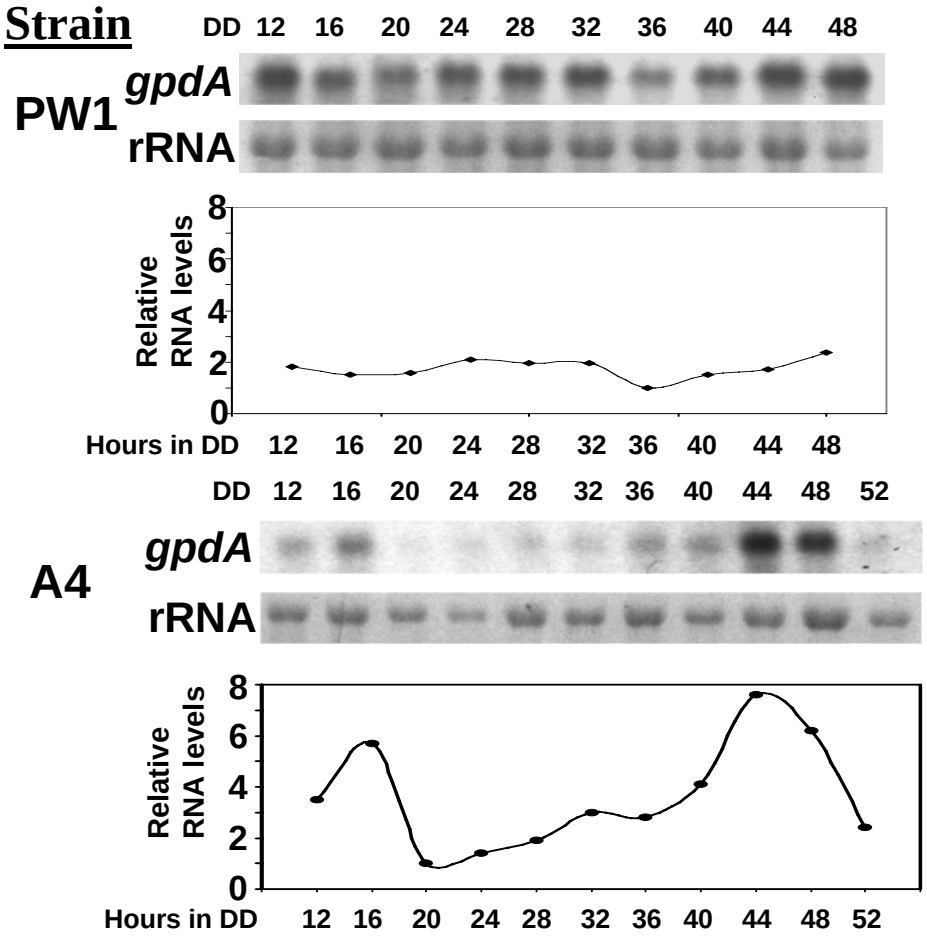

Figure B-1. *gpdA* accumulation is arrhythmic in a *veA1* strain of *A. nidulans*. Total RNA was isolated from *A. nidulans* strain PW1 (*argB2 methG1 biA1 veA1*) or A4 (veA^{+}) grown in DD and harvested every 4 hr. over 2 consecutive days. The northern blots were probed with a PCR product derived from the *A. nidulans gpdA* gene to identify the 1-kb *gpdA* transcript. Ethidium bromide stained rRNA is shown as a loading control. A plot of the relative intensities from the northern blots is shown below each blot. The sample with the lowest *gpdA* level is set to 1.0 for each blot.

Figure B-2. Alignment of the *A. nidulans* and *N. crassa velvet A* proteins. The putative NLS is shown (yellow highlight), as are the PEST domain (green highlight) and putative downstream initiation site used in the *A. nidulans veA1* mutant (arrow). Key: * = identical aa; : = strong similarity; . = weak similarity.

