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Circadian Rhythms in *Neurospora crassa* and Other Filamentous Fungi

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Circadian clocks are endogenous cellular timekeepers that control a wide variety of daily physiological and molecular rhythms in most eukaryotic and some prokaryotic organisms (31, 116). These rhythmic events allow organisms to best adapt to the natural environment on earth (88). All circadian rhythms share three basic properties. First, circadian rhythms persist under constant conditions with a period length of about 24 h. Second, they can be entrained (reset) by environmental cues, with light and temperature being the most important signals. Third, the rhythms are temperature compensated, meaning that the period length of the rhythm is stable over a wide range of physiological temperatures.

The field of chronobiology has recently experienced unprecedented progress in identifying core molecular components involved in the generation of circadian rhythms and is beginning to identify components of the output pathways from these core oscillators in several model organisms, including the filamentous fungus *Neurospora crassa* (31, 32, 116). Investigations of the *Neurospora* circadian clock system have elucidated many of the basic mechanisms that underlie circadian rhythms, including negative feedback, and light and temperature entrainment common to all eukaryotic clocks (32, 49, 72). *Neurospora* continues to be a premier experimental organism for studying circadian rhythms because of its relative simplicity and because it displays an easily assayed circadian rhythm in asexual spore development (conidiation) that has proven extremely useful for measuring the effects of mutations on clock function. The well-described *Neurospora frq*/*wc*-based circadian oscillator (FWO) exhibits remarkable conservation with those of higher eukaryotic organisms, reinforcing its use as a model organism to investigate the workings of the clock.

NEUROSPORA frq/*wc*-BASED CIRCADIAN FEEDBACK LOOPS

Similar to the circadian oscillators in *Drosophila melanogaster* and mammals, the core circadian oscillator of *Neurospora* consists of an autoregulatory negative feedback loop in which FRQ, FRH (an FRQ-interacting RNA helicase), WHITE COLLAR 1 (WC-1), and WC-2 are the core components (49). In this negative feedback loop, a complex of FRQ and FRH (FFC) forms the negative limb of the loop, whereas WC-1 and WC-2, two PER-ARNT-SIM (PAS) domain-containing transcription factors, are the positive elements (2, 14, 25, 32). Our current understanding of the *Neurospora* FWO is depicted in Fig. 1. In constant darkness around subjective late night, WC-1 and WC-2 form a heterodimeric complex (D-WCC) which binds to the Clock box (C box) in the *frq* promoter (Fig. 1), leading to the activation of *frq* transcription (18, 25, 37, 47). *frq* mRNA reaches its peak in the subjective late day, while FRQ protein levels do not peak until 4 to 6 h later (2, 39). After the FRQ protein is synthesized, it dimerizes with itself and forms a complex with FRH (14, 17). In the nucleus, FFC inhibits D-WCC activity, resulting in a decrease in *frq* mRNA levels, which reaches a trough around subjective midnight (2, 81, 84). As soon as FRQ is synthesized, it is progressively phosphorylated by several kinases and dephosphorylated by two phosphatases (71). When FRQ becomes extensively phosphorylated, it interacts with FWD-1, an F box/WD-40 repeat-containing protein and the substrate-recruiting subunit of an SCF-type ubiquitin ligase complex, resulting in the ubiquitination and degradation of FRQ by the proteasome system (45). When FRQ levels drop below a certain threshold, D-WCC is no longer inhibited by FFC, and *frq* transcription is reactivated around subjective late night to start a new cycle. As a result of this autoregulatory negative feedback loop, *frq* mRNA and FRQ protein accumulate with a daily rhythm. These oscillations are critical for the normal circadian behavior of the organism (39).

Multiple lines of evidence indicate a central role for the FWO in the *Neurospora* circadian clock. First, deletion of *frq*, *wc-1*, or *wc-2* or down-regulation of *frh* (an essential gene) leads to arrhythmicity under normal growth conditions (3, 14, 22, 25, 46, 63). Second, mutations in the *frq* or *wc* genes result in short or long periods (ranging from 16 to 35 h), arrhythmia, and/or impaired temperature compensation of the clock (3, 21, 48, 75, 112). Third, as predicted from the negative feedback loop model, the abolishment of *frq* mRNA rhythms by constitutive expression causes arrhythmic development, indicating that *frq* mRNA is essential for normal functioning of the clock. Fourth, FRQ together with FRH represses the transcription of *frq* by inhibiting the activity of D-WCC, a mechanism that closes the negative feedback loop (2, 14, 37, 84). In addition, changes in *frq* mRNA and protein levels by environmental cues (light and temperature) or by experimental manipulation result in a phase shift of the clock, indicating that entrainment of the clock occurs through changes in the levels of the phase-determining, state-variable FRQ (2, 26, 52, 76). Finally, processes that regulate the levels or activities of components of FWO, such as phosphorylation of FRQ and WCs, are critical for period determination and normal functioning of the clock (48, 71, 96, 112–115). Together, these data establish the FWO as a central oscillator of the *Neurospora* circadian clock controlling rhythmic development and gene expression.

