Negative Feedback Defining a Circadian Clock: Autoregulation of the Clock Gene *frequency*

Benjamin D. Aronson, Keith A. Johnson, Jennifer J. Loros, Jay C. Dunlap*

The *frequency* (*frq*) locus of *Neurospora crassa* was originally identified in searches for loci encoding components of the circadian clock. The *frq* gene is now shown to encode a central component in a molecular feedback loop in which the product of *frq* negatively regulated its own transcript, which resulted in a daily oscillation in the amount of *frq* transcript. Rhythmic messenger RNA expression was essential for overt rhythmicity in the organism and no amount of constitutive expression rescued normal rhythmicity in *frq* loss-of-function mutants. Step reductions in the amount of FRQ-encoding transcript set the clock to a specific and predicted phase. These results establish *frq* as encoding a central component in a circadian oscillator.

Circadian rhythms are widespread phylogenetically (*1*) and are essential for proper temporal regulation of a variety of phenomena, ranging from microbial growth and development (*2*), to plant and animal reproduction, and to human sleep-wake cycles and cognitive function (*3*). The cellular machinery that generates this capability is known as the biological clock. Such clocks are intrinsic to the cell (*2, 4, 5*), are endogenous and self-sustaining, and can be reset in response to time cues such as daily light-dark or temperature cycles (*1*).

The molecular mechanisms that constitute clocks are not well described. Therefore, investigators have searched for clock components in genetically and biochemically manipulable microbial systems. Much effort has focused on dissection of the circadian system of *Neurospora crassa*, a filamentous fungus in which the clock regulates a clearly expressed overt rhythm in developmental potential and morphology (*2, 6*). This rhythmicity is manifest through the cyclical production of asexual spores known as conidia. Genetic approaches to the identification of components of the clock in *N. crassa* have identified seven loci, one of which, *frequency* (*frq*), was repeatedly identified. The *frq* gene is a likely candidate for encoding a central component of the oscillator: strains carrying different *frq* alleles are differentially altered in multiple clock properties, including period length and temperature compensation (the ability to maintain period length over a wide range of physiological temperatures), but are otherwise generally morphologically and developmentally normal. Cloning of the locus (*7*) allowed identification of a long open reading frame (*8, 9*) that is phylogenetically conserved (*8*) and that encodes the protein FRQ, the key transacting product of the *frq* gene (*9, 10*). A frameshift mutation within the open reading frame mimics the deletion of *frq* and results in complete loss of stable rhythmicity and compensation (*9*). These observations are compatible with the inclusion of *frq* in the feedback loop that comprises the clock.

A tenet of chronobiology is that feedback is central to the generation of circadian rhythms (*11*). However, although some biochemical and genetic feedback oscillator will likely be a part of the clock, the simple description of a feedback loop does not constitute proof of its involvement in the clock (*2*). Criteria have thus been established for candidate clock components and oscillators (*12*). Briefly, (i) mutations in the component should affect canonical clock properties and null mutations should abolish normal rhythmicity; (ii) the amount (activity) of the component must oscillate in a self-sustained manner with an appropriate periodicity; (iii) induced changes in the amount (activity) of the component must (by feedback) act to change the amount (activity) of the component; (iv) the phase of the component's oscillation must be reset by shifts in the light-dark growth regimen, and conversely, the overt rhythm must be reset by changes in the amount (activity) of the component; and (v) prevention of the component's oscillation should result in loss of the overt rhythm. In particular, there should be no degree of constitutive expression that will support rhythmicity. Thus, to establish the identity of a clock component, it is necessary to demonstrate that an oscillation in the component is necessary for the overt rhythm, that the activity of the component feeds back to affect its own activity, and that step changes in the components activity set the phase of the clock.

Two candidate clock genes fulfill several of the criteria listed above, *frq* and the *Drosophila* gene *period* (*13*). Although feedback regulation of these genes was suspected, the first reported cycling of a candidate clock component involved the PER protein (*14*), which was then extended to the *per* transcript (*15*). These data establish the potential of feedback, but this was not explicitly shown and the other points have not been established. Particularly, in no case has cycling of a putative clock component been established as necessary for circadian function.

