# CONTROL OF RHYTHMIC OUTPUT FROM THE CIRCADIAN CLOCK IN *Neurospora crassa*

A Dissertation

by

ZACHARY A. LEWIS

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

December 2004

Major Subject: Microbiology

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## ABSTRACT

Control of Rhythmic Output by the Circadian Clock in *Neurospora crassa.*

(December 2004)

Zachary A. Lewis, B.S. University of Georgia

Chair of Advisory Committee: Dr. Deborah Bell-Pedersen

Circadian rhythms are visible as daily oscillations in biochemical, physiological, or behavioral processes. These rhythms are produced by an endogenous clock that maintains synchrony with the external environment through responses to external stimuli such as light or temperature. The clock, in turn, coordinates internal processes in a time-dependent fashion. Genetic and molecular analysis of the filamentous fungus *Neurospora crassa* has demonstrated that the products of the *frequency* (*frq*) and *white-collar* (*wc-1* and *wc-2*) genes interact to form an interlocked feedback loop that lies at the heart of the clock in this fungus. This feedback loop, termed the FRQ/WC oscillator, produces a ~24h oscillation in *frq* mRNA, FRQ protein, and WC-1 protein. In turn, the FRQ/WC oscillator regulates rhythmic behavior and gene expression. The goal of this dissertation is to understand how rhythmic outputs are regulated by the FRQ/WC oscillator in *Neurospora.*

To this end, we have taken a microarray approach to first determine the extent of clock-controlled gene expression in *Neurospora.* Here, we show that

circadian regulation of gene expression is widespread; 145 genes, representing 20% of the genes we analyzed, are clock-controlled. We show that clock-regulation is complex; clock-controlled genes peak at all phases of the circadian cycle. Furthermore, we demonstrate the clock regulates diverse biological processes, such as intermediary metabolism, translation, sexual development and asexual development. WC-1 is required for all light- and clock-regulated gene expression in *Neurospora.* We have shown that overexpression of WC-1 is sufficient to activate clock-controlled gene expression, but is not sufficient to induce all light-regulated genes in *Neurospora.* This result indicates that cycling of WC-1 is sufficient to regulate rhythmic expression of a subset of clock-controlled genes. Conversely, a post-translational mechanism underlies WC-1 mediated light signal transduction in *Neurospora.* Finally, we have demonstrated the *Neurospora* circadian system is comprised of mutually coupled oscillators that interact to regulate output gene expression in the fungus.

## DEDICATION

This dissertation is dedicated to my family and Ms. Cory Davis for all of the love and support they have provided throughout my education.

## ACKNOWLEDGEMENTS

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# CHAPTER I

# INTRODUCTION

## Circadian rhythms

Many biological events occur with clock-like regularity throughout the course of the day. The first report of a daily rhythm occurred in 1729 when Jacques de Marian documented leaf movements of mimosa plants housed in a basement (Sweeney, 1987). These rhythmic events, termed circadian rhythms, are evident throughout nature and are exemplified by the daily leaf movements of many plants, daily activity patterns of insects and animals, and the sleep/wake cycle of humans (Pittendrigh, 1993). The biological basis of circadian rhythms has been the subject of intensive study over the last 60 years (Pittendrigh, 1993). Circadian rhythms in behavioral, physiological and biochemical processes have been reported in organisms ranging in complexity from unicellular cyanobacteria to humans (Dunlap, 1999). Despite the tremendous diversity in organisms possessing circadian clocks and even greater diversity in the biological processes regulated by these clocks, shared properties are observed across the kingdoms of life.

Circadian rhythms are endogenous, persisting in the absence of environmental stimuli with a period close to 24 hours (Pittendrigh, 1960). The robust oscillation of circadian clocks in the absence of an environmental cycle is

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This dissertation follows the style of *Molecular Microbiology*.

quite remarkable. Indeed, mice housed in constant darkness and constant temperature have maintained a daily rhythm in wheel-running activity for >2 years (Pittendrigh, 1993). The rhythmic occurrence of any biological event in constant conditions has been termed a "free running rhythm" and the amount of time between two consecutive events is referred to as the "free running period" (FRP) of the rhythm. Although circadian clocks persist in the absence of environmental stimuli, these clocks are reset by environmental cues such as light and temperature (Pittendrigh and Minis, 1964). Responses to these environmental cues occur in a phase-dependent manner allowing synchronization of the internal clock to the external environment. For example, a light pulse administered during the early subjective morning results in a phase advance of the rhythm. Alternatively, a light pulse administered during the subjective evening results in a phase delay of the rhythm. Another remarkable property of circadian rhythms is the stability of the endogenous period across physiological temperature ranges, a phenomenon referred to as temperature compensation (Pittendrigh, 1960; Pittendrigh, 1993). While most biochemical reactions exhibit an increase or decrease in rate as the temperature increases or decreases, the circadian period remains relatively unchanged over an organisms physiological temperature range. In simple terms, we can describe the circadian system as the product of three basic components: a central pacemaker (1) that keeps internal time, input pathways (2) that synchronize the pacemaker to the

external environment, and output pathways (3) that regulate biological processes in a time-of-day dependent manner (Figure 1).

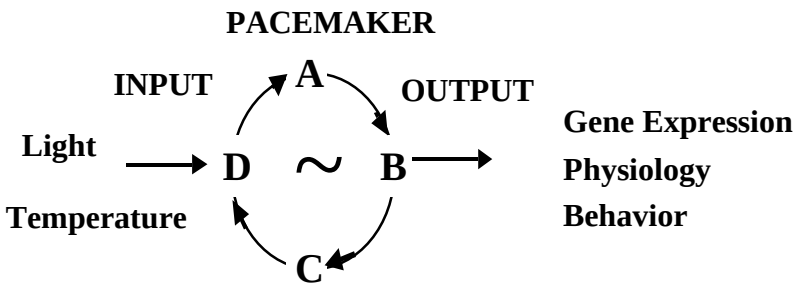


**Figure 1. A simplified view of the circadian clock system.**

The entire circadian system resides within single cells in some organisms (i.e. cyanobacteria, fungi). Alternatively, cell-cell communication within and between tissues is required to relay input information to the pacemaker as well as coordinate output processes from the pacemaker in higher eukaryotes (i.e. humans). Interestingly, the pacemaker is cell autonomous in all organisms that have been studied (for examples see Herzog *et al.*, 1998; Welsh *et al.*, 1995).

The shared properties of circadian systems suggest that the molecular mechanism(s) responsible for rhythm generation may share common properties as well. The use of model organisms has provided a great deal of insight into the molecular workings of circadian clocks. Such studies have identified a number of "clock genes" that are integral components of the time-keeping mechanism. The first "clock genes" were identified through the use of genetic

screens. Mutations in the clock genes *period (per)* in *Drosophila* and *frequency (frq)* in *Neurospora* were described by Ron Konopka and Jerry Feldman, respectively (Feldman and Hoyle, 1973; Konopka and Benzer, 1971). Mutations in these genes can increase or decrease the period length displayed by these organisms. Furthermore, loss of function mutations in these genes abolish rhythms altogether. Subsequent studies demonstrated that clock genes are expressed rhythmically over the course of the day at the mRNA and protein level. Additional molecular analyses have provided a general model describing the time-keeping mechanism in eukaryotes (reviewed by Dunlap, 1999).

This model states that circadian clocks are comprised of transcription/translation feedback loops, whereby transcriptional activators induce expression of their own repressors (Figure 2). The activators or "positive elements" all form heterodimers via PAS domains (CLOCK and BMAL in mammals, CLOCK and CYCLE in *Drosophila,* WHITE COLLAR-1 and WHITE COLLAR-2 in *Neurospora*) and activate transcription of repressors (PERIOD and TIMELESS in *Drosophila,* PERIOD and CRYPTOCHROME in mammals, FREQUENCY in *Neurospora*) by binding to regulatory elements in the promoters of these "negative elements". Following transcriptional activation, the mRNA levels of the negative elements begin to rise followed by an increase in protein levels 4-6 hours later. The negative elements then enter the nucleus and interact with the positive elements, inhibiting transcriptional activation. The negative elements are eventually degraded, resulting once again in their own

transcriptional activation and the start of another cycle. In addition to their activity as transcriptional repressors, the negative elements up-regulate the levels of the positive elements, creating an interlocked feedback loop and adding robustness to the system (reviewed by Dunlap, 1999; Young and Kay, 2001).

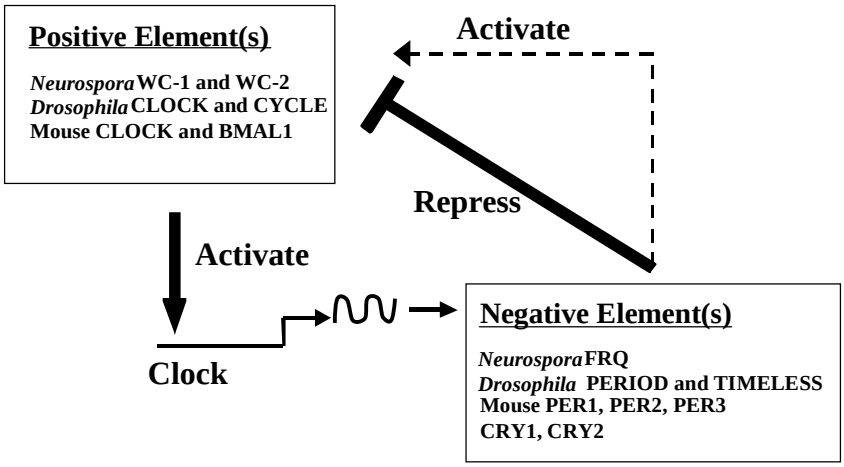


**Figure 2. Common themes in molecular circadian oscillators.**

Light can reset the clock by rapidly altering the levels of at least one component of the feedback loop. In mammals, *period-1* and *period-2* are induced in neurons of the suprachiasmatic nucleus (SCN) following a light pulse (Albrecht *et al.*, 1997; Shearman *et al.*, 1997; Shigeyoshi *et al.*, 1997). In *Drosophila* light-dependent degradation of TIMELESS (TIM) is important for resetting the clock (Hunter-Ensor *et al.*, 1996; Myers *et al.*, 1996; Zeng *et al.*, 1996). In *Neurospora, frequency* (*frq*) mRNA is rapidly induced following exposure to light (Crosthwaite *et al.*, 1995).

Identification of circadian photoreceptors, as well as other components of light input pathways has increased our understanding of how the clock responds to light signals. The CikA protein is required for normal resetting in cyanobacteria (Schmitz *et al.*, 2000). In plants, PHYTOCHROME and CRYPTOCHROME (CRY) proteins relay light information to the pacemaker (Yanovsky *et al.*, 2001). The retinal photopigment melanopsin is the circadian photoreceptor in mammals (Hattar *et al.*, 2003; Panda *et al.*, 2003). Following light absorption by melanopsin, light signals are transduced to neurons in the SCN via direct projections via the retinohypothalamic tract (reviewed by Reppert and Weaver, 2002). In *Drospophila,* CRY mediates the light-dependant degradation of TIM (Emery *et al.*, 2000). CRY proteins in mammals do not appear to be photoreceptors, however. Rather, the three mammalian CRY proteins participate in the negative limb of the circadian feedback loop (Field *et al.*, 2000). WHITE COLLAR-1 in *Neurospora* is a photoreceptor as well as a component of the circadian feedback loop in the fungus (Froehlich *et al.*, 2002; He *et al.*, 2002). Despite advances in our understanding of light input to the circadian clock, much is still unknown about the input pathways. Most notably, the molecular basis of entrainment by temperature is completely unknown. In fact, molecular studies of temperature input to the clock have only been undertaken in *N. crassa* (see below).

**The *Neurospora* circadian clock**

The filamentous fungus *Neurospora crassa* has a long history in the study of circadian clocks (Pittendrigh *et al.*, 1959). *N. crassa* exhibits a circadian rhythm in development of asexual macroconidia (hereafter referred to as conidiation). The conidiation rhythm of *N. crassa* displays all of the formal properties of a circadian rhythm (reviewed by Loros and Dunlap, 2001). Indeed, the conidiation rhythm persists in constant darkness with a period of ~22h. The rhythm can be reset by light and temperature pulses. Lastly, the period of the rhythm is temperature compensated between 20°C and 30°C. Importantly, the rhythm in conidiation is easily monitored in specialized culture tubes containing an agar medium called "race tubes" (Sargent *et al.*, 1966). Mycelia or conidia inoculated at one end of the race tube grow toward the other end at a constant rate (~3.5 cm/ day). The tubes are incubated in constant light at 25°C for 1 day before the race tube is transferred to constant darkness at 25°C to synchronize the clock to dusk. The growth front of the culture is marked every 24 hours under a red light (red light does has no entraining affect on the *Neurospora* clock). In the dark, conidia are formed every ~22h and are visible as fluffy orange bands separated by regions of white mycelia. When the culture reaches the end of the tube, it is simply removed from the dark and the center of the band is marked as the phase reference point. The position of the band relative to the daily marks allows the calculation of the period and phase of the rhythm. The free running period of a strain refers to the amount of time required to form two

consecutive peaks in conidiation (Figure 3). The easily monitored conidiation rhythm and the battery of genetic tools developed for *N. crassa* make the fungus an ideal model system for the study of circadian clocks.

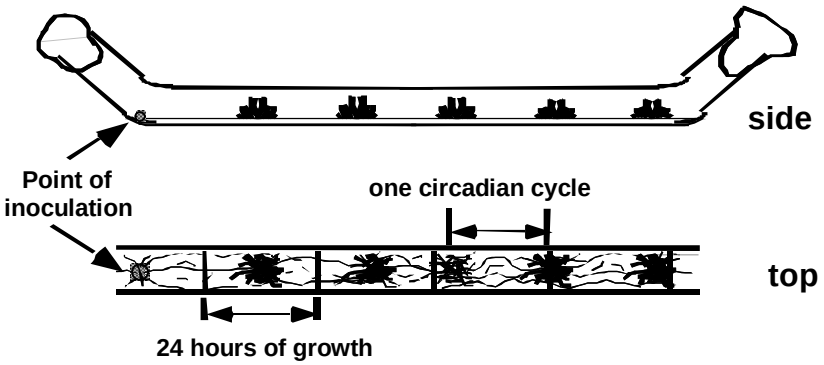


**Figure 3. The race tube assay for circadian rhythms of development in *Neurospora.***

As mentioned above, the *frq* gene of *N. crassa* was identified through genetic studies and subsequent studies have provided evidence that *frq* is a component of the circadian pacemaker in the fungus (reviewed by Bell-Pedersen, 2000; Loros and Dunlap, 2001). In addition, positive activators of *frq*, encoded by the *white collar-1* (*wc-1*) and *wc-2* genes, have been identified (Ballario *et al.*, 1996; Crosthwaite *et al.*, 1997; Linden and Macino, 1997). These three genes interact to form a transcription/translation feedback loop critical for circadian control of the conidiation rhythm (Figure 4). Briefly, the WC-1 and WC-2 proteins heterodimerize through PAS domains to form the White Collar Complex (WCC) (Ballario *et al.*, 1998). WCC binds directly to *cis*-elements

within the *frq* promoter, activating transcription of *frq* mRNA (Crosthwaite *et al.*, 1997; Froehlich *et al.*, 2002). Following activation by WCC, *frq* mRNA accumulates to its highest levels in the early morning followed by a peak in FRQ protein levels about 4-6 hours later (Aronson *et al.*, 1994b; Garceau *et al.*, 1997). FRQ protein enters the nucleus where it interacts with the WCC to inhibit its own transcription. The cycle is renewed when phosphorylation-induced decay of FRQ eventually leads to a decline in FRQ levels and relief of WCC repression (Liu *et al.*, 2000). Although FRQ inhibits the activity of WCC, FRQ protein positively regulates the levels of its activators to create an interlocked feedback loop (Cheng *et al.*, 2001b; Lee *et al.*, 2000). Notably, FRQ promotes rhythmic synthesis of WC-1 (Lee *et al.*, 2000). The resulting WC-1 oscillation is anti-phase to the rhythm in FRQ accumulation. Alternatively, the *wc-1* and *wc-2* mRNA's, as well as the WC-2 protein, do not cycle in constant darkness in the wild type strain (Cheng *et al.*, 2001b; Denault *et al.*, 2001; Lee *et al.*, 2000).

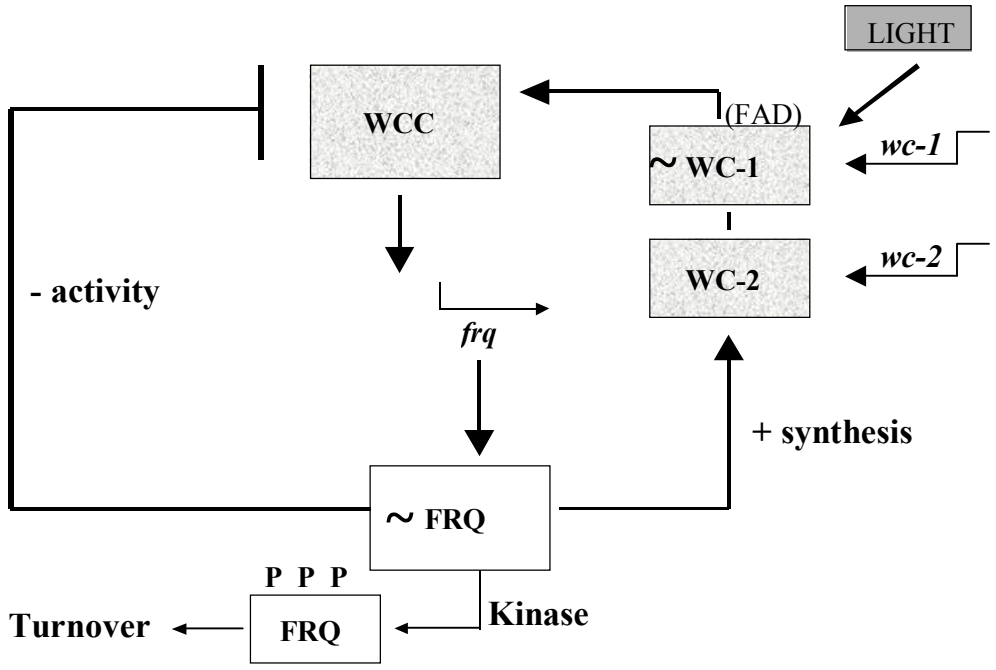


**Figure 4. Current model of the *Neurospora* FRQ/WCC oscillator.**
See text for details, P = phosphorylation; ~ = rhythmic accumulation.

A great deal of work over the last fifteen years has provided a detailed understanding of the intricacy of the FRQ/WC oscillator. As mentioned above, WC-1 mediates light input to the *Neurospora* clock through direct perception of blue light (via a bound FAD chromophore) followed by rapid induction of *frq* mRNA (Crosthwaite *et al.*, 1995; Froehlich *et al.*, 2002; He *et al.*, 2002). Light induction by the WC-1 photoreceptor requires its dimerization partner WC-2 and involves direct interaction of the WCC with the *frq* promoter (Collett *et al.*, 2002; Froehlich *et al.*, 2002). The magnitude of light induction observed for *frq* mRNA is dependent on the time of day the light pulse is percieved. Specifically, maximal light induction of *frq* mRNA occurs when WC-1 protein levels are at their

highest, a phenomenon termed gating (Heintzen *et al.*, 2001; Merrow *et al.*, 2001). Furthermore, *frq* induction is correlated with the direction and magnitude of the phase response of the conidiation rhythm (Crosthwaite *et al.*, 1995). In constant light *frq* is expressed at constitutively high levels resulting in arrhythmic conidiation (Crosthwaite *et al.*, 1995). The effects of constitutive *frq* expression have been simulated using strains harboring an extra copy of *frq* under control of the inducible *quinic acid-2* (*qa-2*) promoter (Aronson *et al.*, 1994b). Overexpression from this promoter results in lower transcript levels from the endogenous promoter, consistent with the role of FRQ as a repressor. Moreover, constitutive expression from the *qa-2* promoter will not rescue the arrhythmic phenotype of the *frq*-null strain, illustrating the importance of *frq* mRNA cycling (Aronson *et al.*, 1994b). The fact that *frq*-null strains show a similar constitutive conidiation phenotype indicates that FRQ does not directly activate or repress conidiation in the fungus (Aronson *et al.*, 1994b).

Temperature regulation of FRQ occurs at the post-transcriptional level. Specifically, two distinct forms of FRQ protein comprise the negative limb of the FRQ/WCC oscillator (Garceau *et al.*, 1997; Liu *et al.*, 1997). Interestingly, a long form (989 AA) is prominent at high temperatures (~30°C), whereas a short form (890 AA) is prominent at low temperatures (~20°C) (Liu *et al.*, 1997). Moreover, strains that produce only the long or short forms conidiate in a constitutive, non-circadian fashion at high or low temperatures, respectively (Liu *et al.*, 1997). These data indicate that the two forms of FRQ may play a role in temperature

compensation. The levels of FRQ protein are also affected by ambient temperature. At high temperatures, FRQ is present at high levels. Alternatively, FRQ is present at low levels when the ambient temperature is low ( i.e. trough levels at high temperature exceed peak levels at low temperatures) (Liu *et al.*, 1998). This observation has led to a model for circadian resetting to step changes in ambient temperature. A step down from high to low temperature produces a new phase where FRQ levels are at the high point of the new cycle. Conversely, a step up from low to high temperature produces a new phase where FRQ levels are at the trough of the new cycle. Despite these advances, the current model for temperature entrainment is insufficient. For example, no genes that affect FRQ temperature regulation have been identified to date.

FRQ protein does not contain many recognizable structural motifs. However, a Nuclear Localization Sequence (NLS) is present and is essential for clock function in *N. crassa* (Lewis and Feldman, 1993; Luo *et al.*, 1998). Also, a PEST domain within the protein and a Serine at residue 513 contribute to phosphorylation-induced decay of the protein (Gorl *et al.*, 2001; Liu *et al.*, 2000). Furthermore, several kinases important for FRQ stability and proper function of the *Neurospora* clock have been identified. Phosphorylation by Casein kinase II and a calcium/ calmodulin-dependent kinase are important for FRQ degradation (Yang *et al.*, 2001; Yang *et al.*, 2002). FRQ also interacts with a Casein kinase I homolog (CKI) *in vivo,* supporting the idea that this kinase is involved in the *Neurospora* clock mechanism (Gorl *et al.*, 2001). CKI has a similar role in

mammals and *Drosophila* where CKI phosphorylation of PER promotes PER turnover (Keesler *et al.*, 2000; Kloss *et al.*, 1998; Lowrey *et al.*, 2000; Vielhaber *et al.*, 2000; Zilian *et al.*, 1999). FRQ degradation is aided by the F-box WD40 repeat protein FWD-1, a homolog of *Drosophila* SLIMB (Grima *et al.*, 2002; He *et al.*, 2003; Ko *et al.*, 2002). SLIMB plays a role in degradation of PER in *Drosophila.* In similar fashion, FWD-1-mediated degradation of FRQ involves protein ubiquitination. The conserved role of FWD-1, CKI, and their homologs suggests that a conserved mechanism is responsible for degradation of negative clock elements across species.

FRQ protein exists in a high molecular weight complex in the cell (Denault *et al.*, 2001). A coiled-coil domain is required for FRQ monomers to self-associate, contributing to the formation of the FRQ complex (Cheng *et al.*, 2001a). Self-association of FRQ is required for the inhibitory interaction of FRQ with the WCC. As mentioned above, FRQ protein also acts to positively regulate the levels of its activators to form an interlocked feedback loop. Indeed, WC-1 and WC-2 levels are reduced in FRQ-null strains (Cheng *et al.*, 2001b; Lee *et al.*, 2000). Moreover, induction of *frq* from the inducible *qa-2* promoter results in a subsequent increase in the levels of WC-1 suggesting that FRQ may drive the rhythm in WC-1 protein accumulation (Lee *et al.*, 2000). The remarkable similarity that the FRQ/WCC oscillator shares with other circadian systems (i.e. *Drosophila,* mammals) reinforces the use of *Neurospora* as a valuable model system for the study of circadian rhythms.

There are still missing pieces in our current model. There is a 4-6 hour lag between the peak in *frq* mRNA levels and the peak in FRQ protein levels; yet, the mechanism responsible for this lag is unknown. Also, we do not yet know if positive regulation of WC-1 and WC-2 by FRQ is direct or indirect. It has been shown that FRQ directly interacts with the WCC to inhibit its activity, but the mechanism of inhibition is not clear. One possibility is that FRQ disrupts interaction of the WCC with other factors that remain uncharacterized. Indeed, several mutant strains have been isolated that display altered period lengths and interact genetically with *frq* (Morgan and Feldman, 1997, 2001). Yet, the genes responsible for these phenotypes have not been cloned. Thus, the existence of unknown players within the circadian system is certain. Continued work with *Neurospora* will undoubtedly provide answers to some of these remaining questions.

**Multiple circadian oscillators**

The well-characterized FRQ/ WCC feedback loop is certainly integral to the circadian mechanism in *Neurospora.* However, there is evidence for additional FRQ-less oscillators (FLO's) within the cell. Mutations in *chain elongation* (*cel*) and *choline-1 (chol*) suppress the arrhythmic conidiation phenotype of FRQ and WC-null mutants under certain conditions (Lakin-Thomas and Brody, 2000). However, the period length of these strains is outside the circadian range and the specific role these lipid biosynthetic enzymes play within

the circadian system is unknown. More compelling evidence comes from work with FRQ-null strains. Indeed, these strains display a rhythm in conidiation in constant conditions (free run) when the medium is supplemented with farnesol or gerianiol (Granshaw *et al.*, 2003). Temperature pulses administered at different phases generate a phase response curve under these conditions, supporting the model that the *Neurospora* circadian system is comprised of multiple oscillators.

Interestingly, species of *Aspergillus* exhibit circadian rhythms in development and gene expression (Greene *et al.*, 2003). Yet, the genomes of these fungi do not contain *frq* homologs, indicating that a FRQ-less oscillator generates rhythms in these fungi (homologs of other negative elements such as PER or TIM are also absent from this genome).

In *Synechococcus* and *Gonyaulax,* multiple FRP's are observed in a single cell, suggesting that multiple oscillators are present within these organisms (Nair *et al.*, 2002; Roenneberg and Morse, 1993). In plants, differential sensitivity of two output genes to temperature pulses can be observed in within a single tissue (Michael *et al.*, 2003). Specifically, an oscillator that regulates expression of CHLOROPHYLL A/B BINDING PROTEIN 2 responds preferentially to light stimuli, whereas a second oscillator that regulates expression of CATALASE 3 responds preferentially to temperature. Moreover, distinct phase response curves to temperature pulses are observed for each gene. These data imply that at least two oscillators reside within a single plant tissue.

The multi-tissue organization of the vertebrate circadian system provides an even greater level of complexity. The activity rhythm of the hamster often splits into two anti-phase components when these animals are housed under conditions of bright light. This is correlated with anti-phase expression of clock genes in the bilateral lobes of the SCN (de la Iglesia *et al.*, 2000). Restricted feeding of mice housed in light:dark cycles results in a new phase relationship of clock gene expression within the liver and the SCN (Damiola *et al.*, 2000; Stokkan *et al.*, 2001). This result indicates that individual tissues may have the capacity to be an oscillator. This is supported by persistent oscillation of a PERIOD2:LUCIFERASE reporter fusion after isolation and maintenance of individual organs in tissue culture (the luciferase reporter is driven by the *per2* promoter) (Yoo *et al.*, 2004). Overt circadian rhythms in many avian species require the function of a pineal oscillator, an oscillator housed within the vSCN (the avian homolog of the mammalian SCN), and an oscillator housed in the retina (reviewed by Gwinner and Brandstatter, 2001). Taken together, these data suggest that circadian output pathways emerge from a complex network of oscillators.

**Output from the clock**

Understanding how output processes are regulated by the pacemaker is fundamental to developing a complete understanding of circadian systems. Due to the complex nature of circadian systems (or in other words, the entire

organism), this has been incredibly difficult. One approach has been to describe the complement of clock controlled genes (ccg's) in various organisms (plants, *Drospophila,* mouse and chick) or tissues (rodent SCN, liver, kidney, heart, hypothalamus and fibroblasts and the chick pineal) using microarrays (Akhtar *et al.*, 2002; Bailey *et al.*, 2003; Claridge-Chang *et al.*, 2001; Duffield *et al.*, 2002; Harmer *et al.*, 2000; McDonald and Rosbash, 2001; Oishi *et al.*, 2003; Panda *et al.*, 2002; Schaffer *et al.*, 2001; Storch *et al.*, 2002). These studies have demonstrated that there is little overlap between clock-controlled genes in various organisms or even among tissues within an organism (~10%). In all of these studies, clock-controlled genes that peak at all phases of the circadian cycle have been identified. The extent of clock-controlled gene expression is also organism-specific. In *Synechococcus* all gene expression is rhythmic with genes peaking at two phases of the circadian cycle (Golden *et al.*, 1997). Experiments in eukaryotic systems have provided estimates that 2% to 20% of genes are controlled by the clock. Despite the small degree of overlap among organisms, certain genes appear to be clock-controlled in all eukaryotes. Notably, genes involved in intermediary metabolism, translation, and protein turnover are clock-controlled in fungi, plants, *Drosophila,* and vertebrates (Akhtar *et al.*, 2002; Bailey *et al.*, 2003; Claridge-Chang *et al.*, 2001; Duffield *et al.*, 2002; Harmer *et al.*, 2000; McDonald and Rosbash, 2001; Oishi *et al.*, 2003; Panda *et al.*, 2002; Schaffer *et al.*, 2001; Storch *et al.*, 2002). Alternatively, many clock-controlled genes reflect the unique lifestyle of the organism. For example, genes

involved in photosynthesis are rhythmically expressed in plants and cyanobacteria, reflecting the dependence of these organisms on light (Golden *et al.*, 1997; Harmer *et al.*, 2000). In multicellular organisms, genes that regulate the physiology of the organism cycle over the course of the day. mRNA's encoding enzymes that participate in the rhythmic synthesis of melatonin accumulated rhythmically in the avian pineal gland (Bailey *et al.*, 2003). In plants, genes involved in the regulation of flowering time and phototropism are rhythmically expressed (Harmer *et al.*, 2000). In flies, genes involved in cuticle formation, pheromone/odorant perception, and neuromodulation are all rhythmically expressed (McDonald and Rosbash, 2001). These examples reflect only a subset of biological processes under control of the circadian clock in these organisms. Although the conidiation rhythm of *Neurospora* is the most observable output from the clock, the identification of clock-controlled genes involved in a wide range of biological processes highlights the importance of the circadian clock in the life of the fungus (Table 1). Indeed, *s*ubtractive hybridization and differential display screens identified clock controlled genes involved in stress responses, sexual development and intermediary metabolism, as well as genes involved in conidiation (Arpaia *et al.*, 1995; Bell-Pedersen *et al.*, 1992; Bell-Pedersen *et al.*, 1996b; Lauter and Yanofsky, 1993; Loros *et al.*, 1989; Loros and Dunlap, 1991; Munger *et al.*, 1987). Notably, the identification of clock-controlled genes in *Neurospora* provided the first description of clock-controlled genes in any organism (Loros *et al.*, 1989; Loros and Dunlap, 1991).

**Table 1. Summary of clock-controlled genes.**

| Gene | Average Peak[a] | Identity[b] | Devel[c] | Light | Reference |
|---|---|---|---|---|---|
| ccg-1 | CT3 | Unknown | + | + | Loros *et al.* (1989) |
| eas (ccg-2) | CT22 | Hydrophobin | + | + | Loros *et al.* (1989); Bell-Pedersen *et al.* (1992); Lauter *et al.* (1992) |
| ccg-4 | CT5 | Pheromone | + | + | Bell-Pedersen *et al.* (1996c) |
| ccg-6 | CT19 | Unknown | + | + | Bell-Pedersen *et al.* (1996c) |
| ccg-7 | CT21 | GAPDH | - | - | Bell-Pedersen *et al.* (1996c); Shinohara *et al.* (1998) |
| ccg-8 | CT20 | Unknown | - | - | Bell-Pedersen *et al.* (1996c) |
| ccg-9 | CT19 | Trehalose Synthase | + | + | Bell-Pedersen et al. (1996c); Shinohara et al. (2002) |
| cmt (ccg-12) | CT18 | CuMT | - | - | Bell-Pedersen *et al.* (1996c); Munger *et al.* (1987) |
| al-3d | CT20 | GGPPS | + | + | Arapiaia *et al.* (1995) |
| con-6 | ZT20 | Unknown | + | + | Lauter and Yonofsky (1993) |
| con-10 | ZT20 | Unknown | + | + | Lauter and Yonofsky (1993) |

[a] The peak in message accumulation varies slightly in different experiments (Bell-Pedersen *et al.;* 1996c) and, with the exception of *al-3, con-6* and *con-10*, was determined from Northern blots of the same rhythmic RNA probed with the indicated *ccg's*. The peak in *al-3* message accumulation was estimated from Northern blots presented in Arapaia *et al*. (1995), whereas *con-6* and *con-10* were shown to peak about 20 h after a light pulse representing zeitgeber time 20 (Lauter and Yanofsky; 1993)

[b] Abbreviations are as follows: GAPDH, glyceraldahyde 3-phosphate dehydrogenase; CuMT, copooer metallothionein; GGPPS, geranylgeranyl pyrophosphate synthase.

[c] Developmental and light regulation of the ccg's. A + indicates increased transcription following developmental induction and light treatment; a - indicates no effect.

[d] Only the longer al-3 transcript has been demonstrated to be rhythmic (Arapaia et al.; 1995)

## Mechanisms of clock-controlled gene expression

A major question in the study of circadian clocks concerns the mechanism by which output gene expression is regulated by the pacemaker. As mentioned above, all gene expression is rhythmic in *Synechococcus,* indicating that there is global regulation of the genome by the circadian clock. Recent studies implicate the four group II sigma factors as part of this global mechanism (Nair *et al.*, 2002). Estimates of the number of rhythmic genes in *Drosophila* are significantly lower (~10%). Yet, rhythmic genes appear to be clustered within the genome. This indicates that regulation of chromatin structure may be a mechanism for coordinating output gene expression in the fruit fly (McDonald and Rosbash, 2001). One simple model for the regulation of output genes involves direct

regulation by components of the known transcription/translation feedback loops. Evidence for this type of clock-controlled gene regulation exists in several systems. In *Drosophila,* the CLK:CYC heterodimer activates transcription of *per* and *tim* by binding to *cis*-elements termed E-boxes within the promoters of these two genes (Hao *et al.*, 1997). Similarly, the mammalian CLK:BMAL1 heterodimer acivates transcription of the *per* and *cry* genes by binding to E-box elements within these promoters (Gekakis *et al.*, 1998). A simple mechanism for regulating output gene expression involves activation of ccg's by interaction of CLK:CYC or CLK:BMAL1 with E-box elements present in the promoters of these genes. This is indeed the case for some outputs in mammals including DBP and vasopressin (Jin *et al.*, 1999; Ripperger *et al.*, 2000) . Recent studies in flies and mammals have used microarrays to examine clock-controlled gene expression in CLK mutant strains. In both of these studies, all *ccg's* showed altered mRNA levels in CLK mutant strains (McDonald and Rosbash, 2001; Oishi *et al.*, 2003). Direct regulation of all *ccg's* by CLK is unlikely, however. E-box elements are not present in the promoters of many *ccg's.* Furthermore, an experiment designed to identify direct targets of CLK only detected nine genes that showed a significant change in transcript levels (McDonald and Rosbash, 2001). Interestingly, many genes that do not cycle were also altered in CLK mutant strains, indicating that CLK may have a role outside the circadian clock.

In *Neurospora,* several genes are both clock-controlled and light regulated (see Table 1). Therefore, it is possible that WC-1 may mediate both

light and clock regulation of these genes. However, promoter analysis of *ccg-2* suggests that this is not the case for this gene. Distinct *cis*-elements within the *ccg-2* promoter are independently responsible for clock and light regulation of the gene (Bell-Pedersen *et al.*, 1996a). Furthermore, the sequence of the clock-element does not contain a WCC binding site.

The presence of multiple oscillators indicates that regulation of many output genes is likely more complicated. Each oscillator within a network of oscillators should have the potential to regulate a subset of clock-controlled genes in an organism. However, this has been difficult to demonstrate experimentally. Most notably, our lack of knowledge regarding the molecular makeup of additional oscillators and the genes that these oscillators control limits experimental design. The best example of this type of multi-oscillator output regulation comes from a recent study in plants. EARLY-PHYTOCHROME-RESPONSIVE1 (EPR1) is rhythmically expressed in *Arabidopsis* (Kuno *et al.*, 2003). Overexpression of this gene results in transcriptional repression at its own promoter, suggesting that EPR1 is a component of an autoregulatory feedback loop. However, overexpression does not affect components of the central clock in *Arabidopsis* placing this gene in a downstream oscillator. ERP1 overexpression alters red light responses and flowering time; importantly, both of these processes are clock regulated in *Arabidopsis.* However, it is unknown if cycling of these processes or any other clock outputs is abolished in ERP1 overexpression strains. Certainly, a better of understanding of the molecular

basis of additional oscillators and identification of genes controlled by additional oscillators will provide insight regarding the regulation of output processes.

**Objectives**

The study of circadian rhythms in *Neurospora crassa* and other organisms has provided an understanding of circadian pacemakers and how these pacemakers are reset by light. However, much work is needed to provide a complete understanding of how these pacemakers regulate overt rhythms. The work presented in this dissertation provides a better understanding of how time information is relayed from the pacemaker to the output processes that it controls. We have tested the following hypotheses in order to address this question:

1. All clock-controlled gene expression in *N. crassa* is morning specific.
   To test this hypothesis, we examined the transcript levels of ~1300 *N. crassa* genes in both wild type and the long period $frq^7$ strain for 1 and ½ days in constant darkness. We identified 150 clock-controlled genes in this study. In contrast to the hypothesis, we identified clock-controlled genes that peaked at all phases of the circadian cycle. We also identified genes that display a rhythm in mRNA accumulation in the FRQ-null strain, indicating that these genes are regulated by an additional oscillator.

2. An increase in WC-1 protein levels will result in the induction of both light-regulated and clock-controlled gene expression in *N. crassa.*

In order to test this hypothesis we have profiled transcript levels of ~1300 *N. crassa* genes using two different experimental treatments. We first exposed *N. crassa* cultures to light in order to identify genes that were light-regulated in the fungus. We then used an inducible promoter to increase the levels of WC-1 in the dark. In contrast to our hypothesis, overexpression of WC-1 activates clock-controlled gene expression but is not sufficient to induce all light-regulated genes. These results demonstrate that additional modification of WC-1 is required for light regulation of genes in *N. crassa.* Furthermore, these results demonstrate that the oscillations in WC-1 protein levels provide a mechanism to regulate clock-controlled gene expression in *N. crassa.*

3. Output from the *N. crassa* circadian system originates from a network of coupled oscillators.

To test this hypothesis we examined expression of *clock-controlled gene-16* in various clock mutant strains. *ccg-16* is rhythmic in the *frq*-null strain indicating that it is regulated by a FLO. However, the rhythm in *ccg-16* mRNA accumulation requires the clock gene *white collar-1.* The phase of the *ccg-16* rhythm is altered in the long period $frq^7$ strain, indicating that the two oscillators are coupled. These results suggest that output from

the *N. crassa* circadian system does indeed originate from a network of coupled circadian oscillators. Furthermore, these results place the FRQ/WCC oscillator at the top of a hierarchical network of oscillators.

The results of this research are presented in the following chapters. Chapter II is a published manuscript (Correa *et al.*, 2004). Chapter III is also a published manuscript (Lewis *et al.*, 2002). Chapter IV represents work that will be submitted for publication (Lewis *et al.*, in preparation). Chapter V is a discussion of these results and the significance of these findings to the field of circadian biology. Appendix A is a published manuscript that describes the light adaptation and circadian clock phenotypes of the *vivid* mutation. Appendix B describes work on two bZip transcription factors. At the beginning of each chapter and the appendix, my contribution to each paper is specified.

# CHAPTER II

# MULTIPLE OSCILLATORS REGULATE CIRCADIAN GENE EXPRESSION IN *Neurospora**

## Overview

High-density microarrays were used to profile circadian gene expression in *Neurospora crassa* cultures grown in constant darkness. We identified 145 clock-controlled genes (ccgs). The ccgs peaked in mRNA accumulation at all phases of the day, with the majority peaking in the late night to early morning. The predicted or known functions of the ccgs demonstrate that the clock contributes to a wide range of cellular processes, including cell signaling, development, metabolism, and stress responses. While the period of the rhythm of most of the ccgs was found to be dependent on the well-characterized FRQ-based oscillator, 3 ccgs appeared to have a rhythm that was significantly short in the long period (29-h) *frq*$^{7}$ mutant strain. These ccgs accumulate mRNA rhythmically with a circadian period in a *frq*-null strain, confirming the existence of a second oscillator in *N. crassa*.

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**Introduction**

Circadian rhythms are endogenous, self-sustaining oscillations that are regulated by a central pacemaker composed of one or more biochemical oscillators (Edmunds, 1988; Young and Kay, 2001). These rhythms are observed in a wide variety of organisms, ranging from daily rhythms in photosynthesis in cyanobacteria and plants, to activity and sleep-wake cycles in rodents and humans. An important aspect of rhythmicity involves control of specific target genes by the oscillators; however, this remains one of the least understood areas in chronobiology.

In *N. crassa,* the well-characterized FRQ-based oscillator consists of an interlocked autoregulatory molecular feedback loop containing positive PAS domain-containing elements (WC-1 and WC-2) and a negative element (FRQ) which depresses the activity of the positive elements (Loros and Dunlap, 2001). WC-1 is a blue-light photoreceptor that links the circadian oscillator to the external environment (Froehlich *et al.*, 2002; He *et al.*, 2002). Using subtractive hybridization and differential screens of time-of-day-specific libraries, eight clock-controlled genes (ccgs) have been identified in *N. crassa* (Loros and Dunlap, 2001). All of the ccgs peak in expression in the late night/early morning, and the associated proteins function in intermediary metabolism, stress responses and development. Mutation of the *frq* gene affects the rhythmic expression of all the known ccgs, suggesting that they are controlled by the FRQ-based oscillator. However, circumstantial evidence suggests that the *N. crassa* clock is built using

more than one oscillator (Iwasaki and Dunlap, 2000; Lakin-Thomas and Brody, 2000; Merrow *et al.*, 1999) and these other FRQ-less oscillators (FLOs) may participate in the regulation of ccgs.

In this study, we used microarray technology to search for ccgs in *N. crassa*. Our data reveal the importance of the clock in the life of the fungus and provide molecular evidence for the existence of the FLO.

## Materials and methods

### *Strains and growth conditions*

The *frq*$^{+}$ strain 87-3 (*bd; a*), the long-period mutant strain 585-70 (*bd; frq7; a*), and the *frq*$^{-}$ strain (*bd; frq*$^{10}$*; a)* were obtained from Jay Dunlap (Dartmouth Medical School, Hanover, NH). The *frq*$^{10}$ mutation was produced by gene replacement resulting in a null allele. The *band* mutation (*bd*) enhances the circadian rhythm of conidiation (Sargent *et al.*, 1966) but does not affect the clock itself. Growth media (Vogel's and Fries minimal media), vegetative growth conditions and crossing protocols are described (Davis and deSerres, 1970).