In addition to its role in repressing D-WCC activity in the circadian negative feedback loop, FRQ promotes the expres-

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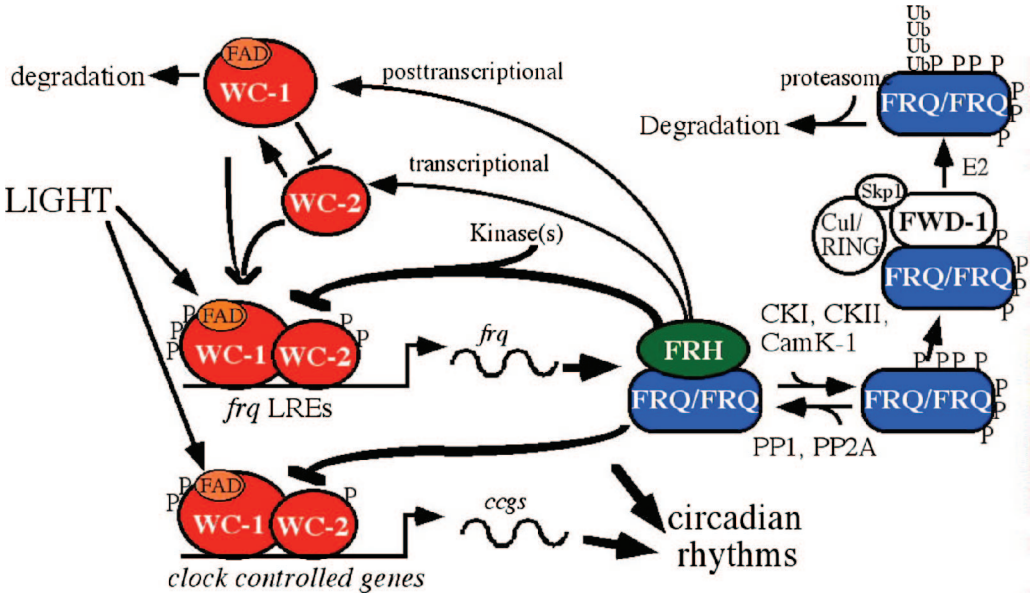

FIG. 1. Current model of the *frq*/*wc*-based circadian oscillator.

sion of WC-1 and WC-2, forming positive feedback loops that are interlocked with the negative loop (18, 19, 64, 83). FRQ regulates WC-1 expression posttranscriptionally, while it promotes *wc-2* transcription (18, 64, 97). Experiments in which *wc-1* or *wc-2* was overexpressed from an inducible promoter to different levels demonstrated that the positive feedback loops are important for maintaining the robustness and stability of the clock (18). Although the mechanism(s) for these regulations has not been elucidated, it was recently shown that phosphorylation of the PEST-2 region of cytoplasmic FRQ (Fig. 2) is required for its function in promoting WC-1 accumulation (97). In addition, mutations of the putative FRQ phosphorylation sites in the PEST-2 region, which are critical for the ability of FRQ to support WC-1 accumulation, result in arrhythmic conidiation, further suggesting an important role for the positive feedback loop in the clock (97). WC-1 and WC-2 also regulate each other to form another interacting feedback loop. WC-1 accumulation in *Neurospora* requires WC-2 and the formation of the WC complex (16). On the other hand, WC-1 negatively regulates *wc-2* expression at the level of transcript abundance (15). The transcription of *wc-1* is regulated by three distinct promoters, and the transcriptional regulation of *wc-1* modulates the phase of circadian conidiation rhythm (55).

REGULATION OF THE *NEUROSPORA* CIRCADIAN NEGATIVE FEEDBACK LOOP

The following three processes are essential for the function of the *Neurospora* circadian negative feedback loop: activation of *frq* expression, inhibition of D-WCC by the FFC, and degradation of FRQ. Each of these processes needs to be properly regulated in order to generate endogenous daily rhythms in development and gene expression.

Activation of *frq* transcription by D-WCC. Both WCs are PAS domain-containing transcription factors (WC-1 contains three PAS domains, while WC-2 contains only one PAS domain) with GATA-type Zn finger DNA binding domains, and the WC proteins are primarily localized in the nucleus throughout the day (4, 70, 99, 105). WC-1 and WC-2 form WC complexes through the PASC domain of WC-1 and the PAS domain of WC-2 (16, 19, 105). In constant darkness, the heterodimeric D-WCC binds to the C box (the distal light-responsive element [LRE], which contains two GATG repeats) in the *frq* promoter (Fig. 2) and activates *frq* transcription (37, 47). During a circadian cycle, D-WCC binding to the C box is rhythmic, and deletion of the C box results in arrhythmic *frq* mRNA accumulation, indicating that binding is essential for

