Neurospora wc-1 and *wc-2*: Transcription, Photoresponses, and the Origins of Circadian Rhythmicity

Susan K. Crosthwaite, Jay C. Dunlap,* Jennifer J. Loros*

Circadian rhythmicity is universally associated with the ability to perceive light, and the oscillators ("clocks") giving rise to these rhythms, which are feedback loops based on transcription and translation, are reset by light. Although such loops must contain elements of positive and negative regulation, the clock genes analyzed to date—*frq* in *Neurospora* and *per* and *tim* in *Drosophila*—are associated only with negative feedback and their biochemical functions are largely inferred. The *white collar–1* and *white collar–2* genes, both global regulators of photoresponses in *Neurospora*, encode DNA binding proteins that contain PAS domains and are believed to act as transcriptional activators. Data shown here suggest that *wc-1* is a clock-associated gene and *wc-2 is* a clock component; both play essential roles in the assembly or operation of the *Neurospora* circadian oscillator. Thus DNA binding and transcriptional activation can now be associated with a clock gene that may provide a positive element in the feedback loop. In addition, similarities between the PAS-domain regions of molecules involved in light perception and circadian rhythmicity in several organisms suggest an evolutionary link between ancient photoreceptor proteins and more modern proteins required for circadian oscillation.

For several billion years, a dependable aspect of living on Earth has been the daily light-dark cycle and its attendant temperature cycle. The endogenous circadian rhythmicity of organisms may have arisen directly as a response to these ubiquitous environmental cycles (*1*). Indeed, in free living cells and in tissues of multicellular organisms—the eyes of marine molluscs such as *Bulla* and *Aplysia*, the avian and lizard pineal, the vertebrate retina (*2, 3*)—there is a correlation between photoresponsiveness and the presence of circadian rhythmicity; even highly specialized rhythmic, but nonphotoreceptive, tissues such as the mammalian suprachiasmatic nucleus have close connections to photoreceptors in the eye (*4*).

In a similar vein, circadian oscillators are based upon analogous transcription-translation–based negative feedback loops (*5*), but candidates for phylogenetically conserved genes and proteins associated with clock function have not been found. For instance, the circadian oscillator in *Neurospora crassa* is built at least in part as a transcription-translation–based autoregulatory loop in which the protein products of the *frequency* (*frq*) gene, the various forms of FRQ, feed back negatively to regulate the amount of *frq* transcript (*5–8*). A similar feedback loop has been described in insects based on the coacting proteins TIM and PER (*9*), but neither FRQ, PER, nor TIM share extended sequence similarities (*10*). Furthermore, although successful circadian oscillation of these loops requires aspects of both negative and positive feedback (*11*), to date no components composing the positive aspect of the loop have been identified. Nor is the true biochemical function of any element in the loop known.

Light resets circadian oscillators by acting rapidly to alter the level of a state variable of the oscillator (*1, 12*), which in *Neurospora* is the amount of *frq* transcript (*13*). Likely candidates for the regulatory elements mediating this increase are the *white-collar* genes (*14*). Strains bearing lesions in either *wc-1* or *wc-2* lack all known photoresponses, including photoinduction of carotenogenesis and light-induced genes, perithecial beak bending, and the induction of rhythmic clock-controlled conidiation following a light-to-dark transfer (*15, 16*). Because of the apparent "blind" phenotype of the *wc* strains, their arrhythmicity has simply been ascribed to an inability to detect or respond to light cues, so that the oscillators in a culture remain asynchronous and the cultures appear to be arrhythmic.

Here, we report an alternative interpretation of the arrhythmicity seen in *wc-1* and *wc-2*: The products of *wc-1* and *wc-2* are required for normal operation of the circadian clock in this fungus. A functional *wc-2* gene is required for the expression of *frq* in the dark at levels sufficient for generation of the circadian oscillation; the *wc-1* gene is required both for normal, robust *frq* cycling in the dark and for light activation of the *frq* transcript in response to the pulses of light that can mediate phase resetting of the biological clock (*13*). Both *white-collar* genes encode DNA binding proteins (*17, 18*) bearing strong similarity to the GATA family of transcription factors of fungi and vertebrates (*17–19*). Also, each has a PAS domain (*17, 18*) similar to that in the clock gene *per* (*20*) and found in photoreceptor molecules from both prokaryotes and eukaryotes (*18, 21, 22*). WC-1 and WC-2 are thought to be the transcription factors required for the induction of all light-regulatable promoters in *Neurospora* (*17, 18*) but had not been expected to have roles in the cell beyond light regulation. We suggest that WC-1 is a clock-associated protein which, although required for sustained rhythmicity in the dark, cannot be considered a component of the feedback loop. In contrast, WC-2 appears to be a clock component required to complete the feedback loop; it would fulfill the requirement for a positive element in the circadian oscillatory loop, and be a clock protein with a known biochemical function. In addition, these data provide both a physical and a conceptual link between photoreception and circadian rhythmicity in *Neurospora*, and a potential evolutionary link (via the PAS domain) between photosensing molecules and molecular components of the clock.

wc-1 but not wc-2 is required for photoinduction of *frq* mRNA. To begin to dissect the light signal transduction pathway from photoreception to *frq* in the oscillator, we asked whether light resetting of the clock shares the same signal transduction pathway as the other photobiological responses of *Neurospora*, thereby requiring *wc-1* and *wc-2* (Fig. 1, top). Consistent with previous results, *frq* transcript (as assayed by Northern analysis) increases approximately 10-fold within minutes after a saturating light pulse in the laboratory wild-type strain *bd* (*frq*$^+$) (*13*). We found *frq* expression was not induced by a 2-min pulse of saturating white light in the *wc-1*$^-$ strain, indicating that the light signaling pathway for the clock is not unique to the clock but has at least one shared component with the signaling pathway for other light-controlled events. Surprisingly however, the acute response of *frq* transcription to light is qualitatively intact in the *wc-2*$^-$ strain (Fig. 1); in both wild-type and *wc-2*$^-$, *frq* expression

The authors are in the Department of Biochemistry, Dartmouth Medical School, Hanover, NH 03755–3844, USA.

*To whom correspondence should be addressed.

increases approximately 6- to 10-fold in response to a 2-min light pulse. This response of *frq* expression to light in the absence of *wc-2* function, and the recently described twofold light induction of *wc-2* itself (*18*), are the only photoresponses detected in a *wc-2*$^-$ strain and confirm the presence of a *wc-2*–independent light transduction pathway (*23*) here used by the clock. Additionally, densitometric analysis of the Northern blot data of these dark-grown control cultures showed that *frq* was expressed at very low levels in both the *wc-1*$^-$ and *wc-2*$^-$ strains; however, upon light induction in the *wc-2*$^-$ strain *frq* RNA reaches a level similar to that seen at the peak of the daily cycle in a *frq*+ dark grown culture (Fig. 1, bottom). Since we know that FRQ corresponding to this amount of *frq* mRNA is sufficient to bring about reduction of *frq* mRNA levels via negative feedback and to affect the oscillator in the wild-type strain in the dark (*7*), we presume that this amount of *frq* mRNA could also be physiologically significant here in terms of setting or resetting the oscillator.