### *Culture harvesting conditions*

For rhythmic RNA analyses, the clock was synchronized by a light-to-dark transition in mycelial mats grown in shaking (100 rpm) liquid culture (Fries minimal media containing 0.03% glucose and 0.05% arginine) at 25°C (Bell-Pedersen *et al.*, 1996b; Loros *et al.*, 1989). The light-to-dark transfer sets the clock to circadian time (CT) 12. Light-to-dark transfer times were such that the

ages of the cultures at harvest were approximately the same, but the circadian times varied (Bell-Pedersen *et al.*, 1996b). Specifically, the mycelia were grown in liquid shaking cultures in the light on day 1 and transferred to the dark on day 1 (for collection at DD (Hunter-Ensor *et al.*) 36, 38, 44, 51) or day 2 (for collection at DD 12, 16, 22, 28, 29, 32) and harvested either at 9 a.m. (DD 12, 16, 22, 36, 38, 44) or 5 p.m. (DD 28, 32, 29, 51) on day 3. Tissue for RNA extraction was harvested after the indicated times in the dark for each experiment.

*Nucleic acid isolation, hybridization and sequencing*

Total RNA isolation and northern blotting were as described (Bell-Pedersen *et al.*, 1996b; Schwerdtfeger and Linden, 2001). Riboprobes were generated by *in vitro* transcription (Maxiscript, Ambion, Austin, TX) in the presence of [α32-P]-UTP (3000 Ci mmol -1, PerkinElmer, Boston, MA) from pLW1K for *ccg-2* (Bell-Pedersen *et al.*, 1996a). DNA probes for clones identified in microarray experiments were generated by random primed DNA synthesis (DECAprime, Ambion, Austin, TX) in the presence of [α32-P]-dCTP (3000 Ci mmol -1, PerkinElmer, Boston, MA), using the appropriate plasmid as a template. The EST clones which corresponded to the ccgs (Supplementary Table 1) were sequenced using the Prism dideoxy sequencing kit (Applied Biosystems) to confirm their identity.

*Microarray construction and hybridization*

Expressed Sequence Tag (EST) libraries corresponding to three stages of the *N. crassa* life cycle (conidial, mycelial and sexual) were generously provided

by the Neurospora genome project at the University of New Mexico (Nelson *et al.*, 1997) (http://www.unm.edu/~ngp/). From this collection, a set of 1343 unique genes was used to construct cDNA microarrays as described (Lewis *et al.*, 2002). Together, the ESTs and negative control spots yielded 1778 probe spots per array. Using the 3DNA Submicro EX Expression array detection kit (Genisphere, Newark, NJ), 5-7 µg of total RNA was reverse transcribed, labeled with Cy3 and/or Cy5 and hybridized to the microarrays according to the manufacturer's instructions. Following hybridization at 62°C for 24 h, the slides were washed and scanned using a Scanarray 5000 scanner (GSI Luminomics, Ottowa, Ontario).

*Data analysis*

Fluorescence data from each microarray was collected using Scanalyze 2.44 (written by Michael Eisen and available online at http://rana.lbl.gov/EisenSoftware.htm). Data normalization and qualitative clustering were performed using GeneSpring software (Silicon Genetics, Redwood City, CA). Values for phase were determined by fitting a cosine function to the normalized microarray data using nonlinear regression with the Prism software package (GraphPad Software, San Deigo, CA). Initially, the background fluorescence from the negative controls was subtracted from the experimental spots. Each spot was divided by the control channel data (Cy5 generated data). The value for each spot was then divided by the 50th percentile of all measurements for that slide to account for differences in overall intensity

between slides. To permit comparisons of the genes on the same scale, each gene was normalized to itself. The average normalized ratio for each gene at each time-point was divided by the median value for that gene. This results in at least one time point value being equal to 1. We imposed a minimum raw intensity value to eliminate DNA spots with low signal intensities from further analysis. These minimum intensity values were chosen to be higher than values observed for the average of negative control spots; the higher the background signal, the greater the minimum signal intensity required. The raw data is available at http://plpa2linux.tamu.edu/Microarray.html.

## Results

### *Circadian clock regulation of mRNA abundance*

To investigate regulation of gene expression by the circadian clock on a global scale in *N. crassa*, cDNA microarrays were generated from Expressed Sequence Tag (EST) libraries corresponding to the major stages of the *N. crassa* life cycle (conidial, mycelial and sexual) (Nelson *et al.*, 1997). The libraries are predicted to represent a comprehensive view of expressed *N. crassa* genes; however, rare transcripts are likely underrepresented in the libraries. The microarrays contained 1778 probe spots representing 1343 unique genes (ca. 14% of the total number of *N. crassa* genes (Galagan *et al.*, 2003)); 401 of these were represented by more than one EST. These repeated

sequences provided one level of control for the reproducibility of the microarray hybridizations.

The microarrays were used to determine the levels of *N. crassa* mRNAs over 1.5 consecutive days. Longer cycle times were not used because in liquid cultures the amplitude of the rhythm begins to damp after 1.5 cycles (Loros *et al.*, 1989). To verify that a given gene was expressed rhythmically, we examined gene expression in both a $frq^{+}$ strain (87-3) that has a period of 22 h and a clock mutant strain, $frq^{7}$ (585-70) that has a period of 29 h in constant darkness at 25°C. The use of these two strains aids in establishing circadian regulation of transcript abundance (Bell-Pedersen *et al.*, 1996b; Dunlap, 1990; Loros *et al.*, 1989); the levels of authentic clock controlled transcripts would cycle in both strains, but due to the difference in circadian period lengths the same mRNA would be expected to cycle at different rates. The microarrays were hybridized with Cy3-labeled probes derived from RNA isolated from cultures kept in constant darkness and harvested at subjective dawn (Circadian Time [CT] 0), noon (CT6), dusk (CT12), midnight (CT18), and CT 0 and CT6 of the next subjective day. CT is used to normalize biological time in strains with different endogenous period lengths to 24 circadian h per cycle. Thus, the two strains were in the dark for different lengths of time to achieve the same CT at the time of harvest (see Materials and Methods) (Loros *et al.*, 1989). The quality of the RNA used to generate the microarray probes and the circadian properties of the cultures were confirmed by northern blots using the previously characterized late

night- to morning-specific clock-controlled *ccg-2* gene as a probe (Figure 5a) (Bell-Pedersen *et al.*, 1996a; Loros *et al.*, 1989). For each time point, the arrays were co-hybridized with a reference sample (CT12) labeled with Cy5. Each slide contains 2 copies of the array and 2 different slides were probed for each time point. Thus, 4 spots representing each gene were analyzed for each time point. The overall cross correlation coefficient between replicate samples in both $frq^{+}$ and $frq^{7}$ strains was ≥ 0.89, indicating a high degree of reproducibility between experiments (see figure on page 49). Following the application of minimum intensity requirements (Lewis *et al.*, 2002), 970 of the 1778 probe sets from $frq^{+}$ and 1090 of the 1778 probe sets from $frq^{7}$ were considered reliable. 760 probe sets passed the restrictions for both strains and were further analyzed. Our filtering parameters excluded genes that were poorly expressed under our growth conditions; thus, it is likely that some genes may be scored circadian by independent methods. For example, *frq* mRNA accumulates rhythmically (Aronson *et al.*, 1994b). While there is sufficient *frq* mRNA for the clock to function normally in our growth conditions (Loros *et al.*, 1989), *frq* mRNA is expressed at low levels and therefore, did not pass our selection criteria.

To determine with confidence which of the 760 genes were expressed under control of the circadian clock, we imposed 3 requirements. First, the expression profile had to have at least one peak in both $frq^{+}$ and $frq^{7}$. Second,

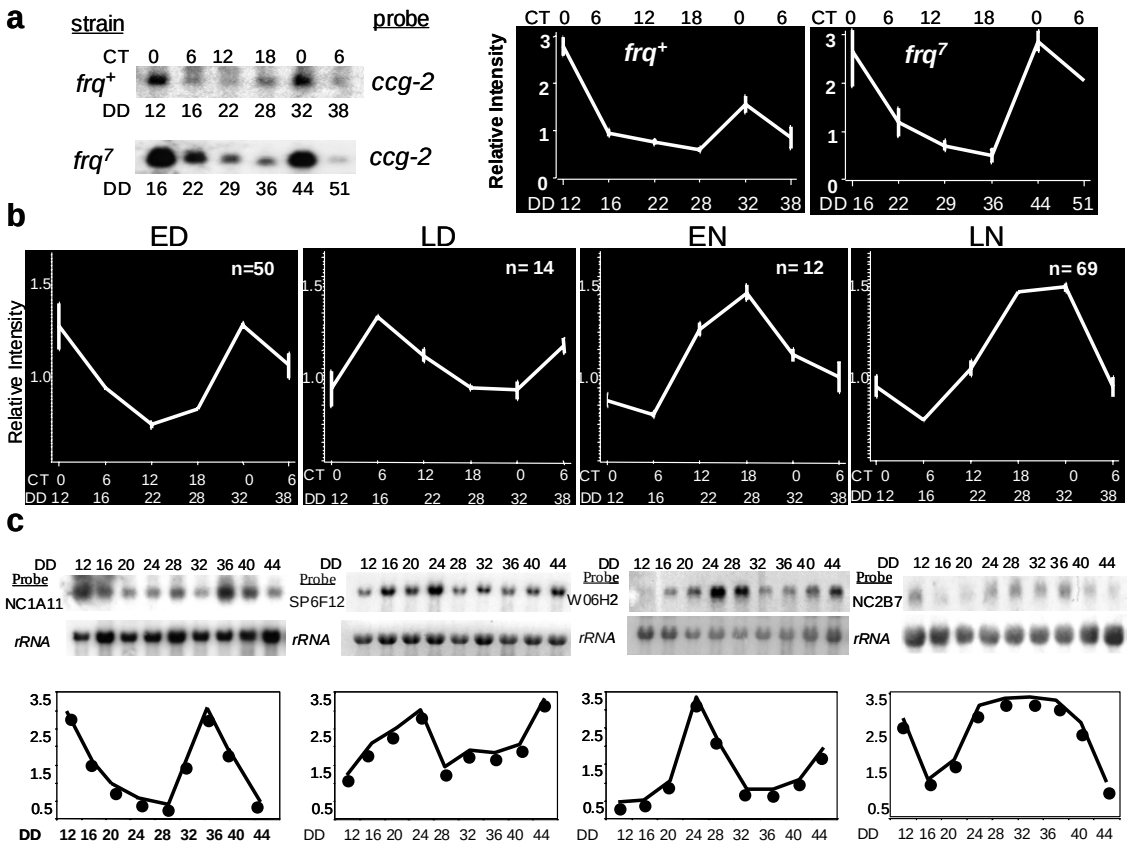


**Figure 5. Microarray analysis of clock-controlled gene expression.**

a. Total RNA was harvested from dark grown clock *frq*$^{+}$ and *frq*$^{7}$ cultures after the indicated times in the dark (DD) and probed with *ccg-2* to verify rhythmicity of the cultures. The corresponding circadian times (CT) are designated at the top. The microarray tracings of *ccg-2* are shown on the right for both strains. The values (means ± SEMs; n=4) for each time point are shown. DD (top) and CT (bottom) times are indicated. b. Circadian clock regulated genes were grouped into 4 classes based on their peak time of mRNA accumulation in *frq*$^{+}$ cells. The values (means ± SEMs) for each time point represent the average of all genes (n) in that class. DD and CT times are indicated below the tracings. c. Total RNA was isolated from cultures after the indicated times in the dark and northern blots were probed with the designated EST clone. rRNA was used as a loading control for each experiment. Relative mRNA levels are plotted as relative band intensity versus time (below).

when two peaks were observed (note that genes which peak in the night have only one peak [Figure 5b]) a period between 18 and 30 hours in *frq*$^{+}$ was required. Third, we required non-overlapping standard errors between the highest and the lowest of the six time points. Using these criteria, 145 genes were classified as being clock-controlled, representing ~19% of the 760 genes that passed our restrictions (Tables 2 and 3). A minimum 1.5-fold increase from the trough to the peak was observed for all selected genes and several of these were independently verified by northern assays (Table 2). Genes previously identified as clock-controlled genes, including *ccg-1*, *ccg-2*, *ccg*-6, *ccg-7* and, *ccg-12* (Bell-Pedersen *et al.*, 1996b; Loros *et al.*, 1989) were scored as rhythmic in the appropriate phase in our data set, further validating our selection criteria (Table 2).

The rhythmically-expressed genes were organized into clusters by peak expression time (Table 3). All possible phases of peak gene expression were represented in the clusters (Fig. 5b). For convenience, each gene was classified as early subjective day (ED), late subjective day (LD), early subjective night (EN) or late subjective night (LN), representing genes whose expression peaked between DD12-14 and DD31-36 (CT22 to 3.99), DD15-19 (CT4 to 9.99), DD20-26 (CT10 to 15.99) or DD27-31 (CT16 to 21.99), respectively, in *frq*$^{+}$ cells. In those cases where ccgs fell into a different class in the *frq*$^{7}$ strain (Table 3), the *frq*$^{+}$ classification was used. Class ED and LN genes are the most highly represented and contain all of the previously characterized ccgs. No genes were

**Table 2. *N. crassa* clock-controlled genes revealed by microarray analysis.**

| EST[1] | ORF[2] | BLAST Match[3] | Organism[4] | Acc. #[5] | ACE[6] | WC-1 BS[7] | Verified by Northern[8] |
|---|---|---|---|---|---|---|---|
| Early Subjective Day Class (ED) | | | | | | | |
| NM7H10 | NCU00061.1 | Beta-glucanase precursor | B. amyloliquefaciens | 121774 | | | |
| W01D7 | NCU00432.1 | Cell wall surface anchor family protein | S. pneumoniae | 15901602 | 1 | | |
| W01E10 | NCU00461.1 | Glutamate dehydrogenase NAD-specific | N. crassa | 11252743 | | | |
| NM1E4 | NCU00477.1 | Serine carboxypeptidase | A. nidulans | 14211580 | | | |
| NM4G6 | NCU00695.1 | Fasciclin domain protein | | PF02469 | | | |
| NP2C1 | NCU00859.1 | Tenascin-X | M. musculus | 2804289 | | | |
| NC1A11 | NCU01317.1 | Probable ribsomal protein L11 | N. crassa | 12718408 | | 1 | + |
| NM6B8 | NCU01517.1 | Glucan 1,4-alpha-glucosidase | N. crassa | 11263739 | | | |
| SM2D7 | NCU01569.1 | WD-40 repeat protein | S. pombe | 1175408 | | | |
| SP1F10 | NCU01613.1 | Related to SHK1 Kinase-binding protein | N. crassa | 11281600 | | | |
| SM2D6 | NCU01754.1 | Alcohol dehydrogenase | N. crassa | 14285342 | | | |
| NM5A10 | NCU01757.1 | Asparaginase related protein | N. crassa | 11359369 | | | |
| NC1H7 | NCU01837.1 | No BLAST Hits | | | | | |
| SP1C3 | NCU01854.1 | Similarity to developmental protein | S. pombe | 19113354 | | | |
| SM2G8 | NCU02116.1 | ymaE | B. subtilis | 16078788 | | | |
| SP1D5 | NCU02470.1 | Fruiting body protein SC7 precursor | S. commune | 548902 | | 1 | + |
| W06E1 | NCU02549.1 | Processing enhancing protein | N. crassa | 168857 | | 1 | |
| NM6E7 | NCU02745.1 | No BLAST Hits | | | | | |

**Table 2 (continued)**

| EST[1] | ORF[2] | BLAST Match[3] | Organism[4] | Acc. #[5] | ACE[6] | WC-1 BS[7] | Verified by Northern[8] |
|---|---|---|---|---|---|---|---|
| W13E1 | NCU02948.1 | Minor allergen ALT A 7 | A. alternata | 1168402 | | 1 | |
| W07E6 | NCU03530.1 | Related to chitinase 3 precursor protein | N. crassa | 11359623 | | | |
| NP5G7 | NCU03753.1 | CCG-1 | N. crassa | 3013 | | | + |
| W01B12 | NCU04387.1 | Vacuolar ATP synthase subunit F | N. crassa | 4426614 | | 1 | |
| SP1D9 | NCU04816.1 | No BLAST Hits | N. crassa | | | | |
| NC1E1 | NCU05429.1 | Probable branching enzyme | N. crassa | 16416077 | | | + |
| NP6H2 | NCU06660.1 | Uncharacterized protein family | | PF01679 | 3 | | + |
| W13B8 | NCU06737.1 | No BLAST Hits | | | | | |
| W15D5 | NCU06910.1/ NCU06432.1 | WSC domain protein/Ribosomal protein L7Ae | | PF01822 | | | |
| NP6G4 | NCU06977.1 | No BLAST Hits | | | 1 | | |
| SM4C4 | NCU07267.1 | BLI-3 | N. crassa | 602074 | | 2 | |
| W07F11 | NCU08409.1 | Tryptophan synthetase (trp3) alpha-2 subunit gene | N. crassa | 168915 | | 1 | |
| SC2A2 | NCU08457.1 | CCG-2 | N. crassa | 2983 | ACE | | + |
| NC1G1 | NCU08500.1 | Probable ribosomal protein Rps8bp | N. crassa | 11276556 | | | |
| SP1D8 | NCU08713.1 | Hypothetical protein B24M22.120 | N. crassa | 11359491 | | | |
| NM2E10 | NCU08791.1 | Catalase I | N. crassa | 13272202 | | | |
| W08H11 | NCU08949.1 | Hypothetical protein SPAC15E1.02c | S.pombe | 7490820 | | | |
| NM1B8 | NCU08950.1 | No BLAST Hits | | | | | |
| SM2B10 | NCU09040.1 | NADP dependent oxidoreductase | S. pombe | 13624910 | | 1 | |
| W10D10 | NCU09041.1 | Related to Sorbitol utilization protein SOU1 | N. crassa | 11595622 | | | |
| NM5G10 | NCU09049.1 | No BLAST Hits | | | | | |
| NC1C9 | NCU09109.1 | Probable ribosomal protein L35Ae | S. cerevisiae | 3045587 | | | |
| SC1E10 | NCU09142.1 | Dynein, cytoplasmic, intermediate chain 2 | M. musculus | 6753658 | | | |
| NM1C3 | NCU09152.1 | No BLAST Hits | | | | | |
| W06B10 | NCU09345.1 | Thiamine biosynthesis protein NMT-1 | N. crassa | 15822512 | | | |
| NC5E9 | NCU09802.1 | No BLAST Hits | | | | | |
| SM3H9 | NCU09873.1 | Phosphoenolpyruvate carboxykinase | A. nidulans | 15983748 | | | |

**Table 2 (continued)**

| EST[1] | ORF[2] | BLAST Match[3] | Organism[4] | Acc. #[5] | ACE[6] | WC-1 BS[7] | Verified by Northern[8] |
|---|---|---|---|---|---|---|---|
| NM7C5 | NCU09929.1 | No BLAST Hits | | | | | + |
| NC2F3 | None | No BLAST Hits | | | | | |
| NM2A8 | None | No BLAST Hits | | | | | |
| SP6H10 | None | No BLAST Hits | | | | | |
| NM3A8 | None | No BLAST Hits | | | | | |
| Late Subjective Day Class (LD) | | | | | | | |
| SP7C6 | NCU00685.1 | Casein kinase I | S. pombe | 729712 | | | |
| NM2B6 | NCU03779.1 | Probable Glyerone Kinase Isoform | N. crassa | 513466 | | 2 | |
| NM7G11 | NCU01207.1 | Vacuolar ATP Synthase Catalytic Subunit A | | | | | |
| NC4B12 | NCU01328.1 | Probable Transketolase | N. crassa | 11595598 | | | + |
| NP6A11 | NCU01504.1 | Nebula related protein | N. crassa | 11289939 | | | |
| NM3F4 | NCU02481.1 | Isocitrate lyase | B. fuckeliana | 3282211 | | | |
| SP6D5 | NCU03193.1 | Hypothetical protein | S. cerevisiea | 6325320 | | | + |
| W06E11 | NCU03396.1 | Probable Nucleolar protein NOP58 | N. crassa | 18376368 | | | |
| W01D5 | NCU04050.1 | CPC-1 | N. crassa | 11115 | | | + |
| NM2B8 | NCU04384.1 | No BLAST Hits | | | | | |
| NC5G11 | NCU04856.1 | Glutamine synthetase | C. gloeosporioides | 2494743 | | | + |
| W07G9 | NCU05969.1 | Related to cel1 protein precursor | N. crassa | 16945376 | | | |
| SC3A6 | NCU06031.1 | Rehydrin protein | C. albicans | 7493810 | | | |
| SP6F12 | NCU10042.1 | Enolase | A. fumigatus | 13925872 | | | + |
| Early Subjective Night Class (EN) | | | | | | | |
| SM1H10 | NCU03501.1 | Hypothetical Protein | Nostoc sp. PCC7120 | 3588 | | 1 | + |
| NM3E11 | NCU02932.1 | Hypothetical protein | S. pombe | 19114179 | | | |
| NC3B3 | NCU03241.1 | FK506-Binding protein 4 | S. cerevisiae | 6323482 | | | |
| NM6A4 | NCU04931.1 | Integral membrane protein | B. graminis | 18033120 | | | |
| W06H2 | NCU05495.1 | No BLAST Hits | | | | | + |
| NM6E2 | NCU07027.1 | Glycogen phosphorylase | S. cerevisiae | 4173 | | | |
| SC2A10 | NCU07318.1 | Mannitol-1-phosphate dehydrogenase (enzyme III) | B. halodurans | 15616413 | | | |
| NM7A9 | NCU07491.1 | No BLAST Hits | | | | | |
| NM5G3 | NCU08847.1 | NF-180 | P. marinus | 2133786 | | | |
| SP4D11 | NCU09057.1 | Late embryogenesis abundant protein-like | A. thaliana | 15241469 | | | |
| W17H1 | NCU09698.1 | High-affinity nicotinic acid transporter | S. cerevisiae | 6321698 | | | |
| SP1B4 | NCU05800.1 | Poly Zinc-Finger Protein 2 | T. cruzi | 204398 | | | |

**Table 2 (continued)**

| EST[1] | ORF[2] | BLAST Match[3] | Organism[4] | Acc. #[5] | ACE[6] | WC-1 BS[7] | Verified by Northern[8] |
|---|---|---|---|---|---|---|---|
| Late Subjective Night Class (LN) | | | | | | | |
| NC2A11 | NCU00043.1 | Serine/threonine protein phosphatase type 1 | N. crassa | 2944404 | | | + |
| SM2A10 | NCU00315.1 | 60S Ribosomal protein L29 | S. frugiperda | 16566716 | 2 | | |
| W08D10 | NCU00464.1 | 60S Ribosomal protein L32 | T. harzianum | 464633 | 1 | 1 | |
| W08E5 | NCU00576.1 | Separation anxiety protein - like | A. thaliana | 15239024 | | | |
| W10A1 | NCU00634.1 | 60S Ribosomal protein L14 | S. pombe | 15214255 | 1 | | |
| W09F2 | NCU00726.1 | Cyclophilin A | N. c rassa | 2998 | 1 | | |
| W06G11 | NCU00979.1 | 60S Acidic ribosomal protein P2 | A. alternata | 1173071 | 3 | | |
| NM7B6 | NCU01162.1 | Glucanosyltransferase | C. immitis | 15824725 | | | |
| NC5G12 | NCU01418.1 | CCG-6 | N. crassa | 3746898 | 1 | | |
| W08B11 | NCU01528.1 | CCG-7 (GAPDH) | N. crassa | 1532188 | 1 | | + |
| NP2H10 | NCU01552.1 | 40S Ribosomal protein S23 | N. crassa | 14285761 | 1 | | |
| NC5E12 | NCU01776.1 | 60S Ribosomal protein L15 | A. niger | 3122672 | 2 | | |
| SP6F4 | NCU01797.1 | NRC-2 protein kinase | N. crassa | 2654105 | | | + |
| W17E10 | NCU01827.1 | 60S Ribosomal protein L27 | S. pombe | 11276862 | 2 | | |
| NC2C12 | NCU01949.1 | 40S Ribosomal protein S9 | P. anserina | 1710780 | | | |
| NC4G10 | NCU01966.1 | 60S Ribosomal protein L37 | A. nidulans | 15214258 | | | |
| NM7C7 | NCU01967.1 | No BLAST Hits | | | | | |
| SC2H9 | NCU02252.1 | Phosphoglyceromutase | A. oryzae | 9955875 | | | + |
| SM2A8 | NCU02635.1 | Probable role in N-glycosylation | S. pombe | 7492940 | | | |
| NM6E1 | NCU02003.1 | Elongation factor 1-alpha | N. crassa | 961481 | | | |
| W07H9 | NCU02806.1 | 14-3-3 protein homolog | T. harzianum | 2492491 | | | + |
| W09G8 | NCU07914.1 | Phosphoglycerate Kinase | N. crassa | 3051 | 1 | 2 | |
| W17E6 | NCU02509.1 | 60S Ribosomal protein L11 | S. pombe | 1710494 | 1 | | |
| SC3B6 | NCU02514.1 | ATP synthase alpha chain atp-1 | N. crassa | 168758 | | | |
| W13A10 | NCU02708.1 | Ribosomal protein L35 | O. ulmi | 14165210 | 1 | | |
| NC2A10 | NCU02736.1 | Glycine-rich RNA binding protein | A. thaliana | 15229525 | | | |
| SP4E7 | NCU03074.1 | No BLAST Hits | | | | | |
| W08C12 | NCU03359.1 | Intermembrane space AAA protease IAP-1 | N. crassa | 12082994 | | | |
| W17H5 | NCU03593.1 | Homeoprotein | P. anserina | 11322493 | 1 | | + |
| W15D3 | NCU03611.1 | Chitin synthase | N. crassa | 168772 | 2 | | |
| NC2B7 | NCU03757.1 | 60S Ribosomal protein L4.e | S. pombe | 542225 | 1 | | + |
| NC3E9 | NCU03806.1 | 60S Ribosomal protein L28 | N. crassa | 132845 | | | |
| W07B11 | NCU03988.1 | 60S Ribosomal protein L18 | S.pombe | 1710503 | 1 | | |
| NC5D11 | NCU04314.1 | No BLAST Hits | | | | | |
| SM2H10 | NCU04553.1/ | Ubiquitin/ribosomal protein S27a fusion protein | N. crassa | 402241 | 3 | | |

**Table 2 (continued)**

| EST[1] | ORF[2] | BLAST Match[3] | Organism[4] | Acc. #[5] | ACE[6] | WC-1 BS[7] | Verified by Northern[8] |
|---|---|---|---|---|---|---|---|
| NC3E3 | NCU04779.1 | 60SRibosomal protein L8 (L7A) | S. pombe | 6094099 | 2 | | |
| W01A5 | NCU05032.1 | Ubiquitin fusion protein | M. grisea | 3047314 | | | |
| W10H8 | NCU05395.1 | Hypothetical protein | N. crassa | 11291652 | | 1 | |
| NC5E10 | NCU05396.1 | No BLAST Hits | | | | | |
| SP4F6 | NCU05498.1 | No BLAST Hits | | | | | |
| SC4A1 | NCU05561.1 | Copper metallothionein (CCG-12) | N. crassa | 2986 | 1 | | + |
| SM1F12 | NCU05599.1 | 40S Ribosomal protein S28 (S33) | S. pombe | 1710760 | 3 | | + |
| SP4F9 | NCU05768.1 | No BLAST Hits | N. crassa | 3044585 | | | |
| NM3B8 | NCU05804.1 | 60S ribosomal protein L19 | S. pombe | 7490016 | | | |
| NC4D7 | NCU05810.1 | CPC-2 | N. crassa | 971565 | | 1 | |
| W17D1 | NCU05816.1 | Cytochrome C oxidase polypeptide | S. cerevisiae | 117096 | | 1 | |
| NC2D3 | NCU06226.1 | 60S Ribosomal protein L23A | P. graminis | 2507312 | | | |
| SP6G3 | NCU06454.1 | GTP-binding protein | M. grisea | 8132884 | | | |
| NC3E8 | NCU06661.1 | Ribosomal L22e protein family | S. pombe | 11276888 | 2 | | |
| NC5F5 | NCU06892.1 | 40S Ribosomal protein S20 | S. frugiperda | 15213828 | 2 | | |
| W08A8 | NCU07024.1 | Osmotic sensitive-2 | N. crassa | 15077323 | | 2 | |
| W08B2 | NCU07033.1 | LysM domain | | PF01476 | 1 | 1 | |
| NC3G11 | NCU07182.1 | 40S Ribosomal protein S24-B | S. pombe | 6093881 | 1 | | |
| NM7A1 | NCU07776.1 | Conserved hypothetical protein | N. crassa | 12718341 | | | |
| W08C5 | NCU08017.1 | No BLAST Hits | | | | | |
| SP4F8 | NCU08093.1 | Vacuolar-type ATPase | S. pombe | 19114095 | 1 | | |
| SM2H2 | NCU08130.1 | No BLAST Hits | | | | | |
| NC1F8 | NCU08332.1 | Probable vacuolar-ATPase, 20K chain | N. crassa | 11359600 | | | + |
| W17G8 | NCU08377.1 | Adenylate cyclase | N. crassa | 399319 | | | |
| W08B8 | NCU08620.1 | 40S Ribosomal protein S16 | C. albicans | 10720257 | 1 | | + |
| NP4F8 | NCU08936.1 | Sporulation-specific protein 2 precursor | S. cerevisiae | 172672 | | | |
| NC4G3 | NCU08963.1 | 60S ribosomal protein L30 | S. pombe | 7490032 | 1 | | |
| SC5B8 | NCU09089.1 | 40S Ribosomal protein S30 | S. frugiperda | 15213844 | 1 | | |
| W01C6 | NCU09285.1 | Alcohol dehydrogenase | S. pombe | 7490087 | | | |
| W13B7 | NCU09711.1 | Vacuole import and degradation | S. cerevisiae | 6324117 | | | not detected |
| W17H12 | None | Putative senescence-associated protein | P. sativum | 13359451 | | | |
| W10C3 | None | Cytochrome 450 monooxygenase | Z. mays | 7489812 | | | |
| W13F6 | None | No BLAST Hits | | | | | |

**Table 2 (continued)**

| EST[1] | ORF[2] | BLAST Match[3] | Organism[4] | Acc. #[5] | ACE[6] | WC-1 BS[7] | Verified by Northern[8] |
|---|---|---|---|---|---|---|---|
| NP3A8 | None | No BLAST Hits | | | | | |

[1] The N. crassa ESTs identified in each class are from the Neurospora Genome Project, University of New Mexico (http://www.unm.edu/~ngp/).
[2] The predicted ORFs are from the Whitehead Institute Neurospora Sequencing Project (http://www-genome.wi.mit.edu/annotation/fungi/neurospora/).
[3] The BLAST matches represent the most significant match in the databases as predicted by the Whitehead Institute (minimum e-value 1e-5) and verified by us using BLAST searches of the NCBI sequence databases.
[4] The organism with the closest BLAST match is indicated
[5] The accession numbers for each BLAST match are from the NCBI sequence database (http://www.ncbi.nlm.nih.gov/).
[6] Number of times that the 8-nt core ACE-like element (TCTTGGCA; P value = 4.3 e-21) is present in the upstream region (1 kb) of the different ORFs.
[7] Number of times that the WC-1 binding site is present in the upstream region (1 kb) of the different ORFs.
[8] Rhythmicity was observed for the indicated genes in the *frq*$^+$ strain.

known in *N. crassa* to cycle with a peak time in the EN. Therefore, to further verify the microarray data we chose candidates from each class and performed northern blot analysis using RNA isolated every 4 h over 2 days from *frq*$^+$ cells. Four representative northern blots are shown in Fig. 5c. Each of the chosen representatives provided a good test of gene discovery using the microarrays, since none of the chosen genes would likely have been predicted to be rhythmically expressed by other means. In all cases, we observed that the array profile closely matched the northern data; however, the amplitudes were typically lower in the array data. In all, we have examined 27 of the candidate genes by

**Table 3. Phase classification of *N. crassa* clock-controlled genes.**

| | Fold Change[1] | | Class[2] | | | Phase[3] | | Normalized Values[4] | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| EST | *frq* $^{+}$ | *frq* $^{+}$ | *frq* $^{+}$ | *frq* $^{7}$ | | *frq* $^{+}$ | *frq* $^{7}$ | *frq* $^{+}$ | | | | | | *frq* $^{7}$ | | | | | |
| | | | | | | | | CT0 | CT6 | CT12 | CT18 | CT0 | CT6 | CT0 | CT6 | CT12 | C18 | CT0 | CT6 |
| NM7H10 | 2.0 | 2.0 | ED | ED | | -1.4 | 0.7 | 1.3 | 0.8 | 0.9 | 1.0 | 1.5 | 0.8 | 1.7 | 1.3 | 1.1 | 0.8 | 1.5 | 0.9 |
| W01D7 | 2.0 | 2.0 | ED | ED | | -0.8 | 1.2 | 1.1 | 0.6 | 0.9 | 1.0 | 1.2 | 1.2 | 1.2 | 0.9 | 0.9 | 0.7 | 1.6 | 1.2 |
| W01E10 | 2.0 | 3.0 | ED | ED | | 1.4 | 1.8 | 1.1 | 0.9 | 0.7 | 0.9 | 1.2 | 1.2 | 1.9 | 1.6 | 0.9 | 0.6 | 1.5 | 0.9 |
| NM1E4 | 2.5 | 2.5 | ED | ED | | -1.0 | 3.4 | 1.1 | 0.8 | 0.6 | 1.0 | 1.5 | 1.0 | 1.7 | 1.5 | 1.0 | 0.7 | 1.0 | 1.5 |
| NM4G6 | 2.0 | 2.5 | ED | ED | | -1.2 | 2.3 | 1.5 | 0.9 | 1.0 | 0.8 | 1.1 | 0.9 | 1.4 | 1.3 | 0.9 | 0.6 | 1.5 | 1.0 |
| NP2C1 | 2.5 | 3.0 | ED | ED | | -1.8 | 3.6 | 1.1 | 0.8 | 0.8 | 1.2 | 2.0 | 1.0 | 1.8 | 1.8 | 0.9 | 0.8 | 0.6 | 1.0 |
| NC1A11 | 3.0 | 6.0 | ED | ED | | 0.7 | 1.3 | 1.7 | 0.9 | 0.8 | 0.6 | 1.1 | 1.0 | 3.1 | 1.4 | 0.8 | 0.6 | 3.7 | 2.7 |
| NM6B8 | 2.0 | 4.0 | ED | ED | | 2.2 | 3.5 | 1.6 | 1.4 | 0.8 | 0.9 | 1.3 | 1.3 | 1.6 | 1.3 | 0.9 | 0.4 | 1.2 | 1.3 |
| SM2D7 | 2.5 | 4.0 | ED | LD | | 1.3 | 4.9 | 1.3 | 1.1 | 0.5 | 0.8 | 1.2 | 1.0 | 1.6 | 1.9 | 0.8 | 0.5 | 1.1 | 2.0 |
| SP1F10 | 1.5 | 3.0 | ED | ED | | 3.4 | 3.8 | 1.2 | 1.2 | 0.8 | 0.8 | 1.0 | 1.1 | 1.3 | 1.4 | 0.6 | 0.9 | 1.2 | 1.8 |
| SM2D6 | 3.0 | 8.5 | ED | ED | | 3.7 | 3.2 | 1.1 | 1.0 | 0.6 | 0.6 | 1.0 | 1.7 | 2.0 | 2.0 | 0.6 | 0.3 | 1.4 | 2.5 |
| NM5A10 | 2.0 | 2.5 | ED | ED | | -2.0 | 2.8 | 1.4 | 0.7 | 0.8 | 1.0 | 1.4 | 0.8 | 1.3 | 1.2 | 0.8 | 0.7 | 1.7 | 1.6 |
| NC1H7 | 3.0 | 3.5 | ED | LD | | 1.0 | 5.1 | 1.5 | 1.2 | 0.5 | 0.5 | 1.1 | 0.6 | 1.8 | 1.3 | 1.0 | 0.5 | 0.7 | 1.8 |
| SP1C3 | 1.5 | 2.0 | ED | ED | | 2.1 | -1.9 | 1.1 | 1.1 | 0.7 | 0.9 | 1.0 | 1.0 | 1.1 | 0.9 | 1.0 | 0.9 | 1.6 | 1.0 |
| SM2G8 | 2.0 | 2.5 | ED | ED | | -1.2 | 4.0 | 0.8 | 1.0 | 0.8 | 1.2 | 1.4 | 1.2 | 1.6 | 1.9 | 0.8 | 0.8 | 1.1 | 1.2 |
| SP1D5 | 2.5 | 5.5 | ED | LD | | -2.0 | 4.2 | 1.1 | 0.7 | 0.6 | 1.0 | 1.5 | 0.8 | 2.0 | 1.8 | 0.8 | 0.4 | 1.3 | 2.2 |
| W06E1 | 1.5 | 1.5 | ED | ED | | 0.8 | 0.9 | 1.2 | 1.2 | 0.9 | 0.9 | 1.2 | 0.8 | 1.2 | 1.2 | 0.8 | 0.9 | 1.3 | 0.9 |
| NM6E7 | 1.5 | 2.0 | ED | LD | | 0.8 | 5.5 | 1.2 | 1.0 | 0.8 | 1.0 | 1.1 | 1.1 | 1.1 | 1.2 | 1.0 | 0.6 | 0.9 | 1.0 |
| W13E1 | 2.0 | 3.5 | ED | ED | | -2.0 | 2.8 | 1.4 | 0.7 | 0.7 | 0.9 | 1.1 | 0.8 | 1.7 | 1.4 | 0.7 | 0.5 | 1.2 | 1.2 |
| W07E6 | 2.0 | 3.5 | ED | ED | | 3.3 | 1.5 | 1.3 | 1.3 | 0.7 | 0.8 | 1.1 | 1.3 | 1.8 | 1.1 | 0.8 | 0.5 | 1.2 | 1.0 |
| NP5G7 | 2.0 | 4.0 | ED | ED | | 2.4 | 4.0 | 1.3 | 1.2 | 0.7 | 0.7 | 1.1 | 1.0 | 1.4 | 1.4 | 0.7 | 0.4 | 1.1 | 1.5 |
| W01B12 | 1.5 | 4.5 | ED | LN | | 3.0 | -7.8 | 1.0 | 1.2 | 0.9 | 0.9 | 1.4 | 1.1 | 0.8 | 1.2 | 1.0 | 2.3 | 0.6 | 0.5 |
| SP1D9 | 1.5 | 2.5 | ED | LD | | -1.9 | 4.8 | 1.1 | 0.8 | 0.9 | 0.9 | 1.3 | 0.9 | 1.2 | 1.2 | 0.8 | 0.5 | 0.8 | 1.2 |
| NC1E1 | 2.0 | 1.5 | ED | # | | -2.0 | # | 0.9 | 1.0 | 0.8 | 1.1 | 1.5 | 0.9 | 0.9 | 1.2 | 0.9 | 0.8 | 1.2 | 1.1 |
| NP6H2 | 2.0 | 2.0 | ED | ED | | -1.7 | 1.8 | 1.3 | 0.6 | 0.9 | 0.9 | 1.3 | 0.9 | 1.3 | 1.2 | 0.7 | 0.7 | 1.5 | 1.1 |
| W13B8 | 1.5 | 3.6 | ED | ED | | -0.3 | -1.8 | 1.1 | 0.9 | 0.8 | 1.1 | 1.3 | 1.2 | 1.0 | 0.7 | 0.8 | 0.9 | 2.5 | 1.1 |
| W15D5 | 1.5 | 2.0 | ED | EN | * | -1.2 | 10.5 | 1.4 | 1.0 | 0.9 | 1.0 | 1.4 | 0.9 | 0.8 | 1.0 | 1.3 | 0.8 | 0.6 | 1.1 |
| NP6G4 | 3.0 | 2.0 | ED | ED | | -1.8 | 1.9 | 1.1 | 0.5 | 0.8 | 1.2 | 1.6 | 1.0 | 1.3 | 1.2 | 0.9 | 0.9 | 1.8 | 1.5 |
| SM4C4 | 2.0 | 2.5 | ED | ED | | -1.3 | 3.4 | 1.3 | 0.7 | 0.7 | 1.2 | 1.4 | 0.9 | 1.3 | 1.2 | 0.8 | 0.6 | 1.3 | 1.4 |
| W07F11 | 2.0 | 3.0 | ED | ED | | -2.0 | -1.7 | 1.0 | 0.9 | 0.8 | 1.0 | 1.5 | 0.9 | 1.0 | 0.8 | 0.8 | 1.0 | 2.3 | 1.2 |
| SC2A2 | 4.0 | 4.5 | ED | ED | | 1.6 | 0.6 | 2.1 | 0.9 | 0.5 | 0.5 | 1.5 | 1.7 | 2.3 | 0.9 | 0.6 | 0.5 | 1.5 | 1.3 |
| NC1G1 | 3.5 | 4.0 | ED | ED | | 0.3 | 2.2 | 1.7 | 1.2 | 0.5 | 0.6 | 1.4 | 0.6 | 1.6 | 1.5 | 0.8 | 0.4 | 1.3 | 0.6 |
| SP1D8 | 2.5 | 5.0 | ED | ED | | -0.8 | 3.1 | 1.2 | 0.8 | 0.6 | 1.1 | 1.4 | 1.1 | 2.0 | 1.9 | 0.7 | 0.4 | 1.4 | 1.3 |
| NM2E10 | 2.0 | 2.5 | ED | LD | | 2.4 | 6.9 | 1.5 | 1.2 | 0.8 | 0.9 | 0.8 | 1.1 | 1.0 | 2.2 | 0.9 | 1.0 | 1.1 | 1.8 |
| W08H11 | 2.0 | 5.5 | ED | ED | | 2.4 | 1.8 | 1.3 | 1.1 | 0.7 | 0.8 | 1.1 | 1.2 | 1.4 | 1.2 | 0.6 | 0.4 | 2.1 | 1.3 |
| NM1B8 | 2.0 | 4.0 | ED | ED | | 0.0 | 2.8 | 1.6 | 0.9 | 0.9 | 0.8 | 1.3 | 1.1 | 1.3 | 1.2 | 0.8 | 0.5 | 1.9 | 1.6 |
| SM2B10 | 2.0 | 2.0 | ED | ED | | -0.4 | 1.2 | 1.6 | 1.0 | 0.9 | 0.9 | 1.0 | 1.1 | 1.5 | 1.0 | 1.0 | 0.9 | 1.3 | 1.3 |
| W10D10 | 2.0 | 2.0 | ED | ED | | 0.0 | 2.7 | 1.6 | 1.0 | 1.0 | 0.9 | 0.8 | 1.1 | 1.1 | 1.1 | 0.9 | 0.8 | 1.7 | 1.5 |
| NM5G10 | 2.0 | 3.0 | ED | ED | | -1.5 | 3.8 | 1.6 | 0.9 | 0.9 | 0.9 | 1.4 | 0.9 | 1.5 | 1.4 | 0.9 | 0.5 | 1.2 | 1.3 |
| NC1C9 | 2.5 | 2.0 | ED | ED | | 1.2 | 3.0 | 1.4 | 1.1 | 0.6 | 0.6 | 1.4 | 0.9 | 1.7 | 1.6 | 0.9 | 0.8 | 1.3 | 1.3 |
| SC1E10 | 3.5 | 7.0 | ED | ED | | -1.1 | 0.8 | 2.6 | 1.1 | 0.9 | 0.8 | 1.0 | 1.1 | 2.6 | 1.1 | 0.7 | 0.5 | 3.4 | 2.1 |
| NM1C3 | 6.5 | 8.0 | ED | ED | | -0.7 | 0.2 | 2.5 | 0.9 | 0.8 | 0.4 | 1.2 | 0.7 | 3.2 | 1.2 | 0.8 | 0.5 | 3.9 | 1.7 |
| W06B10 | 2.0 | 4.0 | ED | ED | | 1.1 | -2.1 | 1.1 | 1.0 | 0.7 | 1.0 | 1.3 | 1.2 | 1.1 | 1.1 | 0.9 | 0.8 | 3.0 | 0.8 |
| NC5E9 | 2.0 | 2.5 | ED | ED | | -0.4 | 1.0 | 1.4 | 0.9 | 0.8 | 1.0 | 1.6 | 1.2 | 1.6 | 1.1 | 0.8 | 0.7 | 1.3 | 1.0 |
| SM3H9 | 3.0 | 10.0 | ED | LN | | -0.8 | -2.9 | 1.2 | 1.2 | 0.8 | 0.9 | 1.5 | 0.5 | 1.2 | 0.9 | 0.9 | 1.0 | 8.0 | 0.8 |
| NM7C5 | 1.5 | 1.5 | ED | ED | | -1.9 | 2.5 | 1.3 | 0.9 | 0.8 | 1.0 | 1.2 | 0.8 | 1.2 | 1.2 | 0.9 | 0.7 | 1.1 | 0.8 |
| NC2F3 | 2.0 | 2.5 | ED | ED | | 1.1 | 3.0 | 1.4 | 1.0 | 0.9 | 0.8 | 1.3 | 1.1 | 1.8 | 1.8 | 0.9 | 0.7 | 1.4 | 1.2 |
| NM2A8 | 2.0 | 2.5 | ED | ED | | 1.2 | 3.8 | 1.3 | 0.9 | 0.6 | 0.7 | 1.0 | 1.0 | 1.6 | 1.6 | 0.8 | 0.6 | 1.3 | 1.6 |
| SP6H10 | 2.0 | 5.0 | ED | ED | | -0.2 | 3.7 | 1.7 | 1.0 | 0.8 | 0.8 | 1.2 | 1.0 | 1.8 | 1.9 | 0.7 | 0.4 | 1.2 | 1.3 |
| NM3A8 | 2.0 | 2.5 | ED | ED | | -1.9 | 3.7 | 1.5 | 0.9 | 0.8 | 1.3 | 1.7 | 0.9 | 1.5 | 1.6 | 0.7 | 0.7 | 0.8 | 0.9 |
| SP7C6 | 2.0 | 2.5 | LD | EN | | 4.1 | -8.4 | 1.0 | 1.3 | 0.6 | 0.8 | 0.9 | 1.1 | 0.8 | 0.8 | 1.2 | 1.9 | 0.7 | 1.0 |
| NM2B6 | 1.5 | 2.0 | LD | LD | | 5.1 | 9.4 | 1.1 | 1.3 | 0.8 | 0.9 | 0.9 | 1.3 | 0.8 | 1.5 | 1.0 | 1.2 | 0.8 | 1.1 |
| NM7G11 | 2.0 | 2.0 | LD | ED | | 7.5 | 2.2 | 1.0 | 1.7 | 1.0 | 0.9 | 1.1 | 1.0 | 1.0 | 1.3 | 0.7 | 0.9 | 1.0 | 0.8 |
| NC4B12 | 1.5 | 2.0 | LD | ED | | 9.9 | 1.8 | 0.9 | 1.2 | 1.2 | 1.0 | 0.8 | 1.1 | 1.3 | 0.9 | 0.8 | 0.9 | 1.4 | 0.9 |
| NP6A11 | 2.0 | 2.0 | LD | LN | * | 9.3 | -7.5 | 0.8 | 1.2 | 1.1 | 0.9 | 0.6 | 1.1 | 1.1 | 0.9 | 0.9 | 1.5 | 0.7 | 1.2 |
| NM3F4 | 3.0 | 2.0 | LD | EN | | 4.6 | -9.2 | 1.3 | 1.7 | 0.8 | 0.6 | 1.1 | 1.2 | 0.8 | 1.0 | 1.3 | 1.4 | 0.7 | 0.7 |
| SP6D5 | 1.5 | 2.0 | LD | LD | | 9.0 | 6.8 | 0.9 | 1.2 | 1.1 | 1.0 | 0.9 | 1.4 | 0.7 | 1.3 | 0.9 | 0.8 | 1.1 | 1.0 |
| W06E11 | 2.0 | 1.5 | LD | ED | | 10.6 | 1.5 | 0.9 | 1.3 | 1.4 | 0.9 | 0.8 | 1.0 | 1.3 | 1.0 | 1.0 | 1.1 | 1.5 | 1.6 |
| W01D5 | 2.0 | 3.0 | LD | # | | 9.6 | # | 0.9 | 1.4 | 1.3 | 0.9 | 0.8 | 1.1 | 1.0 | 1.9 | 1.1 | 0.6 | 1.3 | 0.9 |
| NM2B8 | 2.0 | 3.5 | LD | LN | * | 8.0 | -6.9 | 1.2 | 1.4 | 1.4 | 0.8 | 0.9 | 1.2 | 1.2 | 0.5 | 1.1 | 1.7 | 0.7 | 1.0 |
| NC5G11 | 2.0 | 2.0 | LD | LN | * | 6.0 | -3.8 | 1.2 | 1.3 | 1.1 | 0.7 | 0.9 | 0.8 | 0.9 | 0.9 | 1.0 | 0.9 | 1.4 | 0.8 |
| W07G9 | 2.0 | 2.0 | LD | LN | * | 10.8 | -7.8 | 0.7 | 1.2 | 1.3 | 1.0 | 1.0 | 1.0 | 1.1 | 0.7 | 1.2 | 1.3 | 0.7 | 0.8 |
| SC3A6 | 2.0 | 2.5 | LD | LN | * | 9.3 | -6.0 | 0.8 | 1.6 | 1.0 | 1.0 | 0.8 | 1.1 | 0.9 | 0.6 | 0.9 | 1.6 | 1.1 | 1.3 |
| SP6F12 | 2.0 | 2.5 | LD | LN | * | 10.2 | -4.8 | 0.8 | 1.0 | 1.3 | 1.0 | 1.0 | 1.4 | 0.7 | 0.7 | 1.0 | 1.1 | 1.6 | 1.1 |

