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CIRCADIAN RHYTHMS IN MICROORGANISMS: New Complexities

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■ **Abstract** Recent advances in understanding circadian (daily) rhythms in the genera *Neurospora, Gonyaulax*, and *Synechococcus* are reviewed and new complexities in their circadian systems are described. The previous model, consisting of a unidirectional flow of information from input to oscillator to output, has now expanded to include multiple input pathways, multiple oscillators, multiple outputs; and feedback from oscillator to input and output to oscillator. New posttranscriptional features of the *frq/white-collar* oscillator (FWC) of *Neurospora* are described, including protein phosphorylation and degradation, dimerization, and complex formation. Experimental evidence is presented for *frq*-less oscillator(s) (FLO) downstream of the FWC. Mathematical models of the *Neurospora* system are also discussed.

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GENERAL INTRODUCTION

Definition of Circadian Rhythms

Nearly all life on Earth (with the possible exception of hydrothermal vent animals) has evolved in a cyclic environment, exposed to daily rhythms of light and dark,

temperature, and other environmental variables. These rhythms impose selective pressure on organisms to adapt to, and anticipate, daily environmental changes. The suite of adaptations used by organisms to deal with these daily changes is called the circadian clock (*circa* = about, *dies* = a day). It is a clock in the sense that it gives the organism a mechanism for measuring time, and it is circadian in the sense that it measures time in units of approximately one day. Circadian clocks produce daily rhythmic behavior in the organism, allowing the organism to prepare for, and take advantage of, the daily environmental cycle.

It is remarkable that circadian clocks are found in all eukaryotes and in some prokaryotes (including the cyanobacteria *Synechococcus*). What is even more remarkable is that these circadian clocks continue to generate rhythmic behavior when the organism is held in constant conditions, in the absence of any time-cues from the environment. This property is called self-sustainability and is a primary piece of evidence that circadian rhythmicity is intrinsic to the organism and not merely driven as a direct response to the environment. The circadian clock must therefore include one or more self-sustained oscillators that can produce rhythmic output to drive the rhythmic behavior that we observe even in the absence of external time-cues.

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The Input-Oscillator-Output Structure

Although the circadian clock can continue to produce rhythmic behavior in constant conditions, it must be capable of responding to external cues to allow the organism to remain synchronized to the environmental cycle. These time-cues, or zeitgebers ("time-givers"), reset the circadian clock each day so that the period, which may be several hours different from 24 h under constant conditions, entrains to exactly the period of the external cycle. Entrainment by external time-cues requires an input pathway through which the environmental signals can alter the state of the circadian oscillator.

These properties of circadian rhythmicity have given rise to a model for the circadian clock that has three parts: an input pathway for receiving time-cues from the environment, a self-sustained oscillator that responds to and interprets the time-cues, and an output pathway through which the oscillator influences the behavior of the organism (Figure 1*a*). This venerable model has served circadian biologists well for many years, and we continue to use it as the structure for this review. However, as with most models, it is certainly an oversimplification and the goal of this review is to look at the many ways in which that three-part model has become increasingly inadequate as we learn more about the mechanisms underlying circadian rhythmicity. Our prediction is that if a definitive diagram of a circadian system is ever established for an organism such as *Neurospora*, it will resemble a plate of spaghetti more than a roadmap.

Definition of Complexity

We describe the circadian clock as a suite of adaptations, and this description carries several implications. The first implication is that these adaptations may

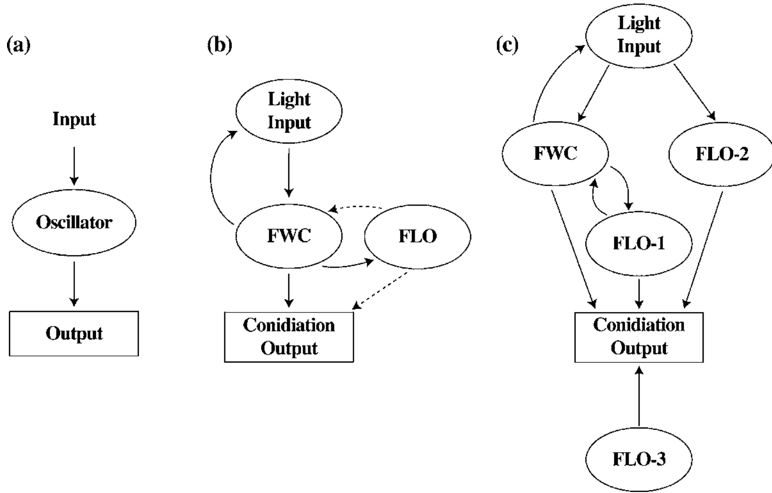

Figure 1 Potential complexity of the circadian system. (*a*) The simple three-part model, input > oscillator > output. (*b*) Complexity in the *Neurospora* system: Two oscillators mutually coupled may drive conidiation rhythms. FWC, *frq/wc* feedback loop; FLO, *frq*-less oscillator. Light input and FWC form a zeitnehmer loop with output from FWC feeding back on the light-input pathway. Coupling between FWC and FLO is indicated with FWC driving FLO under normal conditions. Dotted arrows indicate that FLO is dominant only under unusual conditions, such as in *frq*-null mutants. (*c*) Complexity in the *Neurospora* system: Several independent oscillators may drive conidiation. Direct coupling may occur between some oscillators (FWC and FLO-1) but not others. More than one light-input pathway may connect to more than one oscillator, but some oscillators (FLO-1, FLO-3) may not be light sensitive. Some oscillators (FLO-3) may be independent of environmental input (light) and other oscillators. The relative influence of each oscillator may depend on the conditions.

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have occurred in preexisting metabolic processes and housekeeping functions, so there is no necessity to postulate dedicated "clock molecules" with no function other than timekeeping. The second implication is that adaptations that improved timekeeping may have occurred in many molecular processes. Therefore, it may be impossible to define exactly the boundaries of the circadian clock, with many processes contributing to greater or lesser degrees to its generation and regulation. The third implication is that the particular adaptations may be different in different organisms, depending on the evolutionary history and ecological niche of that organism. These implications increase the opportunities for introducing complexity into the mechanism(s) of circadian rhythmicity.

There are several levels at which complexity might occur. In multicellular organisms, there may be different circadian clocks in different tissues, as has been demonstrated, for example, in *Drosophila* (39) and plants (132). There may be two different clocks within a tissue, as has been proposed for E and M oscillators in the

mammalian suprachiasmatic nucleus (27, 52). These types of complexity do not apply to microorganisms, although the possibility of different clocks in different individual cells in a population might merit serious consideration (see *Gonyaulax*, below). Within the single cell (or syncytium) of a microorganism, there is still the possibility for complexity of several kinds, defined by the following questions: (*a*) How many components are needed to build a fully functional circadian clock within the cell? (*b*) How complex are the interactions and feedbacks among those components? (*c*) How many independent clocks can run simultaneously in one cell?

A recent conference, "Complex Clocks," held in Edinburgh in March 2000, dealt with many of these issues of complexity (59). The proceedings can be found in the *Philosophical Transactions of the Royal Society of London Series B*, vol. 356 (whole issue), and the *Journal of Insect Physiology*, vol. 47 (whole issue). A discussion of complexity within input pathways has also recently appeared (106).