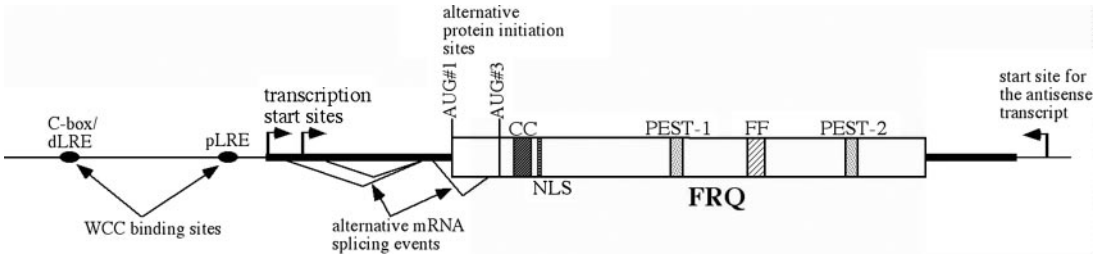

FIG. 2. Graphic depiction of the *frq* locus and its regulation. CC, coiled-coil domain; NLS, nuclear localization signal; FF, FRQ-FRH interaction domain.

the function of FWO (37). *frq* mRNA and FRQ protein levels are extremely low in *wc* null mutants, and the induced expression of WC proteins in *wc* null mutants from an ectopic locus leads to rapid induction of *frq* transcription (18, 25). Together, these data indicate that D-WCC is the primary activator of *frq* transcription.

Both WCs are posttranslationally modified via phosphorylation, which regulates their activity (47, 48, 96, 99, 105). Five major light-independent in vivo WC-1 phosphorylation sites, located immediately downstream of the WC-1 Zn finger DNA binding domain, were identified by mass spectrometry analysis (48). Mutations of these sites suggested that the phosphorylation of WC-1 negatively regulates its activity and is important for its role in the circadian negative feedback loop. In support of this conclusion, it was shown that dephosphorylation of the WCC significantly enhances its DNA binding activity (47). Thus, the regulation of WC phosphorylation is a critical part of the circadian negative feedback loop (see below).

Inhibition of D-WCC by FFC. Two forms of FRQ proteins, large FRQ and small FRQ, which differ by 100 amino acids, are produced as the result of alternative mRNA splicing events (Fig. 2) (23, 29, 39, 73). Although both FRQ forms are able to function in the circadian negative feedback loop, their relative levels are regulated by ambient temperature, allowing the clock to function over a broad range of temperatures (23, 29, 73). FRQ self-associates through its N-terminal coiled-coil domain, and this association is important for its function in the negative feedback loop (17). All FRQ proteins are in complex with FRH, an essential RNA helicase in *Neurospora* (14). The homolog of FRH in *Saccharomyces cerevisiae* is Dob1p/Mtr4p, which has been shown to be a cofactor for the yeast exosome complex, an important regulator of RNA metabolism in eukaryotes. Thus, the association of FRQ with FRH suggests that the complex may also have a role in regulating clock-controlled RNA metabolism. In the frq^9 strain (a strain making a truncated, nonfunctional FRQ) or in a strain in which *frh* is downregulated, normal clock functions are abolished and *frq* mRNA accumulates to high levels (2, 14). In other words, D-WCC constitutively activates *frq* transcription under these conditions. In addition, ectopic expression of FRQ leads to the repression of *frq* transcription and decreased binding between D-WCC and the C box (2, 37). Together, these data demonstrate that the FFC acts as the negative element in the circadian feedback loop.

FRQ physically interacts with the WCC, and disruption of FRQ-WCC interactions abolishes the function of FRQ in the circadian negative feedback loop, suggesting that such an interaction leads to the inhibition of D-WCC (17, 27, 83). The interaction between FRQ and WCC is mediated by FRH (14). Recent evidence suggests that FFC represses D-WCC activity by promoting phosphorylation of D-WCC rather than by the physical interaction alone (13, 47, 48, 96). This conclusion is supported by the lack of a sufficient stoichiometric amount of nuclear FRQ compared to WCC in the nucleus and the substoichiometric interactions between FRQ and WCC (14, 46, 96). In addition, partially purified FFC alone is not sufficient to inhibit D-WCC DNA binding activity (96). Furthermore, WC proteins were found to be hypophosphorylated in an *frq* null mutant, and the phosphorylation state of WC-2 exhibits a circadian rhythm (96). More importantly, the activation of *frq* transcription correlates with the hypophosphorylation of the WCs. These data, together with the observation that WCC phosphorylation negatively regulates its activity, suggest that FFC closes the circadian negative feedback loop by promoting D-WCC phosphorylation (47, 48, 96). Thus, it is likely that FFC recruits a kinase(s) to phosphorylate WCC through its interaction with WCC. The critical kinase(s) involved in this process has not yet been identified.