**Table 3. (continued)**

| | Fold Change[1] | | Class[2] | | | Phase[3] | | Normalized Values[4] | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | *frq* $^{+}$ | | | | | | *frq* $^{7}$ | | | | | |
| EST | *frq* $^{+}$ | *frq* $^{+}$ | *frq* $^{+}$ | *frq* $^{7}$ | | *frq* $^{+}$ | *frq* $^{7}$ | CT0 | CT6 | CT12 | CT18 | CT0 | CT6 | CT0 | CT6 | CT12 | C18 | CT0 | CT6 |
| SM1H10 | 2.5 | 2.0 | EN | LN | | -11.1 | -3.9 | 0.9 | 1.6 | 1.2 | 1.8 | 0.8 | 1.3 | 1.3 | 0.9 | 0.8 | 1.4 | 0.9 | 1.1 |
| NM3E11 | 2.0 | 3.5 | EN | EN | | -8.2 | -8.1 | 1.0 | 0.7 | 1.2 | 1.4 | 0.9 | 1.2 | 0.8 | 1.2 | 1.0 | 2.4 | 0.9 | 0.7 |
| NC3B3 | 1.5 | 3.5 | EN | LN | | -10.8 | -6.7 | 0.8 | 1.0 | 1.2 | 1.1 | 1.1 | 1.0 | 1.0 | 0.9 | 1.0 | 2.3 | 1.1 | 0.7 |
| NM6A4 | 2.0 | 2.0 | EN | LD | | -10.2 | 10.5 | 1.0 | 0.7 | 1.2 | 1.2 | 0.8 | 1.3 | 0.6 | 1.2 | 1.0 | 1.0 | 0.8 | 1.2 |
| W06H2 | 5.0 | 4.5 | EN | # | | -8.2 | # | 0.5 | 0.7 | 1.5 | 2.5 | 1.7 | 0.8 | 0.4 | 0.9 | 1.3 | 1.0 | 0.3 | 1.3 |
| NM6E2 | 2.5 | 2.5 | EN | ED | * | -8.2 | -0.8 | 1.6 | 0.8 | 1.3 | 1.2 | 0.7 | 1.1 | 1.3 | 1.3 | 0.9 | 0.9 | 2.1 | 0.9 |
| SC2A10 | 1.5 | 2.0 | EN | ED | * | -8.2 | -1.2 | 0.8 | 0.9 | 0.9 | 1.3 | 0.9 | 1.0 | 1.0 | 0.9 | 0.7 | 1.2 | 1.3 | 1.1 |
| NM7A9 | 2.0 | 2.0 | EN | LN | | -8.2 | -3.0 | 1.3 | 0.7 | 1.3 | 0.9 | 0.9 | 1.0 | 0.9 | 1.0 | 0.7 | 1.3 | 1.2 | 0.9 |
| NM5G3 | 2.0 | 2.0 | EN | EN | | -11.1 | -8.2 | 0.8 | 0.7 | 1.3 | 1.0 | 1.1 | 1.2 | 0.9 | 0.8 | 1.0 | 1.5 | 0.9 | 1.3 |
| SP4D11 | 1.5 | 2.0 | EN | LN | | -8.1 | -5.7 | 1.1 | 0.9 | 1.2 | 1.4 | 0.9 | 1.0 | 1.2 | 1.1 | 0.8 | 1.7 | 0.9 | 1.2 |
| W17H1 | 1.5 | 2.5 | EN | LN | | -8.2 | -3.9 | 0.9 | 0.9 | 1.1 | 1.4 | 1.0 | 1.1 | 0.7 | 0.7 | 1.1 | 0.7 | 1.8 | 1.0 |
| SP1B4 | 2.0 | 2.0 | EN | EN | | -8.1 | -10.0 | 1.0 | 0.7 | 1.3 | 1.1 | 1.1 | 0.9 | 0.7 | 0.9 | 1.3 | 1.3 | 0.9 | 1.0 |
| NC2A11 | 2.0 | 2.0 | LN | ED | | -5.2 | 1.0 | 1.0 | 1.0 | 1.1 | 1.5 | 1.4 | 0.7 | 1.7 | 1.5 | 0.9 | 1.0 | 1.8 | 1.2 |
| SM2A10 | 2.5 | 2.5 | LN | LN | | -5.9 | -3.0 | 0.8 | 0.8 | 1.0 | 1.9 | 1.6 | 0.9 | 1.2 | 0.9 | 0.8 | 1.7 | 2.0 | 1.2 |
| W08D10 | 2.0 | 2.0 | LN | LN | | -4.7 | -3.9 | 0.9 | 0.8 | 0.9 | 1.5 | 1.5 | 0.7 | 1.2 | 0.9 | 1.0 | 1.8 | 2.0 | 1.1 |
| W08E5 | 3.0 | 2.0 | LN | LN | | -4.0 | -2.1 | 1.0 | 0.5 | 0.9 | 1.3 | 1.4 | 0.8 | 0.9 | 1.0 | 1.0 | 0.9 | 1.6 | 1.0 |
| W10A1 | 2.0 | 2.5 | LN | LN | | -5.0 | -3.8 | 0.9 | 0.8 | 1.0 | 1.4 | 1.4 | 0.7 | 1.2 | 0.8 | 1.3 | 1.1 | 1.8 | 1.0 |
| W09F2 | 2.5 | 3.0 | LN | ED | | -5.2 | 3.6 | 0.9 | 0.6 | 1.1 | 1.5 | 1.5 | 0.8 | 0.7 | 1.0 | 1.2 | 0.7 | 2.2 | 1.5 |
| W06G11 | 2.0 | 2.5 | LN | ED | | -5.7 | 0.0 | 0.8 | 0.7 | 1.0 | 1.4 | 1.3 | 1.1 | 1.5 | 0.8 | 1.0 | 0.9 | 2.0 | 1.5 |
| NM7B6 | 2.0 | 2.0 | LN | LD | * | -3.8 | 8.1 | 1.2 | 0.6 | 1.1 | 1.2 | 1.2 | 0.9 | 1.1 | 1.0 | 1.0 | 0.9 | 0.7 | 1.5 |
| NC5G12 | 2.5 | 2.0 | LN | ED | | -6.6 | 0.0 | 0.6 | 0.6 | 1.2 | 1.4 | 1.5 | 0.9 | 0.9 | 0.7 | 1.3 | 0.9 | 1.0 | 1.2 |
| W08B11 | 2.5 | 5.0 | LN | ED | | -4.3 | -1.1 | 1.0 | 0.7 | 0.9 | 1.5 | 1.6 | 0.8 | 0.8 | 1.0 | 0.9 | 0.6 | 3.1 | 1.1 |
| NP2H10 | 2.0 | 2.0 | LN | ED | | -4.8 | -1.4 | 0.9 | 0.8 | 1.0 | 1.3 | 1.6 | 0.8 | 1.2 | 0.9 | 1.0 | 0.9 | 1.8 | 1.1 |
| NC5E12 | 2.0 | 2.5 | LN | ED | | -4.2 | -0.9 | 1.0 | 0.7 | 1.0 | 1.3 | 1.3 | 1.0 | 1.8 | 0.9 | 1.1 | 0.8 | 1.4 | 1.0 |
| SP6F4 | 2.0 | 2.0 | LN | LD | * | -6.8 | 7.6 | 1.2 | 1.0 | 1.0 | 1.8 | 0.9 | 0.9 | 0.7 | 1.5 | 0.8 | 0.9 | 0.8 | 1.4 |
| W17E10 | 2.5 | 2.5 | LN | LN | | -5.5 | -2.2 | 1.0 | 0.7 | 1.0 | 1.8 | 1.4 | 1.1 | 1.1 | 0.7 | 1.1 | 0.8 | 1.8 | 1.0 |
| NC2C12 | 2.5 | 2.0 | LN | ED | | -4.5 | -1.5 | 0.9 | 0.8 | 1.0 | 1.4 | 2.0 | 0.8 | 1.1 | 0.9 | 0.9 | 0.9 | 1.9 | 1.1 |
| NC4G10 | 1.5 | 2.0 | LN | LN | | -3.8 | -2.7 | 0.9 | 0.7 | 0.9 | 1.1 | 1.1 | 1.0 | 1.2 | 0.7 | 0.9 | 1.2 | 1.3 | 1.0 |
| NM7C7 | 2.0 | 2.5 | LN | ED | | -5.2 | 3.9 | 0.8 | 0.7 | 1.0 | 1.5 | 1.5 | 0.9 | 0.9 | 1.0 | 1.1 | 0.8 | 1.8 | 1.6 |
| SC2H9 | 2.0 | 2.0 | LN | ED | | -6.0 | 0.0 | 0.8 | 1.3 | 1.0 | 1.3 | 1.2 | 0.7 | 1.2 | 1.0 | 1.0 | 1.0 | 1.8 | 1.4 |
| SM2A8 | 2.0 | 3.5 | LN | LN | | -5.6 | -3.3 | 0.9 | 0.6 | 1.1 | 1.1 | 1.1 | 0.9 | 1.7 | 0.5 | 1.0 | 1.2 | 1.1 | 1.0 |
| NM6E1 | 2.5 | 3.0 | LN | ED | | -3.0 | 2.5 | 1.0 | 0.5 | 1.0 | 1.0 | 1.2 | 1.0 | 0.8 | 1.0 | 1.0 | 0.7 | 2.0 | 1.4 |
| W07H9 | 1.5 | 2.0 | LN | ED | * | -5.1 | 3.8 | 0.9 | 0.8 | 1.0 | 1.2 | 1.2 | 1.0 | 1.0 | 1.2 | 1.1 | 0.8 | 1.7 | 1.4 |
| W09G8 | 2.0 | 3.0 | LN | LN | | -3.8 | -2.1 | 1.0 | 0.6 | 1.0 | 1.2 | 1.1 | 1.2 | 1.4 | 0.8 | 1.0 | 0.8 | 1.8 | 0.6 |
| W17E6 | 1.5 | 2.5 | LN | LN | | -6.0 | -2.1 | 0.8 | 0.8 | 0.9 | 1.2 | 1.1 | 1.2 | 1.0 | 0.8 | 1.0 | 0.9 | 1.8 | 1.1 |
| SC3B6 | 2.0 | 3.0 | LN | LN | | -4.4 | -2.8 | 0.9 | 1.0 | 1.0 | 1.5 | 1.6 | 1.2 | 1.1 | 0.9 | 0.9 | 1.0 | 2.4 | 0.8 |
| W13A10 | 2.0 | 2.0 | LN | ED | | -3.3 | 3.9 | 1.1 | 0.6 | 0.9 | 1.2 | 1.2 | 0.9 | 1.3 | 0.9 | 1.1 | 0.9 | 1.5 | 1.9 |
| NC2A10 | 2.0 | 2.5 | LN | ED | | -5.5 | -0.5 | 0.9 | 0.6 | 1.0 | 1.2 | 1.1 | 0.8 | 1.1 | 0.9 | 1.0 | 0.7 | 1.9 | 1.0 |
| SP4E7 | 3.5 | 2.0 | LN | EN | | -4.7 | 11.7 | 0.9 | 0.6 | 1.1 | 1.8 | 2.2 | 0.9 | 0.8 | 0.7 | 1.4 | 0.8 | 1.0 | 1.2 |
| W08C12 | 2.0 | 3.0 | LN | ED | | -3.0 | -1.9 | 0.8 | 1.1 | 0.9 | 1.3 | 1.4 | 1.1 | 1.1 | 0.7 | 0.9 | 0.8 | 2.0 | 1.0 |
| W17H5 | 1.5 | 2.0 | LN | LN | | -3.0 | -4.5 | 1.0 | 0.9 | 0.8 | 1.3 | 1.3 | 1.0 | 1.0 | 0.6 | 1.0 | 1.2 | 1.3 | 0.7 |
| W15D3 | 1.5 | 3.0 | LN | LD | * | -6.3 | 6.8 | 0.9 | 1.0 | 1.1 | 1.4 | 1.3 | 1.0 | 1.1 | 0.6 | 1.2 | 1.0 | 1.3 | 1.9 |
| NC2B7 | 2.0 | 2.0 | LN | LN | | -4.0 | -2.1 | 1.0 | 0.8 | 0.9 | 1.3 | 1.4 | 0.9 | 1.2 | 0.7 | 0.8 | 1.1 | 1.5 | 1.0 |
| NC3E9 | 1.5 | 3.0 | LN | ED | | -3.9 | -1.5 | 0.9 | 0.8 | 1.0 | 1.1 | 1.3 | 1.1 | 1.4 | 0.6 | 0.9 | 0.9 | 1.6 | 1.0 |
| W07B11 | 1.5 | 3.5 | LN | ED | | -7.2 | 0.4 | 0.9 | 0.8 | 1.1 | 1.3 | 1.1 | 1.1 | 1.3 | 0.9 | 1.1 | 0.7 | 2.3 | 1.4 |
| NC5D11 | 2.0 | 2.5 | LN | ED | | -5.2 | 1.5 | 0.8 | 0.7 | 1.0 | 1.4 | 1.4 | 1.0 | 1.1 | 0.8 | 1.0 | 0.7 | 1.9 | 1.5 |
| SM2H10 | 2.0 | 2.0 | LN | ED | | -3.0 | 0.8 | 1.2 | 0.6 | 1.1 | 1.0 | 1.2 | 0.9 | 1.4 | 0.9 | 0.9 | 0.8 | 1.6 | 1.3 |
| NC3E3 | 2.0 | 1.5 | LN | ED | | -5.1 | 2.5 | 1.0 | 0.7 | 0.9 | 1.4 | 1.1 | 1.1 | 1.2 | 1.1 | 1.0 | 0.8 | 1.2 | 1.0 |
| W01A5 | 1.5 | 2.5 | LN | ED | | -5.1 | 1.0 | 0.9 | 0.8 | 1.0 | 1.1 | 1.2 | 0.9 | 1.0 | 1.0 | 0.9 | 0.7 | 1.6 | 1.0 |
| W10H8 | 2.0 | 2.0 | LN | ED | | -3.0 | 3.0 | 1.1 | 0.5 | 0.9 | 1.1 | 0.9 | 1.1 | 1.3 | 0.9 | 1.0 | 0.6 | 1.0 | 1.0 |
| NC5E10 | 2.5 | 2.0 | LN | ED | | -3.0 | -0.5 | 1.3 | 0.6 | 1.0 | 1.3 | 1.4 | 1.0 | 1.0 | 0.8 | 0.6 | 1.0 | 1.2 | 1.0 |
| SP4F6 | 2.0 | 2.0 | LN | ED | | -4.9 | 1.2 | 0.9 | 0.8 | 1.0 | 1.6 | 1.6 | 1.0 | 0.8 | 0.8 | 1.0 | 0.7 | 1.4 | 1.0 |
| SC4A1 | 1.5 | 1.5 | LN | ED | | -4.1 | -1.5 | 1.2 | 0.8 | 0.9 | 1.3 | 1.1 | 0.9 | 1.1 | 0.9 | 0.7 | 1.1 | 1.0 | 1.0 |
| SM1F12 | 2.0 | 2.5 | LN | ED | | -5.8 | -0.9 | 0.9 | 0.8 | 1.1 | 1.7 | 1.5 | 1.0 | 1.1 | 0.9 | 1.0 | 0.8 | 1.9 | 1.1 |
| SP4F9 | 2.0 | 2.0 | LN | ED | | -6.0 | 1.8 | 0.7 | 0.6 | 1.0 | 1.2 | 1.2 | 0.9 | 1.0 | 1.0 | 0.9 | 0.6 | 1.2 | 0.7 |
| NM3B8 | 1.5 | 2.5 | LN | ED | | -6.0 | 1.5 | 1.0 | 0.8 | 1.1 | 1.2 | 1.1 | 0.9 | 1.3 | 0.8 | 0.9 | 0.7 | 1.9 | 1.6 |
| NC4D7 | 2.0 | 3.0 | LN | LN | | -3.5 | -3.3 | 1.0 | 0.6 | 1.0 | 1.0 | 1.2 | 0.9 | 1.0 | 0.9 | 1.2 | 0.8 | 2.1 | 0.7 |
| W17D1 | 1.5 | 2.0 | LN | LN | | -4.2 | -3.6 | 0.9 | 0.9 | 1.0 | 0.9 | 1.2 | 0.8 | 1.0 | 0.9 | 1.1 | 1.0 | 1.6 | 1.0 |
| SP6G3 | 2.0 | 2.0 | LN | LN | | -3.0 | -5.5 | 0.9 | 1.0 | 0.9 | 1.0 | 1.4 | 0.8 | 0.9 | 0.9 | 1.1 | 1.4 | 1.4 | 0.8 |
| NC2D3 | 2.0 | 2.5 | LN | LN | | -5.6 | -2.3 | 0.9 | 0.8 | 1.1 | 1.6 | 1.5 | 1.0 | 1.0 | 0.8 | 1.0 | 0.9 | 2.1 | 1.1 |
| NC3E8 | 2.0 | 2.5 | LN | ED | | -4.6 | -0.3 | 1.0 | 0.7 | 1.0 | 1.3 | 1.3 | 0.8 | 1.1 | 0.9 | 1.0 | 0.7 | 1.8 | 1.0 |
| NC5F5 | 2.0 | 2.0 | LN | ED | | -3.4 | -1.4 | 0.9 | 0.9 | 0.8 | 1.4 | 1.4 | 1.1 | 1.5 | 0.9 | 0.9 | 1.0 | 2.0 | 1.1 |
| W08A8 | 1.5 | 1.5 | LN | EN | | -4.5 | -10.8 | 1.0 | 0.8 | 0.9 | 1.2 | 1.1 | 0.9 | 0.7 | 0.9 | 1.2 | 1.1 | 1.0 | 1.0 |
| W08B2 | 2.0 | 2.0 | LN | LD | * | -3.0 | 5.6 | 1.0 | 0.7 | 0.9 | 1.2 | 1.3 | 1.1 | 0.8 | 0.9 | 1.0 | 0.9 | 1.5 | 1.7 |
| NC3G11 | 1.5 | 2.5 | LN | ED | | -7.2 | 1.2 | 0.9 | 0.8 | 1.2 | 1.2 | 1.2 | 1.1 | 1.1 | 0.8 | 1.0 | 0.6 | 1.6 | 1.1 |
| NM7A1 | 2.0 | 3.5 | LN | LN | | -4.8 | -2.2 | 0.8 | 1.0 | 1.0 | 1.4 | 1.6 | 1.0 | 2.5 | 0.8 | 1.0 | 1.0 | 0.7 | 1.4 |

**Table 3. (continued)**

| | Fold Change[1] | | Class[2] | | | Phase[3] | | Normalized Values[4] | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| EST | *frq* $^{+}$ | *frq* $^{+}$ | *frq* $^{+}$ | *frq* $^{7}$ | | *frq* $^{+}$ | *frq* $^{7}$ | *frq* $^{+}$ | | | | | | *frq* $^{7}$ | | | | | |
| | | | | | | | | CT0 | CT6 | CT12 | CT18 | CT0 | CT6 | CT0 | CT6 | CT12 | C18 | CT0 | CT6 |
| W08C5 | 2.0 | 2.5 | LN | LN | | -4.5 | -3.4 | 1.0 | 0.8 | 1.0 | 1.4 | 1.4 | 1.0 | 0.8 | 0.8 | 0.9 | 1.0 | 1.9 | 1.0 |
| SP4F8 | 1.5 | 2.0 | LN | ED | | -6.8 | 1.9 | 0.9 | 0.8 | 1.2 | 1.2 | 1.2 | 0.9 | 1.0 | 1.1 | 1.0 | 0.8 | 1.7 | 1.2 |
| SM2H2 | 3.0 | 2.5 | LN | ED | | -3.8 | 3.0 | 1.1 | 0.6 | 0.9 | 1.5 | 1.8 | 0.9 | 1.0 | 0.9 | 0.9 | 0.6 | 1.5 | 1.3 |
| NC1F8 | 2.0 | 2.5 | LN | LD | * | -5.6 | 7.1 | 0.8 | 0.9 | 1.1 | 1.3 | 1.5 | 1.1 | 0.9 | 1.4 | 1.2 | 0.8 | 1.3 | 1.9 |
| W17G8 | 2.5 | 2.5 | LN | ED | | -5.8 | 3.7 | 0.8 | 0.9 | 1.3 | 2.0 | 2.0 | 0.9 | 1.1 | 0.9 | 1.2 | 0.6 | 1.6 | 1.5 |
| W08B8 | 3.0 | 3.0 | LN | ED | | -5.2 | -0.9 | 0.9 | 0.7 | 1.0 | 2.0 | 1.8 | 1.0 | 1.1 | 0.8 | 1.2 | 0.7 | 2.2 | 1.2 |
| NP4F8 | 3.5 | 2.0 | LN | ED | | -4.3 | 3.0 | 1.2 | 0.7 | 1.1 | 1.9 | 2.4 | 0.8 | 1.6 | 0.9 | 1.1 | 0.8 | 1.2 | 1.6 |
| NC4G3 | 1.5 | 1.5 | LN | EN | | -4.6 | -9.0 | 1.1 | 0.9 | 1.0 | 1.5 | 1.4 | 1.0 | 0.8 | 0.8 | 1.2 | 1.1 | 1.1 | 1.0 |
| SC5B8 | 3.0 | 2.5 | LN | ED | | -5.9 | 2.2 | 0.8 | 0.7 | 1.1 | 2.0 | 1.7 | 0.9 | 1.2 | 0.8 | 1.2 | 0.8 | 1.9 | 1.7 |
| W01C6 | 2.0 | 2.0 | LN | ED | | -4.1 | 1.4 | 1.1 | 0.6 | 1.1 | 1.1 | 1.3 | 0.8 | 1.2 | 0.9 | 1.0 | 0.8 | 1.7 | 1.4 |
| W13B7 | 1.5 | 2.0 | LN | ED | | -3.0 | 1.1 | 0.9 | 1.0 | 1.0 | 1.4 | 1.2 | 1.2 | 1.4 | 1.4 | 0.9 | 0.9 | 1.6 | 1.0 |
| W17H12 | 2.0 | 2.5 | LN | LD | * | -5.0 | 4.8 | 0.9 | 0.5 | 1.0 | 1.1 | 1.0 | 1.0 | 1.4 | 0.8 | 1.1 | 0.6 | 0.8 | 1.1 |
| W10C3 | 2.0 | 3.0 | LN | LD | * | -3.9 | 7.9 | 1.0 | 0.6 | 1.0 | 1.1 | 1.2 | 0.9 | 1.3 | 0.9 | 1.4 | 0.6 | 0.8 | 1.8 |
| W13F6 | 3.0 | 3.0 | LN | LN | | -5.3 | -6.8 | 1.0 | 0.6 | 1.0 | 1.7 | 1.4 | 0.9 | 1.0 | 0.6 | 1.0 | 1.8 | 0.9 | 1.0 |
| NP3A8 | 2.0 | 3.0 | LN | LN | | -6.0 | -2.8 | 0.9 | 0.6 | 1.0 | 1.2 | 1.0 | 1.0 | 2.2 | 0.7 | 0.9 | 1.4 | 1.0 | 1.4 |

[1] The fold change was determined by dividing the highest normalized data point by the lowest average data point for each strain.

[2] The class was determined from the phase. Classes ED, LD, EN, LN were chosen to represent genes that had a peak phase between -2 to 3.99, 4 to 9.99, 10 to 12 and -12 to -7.99, and -8 to -1.99, respectively. The numbers are CT hours from CT0 to the fitted peak. For example, -5 means the peak occurred 5 CT hours before CT0. Asterisks highlight genes that fell into non-adjacent classes.

[3] The phase was determined by fitting a cosine function to the normalized data using nonlinear regression with the Prism software package (GraphPad Software, San Deigo, CA). # indicates that the gene expression had a shorter than 24 CT hour rhythm in *frq* $^{7}$, and therefore no phase could be assigned

[4] Normalized micorarray values from 4 spots per time point. The complete set of raw data is available at http://plpa2linux.tamu.edu/Microarray.html.

northern assay (Table 2). Rhythmicity was confirmed in 26 out of 27 genes; the one gene that was not confirmed (W13B7) was difficult to detect by northern assay.

Approximately 25% of the 145 ccgs have no predictable function. Many of the identified genes function in previously defined clock-regulated processes including development, stress responses and intermediary metabolism (Table 4 and Table 2). In addition, several genes are predicted to be involved in transcriptional control and signaling. There was some correlation between function and time-of-day expression; 30 of the 145 genes (21%) are involved in translation and 25 of these belong to the LN class (Table 3 and Table 4). Twenty-eight genes encode putative ribosomal proteins; another encodes an

elongation factor-1α and another is NOP58, which is involved in ribosome biosynthesis. Northern analysis of several of the putative ribosomal protein genes confirmed their rhythmic RNA accumulation profile (Fig. 5C, NC2B7 and Table 3).

**Table 4. Classification of clock-controlled genes.**

| Category[1] | Number |
|---|---|
| Cell division | 1 |
| Signaling/communication | 15 |
| Cell structure/cytoskeleton | 8 |
| Cell defense | 4 |
| Development | 7 |
| Gene regulation | 5 |
| Metabolism | 31 |
| Protein processing | 7 |
| Protein synthesis | 30 |
| Unclassified | 37 |

[1]Genes were classified according to their known or predicted functions from the Whitehead Institute Neurospora Sequencing Project (http://www-genome.wi.mit.edu/annotation/fungi/neurospora/).

To identify possible promoter elements that confer circadian rhythmicity on gene expression, we scanned 1 kb of sequence upstream of the predicted transcriptional start site of each of the cycling genes for common sequences within each class, and within the entire rhythmic gene set. Using BioProspector (http://bioprospector.stanford.edu/), we identified an 8-nt element (TCTTGGCA) occurring 42 times in 29 of 69 LN class genes and 5 times in 3 of 50 ED class genes (Table 3). These sequences match the core of the previously characterized activating clock element (ACE) (AACTTGGCCAAGTT) shown to

be necessary and sufficient for cycling of the ED class *ccg-2* transcript (Bell-Pedersen *et al.*, 2001b). We found 4 genes that contain two WC-1 binding sites and 13 genes that contain one WC-1 binding site (Froehlich *et al.*, 2002) in the promoter regions, suggesting that WC-1 may directly regulate some *N. crassa* ccgs (Table 2). However, these data suggested that relatively few ccgs are directly regulated by WC-1, and pointed to the vast majority of ccgs being controlled indirectly by a cascade involving several output pathways. Similarly, using Drosophila microarray analyses, only a small number of genes were identified as being direct targets of the circadian clock transcription factor CLOCK (McDonald and Rosbash, 2001).

*Molecular rhythms in the absence of the FRQ-based oscillator*

Oscillators that are either coupled to, or distinct from, the well-described FRQ-based oscillator may exist in *N. crassa* (Lakin-Thomas and Brody, 2000; Merrow *et al.*, 1999), and may regulate some *N. crassa* ccgs. Consistent with this idea, we identified 3 genes in the microarrays that appeared to have a short period in constant darkness in the $frq^7$ strain suggesting that cycling of these genes transcripts might be independent of the FRQ-based oscillator (Figure 6 and Table 4). For example, in $frq^7$W01D5 mRNA peaked at DD22 (CT6) during the first day and DD44 (CT0) in the second day, whereas *ccg-2*, which is controlled by the FRQ-based oscillator (Loros *et al.*, 1989), peaked at DD16 (CT0) and DD 44 (CT0). However, some level of influence of the FRQ-based oscillator on these genes is evidenced by the differences in phasing of the peaks

in *frq*$^{+}$ versus *frq*$^{7}$ strains following the shift from light to dark. This is particularly visible for W06H2 (Figure 6).

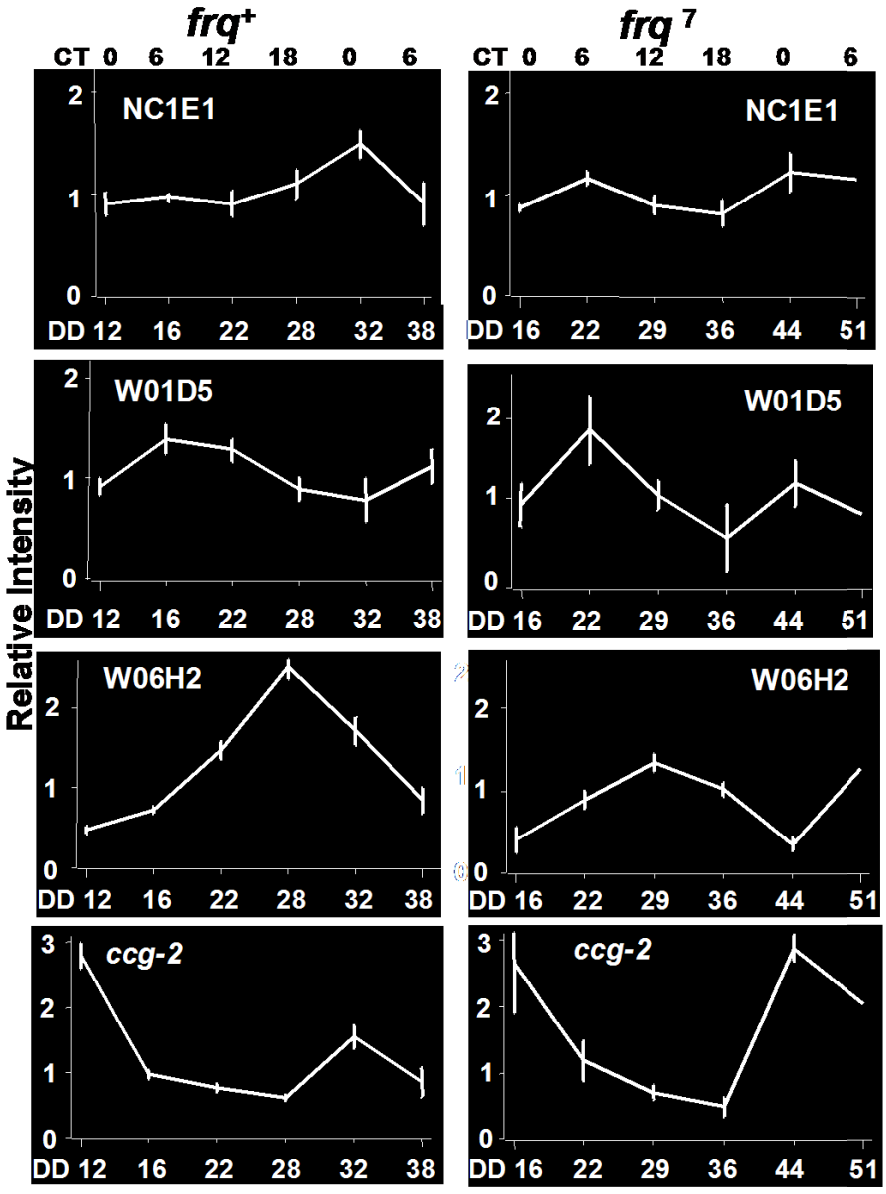


**Figure 6. Microarray tracings of RNA accumulation data from 3 EST clones in clock *frq*$^{+}$ and *frq*$^{7}$ strains.**

The control *ccg-2* tracing is shown for comparison. The values (means ± SEMs; n=4) for each time point are shown. The times in the dark (DD) are shown below each graph and the corresponding CT for each strain is indicated at the top.

To determine whether the rhythmic expression of these genes requires the FRQ-based oscillator, we used northern blots to assay rhythmicity of the same candidates in a $FRQ^{-}$ strain (Aronson *et al.*, 1994a), in conditions where conidiation is arrhythmic (Figure 7). In each case, a rhythm in mRNA accumulation with a period of 20-24 h was observed in cells that were synchronized by a light to dark transfer (Figure 7). Because the cultures were close to the same age at the time of harvest (see Materials and Methods), we can rule out the possibility that the change in mRNA accumulation is due to age, nutritional, or developmental state. These data provide the first molecular evidence of a light-responsive FLO that functions with a 20-24 h period in constant conditions.

The products of the 3 genes regulated by the FLO include CPC-1, a bZIP transcription factor of the GCN4 subfamily that regulates the expression of amino acid biosynthetic genes (Paluh and Yanofsky, 1991), a predicted 1,4 alpha glucan involved in glycogen synthesis (Schulte *et al.*, 2002; Thon *et al.*, 1992) and an unknown protein. The transcripts from these genes peak at different times of day in $frq^{+}$ strains (Figure 7 and Table 5), suggesting that they might be regulated by different output pathways from the FLO.

**Table 5. FRQ-independent ccgs.**

| EST[1] | Class frq[+] | frq7 [2] | ORF[3] | BLAST Match[4] | Organism | Accession #[5] |
|---|---|---|---|---|---|---|
| NC1E1 | ED | 22,44 | 5429.1 | 1,4 glucan branching enzyme | N. crassa | 16416077 |
| W01D5 | LD | 22,44 | 4050.1 | CPC-1 | N. crassa | 11115 |
| W06H2 | EN | 29,51 | 5495.1 | None | | |

[1]The *N. crassa* ESTs identified in each class are from the Neurospora Genome Project, University of New Mexico (http://www.unm.edu/~ngp/).

[2]The peak times in mRNA accumulation in hours after the shift to constant darkness in the *frq*$^{7}$ strain were determined from the microarray data and GeneSpring Software.

[3]The predicted ORFs are from the Whitehead Institute Neurospora Sequencing Project (http://www-genome.wi.mit.edu/annotation/fungi/neurospora/).

[4]The BLAST matches represent the most significant match in the data bases as predicted by the Whitehead Institute (minimum e-value $1X10^{-5}$) and verified by us using BLAST searches of the NCBI sequence databases.

[5]The accession numbers for each BLAST match are from the NCBI sequence database (http://www.ncbi.nlm.nih.gov/).

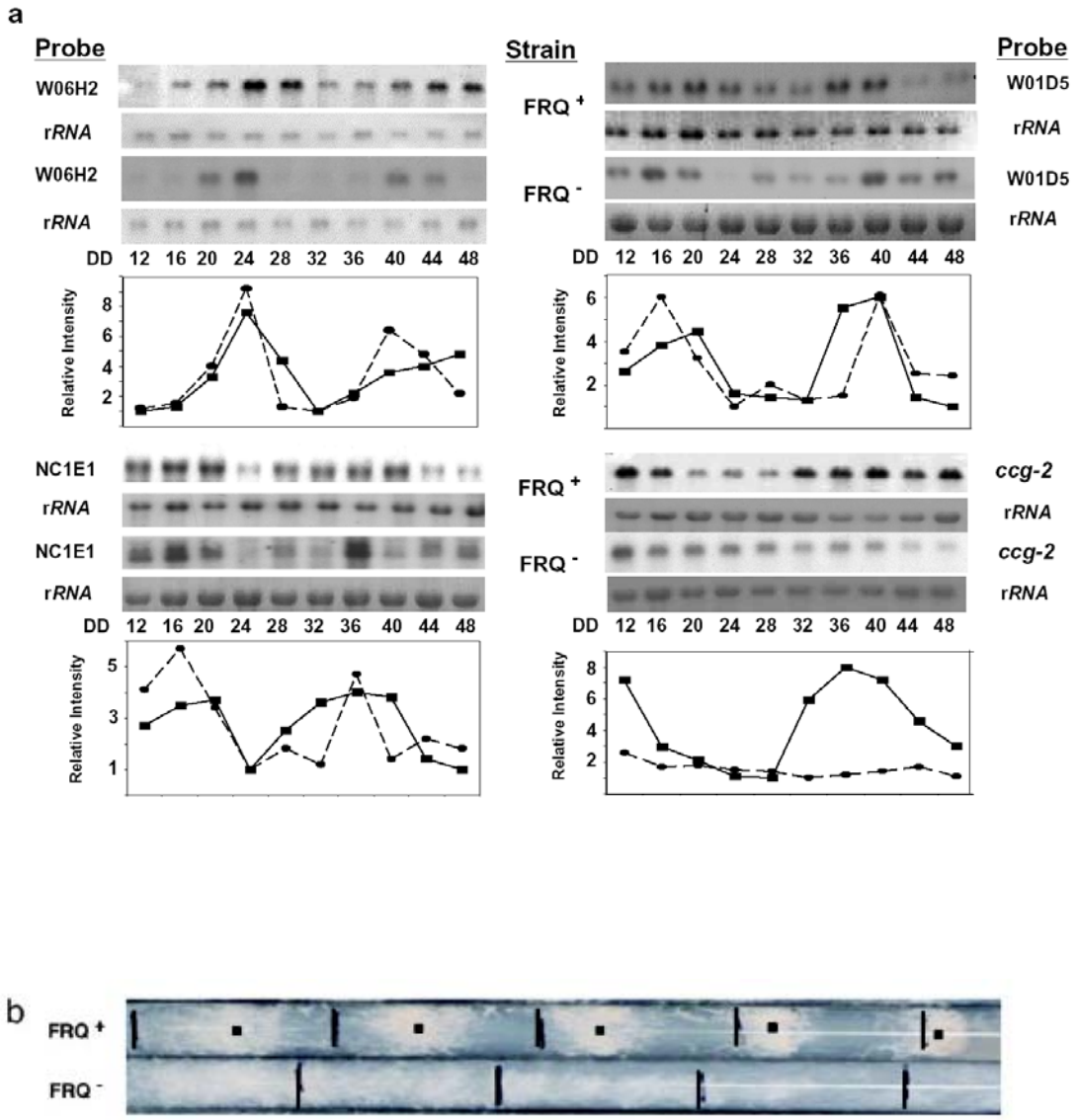


**Figure 7. Rhythmic mRNA accumulation in the FRQ-null strain.**

a. Total RNA was isolated from *frq*$^{+}$ and *frq*$^{-}$ strains harvested after transfer from light to dark (DD) at the indicated times and northern blots were probed with the EST clones. rRNA was used to verify equal loading of RNA. Relative mRNA levels are plotted as relative band intensity versus time in the dark and are shown below the northern blots for each gene. *frq*$^{+}$ is shown as a solid line and *frq*$^{-}$ as a dashed line. For all 4 genes, 2 additional northern blots using RNA isolated from independent time courses gave similar results (not shown). b. Mycelia from each culture were assayed on race tubes grown in constant darkness to verify the circadian phenotype. The growth fronts were marked every 24 h (vertical black lines). The centers of the conidial bands are marked with a black dot.