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FILAMENTOUS FUNGI (*NEUROSPORA* AND *ASPERGILLUS*)

Neurospora

The filamentous fungus *Neurospora crassa* has been used as a model organism for the study of circadian rhythmicity for many years, and this work has been frequently reviewed (5, 6, 69, 83, 90). The most obvious manifestation of the clock in *Neurospora* is the rhythm of conidiation (asexual spore formation), which is observed as a series of dense bands of conidiospores when cultures are grown on solid agar medium. This rhythm is used by all *Neurospora* clock laboratories as the outward marker of the state of the circadian clock, and all clock-affecting mutations were identified and characterized by their effects on the period of this rhythm. We assume this rhythm to be the phenotype on which selection has acted to shape the suite of adaptations that is *Neurospora*'s circadian clock. (However, it should be noted that no one has yet published any field observations of conidiation banding rhythms in *Neurospora* "in the wild," so this banding pattern may be a laboratory artifact.) Our view of the *Neurospora* clock focuses on rhythmic conidiation and how it might be generated and regulated. Several other rhythmic phenomena have been observed in *Neurospora* and were reviewed previously (69), but these rhythms have not been characterized as thoroughly.

The molecular mechanism of the clock in *Neurospora* has been studied primarily through the characterization of clock-affecting mutants. The most important of these are the various mutant alleles of the *frq* (*frequency*) gene, identified by their effects on the period of the conidiation rhythm (38). The *frq* gene has been cloned and sequenced, and a number of molecular studies have provided support for a feedback loop model in which the FRQ protein negatively regulates its own transcription (32). The white-collar proteins (WC-1 and WC-2) also participate in this feedback loop: A complex of the two WC proteins (WCC, white-collar-complex) is required for full transcriptional regulation of *frq*, and the FRQ protein

positively regulates the levels of the WC proteins and negatively regulates the activity of the WCC. Consequently, a null mutant at any of the three loci, *frq*, *wc-1*, or *wc-2*, will have low levels of all three proteins. This interlocked feedback loop of FRQ and WCC (which we abbreviate in this review as the FWC loop) has been proposed as the core of the circadian oscillator in *Neurospora* (32). Much of the "new complexity" we report in this section indicates that this model may not be sufficient to explain the generation of rhythmic conidiation in *Neurospora*.

INPUT The most important zeitgebers for *Neurospora*, as with most organisms, are light and temperature. Input pathways in *Neurospora* have been reviewed recently from the molecular perspective (78). A single input pathway for temperature has not been defined, and for good reason: A change in temperature affects nearly every chemical and physical process in the cell, and it may be impossible to define a single "temperature receptor" or input pathway. The results of a study of the effects of temperature changes on levels of *frq* RNA and FRQ protein (81) were not consistent with a single temperature receptor within the FRQ loop, and indicated complex responses of both transcriptional and translational events. Although rapid temperature changes reset the phase of the rhythm, the period is "temperature compensated" and changes very little when cultures are grown at different constant temperatures. Whether temperature resetting and temperature compensation use the same "temperature sensor" is an open question.

Light, on the other hand, may have a single molecular target in *Neurospora*. All known photoresponses in *Neurospora*, including entrainment of the circadian clock, respond to blue light. Light resets the phase of the conidiation rhythm and activates transcription of many light-induced genes, including *frq* (26). The *frq* gene has two light-response elements (LREs) in its promoter region, either of which can confer light inducibility on a reporter gene, confirming that light induction of *frq* occurs at the level of transcription (34). One of these LREs is also involved in transcriptional regulation of *frq* in the dark (34). The most significant recent development in defining input pathways in *Neurospora* is the identification of the WC-1 protein as the blue-light receptor that also directly regulates gene transcription (34, 46).

The two *white-collar* genes, *wc-1* and *wc-2*, were originally identified by mutations blocking blue-light responses. The gene sequences suggested that these two proteins should be DNA-binding proteins and act as transcriptional regulators (77). Until recently, the blue-light receptor in *Neurospora* was unidentified. It was an open question whether light acted directly on the WC proteins, or whether there was an upstream light receptor that activated the WC proteins through a second messenger (or other signal). This question has now been answered definitively: WC-1 is a light receptor and binds a flavin, FAD (flavin adenine dinucleotide), as the chromophore. This was demonstrated (34) by identifying LREs in the promoter of the *frq* gene and identifying protein complexes that bind to the LREs in vitro. These complexes contain both WC-1 and WC-2. FAD binds to WC-1 (46) and is required for the in vitro DNA-binding reaction (34). WC-1 is hyperphosphorylated

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in response to light and this response is intact in a *wc-2*-null mutant, suggesting that WC-2 is downstream of WC-1 in the light-input pathway (123).

The identity of the kinase that phosphorylates WC-1 has not been positively established. Light-induced phosphorylation of WC-1 appeared to be normal in a casein kinase II mutant (139). A partially purified protein kinase C phosphorylates WC-1 in vitro and protein kinase C inhibitors interfere with light adaptation in vivo, suggesting that protein kinase C may be activated to terminate the light signal by phosphorylating WC-1 (3).

In one sense the identification of WC-1 as the light receptor reduces the potential complexity in *Neurospora*: The input pathway from light to the DNA target is shorter than it might have been. However, other work has shown that light input is more complex than a simple linear pathway: The WC proteins have more than one function in transcriptional regulation, and the activity of the input pathway is rhythmically modulated by output from the circadian clock. He et al. (46) demonstrated that the FAD chromophore binds to a domain, LOV (light-oxygen-voltage sensing), in the WC-1 protein; the putative flavin-binding sites in this domain are important for the light-sensing function of WC-1 (12). The LOV domain is similar to the PAS domains found in many signaling proteins in microorganisms (131), and it is found in plant phototropin proteins that bind another flavin, FMN (flavin mononucleotide). This is where the story becomes more complicated: When the WC-1 LOV domain is deleted, light responses are abolished, including light induction of *frq* transcription, but transcriptional regulation of *frq* in the dark is retained (46). This second function of WC-1 is described below.

Not only does WC-1 play two roles, in both the light-input pathway and dark regulation of *frq*, but the light-input pathway is also more complex than expected. WC-1 is required for light induction of many genes in addition to *frq* (4). Light induction of *frq* has a less stringent requirement for WC-1 function than light induction of other genes: Various mutations in WC-1 that lead to low levels of WC-1 protein (15, 16, 31, 72) or mutations in the LOV domain (16) block light induction of other genes but allow light induction of *frq*.

Genuine knockout mutations of *wc-1* abolish both dark expression and light induction of *frq* (72), and a *wc-2* knockout also abolishes light induction of *frq* (19). However, entrainment of the conidiation rhythm to light/dark cycles has been reported for knockout mutants of both *wc-1* (M. Merrow, personal communication) and *wc-2* (31), suggesting the existence of another (unknown) light-input pathway independent of WC-1 and WC-2. The publication of the draft sequence of the *Neurospora* genome (36) revealed a number of genes that are putative blue-light sensors. The genome (36) also encodes two genes similar to bacteriophytochromes, red-light sensors in prokaryotes; however, no red-light responses have ever been observed in *Neurospora*.

A functional FRQ protein is required for light entrainment of the conidiation rhythm, as no response to light/dark cycles has been found in *frq*-null mutants (11, 67, 88). Light rapidly induces *frq* RNA (26) and FRQ protein increases after 4 to 8 h (19). A constitutively expressed *frq* gene can rescue light entrainment of

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conidiation in a *frq*-null strain (89), suggesting that rhythmic levels of FRQ protein are not required for this light response. However, FRQ protein is not required for light-induced expression of other genes: Light induction of carotenoid synthesis has the same sensitivity in frq^{+}- and a *frq*-null strain, as measured by fluence response curves (89). WC-1 is required for both light entrainment of rhythmicity and carotenoid induction, but these pathways appear to be regulated differently by FRQ.

A further complication comes from rhythmic modulation of the activity of the light-input pathway and the levels of the input pathway molecules. The level of WC-1 protein is rhythmic, although the RNA level is not (89). Light-induced gene expression, measured as the level of RNA increase in response to light, is rhythmic: Induction of *wc-1* and *al* expression, and total carotenoid induction, depend on the time of day (89), as do expression of *frq* and *vvd* (47). This "gating" of the light response by the circadian clock may be mediated by the rhythm in WC-1 levels (89).