To examine the characteristics of phase resetting by light in these strains we needed to be able to synchronize their biological clocks. Although both strains are relatively insensitive to photic cues, we reasoned that exposing the clocks to a nonphotic zeitgeber should be sufficient to synchronize the ensemble of oscillators if they are still intact, and thus to allow the expression of a stable robust overt rhythm. In *Neurospora* and other circadian systems, temperature treatments are strong zeitgebers that are thought to act directly on the oscillator itself, independently of photic transduction pathways (*24, 25*); pulses of high or low temperature reset the oscillator to a new phase that is dependent on the time the temperature pulse is given, and extended incubations at low temperature cause the oscillator to "stop" close to circadian time (CT) 0 or dawn (*25, 26*). For instance, *N. crassa riboflavin* mutants and the *Drosophila subobscura linne* mutant, both unable to respond to light resetting signals, have been synchronized with temperature steps (*27*). Thus, if the effect of the *wc-1*$^-$ or *wc-2*$^-$ mutations are limited to photic input, an extended temperature treatment should synchronize the system and allow expression of the overt rhythm. To this end we assayed the conidiation rhythm in a clock wild-type strain (*bd*) and in both *wc* mutant strains after high and low temperature treatments (Fig. 2). In constant light (LL), all strains fail to express an overt circadian rhythm, and consistent with expectations in constant darkness (DD) the *bd* strain produces a band of conidiation once every 22 hours. In contrast, however, *bd,wc-1*$^-$ and *wc-2*$^-$*,bd* have essentially the same phenotype in DD as in LL. The temperature treatments also reset the *bd* strain appropriately. Specifically, a 35°C step up for 15 hours at CT21 results in a 7-hour phase shift in the wild-type strain, resetting the clock to CT3, and a step down from 25°C to 4°C for 24 hours at CT18 resets the clock to CT0, subjective dawn, consistent with previous data (*26*). Surprisingly however, neither temperature steps up nor down were sufficient to induce rhythmicity in *bd,wc-1*$^-$ or *wc-2*$^-$*,bd*. Rhythmic conidiation is not observable in either *bd,wc-1*$^-$ or *wc-2*$^-$*,bd* mutant strains after entrainment by either light or temperature, both of which apparently fail to synchronize the oscillator.

These results suggested three possibilities: First, the *wc-1* and *wc-2* gene products may be essential for relaying both light and temperature signals to the oscillator, so that mutations in these genes result in desynchronization of the oscillator, and thus the

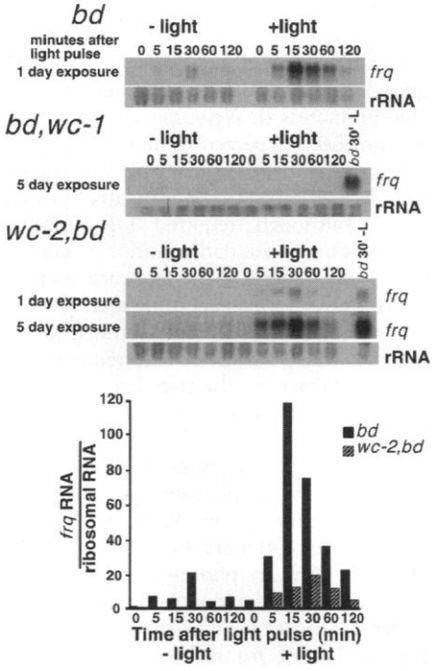

Fig. 1. Light-induced accumulation of *frq* transcript is blocked in a *wc-1* mutant strain. Northern analysis of *frq* and ribosomal RNA levels in control and light-pulsed tissue. Cultures of *bd*, *bd,wc-1*$^-$, and *wc-2*$^-$*,bd* strains (*41*) were grown as described (*8, 13, 42*) and were transferred from constant light to darkness. After 12 hours in the dark a 2-min light pulse (21 μmol photons m^2/s) was given to half of the samples; 5, 15, 30, 60, and 120 min after the light pulse, tissue was harvested, and RNA extracted and analyzed by Northern blotting (*43*). (**Top**) Northern analysis showing *frq* and ribosomal RNA in control and light-pulsed tissue as determined by hybridization of the membranes with *frq*- and rDNA-specific probes (*43*). All Northern blots that are directly compared were hybridized together to the same batch of probe, and the 30 min minus light sample from the *bd, wc*$^+$ panel at the top (*bd* 30′-L) was included at the far right of the *bd,wc-1*$^-$ and *wc-2*$^-$*,bd* panels for comparison. The Northern blots shown are representative of four independent experiments. (**Bottom**) Densitometry showing *frq* transcript levels in control and light-pulsed *bd* and *wc-2*$^-$*,bd* strains.

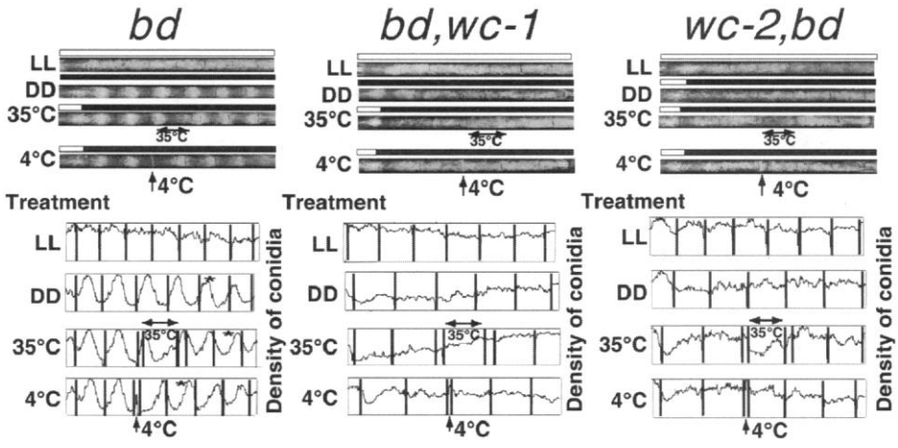

Fig. 2. High and low temperature treatments are unable to elicit rhythmicity in *wc* strains. Race tubes (*44*) were inoculated with the *bd* control strain, the *bd,wc-1*$^-$, or the *wc-2*$^-$*,bd* strains, held in LL (7 to 15 μmol photons m^2/s) for 2 days, and then transferred to DD. After 2 days (DD48) tubes were transferred to 4°C for 24 hours, or after 51 hours of growth to 35°C for 15 hours. At the end of each temperature treatment, the race tubes were returned to 25°C. (**Top**) Photographs of representative race tubes (from three independent experiments) showing growth of the strains under different light and temperature conditions. Bars above the race tubes indicate light (open) and dark (solid) growth conditions. (**Bottom**) Densitometric analysis of growth before and after the different temperature treatments. Vertical black lines, the position of growth fronts marked every 24 hours and at the beginning and end of temperature treatments. Asterisks, the position of the peaks of conidiation to be compared following the resetting stimulus.

overt rhythm in development, from environmental light and temperature cycles. If this is the case, overall *frq* expression levels would be unaltered, but rhythmicity, both molecular and overt, would be masked due to lack of synchronization of the population of oscillators. Alternatively, mutations in *wc-1* and *wc-2* may result in defective control of output from the clock. In this case the oscillator would only appear to be arrhythmic, the underlying rhythmicity being masked as in the *disconnected* and *lark* (*28*) mutants of *D. melanogaster*. In support of this hypothesis, *wc-1* and *wc-2* are required for expression of many light regulatable genes, some of which are also involved in development (*15, 29*). Third, the arrhythmicity in the overt rhythm may be accurately reporting arrhythmicity in the oscillator, suggesting that *wc-1* and *wc-2* encode components required for the assembly or operation of the oscillator. The results in Fig. 1 indicating that *frq* is expressed at very low levels in both of these strains are consistent with this proposal.