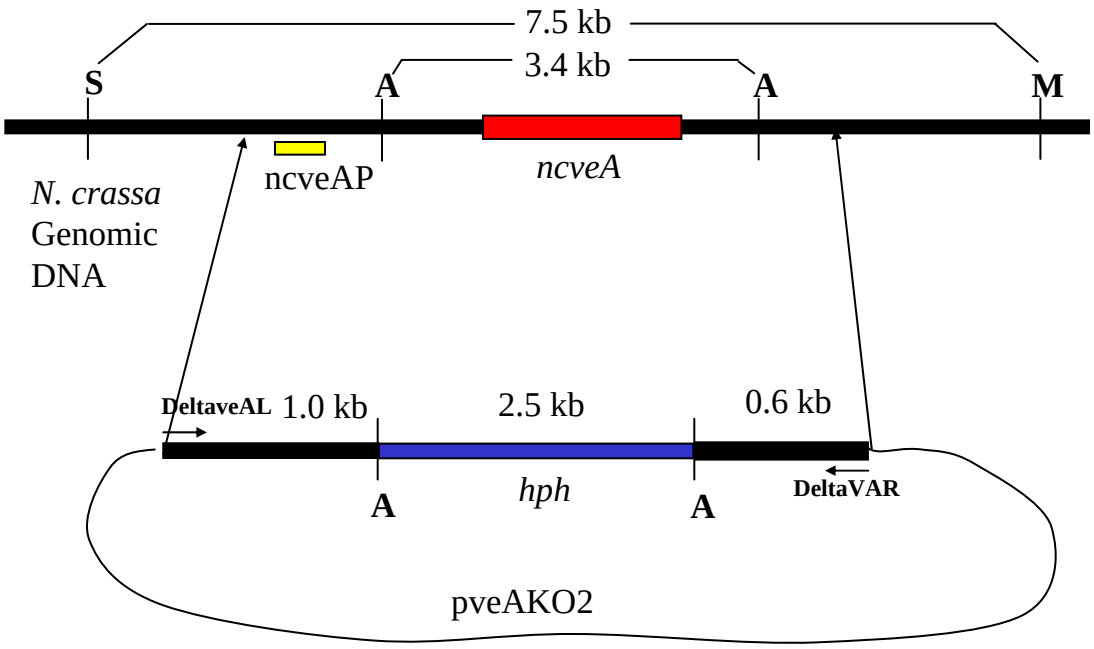

Figure B-3. Schematic representation of the construct for disruption of the *ncveA* ORF. From pVEAKO2, a 4.1 kb PCR fragment, amplified using the primers DeltaveAL and DeltaVAR, containing the *hph* cassette flanked by 1.0 kb of 5' genomic DNA and 0.6 kb of 3' genomic DNA was transformed into *N. crassa* strain FGSC 1858. This resulted in the replacement of a 3.4 kb fragment of genomic DNA, including the entire *ncveA* ORF, with the 2.5 kb bacterial *hph* selectable marker. Also shown is the probe (ncveAP) used in Southern blots (Figure B-4). Key: A = *Avr*II, S = *Sex*AI, M = *Msc*I.