The light-input pathway is subject to downregulation in constant light, a process named adaptation. The VVD (vivid) gene product is an important component of the adaptation response in *Neurospora* (47, 124, 127). In *vvd* mutants the normal downregulation of light-induced genes is inhibited so that RNA levels of light-induced genes, including *wc-1* and *vvd* itself (47), remain high after light exposure. In one report the *vvd* mutation had no effect on the downregulation of *frq* RNA (127), although another group found an increased response of *frq* RNA to light in the *vvd* strain (47); these differences may be due to the use of different strains among laboratories. VVD does not play a major role in the control of rhythmicity, as the conidiation rhythm has a normal period in *vvd* mutants (47, 127) and constant light suppresses rhythmicity normally (127). The phase-resetting effect of light on the rhythm is normal in *vvd* mutants with light intensities above saturation (127) and is stronger with less-than-saturating light pulses (47), as expected if the VVD gene product increases the response of the light-input pathway. The VVD protein contains a LOV domain similar to the flavin-binding LOV domain in WC-1, and it has been proposed that VVD may itself be a light receptor (12).

OSCILLATOR The mechanism that drives rhythmic conidiation in *Neurospora* is more complex than a single, simple feedback loop and may be composed of more than one self-sustained oscillator. New complexities have been reported recently in two areas: the regulation of the FWC feedback loop, and the characterization of rhythmicity in the absence of a functional FWC feedback loop (called the *frq*-less oscillator, or FLO).

***frq/wc* feedback loop** Several research groups have recently focused their attention on the FRQ protein, its structure, posttranslational modification, and turnover. A summary of the structure of FRQ and mutations that have been reported to affect its function is presented in Figure 2.

Two forms of FRQ protein are produced from alternative translation initiation sites (37). These two forms are produced at different temperatures, and either

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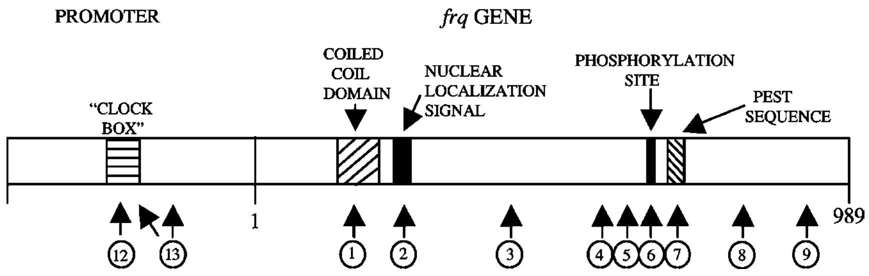

Number	Position	Change	Strain	Period	Remarks	Reference
1	165 169	L → R L → R		Arrhythmic	FRQ dimerization domain	(14)
2	194–199	Deletion	*p*CLIO	Arrhythmic	Nuclear localization signal	(86)
3	364	E → K	frq^3	24 hrs		(1)
4	459	G → D	frq^7	29 hrs	Slower degradation rate	(1)
5	482	G → S	frq^1	16.5 hrs		(1)
6	513 513	S → D S → I		Arrhythmic 35 hrs		(80) (80)
7	540–560	Deletion	ΔPEST-1	Arrhythmic	PEST sequence	(42)
8	662	S → frameshift	frq^9	Arrhythmic	Truncated protein	(1)
9	895	A → T	frq^2	19 hrs		(1)
10	Entire gene	Deletion	frq^{10}	Arrhythmic		(1)
11	211, 240, 311, 394, 601, 627	Either S or T → A	m5	30.7 hrs	Phosphorylation sites	(45)
12	Promoter	78 *bp*	AF26	Arrhythmic		(35)
13	Promoter	2 deletions	AF36		Light-responsive elements	(34)

Figure 2 Summary of FRQ structure and mutations. The table is a partial list of mutations described in the *frq* coding sequence and promoter region. One-letter amino acid abbreviations are used to indicate changes in the coding sequence.

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form can support rhythmicity (79). FRQ localizes to the nucleus immediately after synthesis (92) and this nuclear localization is required for its function (86). The FRQ protein has been found in homodimers, and it is estimated that more than half of all FRQ proteins are dimerized (14). The coiled-coil domain is required for this dimerization, and deleting this domain disrupts rhythmicity. FRQ:FRQ association is not required for nuclear localization, and the mechanism of nuclear transport is still unknown (14).

FRQ is phosphorylated soon after it is synthesized, and continues to accumulate phosphate groups during the circadian cycle until it is degraded (37). These phosphates can influence the turnover rate of FRQ and there is evidence that the turnover rate influences the period of the circadian rhythm (80). Control of phosphorylation of FRQ may therefore be a major factor in the kinetics of the clock. A calcium/calmodulin-dependent kinase phosphorylates FRQ, but disruption of the gene did not have a major effect on the circadian conidiation rhythm or the pattern of FRQ phosphorylation, suggesting that multiple redundant kinases affect FRQ (140). Two casein kinase homologs, CK-1a and CK-1b, have been characterized in *Neurospora* by Görl et al. (42) and are capable of phosphorylating in vitro the PEST-1 and PEST-2 sequences found in FRQ; PEST sequences are associated with protein turnover. When PEST-1 was deleted from FRQ (FRQΔPEST), the degradation rate of the protein decreased and the mutant strain lost the conidial banding rhythm in constant darkness (DD) (42). Surprisingly, both the RNA and protein produced by FRQΔPEST were rhythmic with a long period in spite of the loss of the overt conidiation rhythm. A null mutant of CK-1b showed normal FRQ protein rhythms and phosphorylation patterns (138). Another casein kinase, CKII, also phosphorylates FRQ (139). When the genes for the catalytic or regulatory subunits of CKII were disrupted, or potential CKII phosphorylation sites in FRQ were mutated, FRQ protein was hypophosphorylated, the conidiation pattern was arrhythmic or long-period, and molecular rhythms were absent or long-period (138, 139). The FRQ protein appears to be degraded by the ubiquitination-proteasome pathway, and a candidate ubiquitin ligase (FWD1) has been identified in *Neurospora* (45). When the FWD1 gene was disrupted, FRQ degradation was inhibited and molecular rhythms were lost; conidiation was rhythmic in light/dark cycles but not in constant darkness (45).

Complexity of control of the level of FRQ protein is seen when cultures are subjected to light/dark cycles with different photoperiods (130). The level of *frq* RNA responds rapidly to light with increases and decreases in response to lights-on and lights-off, and there is an additional delayed increase after 6 h in darkness. FRQ protein levels, however, do not follow light-dark transitions so closely and the increase in FRQ levels is not tightly correlated with light transitions or with levels of *frq* RNA (130). These results emphasize the major role played by posttranscriptional regulation of FRQ and the deficiencies of a simple transcription-translation feedback model to explain the kinetics of the molecular rhythms.

Complexity at the *frq* locus is indicated by the existence of two sense and two antisense transcripts, and the role of the antisense transcripts is being investigated

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by Crosthwaite and coworkers (61). The antisense transcripts apparently do not code for proteins, but are rhythmically expressed 180° out-of-phase with the sense transcripts and are light induced. When expression of antisense *frq* is abolished, the phases of the *frq* RNA rhythm and the conidiation rhythm are delayed. Most surprising is the effect on light-induced phase resetting: In the absence of antisense *frq*, light pulses have a larger effect on the phase of the conidiation rhythm in spite of normal effects of light on *frq* RNA and FRQ protein (61). The mechanism of action of antisense *frq* RNA is not yet known.