## Discussion

We used high-density microarrays to identify rhythmically expressed genes in *N. crassa.* We have found the first examples of *N. crassa* genes that are expressed at times other than the late night/early morning, as well as a new class of genes that are rhythmically expressed in a *frq*-null strain.

Using microarrays containing a subset of the genome, 145 genes were shown to be under control of the circadian clock. The percentage of ccgs identified in this study is high compared to the 1-9% observed in microarray studies in other eukaryotes (Akhtar *et al.*, 2002; Ceriani *et al.*, 2002; Claridge-Chang *et al.*, 2001; Duffield *et al.*, 2002; Grundschober *et al.*, 2001; Harmer *et al.*, 2000; Lin *et al.*, 2002; McDonald and Rosbash, 2001; Panda *et al.*, 2002; Storch *et al.*, 2002; Ueda *et al.*, 2002). This may partly reflect use of the customized EST library and the inclusion of low amplitude rhythms. If we imposed a cutoff of a 2-fold increase from the trough to the peak, 113 genes would still be classified as cycling representing 15% of the 760 genes that passed our restrictions. However, northern confirmation of genes with peak to trough differences of lower than 2-fold (Tables 2 and 3) supports the inclusion of low amplitude cycling genes. On the other hand, it is likely that several ccgs were missed in these analyses. Genes that are poorly expressed under the growth conditions used, and transcripts that cycle with a lower than 1.5 fold change from peak to trough levels would not have been detected. Furthermore, a transcript with a circadian rhythm in abundance does not necessarily give rise

to a protein with a rhythm, and conversely, a non-rhythmic RNA does not necessarily mean that the protein is not rhythmic. In summary, while the microarrays provide an effective tool for the discovery of ccgs, they can only be used as a starting point for the analysis of clock control of gene expression. More detailed investigation is needed to validate the involvement of these genes in the clock.

The biochemical functions of the proteins encoded by the ccgs are yielding insights into the diversity of processes that are clock regulated. Several genes encoding enzymes involved in carbon and nitrogen metabolism show circadian rhythms in mRNA accumulation, with peaks occurring in the late-night to early-morning (Table 2). In addition, the genes encoding glycogen phosphorylase and mannitol-1-phosphate dehydrogenase peak in the early night, suggesting that flux into the glycolytic pathway may increase at this time of day. The largest group of coordinately cycling transcripts encodes ribosomal proteins. We found that 36% of the genes in the LN class are involved in protein synthesis. Assuming that the levels of the ribosomal proteins are also rhythmic, these data suggest that the number of ribosomes increase in the late night to prepare for the times of day when the bulk of rhythmic transcripts peak. Finally, several genes encoding transcription factors and proteins with known or suspected roles in signal transduction were found to be rhythmic in *N. crassa*. These genes provide excellent candidates for components involved in signaling time of day information from the oscillator to the output pathways.

The recent use of microarrays to profile rhythmic genes in distinct species and tissues has revealed a wide diversity of clock regulated functions (Akhtar *et al.*, 2002; Ceriani *et al.*, 2002; Claridge-Chang *et al.*, 2001; Duffield *et al.*, 2002; Grundschober *et al.*, 2001; Harmer *et al.*, 2000; Lin *et al.*, 2002; McDonald and Rosbash, 2001; Panda *et al.*, 2002; Storch *et al.*, 2002; Ueda *et al.*, 2002). In general there is little overlap between cycling genes in different organisms and even among tissues (Akhtar *et al.*, 2002; Panda *et al.*, 2002; Storch *et al.*, 2002; Ueda *et al.*, 2002); however, some examples of conservation of clock-controlled processes are becoming evident. Ccgs are involved in protein synthesis and processing, intermediary metabolism, chromatin modification, transcriptional regulation and cellular signaling (Akhtar *et al.*, 2002; Ceriani *et al.*, 2002; Claridge-Chang *et al.*, 2001; Duffield *et al.*, 2002; Grundschober *et al.*, 2001; Harmer *et al.*, 2000; McDonald and Rosbash, 2001; Storch *et al.*, 2002; Ueda *et al.*, 2002). The observation that several genes encoding ribosomal proteins are under clock control in *N. crassa* as well as other organisms (Bailey *et al.*, 2003; Ceriani *et al.*, 2002; Panda *et al.*, 2002; Storch *et al.*, 2002) establishes the generality of clock regulation of protein synthesis and confirms the significance of translational regulation in clock function. Similarly, the role of ubiquitin-mediated protein turnover in clock function is well-established and genes encoding components of the ubiquitin pathway are found to cycle in all organisms examined (Bailey *et al.*, 2003; Ceriani *et al.*, 2002; Claridge-Chang *et al.*, 2001; Duffield *et al.*, 2002; Grundschober *et al.*, 2001; Harmer *et al.*, 2000;

Lin *et al.*, 2002; McDonald and Rosbash, 2001; Storch *et al.*, 2002; Ueda *et al.*, 2002). Expression of transcription factors is found to occur at different phases of the circadian cycle, suggesting that the relay of time information from the clock to these factors coordinates the phase-specific expression of downstream ccgs. Together, these similarities furnish important clues for determining key pathways that are regulated by clocks in diverse organisms.

The observation that FRQ-null strains display conidiation rhythms in temperature cycles led to a model for the *N. crassa* circadian clock that involves multiple oscillators (Merrow *et al.*, 1999). In addition, hyphal branching in *N. crassa* is rhythmic with a period of about 24 h (Sussman *et al.*, 1965), while in the same strains the conidiation rhythm is expressed with a 22 h rhythm (Feldman and Hoyle, 1971). The branching rhythm, unlike the conidiation rhythm, is maintained in constant light, is not entrained to light-dark cycles, and is not well temperature compensated (Feldman and Hoyle, 1971). These differences led to the idea that two separate clock mechanisms control the two rhythms, and predicts that the oscillators that control these rhythms are desynchronized in constant conditions. In the wild; however, the two rhythms would be reset daily and this would likely result in synchronization. A multi-oscillator clock system has also been proposed in gonyaulax, cyanobacteria, Drosophila, and mice (Morse *et al.*, 1994; Nair *et al.*, 2002). However, to date no components of a second oscillator have been described. Using *N. crassa* microarrays, we identified 3 ccgs that appeared to have a short period in the long

period *frq*$^{7}$ mutant strain (Figure 6). These data suggested that under constant environmental conditions the FRQ-based oscillator harboring the *frq*$^{7}$ mutation and the oscillator driving these rhythmic genes have defective coupling and are not synchronized. FRQ-independent rhythmicity of this subset of mRNAs was verified by showing that the rhythms persisted in a FRQ$^{-}$ cultures that were synchronized by a light to dark transfer (Figure 7). These ccgs may themselves be components of a light-responsive FRQ-less oscillator (FLO) or may reside in output pathways from the FLO. Despite the finding that these genes are rhythmic in the absence of FRQ, some level of influence of the FRQ-based oscillator on their expression was suggested by the differences in phasing of the peaks in *frq*$^{+}$ versus *frq*$^{7}$ strains following the shift from light to dark (Figure 6). This may reflect an altered coupling mechanism (Morgan *et al.*, 2000) between the mutated FRQ-based oscillator and the FLO that affects phase but not period. The 3 genes that were found to be rhythmic in the absence of FRQ represented the most obvious genes to investigate from the microarray data; however it is likely that other ccgs are rhythmic in the absence of FRQ. For example, some of the ccgs were found to cycle in opposite phases in *frq*$^{+}$ versus *frq*$^{7}$ strains (Table 3) and these provide excellent candidates for additional ccgs that are outputs from the FRQ-less oscillator. A similar microarray study in Drosophila heads identified 4 out of 22 cycling genes that were rhythmic in a strain that lacked the central oscillator gene *period* and included the clock-associated gene *vrille* (Lin *et al.*, 2002).

In summary, our microarray data reveals that control of outputs by the circadian clock in *N. crassa* is complex, and involves regulation by more than one oscillator. These data show consistent reproducibility (Figure 8). The identification of ccgs that are rhythmic when the FRQ-based oscillator is absent provides powerful new tools for the identification of the FLO components and for understanding how a multi-oscillator system functions to control rhythmic gene expression.

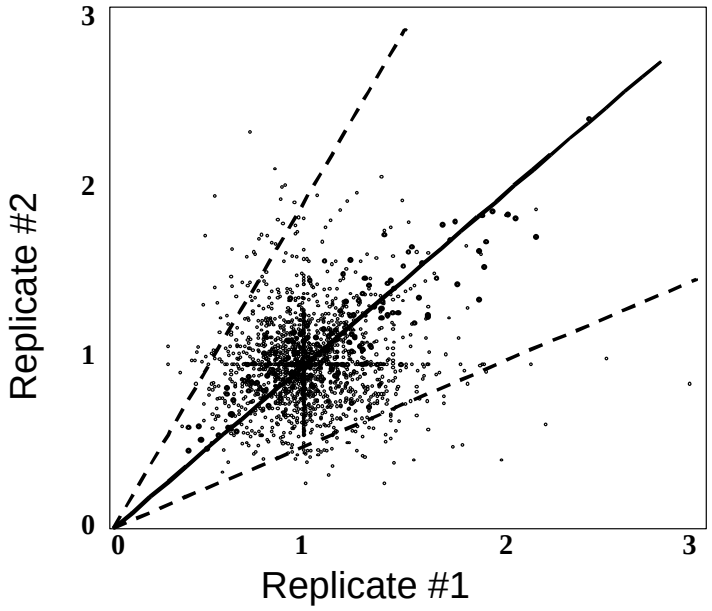


**Figure 8**. **Assessment of microarray reproducibility.**

A representative scatter plot using GeneSpring software showing measurements from the average of two spots (from an individual slide with the duplicated array) from one experiment plotted against the average of two spots from a replicate experiment in *frq*$^{+}$ strains at CT18. The dashed line represents a difference of a factor of 2 in intensity between the two hybridizations. Spots that fall outside of this range did not pass the imposed restrictions and were not analyzed further in this study.

# CHAPTER III

# OVEREXPRESSION OF WHITE COLLAR–1 (WC-1) ACTIVATES CIRCADIAN CLOCK-ASSOCIATED GENES, BUT IS NOT SUFFICIENT TO INDUCE MOST LIGHT-REGULATED GENE EXPRESSION IN *Neurospora crassa*[*]

## Overview

Many processes in fungi are regulated by light, but the molecular mechanisms are not well understood. The WHITE COLLAR-1 (WC-1) protein is required for all known blue-light responses in *Neurospora crassa*. In response to light, WC-1 levels increase and the protein is transiently phosphorylated. To test the hypothesis that the increase in WC-1 levels after light treatment is sufficient to activate light-regulated gene expression, we used microarrays to first identify genes that respond to light treatment. We then overexpressed WC-1 in dark-grown tissue and used the microarrays to identify genes regulated by an increase in WC-1 levels. We found that 3% of the genes were responsive to light, while 7% of the genes were responsive to WC-1 overexpression in the dark. However, only 4 out of 22 light-induced genes were also induced by WC-1 overexpression, demonstrating that changes in the levels of WC-1 are not sufficient to activate all light-responsive genes. The WC proteins are also required for circadian rhythms in dark-grown cultures and for light entrainment of

[*] Reprinted with the permission of Blackwell publishing from: Z.A. Lewis, A. Correa, C. Schwerdtfeger, K.L. Link, X.Xie, R.H. Gomer, T.Thomas, D.J. Ebbole, and D. Bell-Pedersen. (2002) Overexpression of White Collar-1 (WC-1) activates circadian clock-associated genes, but is not sufficient to induce most light-regulated gene expression in *Neurospora crassa*. Molecular Microbiology 45(4), 917-931. © 2002 by Blackwell Publishing

the circadian clock, and WC-1 protein levels show a circadian rhythm in the dark. We found that representative samples of the mRNAs induced by overexpression of WC-1 show circadian fluctuations in their levels. These data suggest that WC-1 can mediate both light and circadian responses, with an increase in WC-1 levels affecting circadian clock-responsive gene regulation and other features of WC-1, possibly its phosphorylation, affecting light-responsive gene regulation.

**Introduction**

The filamentous fungus *Neurospora crassa* is a model organism for studies of blue light perception and signal transduction. Blue light regulates several developmental and morphological processes in *N. crassa*, including the induction of the synthesis of carotenoids in mycelia, the formation of asexual spores, and the resetting of the circadian clock. The regulation of these processes occurs primarily at the level of gene expression, and several blue light-regulated genes have been identified (Lauter, 1997; Linden *et al.*, 1997). Blue light responses are abolished in mutations of two genes, *white collar-1* (*wc-1)* and *wc-2*, demonstrating that the products of these genes function in blue light signal transduction (Ballario and Macino, 1997; Degli-Innocenti and Russo, 1984; Harding and Turner, 1981).

WC-1 and WC-2 are nuclear proteins (Cheng *et al.*, 2001b; Denault *et al.*, 2001; Schwerdtfeger and Linden, 2000) that bear sequence similarity to the GATA zinc-finger family of transcription factors found in fungi and vertebrates

(Ballario *et al.*, 1996; Linden and Macino, 1997). WC complexes bind to consensus GATA elements within the promoters of blue light-regulated genes in *N. crassa* (Carattoli *et al.*, 1994). Both WC-1 and WC-2 contain PAS domains which are required for the proteins to homodimerize and heterodimerize *in vitro* and *in vivo* (Ballario *et al.*, 1996; Ballario *et al.*, 1998; Cheng *et al.*, 2002). The *wc-1* and *wc-2* genes are themselves induced by light (Ballario *et al.*, 1996; Linden and Macino, 1997). This induction results in a transient increase in WC-1 protein levels, but little or no change in the levels of WC-2 (Schwerdtfeger and Linden, 2000; Talora *et al.*, 1999). Several experiments have demonstrated that WC-2 is not limiting in cells (Cheng *et al.*, 2001b; Denault *et al.*, 2001). WC-2 is always present in the nucleus at levels greatly in excess of WC-1 (Denault *et al.*, 2001). Together, these data have supported a model in which heterodimers of WC-1 and WC-2 regulate most, if not all, light-induced gene expression in *N. crassa* (Linden *et al.*, 1997; Talora *et al.*, 1999).

Blue light is also involved in resetting the *N. crassa* circadian clock (Dharmananda, 1980; Fritz *et al.*, 1989; Nakashima, 1982). Circadian oscillators are composed of interlocked feedback loops that contain both positive and negative elements and function to control a wide variety of rhythmic processes in organisms ranging from bacteria and fungi to plants and animals. In *N. crassa*, the rhythmic process that is most widely recognized is the daily production of asexual conidiospores (Pittendrigh *et al.*, 1959; Sargent *et al.*, 1966). Within known oscillators, the positive elements activate transcription of the negative

elements and the negative elements then inhibit their own expression through interference with the activity of the positive elements (Young and Kay, 2001). In *N. crassa*, the blue light phototransduction components, WC-1 and WC-2, constitute the positive elements of the FRQ-based oscillator (Loros and Dunlap, 2001). WC-1 and WC-2 heterodimerize and activate expression of the *frequency (frq)* gene. Two forms of the FRQ protein then feed back to repress transcription of *frq*. This negative regulation likely occurs through interaction of FRQ with the WC proteins, thus blocking their positive activation of *frq* transcription (Cheng *et al.*, 2001b; Denault *et al.*, 2001). Along with the negative role of FRQ in the feedback loop, recent experiments have shown that FRQ positively regulates the levels of WC-1 and WC-2, forming interlocked loops (Cheng *et al.*, 2001b; Lee *et al.*, 2000). This circadian oscillator functions in constant darkness, in the absence of any signals from the external environment. Consistent with a role for the WC proteins in circadian rhythms in the dark, both proteins are found in the nucleus in dark-grown cultures (Schwerdtfeger and Linden, 2000).

In nature, light and temperature cycles act to synchronize the endogenous circadian clock to local time, with light typically considered to be the dominant clock- resetting stimulus (Pittendrigh, 1960). Resetting the clock each day allows organisms to predict and prepare for environmental changes and to coordinate and partition activities to the appropriate times of day. Light acts to reset the clock through a photoreceptor-mediated signal cascade to rapidly alter

the activity of a central clock component. In fungi and mammals, light rapidly induces transcript levels of central oscillator components (Albrecht *et al.*, 1997; Crosthwaite *et al.*, 1995; Shearman *et al.*, 1997; Shigeyoshi *et al.*, 1997), whereas in flies light acts to cause destruction of the clock component TIM (Hunter-Ensor *et al.*, 1996; Myers *et al.*, 1996; Zeng *et al.*, 1996). In *N. crassa*, light acts to rapidly induce the levels of *frq* transcripts and results in setting the clock to subjective day, the time when *frq* message levels peak (Crosthwaite *et al.*, 1995). The light response of *frq* requires the products of the *wc-1* and *wc-2* genes (Collett *et al.*, 2002; Crosthwaite *et al.*, 1997). Thus, *wc-1* and *wc-2* are required for both circadian rhythms in the dark and for light entrainment of the *N. crassa* FRQ-based oscillator.

Recent findings suggest that the WC proteins function differently to regulate light responses of clock-associated genes and other photoinducible genes (Collett *et al.*, 2002; Merrow *et al.*, 2001). Examination of light induction in several *wc-2* alleles, including those resulting in amino acid substitutions within the Zn-finger domain, revealed that *frq* is photoinducible in these strains, while a gene involved in carotenoid biosynthesis (*al-3*) is not (Collett *et al.*, 2002). These data, and observations that mutant strains lacking WC-1 and WC-2 are photoblind (Harding and Jr, 1980; Linden *et al.*, 1997), suggest that while the WC proteins are required for normal light responses, more complex mechanisms may allow discrete regulation of light-controlled gene expression in *N. crassa.* These distinctions may be reflected in the interaction of the WC proteins with

other clock or light signaling factors, or in the state of modification of the proteins. In addition to increasing levels of WC-1, light also results in phosphorylation of both WC proteins (Schwerdtfeger and Linden, 2000). The light-dependent phosphorylation of WC-1 is transient, whereas phosphorylation of WC-2 is stable in constant light. The transient phosphorylation of WC-1 corresponds with the transient induction of some light-regulated genes; however, some light-induced genes are expressed for a long time, corresponding to the length of time that WC-1 levels are high after light induction (Linden *et al.*, 1997; Schwerdtfeger and Linden, 2000, 2001).

To determine if overexpression of WC1 in the dark activates light-responsive genes, we have examined the expression of a subset of the *N. crassa* genome on microarrays using RNA isolated from cells that were photoinduced and in cells that overexpress WC-1 in the dark. We find that most light-induced genes are not affected by WC-1 overexpression and conversely that most genes affected by WC-1 overexpression are not induced by light. These results clearly establish that overproduction of WC-1 is not sufficient for most blue light signal transduction. Several of the genes that responded to WC-1 overexpression were found to express RNAs which accumulate rhythmically, suggesting that they are regulated by the circadian clock. These data provide support for models that invoke distinct mechanisms for regulating clock-specific gene expression and light-induced gene expression in *N. crassa*.

## Results

*Identification of novel light induced genes in* N. crassa

To investigate the dual role of WC-1 in light responses and in circadian rhythms, we used microarrays to identify genes in *N. crassa* that show altered expression patterns following exposure of mycelia to light. Each microarray contained 1778 probe sets representing 1343 unique *N. crassa* genes. This corresponds to roughly 1/7 of the *N. crassa* genes (Nelson *et al.*, 1997).

Microarrays were hybridized with Cy3-labeled cDNA representing mRNA from mycelia kept in the dark (time 0) and mycelia exposed to constant light for 30, 60, 120, and 240 min. The data from two experiments (4 spots) was combined to provide an average intensity, and following application of minimum intensity requirements (see Experimental Proceedures), 669 of the 1778 probe spots were considered reliable and further analyzed. We identified 32 pairs of probe spots that showed at least a 2-fold increase in mRNA levels following exposure to light (Figure 9 and Table 6). Of these 32 probe spots, 10 were either PCR products or plasmids containing *N. crassa ccg-2, con-10* and *con-6* that had been spotted on the arrays as controls and some ESTs were represented more than once. Thus, the 32 probe pairs represented 22 unique genes or approximately 3% of the genes that passed our restrictions. Most of the genes analyzed that were previously identified as being light-inducible genes, and which passed our minimum intensity restrictions, were identified on the arrays as being induced by light, thus validating our experimental methods.

These included *ccg-2, con-10, con-6,* and *vvd* (Heintzen *et al.*, 2001; Linden *et al.*, 1997) (Figure 9A).

Using GeneSpring clustering software, we organized the light-induced genes into one of three classes based on their expression profiles. Class 1L genes (10 probe spots representing 8 unique genes), including *vvd* and *con-10,* were rapidly induced by light, reaching peak levels 30-60 min after transfer to constant light and returning to low levels by 120 min of light exposure (Figure 9B and Table 6). Class IIL genes (12 probe spots representing 9 unique genes, including 1 chimeric clone), including *con-6*, were induced more gradually. These genes peaked 60-120 min after transfer to constant light and began to decline by 240 min after exposure to light (Figure 9B and Table 6). Class IIIL genes (10 probe spots representing 5 unique genes), including *ccg-2*, showed an early peak at 60 min, reduced mRNA levels at 120 min and then increased levels at 240 min following light induction (Figure 9B and Table 6). A fourth class of genes were repressed by light. However, this repression occurred in only one of the two light induction experiments; therefore, these genes were not analyzed further in this study.

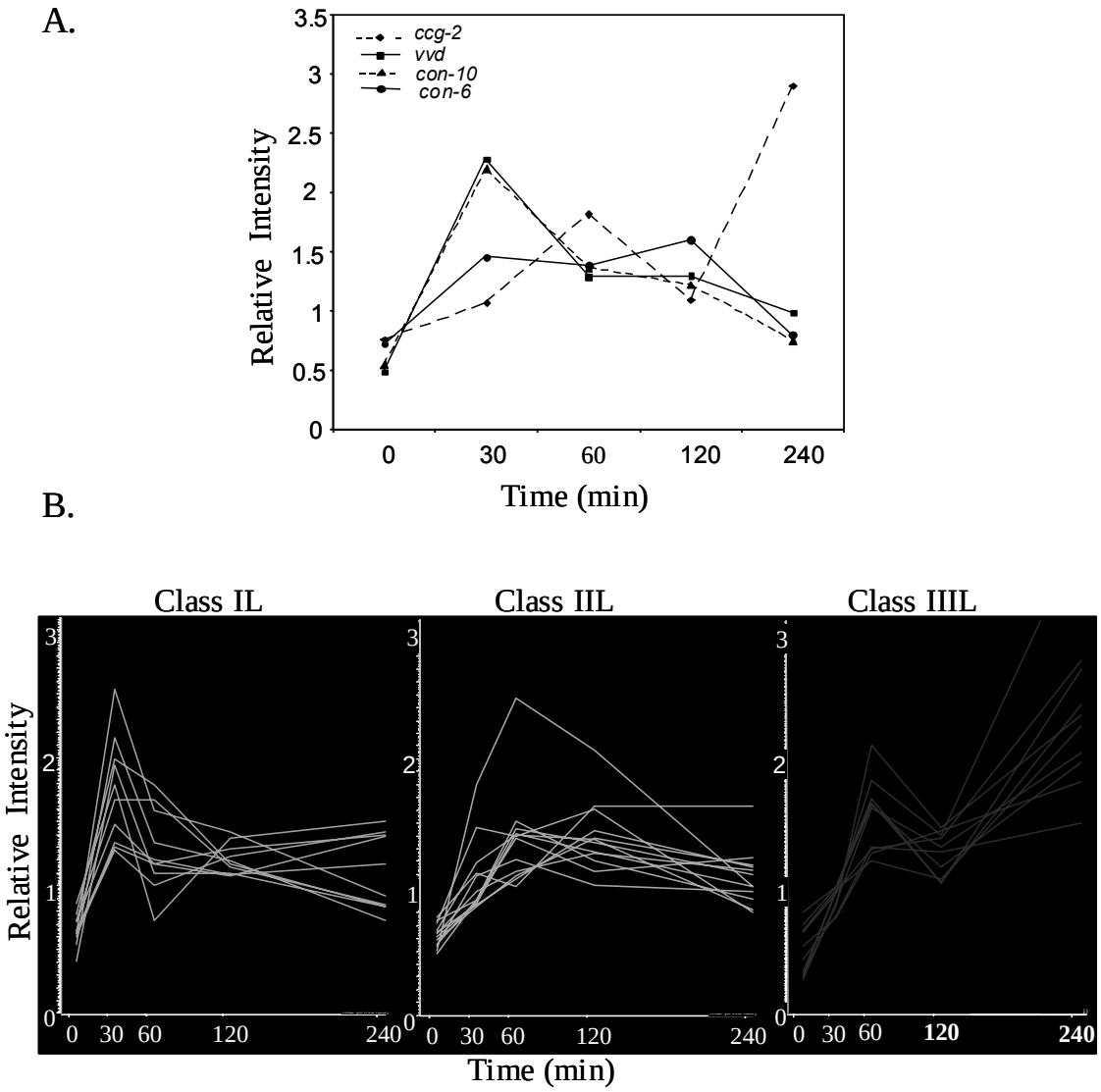


**Figure 9. Light-induced genes are discretely regulated in *N. crassa*.**

A) Microarray expression profiles of the known light induced genes *vvd* (Heintzen *et al.*, 2001), *eas (ccg-2*) (Bell-Pedersen *et al.*, 1996a; Lauter, 1991, 1992), *con-10* (Lauter and Yanofsky, 1993) and *con-6* (Lauter and Yanofsky, 1993) are plotted. Relative mRNA levels determined from microarrays in dark grown cultures (time 0), as well as cultures exposed to light for 30, 60, 120, and 240 min are shown. The values for each time point are the mean of 4 experimental values. B) Light induced genes identified using microarrays were grouped into three distinct classes (IL, IIL, and IIIL) based on their expression profiles. Only those genes showing a significant and minimum 2-fold change in relative mRNA levels following exposure to light were included in the cluster analysis. The values shown for each time point are the mean of 4 experimental values representing 2 spots from duplicate arrays.

We examined the light-induced genes for similar functions within and among the classes; however, no obvious similarities were found. This may reflect the fact that the biochemical functions of many of the proteins encoded by the genes are unknown.

**Table 6. *N. crassa* genes induced following light treatment.**

| CLASS IL[1] | PREDICTED ORF[2] | FOLD CHANGE | BLAST MATCH[3] | ORGANISM | ACCESSION #[4] |
|---|---|---|---|---|---|
| W07H6 | none | 4.736 | VVD | *N. crassa* | 12831202 |
| W17G3 | NCU06420.1 | 4.074 | NO BLAST HITS | | |
| CON-10 | NCU07325.1 | 4.039 | CON-10 | *N. crassa* | 168172 |
| PCON 10 | NCU07325.1 | 2.559 | CON-10 (ENTIRE PLASMID PCON-10) | *N. crassa* | 168782 |
| NC2B7 | NCU03757.1 | 2.306 | RPL4A PROBABLE RIBOSOMAL PROTEIN | *N. crassa* | 12718316 |
| SM2D6 | NCU01754.1 | 2.256 | ALCOHOL DEHYDROGENASE I | *N. crassa* | 14285342 |
| SC5B8 | NCU09089.1 | 2.230 | NO BLAST HITS | | |
| SC2A5 | NCU02431.1 | 2.160 | HYPOTHETICAL ZINC FINGER PROTEIN | *S. pombe* | 7492092 |
| SC5D4 | NCU09089.1 | 2.070 | NO BLAST HITS | | |
| NC1G8 | NCU00567.1 | 2.061 | ARG-6 | *N. crassa* | 1438871 |
| **CLASS IIL** | | | | | |
| SM4C4 | NCU07267.1 | 4.974 | BLI-3 | *N. crassa* | 602074 |
| SM1F5 | NCU06660.1 | 2.990 | HYPOTHETICAL PROTEIN | *C. elegans* | |
| SM3D3 | NCU06660.1 | 2.369 | UPF005 FAMILY PROTEIN | *S. pombe* | 512943 |
| NP6H2 | NCU06660.1 | 2.329 | NO BLAST HITS | | |
| SM1C8 | NCU05166.1 | 2.256 | ABC TRANSPORTER Atr4 | *M. graminicola* | 13183712 |
| SM2A10 | NCU00315.1 | 2.256 | HOMOLOGY TO RAT L29 RIBOSOMAL PROTEIN | *S. cerevisiae* | 14318557 |
| SP1D5 | NCU02470.1 | 2.238 | FRUITING BODY PROTEIN SC7 PRECURSOR | *S. commune* | 548902 |
| CON-6 | NCU08769.1 | 2.224 | CON-6 | *N. crassa* | 415713 |
| PCON 6 | NCU08769.1 | 2.155 | CON-6 (ENTIRE PLASMID PCON-6) | *N. crassa* | 415713 |
| SP1A8 | NCU09917.1 | 2.137 | NO BLAST HITS | | |
| NP4B6 | | 2.025 | CHIMERIC CDNA | | |
| W06G11 | NCU00979.1 | 2.006 | 60S ACIDIC PROTEIN P2 | *A. alternata* | 1173071 |

**Table 6 (continued)**

| CLASS IIIL[1] | PREDICTED ORF[2] | FOLD CHANGE | BLAST MATCH[3] | ORGANISM | ACCESSION #[4] |
|---|---|---|---|---|---|
| CCG-2 | NCU08457.1 | 7.953 | CCG-2 | *N. crassa* | 422231 |
| NC5E3 | NCU08457.1 | 7.631 | CCG-2 | *N. crassa* | 422231 |
| SC2A2 | NCU08457.1 | 3.838 | CCG-2 | *N. crassa* | 422231 |
| CCG-2 1/2 | NCU08457.1 | 6.854 | CCG-2 DNA DILUTION | *N. crassa* | 422231 |
| CCG-2 1/8 | NCU08457.1 | 6.027 | CCG-2 DNA DILUTION | *N. crassa* | 422231 |
| NM1C3 | NCU09152.1 | 3.473 | HYPOTHETICAL PROTEIN B24M22.120 | *N. crassa* | 11359491 |
| NC1A11 | NCU07282.1 | 3.065 | NO BLAST HITS | | |
| W13E5 | NCU08705.1 | 3.541 | RELATED TO SMALL S PROTEIN | *N. crassa* | 11359674 |
| NM1B8 | NCU08949.1 | 8.523 | SPAC15E1.02c HYPOTHETICAL PROTEIN | *S. pombe* | 7490820 |
| W08H11 | NCU08949.1 | 3.472 | SPAC15E1.02c HYPOTHETICAL PROTEIN | *S. pombe* | 7490820 |

[1]The *N. crassa* ESTs identified in each class are from the Neurospora Genome Project, University of New Mexico (http://www.unm.edu/~ngp/).

[2]The predicted ORFs are from the Whitehead Institute Neurospora Sequencing Project (http://www-genome.wi.mit.edu/annotation/fungi/neurospora/).

[3]The blast matches represent the most significant match in the data bases as predicted by the Whitehead Institute (minimum e-value $1e^{-5}$) and verified by us using BLAST searches of the NCBI sequence data bases. minimum e-value $1e^{-5}$

[4]The accession numbers for each blast match are from the NCBI sequence data base (http://www.ncbi.nlm.nih.gov/).

*Identification of genes regulated by WC-1 overexpression*

To identify candidate genes that are regulated by levels of WC-1, we used strain 161-8 *wc-1* (qa-*wc-1*) containing a copy of the *wc-1* gene under control of the quinic acid (QA) inducible qa2 promoter (Cheng *et al.*, 2001b; Giles *et al.*, 1985). Overexpression of WC-1 in the dark results in a transient increase in *frq* mRNA, which peaks 4 h post induction (Cheng *et al.*, 2001b). This is consistent with a model in which a complex of WC-1 and WC-2 act to induce expression of

*frq* and suggests that WC-2 is not limiting in cells (Cheng *et al.*, 2001b; Denault *et al.*, 2001).

The *wc-1* (qa-*wc-1*) strain was incubated in the dark for 24 h and mycelia were harvested before treatment with QA (time 0) and 1, 2, 4, and 10 h following transfer to medium containing QA. Transfer to QA medium resulted in a significant increase in the levels of *wc-1* mRNA in the *wc-1* (qa-*wc-1*) strain, but not in the *bd* strain (Figure 10A). High level QA induction of WC-1 protein in dark grown cultures in this strain has been shown previously (Cheng *et al.*, 2001b). mRNA was isolated from each culture, reverse transcribed, labeled with Cy3 and hybridized to microarrays (Figure 10B). As a control for QA-alone effects on gene expression and to provide an additional control for genes affected by the absence of WC-1 at time 0, RNA was isolated from the *bd* (WC-1+) strain harvested just before (time 0) and 1, 2, 4, and 10 h following transfer to QA medium and was used to probe the microarrays. 940 of the 1778 DNA spot pairs passed our minimum intensity requirements in the *wc-1* (qa-*wc-1*) experiment, and 1013 of the 1778 passed our requirements in the *bd* control experiment (see Experimental Procedures). Importantly, all of the genes that showed a change in *wc-1* (qa-*wc-1*) passed the restrictions in the *bd* strain. Of these genes, 71 ESTs showed at least a 2-fold increase in RNA levels following transfer to QA medium in *wc-1* (qa-*wc-1*), but not in the *bd* strain (Table 7). These 71 ESTs represent 65 unique genes (including 5 chimeras) or 7% of the genes that passed our restrictions for the *wc-1* (qa-*wc-1*) experiment.

The 71 candidate genes were grouped based on their expression profiles into three classes. Class IW genes (9 probe spots representing 8 unique genes) peaked 1-2 h after transfer to QA medium, with RNA levels declining by 10 h (Figure 10B and Table 7). For several genes in this class, a second smaller peak was observed at 4 h. Class IIW genes (38 probe spots representing 35 unique genes) showed a major peak at 4 h, with RNA levels returning to pre-induced levels by 10 h (Figure 10B and Table 7). This latter pattern of expression is similar to that previously reported for *frq* (Cheng *et al.*, 2001b). Although *frq* was represented on the array, expression of *frq* was very low, and thus, the spots were not reliable and failed to pass the minimum value restrictions applied. The Class IIIW genes (24 probe spots representing 22 unique genes) typically peaked at 4 h following induction of WC-1, and their levels remained elevated or even increased at 10 h after transfer to QA medium (Figure 10B and Table 7). As in the light-induction experiments, we identified a group of genes that were down-regulated following QA addition. However, these genes were only repressed in one of the two experiments, and therefore were not analyzed further in this study.

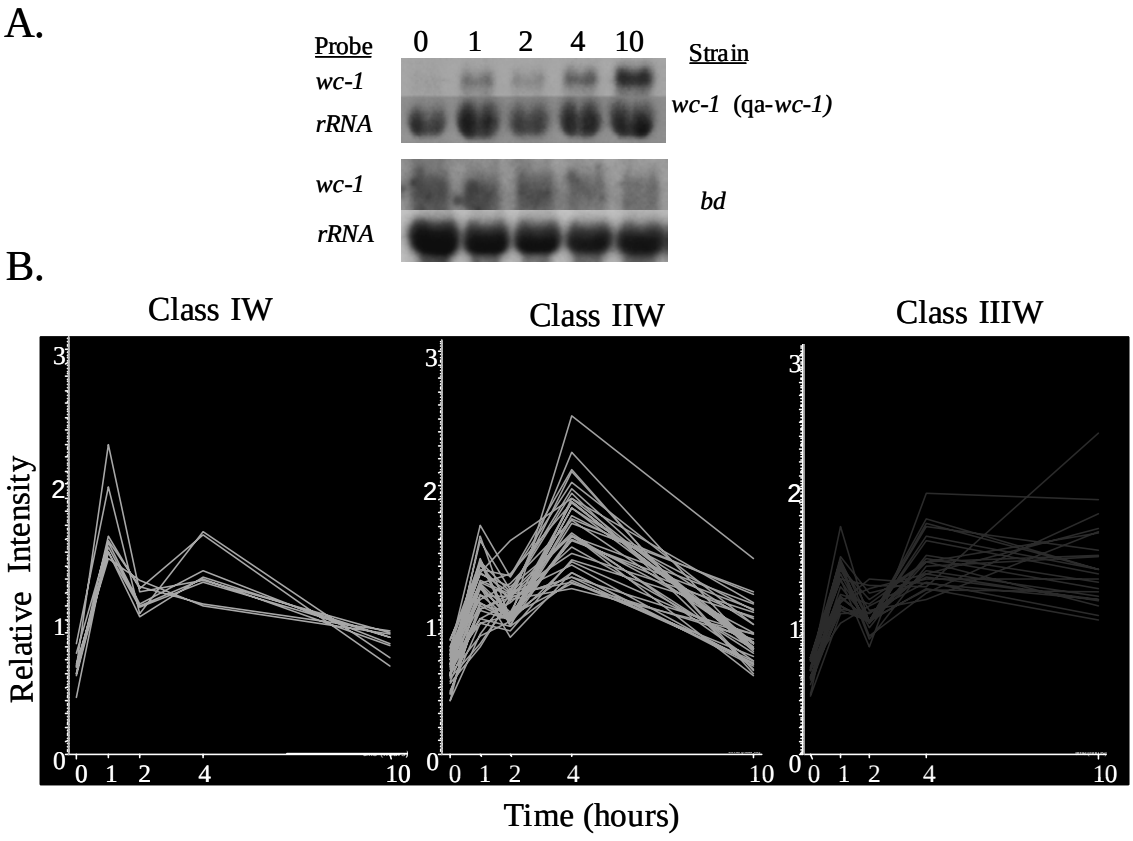


**Figure 10. Overproduction of *wc-1* affects the expression of genes with class-specific profiles in *N. crassa*.**

A) Northern analysis of *wc-1* following the addition of QA to *wc-1* (qa-*wc-1*) (top) and *bd* (bottom) strains. RNA was isolated from mycelia harvested just before transfer to fresh medium containing 10mM QA (0) and 1, 2, 4, and 10 h following induction of *wc-1* by QA. The numbers (in hours) above each lane indicates the time following transfer to QA-containing medium. Each blot also was probed with *rRNA* as a loading control. B) Expression profiles of genes that were induced by QA in the *wc-1* (qa-*wc-1*) strain, but not in the *bd* strain. Genes that showed a minimum 2-fold change following *wc-1* induction by QA were placed into 1 of 3 classes (IW, IIW, and IIIW) based on their expression profiles using cluster analysis. The values shown for each time point represent the mean of 4 experimental values representing 2 spots from duplicate arrays.

No genes, other than *frq*, were known to be directly affected by WC-1 overexpression; however as indicated above, the levels of *frq* expression were very low and so could not be used to verify the array protocol. A few EST's that were duplicated in the unigene set were independently identified in the array probing (e.g. Class IIW EST's encoding a protein with similarity to the *Aspergillus parisiticus* NMT-1 protein are all the same gene) (Table 7).