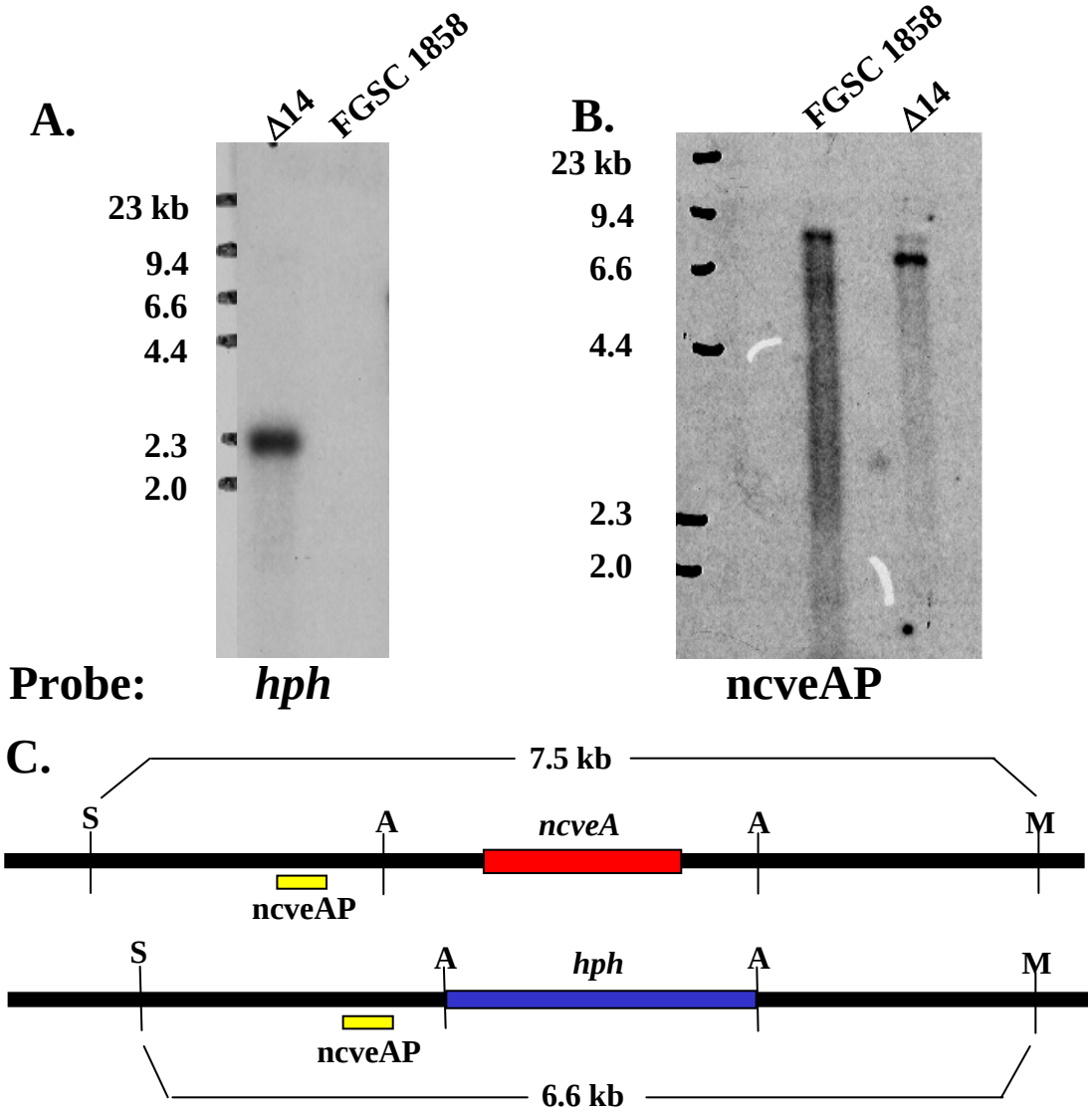

Figure B-4. Confirmation of deletion of *ncveA*. Genomic DNA was isolated from FGSC 1858 and transformant #14, and double digested with *Sex*A1 and *Msc*I or *Avr*II. **A.** Probing of AvrII digested DNA with a probe to the *hph* cassette reveals a 2.5 kb band in the deletion strain, and does not hybridize with FGSC 1858 DNA. **B.** Probing of digested DNA with ncveAP reveals a 7.5 kb band in the wild type, and a 7.5 kb band plus a 6.6 kb band in the transformant, indicating the deletion strain is heterokaryotic. **C.** Schematic representation of the *ncveA* genomic region in wild-type (top) and Δ*ncveA* strains (bottom). Digestion of wild-type DNA with *Sex*AI and *Msc*I yields a 7.5 kb band when probed with ncveAP, while digestion of Δ*ncveA* DNA with *Sex*AI and *Msc*I yields a 6.6 kb band when probed with ncveAP. Key: A = *Avr*II, S = *Sex*AI, M = *Msc*I.

phenotypically indistinguishable from the parental *bd* strain, FGSC 1858. The Δ14 strain was backcrossed to the *bd; a* strain FGSC 1859, and a homokaryotic Δ*ncveA* strain, 434-18 (Δ*ncveA; bd; A*) was obtained from a single ascospore and used in all subsequent experiments.

Growth phenotype of the ΔncveA; bd *strain*

Deletion of the *ncveA* gene allowed for the examination of the role of this gene in *N. crassa* circadian rhythms by assay of the conidiation rhythm on race tubes. When cultured on minimal medium, strain 434-18 displayed a phenotype distinct from that of the *bd* strain. While the parental *bd* strain formed abundant aerial hyphae and conidia, the Δ*ncveA; bd* strain formed little or no aerial hyphae, and instead made a dense mass of conidia clustered at the agar surface (Figure B-5). The Δ*ncveA; bd* strain also had a slight pigmentation defect; it was paler than the *bd* strain. The Δ*ncveA; bd* strain also conidiated more than the *bd* strain on standard race tube medium (0.3% glucose + 0.5% arginine Vogel's), and due to this overconidiation, circadian conidiation bands were difficult to discern (data not shown). Decreasing the amount of glucose in the tubes to 0.05% served to decrease the total amount of conidiation, and on lowered glucose concentrations circadian conidiation bands were more easily observed in the Δ*ncveA; bd* strain, with a period similar to that of the parental *bd* strain (23.1 h in the Δ*ncveA; bd* strain vs. 23.0 h in the *bd* strain) (Figure B-6). However, strain 434-18 grew slightly slower than the *bd* strain (3.0 cm/day vs. 3.5 cm/day). These observations indicated that, while the Δ*ncveA; bd* mutation resulted in increased conidiation in *N. crassa*, deletion of

FGSC 434-18
1858

Figure B-5. Growth phenotype of the homokaryotic *ncveA* mutant. The parental strain FGSC 1858 and homokaryotic deletion strain 434-18 were cultured for 7 days of 2% glucose Vogel's medium. The *ncveA* mutant does not make aerial hyphae, but forms a dense mass of conidia close to the agar surface. The *ncveA* also displays a slight pigment defect, not visible in this photograph.