A major assumption of the FWC model is that the *frq* gene is rhythmically transcribed, but direct experimental evidence has been lacking. Now Froehlich et al. (35) have analyzed the *frq* promoter and have located a "clock box" (or C box) that can drive rhythmic transcription of a reporter gene. This C box is identical to one of the LREs previously identified (34). The WCC binds to this C box in vitro and binding is inhibited by overexpression of FRQ protein. The amount of WCC complex that binds to the C box in vitro depends on the circadian time at which the nuclear extracts were made. If, as predicted by the FWC model (35), FRQ protein inhibits its own transcription by binding to the WCC, then FRQ protein levels should be 180° out-of-phase with the amount of WCC binding to the C box. The data (35) show that, contrary to the prediction, these two rhythms are 90° out-of-phase. The control of *frq* transcription therefore appears to be more complex than a simple correlation between the amount of FRQ and transcriptional repression. A specific phosphorylated form of FRQ, rather than total FRQ, may be the active transcriptional regulator. This would put the emphasis on control of phosphorylation of FRQ (rather than control of total FRQ amount) in regulating the kinetics of the FWC loop. In support of this view, note that it is not the highly phosphorylated forms of FRQ that bind to the WCC, but rather the hypophosphorylated forms, suggesting that phosphorylation may decrease the ability of FRQ to bind to WCC (139).

The FRQ protein regulates not only its own transcription but also the levels of the WC proteins (15). Levels of WC-1 protein are low in a *frq*-null strain (13, 89) and FRQ apparently regulates WC-1 levels posttranscriptionally (13). In contrast, FRQ positively regulates WC-2 levels by increasing the level of *wc-2* RNA (13, 15).

The WC-1 protein functions both as a light receptor and as a regulator of dark transcription, and these functions are carried out by separate protein domains. When the FAD-binding LOV domain of WC-1 is deleted, the levels of *frq* RNA and FRQ protein are similar to wild-type levels in the dark, even though the modified WC-1 protein is found at much lower levels than in wild type (46). This LOV-less strain behaves as if blind and the conidiation rhythm cannot be induced or synchronized by light/dark cycles, but the clock appears to be working because temperature cycles can induce and synchronize the conidiation rhythm (46). Therefore the light-receptor and dark-regulator functions of WC-1 can be separated, and it takes much less WC-1 protein to fulfill the dark regulation function. An analysis of various *wc-1* mutants revealed that the dark regulation of *frq* (but not light

induction) requires the DNA-binding domain and a putative nuclear localization signal, which is unnecessary for nuclear localization (16). The separation of these two functions was also demonstrated by another mutation in *wc-1* that retains light sensitivity for rhythmic conidiation in a light/dark cycle but is arrhythmic in constant dark (133).

The WC-2 protein is more abundant than WC-1 and appears to be in excess, as a low level of WC-2 can still support FRQ expression (15). Mutations in WC-2 can reduce FRQ levels (18, 19), lengthen the conidiation period, and impair temperature compensation (18). It has been proposed that WC-2 is a scaffold mediating the interaction between FRQ and WC-1 to form the WCC (28). The PAS domain of WC-2 is required for the interactions between WC-2 and WC-1 and for the formation of a complex with FRQ (13). WC-1 is required for the interaction of FRQ with WC-2 (16), which argues against a scaffold role for WC-2. The level of WC-2 is regulated by both FRQ protein (which increases WC-2 levels) and WC-1 (which downregulates *wc-2* transcription) (16).

***FLO* (*frq-less oscillator*)** The FRQ-WCC loop is clearly important in the control of rhythmic conidiation in *Neurospora*: Mutations in *frq*, *wc-1*, or *wc-2* can alter the period, light sensitivity, temperature compensation, and expression of the conidiation rhythm. However, an active FRQ-WCC is not required for rhythmic conidiation, as evidenced by a growing number of conditions under which rhythmicity can still be observed in null mutants missing FRQ-WCC activity. A summary of the conditions under which conidiation rhythms can be seen in null FWC strains is found in Table 1.

Rhythmicity in *frq*-null mutants was demonstrated first with the *frq*^{9} strain (84, 85) and later the *frq*^{10} strain (1). Conidiation rhythms in these strains are seen after several days of growth in extra-long race tubes and in some but not all cultures, and the rhythms have poor temperature and nutritional compensation. Rhythmic conidiation can also be seen in *wc-1* and *wc-2* mutants with a combination of glucose-free growth medium and long race tubes, which allow the rhythm to develop after several days of growth (31). Under these conditions the period is circadian but more variable than the wild type.

More reliable rhythmicity can be seen in *frq*- and *wc*-null mutants when they are combined in double-mutant strains with mutations in either *cel* or *chol-1*, two mutations that affect lipid synthesis. When *cel*, which is defective in fatty acid synthesis, is grown on media containing unsaturated fatty acids, the period can be as long as 40 h and temperature compensation is poor (87). Similarly, when *chol-1* is deprived of choline, which it requires for normal growth, the period can be about 60 h and again temperature compensation is poor (63). In both strains, robust long-period rhythmicity is still seen in the absence of functional *frq*, *wc-1*, or *wc-2* genes (67). Note that the long-period rhythmicity in both *cel* and *chol-1* is not simply independent of FRQ function: Both long- and short-period *frq* mutations affect the long period of *cel* (66), while short-period *frq* mutations significantly shorten the long period of *chol-1* (63).

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TABLE 1 Free-running conidiation rhythms in strains carrying null mutations in *frq*, *wc-1*, or *wc-2*

Strain[a]	Property	Control conditions[b]	Extra-long race tubes[c]	Extra-long race tubes, no carbon source	*cel*+ 18:2[d]	*chol-1*, no choline[e]	Geraniol or farnesol[f]
Reference(s)			(1, 84, 88)	(31)	(67)	(67)	(43)
Wild-type (*bd* or *bd csp-1*)	Period (h):	21 at 25°C	21 at 25°C	23 at 25°C	34 at 22°C	65 at 22°C	22–23 at 20°C
	Light-sens.[g]:	Yes	Yes	Yes	Yes	Yes	—
	Temp. comp.[h]:	Yes	Yes	—	No	No	—
frq[9]	Period (h):	NR[i]	17–22 at 25°C	—	—	67–83 at 22°C	Similar to *frq*[10]
	Light-sens.:		No (88)	—	—	No	
	Temp. comp.:		No	—	—	—	
frq[10]	Period (h):	NR	17–22 at 25°C	—	32–37 at 22°C	64–67 at 22°C	22–30 at 20°C
	Light-sens.:		No (88)	—	No	No	No
	Temp. comp.:		No	—	—	No	No
wc-1	Period (h):	NR	NR[j]	22–29 at 25°C	—	63–71 at 22°C[l]	25–27 at 20°C[l]
	Light-sens.:			Yes[k]	—	No	—
	Temp. comp.:			—	—	No	—
wc-2	Period (h):	NR	NR[j]	25–35 at 25°C	33–36 at 22°C	77–87 at 22°C	21–25 at 20°C
	Light-sens.:			Yes	No	No	—
	Temp. comp.:			—	—	No	No

[a]All strains carry the *bd* mutation, and some also carry *csp-1*.

[b]30-cm race tubes, standard media without additional supplements.

[c]Race tubes longer than 30 cm. Rhythmicity typically begins after 5–7 days of growth.

[d]Double-mutant strains carrying the *cel* mutation, grown on media supplemented with linoleic acid (18:2).

[e]Double-mutant strains carrying the *chol-1* mutation, grown on media without choline supplementation.

[f]Grown on media with geraniol or farnesol supplementation.

[g]Light sensitivity: Is rhythmicity damped by constant light or entrained by light/dark cycles?

[h]Temperature compensation: Is temperature compensation of the period similar to wild type?

[i]Not rhythmic under these conditions.

[j]S. Brody, unpublished data.

[k]M. Merrow, personal communication.

[l]This allele, *wc-1*^{ER53}, produces a truncated protein with some residual activity (72).