**Table 7. *N. crassa* genes induced during WC-1 overexpression in the dark.**

| CLASS IW[1] | PREDICTED ORF | FOLD CHANGE | BLAST MATCH | ORGANISM | ACCESSION # |
|---|---|---|---|---|---|
| W17B4 | NCU06185.1 | 2.548 | CHIMERIC CDNA | | |
| NC4A2 | NCU05526.1 | 2.400 | HOMOCITRATE SYNTHASE | *N. crassa* | 7672738 |
| SC2F2 | NCU03608.1 | 3.528 | ilv-2 GENE (ALPHA-KETO-BETA-HYDROXYACYL REDUCTOISOMERASE) | *N. crassa* | 168821 |
| W01G11 | NCU04502.1 | 2.092 | NO BLAST HITS | | |
| SC3E2 | NCU05259.1 | 2.543 | OLE-1 GENE | *H.capsulatum* | 757859 |
| SC2H9 | NCU02252.1 | 3.718 | PHOSPHOGLYCEROMUTASE | *A. oryzae* | 9955875 |
| NC2D12 | NCU02252.1 | 2.156 | PHOSPHOGLYCEROMUTASE | *A. oryzae* | 9955875 |
| NC2D1 | NCU07914.1 | 2.270 | PKG GENE (ATP;3-PHOSPHO-D-GLYCERATE-1-PHOSPHOTRANSFERASE) | *N. crassa* | 3051 |
| W01H10 | NCU06464.1 | 2.713 | Yk1056cp | *S. cerevisiae* | 6322794 |
| **CLASS IIW** | | | | | |
| NP3F8 | NCU06110.1 | 4.736 | CY PBP37 PROTEIN | *N. crassa* | 9801262 |
| SC2B5 | NCU06432.1 | 4.224 | 40S RIBOSOMAL PROTEIN | *B. graminis* | 12229899 |
| NC3E8 | NCU06661.1 | 3.579 | 60S RIBOSOMAL PROTEIN L22 | *S. pombe* | 12644397 |
| NC1C9 | NCU09109.1 | 2.766 | 60S RIBOSOMAL PROTEIN L33-A | *S. cerevisiae* | 15214241 |
| NM5F12 | | 2.389 | CHIMERIC CDNA | *S. pombe* | 6094179 |
| W10A5 | NCU03748.1 | 2.182 | CHIMERIC CDNA | | |
| NC1A10 | | 2.089 | CHIMERIC CDNA | | |
| NM6F5 | NCU01692.1 | 2.264 | CITRATE SYNTHASE | *N. crassa* | 168774 |
| NC1D10 | NCU06512.1 | 2.350 | COBALAMIN-INDEPENDENT METHIONINE SYNTHASE | *A. nidulans* | 8927554 |
| W10F8 | NCU05810.1 | 2.199 | CPC-2 | *N. crassa* | 971565 |
| NC3B5 | NCU06110.1 | 3.495 | CY PBP37 PROTEIN | *N. crassa* | 9801262 |
| W01F1 | NCU03038.1 | 2.745 | CYTOPLASMIC RIBOSOMAL PROTEIN S13 | *P. ginseng* | 8131699 |

**Table 7 (continued)**

| CLASS IIW[1] | PREDICTED ORF | FOLD CHANGE | BLAST MATCH | ORGANISM | ACCESSION # |
|---|---|---|---|---|---|
| W17B4 | NCU06185.1 | 2.548 | CHIMERIC CDNA | | |
| SC5E7 | NCU10051.1 | 2.306 | FLAVOHEMOGLOBIN | *F. oxysporum* | 3551511 |
| SC7G7 | NCU09269.1 | 2.133 | Gsp1 | *C. albicans* | 8698689 |
| W09C7 | NCU02905.1 | 3.406 | HOMOLOGY TO E.COLI L14 | *S. cerevisiae* | 6319384 |
| NM5H11 | NCU00475.1 | 2.569 | HOMOLOGY TO RAT RIBOSOMAL PROTEIN S18 AND E.COLI S13 | *S. cerevisiae* | 6320658 |
| NC5F2 | NCU06743.1 | 2.353 | HOMOLOGY TO RAT S10 RIBOSOMAL PROTEIN | *S. cerevisiae* | 6323886 |
| NC3G11 | NCU07182.1 | 2.568 | HOMOLOGY TO RAT S24 RIBOSOMAL PROTEIN | *S. cerevisiae* | 6320918 |
| W01G6 | NCU04142.1 | 2.343 | HSP-80 | *N. crassa* | 4241664 |
| SC2A5 | NCU02431.1 | 2.121 | HYPOTHETICAL ZINC FINGER PROTEIN | *S. pombe* | 7492092 |
| NC1C6 | NCU07318.1 | 2.221 | MANNITOL 1-PHOSPHATE-5-DEHYDROGENASE | *N. crassa* | 400286 |
| W07C10 | NCU09345.1 | 4.085 | NMT-1 PROTEIN | *A. parasiticus* | 1171741 |
| W09C3 | NCU09345.1 | 3.121 | NMT-1 PROTEIN | *A. parasiticus* | 1171741 |
| W06B10 | NCU09345.1 | 2.693 | NMT-1 PROTEIN | *A. parasiticus* | 1171741 |
| W10E9 | NCU09345.1 | 2.086 | NMT-1 PROTEIN | *A. parasiticus* | 1171741 |
| NM5B6 | NCU00489.1 | 2.949 | NO BLAST HITS | | |
| SC5D4 | NCU09089.1 | 2.169 | NO BLAST HITS | | |
| W07A4 | NCU06326.1 | 2.672 | PECTATE LYASE | *C. gloeosporioides* | 5453412 |
| NC2B7 | NCU03757.1 | 3.438 | PROBABLE RIBOSOMAL PROTEIN RPL4A | *N. crassa* | 12718316 |
| SC6A9 | NCU08344.1 | 3.328 | PUTATIVE 60S RIBOSOMAL PROTEIN | *C. gloeosporioides* | 11125659 |
| W13A10 | NCU02708.1 | 3.155 | RIBOSOMAL PROTEIN L35 | *O. ulmi* | 14165210 |
| NC3G1 | NCU06226.1 | 2.372 | RIBOSOMAL PROTEIN S23A | *H. sapiens* | 404015 |
| SC6E7 | NCU02181.1 | 3.041 | RIBOSOMAL PROTEIN S4 HOMOLOG | *Y. lipolytica* | 6094184 |
| W10G12 | NCU02274.1 | 2.176 | SERINE HYDROXYMETHYL TRANSFERASE | *N. crassa* | 168906 |
| W07H5 | NCU08976.1 | 3.139 | SUR PROTEIN FAMILY MEMBER | *C. elegans* | 6434519 |
| W07F11 | NCU08409.1 | 2.354 | TRP-3 (TRYPTOPHAN SYNTHASE) | *N. crassa* | 168915 |
| NC1A3 | NCU01207.1 | 2.532 | vma-1 (VACUOLAR ATPASE SUBUNIT) | *N. crassa* | 168925 |
| **CLASS IIIW** | | | | | |
| SC2G2 | none | 3.980 | NO BLAST HITS | | |
| W08B8 | NCU08620.1 | 2.410 | 40S RIBOSOMAL PROTEIN S16 | *S. pombe* | 6094158 |
| W09H4 | NCU05599.1 | 3.381 | 40S RIBOSOMAL PROTEIN S28 | *S. pombe* | 1710760 |
| SM1F12 | NCU05599.1 | 2.130 | 40S RIBOSOMAL PROTEIN S33 | *S. pombe* | 1710760 |
| NC2C12 | NCU01949.1 | 2.750 | 40S RIBOSOMAL PROTEIN S9 | *P. anserina* | 1710780 |
| W01B9 | NCU01949.1 | 2.429 | 40S RIBOSOMAL PROTEIN S9 | *P. anserina* | 1710780 |

**Table 7 (continued)**

| CLASS IIIW[1] | PREDICTED ORF | FOLD CHANGE | BLAST MATCH | ORGANISM | ACCESSION # |
|---|---|---|---|---|---|
| W17E6 | NCU02509.1 | 2.312 | 60S RIBOSOMAL PROTEIN L11 | *S. pombe* | 5738531 |
| NC3C10 | NCU06843.1 | 2.924 | CHIMERIC CDNA | | |
| W13D4 | NCU07014.1 | 2.428 | CRP-3 RIBOSOMAL PROTEIN | *N. crassa* | 168795 |
| NC4G3 | NCU08963.1 | 2.271 | F28KF19.15 | *A. thaliana* | 6573778 |
| NC4F12 | NCU07807.1 | 2.141 | FRUCTOSE-BISPHOSPHATE ALDOLASE | *N. crassa* | 1703247 |
| SM2A10 | NCU00315.1 | 2.752 | HOMOLOGOUS TO RAT L29 RIBOSOMAL PROTEIN | *S. cerevisiae* | 14318557 |
| W10A1 | NCU00634.1 | 2.681 | HOMOLOGY TO MAMMALIAN L14 | *S. cerevisiae* | 6321786 |
| W01G9 | NCU08500.1 | 2.709 | HOMOLOGY TO MAMMALIAN S8 | *S. cerevisiae* | 6319399 |
| NC5E12 | NCU01776.1 | 2.018 | HOMOLOGY TO RAT L15 RIBOSOMAL PROTEIN | *S. cerevisiae* | 6323769 |
| W13B7 | NCU09711.1 | 2.125 | HYPOTHETICAL PROTEIN | *S. Pombe* | 6855453 |
| SC1F6 | none | 2.805 | NO BLAST HITS | | |
| W08C5 | NCU08017.1 | 2.103 | NO BLAST HITS | | |
| W08B2 | NCU07033.1 | 2.004 | NO BLAST HITS | | |
| W13A6 | NCU03702.1 | 2.101 | nop-1 (PROBABLE FIBRILLIN) | *N. crassa* | 11595586 |
| SC5A7 | NCU04656.1 | 2.361 | PUTATIVE TRANSPORTER | *S. pombe* | 3183364 |
| NC3E3 | NCU04779.1 | 2.284 | RIBOSOMAL PROTEIN L8 | *S. pombe* | 6094099 |
| SC1G8 | NCU07550.1 | 2.796 | TRIOSE PHOSPHATE ISOMERASE | *A. oryzae* | 9955867 |
| W17G06 | NCU03989.1 | 2.312 | Ypr011cp HYPOTHETICAL PROTEIN | *S. cerevisiae* | 6325268 |

[1]The columns in this table are as in Table 1.

These data provided some verification of our methods. However, to further validate the WC-1 overexpression microarray data, we chose representative candidates from each expression class and performed northern blot analysis. The Class IW gene NC4A2 encoding homocitrate synthase (Table 7) was chosen for northern analysis. Because regulation of homocitrate synthase by WC-1 would not have been expected, this provided a good test case for gene discovery using the microarrays (the same reasoning was used to select representative Class IIW and Class IIIW ESTs, below). The expression

profile from the array is typical of Class I genes (Figure 11A left panel). Northern analysis of NC4A2 expression in the *wc-1* (qa-*wc-1*) strain is in good agreement with the expression observed from the WC-1 array experiments and showed that this gene did not respond to the addition of QA in the *bd* strain (Figure 11B and C). These data suggest that NC4A2 is either directly or indirectly regulated by WC-1.

The EST NC1A3 was chosen as a representative Class IIW gene (Table 7). It is the *N. crassa vma-1* gene, encoding a subunit of the vacuolar ATPase (Bowman *et al.*, 1988). The microarray expression profile of NC1A3 from the WC-1 induction experiments is shown in Figure 11A (middle panel). Northern analysis in the *wc-1* (qa-*wc-1*) strain confirmed the expression pattern observed from microarray analysis (Figure 11B and C).

The Class IIIW EST SC1G8 shows similarity at the amino acid level (p= 4.00 $e^{-68}$) to *Aspergillus oryzae* triose phosphate isomerase, a glycolytic enzyme (Nakajima *et al.*, 2000). The microarray profile of SC1G8 is shown in Figure 11A (right panel).

Northern analysis of SC1G8 in the *wc-1* (qa-*wc-1*) strain revealed a significant induction in mRNA levels following WC-1 induction (Figure 11B). The levels at 10 h were slightly lower than that observed in the array data; however, the overall trend was similar to the microarray profile. Again, there was little increase in mRNA levels following transfer of the wild-type *bd* strain to QA medium suggesting this gene is indeed regulated by WC-1. In all cases, the

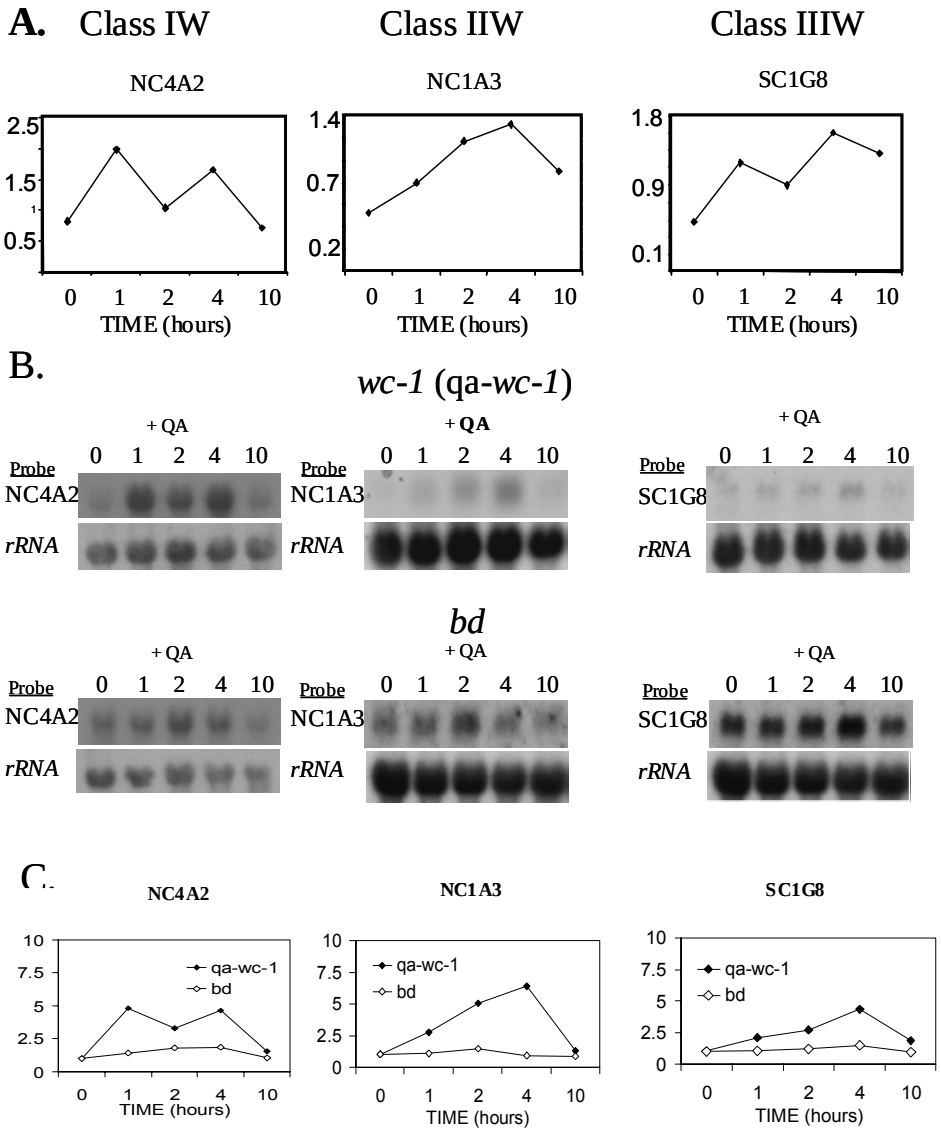


**Figure 11. Gene expression analyses of candidate *wc-1*-induced genes.**

A) The microarray expression profiles of a Class IW (left), a Class IIW (middle), and a Class IIIW (right) *wc-1*-induced candidate gene are shown. Relative mRNA levels were plotted as the mean value from 4 experimental values. B) Northern analyses of the candidate genes from A. Northern blots of Class I gene NC4A2 (left), Class IIW gene NC1A3 (middle), and Class IIIW gene SC1G8 (right) in *wc-1* (qa-*wc-1*) (top row), and in the wild-type *bd* strain (bottom row). RNA was isolated from mycelia harvested immediately before transfer to QA medium (0), and 1, 2, 4, and 10 h following transfer to QA medium. *rRNA* is shown as a loading control for each experiment. Northern blots were performed at least twice with similar results. C) Relative mRNA levels from the northern blots in panel B were quantitated by densitometry, normalized to rRNA, and plotted as relative band intensity versus time before or after QA induction.

high level of mRNA in the *bd* strain at time 0 compared to the *wc-1* strain (a time at which no WC-1 protein is produced) independently demonstrates that WC-1 is required for expression of these genes. No difference in the mRNA levels of a gene (*ccg-2)* that did not respond to WC-1 overexpression was observed at time 0 in the *bd* and *wc-1* (qa-*wc-1*) strains (data not shown). These data rule out the possibility that the differences at time 0 are the result of strain variation, but rather are due to the presence or absence of WC-1.

*Light-induced gene expression in* N. crassa *is not mediated by an increase in WC-1 levels*

To determine if light induces gene expression by simply increasing WC-1 levels, we compared the set of IL, IIL and IIIL genes with the set of IW, IIW and IIIW genes. All of the light-induced genes passed our minimum intensity criteria for inclusion in the WC-1 experiment with the exception of *vvd*, a gene known to be repressed in the dark (Heintzen *et al.*, 2001). We found that only 4 of the 22 unique light-induced genes were induced by WC-1 overexpression (Table 8). Three of the four light-induced genes were Class IL and Class IIW. The other gene, SM2A10 was Class IIL and Class IIIW.

**Table 8. Genes affected by both light treatment and by overexpression of WC-1 in the dark.**

| EST | CLASSES[1] | BLAST MATCH | ORGANISM | ACCESSION # |
|---|---|---|---|---|
| NC2B7 | IL, IIW | RPL4A PROBABLE RIBOSOMAL PROTEIN | *N. crassa* | 12718316 |
| SC2A5 | IL, IIW | HYPOTHETICAL ZINC FINGER PROTEIN | *S. pombe* | 7492092 |
| SC5D4 | IL, IIW | NO BLAST HITS | | |
| SM2A10 | IIL, IIIW | HOMOLOGY TO RAT L29 RIBOSOMAL PROTEIN | *S. cerevisiae* | 14318557 |

[1]The class designation of the light- and WC-1-responsive genes are described in the text and are shown in Figures 1 and 2.

We used northern assays to verify the comparisons of the light and WC-1 responsive genes. Class IIIL gene *ccg-2,* a well-described clock-controlled gene (Bell-Pedersen *et al.*, 1992) was induced following light treatment (Figure 12A left panel), but the levels of RNA did not increase following overexpression of WC-1 (Figure 12B left panel). Class IIIW gene, SM1F12 responded to WC-1 overexpression, peaking at 4 h (Figure 12B middle panel), but was unresponsive to light treatment (Figure 12A middle panel). In contrast, Class IL and IIW gene NC2B7 responded to light treatment, peaking at 30 min (Figure 12A right panel) and to WC-1 overexpression, peaking at 4 h (Figure 12B right panel).

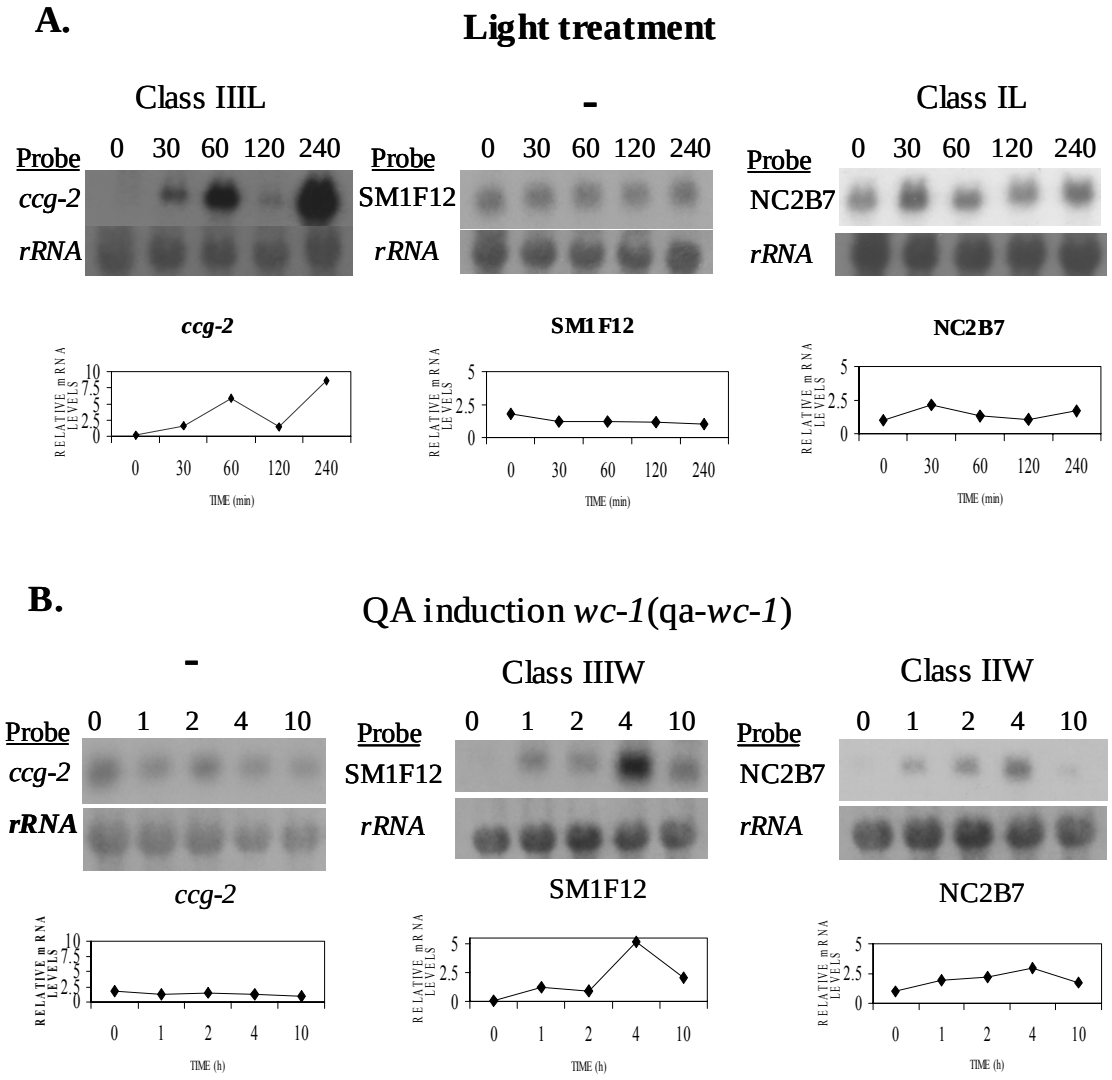


**Figure 12. Representative northern analysis of genes regulated by light, WC-1 overexpression or both.**

A) Northern blot assays of Class IIIL *ccg-2* (left), and Class IL NC2B7 (right) and the non-responsive gene SM1F12 (middle). Total RNA was isolated from dark grown cultures (time 0), as well as from cultures exposed to light for 30, 60, 120, and 240 min and hybridized with the indicated probes. *rRNA* was used as a loading control for each blot. The relative mRNA levels are plotted and are shown below each set of northerns. B) Northern blots of Class IIIW SM1F12 (middle), Class IIW NC2B7 (right), and the non-responsive *ccg-2* gene (left) are shown. RNA was isolated from mycelia harvested immediately before transfer to QA medium (0), and 1, 2, 4, and 10 h following transfer to QA medium. *rRNA* is shown as a loading control for each experiment, and the relative mRNA levels from the northerns are plotted below. For A and B, northern blots were performed at least twice with similar results.

In addition to light responses, WC-1 is involved in the circadian clock. Since most of the Class IW, IIW, and IIIW genes were not light-responsive, we examined whether they might be regulated by the circadian clock. As shown in Figure 13, four out of four selected WC-1-responsive genes (two from Class IIW and two from IIIW) had expression levels that were clearly rhythmic with a 22 h period in the dark. This is the period of the free running *N. crassa* clock (Loros and Dunlap, 2001). Furthermore, all of the genes peaked in the early morning, around CT0. Previous studies indicated that approximately 10% of the *N. crassa* genes are regulated by the clock (Bell-Pedersen *et al.*, 1996b). Thus, the probability that four out of four genes would be rhythmic is 0.01%. We also examined the Class IW-IIIW genes for rhythmic expression in microarrays assayed for rhythmically expressed genes (Correa, A., Lewis, Z. and Bell-Pedersen, D., unpublished data), and found that 51 out of the 71 ESTs showed patterns consistent with rhythmic-expression (data not shown). However, not all rhythmically expressed genes are affected by WC-1 overexpression as exemplified by *ccg-2*. We observed that *ccg-2* is slightly repressed following the addition of QA in the *wc-1* (qa-*wc-1*) strain (Figure 12B); however, this same repression occurs in the *bd* strain (data not shown), indicating that the response is due to the inducer, rather than WC-1 protein.

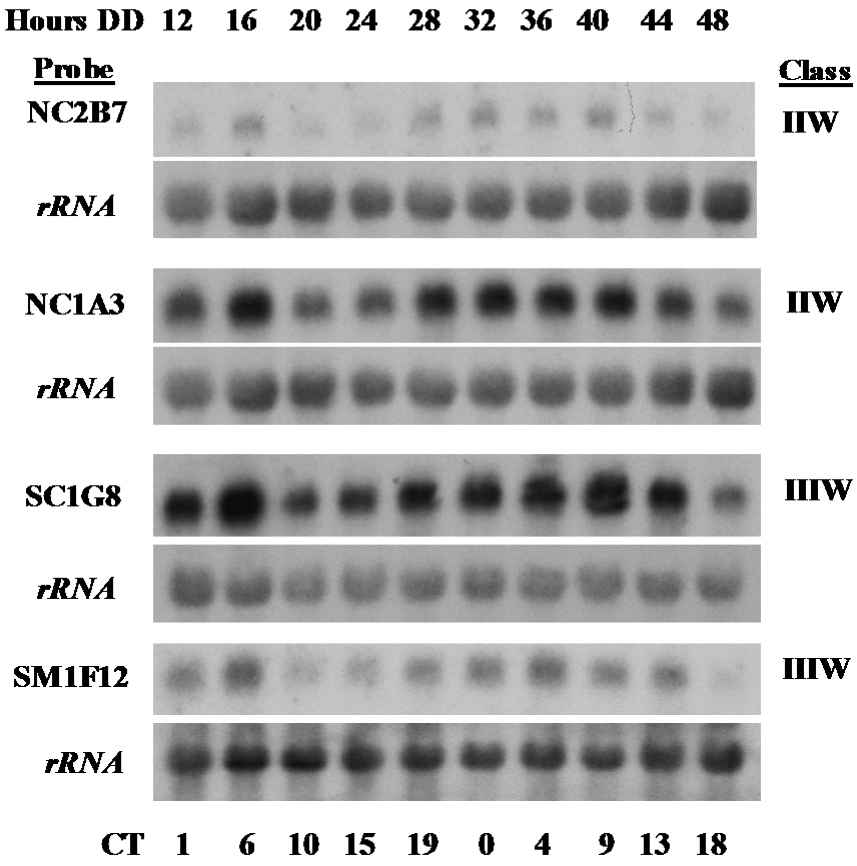


**Figure 13. The majority of WC-1 responsive genes are clock regulated.**

The steady-state levels of representative Class IIW (NC2B7 and NC1A3) and IIIW (SC1G8 and SM1F12) genes were assayed by northern blot analyses for rhythmicity. Liquid cultures of mycelia were grown in constant darkness and harvested after the indicated hours in the dark (Hours DD). The approximate circadian times (CT) at the time of harvests are indicated below the autoradiograms. Circadian time is used to normalize biological time in strains with different endogenous period lengths to 24 circadian hours per cycle. By convention, CT0 represents subjective dawn and CT12 represents subjective dusk. The probes are indicated on the right of each blot. *rRNA* is shown as a loading control. Similar results were obtained in at least 2 separate repetitions of these experiments.

## Discussion

In this study, we tested whether or not increased levels of WC-1 are sufficient to activate light responsive genes. Microarrays were used to identify genes that respond to light treatment and to overexpression of WC-1 in the dark. We identified genes that are induced by light and genes that are induced following overexpression of WC-1 in the dark. Surprisingly, only 4 genes were found to be regulated under both conditions, demonstrating that increased levels of WC-1 in cells are not sufficient to activate all aspects of the phototransduction pathway.

Our results suggested that about 3% of the *N. crassa* genome is responsive to blue light, demonstrating the importance of light in the life of the fungus; however, this estimate may be low. Several genes that are known to be light-induced did not pass our minimal expression restrictions, and therefore, were not further analyzed. In one experiment, we found several genes that were repressed following light treatment; however, the fold repression was not consistent between biological replicates. Therefore, we did not investigate these genes further in this study. Similar to *N. crassa*, light plays a critical role in growth and development in *Arabidopsis thaliana*. Recent microarray experiments in *A. thaliana* have shown that 34% out of approximately 6000 unique genes are responsive to light (Ma *et al.*, 2001). Out of these genes, 24% were induced and 10% were repressed by blue light treatment. Both Class IL and IIL genes showed light adaptation responses, whereby transcription was

induced transiently in response to the light signal. This pattern of regulation is typical for most of the known light-regulated genes of *N. crassa* (Linden *et al.*, 1997), further supporting the existence of a general mechanism that allows cells to adapt to a light stimulus (Schwerdtfeger and Linden, 2000; Shrode *et al.*, 2001). However, within the 4-h time limit used in these analyses, adaptation responses were not observed for the Class IIIL genes.

The close relationship between light and circadian rhythmicity prompted us to examine whether genes that were affected by overexpression of WC-1 in the dark were rhythmically expressed, and thus, linked to the circadian clock. WC-1 is essential for circadian rhythms in cultures grown in constant dark and temperature (Crosthwaite *et al.*, 1997). Despite findings demonstrating that *wc-1* mRNA levels do not cycle, WC-1 protein levels are rhythmic in dark-grown cultures (Cheng *et al.*, 2001b; Lee *et al.*, 2000; Merrow *et al.*, 2001). Mutations of *frq* affect both the cycling and levels of WC-1, suggesting that rhythmically-produced FRQ acts at the posttranscriptional level to regulate the amount of WC-1 (Cheng *et al.*, 2001b; Lee *et al.*, 2000). We assayed several of the WC-1-induced genes for clock regulation and found that mRNA from four out of four genes accumulated rhythmically (Figure 13). In addition, we found that greater than 70% of the Class IW-IIIW genes were rhythmic in microarray experiments assayed using RNA isolated from cultures grown in the dark and harvested every 6 h for 2 consecutive days (Correa, A., Lewis, Z. and Bell-Pedersen, D., unpublished data). These results support the idea that WC-1 has at least two

roles in the cell, one involved in light responses and the other involved in the regulation of clock-controlled genes.

One possible explanation for WC-1 being able to regulate two separate classes of genes is that WC-1 is transiently phosphorylated in response to light (Schwerdtfeger and Linden, 2000; Talora *et al.*, 1999) and this modification may constrain its activity to the light pathway. In the absence of phosphorylation, the WC complex may function in the nucleus to regulate different sets of genes, including clock-controlled genes. The non-phosphorylated WC complex can enter the nucleus (Schwerdtfeger and Linden, 2000) and is believed to bind directly to the *frq* promoter to activate *frq* transcription (Loros and Dunlap, 2001). Following *frq* transcription, FRQ protein is produced and FRQ proteins homodimerize and interact with the WC heterodimers to inhibit the positive activity of the WC complex on *frq* transcription. The *frq* gene is itself light-induced, and light induction requires the WC complex (Collett *et al.*, 2002; Crosthwaite *et al.*, 1997). In this case, it is possible that light, by phosphorylation of WC-1 and WC-2, releases the WC complex from the inhibitory action of FRQ. This release of inhibition is consistent with the observed correlation between *frq* photoinduction and resetting of the circadian clock by light through changes in the dynamics of the *frq* feedback loop (Crosthwaite *et al.*, 1997).

Of the genes that were induced by both light and by WC-1 overexpression, three out of the four of these genes were Class IL (peaking transiently soon after light treatment) and Class IIW (peaking about 4 h after QA

induction of WC-1) (Table 8). Of these genes, one is of unknown function, one is probably a ribosomal protein and the other is a possible zinc finger protein. The Class IIL/IIIW gene is similar to a rat L29 ribosomal protein. Thus, although all 4 of the genes induced by both light and WC1 overexpression showed a faster induction by light then by an increase in WC-1 levels in the dark, they have no obvious common function. Similar to *frq*, which is induced by light and is rhythmically expressed, all four light- and WC-1-responsive genes accumulate mRNA rhythmically (Figure 12 and data not shown). Together, these data suggest that the dual-regulated genes identified in this study contain distinct promoter elements that mediate clock and light regulation through direct or indirect action of modified or unmodified WC-1 complexes.

WC-1 shares functional and sequence resemblance to the mammalian BMAL protein involved in circadian rhythmicity (Lee *et al.*, 2000). Like WC-1, BMAL is a PAS containing protein (Gekakis *et al.*, 1998). BMAL interacts with CLOCK to activate the expression of the clock genes *mper* and *mcry* (Young and Kay, 2001). Recent experiments have shown that BMAL/CLOCK heterodimers are also directly involved in regulating output pathway gene expression (Jin *et al.*, 1999; Ripperger *et al.*, 2000). Our results suggest that in the dark WC-1/WC-2 heterodimers may function not only to regulate clock gene expression (*frq)*, but may also regulate some, but not all, aspects of rhythmic output gene expression. In this regard, we examined the Class IW-IIIW genes for common promoter elements using AlignAce (http://atlas.med.harvard.edu/download/) (Roth *et al.*,

1998) that might provide a clue as to the regulation by WC-1 complexes. However, no obvious elements were observed between or among the classes. This may be because not all of the genes are direct targets of WC-1 or a WC complex. In support of indirect regulation of genes by WC complexes, none of the known clock output genes that are represented on the array (such as *ccg-2*) were affected by overexpression of WC-1 (Table 7 and Figure 12). Of particular interest for potential direct targets of WC-1 are the Class IW genes because they peak early, and the IIW genes since these genes peak in mRNA accumulation at about the same times as *frq* mRNA following WC-1 induction in the dark (Cheng *et al.*, 2001b).

The *N. crassa* circadian clock plays an important role in development and metabolism (Bell-Pedersen *et al.*, 2001a; Loros and Dunlap, 2001; Shinohara *et al.*, 2002). Thus, it was not surprising to find that many of the genes induced following overexpression of WC-1 and associated with circadian rhythms encode metabolic functions. What was surprising was that many, but not all, of the putative ribosomal protein genes present on the microarrays were found to be upregulated by WC-1 overexpression (24 ESTs, Table 7). Thus, an intriguing possibility is that WC-1 might also regulate protein translation by regulating ribosomal protein mRNAs.

In summary, our data supports two roles for the WC-1 protein. One is involved in circadian clock-responsive gene regulation in constant conditions, and the other in blue light signal transduction. Several challenges lie ahead,

particularly with regard to the underlying biochemistry of the interactions of the WC proteins and FRQ when cultures are exposed to light or grown in the dark.

## Experimental procedures

*Strains and growth conditions*

The wild type Oak Ridge strain 74OR23-1A (Fungal Genetics Stock Center (FGSC), University of Kansas, Kansas City, #987) was used for light induction experiments. All other experiments used strain 30-7 (*A; bd*) as the clock wild-type control. The *bd* mutation enhances the visibility of the circadian rhythm in asexual spore development in closed culture tubes (Sargent *et al.*, 1966). WC-1 over-expression was accomplished using strain 161-8 (*his-3* [*his-3*$^{+}$*qa-2pwc-1*]*, A, bd, wc-1* $^{ER53}$) (obtained from Dr. Yi Liu, University of Texas Southwestern Medical School) in which the *wc-1* gene is under control of the quinic acid-inducible promoter and inserted into the *his-3* locus (Cheng *et al.*, 2001b). The *wc-1* $^{ER53}$ allele (FGSC#4397) produces a truncated WC-1 protein (Cheng *et al.*, 2002). For simplicity, we have designated strain 161-8 as *wc-1* (qa-*wc-1*) and 30-7 as *bd* throughout the rest of the manuscript.

Cultures were grown as previously described (Davis and deSerres, 1970). For the light induction experiments, 74OR23-1A was grown in liquid Vogel's minimal medium containing 2% sucrose and harvested after light treatments as described (Schwerdtfeger and Linden, 2001). For quinic acid (QA) induction experiments, conidia were inoculated into 25 ml of high glucose liquid minimal

medium (1X Fries salts, 0.5% arginine, 0.3% glucose, 5 ng/ml biotin) until mycelial mats formed (~36 h). Mycelial disks (6mm diameter) were cut from the mats and transferred into 5 flasks containing low glucose minimal medium (1X Fries salts, 0.05% arginine, 0.03% glucose, 5 ng/ml biotin). Cultures were grown in the light for 2 h before transfer to constant darkness and 25°C for 24 h. After 24 h in the dark, mycelia were collected under a red safe light and frozen in liquid nitrogen (time 0) or placed into fresh medium containing 10mM QA and harvested after 1, 2, 4, and 10 h in the dark. This concentration of QA results in high level QA-induced transcription (Giles *et al.*, 1985). For rhythmic RNA analysis, the clock was synchronized by a light-to-dark transition of mycelial mats grown in shaking cultures (100 r.p.m.) in low glucose minimal medium at 25ºC (Loros *et al.*, 1989; Loros and Dunlap, 1991). This light-to-dark transition sets the oscillator to dusk or circadian time (CT) 12. Circadian time (CT) is used to normalize biological time in strains or organisms with different endogenous period lengths to 24 circadian h per cycle. Light-to-dark transfer times were such that the ages of the cultures at harvest were similar, but the circadian times varied (Bell-Pedersen *et al.*, 1992). All liquid cultures were maintained in constant light for at least 2 h prior to transfer to the dark.

*Nucleic acid isolation, hybridization and sequencing*

Total RNA was isolated as described previously (Bell-Pedersen *et al.*, 1996b; Schwerdtfeger and Linden, 2001). mRNA for microarray hybridization was selected from total RNA using the Oligotex messenger RNA purification kit

(Qiagen, Valencia, CA). Conditions for northern transfer, hybridization and washing were as previously described (Bell-Pedersen *et al.*, 1996b). Riboprobes for *eas (ccg-2)* and *wc-1* were generated by *in vitro* transcription (Maxiscript, Ambion, Austin, TX) in the presence of [$\alpha^{32}$-P]-UTP (3000 Ci mmol $^{-1}$, PerkinElmer, Boston, MA) from templates pLW1 (Bell-Pedersen *et al.*, 1996b) and pGEM4wc-1 (Merrow *et al.*, 2001) respectively. DNA probes for clones identified in microarray experiments were made by random primed DNA synthesis (DECAprime, Ambion, Austin, TX) in the presence of [$\alpha^{32}$-P]-CTP (3000 Ci mmol $^{-1}$, PerkinElmer, Boston, MA), using the appropriate plasmid as a template. Automated sequencing of plasmid DNA was accomplished using the Prism Di-deoxy sequencing kit (Applied Biosystems Inc, Foster City, CA) with T3 and T7 primers.

*Microarray construction and hybridization*

Expressed Sequence Tag (EST) libraries corresponding to three stages of the *N. crassa* life cycle (conidial, mycelial and sexual) were generously provided by the Neurospora genome project at the University of New Mexico (Nelson *et al.,* 1997; http://www.unm.edu/~ngp/). From this collection, a set of 1764 EST clones was assembled using stackPACK™ (Electric Genetics, Capetown South Africa). Several clones were re-sequenced and we found greater than 90% to be correct. A listing of the unigene set is available upon request and at http://www.tamu.edu/clocks/bellpedersen. html. While most genes are represented only once in our unigene set, as would be expected using EST

sequence comparisons, some genes were found to be present more frequently. Subsequent to the construction of the microarrays, we compared the EST sequences to the most recent release of the *N. crassa* genome sequence (release 3, http://www-genome.wi.mit.edu/annotation/fungi/ neurospora/) where we determined that the 1764 ESTs actually represented 1343 unique genes. Following assembly of the unigene set, cDNA inserts were amplified by PCR using primers to T3 and T7 promoter sequences, ethanol precipitated, and re-suspended in water to a final volume of 70 μl at a concentration of 25-100 mg/ml. For greater than 96% of the clones, only one PCR product was produced. The DNA was isolated using 96-well GenElute spin columns (Sigma-Aldrich, St. Louis, MO) and SSC was added to a final concentration of 3X SSC. The DNA was printed in duplicate on poly-lysine coated glass slides (Cel Associates Inc., Pearland, TX) using a Gene Machines OmniGrid arrayer (San Carlos, CA). In addition to *N. crassa* EST sequences and a few select *N. crassa* clones, 6 negative control spots were included on each array. These included *Aspergillus nidulans verA* (Keller *et al.*, 1994) and *aflR* (Yu *et al.*, 1996) and *Arabidopsis thaliana pap-1* (GenBank Accession #ATU48448)*, dyskerin* (GenBank Accession #AY050833)*, aap* (T. McKnight, personal communication)*,* and *trp-1* (Chen *et al.*, 2001) (GenBank Accession #Y17722). Together, the ESTs and control spots yielded 1778 probe spots per array.

Using the 3DNA Submicro EX Expression array detection kit (Genisphere, Newark, NJ), 300ng of messenger RNA was reverse transcribed, labeled with

Cy3 and hybridized to the microarrays according to the manufacturer's instructions. Prior to hybridization, the slides were processed according to the Genisphere kit instructions (Appendix C). Following hybridization at 60°C for 24-36 h, the slides were washed and scanned using a Scanarray 5000 scanner (GSI Luminomics, Ottowa, Ontario).

*Data analysis*

Fluorescence data from each microarray was collected using Scanalyze 2.44 (written by Michael Eisen and available online at http://rana.lbl.gov/EisenSoftware.htm). Due to inconsistencies in signal intensities using Cy-5-labeled probes as a result of photobleaching, we chose to analyze the single Cy3 data. This method has been used successfully by several groups to detect changes in gene expression using microarrays (Harmer *et al.*, 2000). Raw fluorescence data was then exported as a text file using Microsoft Excel (Microsoft, Redmond, WA). All higher level analyses, including data normalization and clustering, were performed using Genespring software (Silicon Genetics, Redwood City, CA). Initially, the background fluorescence (obtained by using the median intensity obtained from the 12 negative control spots on the array) was subtracted from the experimental spots. Each spot was then divided by the 50$^{th}$ percentile of all measurements for that slide to account for differences in overall intensity between slides. To permit comparisons of the genes on the same scale, each gene was normalized to itself. The average normalized intensity for each gene at each time-point was divided by the median

value for that gene. This results in at least one time point value being equal to 1. We imposed a minimum raw intensity value to eliminate DNA spots with low signal intensities from further analysis. These minimum intensity values were chosen to be higher than values observed for negative control spots; the higher the background signal, the greater the minimum signal intensity required. For light induction experiments, a minimum average pixel intensity of 5000 was required for each gene in at least 2 of the 5 conditions tested. For QA induction experiments, a minimum average pixel intensity of 4000 was required for each gene in at least 2 of the 5 conditions tested. Genes that showed a minimum 2-fold change in relative intensity following addition of QA or after light treatment were selected. From this collection, only those genes in which the standard deviation of the pre-induced mean value did not overlap with standard deviation of the highest post-induction mean value were included in the final analysis.

# CHAPTER IV

# MULTIPLE COUPLED OSCILLATORS DIFFERENTIALLY REGULATE CIRCADIAN RHYTHMICITY IN *Neurospora crassa*

## Introduction

Organisms from bacteria to humans use an endogenous timekeeping mechanism, called the circadian clock, to regulate daily rhythms in a wide range of biochemical, physiological, and behavioral activities. At the core of all circadian clocks is a pacemaker made up of one or more circadian oscillators. Circadian oscillators that have been described to date are composed of positive and negative elements that form feedback loops (Dunlap, 1999; Hall, 1998; Harmer *et al.*, 2001; Johnson and Golden, 1999; King and Takahashi, 2000; Reppert and Weaver, 2002; Young and Kay, 2001). In these circuitry's, the positive elements of the loop activate transcription of the "clock genes" that encode the negative elements. The concentration of the negative elements rise, and then the negative elements physically interact with the positive elements to inhibit their activity. This reduces transcription of the clock genes. Phosphorylation-induced decay of the negative elements decreases their concentration leading to reactivation of the positive elements allowing the cycle to start again. In these molecular oscillators, the negative elements also activate the expression of one or more of the positive elements forming coupled positive and negative feedback loops that are important for maintaining the stability and robustness of the clock. Components of these loops direct the transduction of

time information through the output pathways to regulate rhythmic *clock-controlled gene* (*ccg)* expression and overt rhythmicity.

In several organisms, there is evidence for multiple autonomous oscillators, as well as dependent slave oscillators, controlling different rhythmic outputs. For example, in the marine dinoflagellate *Gonyaulax polyedra*, two oscillators that are responsive to different wavelengths of light were identified, with one oscillator regulating rhythms in bioluminescence and the other controlling aggregation (Morse *et al.*, 1994). Similarly, in *Synechococcus elongatus,* rhythms with different periods can be observed in a single cell grown in constant conditions, suggesting that different endogenous oscillators regulate these rhythms (Nair *et al.*, 2002). In plants, differential sensitivity of two different clock-controlled genes to temperature pulses can be observed within a single tissue supporting the existence of a multi-oscillator system (Michael *et al.*, 2003).

The multi-tissue organization of the vertebrate circadian system provides an even greater level of complexity. In hamsters and ground squirrels kept in constant bright light, locomoter activity rhythms sometimes split into 2 separate components, each with different periods (Pittendrigh, 1960; Pittendrigh and Daan, 1974). This splitting has been correlated with anti-phase expression of clock genes in the bilateral lobes of the suprachiasmatic nucleus of the hypothalamus (SCN) (de la Iglesia *et al.*, 2000). Furthermore, restricted feeding of mice housed in light:dark cycles results in a new phase relationship of clock gene expression within the liver and the SCN (Damiola *et al.*, 2000; Stokkan *et*

*al.*, 2001). These results indicate that individual tissues have the capacity for autonomous molecular oscillators. This prediction is supported by persistent oscillation of luciferase from a clock gene PERIOD2:LUCIFERASE reporter fusion in individual organs in tissue culture (Yoo *et al.*, 2004). In birds and reptiles, pinealectomy revealed separate oscillators in the retina and brain that regulate different overt circadian behaviors (Bartell *et al.*, 2004; Takahashi and Menaker, 1982). Together, these data have led to the current view of the vertebrate circadian system as being composed of multiple circadian oscillators in both the brain and periphery, with the suprachiasmatic nucleus of the hypothalamus (SCN) sitting at the top of the organizational hierarchy. Thus, one of the major challenges of the field is to determine the details of this organization.