We now show the existence of a molecular feedback loop in which the product of *frq* satisfies all of the criteria expected for a central clock component. The *frq* gene encodes a product that negatively feeds back to regulate its own transcript, resulting in a daily oscillation of *frq* transcript amount with a peak in the subjective morning. Because *frq* and its product function within individual cells or syncytia rather than intercellularly, these results establish *frq* as encoding a central component, a state variable, in a cellular circadian oscillator.

Rhythmic expression of *frq* mRNA. Northern (RNA) analysis was done to examine *frq* mRNA at 4-hour intervals over 48 hours under constant conditions (Fig. 1A). In a clock wild-type genetic background (*16*), the transcript showed a rhythmic accumulation that peaked at 12 to 16 and 32 to 36 hours in the dark. This corresponds to circadian time (CT) 0 to 5, that is, subjective morning (*17*). Conversely, the trough in mRNA abundance was at 0 to 4 hours and again at 24 to 28 hours in the dark (subjective early evening). The period length of the mRNA rhythm was approximately 22 hours, the same period as the *N. crassa* conidiation rhythm (*2*). If *frq* cycling is regulated by the cellular oscillator, then the period length of the *frq* RNA rhythm should reflect the period length of the overt conidiation rhythm. This was shown to be the case when RNA isolated from a frq^7 strain (29-hour period length) was used in an identical RNA hybridization experiment (Fig. 1B). Under the culture conditions used, the amount of *frq* transcript at the peak was about 5 to 10 times the amount at the trough in both frq^+ and frq^7, although expression in frq^7 was reproducibly greater than in frq^+. The amplitude and phase of the RNA rhythm of *frq* was

B. D. Aronson is in the Department of Biochemistry and Cell Biology, State University of New York, at Stony Brook, Stony Brook, NY 11794, USA. K. A. Johnson is in the Department of Biochemistry and Cell Biology, Rice University, Houston, TX 77251, USA. J. J. Loros and J. C. Dunlap are in the Department of Biochemistry, Dartmouth Medical School, Hanover, NH 03755–3844, USA.

*To whom correspondence should be addressed.

similar to that of two other *N. crassa* clock-controlled genes, *ccg*-1 and *ccg*-2 (*18*). Thus, *frq* is a morning-specific clock-controlled gene that acts to control the period length of the clock, and thus the timing of its own expression.

If *frq* RNA cycling is necessary for overt rhythmicity, then mutations in *frq* that result in loss of normal rhythmicity should result in loss of *frq* RNA cycling. This was confirmed with RNA harvested from a strain bearing frq^9, a recessive loss-of-function allele that displays reduced conidiation and no stable circadian rhythmicity (*19*). The pattern of *frq* mRNA expression in this strain was distinct from the frq^+ and frq^7 cases; *frq* RNA did not cycle in the frq^9 background, although the amounts did fluctuate (Fig. 1B) in a manner reminiscent of the unstable conidial rhythms observed in such *frq* null backgrounds (*9, 19*). In frq^9, *frq* mRNA was two to three times more plentiful than the peak amounts in a frq^+ strain (Fig. 1B). The observation that *frq* mRNA in a loss-of-function mutant did not show a stable rhythm and was elevated as compared to the peak of wild-type expression was consistent with a model in which the cycling of the *frq* transcript is the result of negative feedback regulation, in a manner similar to that suggested for the *per* transcript (*14, 15*). Here then, the protein FRQ appears to act directly or indirectly to depress the abundance of its own mRNA, thus creating a feedback loop with a period length of approximately 1 day.