Robust rhythmicity can also be induced in *frq*-, *wc-1*-, and *wc-2*-null mutants by adding farnesol or geraniol to the growth medium (43). These two compounds are intermediates in the steroid synthesis pathway, but their molecular targets in this case are unknown. Rhythmicity on farnesol or geraniol shows poor temperature compensation and can be abolished by substituting acetate for the usual maltose as carbon source in the growth medium (43).

A different method for detecting a "cryptic" oscillator is to look for entrainment of conidiation by external cycles (88). The *frq*-null strains do not entrain to light/dark cycles, but they do produce rhythmic conidiation in temperature cycles. The phase relationship between the conidiation rhythm and the temperature cycle depends on the length of the cycle, suggesting that rhythmicity under these conditions is due to true entrainment of an oscillator and not a direct driven response of conidiation to temperature changes (88).

In addition to rhythmic conidiation, rhythms at the molecular level can also be found in FWC-null strains. Rhythms in nitrate reductase activity (17), diacylglycerol levels (105), and gene expression (25) have been found in *frq*- and *wc-1*-null mutants and are described below.

Evidence that the FRQ-WCC loop is not required for rhythmicity has led to the concept of a FRQ-independent oscillator named FLO (*frq*-less oscillator) (49). The FLO can be conceived of as an "opportunistic" rhythmicity that appears when the FRQ-WCC loop is missing, but has no normal function in the intact organism (Figure 1*b*); or as an oscillator coupled to FRQ-WCC that is seen only when FRQ-WCC is defective, but is required for normal circadian properties; or as a redundant oscillator working in equal partnership with FRQ-WCC; or as the central rhythm generator to which a rhythmically modulated input pathway (FRQ-WCC) is attached.

This fourth possibility receives support from several sources. It is clear from the work on light reception in *Neurospora* that WC-1 is firmly located in the light-input pathway. FRQ also appears to be located either in the input pathway or on the input side of a multi-oscillator system. Strains missing FRQ or WC-1 function are blind to the effects of light on rhythmic conidiation: *frq*-null strains cannot be entrained to light/dark cycles, either on normal media (11, 88) or on farnesol or geraniol (43), nor can light entrain *frq* or *wc* double mutants with *chol-1* or *cel* (67). The concept of a rhythmically modulated input pathway interacting with a central oscillator was explored in a theoretical model by Roenneberg & Merrow (109) (see Mathematical Models, below).

Although FRQ appears to be essential in the light-input pathway for rhythmic conidiation, new evidence suggests the existence of a FRQ-independent light-input pathway for entrainment of rhythmic gene expression. Using microarray analysis, Correa et al. (25) found several genes that are rhythmically expressed in *frq*-null cultures phase-set by transfer from light to dark, indicating that light input can reach a FLO in the absence of FRQ. (This proposed pathway would correspond to FLO-2 in Figure 1*c*.) These results contrast with those of Nowrousian et al. (103), who failed to find rhythmic gene expression in *frq*-null cultures

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exposed to temperature cycles. Note that in both cases, cultures were grown in liquid medium in which the conidiation pathway is suppressed, and therefore effects of light or temperature entrainment on the conidiation rhythm could not be assayed.

If the FRQ gene product is not required for rhythmicity and may not be an essential component of a central rhythm-generating mechanism, how should we describe its function? It has been suggested (64, 65, 68) that FRQ may be an environmental sensor, integrating signals from light, temperature, and nutrition. This model is based partly on the role of FRQ in temperature compensation of the period: *frq* mutants have altered temperature compensation, suggesting that FRQ may be a temperature sensor, adjusting the oscillator characteristics in response to different ambient temperatures.

Mutations in the *frq* gene affect not only temperature compensation but also "metabolic" compensation. The period of the wild type is not significantly affected by changes in the composition of the growth medium, but the period of the null mutant *frq*^{9} depends on the carbon source (85). Ruoff et al. (115) have found that the period of the wild-type *frq*$^{+}$ is pH compensated such that the pH of the medium has little effect on period, but *frq* mutations lose pH homeostasis in parallel with the loss of temperature compensation. The *cel* and *chol-1* strains, although they have lost temperature compensation, still demonstrate pH compensation, indicating separate mechanisms for these two types of rhythm homeostasis (118).

The role of the *frq* gene in rhythmicity might be clarified by a comparative analysis of its role in other organisms. *frq* homologs have not been found in other organisms, except in fungi closely related to *Neurospora* (75, 91). The *frq* gene has not been found in some other fungi, including *Aspergillus*, which has a functional circadian oscillator (44) (as described below). On this basis, it has been proposed that the FLO may be ancestral to the FRQ-based oscillator in fungi (44).

Many other genes affect circadian rhythmicity in *Neurospora*; however, this is sometimes obscured by the attention given to the *frq* gene. Several recent papers and reviews have sought to redress the balance by taking another look at these other genes (5, 23, 95, 96). Approximately 25 genes can be listed as clock-affecting, depending on how the term is defined (5, 23, 96). Interactions between subsets of these clock-affecting genes have been assayed in double-mutant strains. In most cases the mutations have independent effects on period, but several cases of gene interactions have been found. For example, *prd-2*, *prd-3*, *prd-6*, and *frq*^{7} mutually interact (95). Interactions between *prd-1* and *cel*, and between *cel* and *chol-1* and *frq*, have also been described (5, 23, 96). These interactions form the basis for models of the circadian system incorporating FRQ/WC as a light-input pathway; a metabolic oscillator (FLO) including *cel* and *chol-1*; and a link between the two involving *prd-2*, *prd-3*, and *prd-6* (23, 96). These mutations affect the property of temperature compensation, and in double-mutant strains this property can also demonstrate genetic interactions. Temperature compensation is therefore incorporated into these models (23, 96) as a product of the integration of the functions of many gene products in the complete system.

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What is the relationship between FWC and FLO? Does FWC drive FLO, or does FLO drive FWC, or are they mutually coupled? Under "normal" circumstances, in the wild-type strain, the period and expression of the conidiation rhythm are strongly influenced by FRQ and WCC functions: Mutations in FRQ affect the period, and *frq*- and *wc*-null mutants do not express normal rhythmicity. In the lipid-deficient *cel* and *chol-1* strains, long-period rhythms continue in the absence of FWC function and therefore must be driven by a FLO; these long-period rhythms can be modulated by mutations in *frq*, but the long period of the FLO is predominant in both *frq*$^+$- and *frq*-null backgrounds. FWC and FLO may be mutually coupled to produce a rhythmic output driving conidiation (as in Figure 1*b*).

However, it may be more useful to think in terms of many oscillators contributing to an output that integrates multiple sources of information to produce rhythmic conidiation (Figure 1*c*). The relative influence of different oscillators would vary depending on mutations, metabolic state, developmental state, and other factors. For example, the signal from the lipid-deficient FLO (corresponding to FLO-1 in Figure 1*c*) can override the FWC signal, producing long periods in *frq*$^+$ and *frq* mutants, although a weak signal from FWC can still be seen as a small effect of *frq* mutations on the long period. On the other hand, the FLO that drives conidiation in *frq*-null mutants in the presence of farnesol or geraniol must produce a weak signal that is overridden by FWC in wild-type strains, since farnesol or geraniol has no effect in wild type.

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OUTPUT The rhythm of conidiation is the most obvious output in *Neurospora* and is probably the most significant target for natural selection. Therefore the most significant question about output is, How does the endogenous oscillator(s) drive rhythmic conidiation? The answer is likely to involve the rhythmic control of transcription factors that regulate the expression of conidiation-specific genes.