The model organism *Neurospora crassa* has helped to elucidate many of the basic mechanisms underlying circadian rhythms, including negative feedback and light entrainment common to all clocks (Aronson *et al.*, 1994b; Crosthwaite *et al.*, 1995). The *N. crassa* clock controls several rhythmic processes, including the daily production of asexual conidiospores (Pittendrigh *et al.*, 1959). In *N. crassa,* the well described FRQ/WC oscillator that directs these rhythms has the basic signature features of oscillators in other model systems, including *Drosophila* and mouse. In constant dark and temperature, at subjective dawn, both *frequency (frq)* mRNA and FRQ protein levels are low (Aronson *et al.*, 1994b; Garceau *et al.*, 1997). White Collar-1 (WC-1) and WC-2 dimerize through

their PAS domains (forming a WHITE COLLAR complex WCC) and directly activate *frq* transcription (Ballario *et al.*, 1998; Cheng *et al.*, 2002; Denault *et al.*, 2001; Froehlich *et al.*, 2002; Froehlich *et al.*, 2003). About 4-5 hours later, *frq* mRNA levels reach their peak, and FRQ protein accumulates and enters the nucleus with about a 4 hour delay (Luo *et al.*, 1998; Merrow *et al.*, 1997). Negative feedback occurs by the interaction of FRQ with the WCC, which interferes with the ability of the WCC to activate *frq* transcription. (Aronson *et al.*, 1994b; Cheng *et al.*, 2001a; Denault *et al.*, 2001). For the rest of the day and into the early 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 using two different proposed mechanisms: FRQ is hypothesized to positively regulate WC-1 postranscriptionally leading to its rhythmic expression, and to increase the steady state levels of *wc-2* mRNA (Cheng *et al.*, 2001b; Lee *et al.*, 2000). Thus, the current model suggests that FRQ serves at least two roles in the feedback mechanism, interlocking the repression of its own transcription with the up-regulation of the levels of the WC proteins. However, current studies have not revealed how FRQ exerts a positive effect on the WC proteins. Completion of the feedback loop is achieved by removal of FRQ from the nucleus, ultimately resulting in activation of *frq* transcription by the WCC.

There is also evidence for multiple oscillators within the *N. crassa* cell. This evidence comes primarily from studies of strains that lack FRQ, and thus lack a functional FRQ/WC oscillator. FRQ-null strains can sometimes display a

free running rhythm in conidiospore development in constant darkness, but the period is variable ranging from 12-35 h (Aronson *et al.*, 1994a; Loros and Feldman, 1986). The conidiation rhythm can be stabilized by the addition of farnesol or gerianiol to the growth medium. Here, the rhythm is not sensitive to light dark cycles and is not temperature compensated, but phase shifting by temperature pulses has been demonstrated (Granshaw *et al.*, 2003), suggesting that this FRQ-less oscillator (FLO) is responsive to temperature. Furthermore, robust conidiation rhythms are observed in FRQ-null strains in cultures subjected to 5°C temperature cycles in constant light or darkness (Merrow *et al.*, 1999); however, this rhythm appears to be due to the direct affect of temperature on development (Jay Dunlap, personal communication). These results, along with the evidence for multiple oscillators in other organisms, are moving the model paradigm away from a simple negative feedback loop to a molecular network circadian system. The simplicity of *N. crassa* will likely prove key for elucidating the mechanisms and interactions of this circadian network.

Molecular components of the *N. crassa* FLO's have not yet been identified. In previous studies, we isolated 3 evening-specific genes that have a daily rhythm in mRNA accumulation in the absence of the key oscillator component FRQ (Correa *et al.*, 2003). However, some level of influence of the FRQ/WCC oscillator on expression of these genes was suggested because the phasing of the peaks in mRNA accumulation differed in wild type versus strains that carried a mutation in the *frq gene* that caused a long 29-h period. These

ccgs were predicted to be either components of a FLO that is coupled to the FRQ/WCC oscillator, or reside in output pathways from the FLO.

To begin to describe the multi-oscillator system of *N. crassa*, we have further characterized one of these genes, the evening-specific *ccg-16* gene. Consistent with our previous data, we show that *ccg-16* is rhythmic in the absence of FRQ and under conditions in which the conidiation rhythm is abolished, such as constant light. Interestingly, robust *ccg-16* rhythms require a functional WC-1 protein, suggesting that WC-1 couples the two oscillators. In contrast to current models of the FRQ/WC oscillator, we show that WC-1 protein accumulates rhythmically in the absence of FRQ. Taken together, our data reveal that the circadian clock of *N. crassa* is a network composed of at least two coupled oscillators that function to regulate different output pathways.

## Materials and methods

### N. crassa *strains and growth conditions*

Growth media (Vogel's minimal media), vegetative growth conditions and conditions for sexual crosses are described (Davis and deSerres, 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 conidation rhythm in race tube cultures (Sargent *et al.*, 1966). The *frq*$^{10}$ and wc-1$^{KO}$ mutations were produced by gene replacement resulting in a null allele 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) 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 rhythmic 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). For constant dark (DD) experiments, cultures were synchronized by transfer from constant light (LL) to DD, from 30°C; LL to 25°C; DD or from 30°C; DD to 25°C; DD as indicated in the text. For LL experiments, cultures were synchronized by transfer from DD to LL or from 30°C;LL to 25°C; LL as indicated in the text. 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 (Loros et al, 1989; Correa et al, 2003). For rhythmic 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, 0.5% Arginine).

*Nucleic acid isolation and hybridization*

Total RNA isolation and northern blotting was performed as previously described (Bell-Pedersen *et al.*, 1996b). For northern analysis of *ccg-1*, radioactive riboprobes were synthesized from pLK119 by *in vitro* transcription (Maxiscript, Ambion Inc., Austin, TX) in the presence of [$\alpha^{32}$P]UTP [3000 Ci mmol$^{-1}$] (Perkin Elmer Inc.) For northern analysis of *ccg-16*, T3 and T7 primers were used to amplify the *ccg-16* coding sequence by polymerase chain reaction 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 [$\alpha^{32}$P]CTP [6000 Ci mmol$^{-1}$] (Perkin Elmer Inc.).

*Protein isolation and analysis*

Total protein isolation and western blotting was performed as previously described (Garceau *et al.*, 1997). Antibodies to FRQ (polyclonal) and WC-1 (monoclonal) were generously provided by Drs. Martha Merrow and Till Roenneberg (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 bacterial *hygromycin phosphotrasferase* (*hph)* gene. PCR was used to produce split marker fragments from the knockout plasmid for transformation into *N. crassa* (Aronson *et al.*, 1994c). 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 (Stabin *et al.*, 1989) (hphF-ACATACGATTTAGGTGACACTATAGAACGCCCGTCGACAGAAGATGATATT GAAGGAGC hphR-AGCTGACATCGACACCAACG) and pRS416 was digested with XbaI and XhoI. 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 transformants 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).

## Results

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

In constant darkness and temperature, the wild type clock strain *bd* 40-9 (hereafter referred to as wild type) displayed 22-h rhythms in conidiospore production as viewed on race tubes (Figure 14A). However, under the same conditions, the FRQ-null strain, *bd, frq$^{10}$; a* (40-2), was arrhythmic. Similarly, the well-characterized, morning-specific clock-controlled gene, *ccg-1* mRNA accumulates rhythmically in wild type strains peaking in the subjective morning (DD 12 and DD36) in cultures that were synchronized by a light (30ºC) to dark (25ºC) transition (Loros *et al.*, 1989), but was arrhythmic in the FRQ-null strain (Figure 14B, C). However, consistent with previous results (Correa *et al.*, 2003), a ~24 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 14). In some experiments, a phase difference between wild type and the FRQ-null strain was observed (for example, an 4 hour phase difference is shown in Figure 14B), suggesting that the oscillation in *ccg-16* mRNA levels is not as stable in strains that lack the FRQ/WC oscillator as compared to wild type.

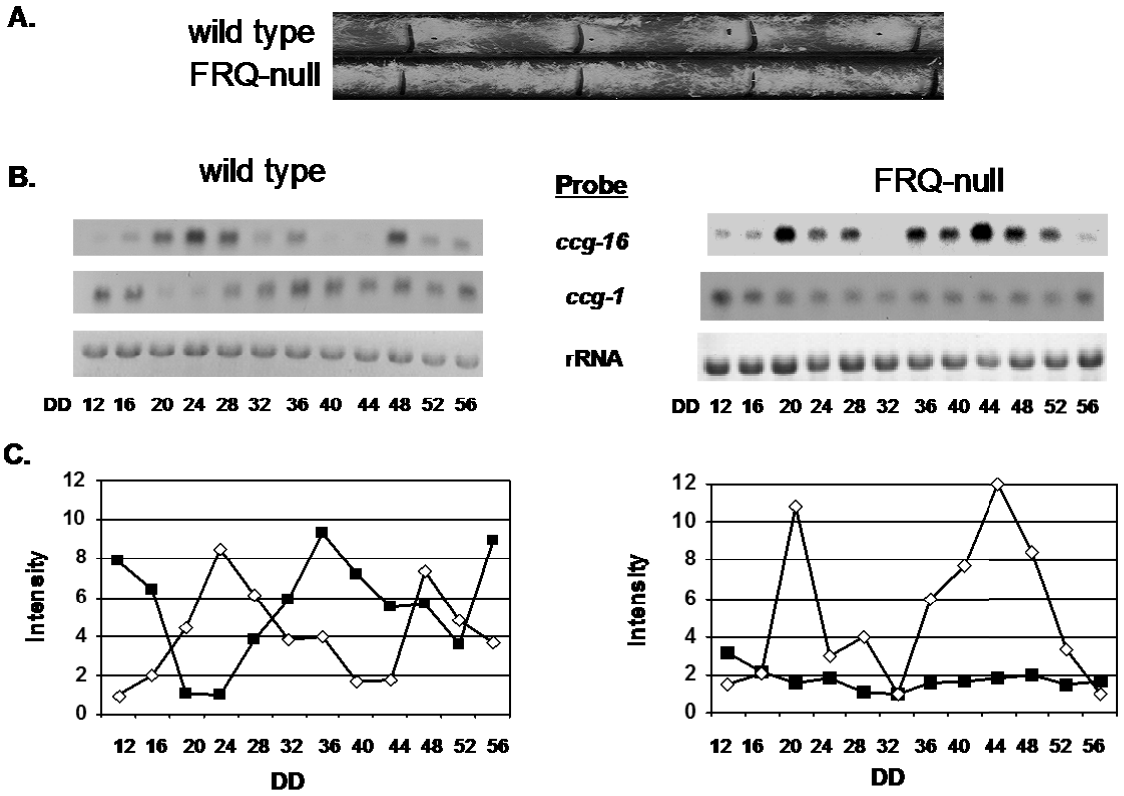


**Figure 14. Circadian accumulation of *ccg-16* mRNA in wild type and *frq*$^{10}$ strains in constant darkness.**

(A) Race tube cultures of wild type (top) and *frq*$^{10}$ (bottom) strains were grown in DD at 30°C for 24 hours before transfer to 25°C DD. The marks represent 24 hours of growth. (B) Representative northern blots of *ccg-16* and *ccg-1* in constant darkness are shown. Wild type and FRQ-null strains grown in constant darkness (DD) at 30°C before transfer to 25°C DD. Cultures were harvested after transfer to 25°C DD at the indicated times. Northern blots of RNA from wild type (left) and FRQ-null (right) strains were probed with radioactive *ccg-16* and *ccg-1* probes as indicated. rRNA is shown to verify equal loading of all lanes. (C) Relative mRNA levels (band intensity/rRNA) of *ccg-16* and *ccg-1* from wild type (left) and *frq*$^{10}$ (right) are plotted versus time. *ccg-16* is represented by open diamonds. *ccg-1* is represented by solid squares.

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). Alternatively, wild type strains 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 suggested that a FRQ-less oscillator (FLO) is responsible for generating the *ccg-16* rhythm and that this oscillator responds better to temperature than light cues for synchronization.

We hypothesized that if *ccg-16* rhythms are truly independent of FRQ, 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 15A). Under these conditions, *frq* mRNA is induced to high levels and does not accumulate rhythmically (Crosthwaite *et al.*, 1995). Also in constant light, FRQ protein is heavily phosphorylated and protein levels are high and non-circadian as compared to rhythmic FRQ accumulation in constant dark (Figure 15B). Consistent with FRQ-independent *ccg-16* rhythmicity, the levels of *ccg-16* mRNA oscillate with a ~24h period in constant light in both wild type and FRQ-null strains (Figure 15C, D). In the FRQ-null strain, the period and phase of the *ccg-16* mRNA rhythms showed variations, similar to DD experiments when FRQ-null cultures are synchronized by light. In contrast, the FRQ/WC oscillator-dependent gene *ccg-1* was not rhythmically expressed in constant light in either strain. These data confirm that *ccg-16* is controlled by a FLO; *ccg-16* mRNA accumulation is rhythmic in the absence of FRQ protein and

in conditions where both the conidiation rhythm is abolished and FRQ is expressed at high levels in a non-circadian fashion.

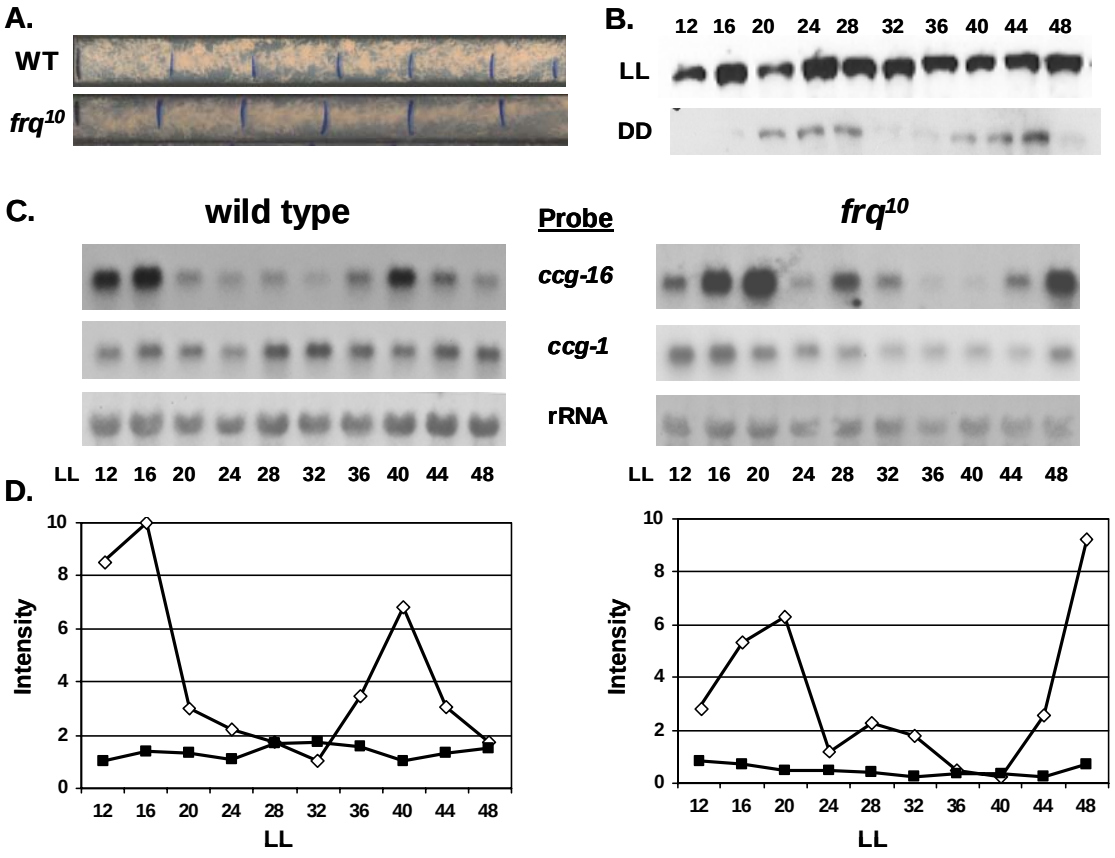


**Figure 15. Circadian accumulation of *ccg-16* mRNA in wild type and *frq*$^{10}$ strains in constant light.**

(A) Race tube cultures of wild type (top) and *frq*$^{10}$ (bottom) strains were grown in the dark for 24 hours before transfer to constant light (LL). The marks represent 24 hours of growth. (B) Total protein was isolated from wild type cultures after transfer from dark to light (LL) or light to dark (DD) at the indicated times. Western blots were probed with antibodies to FRQ. (C) Representative northern blots of *ccg-16* and *ccg-1* in constant light are shown. Total RNA was isolated from wild type and FRQ-null strains after transfer from dark to light at the indicated times. Northern blots of RNA from wild type (left) and FRQ-null (right) strains were probe with radioactive *ccg-16* and *ccg-1* probes. rRNA is shown to verify equal loading of each lane. (D) Relative mRNA levels (band intensity/rRNA) of *ccg-16* and *ccg-1* from wild type (left) and *frq*$^{10}$ (right) are plotted versus time. *ccg-16* is represented by open diamonds. *ccg-1* is represented by solid squares.

*The FLO regulating* ccg-16 *rhythmicity is coupled to the FRQ/WC oscillator*

It is possible that the FLO that regulates *ccg-16* expression is independent of the well-characterized FRQ/WCC oscillator. Alternatively, the FRQ/WCC oscillator may communicate with the FLO when the circadian system is complete. In northern assays we noticed that *ccg-16* mRNA levels were always reduced in the FRQ-null strain relative to wild type. Therefore, we re-examined *ccg-16* levels in wild type and FRQ-null strains at DD20 and DD32, when *ccg-16* is near its peak and trough levels, respectively. The levels of *ccg-16* mRNA were significantly reduced in the FRQ-null strain relative to wild type at both times of the day (wild type levels are ~2.5 fold higher at DD20 and ~4.5 fold higher at DD32) (Figure 16A).

This requirement of FRQ for normal expression levels of *ccg-16* indicated that the FLO and the FRQ/WC oscillator may be coupled. This hypothesis predicts that mutations in FRQ that alter the period of the conidiation rhythm should also affect the period or phase of the *ccg-16* rhythm. To test this prediction, we examined *ccg-16* accumulation in a strain harboring the $frq^7$ mutation, which lengthens the period of the FRQ/WC feedback loop and results in a long 29-h conidiation rhythm in constant darkness. In the $frq^7$ mutant strain, peak *ccg-16* mRNA levels are delayed ~8h relative to wild type, similar to that observed for the FRQ/WC-dependent *ccg-1* gene (Figure 16B, C). These data support the notion that the FRQ/WCC oscillator is coupled to the FLO.

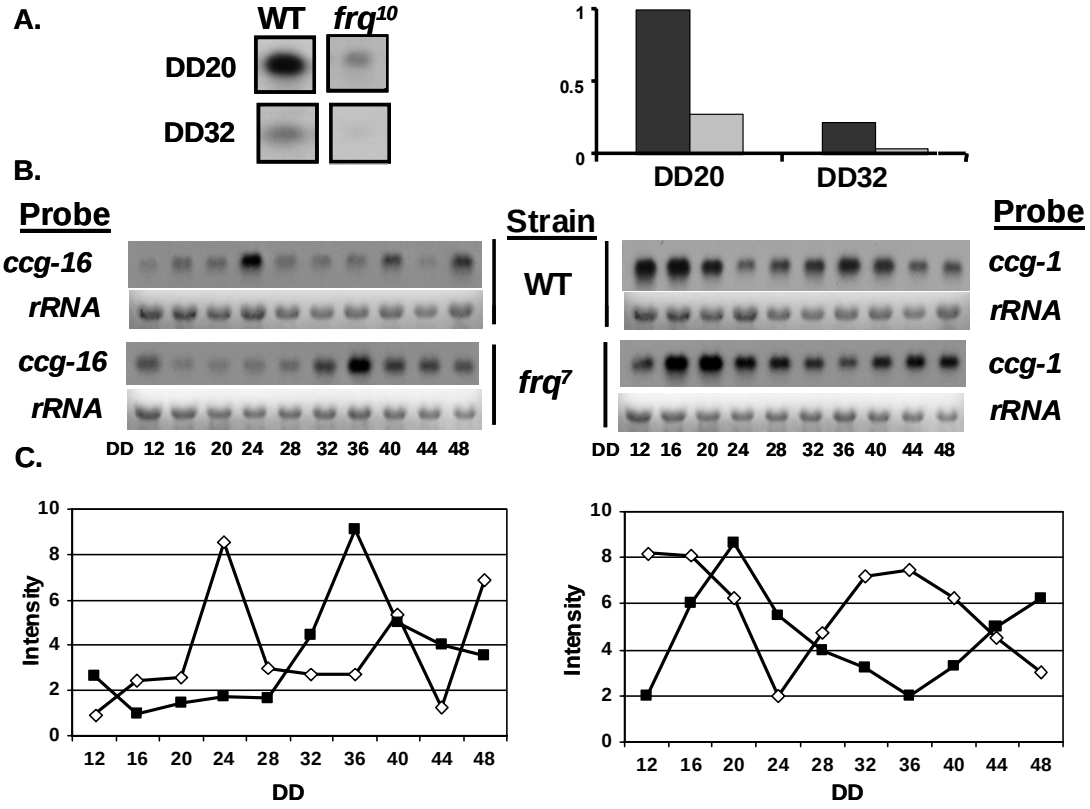


**Figure 16. The FRQ/WC-oscillator is coupled to the FLO.**

(A) Total RNA was isolated from wild type and *frq*$^{10}$ strains harvested following transfer from 30°C constant darkness (DD) to 25°C DD after 20 (DD20) and 32 (DD32) hours as indicated. Northern blots were probed with a radioactive *ccg-16* probe(left). Relative mRNA levels (band intensity/ rRNA) are plotted for each time (right). *ccg-16* levels are shown as black bars for the wild type strain and gray bars for the FRQ-null strain. (B) Total RNA was isolated from wild type and *frq*$^{7}$ strains after transfer from light to dark at the indicated times. Northern blots were probed with radioactive *ccg-16* (left) and *ccg-1* (right) probes. rRNA is shown to verify equal loading of each lane. (C) Relative mRNA levels (band intensity/rRNA) of *ccg-16* (left) and *ccg-1* (right) in wild type and *frq*$^{7}$ are plotted versus time. wild-type is represented by open diamonds. *frq*$^{7}$ is represented by solid squares.

*WC-1 couples the FRQ/WC oscillator to the FLO regulating* ccg-16 *rhythmicity*

We examined the possibility that WC-1 might be involved in coupling the two oscillators. A WC-1-null strain *bd; wc-1$^{KO}$; A* (418-3) grown in constant darkness was used to examine *ccg-16* rhythmicity following temperature synchronization of the cultures (30°C LL to 25°C DD). While the levels of both *ccg-16* and *ccg-1* fluctuated slightly over the course of the day, the mRNA levels were not rhythmic for either gene in 6/6 independent experiments (Figure 17A, B). Similarly, *ccg-16* and *ccg-1* mRNA accumulation in the WC-1 null strain in constant light (30°C DD to 25°C LL) were arrhythmic. Together these data suggest that WC-1 is required for rhythmic expression of *ccg-16* and involved in coupling the two oscillators.

In wild type strains, *wc-1* mRNA is expressed constitutively in constant darkness, whereas WC-1 protein accumulates with a circadian rhythm and peaks in the subjective morning, antiphase to FRQ protein peak accumulation (Lee *et al.*, 2000). Previous studies have suggested that rhythmic WC-1 requires FRQ protein, since WC-1 accumulation is arrhythmic in FRQ-null strains in cultures grown in constant dark and synchronized by a light to dark transition at 25ºC (Lee *et al.*, 2000). Furthermore, current models propose that FRQ protein drives the rhythm in WC-1 accumulation using a posttranscriptional mechanism (Cheng *et al.*, 2001b; Lee *et al.*, 2000). If indeed WC-1 is required for rhythmic *ccg-16* accumulation in LL and DD following a temperature shift, we hypothesized that WC-1 protein levels might also be rhythmic under these same conditions.

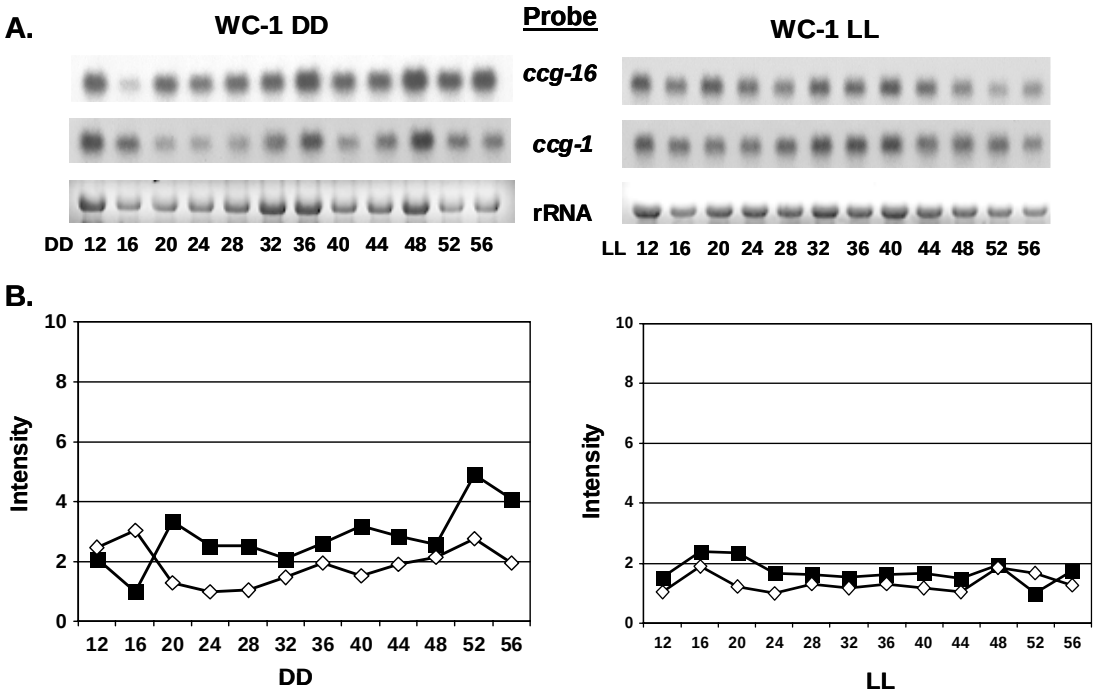


**Figure 17. *wc-1* is required for *ccg-16* rhythms.**

(A) Representative northern blots of *ccg-16* and *ccg-1* in the *wc-1*-null strain are shown. Total RNA was isolated from the *wc-1*-null strain after transfer from 30°C LL to 25°C DD (left) or from 30°C DD to 25°C LL (right) at the indicated times. Northern blots of RNA from the *wc-1*-null strain were probed with radioactive *ccg-16* and *ccg-1* probes. rRNA is shown to verify equal loading of each lane. (D) Relative mRNA levels (band intensity/rRNA) of *ccg-16* and *ccg-1* from the *wc-1*-null strain in constant dark (left) and constant light (right) are plotted versus time. *ccg-16* is represented by solid sqares. *ccg-1* is represented by open diamonds.

We examined WC-1 protein accumulation in wild type and FRQ-null strains grown in constant light (LL) or dark (DD) and synchronized by temperature (30°C LL to 25°C LL or 30°C DD to 25°C DD). A robust rhythm in WC-1 protein levels was observed in both wild type and FRQ-null strains after a temperature synchronization in DD (data not shown). In constant light, however,

rhythmicity was observed in FRQ-null cultures but not wild type cultures (Figure 18). It is unknown if *wc-1* mRNA is rhythmic in the FRQ-null strain. Consistent with previous results, the overall levels of WC-1 were significantly lower in the FRQ-null strain (data not shown). Taken together, these results suggest that while FRQ is involved in maintaining the overall levels of WC-1 protein in the cell, FRQ is not essential for rhythmic accumulation of WC-1. Thus, other undescribed factors must be involved in regulating the circadian expression of WC-1 following temperature entrainment. Furthermore, these data indicate that WC-1 rhythmicity is not essential for *ccg-16* rhythmicity; whereas *ccg-16* is rhythmic in wild type strains in constant light, WC-1 accumulates at a constant level under these same conditions).

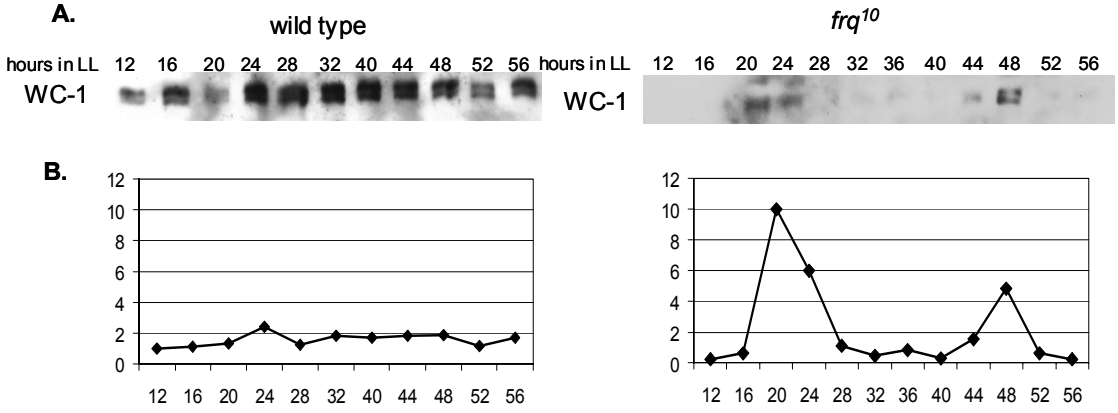


**Figure 18. WC-1 is rhythmic in the FRQ-null strain.**

(A) Total protein was isolated from wild type and *frq*$^{10}$ cultures that were collected after transfer from 30°C DD to 25°C LL at the indicated times. Western blots were probed with antibodies to WC-1. (B) Relative WC-1 levels ( WC-1/ total protein) are plotted below each blot. LL indicates time in constant light.

*The* ccg-16 *gene does not affect circadian conidiation*

The CCG-16 predicted coding sequence consists of 125 amino acids and is comprised of two repeated domains (not shown). BLAST searches using the predicted CCG-16 amino acid sequence failed to identify any conserved domains in CCG-16. Similar predicted sequences were identified only in the genomes of other filamentous ascomycetes, indicating that CCG-16 is a fungal-specific protein.

BLAST searches did not provide clues about the potential function of CCG-16. However, similar proteins are found in the genomes of other filamentous fungi (Figure 19). To determine if *ccg-16* is required for normal circadian rhythmicity and possibly functions within the FLO mechanism, we created a knockout strain by replacing the predicted coding sequence with the bacterial *hygromycin phospotransferase* (*hph*) gene in a $FRQ^{+}$ strain. We observed normal circadian rhythms in development in the $FRQ^{+}\Delta$*ccg-16* strain in constant dark (data not shown).


**Figure 19. Sequence alignment of CCG-16.**

ClustalW alignment of the CCG-16 predicted coding sequence (NCU05495.1) and putative homologous sequences from *A. nidulans* (AN3481.2), *F. gramineurium* (FG04975.1), *M. grisea* (MG04913.1). The consensus sequence is shown below the alignment.

## Discussion

The complexity of the circadian clock system in organisms is becoming increasingly apparent as additional components required for normal circadian rhythmicity are identified and their functions investigated. It is now clear that the clock mechanism involves multiple interlocked and autonomous feedback loops, involving both transcriptional and postranscriptional regulation. The traditional view of an input pathway to a single circadian oscillator that generates the rhythms to unidirectional output pathways can no longer adequately describe the circadian timing mechanism. Yet, this simplified view was crucial for the initial identification and description of key clock components now fully engrained in the models for circadian clocks.

Studies in *Neurospora* identified FRQ, WC-1 and WC-2 as central clock components required for the organism to have complete circadian properties. This was based on monitoring the rhythm in development, along with rhythmicity of two morning-specific clock-controlled genes, *ccg-1* and *ccg-2* (Aronson *et al.*, 1994b; Bell-Pedersen *et al.*, 1996b; Crosthwaite *et al.*, 1997). It was initially thought that the *Neurospora* clock only regulated morning-specific events since all of the clock-controlled genes found peaked in the late-night to early morning (Bell-Pedersen *et al.*, 1996b; Loros *et al.*, 1989; Loros and Dunlap, 1991; Nowrousian *et al.*, 2003). However, using microarrays we found that the clock regulates genes at all phases of the day (Correa *et al.*, 2003). Thus, the identification of additional ccgs, or readouts from the clock, allowed further investigation of the roles of the FRQ/WC oscillator in generating overt circadian rhythms.

In this study, we show that the evening-specific gene *ccg-16* accumulates mRNA with a circadian period in strains that lack FRQ and when grown in constant light when FRQ protein accumulation is high and arrhythmic. These data show that the oscillator that regulates *ccg-16* circadian rhythms is a FRQ-less oscillator (FLO) that can function without FRQ. However, the phase of the *ccg-16* rhythm is altered in the long period (29-h) *frq7* strain, suggesting that under normal circumstances, the FLO and the FRQ/WC oscillator are coupled. Our data suggests that WC-1 is involved in this coupling, since *ccg-16* is

arrhythmic in a WC-1-null mutant strain. Furthermore, our data demonstrate that the FLO provides feedback to the FRQ/WC-oscillator by regulating WC-1.

Early studies on entrainment by light and temperature in *Drosophila* gave rise to models in which a pacemaker served to drive a slave oscillator that directly regulated overt rhythmicity of one or more functions. In describing the nature of slave oscillators in 1981, Pittendrigh noted that the use of multiple slave oscillators to regulate aspects of output leaves the circadian program open to adjustment of one component part without interference with the others. In addition, he suggested that slave oscillators need not have all of the circadian properties ascribed to the pacemaker since they will normally be coupled to and entrained by the pacemaker (Pittendrigh, 1981). Indeed, he noted that any biochemical feedback loop in an organism is a potential slave oscillator, where if the pacemaker can make an input to the loop, the slave will assume a circadian period. Our results with *ccg-16* are reminiscent of a pacemaker-slave relationship in which an oscillator that regulates *ccg-16* is coupled to and entrained by the FRQ/WC oscillator.

However, the fact that WC-1 exhibits a rhythm in the FRQ-null strain indicates that the FLO provides feedback to the FRQ/WC oscillator, and therefore, indicates that the two oscillators are mutually coupled. While it is clear that the FLO does feedback to regulate WC-1 rhythms, the extent that each oscillator influences the WC-1 rhythm is not clear. A rhythm in WC-1 is not observed when the wild type strain is grown in constant light, indicating that FRQ

has a stronger effect on WC-1 than the FLO. Feedback from the FLO may act to "fine-tune" the FRQ/WC oscillator, and consequently, contribute to the maintenance of a stable phase relationship between the two oscillators. The ability of FRQ to positively regulate WC-1 has been clearly demonstrated (Cheng *et al.*, 2001b; Lee *et al.*, 2000). However, the mechanism by which WC-1 is regulated by the FLO is currently unknown. The FLO may promote synthesis of WC-1 in the same manner that FRQ does. Alternatively, the FLO may promote degradation of WC-1, providing yet another level of regulation within the FRQ/WC oscillator.

Understanding how circadian oscillators are coupled to peripheral oscillators is fundamental to understanding circadian systems. The data presented here clearly demonstrate the importance of WC-1 in coupling the FRQ/WC oscillator with the FLO. Interestingly, while WC-1 protein is required for the FLO to generate a rhythm in *ccg-16* mRNA, rhythmic accumulation of WC-1 protein does not appear to be necessary. This suggests that WC-1 may interact with a rhythmic component of the FLO. If this is indeed the case, this interaction would provide a mechanism for entrainment of the FLO by the FRQ/WC oscillator. The molecular components of the FLO are currently unknown. Genetic and biochemical analysis of FLO components will be necessary to determine how the FLO is coupled to and entrained by the FRQ/WC oscillator.

A prominent multiple oscillator model for the vertebrate circadian system involves morning and evening oscillators (Pittendrigh and Daan, 1974). The fact

that *ccg-16* levels peak in the evening while the known FRQ-regulated ccg's peak in the morning is reminiscent of this type of organization. However, the extent of clock-controlled genes that are regulated by the FLO is unknown. It is possible that the FLO may regulate evening-specific gene expression in the fungus, while morning-specific genes are regulated by the FRQ/WC oscillator. Alternatively, both oscillators may regulate output gene expression at both phases of the cycle.

Physiological data have provided evidence that the conidiation rhythm is controlled by a FLO (Aronson *et al.*, 1994a; Granshaw *et al.*, 2003; Loros and Feldman, 1986; Merrow *et al.*, 1999). Currently, it is not clear if the FLO that regulates *ccg-16* is identical to the FLO that regulates conidiation. The conidiation rhythms observed when the medium is supplemented with geraniol or farnesol are not dependent on WC-1, indicating that this oscillator is distinct from the FLO that regulates *ccg-16* (Granshaw *et al.*, 2003). However, the *wc-1* allele used in this study likely produces a partially functional WC-1 protein (Cheng *et al.*, 2003). Additional experiments will be required to resolve this issue. Despite this, the idea that the FRQ/WC oscillator rests at the top of a network of oscillators is intriguing. Furthermore, this situation bears a striking similarity to the organization of the vertebrate system, where the SCN regulates a multitude of oscillators throughout the periphery.

The data presented here provide strong evidence for multiple oscillator organization of the *Neurospora* circadian system. This finding provides a

foundation for understanding several fundamental aspects of eukaryotic circadian organization. The well-established genetics of *Neurospora* leave this system well-suited for developing a more sophisticated understanding of how multiple oscillators are coupled as well as how coupled oscillators interact to coordinate output.

# CHAPTER V

# SUMMARY

The results presented in this dissertation have provided additional insight into the organization of the *Neurospora* circadian system. Here, we have demonstrated that output gene expression from the *Neurospora* clock is widespread and complex; 20% of the genes we examined were rhythmic with genes peaking at all phases of the circadian cycle. We have examined the divergent roles that WC-1 plays in light- and clock-controlled gene regulation. Lastly, we have provided evidence that the *N. crassa* circadian system is composed of interconnected oscillatory networks that produce rhythmic gene expression and behavior.

## Output from the *Neurospora* clock

The identification of 145 clock-controlled genes (chapter II) has provided an increased understanding of the complexity of the *Neurospora* circadian system. The identification of clock-controlled genes involved in diverse biological processes demonstrates that the circadian clock influences all aspects of the *N. crassa* life cycle, not just asexual development (chapter II). Furthermore, many clock-controlled genes show peak mRNA levels during the late-day and early-evening (chapter II). This result is the first demonstration that the *N. crassa* clock regulates gene expression at all phases of the circadian

cycle. At this time, we are only beginning to construct a model to describe how the clock may accomplish this.

In chapter II, we identified a putative serine/threonine kinase, *non-repressible conidiation-2* (*nrc-2*), that is under control of the clock. Loss of function mutations in *nrc-2* result in arrhythmic expression of all morning-specific genes that have been analyzed. However, the evening-specific gene *ccg-16* (chapter II and chapter IV) is still rhythmically expressed in the *nrc-2* mutant. Importantly, cycling of the clock proteins WC-1 and FRQ are unaffected in the *nrc-2* mutant, placing NRC-2 in an output pathway (A. Greene, A. Correa, and D. Bell-Pedersen, in preparation). This may indicate that all morning-specific gene expression is regulated by the FRQ/WC-oscillator through the NRC-2 pathway (Figure 20). In chapters II and IV, we describe a second oscillator that regulates expression of the evening-specific gene *ccg-16.* It is possible that all evening-specific genes are controlled by this oscillator. In chapter II, NC1E1 was identified as a morning-specific gene that is controlled by the FLO. However, northern analysis suggests that this gene peaks in the late day. Therefore, this result is consistent with a role for the FLO in regulating evening-specific ccg expression. Alternatively, the FRQ/WC-oscillator and the FLO may both regulate clock-controlled gene expression at multiple phases of the circadian cycle.

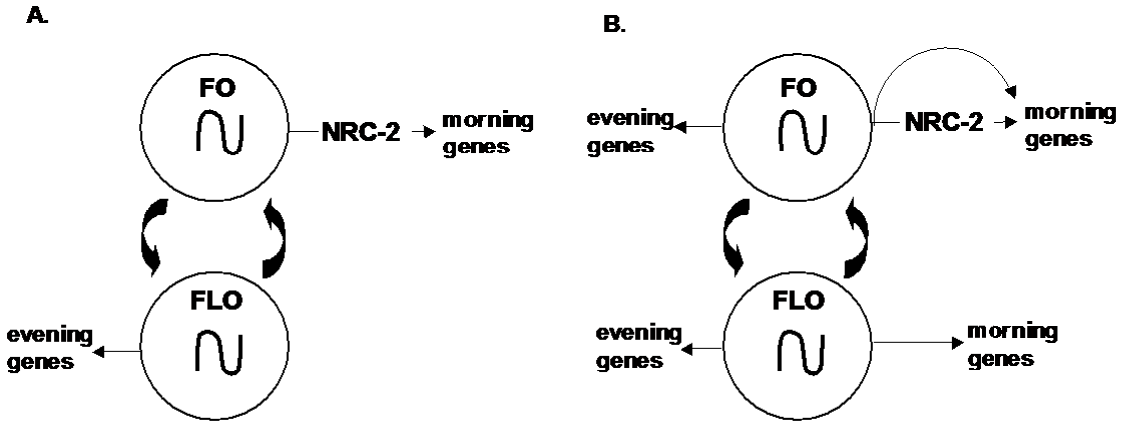


**Figure 20. Models for regulation of output genes in *Neurospora.***

See text for description. ~ = rhythmic.

The models proposed above can be easily tested using a microarray approach. Currently, whole-genome oligonucleotide arrays are under construction for use by the *Neurospora* community. Profiling circadian transcript accumulation in the *frq*-null strain will allow determination of the extent of clock-controlled gene expression that is regulated by the FLO. Model A predicts that all FLO-regulated genes would peak in the evening phase of the cycle. Alternatively, model B predicts that FLO-regulated genes would peak at both phases of the cycle. Similarly, profiling transcript levels in the *nrc-2* mutant would allow determination of the extent of clock-controlled genes regulated by the NRC-2 pathway. Model A predicts that rhythmic accumulation of all morning-specific genes would be eliminated in this mutant. In model B, morning-specific genes may be regulated by the FLO or by an NRC-2 independent pathway.

When considering the evolution of circadian clocks, one reasonable assumption is that individuals that have evolved a mechanism to temporally

separate competing biochemical processes may be provided with a selective advantage over individuals that have not. Evidence of temporal separation of competing pathways can be found in *Neurospora.* For example, two genes with opposing roles in ammonia metabolism are expressed at discreet phases within the circadian cycle. Ammonia is assimilated through a pathway that utilizes an NADPH-specific glutamate dehydrogenase to produce Glutamate from ammonia and 2-oxoglutarate. In the terminal step of this pathway, Glutamine is synthesized from glutamate and ammonia by Glutamine synthase. Conversely, Glutamate is broken down into ammonia and 2-oxoglutarate by an NAD-specific Glutamate dehydrogenase. While the mRNA for Glutamine synthase oscillates with a peak in the late day, the mRNA for the catabolic NAD-dependent Glutamate dehydrogenase has a peak in the early day.