Degradation and phosphorylation of FRQ. The amount of FRQ determines the level of FFC in the cell, and the degradation and posttranslational modifications of FRQ play an essential role in period length determination and the function of the circadian negative feedback loop (14, 71, 75). FRQ is progressively phosphorylated by several kinases, and its phosphorylation triggers its degradation through the ubiquitin-proteasome pathway (45). Mutation of FRQ phosphorylation sites was found to lengthen the period of the clock, suggesting that phosphorylation of FRQ promotes its turnover (41, 75, 112). Interestingly, the phosphorylation events appear to be independent of each other, suggesting that FRQ is phosphorylated by multiple kinases at multiple independent sites. Three kinases, casein kinase I (CK-1a), CKII, and CAMK-1 (a calcium/calmodulin-dependent kinase), have been identified as kinases that phosphorylate FRQ (41, 112–114). However, among these kinases, the in vivo physiological roles in the clock have been established only for CKII. CKII was biochemically purified based on its FRQ-phosphorylating activity (113). Disruption of the CKII catalytic subunit (*cka*) in *Neurospora* results in high levels of hypophosphorylated FRQ and a loss of normal circadian rhythmicity, indicating that CKII is an essential clock component. In addition to its role in promoting FRQ degradation, CKII was shown to be important for the negative activity of FRQ in the FWO; high levels of *frq* mRNA were observed in the mutant strain despite abundant FRQ levels. These conclusions were further supported by the disruption of *ckb1* (a CKII regulatory subunit gene), which results in a partially functional CKII holoenzyme (112).

Neurospora CK-1a is homologous to the *Drosophila* clock protein DOUBLETIME, and CK-1a can phosphorylate the two PEST regions of FRQ (Fig. 2) in vitro (41). Importantly, CK-1a was found to be associated with FRQ in vivo, suggesting that it might be a FRQ kinase (14, 41). Deletion of the PEST-1 region of FRQ results in a slowdown of FRQ degradation, further supporting the idea that phosphorylation mediates FRQ degradation (41). On the other hand, the mutation of putative CK-1a PEST-2 phosphorylation sites abolishes FRQ's role in promoting WC-1 expression (97). The association of CK-1a with FRQ also suggests that it might be a kinase recruited by FRQ to phosphorylate WCC. However, in vivo evidence for the involvement of CK-1a in the clock is not currently available due to the requirement of CK-1a for cell survival in *Neurospora*.

In addition to the kinases, two protein phosphatases, PP1 and PP2A, have been shown to contribute to the control of FRQ phosphorylation (115). PP1 regulates the stability of FRQ, whereas PP2A is important for the function of the negative feedback loop. PP2A was also recently found to regulate the phosphorylation state of WC-1, a process that affects the activity of WCC (96).

The phosphorylation-dependent degradation of FRQ is me-

diated by FWD-1, an F box/WD-40 repeat-containing protein and the *Neurospora* homolog of the *Drosophila* protein Slimb (43, 45). FWD-1 physically interacts with phosphorylated forms of FRQ and serves as the substrate-recruiting subunit of an SCF-type ubiquitin (E3) ligase (SCF^{FWD-1}) to mediate FRQ ubiquitination. In an *fwd-1* mutant strain, circadian rhythms are abolished and FRQ protein accumulates to high levels in its hyperphosphorylated state. In addition, the COP9 signalosome, a conserved multisubunit complex in all eukaryotes, was shown to be important for clock function by regulating the stability of the SCF^{FWD-1} complex in *Neurospora* (44). Taken together, these data suggest that the progressive phosphorylation of FRQ, regulated by multiple kinases and phosphatases at multiple independent sites, fine-tunes the stability of FRQ and is a major determinant of the period length of the clock. A recent study also suggested that the degradation process of FRQ may have an important role in determining the temperature compensation of the circadian clock (94).

CONSERVATION OF EUKARYOTIC CIRCADIAN OSCILLATORS

Despite the evolutionary distance between *Neurospora* and higher eukaryotes and the uniqueness of each circadian system (51), remarkable conservation exists between the circadian oscillators of *Neurospora* and those of *Drosophila* and mammals. The conservation is observed at three levels, namely, mechanistic organization, regulation, and components.

Conservation at the mechanistic level. First, the circadian oscillators of *Neurospora*, *Drosophila*, and mammals that have been described are all based on autoregulatory negative feedback loops, and these loops are essential for normal functioning of the clocks (31, 116). Second, in these three circadian negative feedback loops, there are positive and negative elements, and the positive elements are all heterodimeric complexes consisting of two PAS domain-containing transcription factors, i.e., WC-1 and WC-2 in *Neurospora*, dCLOCK and CYCLE in *Drosophila*, and CLOCK/NPAS2 and BMAL1 in mammals. These positive elements all activate the transcription of the negative elements by directly binding to the promoters of the genes encoding the negative elements. Third, the negative elements (FFC in *Neurospora*, PERIOD [PER] and TIMELESS in *Drosophila*, and PER and CRYPTOCHROME in mammals) all close the negative feedback loops by inhibiting the activities of the positive elements through their physical interactions and by recruiting kinases (57, 108, 117). Fourth, in addition to the negative feedback loops, interlocked positive feedback loops are found in all three systems, and they share a role in promoting the robustness and stability of the clocks (18, 40, 56, 64, 90, 101).