Because *frq* was a central component in this feedback loop, and mutations in *frq* lead to alterations in the circadian clock (*2*), *frq* is a candidate for being or encoding a state variable in the circadian oscillator. However, these and similar data (*15, 20*) are equally compatible with genes such as *frq* providing a necessary support function to a separate oscillator, analogous to an enzyme supplying a critical substrate for a clock protein or a channel required to maintain a necessary intracellular ionic environment. If *frq* is a central component of the clock rather than an auxiliary gene, then normal rhythmic expression should be required for overt rhythmicity. Three predictions follow from this: (i) just as loss-of-function mutations eliminate normal circadian rhythmicity, constitutive elevated expression from a heterologous inducible *frq* gene should eliminate rhythmicity by silencing expression from the endogenous, feedback-regulated copy, thereby short-circuiting the autoregulatory loop; (ii) constitutive *frq* expression, in any amount, should not rescue overt rhythmicity in a *frq*-null strain; (iii) the phase of the rhythmic expression of *frq* must define the phase of the overt rhythm. Thus, in a frq^+ strain bearing a second inducible copy of a FRQ-encoding gene, a step from constitutive elevated inducible *frq* expression to the normal feedback-regulated amount of *frq* mRNA should reset the clock to a phase corresponding to the low point in the normal oscillation. Each of these three predictions was tested.

Requirement for oscillating quantities of *frq* mRNA. The prediction that *frq* RNA cycling, rather than simply its expression, is necessary for proper operation of the circadian clock was tested by fusion of the heterologous, inducible *N. crassa* promoter, *qa*-2 (*21*), to the *frq* open reading frame (Fig. 2A) (*22*). A construct, designated *qa*-2pFRQ, was made in which expression of the FRQ open reading frame was dependent on the presence of an inducer, quinic acid. The *qa*-2pFRQ construct was transformed (*23*) into a frq^+ strain and examined for a conditional quinic acid–dependent phenotype by the race tube assay (Fig. 2B). Nine transformed strains were extensively analyzed (*24*) to test if constitutive expression of FRQ-encoding RNA eliminated overt rhythmicity. All nine were internally consistent: frq^+ strains transformed with the *qa*-2pFRQ construct showed wild-type rhythmicity in the absence of the inducer; however, in the presence of 10^{-5} M quinic acid (Fig. 2B) or greater (up to 10^{-2} M was tested), no rhythmic conidial banding was observed in any of nine independent *qa*-2pFRQ transformants. Instead there was constant conidiation down the length of the race tube. This confirms the first prediction, that constitutive elevated expression of FRQ-encoding RNA should result in arrhythmicity.

The threshold concentration of inducer that was required to observe the constant conidiation phenotype was determined. Complete arrhythmicity of all frq^+(*qa*-2pFRQ) isolates (*24*) (Fig. 2B) was observed at quinate concentrations (10^{-5} M) that induced the heterologous *qa*-2pFRQ transcript to concentrations lower than the trough amounts of endogenous *frq*. The phenotypic response to increasing amounts of inducer is shown for one representative strain, frq^+(*qa*-2p$FRQ^{ec\#10}$) (Fig. 2B). In some cases, at inducer concentrations below 10^{-5} M, several days of growth on the race tube were required before arrhythmic constant conidiation was evident. At inducer concentrations below the threshold value required to observe complete arrhythmicity, the period length and phase of conidial banding was unaffected by the inducer.

To test the second prediction, frq^9 null mutants transformed with the *qa*-2pFRQ construct were examined for inducer-dependent rescue of normal rhythmicity (*23, 24*). Four *his*-3–targeted transformants and four ectopic insertion strains were examined for conditional rhythmicity by serially diluting the inducer over a range of concentrations (10^{-2} M to 10^{-7} M) that spanned the dynamic range of the promoter (*21, 25*); rhythmicity was not restored under these conditions. The four *his*-3–targeted strains (*24*) were then subjected to a more