The genes involved in glucose utilization and synthesis may provide another example of this type of regulation. Three of the four glycolytic genes that we identified as clock-controlled are co-regulated (chapter II). Glyceraldahyde 3-phoshpate dehydrogenase, phosphoglycerate kinase, and phosphoglycerate mutase all have peak mRNA levels in the late night. Interestingly, the mRNA encoding the glycolytic gene enolase peaks out of phase in the early night. The biological basis for this divergent regulation of enolase is unknown. One possibility is that enolase may have a function outside of glycolysis. Indeed, enolase is a component of an mRNA degradation complex

in bacteria. A glycolysis-independent role for enolase has not yet been reported in eukaryotic cells; however, this remains an exciting possibility.

While glycolytic genes that break down glucose show peak mRNA levels in the late night, genes involved in glucose production peak at other times within the circadian cycle. The mRNA for phosphoenolpyruvate carboxykinase, a gene that catalyzes a key reaction in the gluconeogenesis pathway, peaks in the early day. Similarly, the mRNA for Isocitrate lyase, an enzyme of the glyoxylate cycle, oscillates with peak mRNA levels occurring in the late day. The glyoxylate cycle converts acetyl-CoA produced during fatty acid catabolism into oxaloacetate and eventually glucose via gluconeogenesis. Also consistent with this hypothesis, the mRNA for glycogen phosphorylase peaks in the early night (glycogen phosphorylase releases glucose from glycogen polymers for breakdown by glycolysis). There is one problem with concluding that the genes involved in glucose utilization and synthesis are regulated as two competing pathways. All of the glycolytic genes discussed above catalyze reversible reactions that participate in both glycolysis and gluconeogenesis. Unfortunately, Phosphofructokinase, an enzyme responsible for regulating flux through glycolysis is not represented on our microarray. The genes encoding hexokinase and pyruvat kinase were identified as rhythmic in the array experiments but failed to pass all the criteria for rhythmic gene classification. The circadian separation hypothesis predicts that these genes would exhibit

peak mRNA levels in the late night, out of phase with glucose synthesis genes. This should be confirmed by northern analysis.

Our understanding of how the clock may regulate competing pathways is limited. The necessary next step will be to determine if the circadian clock directly regulates all or only some of the genes within a set of competing pathways. It is possible that the clock regulates only a few (rate-limiting) genes in a pathway, allowing catabolite repression or allosteric mechanisms to drive oscillations in other pathway genes. For each set of competing pathways, the specific *cis*-elements responsible for rhythmic expression of each gene must be identified. Bioinformatic approaches may be useful in this endeavor. The glycolysis/gluconeogenesis pathways would serve as an excellent starting point for identifying promoter elements that regulate circadian separation of these two pathways. Moreover, study of this well-characterized set of competing biochemical pathways may provide an increased understanding of how clocks regulate competing biochemical pathways in general.

A sophisticated method for measuring fitness has not been developed in *Neurospora.* Without this, it is difficult to test the evolutionary significance of circadian separation of competing pathways. Initially, it is necessary to determine the relative fitness of clock mutant strains compared to wild type clock strains. Subsequent experiments could then be designed to determine how circadian separation of specific sets of competing pathways contributes to the overall fitness of the organism.

**The role of WC-1 in light- and clock-regulation of gene expression**

In chapter III, we examined the role of WC-1 in the regulation of clock-controlled gene expression. WC-1 is required for all blue light photoresponses in *N. crassa* (Degli-Innocenti and Russo, 1984). In addition, WC-1 is required for overt circadian rhythms in the fungus (Crosthwaite *et al.*, 1997). A transient increase in the levels of WC-1 has been reported following exposure of *N. crassa* to light (Schwerdtfeger and Linden, 2000). Similarly, the levels of WC-1 accumulate in a circadian fashion when *N. crassa* cultures are grown in constant darkness (Denault *et al.*, 2001; Lee *et al.*, 2000). These observations provided a testable hypothesis that was addressed in chapter III of this dissertation. Specifically, if an increase in the levels of WC-1 is sufficient to regulate transcription from all WC-1-dependent promoters, then the complement of light-regulated genes should be similar to those genes induced following WC-1 overexpression. This was not the result we observed and describe in chapter III. Conversely, we demonstrated that overexpression of WC-1 is not sufficient to regulate all light-induced genes in the fungus. WC-1 overexpression does activate clock-controlled gene expression, however.

This result supports a model in which modification of WC-1 is required for light-regulated gene expression. Alternatively, circadian expression of WC-1-regulated genes occurs simply due to changes in the levels of WC-1 over the course of the day. In response to light, WC-1 is hyperphosphorylated relative to

the low levels of phosphorylation observed in constant darkness (Lee *et al.*, 2000; Schwerdtfeger and Linden, 2000). The differential phosphorylation of WC-1 may be important for the divergent regulation of light- and clock-regulated gene expression by this protein. However, no phosphorylation sites within the WC-1 protein have been described to date. Certainly, additional analysis will provide an increased understanding of WC-1 phosphorylation and its role in photo-regulation. This may be accomplished by mutant screens for additional genes that abolish or diminish light responses in *Neurospora.* However, this avenue may have been exhausted with little success. This may indicate that many genes involved in photoreception are essential. For this reason, biochemical methods employed to identify kinases that phosphorylate WC-1 would likely be more productive.

Recent biochemical data have provided evidence that the WCC undergoes oligomerization upon exposure to light (i.e. gel shifts reveal that a slower migrating complex interacts with *frq* promoter DNA when the WC proteins are exposed to light) (Froehlich *et al.*, 2002). Although this high molecular weight complex is not observed in protein extracts from dark grown cultures, a low molecular weight WCC does interact with the *frq* promoter in the dark (Froehlich *et al.*, 2002). A model in which different WC-1 containing complexes are responsible for differential regulation of light- and clock-regulated gene expression is consistent with our results. Detailed analyses of proteins that interact with the WCC in the dark and in the light will provide a more complete

understanding of the multiple roles of the WCC. In addition, identification of WC-1 interacting proteins may identify genes that participate in additional circadian feedback loops (see below).

When we compare the list of genes that respond to WC-1 overexpression (chapter III) with the list of clock-controlled genes (chapter II), there are two general observations that we can make. First, overexpression of WC-1 is not sufficient to induce all clock-controlled genes. Whereas we identified only 65 genes that respond to WC-1 overexpression, we identified 150 rhythmic genes in chapter II. Of the 65 genes that were induced following WC-1 overexpression in chapter III, 26 were identified as rhythmic in chapter II (Table 9). This result must be interpreted with caution. It is possible that we did not identify all WC-1-regulated genes due to the limitations of microarray technology. Also, we only monitored WC-1 regulated gene-expression for 13 hours after WC-1 induction. Therefore, it is possible that other clock-controlled genes would have been induced if we extended our time course for a full 24 hours. However, the dramatic difference suggests that WC-1 most likely only regulates a subset of clock-controlled gene expression. This conclusion is supported by the results obtained in chapter IV; although a WC-1 rhythm is not necessary for rhythmic *ccg-16* accumulation, WC-1 rhythms are not sufficient to generate a rhythm in *ccg-1* expression. Of the 27 rhythmic genes induced by WC-1, all but 2 had peak mRNA levels in the late night or early morning, a time when WC-1 levels are high.

A second observation supports a clock-independent function for WC-1 in the dark. Thirty-five genes were identified as WC-1-induced in chapter III, but were not identified as clock-controlled genes in chapter II. Again, this result must be interpreted with caution due to the limitations of microarray technology. It is possible that these genes are indeed rhythmic but were not identified in chapter II. For example, SC1G8 encodes the glycolytic gene triose phosphate isomerase. Triose phosphate isomerase was induced by WC-1 in chapter III but was not identified as clock-controlled in chapter II. Yet, northern blots demonstrate that the mRNA accumulates in a circadian fashion (chapter III). Despite the technological limitations that must be considered when interpreting these data, these two general trends should be noted when comparing the data from chapters II and III. In the future, confirmation of these observations using northern blot analysis must be done to clearly illustrate these trends.

**Table 9. Summary of rhythmic genes induced by WC-1.**

| EST[a] | Predicted ORF[b] | BLAST Match[c] | Organism[d] | Accession numbe[e] | WC-1 class[f] | Phase[g] |
|---|---|---|---|---|---|---|
| SM2A10 | NCU00315.1 | HOMOLOGOUS TO RAT L29 RIBOSOMAL PROTEIN | S. cerevisiae | 14318557 | IIIW | LN |
| W10A1 | NCU00634.1 | HOMOLOGY TO MAMMALIAN L14 | S. cerevisiae | 6321786 | IIIW | LN |
| NC1A3 | NCU01207.1 | vma-1 (VACUOLAR ATPASE SUBUNIT) | N. crassa | 168925 | IIW | LD |
| NC5E12 | NCU01776.1 | HOMOLOGY TO RAT L15 RIBOSOMAL PROTEIN | S. cerevisiae | 6323769 | IIIW | LN |
| NC2C12 | NCU01949.1 | 40S RIBOSOMAL PROTEIN S9 | P. anserina | 1710780 | IIIW | LN |
| NC2D12 | NCU02252.1 | PHOSPHOGLYCEROMUTASE | A. oryzae | 9955875 | IW | LN |
| W17E6 | NCU02509.1 | 60S RIBOSOMAL PROTEIN L11 | S. pombe | 5738531 | IIIW | LN |
| W13A10 | NCU02708.1 | RIBOSOMAL PROTEIN L35 | O. ulmi | 14165210 | IIW | LN |
| NC2B7 | NCU03757.1 | PROBABLE RIBOSOMAL PROTEIN RPL4A | N. crassa | 12718316 | IIW | LN |
| NC3E3 | NCU04779.1 | RIBOSOMAL PROTEIN L8 | S. pombe | 6094099 | IIIW | LN |
| W09H4 | NCU05599.1 | 40S RIBOSOMAL PROTEIN S28 | S. pombe | 1710760 | IIIW | LN |
| W10F8 | NCU05810.1 | CPC-2 | N. crassa | 971565 | IIW | LN |
| NC3G1 | NCU06226.1 | RIBOSOMAL PROTEIN S23A | H. sapiens | 404015 | IIW | LN |
| SC2B5 | NCU06432.1 | 40S RIBOSOMAL PROTEIN | B. graminis | 12229899 | IIW | ED |
| NC3E8 | NCU06661.1 | 60S RIBOSOMAL PROTEIN L22 | S. pombe | 12644397 | IIW | LN |
| W08B2 | NCU07033.1 | NO BLAST HITS | | | IIIW | LN |
| NC3G11 | NCU07182.1 | HOMOLOGY TO RAT S24 RIBOSOMAL PROTEIN | S. cerevisiae | 6320918 | IIW | LN |
| NC1C6 | NCU07318.1 | MANNITOL 1-PHOSPHATE-5-DEHYDROGENASE | N. crassa | 400286 | IIW | EN |
| NC2D1 | NCU07914.1 | PKG GENE (ATP;3-PHOSPHO-D-GLYCERATE-1-PHOSPHOTRANSFERASE) | N. crassa | 3051 | IW | LN |
| W08C5 | NCU08017.1 | NO BLAST HITS | | | IIIW | LN |
| W07F11 | NCU08409.1 | TRP-3 (TRYPTOPHAN SYNTHASE) | N. crassa | 168915 | IIW | ED |
| W01G9 | NCU08500.1 | HOMOLOGY TO MAMMALIAN S8 | S. cerevisiae | 6319399 | IIIW | ED |
| NC4G3 | NCU08963.1 | F28KF19.15 | A. thaliana | 6573778 | IIIW | LN |
| SC5D4 | NCU09089.1 | NO BLAST HITS | | | IIW | LN |
| NC1C9 | NCU09109.1 | 60S RIBOSOMAL PROTEIN L33-A | S. cerevisiae | 15214241 | IIW | ED |
| W07C10 | NCU09345.1 | NMT-1 PROTEIN | A. parasiticus | 1171741 | IIW | ED |
| W13B7 | NCU09711.1 | HYPOTHETICAL PROTEIN | S. Pombe | 6855453 | IIIW | LN |

[a] The N. crassa ESTs identified are from the Neurospora Genome Project, University of New Mexico (http://www.unm.edu/~ngp)
[b] The predicted ORFs are from the Broad Institute Neurospora Sequencing Project (http://www-genome.wi.mit.edu/annotation/fungi/neurospora/
[c] The BLAST matches represent the most significant match in the database as predicted by the Broad Institute (minimum value <e-5).
[d] The organism with the closest BLAST match is indicated.
[e] The accession numbers for each BLAST match are from the NCBI sequence database (http://www.ncbi.nlm.nih.gov/)
[f] Indicates the class that the gene was placed in in chapter III
[g] Indicates the phase of peak expression that the gene exhibited in chapter II.

## Multiple circadian oscillators

Much work over the last five years has provided physiological evidence for multi-oscillator organization of the *N. crassa* circadian system (Granshaw *et al.*, 2003; Lakin-Thomas and Brody, 2000; Merrow *et al.*, 1999). In chapter II we described three genes that display a rhythm in mRNA accumulation in a *frq*-null strain. This result demonstrates that an additional oscillator is in fact present within the *N. crassa* circadian system. In chapter IV we further characterized the properties of this oscillator by examining expression of *ccg-16* in different genetic

backgrounds under various conditions. We provided evidence that *ccg-16* is indeed controlled by a FLO. We demonstrated that *ccg-16* mRNA accumulates in a circadian fashion in a *frq*-null strain. Also, *ccg-16* mRNA accumulates rhythmically in constant light when FRQ protein levels are constitutively high and arrhythmic. The phase of *ccg-16* is altered in the long period $frq^7$ strain indicating that the FLO that regulates *ccg-16* is coupled to the FRQ/WC-oscillator. Interestingly, WC-1 is required for *ccg-16* mRNA rhythms. Most notably, we further demonstrated that WC-1 levels oscillate in a *frq*-null strain. This result contradicts a previous model in which FRQ protein is required for the WC-1 rhythm. Although we observed a rhythm in WC-1 in the $frq^{10}$ strain, we demonstrated that a rhythm in WC-1 is not required for rhythmic accumulation of *ccg-16.* These data provide the most detailed molecular characterization of a FLO to date.

Based on the results reported in chapter IV, we propose a two-oscillator model for regulation of output gene expression in *Neurospora* (Figure 21). In this model, the FRQ/WC-oscillator is coupled to and drives the FLO that regulates *ccg-16.* Two pieces of evidence support this hierarchal organization: 1) The long period $frq^7$ strain alters the phase of the *ccg-16* rhythm. 2) WC-1 protein accumulates rhythmically in a *frq*-null strain but not a wild type strain under constant light conditions. This second result indicates that FRQ has a stronger affect on WC-1 protein than the FLO. On the other hand, the observation that WC-1 levels oscillate in a *frq*-null strain demonstrates that the

FLO does contribute to the rhythmic accumulation of WC-1. This indicates that communication between the FRQ/WC-oscillator and the FLO occurs in both directions; in other words, there is mutual coupling of the two oscillators.

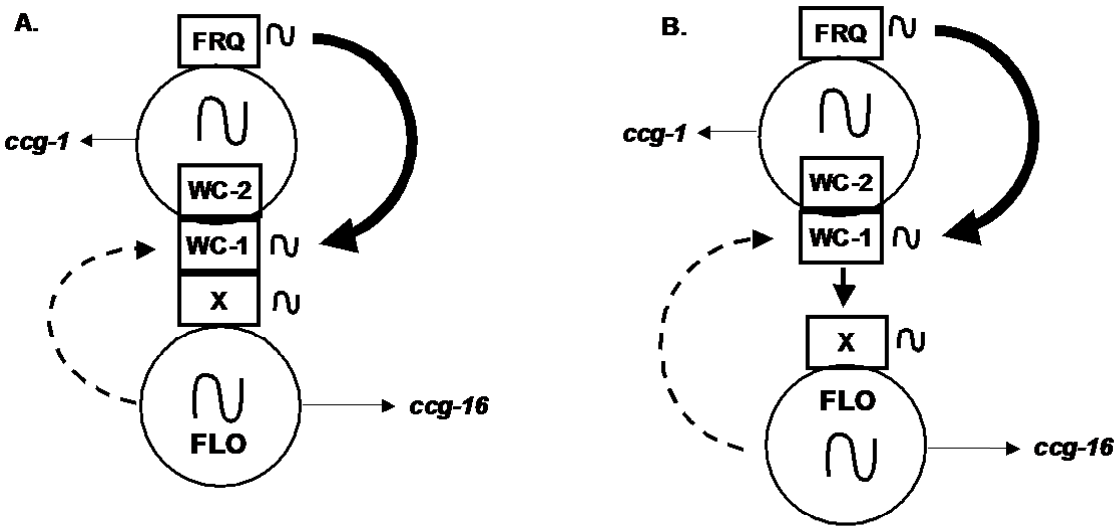


**Figure 21. Models for multiple oscillator organization in *Neurospora.***

See text for description. ~ = rhythmic.

There are several aspects of the model that must be addressed in the future. For instance, the role of WC-1 in the FLO remains unclear. While WC-1 protein is required for the FLO to generate rhythmic gene expression, rhythmic accumulation of WC-1 protein does not appear to be necessary (*ccg-16* is rhythmic in wild type strains in constant light while WC-1 is not rhythmic). This result can be explained if WC-1 interacts with a component of the FLO that accumulates rhythmically (model A). Importantly, this scenario provides a mechanism for entrainment of the FLO by the FRQ/WC-oscillator. Alternatively, WC-1 may only be required to activate a component of the FLO. This

component may itself be rhythmic (through a post-transcriptional mechanism), or this component may interact with a second rhythmic component (model B).

Biochemical approaches or a yeast two-hybrid screen to identify FLO components that interact with WC-1 may help distinguish between these two possibilities. Alternatively, bioinformatic analysis of the genome may help identify direct transcriptional targets of WC-1, and therefore, may help identify key FLO components. Genetic screens to identify FLO components will likely be a productive means to understand the mechanism of rhythm generation by the FLO. Undoubtedly, characterization of FLO components and how they interact genetically and biochemically with the FRQ/WC-oscillator will provide a general understanding of how these two oscillators interact. Currently, technical limitations make these screens difficult if not impossible. Attempts to generate stable rhythmic LUCIFERASE reporter strains with the *ccg-16* promoter have been unsuccessful. Recently, GFP has been used as a reporter in several *Neurospora* labs (Freitag *et al.*, 2004). This may provide the needed reporter system to design the necessary genetic screens. A CCG-16:GFP reporter could be used to isolate mutants that show arrhythmic CCG-16 expression in the *frq*-null background. In addition, screens for strains that alter the period or phase of *ccg-16* but do not affect *frq*-regulated output should identify components of the FLO.

At this time, we can not be certain if feedback from the FLO significantly affects the FRQ/WC-oscillator in a wild type cell. To determine the relative

effects of the FRQ/WC-oscillator and the FLO on the *ccg-16* rhythm, the phase response curves (PRC's) exhibited by *ccg-16* must be determined experimentally for both light and temperature pulses. If the FLO is entrained exclusively by the FRQ/WC-oscillator, the PRC's should be identical to those displayed by the conidiation rhythm. If the FLO itself receives environmental input, then discreet PRC's for *ccg-16* and conidiation will be observed. These experiments will also require a reliable reporter system such as a CCG16:GFP fusion.

It is possible that the FLO that regulates *ccg-16* is identical to the oscillator that generates conidiation rhythms in the *frq*-null strain (i.e. when media is supplemented with farnesol or geraniol). If this is the case, then two experimental predictions can be made. 1) The *wc-1* deletion strain will not conidiate rhythmically when supplemented with these compounds, and 2) WC-1 levels will be elevated when the *frq*-null strain is grown in media supplemented with farnesol and geraniol. If these predictions do not hold, it is likely that the FLO that regulates *ccg-16* is separate from the previously hypothesized FLO.

Multiple oscillator organization is widespread in metazoan organisms. Circadian networks are composed of coupled autonomous oscillators that interact to drive rhythmic outputs. The neuro-endocrine loop model illustrates the multi-oscillatory network that comprises the avian circadian system; here, coupled oscillators in the pineal gland and the hypothalamus (vSCN) interact to drive rhythmic behavior (Cassone and Menaker, 1984). In the house sparrow for example, pinealectomy results in arrhythmic behavior. Moreover, transplantation

of the pineal gland to a pinealectomized recipient restores rhythmic behavior and imposes the phase of the pineal donor. This clearly implicates the pineal as a master oscillator. However, ablation of the avian homolog of the SCN (vSCN) also produces arrhythmic behavior, indicating that this structure is involved in the circadian system as well. Furthermore, pinealectomy-induced arrhythmia is observed days after transfer of the animals into constant darkness. This is likely due to the residual activity of a damped circadian oscillator, probably the vSCN. Consistent with this, pinealectomized animals undergo systematic circadian entrainment to light:dark cycles, indicating a light entrainable oscillator is present in these animals (Pittendrigh, 1981). Taken together, these data suggest that circadian behavior in the house sparrow emerges from mutually coupled circadian oscillators.

Alternatively, early work with *Drosophila* led to a model in which a master oscillator drives a second slave oscillator to control circadian eclosion in the fly (Pittendrigh, 1960). Differential responses to light and temperature imply that a temperature responsive slave oscillator directly controls eclosion. However, this oscillator is entrained to light:dark cycles only through entrainment by the master oscillator. Furthermore, the second oscillator provides little feedback onto the master oscillator when competing light:dark and temperature cycles are imposed on the system. The *Neurospora* system more closely resembles this type of organization. In all cases that have been reported, *Neurospora* FLO's do not exhibit circadian properties such as entrainment to light:dark, temperature

compensation, or frequency demultiplication , (Aronson *et al.*, 1994a; Granshaw *et al.*, 2003). Moreover, when intact, the FRQ/WC-oscillator imposes its period on the FLO (L.W. Morgan, Z.A. Lewis, and D. Bell-Pedersen, in preparation). There is one exception addressed in appendix B. However, this requires further investigation.

A third prominent model for multiple oscillator organization involves morning and evening oscillators (Daan *et al.*, 2001). When certain nocturnal mammals are housed in constant light, the activity rhythm often splits into two components that eventually become locked in a stable anti-phase relationship. This "splitting" phenomenon was the basis the morning / evening oscillator model. The model is particularly attractive because it provides a means to measure day length, and therefore provides a mechanism for circadian regulation of photoperiodic responses (i.e. seasonal reproduction). Recently, splitting has been shown to result from anti-phase oscillation of clock genes in the two bilateral lobes of the SCN (de la Iglesia *et al.*, 2000). The morning and evening oscillator model remains a viable paradigm, however. This model is used to explain differential phase shift responses of morning and evening components of multiple rhythmic events. For example, coronal SCN slices maintained in culture exhibit two peaks of electrical activity each day, one in the early subjective day and another in the late subjective day (morning and evening) (Jagota *et al.*, 2000). Glutamate infusion (which simulates a light pulse) in the early subjective morning causes a rapid phase advance of the morning

peak, but the evening peak remains unchanged. On the other hand, glutamate infusion in the early night causes a phase delay of the evening peak, while the morning peak remains unchanged.

Evening-specific regulation of *ccg-16* by a second oscillator could indicate that the *Neurospora* clock is comprised of morning and evening oscillators. While the data presented in this dissertation do not support or oppose this idea, the discovery of evening-specific genes provides a means to test this possibility. One prediction of such a model is that the phase relationship of morning- and evening-specific genes would be altered by changes in photoperiod (the peaks should drift apart as photoperiod is increased). One might also predict that phase advances would occur more rapidly for morning- specific genes while phase delays would occur more rapidly for evening-specific genes. Unlike in metazoan systems however, phase shifts of the conidiation rhythm occur immediately following a light or temperature pulse (transients are not observed) (reviewed by Lakin-Thomas *et al.*, 1990). Based on this, differential phase responses of morning and evening genes seems unlikely.

In summary, the work presented here greatly expands our knowledge of the *Neurospora* circadian system. Models for the *Neurospora* clock must now incorporate a mechanism for clock-controlled gene regulation at all phases of the circadian cycle. In addition, models must include a mechanism for interaction of the FRQ/WC-oscillator with the FLO. Most generally, the work presented here

provides a solid foundation for understanding the complexity and organization of a simple circadian system.

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# APPENDIX A

## *vvd* IS REQUIRED FOR LIGHT-ADAPTATION OF CONIDIATION-SPECIFIC GENES OF *Neurospora crassa,* BUT NOT CIRCADIAN CONIDIATION*

This appendix contains a published manuscript that is the result of a collaboration between the laboratories of Deborah Bell-Pedersen and Daniel Ebbole at Texas A&M University. My specific contribution to this paper includes assays for a functional circadian clock in the *vvd* strain and northern analysis of the *frequency* gene.

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# *vvd* Is Required for Light Adaptation of Conidiation-Specific Genes of *Neurospora crassa*, but Not Circadian Conidiation

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**Shrode, L. B., Lewis, Z. A., White, L. D., Bell-Pedersen, D., and Ebbole, D. J. 2001. *vvd* is required for light adaptation of conidiation-specific genes of *Neurospora crassa*, but not circadian conidiation. *Fungal Genetics and Biology* 32, 169–181. *con-10* and *con-6* are two of the *conidiation* (*con*) genes of *Neurospora crassa* that were identified based on their preferential expression during macroconidiophore development. They are also regulated by several other environmental stimuli independent of development, including a transient induction by light. We identified an allele of *vivid* (*vvd*) in a mutant screen designed to obtain strains with altered expression of *con-10*. *vvd* mutants display enhanced carotenoid pigmentation in response to light. In addition, *con-10* and *con-6* show a heightened response to photoinduction. We tested the function of the light-responsive circadian clock in the *vvd* mutant and found no major defect in the circadian rhythm of conidiation or light regulation of a key clock component, *frequency* (*frq*). We conclude that *vvd* is primarily involved in a process of light-dependent gene repression, called light adaptation. Although a number of gene products are known to control light induction in fungi, *vvd* is the first gene shown to have a role in adaptation to constant light.** © 2001 Academic Press

*Index Descriptors:* sporulation; *Neurospora crassa;* light regulation; carotenoid; circadian rhythm.

*Neurospora crassa* is a filamentous ascomycete that can undergo three distinct sporulation pathways, with the choice dependent on environmental conditions (Harding and Melles, 1983; Linden *et al.,* 1997a; Ricci *et al.,* 1991; Springer, 1993). *N. crassa* can undergo sexual development leading to the production of ascospores and two forms of asexual reproduction leading to the formation of microconidia and macroconidia (Springer, 1993; Turian and Bianchi, 1972). Macroconidiation is induced by exposing vegetative hyphae to air or by starving for carbon or nitrogen (Turian, 1973; Turian and Bianchi, 1972). Conidiation is also regulated by the endogenous circadian clock (Pittendrigh *et al.,* 1959; Sargent *et al.,* 1966). Once conidiation commences, vegetative hyphae that are growing by apical elongation form branches that switch to an apical budding form of growth (Springer and Yanofsky, 1989). This budding process ultimately produces the orange-pigmented, multinucleate conidia. Blue light regulates the production of carotenoid pigment, regulates the formation of conidia, and resets the circadian clock of the fungus (Harding and Turner, 1981; Linden and Macino, 1997; Sargent and Briggs, 1967).

Investigations by Berlin and Yanofsky (1985a) established that changes in gene expression and protein accumulation occur throughout conidiation. A number of genes were identified that were preferentially expressed during conidiation called *conidiation* (*con*) genes (Berlin and Yanofsky, 1985b). *con-10* is expressed in all three sporulation pathways and is subject to other forms of regulation that are independent of development (Corrochano *et al.,* 1995; Lauter and Yanofsky, 1993; Lee and Ebbole, 1998b; Springer and Yanofsky, 1992). Light, the circadian clock, nitrogen starvation, carbon starvation, and

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heat shock have all been shown to regulate *con-10* expression (Lauter and Yanofsky, 1993; Lee and Ebbole, 1998a,b). The *con-10* gene encodes a small stress-response protein of unknown function that is conserved in a number of bacterial, fungal, and plant species (Lee and Ebbole, 1998b; Roberts *et al.,* 1988). Its promoter has also been examined in considerable detail and these studies identified multiple sites for repression and activation of *con-10* transcription (Corrochano *et al.,* 1995; Lee and Ebbole, 1998a,b).

The *con-6* gene also encodes a small protein of unknown function (White and Yanofsky, 1993). It is expressed slightly earlier during conidiation than *con-10* (Springer and Yanofsky, 1989) and, similar to *con-10,* is regulated by light and the circadian clock (Lauter and Yanofsky, 1993).

Both *con-6* and *con-10* are transiently induced following a light pulse or when cultures are transferred from the dark to continuous light (Lauter and Yanofsky, 1993). This pattern of regulation is typical for most of the known light-regulated genes of *N. crassa* (Linden *et al.,* 1997a), suggesting that a general mechanism exists that allows cells to adapt to a light stimulus.

Mutagenesis experiments have been performed to obtain strains that have altered regulation of *con-10* gene expression (Madi *et al.,* 1994). The *regulator of conidiation-1* (*rco-1*) and *rco-3* mutants were isolated by this approach (Madi *et al.,* 1997; Yamashiro *et al.,* 1996). RCO1 shares sequence similarity to yeast Tup1 and is thought to be a cell type-specific repressor of *con-10* expression (Lee and Ebbole, 1998b; Yamashiro *et al.,* 1996). RCO3 appears to act as a glucose sensor that plays a role in conidiation, sugar transport, and glucose repression (Madi *et al.,* 1997).

Here, we identified an additional mutant that displayed altered *con-10* expression and enhanced carotenoid pigmentation in response to light. This mutant contains a recessive mutation that is allelic to *vivid* (*vvd*) (Perkins *et al.,* 1997). *vvd* mutations result in a longer and stronger response of *con* gene expression to photoinduction. Based on these findings we propose that the *vvd* locus encodes a protein involved in repression of gene expression in a light-dependent manner. However, not all light-regulated genes are equally affected by *vvd.* The *frequency* (*frq*) gene encodes a major component of the circadian clock (Aronson *et al.,* 1994a,b; Dunlap, 1996) and is light induced. Unlike *con-10* and *con-6, frq* transcripts remain elevated following transfer of cultures from the dark to continuous light (Crosthwaite *et al.,* 1995). Here, we show that the circadian rhythm of conidiation is not disrupted and light regulation of *frq* appears similar in *vvd.* Together, these results indicate a specific role for *vvd* in photoregulation of light-adapted genes.

## MATERIALS AND METHODS

### *Fungal Strains and Plasmids*

Unless otherwise noted, strains were obtained from the Fungal Genetics Stock Center (FGSC),[1] Department of Microbiology, University of Kansas Medical Center, Kansas City, Kansas. The *vvd* strain (*vvd*(P4246), FGSC No. 7855; Perkins *et al.,* 1997) was provided by Dr. David Perkins, Stanford University.

An *adenine-7* (*ad-7* (Y112M33)), *histidine-3* (*his-3* (1-234-1438)) strain was transformed with a plasmid containing a translational fusion of *con-10* to the hygromycin phosphotransferase gene (*hph*) (Cullen *et al.,* 1987). The fusion gene contains a *con-10* DNA fragment from positions 1139–1935 (Roberts *et al.,* 1988). This fragment extends from −520 upstream of the major transcription initiation site to codon 40 of *con-10.* The plasmid containing this fusion, pCH10 (Madi *et al.,* 1994), was targeted for integration at the *his-3* locus. The resulting strain, LBS124, was found to be sensitive to 200 μg/ml hygromycin when grown on adenine-supplemented minimal medium in constant light.

Strain 30-7 (*bd; A*) was crossed with the *vvd*(P4246) strain to produce strains 17.1 (*bd;vvd;A*) and 17.2 (*bd;vvd; a*). The *bd* allele allows visualization of the circadian rhythm of conidiation on closed growth (race) tubes (Sargent and Kaltenborn, 1972).

The plasmid used for the insertional mutagenesis experiment, pAD744, contains a 4.4-kb fragment of *N. crassa* genomic DNA from cosmid X24:H5 of the Orbach/Sachs cosmid library (Orbach and Sachs, 1991) that complements the *ad-7* mutation.

Protoplasts of LBS124 were transformed with pAD744 as described (Vollmer and Yanofsky, 1986). Integration of DNA into the *N. crassa* genome frequently occurs by ectopic integration and can result in insertional inactivation of genes at the site of integration (Asch and Kinsey, 1990). Transformed protoplasts were resuspended in 50 ml of regeneration agar and then aliquoted to five petri dishes and the agar was allowed to solidify. Agar (20 ml for

[1] Abbreviations used: CT, circadian time; FGSC, Fungal Genetics Stock Center; RIP mutation, repeat induced point mutation.

each plate) containing 2% sorbose, 0.05% fructose, 0.05% glucose, and 200 μg/ml hygromycin was poured on top of the solidified medium containing the transformed protoplasts. Plates were incubated at 34°C in constant light for 3 to 7 days. Prototrophic transformants with resistance to hygromycin were isolated as putative mutants and then screened for visual phenotypes.

The original pAD744 transformant (AUB1) was crossed with a *fluffy* (*fl*) mutant (FGSC No. 45). Because the allele of *vvd* in AUB1 was found to differ in phenotype from the *vvd*(P4246) allele, we will designate the *vvd*(P4246) allele as $vvd^{P}$ and the allele derived from strain AUB1 as $vvd^{aub}$. The $vvd^{aub}$ strain derived from this cross, AUB2, was backcrossed to *white collar-1* ($wc\text{-}1^{P826}$) (FGSC No. 128) and backcrossed two additional times with the wild-type strain 74-OR23-1VA (FGSC No. 2489). The segregation of $vvd^{aub}$ to $vvd^{+}$ in this final cross was 1:1. AUB4, an offspring of the final cross, was the $vvd^{aub}$ strain used for all experiments reported in this paper unless otherwise noted. During this genetic characterization it was found that the mutation in *vvd* was not linked to the integrated *ad-7* plasmid and the *vvd* mutation apparently arose spontaneously.

## Race Tube Assay

Measurement of circadian period was performed on race tubes containing glucose–arginine medium (Sargent and Kaltenborn, 1972). Analysis of phase response to light was performed on race tubes containing acetate–casamino acid medium (Feldman and Hoyle, 1973), which results in condensed conidial bands and a more accurate measure of phase response. Calculation of circadian period length and phase was performed as previously described (Dharmananda and Feldman, 1979). Phase shifting by light was evaluated by placing race tube cultures in a controlled environment chamber (Percival Scientific, Inc.) containing fluorescent lights (Phillips F20T12 CW 20 W). Light pulses of 15 min were given to cultures after 120 or 136 h of growth in the dark and were of an intensity of 44.8 μmol photons/$m^2$/s (1700 lux). Light intensity was measured using two different meters: a quantum sensor to measure photons (Biospherical Instruments) and a dual-range light meter (Traceable Instruments) to measure lux. In these experiments, the light intensity was above the level of saturation required to produce a maximal phase shift of the conidiation rhythm (estimated at 100 lux for a 5-min light pulse (Dharmananda and Feldman, 1979)). Constant light treatments of race tube cultures were carried out in bright light (1000 lux) or dim light (40 lux, data not shown). Both the bright and the dim light conditions allowed observation of the conidiation rhythm in the light-insensitive *lis-2* mutant strain (Paietta and Sargent, 1983) (data not shown).

## Heterokaryon Tests for Complementation and Dominance

AUB4 was crossed with a *pyrimidine-3* (*pyr-3*) strain (FGSC No. 937) and the $vvd^{P}$ strain was crossed with a *histidine-5* (*his-5*) strain (FGSC No. 456). Double mutants from each cross, *pyr-3;* $vvd^{aub}$ and *his-5;*$vvd^{P}$, were obtained in order to perform heterokaryon tests. Heterokaryon complementation testing was performed by spreading conidia from *pyr-3;*$vvd^{aub}$ and *his-5;*$vvd^{P}$ on a slant of minimal medium. Heterokaryon dominance tests were carried out by spreading conidia from *pyr-3;*$vvd^{aub}$ and *ad-3* (FGSC No. 6656) or *his-5;*$vvd^{P}$ and *ad-3* on minimal medium.

## Light Induction Experiments

*N. crassa* strain 74-OR23-1VA was grown for 7–10 days on solid minimal medium N (Davis and DeSerres, 1970). Conidia were suspended in water and then filtered through four layers of cheesecloth to remove mycelial fragments. Conidia were inoculated into 50-ml cultures of minimal medium in 125-ml flasks at a concentration of $1 \times 10^6$ conidia/ml. These cultures were grown in the dark at 34°C (200 rpm) for 12, 24, 36, 48, or 60 h. At each of these times, one flask was harvested as a control and six flasks were moved into constant light at 34°C (200 rpm) for light induction experiments. At 0.5, 1, 1.5, 2, 3, and 4 h after the cultures were moved into the light, one sample from the light and one sample from constant darkness were harvested and quickly frozen in liquid nitrogen. Experiments to compare light-regulated gene expression in the $vvd^{aub}$, $vvd^{P}$, and 74-OR23-1VA strains were performed using this nonharvested liquid culture method.

In contrast to the experiments described above, a second method was used to examine light induction in mycelia harvested on Whatman No. 1 filter paper at each of the indicated times. One pad was frozen immediately, and the remaining six pads were cut in half. One pad was placed in constant light at 34°C and the other remained in the dark at 34°C. Minimal medium (1 ml) was added to the samples every 30 min to ensure that they did not desiccate. After 0.5, 1, 1.5, 2, 3, and 4 h, harvested samples from the light and the dark were frozen in liquid nitrogen.

A third growth regime was used to assess light induction of *frq* and circadian responses. Conidia were inoculated directly into petri dishes containing 25 ml of minimal salts, 1% glucose, 0.5% arginine, and 100 ng/ml biotin (Sargent and Kaltenborn, 1972) and allowed to grow in constant light at 25°C for 24–48 h. Then, approximately 1-mm mycelial disks were isolated and placed into 50 ml of fresh medium in 125-ml Erlenmeyer flasks. The flasks were placed on a rotary shaker at 150 rpm and maintained at 25°C in constant light for at least 1 h before transfer to constant darkness. This transfer synchronizes the cultures and sets the circadian clock to dusk (circadian time, CT12). CT is used to normalize biological time in strains or organisms with different endogenous period lengths to 24 circadian hours per cycle. By convention, CT0 represents subjective dawn and CT12 represents subjective dusk. After remaining in constant darkness for 24 h, half of the cultures were given a light pulse (44.8 $\mu$mol/m$^2$/s (1700 lux)), while the second half remained in constant darkness. Mycelial pads were collected just before the light pulse (time 0) and one mycelial pad for each experiment was harvested after 5, 15, or 30 min in the light. The remaining cultures (that were exposed to 30 min of light) were returned to the dark and harvested 60, 90, or 120 min after the start of the experiment (time 0). Light treatments were carried out as described above for the race tube analysis.

### Constant Light Experiments

Strains 74-OR23-1V*A*, *vvd*$^{sub}$, and *vvd*$^{P}$ were grown on agar minimal medium for 7 days and the conidia were harvested (Davis and DeSerres, 1970). Four 125-ml flasks of minimal medium (50 ml) were inoculated with $1 \times 10^6$ conidia/ml for each strain. Three cultures of each strain were grown in constant light, while the remaining culture of each was grown in constant darkness (34°C at 200 rpm). Cultures grown in constant light were harvested and frozen in liquid nitrogen at 24, 48, and 72 h while the cultures grown in the dark were harvested at 72 h.

### RNA Extraction and Analysis

RNA extraction and analyses were performed as previously described (Madi *et al.*, 1994). An actin cDNA clone (provided by Dr. M. Plamann, University of Missouri, Kansas City) or rDNA was used as template for generating probes used as loading controls (Bell-Pedersen *et al.*, 1996; Tinsley *et al.*, 1998). Radiolabeled probes for *con-10* and *con-6* were prepared from cDNA clones from plasmids pBW100 and pCON6-6, respectively (Madi *et al.*, 1994), by random-primed synthesis (Rediprime, Amersham–Pharmacia, Inc.) or as riboprobes using the MAXIscript *in vitro* transcription kit (Ambion, Inc.). *frq* riboprobes were synthesized from plasmid pKAJ106 (Aronson *et al.*, 1994a).

### Densitometry

Autoradiographs of Northern blot hybridizations were scanned using the UltraScan XL Laser Densitometer (Pharmacia LKB Biotechnology AB) to estimate mRNA levels for the *con-6, con-10,* and actin (*act-1*) genes. Data were analyzed using the GelScanXL Software package (Pharmacia). The signal obtained from 20 $\mu$g of RNA hybridized with *act-1* from one sample on each blot was used to normalize RNA loading by comparing *act-1* mRNA signals for each lane. The *con-6* and *con-10* mRNA levels were defined by the ratio of their mRNA levels to *act-1* mRNA. All blots for an experiment were probed together and direct comparisons of hybridization intensities were made. Two independent experiments were performed.

## RESULTS

### Selection of Mutants with Altered con-10 Expression

*con-10* expression is regulated by development, stress, light, and the circadian clock. Under constant light conditions, *con-10* is not normally expressed in mycelia (Lauter and Russo, 1991). A promoter fusion was engineered and used to isolate mutants with altered *con-10* expression (Madi *et al.*, 1994). Increased expression of the *con-10–hph* fusion leads to higher levels of resistance to hygromycin. In this experiment, the selection was carried out in an incubator with constant light conditions. Since *con-10* is highly repressed in constant light in the absence of development, we expected to identify *trans*-acting mutations affecting developmental, stress, light, or circadian clock regulation of the *con-10–hph* fusion. LBS124 is an *ad-7* mutant containing a single copy of the *con-10–hph* fusion integrated at the *his-3* locus. Protoplasts of LBS124 were transformed with a plasmid, pAD744, containing DNA that complements the *ad-7* mutation. Because the majority of transformation events result from nonhomologous integration of the plasmid throughout the genome, we

expected some fraction of transformants to generate insertional mutations (see Materials and Methods). Eighty-one transformants that grew on minimal medium in constant light containing selective levels of hygromycin (200 $\mu$g/ml) were chosen for further analysis.

While the majority of the 81 strains were not visibly distinguishable from wild-type, 9 isolates displayed discernible phenotypes. One strain, designated AUB1, displayed a striking pigmentation phenotype. The enhanced pigmentation of AUB1 was also seen in submerged cultures that were grown in constant light for 48 h. When the AUB1 strain was grown in the dark, no carotenoid pigmentation was observed, suggesting that the pigmentation phenotype was light-regulated. We measured the level of *con-10* mRNA in conidia of the AUB1 strain by Northern blot analysis. AUB1 displayed an approximately threefold increase of *con-10* expression in conidia when compared to wild-type (data not shown).

The phenotype of the AUB1 strain suggested that light or developmental regulation could be defective since *con-10* and carotenoid biosynthesis are regulated by both light and development. In a cross of AUB1 to the aconidial mutant *fluffy* (*fl*) (Bailey and Ebbole, 1998; Perkins *et al.*, 1982) an aconidial class of progeny was obtained that showed enhanced carotenoid pigmentation. One of the progeny from this cross that resembled the AUB1 parent, AUB2, was crossed to *white collar-1* (*wc-1*) to obtain a double mutant. *wc-1* is required for carotenoid induction in response to light (Nelson *et al.*, 1989). The double mutant strain did not accumulate carotenoids in mycelia when grown in constant light. Thus, *wc-1* is epistatic to the mutation in AUB1, but *fl* is not.

An offspring of a fourth-generation backcross, AUB4, was used for genetic analyses and further characterization. Southern blot analysis of progeny from this fourth-generation backcross showed that the mutation was not tagged with the pAD744 plasmid, but apparently was generated spontaneously (data not shown).