The transcription factors regulating circadian gene expression have not yet been identified. The WC-1 protein is a candidate for circadian control of gene expression, in addition to its established role as a light receptor activating transcription of light-induced genes. Using microarray analysis, Lewis et al. (76) found that overexpression of WC-1 increased the RNA levels of a number of genes and that most of these genes were different from those genes induced by light. A sample of the genes induced by WC-1 overexpression was rhythmically expressed. These results may indicate that changes in WC-1 levels mediate circadian control of gene expression. Light induction may depend not on WC-1 levels, but on phosphorylation or some other feature of the WC-1 protein (76).

Although WC-1 levels may mediate rhythmic gene expression, the downstream output pathways are divergent and complex. WC-1 itself may not necessarily bind directly to the promoters of clock-controlled genes (ccgs): An analysis of rhythmic expression of *ccg-2* has shown that transcriptional complexes binding to the "positive-acting clock element" (ACE) of *ccg-2* probably do not contain either FRQ or WC-1 proteins (8). Similar ACEs were found in the promoter regions of

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32 of 119 rhythmically expressed genes (25). Only 17 of 119 genes had WC-1-binding sites, suggesting that WC-1 directly regulates relatively few ccgs (25).

The regulation of ccgs by the circadian oscillator differs from gene to gene. In a casein kinase II mutant that is arrhythmic for conidiation, the RNA levels for *ccg-1* are constantly high, whereas the levels of *ccg-2* are low (139), suggesting that *ccg-1* expression may be repressed by signals from the oscillator and that *ccg-2* expression is activated. The *ccg-1* and *ccg-2* genes are regulated by the conidiation developmental pathway in addition to the clock, and their levels are low in strains carrying mutations that block the conidial development pathway (24). However, the clock in these developmental mutants still rhythmically regulates these genes (24). This indicates that rhythmic control of conidiation is not simply the throwing of a master developmental switch, but that genes in the developmental pathway are individually clock-controlled.

Although the conidiation rhythm is the most obvious clock output in *Neurospora*, there may be other outputs driven independently of conidiation. Genes for yeast-like mating pheromones have been identified in *Neurospora* (10). Both genes are under circadian control, and one gene is identical to a previously identified clock-controlled gene (*ccg-4*), suggesting that mating behavior is under clock control in *Neurospora* (10). Other ccgs have been identified as glyceraldehyde-3-phosphate dehydrogenase (*ccg-7*) (126), copper metallothionein (*ccg-12*) (9), and trehalose synthase (*ccg-9*) (125), suggesting that the clock regulates aspects of metabolism and stress responses not necessarily connected to conidiation.

A number of other genes are rhythmically expressed in *Neurospora*, by the criterion of rhythmic levels of mRNA. By the year 2000, 11 such ccgs had been identified (5). Recently, a library of 1431 expressed sequence tags (ESTs) was screened for differences in expression between morning and evening, and 26 clones were identified, of which 4 clones were confirmed as new ccgs by Northern assays (141). These results indicate that a large proportion of genes in *Neurospora* may be rhythmically expressed.

More ccgs were identified in a microarray screen by Nowrousian et al. (103) using a temperature entrainment protocol. Of approximately 1100 genes, they identified 27 genes whose RNA levels are rhythmic in DD and constant temperature in frq^{+} and can therefore be classified as clock-controlled. Those genes whose functions could be identified belonged to a wide range of cellular processes. Fourteen of these same genes were also rhythmic in a temperature cycle; no other genes were rhythmic in temperature cycles and arrhythmic in DD. None of these genes were rhythmic in a frq^{10}-null strain in temperature cycles.

Until recently, all known ccgs were classified as morning specific, with RNA levels that peak in late night or early morning. Correa et al. (25) used microarrays constructed from EST libraries and found that approximately 145 of 760 genes were rhythmically expressed. They found all possible phases of peak expression, although the late night–early morning phase was most highly represented. As in other studies, the sequences of the rhythmic genes suggested a wide range of predicted functions.

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The study of circadian outputs and clock control of transcription will be revolutionized in *Neurospora* by the recent achievement of introducing an easily assayed reporter gene into the fungus. It has been something of an embarrassment to *Neurospora*-ologists that, unlike other clock model organisms, *Neurospora* resisted attempts at expression of luciferase. Morgan et al. (97) have now reported clock-controlled expression of luciferase under control of the *Neurospora ccg-2* promoter in both *Neurospora* and *Aspergillus*. This technical achievement will open up many new experimental avenues with these organisms.

Beyond rhythmic gene expression, other outputs can also be defined. Rhythms in macromolecule levels, enzymatic activities, ionic concentrations, fatty acid desaturation levels, and CO_2 output have been described (69). Recently a rhythm in nitrate reductase activity has also been described (17), and this rhythm persists in null mutants of *frq* and *wc-1*. Rhythmic conidiation can be divided into two output processes: the determination of the spatial pattern of bands and interbands, and the differentiation of aerial hyphae and conidiospores. Time-lapse video analysis of cultures growing on solid agar, first described by Gooch and colleagues (70), revealed a surprising temporal separation of these two processes. In long-period *chol-1* strains, these two processes seem to be only loosely coupled to each other, and the phase relationship between them is variable. A rhythm in the levels of the neutral lipid diacylglycerol has also been described in *Neurospora* (105) and it persists with a 12-h period in a null *frq* mutant. This rhythm also seems to be only loosely coupled to the conidiation rhythm, sometimes displaying bimodal peaks and a phase relationship to the conidiation bands that is not always constant (105). Whether this indicates a complex output pathway or the existence of multiple oscillators has yet to be determined.

Another function for the *Neurospora* clock has been revealed by the discovery that, like many higher organisms, *Neurospora* displays photoperiodic control of development (130a). Both asexual conidial formation and sexual protoperithecial formation show peaks of production at photoperiods around 12 to 15 h; carotenoid pigment production was also affected by photoperiod. In all cases the photoperiodic effect was missing in a null *frq*^{10} strain. This may mean that the *frq* gene product is required for the clock function controlling the photoperiodic response, or simply that these strains are blind to the light/dark cycles. In any case, these results open the way for the use of *Neurospora* as a convenient model organism for the molecular dissection of photoperiodism.

MATHEMATICAL MODELS The *Neurospora* clock has been modeled by several groups using equations relating the concentrations and rates of production and degradation of *frq* RNA and FRQ protein and including the inhibition of *frq* transcription by FRQ protein. A generic transcription-translation negative feedback loop was first suggested by Goodwin (41) as the basis for a circadian oscillator, before the autoregulatory properties of clock proteins were described.

The Goodwin model has been most extensively developed by Ruoff and coworkers in a series of papers looking at the effects of FRQ protein stability on period

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and temperature compensation (116, 117, 119–121). This model has three variables (*frq* RNA, cytosolic FRQ protein, and nuclear FRQ protein) and a set of reaction kinetic equations describing their rates of production and degradation. What is surprising in this model is that the period is highly dependent on the rates of degradation of the molecular species, but is relatively insensitive to rates of synthesis. With appropriate choice of parameters, this model can simulate temperature compensation, and it generates the prediction that changes in period by mutations that change the degradation rate of the FRQ protein would affect period and temperature compensation in parallel. This prediction is in agreement with experimental work on the half lives and periods of several FRQ mutants (42, 80; P. Ruoff, personal communication). This model can also simulate the phase-shifting effects of the protein synthesis inhibitor cycloheximide (CHX), but only if the inhibition of protein degradation by CHX is also included in the model (119). This theoretical work has focused additional attention on the important role of protein degradation in the *Neurospora* clock mechanism.

Goldbeter and colleagues (74, 40) have developed a model for the *Neurospora* clock based on their model for the *Drosophila* oscillator (73). In this model (74) a limit-cycle oscillator is constructed using three sets of kinetic equations that govern the levels of *frq* RNA, cytosolic FRQ protein, and nuclear FRQ protein. The model includes activation of *frq* transcription by light and can generate a phase-response curve similar to the in vivo results. A version of the *Drosophila* model that can also be applied to *Neurospora* has been used to study the effects of stochastic noise on a small number of molecules of RNA and protein (40), demonstrating that oscillations can still occur with the low numbers of molecules expected in vivo.