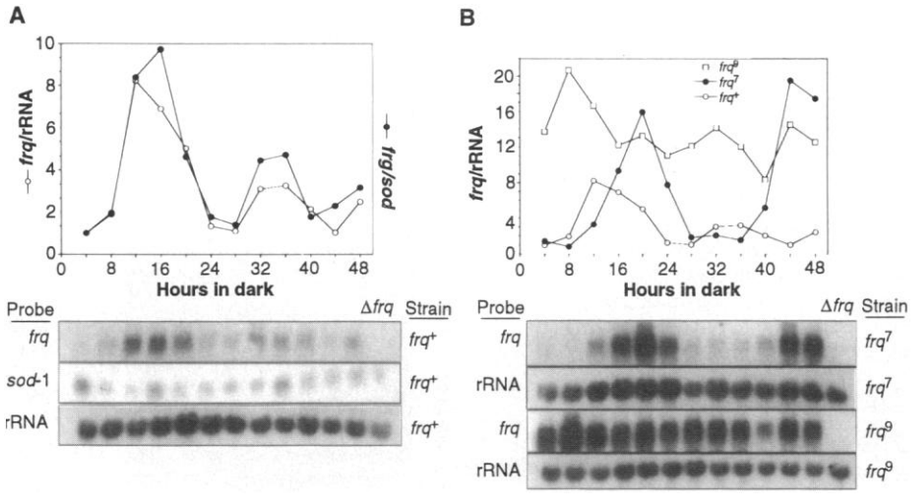

Fig. 1. Circadian clock–dependent expression of *frq* mRNA. Tissue was harvested at 4-hour intervals after transfer from light to dark, total RNA was isolated, and RNA (10 μg) was separated by electrophoresis on formaldehyde-agarose gels and blotted (*18, 30*). (**A**) Northern (RNA) analysis of frq^+ RNA with probes specific for *frq*, *sod*-1 (Cu^{2+}-Zn^{2+} superoxide dismutase), and ribosomal RNA (*45*). Signals were quantitated by densitometry. The frq^+ rhythm was normalized to the constitutively expressed *sod*-1 transcript and to ribosomal RNA. The final lane in each panel, Δ*frq*, is RNA-isolated from a *frq* strain in which the open reading frame was deleted by gene replacement (*9, 46*). (**B**) Expression of the *frq* mRNA in the frq^7 (29-hour mutant strain) and frq^9 (*frq* null strain) genetic background. Northern signals were generated by hybridization with the *frq*-specific RNA probe as in (A), quantitated by densitometry, and normalized to ribosomal RNA. Densitometry of the frq^+ data in (A) is plotted in (B) for ease of comparison.

refined analysis. The inducer concentration was gradually raised in twofold steps, from a basal concentration of 3×10^{-7} M quinic acid to 10^{-5} M; this covered the entire range of inducer in which the conditional phenotype of *frq*$^+$(*qa*-2pFRQ*his*-3) strains was observed. There were no concentrations of inducer at which stable overt rhythmicity was seen (Fig. 2B). These data suggest that the lack of complementation of the *frq*^{9} phenotype by the *qa*-2pFRQ construct results from a lack of rhythmic expression of the mRNA, consistent with the second prediction that rhythmic expression is essential for overt rhythmicity.

Feedback repression by *qa*-2pFRQ. The effect of expression of the inducible *frq* gene on endogenous *frq* mRNA was examined in both a *frq*$^+$ and a *frq*^{9} background (Fig. 3). At 1.5×10^{-2} M quinic acid and 2 percent glucose the inducible transcript was present and expression of the endogenous gene was depressed by approximately 70 percent; this concentration of inducer had no effect on the *frq* mRNA in the *frq*$^+$ control (Fig. 3A). A sharper but similar repression of *frq* mRNA by induction of *qa*-2pFRQ was observed in the *frq*^{9} background (Fig. 3B) although higher concentrations of inducer were needed. The endogenous transcript was not detected at the highest inducing conditions (1.5×10^{-2} M quinic acid, no glucose) in any of the three *frq*^{9}(*qa*-2pFRQ*his*-3) isolates examined. The higher concentrations of inducer necessary to observe the repression of the endogenous *frq* mRNA in the *frq*^{9} background may be related to the higher endogenous *frq* expression in the *frq*^{9} strain (Fig. 1B). Also, unlike the *frq*$^+$ case, there was no contribution to overall functional FRQ concentrations by the endogenous *frq*^{9} gene. Induction of *frq* from a heterologous promoter thus acted to repress expression of the endogenous copy, a finding consistent with the *frq*^{9} rhythmic Northern blot result (Fig. 1B) and in support of the interpretation that *frq* is regulating its own expression. The *frq* mRNA is part of a feedback loop, apparently with the protein FRQ acting in a negative fashion to control the abundance of its own mRNA.