WC-1 and WC-2 are required for the circadian rhythm in *frq* expression. In order to distinguish among these three possibilities, and to ask whether mutation of either WC-1 or WC-2 was affecting cyclic expression of *frq* mRNA, we monitored the circadian oscillator directly by measuring *frq* transcript and FRQ protein levels, since these oscillations in part comprise the oscillator (*8*). If the clock is operational in either of the *wc* mutant strains then levels of *frq* transcript and FRQ will cycle, whereas if the oscillator is disrupted, they will not. We monitored the oscillator in clock wild-type (*bd*), *bd,wc-1*$^-$, and *wc-2*$^-$,*bd* mutant strains after treatment either with saturating light (Fig. 3, A and B) or with strong temperature-resetting stimuli (Fig. 4, A and B).

Light treatments failed to elicit a rhythm (Fig. 3). In the control strain, the light to dark transfer sets *frq* RNA and protein to CT12 levels, equivalent to dusk, and the *frq* transcript and protein oscillate in abundance over the course of the circadian cycle (Fig. 3, A and B) as expected (*5–7*). However, in both the *bd,wc-1*$^-$ and *wc-2*$^-$,*bd* mutant strains after a light to dark transfer, *frq* RNA is difficult to detect and FRQ protein is low to undetectable. No evidence for cycling of either *frq* RNA or FRQ protein was found after a light to dark step in either of the *wc* mutant strains, despite the fact that light was previously shown to induce *frq* mRNA to physiologically effective levels in *wc-2*$^-$ (Fig. 1). To confirm that overall transcription is unaffected in these strains, we verified that overall amounts of expression of the clock-controlled output gene *ccg-2* (*30*) were unaffected by mutation of *wc-2* or *wc-1* (Fig. 3A).

Similar to the results with light to dark steps, examination of molecular rhythms of *frq* mRNA and protein after strong temperature treatments revealed extremely low levels of expression with no evidence for cycling in either of the *white-collar* mutants (Fig. 4, A and B). In a wild-type strain exposed to low temperature (a drop from 25°C to 4°C lasting 24 hours), *frq* mRNA increased to levels comparable to those induced by light (*31*); on return to higher temperatures FRQ increases and the molecular transcript cycle begins from this high level, which corresponds to that seen at about CT0 (subjective dawn) and peaks again approximately 22 hours later (Fig. 4A). These molecular data confirm the results of physiological analyses demonstrating that long-duration, low-temperature treatments reset the clock to approximately CT0 (*26*). In contrast to the elevated expression of *frq* elicited by the temperature treatment in the *bd* strain, *frq* does not accumulate during growth at low temperature in either the *bd,wc-1*$^-$ or *wc-2*$^-$,*bd* mutant strains. Interestingly however, in the *wc-2*$^-$,*bd* strain (but not in *bd,wc-1*$^-$), a single anomalous *frq* mRNA peak of reduced magnitude is consistently observed 6 hours after transfer to higher temperatures, followed 4 hours later by a single appearance of FRQ protein. Despite the appearance of this peak, which can correspond approximately to the amount of *frq* seen at the peak of a normal cycle in darkness (Fig. 4A) or to the amount induced by light in the *wc-2*$^-$ background, no evidence for sustained rhythmicity has been found in a *wc-2* mutant background. If the absence of overt rhythmicity in either *wc* mutant strain was the result of a lack of synchronized molecular rhythmicity, then both *frq* mRNA and FRQ should have been present at average wild-type levels in the mutant strains, although expression would appear arrhythmic. This is not the case; instead *frq* and FRQ were expressed at very low to undetectable levels in these mutants, and in both strains the oscillator was incapable of sustained rhythmicity.

Transient oscillation can be induced in the absence of functional WC-1. These data demonstrated that a circadian oscillator cannot be established or sustained in a *wc-2*$^-$ background despite the presence of a wild-type *frq* locus and induced expression of *frq* mRNA and *FRQ* after light or temperature treatment. However, a caveat to reaching the same conclusion for *wc-1*$^-$

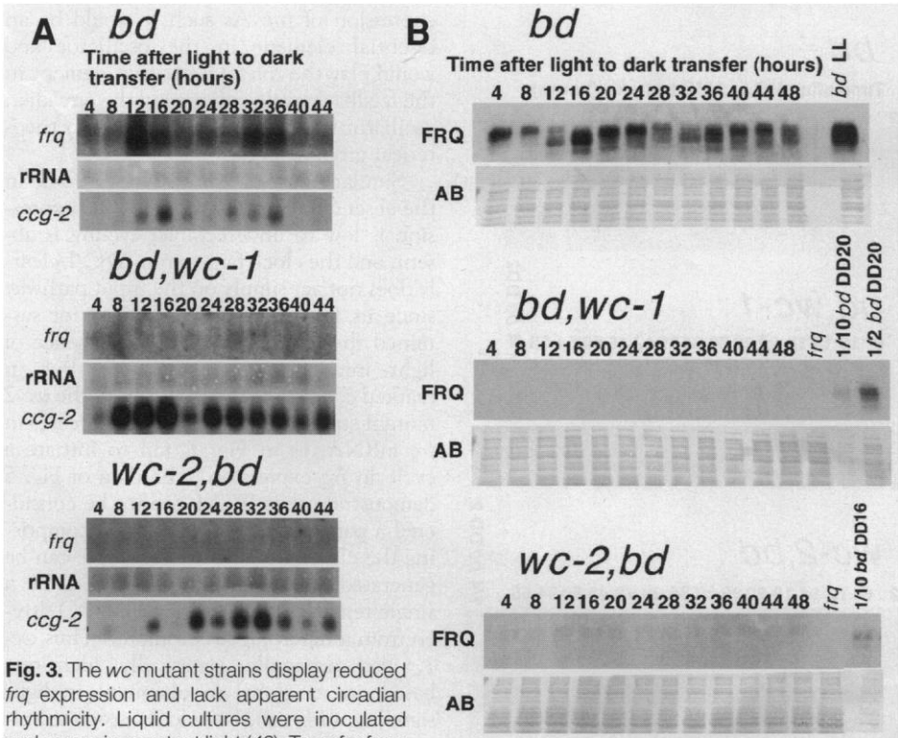

Fig. 3. The *wc* mutant strains display reduced *frq* expression and lack apparent circadian rhythmicity. Liquid cultures were inoculated and grown in constant light (*42*). Transfer from LL to DD was staggered so that cultures grown in DD at 25°C from 4 to 48 hours could be harvested within an 8-hour period to equalize developmental age. (**A**) Northern blots showing *frq*, ribosomal RNA, and *ccg-2* transcript levels in the *bd* control strain, or the *bd,wc-1*$^-$, or the *wc-2*$^-$,*bd* strains at 4-hour intervals for 44 hours of growth in DD. Although in the *wc* mutants *frq* is poorly expressed, *ccg-2* levels are approximately normal although lacking in apparent rhythmicity. (**B**) Western analysis of protein extracts prepared from the same tissue and probed with an antibody to FRQ (*45*), and Western blots stained with Amido Black (AB). Data representative of three independent experiments are shown.