A more elaborate model that includes WCC and FRQ phosphorylation has been developed by Smolen et al. (128). The effect of FRQ on WCC levels is modeled as a positive feedback loop interlocked with the negative feedback of FRQ on its own transcription. This model adequately simulates molecular oscillations, light entrainment, and changes in period produced by changes in FRQ phosphorylation rates. The most interesting finding from this model is that the positive feedback loop can be eliminated without destroying circadian rhythmicity.

A different perspective on modeling the *Neurospora* clock has been provided by Roenneberg & Merrow (109). These authors have modeled the central oscillator as an abstract two-component feedback loop without identifying the molecular players. They have added an input pathway that is itself rhythmically modulated by output from the central oscillator, which they call a zeitnehmer, or time-taker. This input pathway is an autoregulatory loop, with a protein feeding back to inhibit its own transcription. Changes in the activity of this input pathway can affect the period and temperature compensation of the total system in a way that is indistinguishable from changes in a component of the central oscillator. In other words, this model demonstrates that a gene such as *frq* could function in either a central oscillator or an input pathway, and it would be very difficult to distinguish these two roles experimentally.

What can these models tell us about complexity in the *Neurospora* clock? All these models lag behind the biochemical data in complexity, and they have not incorporated all the molecular details such as changes in phosphorylation of FRQ and WC-1 and complexes between FRQ and WCC. Nevertheless, these oversimplified models are capable of mimicking the major properties of the clock, such as self-sustained rhythmicity, entrainment, and temperature compensation, indicating that circadian rhythmicity does not require a highly complex system.

UNANSWERED QUESTIONS The recent work on the circadian system of *Neurospora* raises a number of questions that should be addressed by future research.

FWC loop The mathematical models discussed above tell us that a highly complex system is not necessary for generating rhythmicity. Therefore chief among our unanswered questions is, What is the exact role of the FWC loop? It is dispensable for rhythmic conidiation under some conditions. Which components of the loop are required for normal rhythmicity? Although rhythmic expression of *frq* and/or *wc-1* appears to be required for clock function (2), is there a level of constitutive expression of *frq* and/or *wc-1* that supports normal rhythmicity? In light of the lack of *frq* in *Aspergillus*, why does *Neurospora* have a *frq*-based oscillator as well as FLO(s)?

With respect to FWC, what are the biochemical details of the FWC loop? What are the posttranslational modifications that are important for the key proteins? How important is rhythmic phosphorylation and degradation of FRQ and what are the kinases involved? What are the kinetic parameters of the key reactions in the FWC loop, and can they be used in a mathematical model to generate a realistic 22-h oscillation?

FLO The major question is, of course, what is the FLO? Can components of the FLO be identified by looking for treatments or mutations that affect the period of rhythms expressed in FWC-null strains, such as in the presence of farnesol or geraniol? Can mutations in FLO be found with appropriate selection regimes? Are there many FLOs? Are there differences between the rhythms seen in FWC-null strains under different conditions that might point to a multitude of potential FLOs?

We are also lacking information on how FLO(s) interacts with FWC. Can FLO(s) and FWC oscillate independently of each other in the same mycelium? Can gene expression rhythms and conidiation rhythms be uncoupled?

Input Several interesting questions arise when considering environmental input to the clock. First, why is the light-input pathway rhythmic? Did the autoregulatory FWC loop evolve from an adaptation mechanism to downregulate the light-input pathway during prolonged light exposure? Second, where is the property of temperature compensation located? Is it a specific input from one oscillator, FWC or FLO, or is it a property of the entire system?

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Output A complete definition of an output pathway is still lacking. What is the relationship between the conidial banding rhythm and the molecular rhythms of the FWC loop? What are the outputs (from FWC or FLO) that drive rhythmic conidiation? Finally, is there spatial complexity in *Neurospora*? There is evidence for independent oscillators in all parts of the mycelium (6); are they coupled, and do they use the same oscillator mechanism?

Aspergillus

Neurospora is not the only fungus to display circadian rhythms (7), but it is the only system that has been systematically investigated. A comparative study of circadian clocks among fungi has been initiated with the characterization of circadian rhythmicity in *Aspergillus* species (44). Bell-Pedersen and colleagues (44) have found a developmental rhythm of sclerotium production in *Aspergillus flavus*; sclerotia are reproductive and survival structures larger than conidiospores. Bands of sclerotia were formed under constant conditions with a temperature-compensated period of about 33 h, and they could be entrained to LD cycles and temperature cycles (44). In *A. nidulans*, no free-running developmental rhythms could be seen, but a rhythm in the level of RNA transcript from the *gpdA* gene was found; this rhythm could be entrained by a combined light and temperature cycle (44). This study of *Aspergillus* has barely begun and is already turning up surprises: No ortholog of the *Neurospora frq* gene could be found in the *Aspergillus* genome, although both *wc-1* and *wc-2* orthologs were found (44).

OTHER MICROORGANISMS

A comprehensive review of other single-celled clocks might easily fill up a good portion of this volume. In addition to the organisms mentioned here, clocks have also been described in the genera *Acetabularia, Chlamydomonas, Chlorella, Euglena, Paramecium, Physarum, Saccharomyces, Tetrahymena*, and many others (33). The *Gonyaulax* and *Synechococcus* systems are the best characterized and that work is examined briefly below. For more detailed treatment the reader is referred to several excellent reviews (30, 53, 93).

Gonyaulax

Studies on the biological clock of *Gonyaulax* (now known as *Lingulodinium polyedrum*), the marine dinoflagellate, were initiated in the 1950s (129). However, the lack of a good genetic system and the relative difficulties in applying biochemical and molecular techniques have limited the use of *Gonyaulax* compared to more manipulable organisms. For example, in *Gonyaulax*, there are no clock mutants that are currently employed, nor have any of the clock circuitry components been positively identified. It is likely that protein phosphorylation and degradation are involved in the clock mechanism, and evidence for a role for protein kinases and

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phosphatases comes from the effects on the clock of inhibitors of these enzymes (20–22). Complexity in the *Gonyaulax* clock is evident from physiological and cell biological studies at the input, oscillator, and output levels, and is discussed in recent reviews (110, 111).

Complexities in the input side of the system were introduced by several sets of findings. *Gonyaulax* shows different responses to blue light versus red and blue light (107, 108, 114), indicating at least two light-input pathways. In addition, this organism's clock is sensitive to pulses in its nitrogen source, and the response of its clock to nitrogen is quite different from the light response (113). Furthermore, evidence has been presented which suggests that there is feedback from the oscillator to the input, as shown by circadian modulation of the light-input pathway(s) (29, 107, 114).

Evidence for complexity in the basic oscillator structure of *Gonyaulax* comes from experiments that showed two separate rhythms in this organism, one of bioluminescence and one of aggregation (112). These rhythms run with different periods and their periods can be independently altered. This was the first well-documented report to suggest this type of complexity in a single-celled organism. Because there are not yet any molecular details for the *Gonyaulax* oscillators, or the FLO of *Neurospora*, it is too soon to tell whether they share common mechanisms. There is even the possibility that each of the two cellular oscillators in *Gonyaulax* is composed of more than one molecular oscillator.