Appropriate conditional production of the *qa*-2pFRQ transcript in the *frq*^{9} background is shown in Fig. 3C; similar results were observed in the *frq*$^+$ background. The production of an inducible transcript at amounts as great as the peak of wild-type *frq* mRNA, a conditional circadian clock phenotype, and repression of the endogenous *frq*^{9} mRNA all show that a functional FRQ-encoding mRNA must be produced by the *qa*-2pFRQ construct in the *frq*^{9} background. Therefore it is most likely that the lack of *frq*$^+$ strain results in an arrhythmic phenotype, but constitutive expression in *frq*^{9} cannot rescue normal rhythmicity. Rhythmicity of strains bearing ectopic insertions of the *qa*-2pFRQ construct was analyzed (*19, 47*) by inoculating strains onto race tubes containing increasing amounts of inducer (quinic acid, QA). Normal rhythmic expression of the experimental strain, *frq*$^+$(*qa*-2pFRQ$^{ec\#10}$), labeled as *frq*$^+$(*qa*-2pFRQ), was observed under conditions where inducer was below 6×10^{-7} M. As the concentration of inducer increased, expression of the overt rhythm of conidiation was gradually lost. Arrhythmic behavior of the strain can be observed at inducer concentrations of 6×10^{-7} M and greater. Control *frq*$^+$ and *frq*^{9} strains, as well as a representative *frq*^{9}(*qa*-2pFRQ) strain, *frq*^{9}(*qa*-2pFRQ*his*-3#6), are also shown under the boundary conditions (no inducer and 10^{-5} M inducer) used in this experiment (*47*).

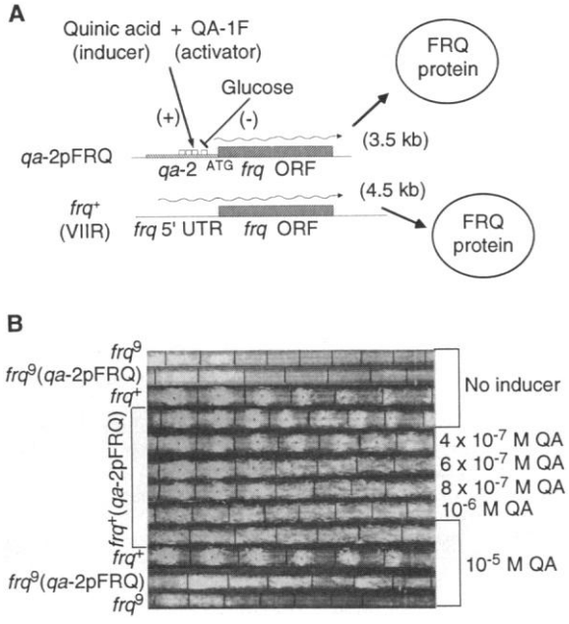

Fig. 2. Expression of FRQ-encoding RNA from the inducible *qa*-2pFRQ construct. (**A**) To generate a strain in which an FRQ-encoding RNA transcript could be both inducible and adjustable, the quinic acid–inducible *N. crassa* promoter, *qa*-2, was positioned in front of the *frq* open reading frame (ORF) (*22*). In the presence of inducer, the *qa*-2 promoter is activated by the protein QA-1F (*21*), which binds DNA elements depicted by small boxes shown in the *qa*-2 promoter region. Here the presence of inducer led to the production of an mRNA product encoding the FRQ protein. This heterologous *frq* mRNA lacked the *frq* 5′ untranslated region (UTR), and its size is 3.5 kb, whereas the endogenous *frq* mRNA is about 4.5 kb. The endogenous feedback-regulated *frq* is located on VIIR. (**B**) Expression of heterologous *frq* mRNA in a