## Enhanced Pigmentation Is Caused by Mutation of vvd

*vvd* strains have greatly enhanced orange pigmentation and the gene maps to the left arm of chromosome VI (Perkins *et al.*, 1997). To compare the phenotypes of wild-type, the *vvd*$^{p}$ strain, and AUB4, we inoculated minimal medium slants and liquid cultures of each strain and grew them in constant light. The AUB4 strain had pigmentation that was similar to the *vvd*$^{p}$ strain (Fig. 1). After 48 h in liquid culture, the mycelium of AUB4 and the *vvd*$^{p}$ mutant had enhanced pigmentation, while wild-type had little pigmentation (data not shown). To determine whether any relationship existed between these two genes affecting pigmentation, we crossed AUB4 to the *vvd*$^{p}$ mutant and examined 300 offspring. All the 232 viable progeny had enhanced carotenoid pigmentation, suggesting that the mutations are closely linked.

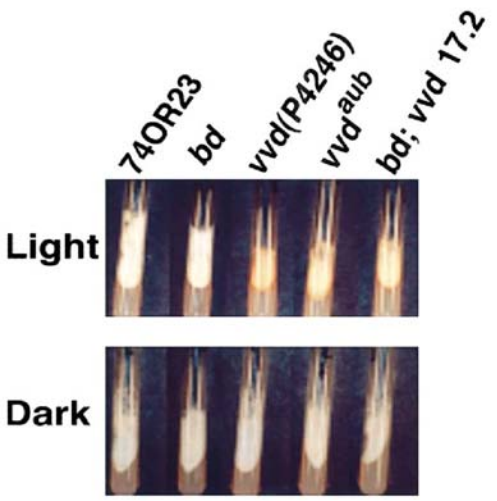


FIG. 1. Enhanced pigmentation of *vvd* alleles. The indicated strains were grown on minimal agar slants for 7 days in the light (top) or the dark (bottom) and photographed.

Complementation and dominance of genes can be studied by making forced heterokaryons since nuclei originating from different strains share a common cell and cytoplasm (Davis and DeSerres, 1970). Auxotrophic markers can be used to select for heterokaryons. We crossed AUB4 with a *pyr-3* strain and the *vvd*$^{p}$ strain with a *his-5* strain to obtain the double mutant strains for heterokaryon analysis. The pigmentation of the forced heterokaryon was indistinguishable from the pigmentation of the AUB4 and *vvd*$^{p}$ strains. Because these genes did not complement one another in the forced heterokaryon and they are tightly linked, we conclude that they are allelic. The allele of *vvd* in AUB4 was designated *vvd*$^{aub}$. Forced heterokaryons of *pyr-3; vvd*$^{aub}$ and *his-5; vvd*$^{p}$ with *ad-3* resulted in normal pigmentation in the heterokaryons, indicating that *vvd*$^{p}$ and *vvd*$^{aub}$ are recessive (data not shown).

### Measurement of Light Induction

Although *con-10* and *con-6* transcripts are not normally detectable in mycelia grown in constant darkness or constant light, previous work has shown that these transcripts are detected transiently after a shift from dark to light (Lauter and Russo, 1991; Lauter and Yanofsky, 1993). Many other environmental factors also influence *con* gene expression. Therefore, before testing the effect of *vvd* on *con-6* and *con-10* gene expression, we examined light induction in wild-type cultures grown to different cell densities. Light induction experiments were carried out by two methods. In the first method, liquid cultures were grown at 34°C (200 rpm) in constant darkness and then shifted to constant light. The second method was similar to that described by Lauter and Yanofsky (1993) where light induction experiments were accomplished using harvested mycelial pads exposed to air (see Materials and Methods).

Results from these experiments revealed that the culture age had a significant effect on light induction of *con-10* and *con-6*. Light induction of *con-10* or *con-6* was not observed in liquid cultures 12 h after inoculation with conidia (data not shown). Although light induction of *con-10* was detected in the 12-h harvested samples (Fig. 2), the level of expression was about 10-fold lower than the induction observed in the 24-h harvested cultures. In both 24-h harvested and nonharvested cultures, expression of *con-10* was induced within 30 min after exposure to light before declining sharply (Fig. 2). In a number of experiments we detected *con-10* mRNA after 1 h of exposure to light; however, the level of *con-10* gene expression was always lower than at the 30-min exposure time (Fig. 2). As the age of the culture increased, the ability to distinguish light regulation from other forms of regulation decreased and variability between samples harvested from different flasks was observed as illustrated by the 48-h nonharvested sample (Fig. 2). Similar results were obtained with *con-6* (data not shown). We chose to carry out light induction experiments with $vvd^{p}$ and $vvd^{aub}$ strains in liquid cultures grown for 24 h in the dark without harvesting so that light would be the only obvious variable in the experiment.

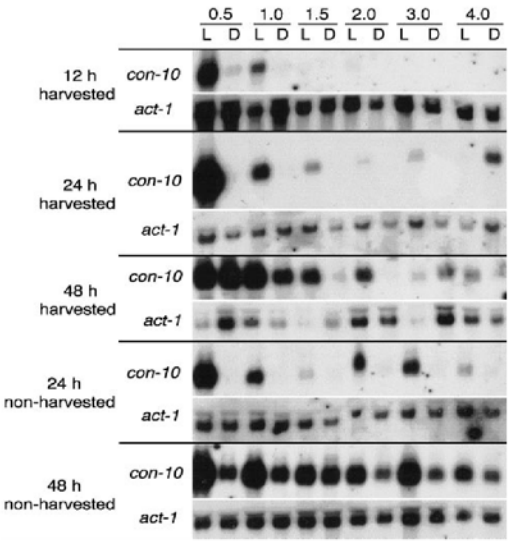


FIG. 2. Optimizing growth conditions for light induction of *con-10*. Cultures were grown in minimal medium for 12, 24, and 48 h and light induction for 0.5, 1.0, 1.5, 2.0, 3.0, and 4.0 h was examined in cultures that were first harvested as mycelial pads or left in culture (nonharvested). Cultures were exposed to constant light, L, or remained in the dark, D, for the times indicated. Here we show Northern blots for the 12- and 24-h harvested and the 24- and 48-h nonharvested samples probed with *con-10* and actin (*act-1*).

### Expression of con-10 and con-6 in Response to Light Is Altered in vvd

To examine expression of light-inducible genes in the *vvd* strains, we performed Northern blot analysis of RNA isolated from mycelia exposed to light for 0.5 to 4 h. Enhanced photoinduction of *con-10* mRNA was observed primarily at the 0.5-h time point in $vvd^{aub}$ relative to wild-type during the first 1.5 h after exposure to light. In $vvd^{aub}$, *con-6* mRNA was detected over a longer period of time than *con-10* (Fig. 3). The $vvd^{p}$ strain showed a more pronounced response to light than $vvd^{aub}$ (Fig. 3). The accumulation of *con-10* and *con-6* mRNA was not inhibited prior to 4 h in the light in $vvd^{p}$ as it was in the wild-type and $vvd^{aub}$ strains after 1 and 2 h, respectively (Fig. 3). Since the blots shown in Fig. 3 were probed together and exposed to film for the same length of time, a direct comparison of the change in gene expression could be made. We estimated the level of mRNA by densitometry of the autoradiographs and found that expression in $vvd^{p}$ was elevated at least 3-fold for *con-6* and 30- to 40-fold for *con-10* relative to the wild-type at the 0.5- and 1.0-h time points. At later time points, expression of both genes was elevated more than 100-fold. This value likely underrepresents the increase in the level of gene

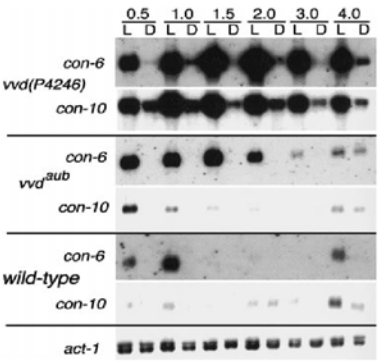


FIG. 3. Light regulation of *con-10* and *con-6* is altered in $vvd^{aub}$ and $vvd^{p}$. Time 0 represents gene expression after 24 h of growth in the dark. Nonharvested cultures were exposed to constant light, L, or remained in the dark, D, for the times indicated. RNA was hybridized to *con-6* or *con-10*.

expression because the $vvd^{p}$ autoradiograph was somewhat overexposed. These data indicate that *vvd* plays a role in light-dependent repression of light-inducible genes.

## *con-10 and con-6 Are Expressed in vvd Strains Grown in Constant Light*

Enhanced pigmentation in the mycelium of the *vvd* strains was observed when cells were grown in constant light. We also examined *con-10* and *con-6* expression in constant light for up to 72 h. The most significant finding is that after 24 h in constant light, *con-10* mRNA was detected in both $vvd^{p}$ and $vvd^{aub}$ strains, but not the wild-type control (Fig. 4). The level of *con-10* expression increased significantly in the 48-h samples; however, some expression of *con-10* was detected in the wild-type strain after 48 h, indicating that *con-10* expression was beginning to be influenced by other factors. The fact that *con-10* expression was induced in older cultures was illustrated dramatically in the 72-h cultures grown in the light and dark (Fig. 4). Increased expression of *con-10* in the 48- and 72-h wild-type samples was likely due to nutritional stresses and conidiation rather than light since the levels of *con-10* mRNA in the samples grown in the dark were also elevated. We have never observed *con-10* expression in $vvd^{aub}$ strains grown in constant darkness for 24 h; however, we sometimes observe elevated expression of *con-10* in $vvd^{p}$ after growth for 24 h in constant darkness (Fig. 3). The expression of *con-6* in constant light was very similar to that of *con-10* in each of the strains we examined (data not shown).

## *The Circadian Rhythm of Conidiation Is Not Severely Affected by the vvd Mutation*

The circadian clock relies primarily on light or temperature signals to maintain a proper phase relationship with the external environment (Pittendrigh, 1960). Because the light induction of several genes was significantly altered in the *vvd* mutants, we wanted to determine whether the *vvd* mutation has a discernible effect on the circadian clock. We constructed *bd;*$vvd^{p}$ strains to observe the circadian rhythm of conidiation on a race tube (see Materials and Methods). The *bd;*$vvd^{p}$ strains were also highly pigmented (Fig. 1). Two isolates were examined on race tubes and compared to *bd* strain 30-7. Both *bd;vvd* isolates (17.1 and 17.2) consistently expressed a circadian rhythm of conidiation with a period length of 22.1 ± 0.5 h ($n$ = 90), similar to that of the *bd* strain (21.3 ± 0.5 ($n$ = 108); Figs. 5A and 5B; and data not shown).

It is well established that the phase of the circadian clock can be shifted by a light pulse (Pittendrigh, 1960). The magnitude, as well as the direction, of the phase shift (advance or delay) is dependent upon the time at which the light pulse is administered. The magnitude of the response is also dependent on the duration and intensity of the light pulse. To examine whether the *vvd* mutation affects phase shifting of the conidiation rhythm by light, a saturating 15-min light pulse was given to race tubes of

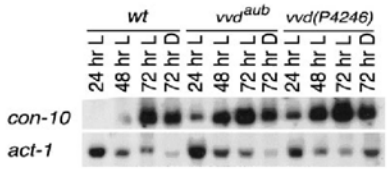


FIG. 4. Northern blot analysis of *con-10* expression in wild-type (wt), $vvd^{aub}$, and $vvd^{p}$ strains grown in constant light. Four flasks of minimal medium were inoculated with $1 \times 10^{6}$ conidia/ml of wt, $vvd^{aub}$, or $vvd^{p}$ and grown for 24, 48, and 72 h in constant light (L) and 72 h in constant darkness (D). Twenty micrograms of total RNA was loaded in each lane. *act-1* was used as a probe to control for mRNA abundance.

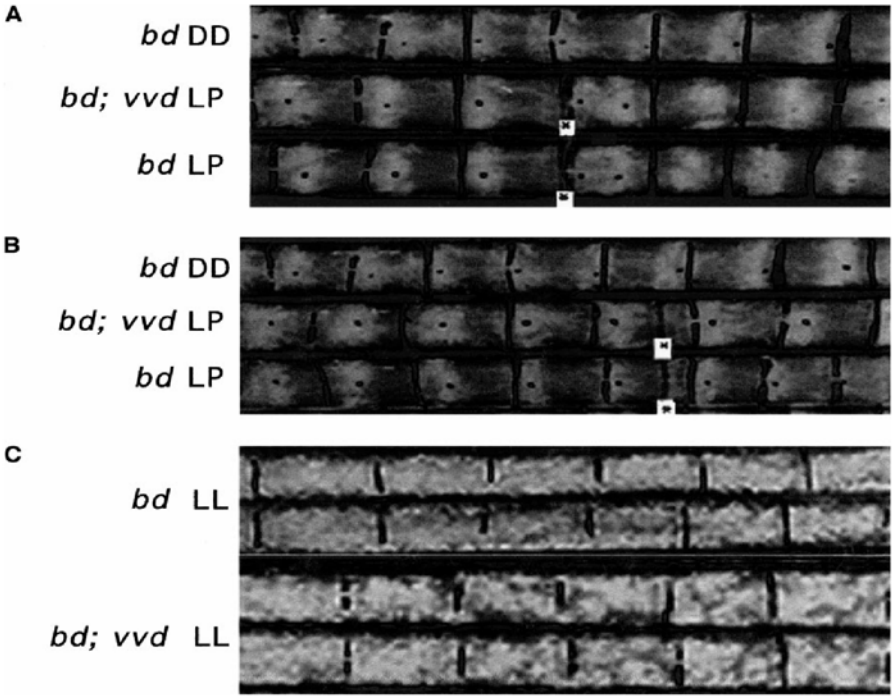


FIG. 5. The *vvd* mutation does not significantly alter the free-running conidiation rhythm or the response of the clock to light pulses. (A) Race tube cultures were given a 15-min saturating light pulse (LP) after 120 h in constant darkness, a time corresponding to subjective morning. (B) Race tube cultures were given a 15-min saturating light pulse after 136 h in constant darkness, a time corresponding to subjective night. In A and B, asterisks indicate the time at which the light pulse was given. Comparison of the conidiation pattern before the light pulse shows that the periods of the rhythms are the same. Control *bd* cultures that stayed in constant darkness (DD) are shown. (C) Race tubes grown in constant fluorescent light (LL). Duplicate race tubes of each strain are shown. The location of the growth front was marked every 24 h (vertical black lines).

each strain. The *bd;vvd* strain 17.2 showed a phase response similar to that of the control *bd* strain 30-7. A light pulse given to cultures after 120 h in the dark (DD120) results in about a 12-h change in the phase of the rhythm (either advance or delay) (Fig. 5A) and a light pulse given at DD136 results in about a 2-h phase delay (Fig. 5B) in both *bd* and *bd;vvd* strains. Under these saturating conditions, *vvd* does not have a significant role in modulating the phase response at the level of resolution of conidiation banding patterns in race tubes. However, subtle differences that might be uncovered using nonsaturating light pulses would have been missed in these analyses.

When *N. crassa* strains containing the *bd* mutation are grown on race tubes in constant light, the conidiation rhythm is lost (Sargent and Briggs, 1967). We found that *vvd* had no effect on photosuppression of the conidiation rhythm (Fig. 5C). Both the *bd* and the *bd;vvd* strains conidiated similarly in constant bright light. We also examined photosuppression of these strains in dim light and found no effect of the *vvd* mutation on photosuppression of the conidiation rhythm (data not shown).

The *frq* gene is required for circadian rhythmicity in *N. crassa* (Aronson *et al.*, 1994b). The *frq* gene is induced rapidly in response to light and the magnitude of a phase

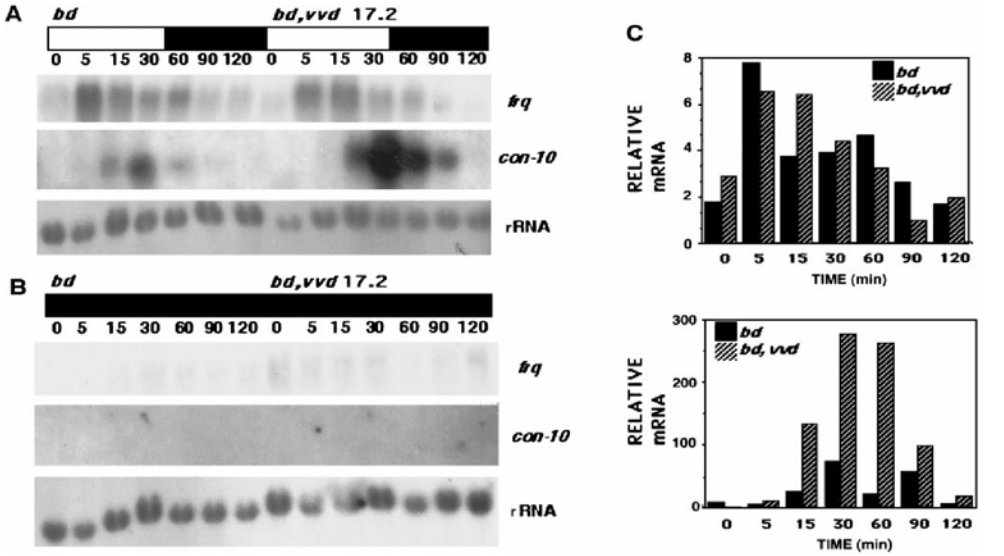


FIG. 6. The *vvd* mutation does not significantly alter the response of *frq* to light. (A) Both *bd* (30-7) and *bd;vvd* (17.2) strains were harvested after 24 h of growth in the dark (0), following a 5-, 15-, or 30-min light pulse (5, 15, 30), or upon return to the dark after the start of the 30-min light pulse (60, 90, 120 min) (see Materials and Methods). RNA isolated from the cultures was probed with *frq* (top) and *con-10* (center). A *rRNA*-specific probe was used as a control for RNA loading and to normalize total RNA in each lane (bottom). (B) Hybridization of *frq*, *con-10*, and *rRNA* to total RNA isolated from cultures grown at the same time as in A under conditions of constant darkness (no light pulse). Bars above designate light (white bars) versus dark (black bars) conditions. (C) The top graph represents the relative levels of *frq* mRNA from A and the bottom graph represents the relative levels of *con-10* mRNA from A. A shorter exposure of the *con-10* autoradiograph shown in A was used for quantification. mRNA levels were normalized to the level of rRNA. Numbers on the *X* axis indicate the time at which the culture was harvested after the beginning of the light pulse.

shift produced by a light pulse is correlated with the level of *frq* induction (Crosthwaite *et al.*, 1995). Furthermore, *frq* does not exhibit a transient light response similar to that of *con-6* and *con-10,* but instead remains elevated in prolonged light periods (although with some variability in levels). To determine whether *vvd* has any effect on *frq* light induction, a 5- to 30-min light pulse was given to *bd* and *bd;vvd* cultures after growth for 24 h in the dark. The time of the start of the light treatment corresponds to subjective evening (CT15), a time when *frq* mRNA levels are low (Aronson *et al.*, 1994b). Light induction of *frq* mRNA in the *bd* and *bd;vvd* strains was examined by Northern analysis (Fig. 6). Consistent with previously published results (Crosthwaite *et al.*, 1995), *frq* mRNA levels increased rapidly, peaked after 5 min of light treatment, remained elevated (although not to the same levels observed for the 5-min light pulse), and then declined to preinduced levels by 60 min after the cultures were returned to the dark (90 min). A similar trend was observed in the *bd;vvd* strain. As a control for this experiment, we probed the same filters with *con-10*. Drastic changes in both the overall levels of induction of *con-10* and the time required to return to the noninduced levels were observed in the *bd;vvd* strain compared to the *bd* strain. Very low levels of mRNA were seen for both *frq* and *con-10* in the noninduced samples (Fig. 6B). This behavior was observed using three slightly different light regimens (including longer exposure to light) and culture medium (data not shown). In these experiments, the light response of *frq* in the *bd;vvd* strain was similar to that in the *bd* strain,

although a difference in transcript accumulation was observed after the 15-min light treatment (Fig. 6A). However, this difference was not detected in all experiments and expression of *frq* mRNA was not altered to the same degree as was *con-10* in the *vvd* strain. In any case, we cannot rule out the possibility that *vvd* may have minor effects on *frq* light responsiveness.

## DISCUSSION

*con-10* and *con-6* were isolated based on their preferential expression during conidiation, but are also directly regulated by light. The $vvd^{aub}$ strain was isolated in an experiment designed to obtain mutant strains that are altered in *con-10* regulation. Here, we reported the characterization of two strains containing different alleles of the *vvd* gene, $vvd^{p}$ and $vvd^{aub}$. VVD appears to be involved in regulating the production of carotenoid pigment, as well as regulating expression of two light-adaptable genes, *con-10* and *con-6,* in response to light.

Blue light regulation has been studied extensively in *N. crassa,* and several mutants that are defective in this process have been isolated. Phenotypes of these mutants range from strains displaying almost no blue light-induced processes to strains with constitutive expression of blue light-induced genes. The *white collar* mutants (*wc-1* and *wc-2*) are "blind" to blue light and almost all blue light-induced processes are defective (Arpaia *et al.,* 1995; Degli-Innocenti and Russo, 1984; Harding and Melles, 1983; Lauter and Russo, 1991; Linden *et al.,* 1997a,b; Nelson *et al.,* 1989; Sommer *et al.,* 1989).

Cloning and characterization of *wc-1* and *wc-2* revealed that they encode zinc finger GATA factors believed to be involved in transcriptional control of light-regulated genes and blue light signaling (Ballario *et al.,* 1996; Linden and Macino, 1997). Carattoli *et al.* (1995) isolated two mutants, *blr-1* and *blr-2,* that have a pale-orange color and reduced levels of light-induced expression of *con-10, con-8, al-1,* and *al-3.* In contrast to these mutants, there are constitutive carotenoid biosynthesis mutants, *ccb-1* and *ccb-2,* that accumulate carotenoids in cultures grown in constant dark and constant light (Linden *et al.,* 1997b). In response to light, the *ccb-1* strain had a threefold increase in the expression of carotenoid biosynthetic genes when compared to wild-type, but this increase was not observed for *con-10* or *con-8* (Linden *et al.,* 1997b). It was proposed that the product of *ccb-1* functions as a general repressor of transcription of some light-regulated genes.

Lauter and Yanofsky (1993) showed that *con-10* and *con-6* are not expressed in mycelia grown in constant darkness or constant light, but they are expressed transiently in response to light after a minimum 5-h incubation in the dark. They suggested that this transient response is due to the inhibition of accumulation of these transcripts by light. As a result of these data, a model of light regulation was proposed in which light has both an activating and a repressing effect on transcription (Lauter and Yanofsky, 1993). We refer to this repressing effect of light on light-inducible genes as light adaptation.

Since the enhanced carotenoid pigmentation and the altered expression of *con-10* and *con-6* in *vvd* strains requried the presence of light, we conclude that the product of *vvd* is required for light adaptation. In general, *vvd* strains do not express high levels of *con-10* or *con-6* in constant darkness (Figs. 3 and 6). However, we have noted a level of *con-10* expression in the dark in the $vvd^{p}$ strain that is higher than the wild-type in some (Fig. 3), but not all (Fig. 6), experiments. Although this hints that *vvd* mutants may be defective in some additional aspects of repression of *con-10,* the main effect is clearly on the ability of cells to reduce both *con-6* and *con-10* gene expression in constant light (Figs. 3 and 4).

Perkins *et al.* (1997) found a high frequency of *vvd* mutants produced in crosses of strains bearing partial duplications of a region of the right arm of LGVI. Presumably these mutants arose as repeat induced point (RIP) mutations (Selker, 1990). RIP mutations frequently result in null alleles and this suggests that null mutants of *vvd* are defective in repressing *con-10* and *con-6* expression and carotenoid levels. The finding that both *vvd* alleles are recessive is consistent with the view that they result from complete or partial loss of function mutations. Since $vvd^{aub}$ has a less severe defect in repressing *con-10* and *con-6* expression in constant light than $vvd^{p}$, we propose that $vvd^{aub}$ is only partially defective.

Analyses of the *con-10* promoter have revealed a striking degree of regulatory complexity (Corrochano *et al.,* 1995; Lee and Ebbole, 1998a,b). *Cis*-acting elements in the promoter that are important for dark repression and mycelial repression have been identified (Lee and Ebbole, 1998b). The RCO1 protein is a tissue-specific repressor (the mycelial repressor) that is thought to mediate its effect at the *con-10* promoter between −778 and −353 relative to the transcription initiation site when the fungus is growing as vegetative hyphae (Corrochano *et al.,* 1995; Lee and Ebbole, 1998b). The basal level of *con-10* expression is 10-fold higher in the *rco-1* mutant and, unlike the wild-type, is highly inducible when the fungus is exposed

to several stress conditions (Lee and Ebbole, 1998b). The lack of light adaptation is one of the most striking phenotypes of the *vvd* mutant. Thus, VVD may represent another repressor of *con-10* gene expression that also regulates *con-6* expression. *Cis*-acting elements involved in light-dependent repression were not defined in the *con-10* promoter. This form of regulation would have escaped detection because light adaptation was not analyzed in these promoter studies.

The *con-10–hph* fusion used in the selection scheme contained only 520 bp of *con-10* DNA upstream of the transcription initiation site. If the hygromycin resistance in strain AUB1 is due to the $vvd^{inib}$ mutation, VVD may act through an element downstream of position 520. However, the original mutant strain may have contained additional mutations that could affect hygromycin resistance. Therefore, construction of new *con-10–hph* or *con-10–lacZ* fusion strains would be required to localize the promoter elements through which VVD effects repression of *con-10* in the light.

It has been shown previously that *frq* mRNA levels remain elevated in constant light (Crosthwaite *et al.,* 1995) and hence *frq* is not subject to a light adaptation mechanism analogous to that observed for *con-6* and *con-10.* We note that after an initial light treatment, the level of *frq* transcript is reduced in *vvd;* however, unlike *con-6* and *con-10,* it does not return to preinduced levels until the cultures are returned to the dark. The finding that light induction and dark repression of *frq* are not severely affected in *vvd,* and that the conidiation rhythm of the *vvd* mutant is normal, indicates that VVD is not required for dark to light or light to dark synchronization of the cultures. While subtle differences in the light responsiveness of the clock may have been missed due to the use of saturating light treatments in these studies, our results demonstrate that *vvd* does not play a major role in the normal operation of the clock or in *frq* responsiveness to light. In contrast, both WC-1 and WC-2 were shown to be required for conidiation rhythms, as well as induction of *frq* expression by light and for the circadian-regulated increase of *frq* expression in the dark (Crosthwaite *et al.,* 1997).

VVD may act by binding directly to the promoters of light-regulated genes or by affecting the ability of WC-1 and WC-2 to stimulate expression of light-regulated genes. Because *frq* regulation is not strongly affected by *vvd* and both *wc-1* and *wc-2* are required for normal regulation of *frq,* and *wc-1* is epistatic to *vvd,* we favor the view that VVD acts downstream of WC-1 and WC-2 to repress *con-6* and *con-10* expression.

VVD appears to play an important role in limiting induction of genes in response to light. Previous studies of light regulation have focused on induction of genes in response to light and components of this pathway have been identified. *vvd,* on the other hand, is the first gene shown to be involved in light adaptation in *N. crassa.* Light-adaptive responses have long been recognized in the vertebrate retina, and this response involves both calcium-dependent and -independent mechanisms (Pugh *et al.,* 1999). The ability of cells to adapt to extracellular stimuli, such as light, likely involves interactions between different signaling pathways. How these pathways integrate complex information and respond appropriately is unknown. Thus, further analysis of the *vvd* mutant and molecular characterization of the *vvd* gene will likely yield important insights into the light-adaptive responses of *N. crassa* and provide a paradigm for the dissection of the phototransduction signaling pathways required for this response.

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# APPENDIX B

The bZip transcription factor *cross-pathway control-1* (*cpc-1*) is a homolog of yeast Gcn4p and a master transcriptional regulator in the filamentous fungus *Neurospora crassa* (Ebbole *et al.*, 1991; Paluh *et al.*, 1988; Paluh and Yanofsky, 1991) . *GCN4* and *cpc-1* mRNA's contain 4 upstream open reading frames that prevent translation of CPC-1 when amino acids are in excess. However, amino acid limitation leads to translation of CPC-1 which can then enter the nucleus and activate transcription of amino acid biosynthetic genes (Hinnebusch, 1997; Luo *et al.*, 1995).

In chapter II, we identified an EST corresponding to *cpc-1*, W01D5, that was expressed with a 22 hour period in the wild type strain. Interestingly, the period of the *cpc-1* mRNA rhythm appeared to be 22 hours in the long period $frq^7$ strain (the conidiation rhythm of $frq^7$ has a period of 29 hours). This result suggested that *cpc-1* may be controlled by a FLO in the fungus. In order to examine this in greater detail, northern blot analysis was performed to confirm that *cpc-1* was indeed expressed with a 22 hour period in the $frq^7$ strain. In three independent experiments, *cpc-1* mRNA did accumulate in a circadian fashion. However, the results were inconclusive when attempting to determine the period of the *cpc-1* oscillation. In order to investigate this further, a translational fusion was constructed in which CPC-1 was fused to LUCIFERASE (LUC) at the translational start site; this construct contains the *cpc-1* promoter and all four

upstream open reading frames. The CPC-1-LUC was placed at the *Neurospora his-3* locus by homologous recombination. Transformants expressing detectable luciferase activity were identified in both the wild-type and the short period $frq^1$ strains. Transformants were then inoculated in 96 well plates and luciferase activity was monitored for several days using a Packard topcount luminometer. In wild-type strains, light emission was rhythmic with a period of approximately 22 hours. Consistent with the microarray results, light emission from the CPC-1-LUC fusion was rhythmic with a period of approximately 22 hours in the short period $frq^1$ strain as well. This result suggested that CPC-1 translation may be regulated independently of the FRQ/WC-oscillator The results obtained with the CPC-1:LUC fusion must be interpreted with caution, however. The reproducibility of these experiments was poor. This was primarily due to a loss of luciferase activity in subsequent experiments. The reason for this is not clear. One possibility is RNA silencing, which is efficient in *Neurospora* (Pickford and Cogoni, 2003). Alternatively, DNA methylation may be responsible for silencing of the luciferase transgene. Silencing of transgenes is observed frequently in *Neurospora* (E. Selker, personal communication). Also, transformation of a CCG-2-LUC reporter (Morgan *et al.*, 2003) showed higher expression levels when transformed into a strain containing a mutation in the *Neurospora* DNA methyltransferase *dim-2* (*defective in methylation-2*). Attempts have been made to generate *bd;* $frq^1$ *dim-2; his-3* strains to repeat this experiment. This has been difficult due to extremely tight linkage of the *frq* and *dim-2* loci (~1%). In order to

clearly demonstrate that CPC-1 accumulates with a period that is independent of the *frq* allele, a reliable LUC reporter strain must be constructed. However, these data are encouraging and suggest that CPC-1-LUC may provide a needed reporter to monitor rhythms from the FLO.

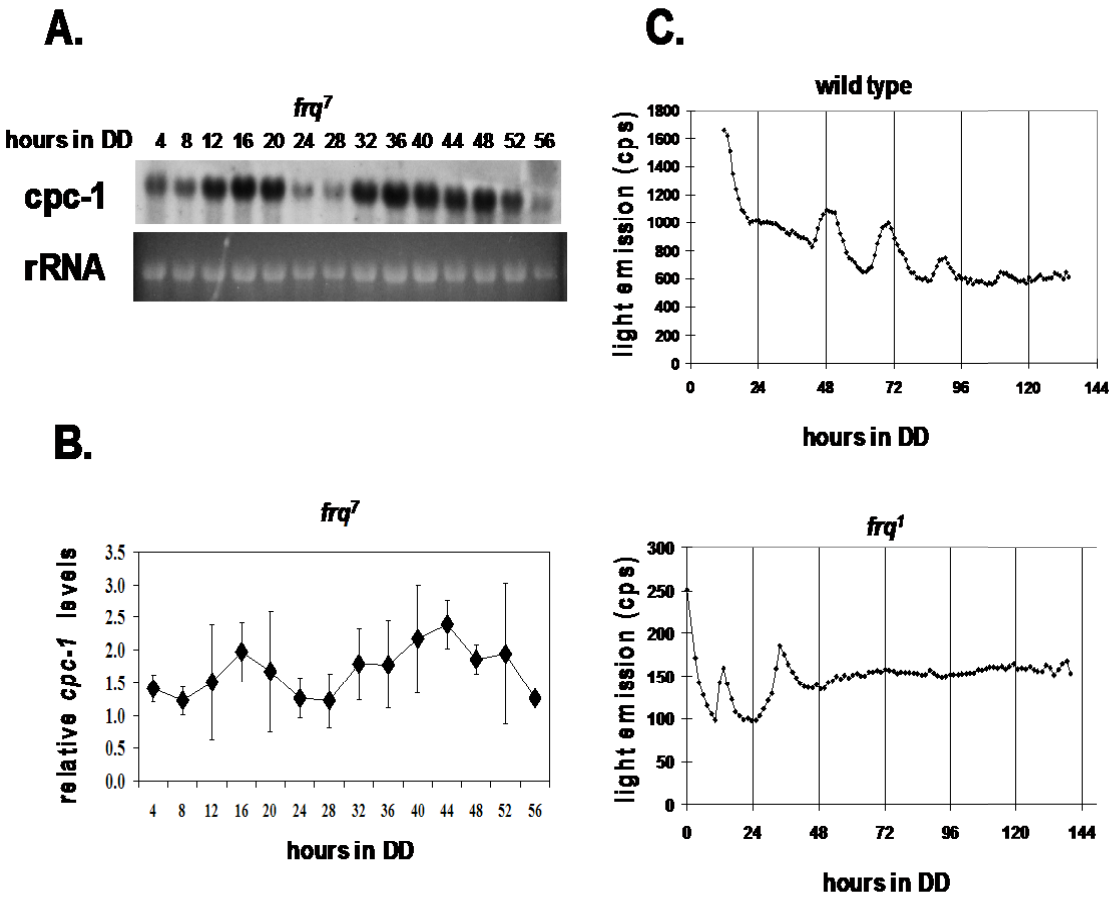


**Figure B.1 Rhythmic accumulation of *cpc-1* and CPC-1.**

The *frq*$^7$ strain was grown in constant light before transfer to constant darkness (DD). Cultures were harvested after transfer to DD at the indicated times. (A) Northern blots of RNA from *frq*$^7$ were probed with a radioactive *cpc-1* probe as indicated. rRNA is shown to verify equal loading of all lanes. (B) Relative mRNA levels (band intensity/rRNA) of *cpc-1* are plotted versus time as the mean +/- the standard deviation for three independent experiments. (C) Light emission (counts per second) from wild type (top) and *frq*$^1$ (bottom) strains harboring the CPC-1:LUC reporter is plotted versus time.

In order to determine if *cpc-1* plays a role circadian clock function in *Neurospora*, we crossed a *cpc-1* deletion strain (provided by Dr. Matt Sachs) to our wild type clock strain (*bd*) to obtain progeny that were *bd; Δcpc-1* (the *cpc-1* coding sequence is replaced with a gene conferring hygromycin resistance). *Δcpc-1* (*bd; Δcpc-1*) progeny typically exhibited period lengths that were approximately one hour longer than wild type (*bd*) progeny (*bd* = 22.0 +/- 0.3 hours; *bd;Δcpc-1* = 23.3 +/- 0.5 hours) . One of these progeny was backcrossed to the long period $frq^7$ strain and progeny from this cross were analyzed on race tubes. We observed that progeny containing the *cpc-1* deletion typically increased the period of both wild type and $frq^7$ strains. We did observe a range of period lengths for each genotype. These results are plotted in figure 2.

Since *cpc-1* appears to effect the period length of the circadian clock in *Neurospora,* we decided to test another component of the cross pathway control pathway. The CPC-3 protein kinase regulates translation of CPC-1 in response to amino acid limitation (Sattlegger *et al.*, 1998). In CPC-3, an increase in CPC-1 levels is not observed following amino acid limitation. We obtained a strain in which *cpc-3* coding sequence had been replaced with a gene conferring hygromycin resistance. This strain (*Δcpc-3* ) (Sattlegger *et al.*, 1998) was crossed to our wild type clock strain (*bd*) and progeny were analyzed on race tubes. Surprisingly, *bd; Δcpc-3* progeny all had period lengths that were virtually identical to wild type progeny (*bd* = 21.9 +/- 0.5 hours; *bd; Δcpc-3* = 22.3 +/- 0.2 hours) . Since *cpc-3* does not affect period length and CPC-1 protein is

rhythmically expressed, we must conclude that circadian control of CPC-1 translation is regulated by a CPC-3 independent mechanism.

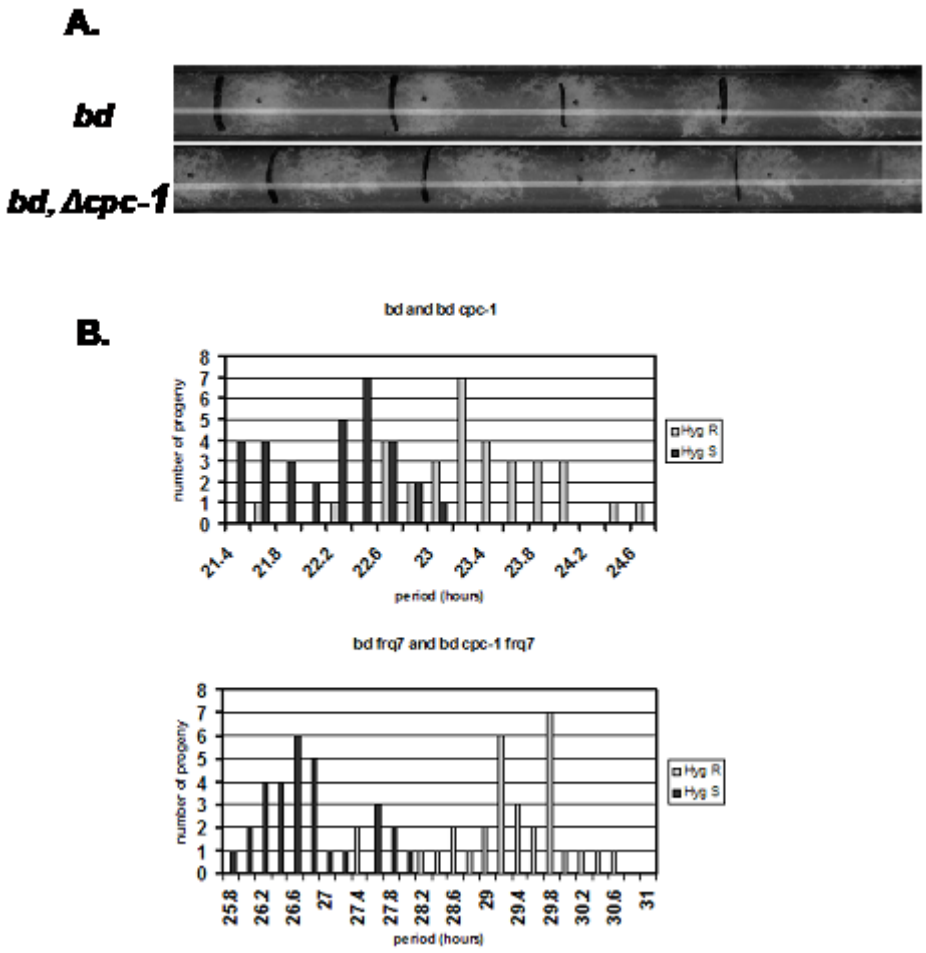


**Figure B.2. *cpc-1* mutants display an increased period length.**

(A) Race tube cultures of wild type (top) and *frq*$^{10}$ (bottom) strains were grown in DD at 30°C for 24 hours before transfer to 25°C DD. The marks represent 24 hours of growth. (B) The period lengths of progeny from a cross between *bd; cpc-1* and *bd; cpc-1; frq*$^{7}$ are plotted. Progeny with a wild type *frq* allele are plotted on the top graph. Progeny carrying the *frq*$^{7}$ allele are plotted on the bottom graph. Black bars indicate hygromycin sensitive *cpc-1*$^{+}$ progeny. Gray bars indicate hygromycin resistant Δ*cpc-1* progeny. The graphs depict the number of progeny (y-axis) versus period lenght (x-axis).

**Circadian regulation of a second bZip transcription factor**

During the course of our work on *cpc-1,* we examined the *N. crassa* genome for the presence of additional bZip transcription factors. One gene was of particular interest. bZip-1 (NCU08055.1) has a DNA binding domain that is very similar to the C/EBP binding protein subfamily of bZip transcription factors (Figure A-1). The concensus binding sequence of this protein subfamily is an inverted repeat sequence (TTGCGCAA) that is similar to the previously identified Activating Clock Element (ACE) (TTGGCCAA) (Figure A-1) (Bell-Pedersen *et al.*, 2001b; Fujii *et al.*, 2000). The ACE is necessary for rhythmic expression of the well characterized *ccg-2* gene. Moreover, the ACE is sufficient to drive rhythmic expression of a reporter gene (Bell-Pedersen *et al.*, 1996a). Thus, identification of protein factors that bind this sequence would provide further insight into the mechanism of clock-controlled gene regulation.

Northern analysis of bZip1 demonstrates that the mRNA accumulates in a circadian fashion. The mRNA peaks in the evening at CT12. Further analysis of the role of this gene in circadian clock function is necessary. Attempts to generate a knockout strain are currently underway in the lab of Dr. Dan Ebbole. Racetube analysis of the knockout strain should determine if this gene plays an essential role in the *Neurospora* time keeping mechanism. Additionally, it will be necessary to perform northern blot analysis to determine if rhythmic expression of *ccg-2* is abolished in the bZip1 deletion strain.

**A.**

```
C/EBP    VRRERNNIAVRKSR-DDAD--QR--
bZip1    IKRQRNNIAAKKYRQKKIDRIQELE
```

**B.**

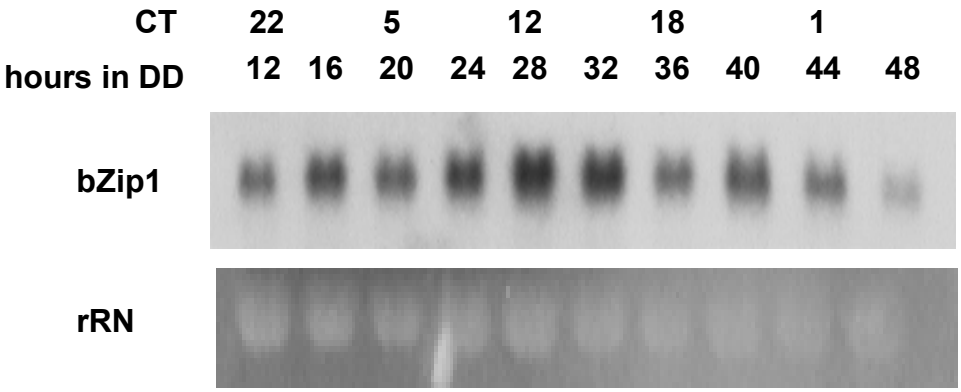


**Figure B.3. mRNA for bZip1 accumulates rhythmically.**

(A) Clustal W alignment of the bZip1 DNA binding domain with the consensus C/EBP DNA binding domain. Blue letters represent similar amino acids. Red letters indicate identical amino acids. (B) The *frq*$^{7}$ strain was grown in constant light before transfer to constant darkness (DD). Cultures were harvested after transfer to DD at the indicated times. (B) Northern blots of RNA from *frq*$^{7}$ were probed with a radioactive *bZip-1* probe as indicated. rRNA is shown to verify equal loading of all lanes.

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## Publications

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