Conservation of regulation. Similar to FRQ and the WCs, the core clock proteins in *Drosophila* and mammals are posttranslationally regulated by phosphorylation. Just like FRQ, PER proteins in animals are progressively phosphorylated, which ultimately triggers their ubiquitination and proteasome-mediated degradation (33, 43, 45, 59, 116). In addition, the phosphorylation state of *Drosophila* CLOCK, like that of WCs, exhibits a circadian rhythm, and CLOCK phosphorylation is dependent on PER (57, 117), suggesting similar mechanisms for closing the negative feedback loop in *Neurospora* and *Drosophila*. Similar to the association between FRQ and FRH, an RNA binding protein was found to be associated with PER and to regulate clock functions in mammals and *Drosophila*, suggesting that the regulation of RNA metabolism may be another common thread among these clock systems (11).

Conservation of regulatory components. The regulatory components that are critical for posttranslational regulation are highly conserved from *Neurospora* to mammals. Although FRQ and PER proteins are not sequence homologs, they are phosphorylated by the same kinases (CKI and CKII) and dephosphorylated by the same phosphatases (PP2A) (1, 41, 58, 68, 80, 86, 91, 95, 110, 112, 113, 115, 116). In addition, phosphorylation in both cases promotes protein degradation and is important for FRQ and PER repressor activities. Furthermore, the phosphorylation-dependent ubiquitination and degradation of PER and FRQ are mediated by a conserved SCF E3 ligase, with FWD-1 homologs as the substrate-recruiting subunits (33, 43, 45, 59).

Together, the levels of conservation observed among the eukaryotic circadian oscillators highlight the importance of using *Neurospora* as an instructive model system for eukaryotic circadian systems.

LIGHT INPUT INTO THE *NEUROSPORA* CLOCK

Among all environmental factors, light and temperature are two of the most important environmental inputs for entrainment of circadian clocks. *Neurospora* has the best-understood circadian system in terms of environmental inputs (72). In addition, *Neurospora* has served as the model system for the understanding of light responses in fungi (74). Because this topic has been reviewed extensively (12, 13, 49, 72, 78, 94), we only summarize the light input pathways of the clock, focusing primarily on recent results.

WC-1 is the blue light photoreceptor for the circadian clock and other light responses, while WC-2 is its partner in the light signaling pathway. Almost all known *Neurospora* light responses, including light resetting of the clock, are mediated by blue light (74). In addition to WC-1 and WC-2's roles in the circadian feedback loops, these two transcription factors are essential for all known *Neurospora* light responses (4, 69, 70, 74). WC-1 gained photoreceptor status because of its N-terminal LOV (light-oxygen-voltage sensing) domain, which is a specialized PAS domain that binds flavin (36, 46). Purified WC complexes from *Neurospora* or from a heterologous expression system are associated with flavin adenine dinucleotide, and mutations in the WC-1 putative flavin binding abolish its light functions (15, 46, 47). Similar to other photoreceptors, WC-1 has a photocycle in vivo, but the half-life of its photocycle is very long (>1 h), suggesting that WC-1 cannot be used efficiently for repeated photoactivation (47). Although the DNA binding domain of WC-1 is required for its function in the circadian negative feedback loop, it is not required for its light function (19). Consistent with this notion, several fungal WC-1 homologs which are important for light responses in other fungi lack the DNA binding domain (53).

Like WC-1, WC-2 is required for all light responses (74). The facts that WC-1 can only exist in the cell in a WC complex and that the WC-1 DNA binding domain is not required for

light responses indicate that WC-2 is an essential partner for WC-1 in the light signaling pathway (16).

How light resets the clock and triggers light-induced transcription. The discovery by Crosthwaite et al. that light rapidly induces the transcription of *frq* provided a beautifully simple molecular explanation for light-induced clock resetting behavior in eukaryotes: a light-induced change in the level of a key oscillator component (in this case, *frq* mRNA) leads to a phase shift of the clock (26). Their subsequent finding that the *wc* genes are required for light induction of *frq* demonstrated an essential role for the WCs in light input of the clock (25). Studies over the last 4 years have led to a description of the light signaling pathway resulting in the activation of *frq* transcription. After light exposure, at any time of day, a large WC complex (L-WCC) is activated through the WC-1 photosensory LOV domain (36). L-WCC is considerably larger than D-WCC, consisting of only WC-1 and WC-2, but with more than one WC-1 molecule (19, 36, 47). The light-activated L-WCC then binds to the two LREs (each contains two GATX repeats) on the *frq* promoter (Fig. 2), leading to the activation of *frq* transcription (36, 47). The light-induced transcriptional activation of *al-3* and *vvd*, two other *Neurospora* immediate light-inducible genes, was shown to use a similar mechanism to that for *frq*, suggesting a common molecular basis for light responses in *Neurospora* (47).