strains is that we had, in all cases, failed to elicit significant *frq* expression in the absence of functional WC-1, so the possibility existed that a functional *frq* autoregulatory circadian oscillator could be run in this strain if only FRQ was produced once at levels sufficient to synchronize the oscillator and initiate the feedback loop. To examine this possibility we utilized a construction, *qa*-2pFRQ, in which expression of just the *frq* open reading frame is driven by the strong, inducible, and highly regulated *qa-2* promoter (*32*). [This construction was previously used to establish that rhythmic expression of *frq* was an essential part of the clock and that a step change in the amount of FRQ-encoding mRNA would reset the clock (*8*)]. Control experiments (Fig. 5A) recapitulate the expected result in a clock wild-type strain, namely that induction of the *qa*-2pFRQ promoter results in abundant *frq* expression that disappears upon removal of the inducer QA and that the phase of the subsequent circadian oscillation is determined by the time at which the inducer is removed and FRQ expression levels drop: In these experiments the removal of the inducer is mimicking the light to dark transfer, and the oscillation of FRQ in the cultures transferred from high to low QA (Fig. 5A, middle) are approximately 8 hours out of phase with that seen in the minus-QA control (Fig. 5A, top). We confirmed that the inducer was thoroughly washed out of the culture by verifying that *qa-2* mRNA itself immediately dropped to low levels and remained there for the duration of the experiment (Fig. 5A, bottom).

When the same experiment was then carried out in a *wc-1*$^-$ strain (Fig. 5B), an unexpected result was obtained. The data in Figs. 1 through 4 suggested that *frq* expression would be initially high in an induced *wc-1*$^-$ strain carrying *qa*-2pFRQ and that, upon removal of the inducer, *frq* transcript and FRQ levels would decay and remain low thereafter. Indeed, upon removal of the inducer, *frq* mRNA and FRQ levels did drop with normal kinetics. However, instead of remaining low, *frq* mRNA and FRQ levels rose again and then fell, completing a second full circadian cycle (Fig. 5B) in the absence of QA-induced *frq* expression and in the *wc-1*$^-$ background. The initial bolus of elevated *qa*-2pFRQ-driven expression seemed sufficient to "jump-start" the oscillator but not to keep it running. *wc-1* is essential for sustained overt rhythmicity in the dark so that *wc-1* loss-of-function mutants would appear, albeit incorrectly, to be identifying components of the oscillator; instead WC-1 does not appear to be a required component of the loop. These data are consistent with a model in which the products of *wc-1* are required to drive expression of genuine clock components at levels sufficient to complete the feedback loop. In their absence the clock may be able to run for one or a few cycles but this would gradually give way to arrhythmicity. This is in some respects reminiscent of a phenotype observed in strains of mice homozygous for a mutation in the *clock* gene (*33*).

Requirement of two DNA binding proteins involved in light signaling for circadian clock function. Our data demonstrate that WC-2 is required for normal operation of a functional circadian clock in addition to its requirement for general light signal transduction, separate from the clock, that served as the basis for its original genetic isolation and more recent cloning (*18*). The failure of light and temperature treatments to induce either sustained overt or molecular rhythmicity, while either treatment alone is capable of inducing transient expression of *frq* products and entraining normal clocks, combined with the extremely low expression levels of *frq* mRNA and FRQ in constant conditions, are consistent with a role for WC-2 in promoting the expression of *frq*. As such it would be an essential element in the oscillator and would play the role of a positive element in the feedback cycle comprising the circadian oscillator that has been predicted on theoretical grounds (*11*).

Similar to the findings with WC-2, in the absence of functional WC-1 *frq* expression is low to undetectable, cycling is absent, and the clock fails to run. WC-1 clearly does not act simply on the input pathway since its function is still required for sustained rhythmicity even in the absence of light information to transduce. But in marked contrast to the results with the *wc-2* mutant strains (where transient increases in *frq* mRNA, as in Fig. 1, fail to initiate a cycle in *frq* expression), the data of Fig. 5 demonstrate that WC-1 cannot be considered a part of the oscillatory loop comprising the clock since a full FRQ cycle can be generated in the absence of WC-1 by a single temporary introduction of FRQ driven from a heterologous promoter. Thus *wc-1*, also originally genetically identified based on its global involvement in light signaling (*14*, *34*), is a clock-associated element in the circadian system in *Neurospora*. WC-1 is absolutely required for the induction of *frq* gene expression by light, thus linking photoreception to the oscillator, and light can induce *frq* at any time of day (*13*). This suggests that WC-1 activity is always present at some level in the cell,

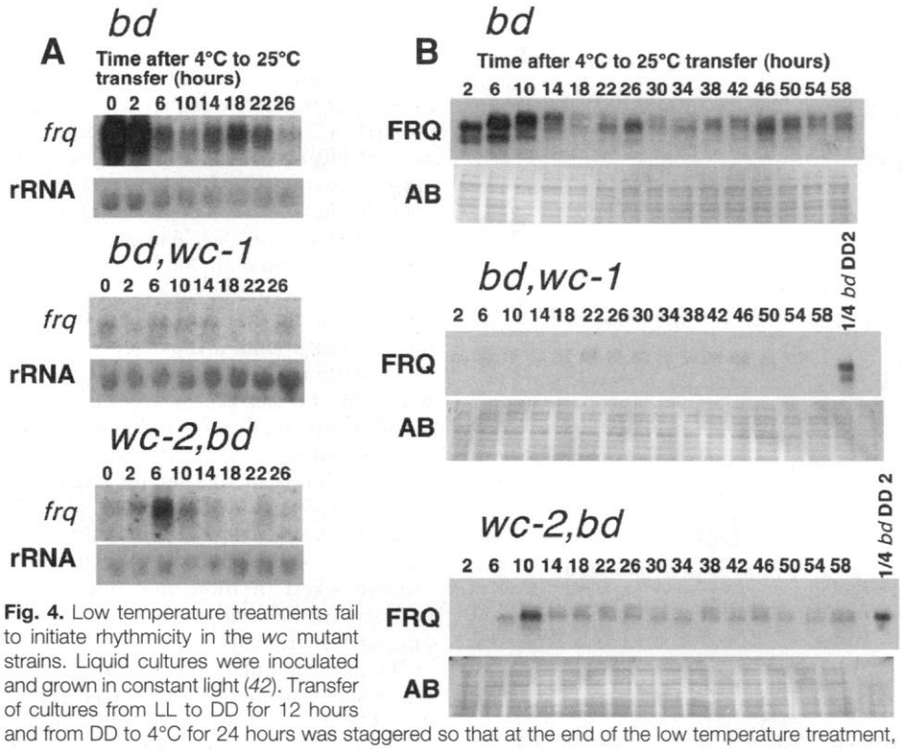

Fig. 4. Low temperature treatments fail to initiate rhythmicity in the *wc* mutant strains. Liquid cultures were inoculated and grown in constant light (*42*). Transfer of cultures from LL to DD for 12 hours and from DD to 4°C for 24 hours was staggered so that at the end of the low temperature treatment, samples incubated from 2 to 58 hours in DD after the low temperature treatment were all approximately the same developmental age (8-hour range). (**A**) Northern analysis showing levels of *frq* and ribosomal RNA after a low temperature treatment. A reproducible single peak of *frq*mRNA is seen in *wc-2*$^-$,*bd* at 6 hours after the 4°C to 25°C shift. (**B**) Western analysis of protein extracts prepared from the same tissue and probed with an antibody to FRQ (*45*) and stained with Amido Black (AB). Data representative of three independent experiments are shown.