Output pathways in *Gonyaulax* show several types of complexity. Photosynthesis (both carbon fixation and oxygen evolution) is rhythmic in *Gonyaulax*, as in many other photosynthetic organisms, but the molecular basis for this rhythmicity is not yet known. This photosynthetic rhythm (a circadian output) feeds back on the input side and modifies the red/blue-light response (56). The enzyme Rubisco is responsible for carbon fixation, but neither its activity in extracts nor its amount in vivo is rhythmic (101). What is rhythmic is the subcellular location of Rubisco, and this change in location may change the in vivo activity of the enzyme by altering the local environment near the enzyme (101). A likely candidate for driving rhythmic oxygen evolution is the light-harvesting protein PCP. Le et al. (71) found that synthesis rates of PCP isoforms are indeed rhythmic, but the amount of PCP did not change and could not account for the rhythm in oxygen evolution. Moreover, mRNA levels were not rhythmic and therefore PCP protein rhythms must be driven by rhythmic translation.

Other rhythmic *Gonyaulax* proteins, notably the two proteins involved in generating bioluminescence, are also controlled by translational as opposed to transcriptional mechanisms (93). This control may be exercised by an RNA-binding protein that rhythmically binds to the mRNA to inhibit translation (93). A similar RNA-binding protein has been found in *Chlamydomonas*, indicating that translational control may also act in the clock output pathway of this organism (94, 135). These results are a reminder that changes in cellular activities are not always directly driven by changes in gene transcription and should sound a note of caution when we consider the significance of the microarray data now coming out of

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various laboratories looking for rhythmic gene expression. Both posttranscriptional and posttranslational mechanisms may override the patterns seen on microarrays, and it is a long way from RNA to cellular function.

Cyanobacteria (*Synechococcus*)

Studies on the biological clock of prokaryotes got a late start compared to studies on other single-celled organisms because of several factors: (*a*) the prevailing myth that organisms with a cell division time less than 24 h could not sustain a circadian rhythm; and (*b*) the myth that prokaryotes could not sustain rhythmicity because cellular complexity and organelles, particularly a membrane-bound nucleus, were required. Studies on cyanobacteria have dispelled both myths, and now that there are well-documented rhythms in cell division, nitrogen fixation, and gene expression, the field has quickly caught up. Cyanobacteria have also contributed the best-documented evidence from any organism for the adaptive significance of circadian rhythms (104). The reader is referred to several excellent reviews (30, 54, 55, 93), and only those recent results relevant to our theme of complexity are briefly described here.

A fairly comprehensive picture of the *Synechococcus* oscillator has emerged, with three genes, *kaiA*, *kaiB*, and *kaiC*, as the central players (48). Clock mutants are known in all three genes, and deletion of any *kai* gene leads to arrhythmicity. The levels of KaiB and KaiC proteins are rhythmic (136) and KaiC protein is rhythmically phosphorylated (50). The molecular techniques available in *Synechococcus* have made it possible to investigate *kai* gene promoter function, and protein function at the level of domains and individual amino acids. These genes are not structurally related to clock genes in eukaryotes and several features of the *Synechococcus* oscillator make it strikingly different from the eukaryotic systems: (*a*) The Kai proteins are much smaller than the eukaryotic clock proteins, but they aggregate into very large complexes. (*b*) The KaiB protein rhythmically moves between the cell membrane and the cytosol (60). (*c*) The KaiC protein has domains for ATP binding and motifs found in GTPase proteins, and binds ATP and GTP in vitro (98, 102). (*d*) The KaiC protein autophosphorylates in vitro (102) and in vivo (137) and no other kinases have been implicated. (*e*) Both the phosphorylation state and degradation rate of KaiC affect the period, but these two processes are unlinked (137). (*f*) Although feedback inhibition of Kai proteins on transcription is observed, this does not require *kai*-specific promoters (30, 137). In summary the *Synechococcus* oscillator seems to rely heavily on autophosphorylation for its kinetics, with little reliance on transcription events for timing.

At the input level, complexities have arisen in the analysis of mutations affecting input pathways. (*a*) The *lpd* (light-dependent period) gene apparently acts in a light-input pathway and modifies the effects of light intensity on period (57). This gene may be a redox sensor influenced by photosynthesis, making a connection between cellular metabolism and the clock (57). (*b*) The *pex* (period extender) gene lengthens the period when overexpressed and affects expression of KaiA

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(62). (*c*) Mutations in the *cikA* (circadian input kinase) gene lead to a "blind" and "numb" phenotype that is insensitive to both dark pulses and temperature pulses, thus identifying this gene as a key player in the input pathway to the oscillator (30, 122). (*d*) The KaiA protein may be the target for input pathways impinging on the oscillator: The protein sequence contains a "pseudo-receiver" domain similar to two-component signal transduction systems and may respond to protein-protein interactions (30).

On the output side of the oscillator, there are several fascinating new complexities. (*a*) Essentially the entire genome is under transcriptional control by the clock (30, 82, 98). There may be global control of transcription, either by circadian changes in the activity of sigma factors (subunits of RNA polymerase) such that the entire transcriptional apparatus oscillates (30, 100), or by global changes in chromosome condensation mediated by complexes of KaiC protein (98, 99). (*b*) Feedback from output to oscillator is indicated by period and phase effects of mutations in output pathways. The SasA protein appears to be a histidine kinase and physically interacts with KaiC protein; disruption of *sasA* shortens the period and reduces the amplitude of *kaiBC* expression, while most other gene expression becomes arrhythmic (51). Inactivation of the *cpmA* gene changes the phase of expression of some genes, but not others (58). (*c*) Mutations in sigma factors can affect the period of expression of some genes (134) while leaving others unchanged (100).

This result (100) has been interpreted as evidence for complex feedback from output to oscillator. If this result were confirmed, it would also suggest that separate oscillators with different periods could operate within a single cell. This is reminiscent of the multiple oscillators observed in *Gonyaulax* (112). What has not been found in *Synechococcus* is any rhythmicity in the absence of the *kai*-based oscillator. No residual rhythmicity has been seen in *kai*-null mutants, and no suppressors of *kai*-null mutants have been found.

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CONCLUSIONS

Microorganisms may be relatively simple in morphology and development compared with higher organisms, but they have demonstrated a wealth of complexities in their circadian organization. At the input level, both *Neurospora* and *Gonyaulax* may have more than one light-input pathway, with different spectral sensitivities in the case of *Gonyaulax*. Feedback from the oscillator(s) to the input pathway(s) creates rhythmic light sensitivity, and the activity of input pathways modifies the period of the system as a whole. At the oscillator level, *Neurospora*, *Gonyaulax*, and *Synechococcus* provide evidence for multiple oscillators within the same cell. Output pathways must also be complex with control of gene expression at multiple levels, transcriptional and posttranscriptional.

In *Neurospora* the three key clock proteins coded for by *frq*, *wc-1*, and *wc-2* are interrelated by positive and negative feedback loops, designated the FWC loop.

New information on phosphorylation and degradation of clock proteins is shifting the emphasis from transcriptional events to posttranscriptional events in the control of clock kinetics. This is most vividly demonstrated by the *Synechococcus* Kai-based oscillator, which appears to be based on autophosphorylation events and does not rely on *kai*-specific promoters for timing.

The presence of conidiation rhythms and molecular rhythms in the absence of a functional FWC loop in *Neurospora* led to the proposal of a second oscillator, the FLO (*frq*-less oscillator). In the future, genetic studies of the FLO will focus on the search for epistatic mutations and/or new mutants that knock out the FLO mechanism. More information about the relationship between FWC and FLO will help determine whether some features of the circadian system, such as light sensitivity or temperature compensation, reside in one oscillator or the other.

The old model of a self-contained clock mechanism that unidirectionally received input from the environment and drove gene expression as output is no longer adequate. The existence of other oscillators raises the possibility of feedback from cellular metabolism interacting with an oscillatory module in the clock mechanism. A second FLO-type oscillator may serve as a sensor of the internal environment (sie) of the cell, while a FWC-type oscillator may be primarily a sensor of the external environment (see). Our search for mutants in the FLO(s) must take into account the possibility of dual participation of genes in both key metabolic functions and the clock mechanism.

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