In addition to being phosphorylated in the dark, WC proteins become hyperphosphorylated after light exposure (99, 105). Similar to its function in the dark, light-induced WC phosphorylation inhibits L-WCC DNA binding activity (47). In addition, light-induced WC phosphorylation triggers the degradation of WC proteins (47, 64). As a result of these events, L-WCC only binds to the LREs transiently, a mechanism that contributes to the photoadaptation process (47).

An antisense *frq* transcript, initiated downstream of *frq*, was found to regulate proper light entrainment of the *Neurospora* clock (60). The elimination of this transcript, which is not predicted to encode any functional protein, resulted in altered light-resetting behavior of the clock. The mechanism for its function is unknown.

Photoperiodic responses in *Neurospora* and the role of VVD in clock entrainment. VIVID (VVD) is a small LOV/PAS domain-containing protein and a WC-dependent blue light photoreceptor in *Neurospora* (15, 50, 100). It plays important roles in regulating light responses and photoentrainment of the clock (50, 98, 104). In *vvd* mutants, light responses are elevated and photoadaptation is partially lost, indicating that VVD functions as a general repressor of light responses. While VVD is not essential for the circadian clock, *vvd* mutants exhibit a delay in the phase of the circadian conidiation rhythm and increased sensitivity of light-induced phase shifting of the clock. Recently, it was shown that VVD also plays a role in entraining the clock to photoperiods. In light-dark cycles, VVD reduces the light resetting at dawn, probably by inhibiting WCC activity, while it promotes resetting of the clock at dusk by accelerating the decrease of *frq* RNA levels (34). Thus, VVD may help to entrain the *Neurospora* circadian clock properly with natural photoperiods.

Photoperiodic responses allow organisms to adapt to all seasons, and one role for circadian clocks is to provide organisms with the ability to measure changes in day length. Photoperiodic production of sexual spores and asexual spores and synthesis of carotenoid was recently observed in *Neurospora* (107). Similar to those in other organisms, these photoperiodic responses were abolished in strains lacking one or more key oscillator components, indicating that seasonal responses require a functional clock. At the molecular level, it was found that with different photoperiods, although the phase of *frq* RNA is locked by the light-dark transitions, the phase of FRQ protein is not, suggesting a posttranscriptional mechanism regulating the level of FRQ (106).

OUTPUT OF THE CLOCK

While much attention in the study of the circadian clock system has focused on the mechanisms by which circadian time is generated, the observable or overt rhythms in organisms were what first caught the attention of biologists who pioneered the field of chronobiology. For *Neurospora* and other fungi, rhythms in several physiological properties have been described (8, 61), but it was the easily observable daily rhythm in the development of conidiospores that first led to the investigation of the *Neurospora* clock (89). The study of output pathways was critical for the initial identification of FWO components in *Neurospora* (35), and the identification of genes that are regulated by the circadian clock has recently confirmed the existence of FRQ-independent circadian oscillators in the *Neurospora* cell (24). Thus, the study of circadian output pathways not only has yielded information on which cellular functions are regulated by the clock and how this regulation occurs but has also been instrumental in the discovery of oscillator components.

Identification and characterization of clock-controlled genes. To begin to characterize circadian output pathways at the molecular level in *Neurospora*, genes that are rhythmically expressed, and thus likely under control of the clock, were isolated. Such studies were the first for any organism to specifically target genes that have rhythms in mRNA abundance, and the term clock-controlled gene (ccg) was used to describe the genes (77). In the initial screens for ccg's, 12 rhythmic genes that peak in the late night to early morning were identified (9, 77, 118). Verification of clock regulation of the ccg's, as opposed to some other form of temporal regulation, was achieved by demonstrating that the period of the ccg mRNA abundance rhythm equaled the period of the strain examined. Specifically, for the long (29-h)-period frq^7 mutant strain, the period of the ccg mRNA rhythms was 29 h. In all cases examined, the FWO functioned normally in strains containing inactivated copies of the ccg's, demonstrating that they are part of an output pathway and are not involved in oscillator function (7, 102, 103). Such loss-of-function assays have been critical for helping to distinguish whether a gene that is rhythmically expressed is an output from the clock or is part of the oscillator mechanism. For example, *frq* mRNA cycles in abundance, but since inactivation of *frq* abolishes the developmental rhythm and disrupts the FWO, it is not considered an output gene (2, 3).

The recent use of microarrays to profile rhythmic genes has greatly accelerated the search for ccg's, and to date more than 150 ccg's have been identified (24, 87). These studies have shown that while the dominant peak in rhythmic expression for

TABLE 1. Summary of *Neurospora* ccg's

Functional category[a]	No. of ccg's	No. of rhythmic genes in cells that lack the FWO[b]
Cell division	1	
Signaling/communication	16	1
Cell structure/cytoskeleton	8	1
Cell defense	4	
Development	11	
Gene regulation	5	
Metabolism	42	3
Protein processing	10	
Protein synthesis	33	
Unclassified	50	2

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

[b] Information in this table is expanded from that presented previously (24) to include all known ccg's.

most of the ccg's anticipates dawn, the *Neurospora* clock regulates ccg's at all possible phases of the day (24).