although the fact that Macino and colleagues have shown the *wc-1* gene to be a strong positive autoregulator (*17, 18*) suggests that even a low (and not necessarily constant) level of WC-1 would be sufficient to mediate the activation of its own gene and of *frq*. It is therefore too soon to decide whether WC-1 could be classified as a parameter of the oscillator, since nothing about our data precludes the possibility that it is rhythmically regulated around a finite average level of activity and in this way could be formally connected to the driving oscillator. In summary, WC-1 may be the transcriptional activator most responsible for the photoinducible expression of *frq* that drives phase resetting of the *Neurospora* oscillator, and it appears likely that WC-1 is required to sustain but not to generate rhythmicity in the dark.

WC-2 and WC-1 are both transcription factors of the GATA factor family (*17, 18*). Like similar fungal proteins, including NIT-2 from *Neurospora* and AREA from *Aspergillus*, they contain zinc fingers, bind to DNA in the promoters of induced genes, and show distinct similarity in structure and action with vertebrate transcriptional activators of the GATA factor family (*19, 35*). Both frame shift mutations in *wc-2*, which result in premature termination and missense mutations in the zinc finger (*36*), result in a failure to induce light-responsive genes (*15, 18*) and in a failure to express *frq* at normal levels in the dark. These data bolster the idea that in the dark WC-2 is part of a transcriptional activation complex required either directly or indirectly to stimulate expression of *frq*. Our data are consistent with the model of Macino and colleagues (*17, 18, 37*) in which WC-2 normally acts in conjunction with WC-1, perhaps as a heterodimer in a two-component transcriptional activation complex for blue-light regulated gene expression in *Neurospora*.

WC-1 and WC-2 also contain PAS domains (*18*), which are thought to mediate protein-protein interaction (*20*); for instance, the PAS domain in the *Drosophila* clock protein PER is important for PER-TIM dimerization, which controls the stability of PER and the entry of the PER-TIM complex into the nucleus (*38*). Generally, PAS domains are paired as repeats, although both WC-1 and WC-2 have single PAS regions that share about 40% similarity with the PAS B domain of PER and other vertebrate and insect PAS domains (*18*). Our inference that WC-2 is a clock protein and contains a PAS domain supports a distinct molecular and evolutionary connection among circadian clock molecules. Equally important, the identification of a PAS domain in WC-1, which we have shown is not absolutely required to complete the oscillatory feedback loop, shows that not all clock-associated proteins containing PAS domains can be assumed to be clock components. Finally, the WC-2 PAS domain is 45% similar to a domain comprising half (60 out of 125 amino acids) of photoactive yellow protein (PYP), a prokaryotic photoreceptor protein, and is also similar to an internally repeated domain of about 60 amino acids found in higher and lower plant phytochromes (*39*). Figure 6 shows the PAS domains of a series of proteins associated with light reception, signal transduction, and light regulation in bacteria, plants, and fungi (*18, 22*) aligned with the PAS domains common to proteins associated with circadian rhythmicity in fungi and insects. These two separate lines of phylogenetic and physiological relatedness meet uniquely in the two *wc* genes of *Neurospora*. Since PAS domains are fairly rare in proteins but are not restricted phylogenetically (having been found in vertebrates, insects, algae, and fungi, and in the eukaryotes and prokaryotes), our data suggest an evolutionary link among some modern clock molecules and between clock molecules and more ancient proteins that detected or transduced environmental signals.

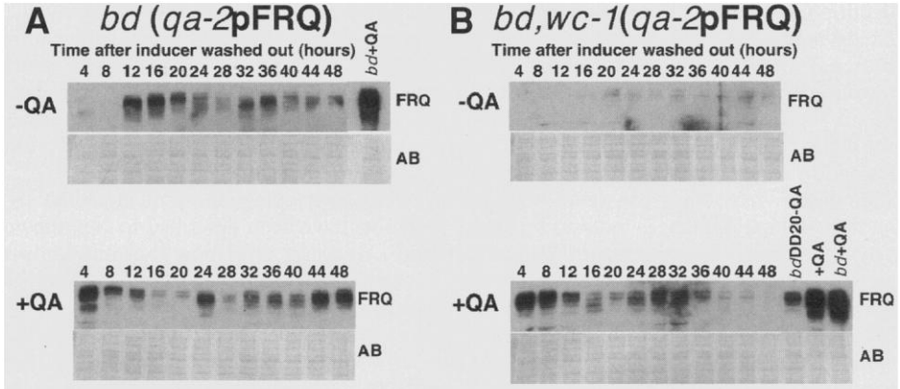

Fig. 5. Expression of FRQ from a heterologous promoter induces the expression of FRQ from the endogenous *frq* locus in the *bd,wc-1*$^-$ mutant strain. FRQ protein was monitored in *bd* and *bd,wc-1*$^-$ strains containing a copy of the FRQ ORF fused to the inducible *qa-2* promoter, and QA treatments are described in (*46*). (**A, top and middle**) Western analysis showing FRQ levels in control and QA-treated samples in the clock wild-type strain *bd*(*qa*-2pFRQ). Western blots stained with Amido Black (AB) are shown below. (**A, bottom**) Northern analysis showing level of the *qa-2* transcript in the presence of inducer and at times after the inducer was removed. (**B**) FRQ levels in control and QA-treated samples in the *bd,wc-1*$^-$ (*qa*-2pFRQ) strain. Three lanes at the right showing the amount of FRQ in the *bd* control from (A), and in the fully QA-induced *bd* [*qa*-2pFRQ] or *bd,wc-1*$^-$ [*qa*-2pFRQ] strains (marked *bd*+QA and +QA respectively) are included for comparison. Data representative of three independent experiments are shown.