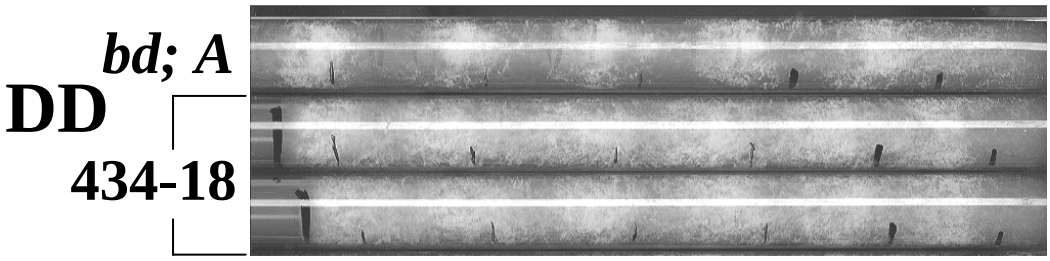

Figure B-6. The *ncveA* deletion strain conidiates rhythmically in DD. Race tube assays of FGSC 1858 (*bd, A*) and 434-18 (Δ*ncveA; bd; A*) are shown. Tubes were grown in LL at 25°C for 24 hr prior to transfer to DD at 25°C. Growth fronts were subsequently marked every 24 hr (short black lines). Growth medium is 0.05% glucose + 0.5% arginine Vogel's medium.

this gene had no affect on free running circadian rhythmicity. Entrainment of the conidiation rhythm to 12:12 L:D cycles was also tested, and the Δ*ncveA; bd* strain entrained to these cycles in a manner indistinguishable from the *bd* strain, indicating that *ncveA* does not play a role in circadian entrainment to light (data not shown).

A final phenotype we assayed is the ability of homokaryotic Δ*ncveA; bd* strains of opposite mating type to mate. We found that, independent of whether the strains were co-inoculated on crossing medium or one was allowed to grow and form protoperithecia (the "female" in the cross) prior to addition of conida of opposite mating type, the homokaryotic Δ*ncveA; bd* strains were able to function as both male and female, and cross to each other and produce viable ascospores. These results indicated that the function of the *ncveA* gene is distinct from that of its *Aspergillus* ortholog, at least with respect to sexual development.

Conclusions and future directions

The data presented in this appendix describe the deletion and preliminary characterization of the *N. crassa* homolog of the *A. nidulans veA* gene. Our data indicate that the function of *ncveA* is distinct from that of the *veA* gene, as the *N. crassa* Δ*ncveA; bd* strain is functional as both male and female in sexual crosses and is able to respond to light signals, as assayed by entrainment of the conidiation rhythm. A possible explanation for this is that the *ncveA* gene is similar primarily to the N-terminus of its *Aspergillus* counterpart; the C-terminal region of the protein, including the PEST domain, is not as well conserved between the two species.

While our data indicate that the Δ*ncveA; bd* strain is able to respond to light, it is possible that other light responses are affected by the Δ*ncveA* mutation. A more rigorous test of the light responses of this strain would require the assay of light induced gene expression, carotenoid biosynthesis, and ascospore discharge, among other light mediated responses. The slight carotenoid defect seen in the Δ*ncveA; bd* strain may indicate a defect in light perception or response, and this should be examined further. In addition, the Δ*ncveA; bd* strain makes few aerial hyphae and forms conidia close to the agar surface, suggesting that NCVEA may play an activator role in the transition from surface to aerial growth. The overconidiation phenotype observed suggests that NCVEA is invloved in negatively regulating conidiation in *N. crassa*; a similar phenotype is seen in the *veA1* strain of *A. nidulans*, which conidiates in larger quantities than the wild-type. This suggests a possible conservation of function between the *A. nidulans* and *N. crassa velvetA* genes.

Finally, the *A. nidulans veA1* strain used in this study is an auxotroph, requiring arginine, methionine and biotin for growth. While auxotrophic mutants of *N. crassa* do not abolish free running conidiation rhythms, it is possible that these mutations do affect rhythmicity in *Aspergillus*. Recent results with a *pyrG* strain (requiring uridine and uracil) of *A. flavus* 12S support this notion; the *pyrG* strain does not display free running sclerotium rhythms (Greene and Keller, unpublished observations). Future studies will be necessary to clarify this situation; development of an easily assayable reporter in *Aspergillus* will greatly facilitate this line of inquiry.

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Publications

Greene AV, Keller NP, Haas H and Bell-Pedersen D. *Eukaryot Cell*. 2003 Apr; **2** (2):231-7. A Circadian Oscillator in *Aspergillus spp.* Regulates Daily Development and Gene Expression.

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