The predicted biochemical functions of the proteins encoded by the *Neurospora* ccg's provide critical insights into the diverse processes that are clock regulated (Table 1). Notably, several genes encoding transcription factors and proteins with known or suspected roles in signal transduction are rhythmic, but with peak levels occurring at different phases of the circadian cycle, suggesting that the relay of time information from the FWO to these factors may have a role in orchestrating phase-specific expression of downstream ccg's. These genes provide excellent candidates for components involved in signaling time-of-day information from the FWO to the output pathways.

Because organisms use their clocks to control species-specific events, it may not be surprising that little overlap exists between cycling genes in different organisms (30), yet some aspects of clock control are universally conserved. There are ccg's in nearly all organisms examined that are involved in protein synthesis and processing, intermediary metabolism, chromatin modification, transcriptional regulation, and cellular signaling (30). An interesting observation is that several genes encoding ribosomal proteins are under clock control in multiple organisms. In *Neurospora*, the largest group of coordinately cycling transcripts encode ribosomal proteins, and most of these genes peak in the late night. Assuming that the levels of the ribosomal proteins are also rhythmic (this has not been tested), these data imply that the number of ribosomes increases in the late night to prepare for the times of day when the bulk of rhythmic transcripts peak.

Importantly, the search for ccg's has also uncovered evidence for additional oscillators in the *Neurospora* cell. Multiple oscillators were predicted early on because strains that lack components of the FWO can display rhythmic development under certain growth conditions, although the rhythms are lacking in at least one circadian property (3, 62, 79, 82). Furthermore, rhythms in nitrate reductase activity (20) and diacylglycerol levels (92) have been observed in the absence of FWO components. Together, these data have suggested the existence of one or more FRQ-less oscillators (FLOs) in the *Neurospora* cell (54), which likely require the FWO for full circadian properties. The presence of multiple oscillators within the cell may contribute to the diverse rhythmic processes under clock control, such as conidiation versus the expression of genes unrelated to development. Despite this speculation, genetic details are only known for the core FWO, and it is still not known if the FLOs play a major role in circadian oscillations in a wild-type strain; no data exist on the components of the putative FLOs, nor do we know if the FRQ-less oscillations are controlled by one or more distinct oscillators. Recent results from the study of output pathways may help to answer these questions.

A new class of ccg's that cycle in expression in a strain that lacks FRQ was identified from microarray experiments (24). Characterization of one of these genes, *ccg-16*, has demonstrated that *ccg-16* mRNA rhythms are generated by a temperature-responsive, temperature-compensated circadian FLO that, similar to FWO, requires functional WC-1 and WC-2 proteins for activity (28). These data suggest the possibility that the FWO and FLO that regulate *ccg-16* rhythmicity may interact with each other through the shared WC proteins. In any case, the identification of distinct FLO-dependent ccg's provides critical molecular tools for identifying components of the FLO(s) and for eventually understanding how a multioscillator system, considered to be common in eukaryotic circadian clock systems (5), functions to differentially control rhythmic gene expression.

Clock control of ccg mRNA rhythms. A fundamental question that still remains is how oscillator components signal time information through the output pathways to regulate rhythmic gene expression. One mechanism by which some ccg's would be predicted to be rhythmically controlled is by direct activation by components of the FWO. The C box [CGAT(N)C CGCT] in the *frq* promoter, which is bound by the WCC and required for rhythmic *frq* expression (36, 37), was identified in the promoters of 19 ccg's, suggesting that these genes may be direct targets of the WCC (24). Two of the 19 ccg's are themselves putative transcription factors that would, in turn, be predicted to control a subset of downstream ccg's. Also consistent with the idea that the WCC can mediate the transduction of time information from the FWO to the output pathways is the observation that several ccg's are rapidly induced in response to overexpression of WC-1 in cultures grown in the dark (67).

An 8-nucleotide element (TCTTGGCA) was found to occur 49 times in 39 of 59 *Neurospora* late-night-specific genes identified from microarrays (24). These sequences are very similar to the core of a 45-bp fragment in the *ccg-2* promoter located near the start of transcription that contains a positive *a*ctivating *c*lock *e*lement (ACE) (6). The ACE was shown to be both necessary and sufficient for rhythmicity. A probe containing the ACE identified factors present in nuclear extracts that interact specifically with these sequences. Examination of the binding factors revealed that the amount of binding and the mobility of the complexes change over the course of the day. These data suggested that the amount or activity of the factors, modification of the factors, or addition of accessory factors is rhythmic, consistent with these proteins having a role in clock control of the *ccg-2* gene. Experiments to identify the factors, including biochemical purification and yeast one-hybrid assays, have ruled out binding of the ACE directly by WC-1 and FRQ,

suggesting that a novel factor(s) controls the rhythmic expression of *ccg-2* (D. Bell-Pedersen, unpublished data).