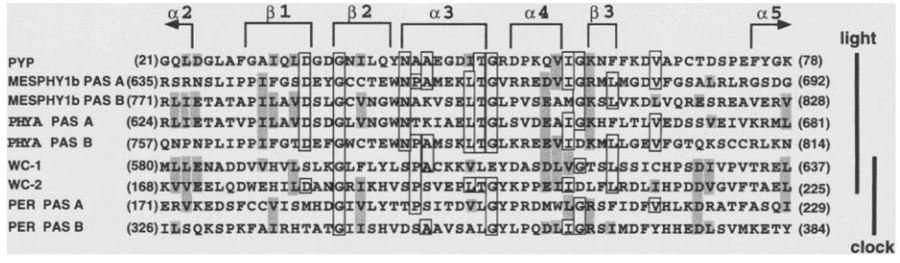

Fig. 6. The PAS domain is a shared motif among polypeptides involved in light signaling and circadian rhythmicity. A multiple alignment of the PAS region from WC-2 (*N. crassa* Gb:Y09119) with PYP (*E. halophila* Sw:P16113), MESPHY1b (phytochrome from *M. caldariorum* Gb:U31284), PHYA (phytochrome from A thaliana SW:P14712), WC-1 (*N. crassa* Gb:X94300) and the PAS A and PAS B repeats from PER (period clock protein from *D. melanogaster* Gb:PO7663). The internal repeat regions from the two phytochromes are here called PAS A and PAS B for consistency. Amino acid residue numbers are shown in parenthesis before and after each sequence. Boxed residues indicate identical sites in more than 50% of the sequences shown. Shaded areas indicate a greater than 50% conservative similarity between residues at a particular site. Shown above the sequence alignment are regions of secondary structure for the PYP, as determined by x-ray crystallography (*39*).

PAS-containing proteins comprise an interesting and diverse class of molecules. The PYPs are small (about 14 kD), highly conserved, water-soluble proteins associated with blue-light photoreception, light harvesting, and signal transduction that have been identified in three different families of bacteria. These proteins capture light energy via a chromophore, directing the captured energy into a photocycle, and the protein structure then undergoes a conformational or chemical change that leads to signal transduction. The PYP structure [known at 1.4 Å resolution (*39*)] includes an unusual α/β fold with remarkable structural similarity to eukaryotic profilin and the SH2 domain involved in signal transduction (*39*). The PYPs are structurally related via the PAS domain to a number of bacterial two-component regulatory proteins and also to plant phytochromes (*21*, *22*), a family of photoreceptors that sense the light environment, transduce light information to yield changes in gene expression, and thereby effect the developmental and physiological changes induced by light. Phytochromes allow organisms to appropriately adapt to prevailing environmental conditions, a use similar to that imagined for internal clock regulation. Phylogenetically widespread among photosynthetic organisms, phytochromes have been proposed (based in part on the above-mentioned similarities) to function as light-activated protein kinases similar to the two-component systems found in bacteria (*21*). Genetic analysis of the regulatory and signal transduction (as distinct from photoreception) aspects of phytochrome action have identified clusters of mutations in a small, COOH-terminal region similar to PAS domains (*40*). The light-association, signal transduction, and transcriptional activities associated with phytochrome action are similar to the activities associated with the *wc* gene products, and they share sequence similarity via the PAS domain (*17*, *18*) (Fig. 6). Moreover, the similarity between the putative clock components WC-2 and PER is noteworthy; both the *Neurospora* and *Drosophila* oscillators are transcription-translation–based negative feedback loops (*3*, *5*–*9*), but these similarities are the first to be confirmed among actual components of the two loops. The similarities in primary sequence in the PAS region between these two phylogenetically distinct clock molecules suggest that the association between clock molecules and PAS domains may extend well beyond the fungi and invertebrates. Finally, the widespread occurrence of PAS domains in light- and clock-associated proteins, combined with the predicted ancient origins of circadian rhythmicity (*1*), suggest that circadian clocks may have arisen from the cellular processes associated with the perception of the daily light-dark cycle and the transduction of this information within the cell to regulate metabolism in response to light.

Although components of circadian oscillators have been described in *Neurospora* and *Drosophila*, until now the only known functions of these molecules have been specifically associated with time-keeping. WC-2 represents a departure from this precedent, in that it plays a clear and distinct role in many photoresponses in the organism, and yet is additionally required for the operation of the circadian clock in constant darkness. Figure 7 provides a simple heuristic picture of its actions. A dual function for WC-2 can explain the pleiotropic effects of mutations in this gene, including the loss of photoresponses and of rhythmicity and the effect on sexual development (*15*, *16*). It is easy to predict that a mutation in a component of the light signal transduction pathway to the oscillator would result in arrhythmic behavior. That the same component would also be necessary for operation of the circadian oscillator in the dark was unexpected. Whether this is a general feature of circadian oscillators is not yet known, but the general coincidence of photoresponsive organisms or cells with circadian rhythmicity, and the anatomical coincidence of photoresponsive tissues and oscillator functions within vertebrates and invertebrates is striking (*1*–*3*, *5*). Our results with *wc-2* represent a sound molecular corollary to these anatomical, physiological, and potential evolutionary connections. An organism's ability to respond to light and its ability to keep time are inextricably linked.

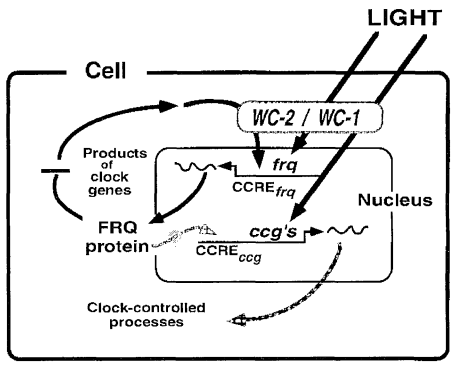

Fig. 7. A model of a rhythmic, light responsive cell. The *frq* gene participates in an autoregulatory feedback oscillator. Activation of *frq* mRNA requires the putative transcription factor WC-2. *frq* mRNA then encodes two forms of the FRQ protein (*7*) that subsequently feed back, either directly or indirectly, to downregulate the amount of *frq* mRNA, resulting in a daily oscillation. WC-1 is necessary for the sustained cycling of *frq* mRNA and FRQ, although a single FRQ cycle can be completed in the absence of this putative transcriptional activator. An additional activity of the *frq* gene is to confer rhythmicity on transcription rate and accumulation of output clock-controlled genes, the *ccg's*, probably by indirect regulation of CCRE's, the circadian clock responsive elements of these genes. Many of these *ccg's* are independently light responsive, requiring both WC-1 and WC-2 proteins for light response. Gene expression of *frq* is also induced by light (*13*), and this induction can take place in the absence of functional WC-2 but requires the WC-1 protein.