Many of the ccg's lack both the ACE and the C box, consistent with the existence of other clock control regulatory elements and a hierarchical organization for the regulation of ccg's (24). This idea is supported by the observation that two of the morning-specific genes, *ccg-1* and *ccg-2*, are regulated differently by the FWO (6, 109, 113). The levels of *ccg-1* are constitutively high in mutant strains that lack a functional FWO, whereas the levels of *ccg-2* are constitutively low, and deletion of the ACE element results in constitutive low-level expression of *ccg-2* (6). The simplest model that fits these observations is that the FWO regulates a repressor of *ccg-1* and an activator of *ccg-2*. To identify the output regulators, genetic selection for mutations that affect the expression of *ccg-1* and *ccg-2* was initiated (109).

In the selection, three mutant strains were found to affect the rhythmicity of both *ccg-1* and *ccg-2*, suggesting a bifurcated output pathway from the FWO and that the mutations affected components upstream of the bifurcation. Another mutant strain had altered expression of *ccg-1* mRNA but retained normal *ccg-2* levels, suggesting that the mutation affected a component that resides downstream of the bifurcation leading to rhythmic *ccg-1* expression. Together, the identification of candidate transcription and signaling components from the microarrays, the availability of gene knockouts from the *Neurospora* genome project for their study (http://www.dartmouth.edu/~neurosporagenome/), the development of the genetic selection scheme, and the recent use of luciferase as a reporter gene to monitor ccg promoter-driven rhythmic gene expression (85) hold promise for rapid progress in the identification of key components of the output pathways.

CIRCADIAN RHYTHMS IN OTHER FILAMENTOUS FUNGI

As already suggested, one goal of studying the circadian clock in model organisms is to determine if the properties of the clock in one organism are conserved in other organisms. Eventually, such conserved features will likely provide important clues for the function of the human clock and provide ideas for therapies for clock dysfunction. As one step towards this goal, circadian clocks were investigated in two other species of filamentous fungi that are easily cultured in the laboratory and whose genomes are sequenced, i.e., *Aspergillus flavus* and *Aspergillus nidulans* (38, 42). In *A. flavus*, the clock was shown to control daily rhythms in the development of sclerotia, which are large survival structures produced by many fungi. This developmental rhythm exhibits all of the principal clock properties: the rhythm is maintained under constant environmental conditions, with a period of about 30 h at 30°C, it can be entrained by environmental signals, and it is temperature compensated. Interestingly, this endogenous 30-h period is one of the longest natural circadian rhythms reported for any organism, and this likely contributes to some unique responses of the clock to environmental signals. In *A. nidulans*, no obvious rhythms in development were observed. However, a free-running and entrainable rhythm in the accumulation of *gpdA* mRNA (encoding glyceraldehyde-3-phosphate dehydrogenase) was shown, suggesting the presence of a circadian clock in this species.

The *Ascomycete* subgroup *Euascomycete* radiated into several monophyletic groups (estimated at about 240 million years ago), including the *Plectomycetes* group, which contains *Aspergillus*, and the *Pyrenomycetes* group, which contains *Neurospora* (10). *frq* homologs have been identified in several species of *Pyrenomycetes*, but *frq* has not been found in members of the *Plectomycetes* (65, 66). *Aspergillus* lacks a detectable *frq* gene, suggesting that the *Aspergillus* clock differs from *Neurospora* FWO. However, homologs of *Neurospora wc-1* and *wc-2* are present in *A. nidulans*, *Aspergillus fumigatus*, and *A. flavus.* Because *Aspergillus* displays blue light responses (111), it is possible that the primary evolutionary force for maintaining the *wc* genes in the ascomycetes was to allow blue light sensing. It is also possible that the *Aspergillus* WC proteins function in an oscillator that uses a different negative component that substitutes for FRQ. Alternatively, the absence of FRQ, along with the unusual properties of the circadian clock in *A. flavus*, might suggest that the *Aspergillus* clock and *Neurospora* FLO are related and that FLO is ancestral to FWO. Ultimately, comparisons between the *Neurospora* and *Aspergillus* oscillators will allow investigation of whether circadian clocks have diverse evolutionary origins or whether molecular adornments have been added to a common ancestral mechanism.

Finally, circadian clocks in fungi are not limited to the ascomycetes. Fungal rhythms in spore development and discharge are widespread; however, the circadian nature of these rhythms has only been investigated in a few species. Despite this, circadian rhythms have been documented in species that span the fungal kingdom, including the zygomycete *Pilobolus* sp. and the basidiomycete *Pellicularia filamentosa* (8). The extent of clock control of spore development and release across the fungal kingdom indicates a selective advantage for regulation of these events at specific times of the day or season (93). Therefore, what we learn about the clock mechanisms in *Neurospora* and *Aspergillus* will hopefully encourage investigations of circadian rhythms in development and gene expression and comparative analyses of circadian clocks in other fungal species.

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