REFERENCES AND NOTES

1. C. S. Pittendrigh, *Annu. Rev. Physiol.* **55**, 17 (1993); M. Zatz, Ed., *Circadian Rhythms, Discuss. Neurosci.* **8** (Elsevier, Amsterdam, 1992).
2. M. S. Grace, V. Alones, M. Menaker, R. G. Foster, *J. Comp. Neurol.* **367**, 575 (1996); G. Block, M. Geusz, S. Khalsa, S. Michel, D. Whitmore, *Ciba Foundation Symp.* **183**, 51 (1995); M. H. Hastings *et al.*, *ibid.*, p. 175; G. D. Block, M. Geusz, S. Khalsa, S. Michel, *Sem. Neurosci.* **7**, 37 (1995); G. Tosini and M. Menaker, *Science* **272**, 419 (1996); U. Raju, C. Koumenis, M. Nunez-Reguiero, A. Eskin, *ibid.* **253**, 673 (1991); M. Zatz, *Brain Res. Rev.* **18**, 326 (1993); G. Cahill and J. C. Besharse, *Prog. Retinal Eye Res.* **14**, 267 (1995); M. Menaker and S. Wisner, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 6119 (1983); T. Roennberg, *Ciba Foundation Symp.* **183**, 177 (1995).
3. K. Siwicki, Ed., *Sem. Neurosci.* **7**, 1 (1995).
4. R. Moore, *Discuss. Neurosci.* **8**, 26 (1992); R. Y. Moore, *Ciba Foundation Symp.* **183**, 88 (1995); R. Silver, J. LeSauter, P. A. Tresco, M. N. Lehman, *Nature* **382**, 810 (1996); W. Schwartz, N. Aronin, J. Takeuchi, M. Bennett, R. Peters, *Sem. Neurosci.* **7**, 53 (1995); P. Sassone-Corsi, *Cell* **78**, 361 (1994).
5. J. C. Dunlap, *Annu. Rev. Genetics* **30**, 579 (1996); J. C. Hall, *Neuron* **17**, 799 (1996); J. J. Loros, *Sem. Neurosci.* **7**, 3 (1995); D. Bell-Pedersen, N. Garceau, J. J. Loros, *J. Genet.*, in press.
6. N. Garceau, Y. Liu, J. J. Loros, J. C. Dunlap, *Cell*, in press.
7. M. Merrow, N. Garceau, J. C. Dunlap, *Proc. Natl. Acad. Sci. U.S.A.*, in press.
8. B. Aronson, K. Johnson, J. J. Loros, J. C. Dunlap, *Science* **263**, 1578 (1994).
9. J. C. Hall, *Trends Neurosci.* **18**, 230 (1995); P. E. Hardin, J. C. Hall, M. Rosbash, *Nature* **343**, 536 (1990); P. Hardin and K. Siwicki, *Sem. Neurosci.* **7**, 15 (1995); S. M. Reppert and I. Sauman, *Neuron* **15**, 983 (1995); A. Sehgal, *Sem. Neurosci.* **7**, 27 (1995); A. Sehgal *et al.*, *Science* **270**, 808 (1995).
10. Although statistically significant similarities between FRQ in *N. crassa* and PER in *D. melanogaster* exist in the region of the Thr/Gly repeats of the two proteins [C. McClung *et al.*, *Nature* **339**, 558 (1989)], subsequent molecular analyses of PER homologs from different insects and FRQ homologs from different fungi have failed to support the significance of this similarity. However, the region retains interest in the context of temperature compensation and natural selection [X. Costa *et al.*, *Proc. R. Soc. London* **250**, 43 (1992)], and WC-2 contains a Thr/Gly repeat.
11. A. Goldbetter, *Biochemical Oscillations and Cellular Rhythms* (Cambridge Univ. Press, Cambridge, 1996); B. C. Goodwin, *Temporal Organization in Cells* (Academic Press, London, 1963).
12. C. S. Pittendrigh, in *The Neurosciences Third Study Program*, F. O. Schmitt and F. G. Worden, Eds. (MIT Press, Cambridge, MA, 1974), pp. 437–458.
13. S. C. Crosthwaite, J. J. Loros, J. C. Dunlap, *Cell* **81**, 1003 (1995).
14. R. W. Harding and W. J. Shropshire, *Annu. Rev. Plant Physiol.* **31**, 217 (1980).
15. G. Arpaia, J. J. Loros, J. C. Dunlap, G. Morelli, G. Macino, *ibid.* **102**, 1299 (1993).
16. F. Degli Innocenti and V. E. A. Russo, in *Blue Light*

Effects in Biological Systems, H. Senger, Ed. (Springer Verlag, Berlin, 1984), pp. 213–225; F.-R. Lauter and V. E. A. Russo, *Nucl. Acids Res.* **19**, 6883 (1991); T. Sommer, J. A. A. Chambers, J. Eberle, F. R. Lauter, V. E. A. Russo, *ibid.* **17**, 5713 (1989); R. Harding and S. Melles, *Plant Physiol.* **72**, 745 (1983); V. Russo, *J. Photochem. Photobiol. B.* **2**, 59 (1988).
17. P. Ballario *et al.*, *EMBO J.* **15**, 1650 (1996).
18. H. Linden and G. Macino, *ibid.* **16**, 98 (1997).
19. S. H. Orkin, *Blood* **80**, 575 (1992); G. A. Marzluf, *Adv. Genet.* **31**, 187 (1994).
20. Z. J. Huang, I. Edery, M. Rosbash, *Nature* **364**, 259 (1993).
21. P. Quail *et al.*, *Science* **268**, 675 (1995); H.A.W. Schneider-Poetsch, B. Braun, S. Marx, A. Schaumburg, *FEBS' Lett.* **281**, 245 (1991).
22. D. M. Lagarias, W. Shu-Hsing, J. C. Lagarias, *Plant Mol. Biol.* **29**, 1127 (1995).
23. Although *wc-2* is normally required for light inducibility of clock-controlled gene *ccg-1*, *wc-2*–independent induction occurs [G. Arpaia *et al.*, *Mol. Gen. Genet.* **247**, 157 (1995)] in a mutant background of the *cot-1* gene that encodes cAMP-dependent protein kinase [O. Yarden *et al.*, *EMBO* **11**, 259 (1992)].
24. B. Sweeney and J. W. Hastings, *Cold Spring Harbor Symp. Quant. Biol.* **25**, 87 (1960).
25. Y. Liu, N. Garceau, J. Loros, J. C. Dunlap, *Cell*, in press.
26. C. Francis and M. L. Sargent, *Plant Physiol.* **64**, 1000 (1979); V. D. Gooch, R. A. Wehseler, C. G. Gross, *J. Biol. Rhythms* **9**, 83 (1994).
27. J. Paietta and M. Sargent, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 5573 (1981); P. Lankinen, *Behav. Genet.* **23**, 359 (1993).
28. P. E. Hardin, J. C. Hall, M. R. Rosbash, *EMBO J.* **12**, 1 (1992); L. M. Newby and F. R. Jackson, *Genetics* **135**, 1077 (1993).
29. L. M. Corrochano, F. R. Lauter, D. E. Ebbole, C. Yanofsky, *Dev. Biol.* **167**, 190 (1995).
30. J. J. Loros, S. A. Denome, J. C. Dunlap, *Science* **243**, 385 (1989); D. Bell-Pedersen, J. C. Dunlap, J. J. Loros, *Genes Develop.* **6**, 2382 (1992).
31. M. Merrow, J. J. Loros, J. C. Dunlap, in preparation.
32. N. H. Giles *et al.*, *Microbiol. Rev.* **49**, 338 (1985).
33. M. Hotz-Vitaterna *et al.*, *Science* **264**, 719 (1994).
34. D. D. Perkins, M. Glassey, B. A. Bloom, *Can. J. Genet. Cytol.* **4**, 187 (1962).
35. B. Kudla *et al.*, *EMBO J.* **9**, 1355 (1990); T. Y. Chiang and G. A. Marzluf, *Biochemistry* **33**, 576 (1994).
36. In all of the mutant *wc-2* alleles analyzed by sequence to date, including those in (*17*), the zinc-finger DNA binding domain of WC-2 has been either deleted or disrupted. In ER33, the allele used in this study, Ser takes the place of conserved Gly^{485} in the zinc-finger domain.
37. G. Macino, personal communication.
38. H. Zeng, Z. Qian, M. Myers, M. Rosbash, *Nature* **380**, 129 (1996); L. Saez and M. W. Young, *Neuron* **17**, 911 (1996); N. Gekakis *et al.*, *Science* **270**, 811 (1995).
39. M. Koh *et al.*, *Biochemistry* **35**, 2526 (1996); G. E. Borgstahl, D. R. Williams, E. D. Getzoff, *ibid.* **34**, 6278 (1995).
40. J. W. Reed, A. Nagatani, T. D. Elich, M. Fagan, J. Chory, *Plant Physiol.* **104**, 1139 (1994); T. D. Elich and J. Chory, *Plant Mol. Biol.* **26**, 1315 (1994); B. M. Parks and P. H. Quail, *Plant Cell* **5**, 39 (1993); D. Wagner and P. H. Quail, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 8596 (1995); Y. Xu, B. M. Parks, T. W. Short, P. H. Quail, *Plant Cell* **7**, 1433 (1995).
41. *White collar-1* (FGSC# 4397, allele ER53) and *white collar-2* (FGSC# 4408, allele ER33) were obtained from the Fungal Genetics Stock Center. These strains were crossed to a strain carrying the *band* (*bd*) mutation (Oakridge), and one of double mutant progeny from each cross were back crossed to the wild-type *74OR23*. In strains *his-3,bd*(*his-3*$^{+}$ *qa-2*pFRQ) and 161-8 *his-3bd,wc-1,*(*his-3*$^{+}$ *qa-2*pFRQ) *qa-2*pFRQ (8) has been inserted at the *his-3* locus, and the *qa-2* promoter drives expression of the *frq* ORF. In the *frq* null strain, *bd;frq*^{10} [B. Aronson *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 7683 (1994)], 5.3 kb of the *frq* locus has been deleted.
42. General conditions for growth and manipulation of *Neurospora* have been described by R. L. Davis and D. deSerres [*Methods Enzymol.* **27A**, 79 (1970)]. Culture conditions used for the liquid culture experiments are as described (*8, 13*), except that Vogel's salts not Fries salts were used. A light to dark transfer synchronizes the cultures by setting the oscillator to CT12. Circadian time is a formalism whereby clocks from different organisms having different endogenous periodicities can be compared. The circadian day (about 22 hours in length in *Neurospora*) is divided in 24 equal parts, circadian hours. By convention CT0 corresponds to subjective dawn and CT12 to subjective dusk. Light and dark treatments were carried out in controlled environment chambers (Percival) containing fluorescent lamps (Phillips F20T12 CW 20W). Light intensity was measured with a LI-COR LI-189 quantum sensor.
43. *frq*-specific riboprobes were made with a plasmid pKAJ106 containing a 1.806-kb fragment of the *frq* ORF (coordinates 1389–3195) (8). Probes were labeled with NEN [α-^{32}P]dUTP (6000 Ci/mmol) to a specific activity of at least 10^9 cpm/μg. Hybridization was carried out at 65°C, and blots were washed at high stringency (68°C, 0.1× SSC, 0.1% SDS). To detect the *qa-2* transcript, riboprobes were made with a plasmid containing 86 bp of the *qa-2* 5′-UTR (*7*). Blots were hybridized with probe at 58°C and washed at 68°C and 76°C. *ccg-2*-specific riboprobes were made with the plasmid pLW1 (*30*) and hybridization and washing was carried out at 50°C. To control for RNA loading, Northern blots were also probed with randomly primed ribosomal DNA (rDNA); *frq* mRNA signals were normalized to 26S rRNA as described (*13*). Hybridization of blots with rDNA was carried out at 42°C. Blots were washed at 42°C with 0.5× SSC, 0.1% SDS. All Northern blots were exposed to Dupont x-ray film. All Northern blots that were directly compared to establish relative levels of *frq* expression were hybridized together to the same preparation of probe and placed on film at the same time.
44. *Bd* (clock wild-type), *bd*, *wc-1*$^{-}$, and *wc-2*$^{-}$,*bd* were grown on race tubes in constant light. After 2 days of growth the tubes were transferred into constant darkness. After 51 hours in constant darkness, a fraction of the tubes were transferred to a growth chamber at 35°C for 15 hours and then returned to constant darkness at 25°C. Another fraction of tubes were transferred to 4°C for 24 hours at DD48 and then returned to 25°C. Temperature treatments were carried out in the same chambers (35°C) or in a cold room 4°C ± 1°C. Densitometric analysis of race tubes to determine growth patterns and to look for low amplitude rhythms was carried out with NIH Image 1.59.
45. For Western blot analysis, tissue was ground in liquid nitrogen with a mortar and pestle and suspended in ice-cold extraction buffer (50 mM Hepes, pH 7.4, 137 mM KCl, 10% glycerol containing 10 mM diisopropyl-fluorophosphate, 1 mM EDTA, 1 μg/ml pepstatin A, and 1 μg/ml leupeptin) at a ratio of 1 ml of buffer per 0.2 g of tissue (wet mass). Equal amounts of protein (150 μg) per lane were subjected to 7.5% SDS-PAGE, transferred to PVDF membrane (Immobilon-P, Millipore) in 384 mM glycine, 50 mM Tris (pH 8.4), 20% methanol at 400 mA for 2.5 hours and the membrane was then blocked with phosphate-buffered saline (PBS), 5% milk, 0.3% Tween 20. Next, the membrane was probed sequentially with 1:3000 dilutions of antibody to FRQ1-989 and goat antibody to rabbit IgG–horseradish peroxidsae diluted in PBS, 5% milk, 0.3% Tween 20, and the blot was developed by chemiluminescence (ECL, Amersham). X-ray films were scanned with a Silver Scanner III equipped for transparency scanning, and densitometry was performed with NIH Image 1.59. FRQ was normalized against total protein by densitometry of Amido Black stained Western blots.
46. 5×10^4 conidia per milliliter were inoculated into petri dishes containing 30 ml of media (Vogel's salts, 0.1% glucose, 0.17% arginine, and biotin) and left to form mycelial mats at 25°C in LL. Nine-millimeter disks were transferred into 100 ml of fresh media in 125-ml Erlenmeyer flasks and shaken at 150 rpm in LL at 21°C. Addition of the inducer quinic acid (QA) (pH 5.8) and transfer of cultures from LL to DD was staggered so that control and QA-treated samples (from which the inducer had been absent for between 4 and 48 hours) could be harvested within an 8-hour period. QA was added to a final concentration of 10^{-2} M 2 hours before the light to dark transfer; 12 hours after addition of QA, the inducer was washed out by blotting excess media off each disk, transferring each disk to 100 ml fresh media lacking QA and shaking for 15 min. Excess media was again blotted off before placing each disk into 100 ml of fresh media without inducer. Samples were harvested at 4-hour intervals.
47. We thank S. Kay for seminal discussions and members of our laboratories for advice, especially N. Garceau for help on Westerns and D. Bell-Pedersen for critical reading of the manuscript. Supported by grants from the AFOSR (F49620-94-1-0260 to J.J.L.), the National Science Foundation (MCB-9307299 to J.J.L.), the National Institute of Health (GM 34985 and MH01186 to J.C.D. and MH44651 to J.C.D. and J.J.L.), and the Norris Cotton Cancer Center core grant at Dartmouth Medical School.

9 April 1997; accepted 15 April 1997