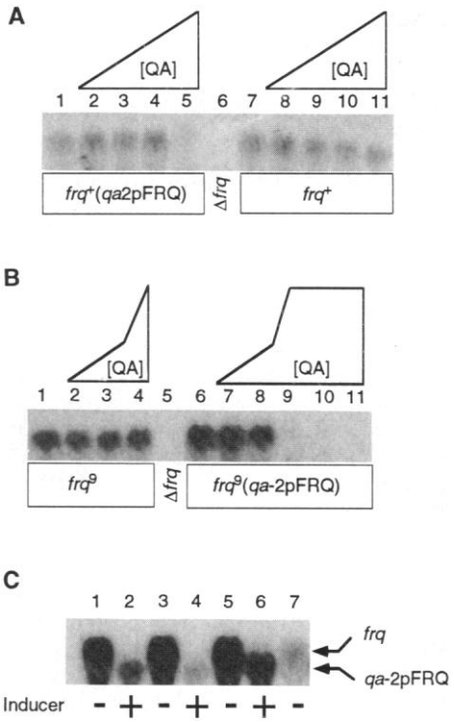

Fig. 3. The amount of endogenous *frq* mRNA is reduced after induction of *qa*-2pFRQ (*48*). (**A**) Repression by *qa*-2pFRQ of endogenous *frq*$^+$ mRNA. RNA from *frq*$^+$(*qa*-2pFRQ$^{ec\#10}$) was in lanes 1 to 5; RNA from Δ*frq*, a *frq* deletion strain (*frq*^{10}) (*9*) grown in the absence of inducer, is in lane 6; and RNA from a control *frq*$^+$ strain is in lanes 7 to 11. Northern hybridizations were done with a riboprobe that detects endogenous *frq* mRNA, but not *qa*-2pFRQ mRNA (*48*). Inducer concentrations were as follows: Lanes 1 and 7, no inducer; lanes 2 and 8, 3×10^{-6} M; lanes 3 and 9, 10^{-5} M; lanes 4 and 10, 3×10^{-5} M; lanes 5 and 11, 1.5×10^{-2} M. The *frq*$^+$(*qa*-2pFRQ) strain used in this experiment is the same isolate used in Figs. 2 and 4. (**B**) Repression by *qa*-2pFRQ of the endogenous *frq*^{9} mRNA. RNA from strains grown in the presence of increasing inducer concentration was separated and hybridized with the same probe as in (A). Lanes 1 to 4, *frq*^{9}; lane 5, the *frq* deletion strain Δ*frq*; lanes 6 to 9, *frq*^{9}(*qa*-2pFRQ*his*-3-6); lanes 10 and 11, two additional isolates of *frq*^{9}(*qa*-2pFRQ*his*-3), numbers 1 and 4. All cultures, with the exception of lanes 4 and 9 to 11, were grown in 2.0 percent glucose; inducer concentrations were: lanes 1 and 6, no inducer; lanes 2 and 7, 3×10^{-5} M; lanes 3 and 8, 1.5×10^{-2} M; lanes 4 and 9 to 11, 1.5×10^{-2} M (no glucose). The *frq*^{9}(*qa*-2pFRQ*his*-3#6) isolate in lanes 6 to 9 is the same strain as in Fig. 2B. (**C**) Expression of *qa*-2pFRQ mRNA. Cultures were grown the same as in (A). The RNA was harvested from uninduced and induced (1.5×10^{-2} M quinic acid, no glucose) cultures of three isolates of *frq*^{9}(*qa*-2pFRQ*his*-3) and hybridized with an RNA probe that detected both endogenous and *qa*-2pFRQ mRNA: Isolate number 1, uninduced (lane 1) and induced (lane 2); isolate number 4 uninduced (lane 3) and induced (lane 4); isolate number 6, uninduced (lane 5) and induced (lane 6); *frq*$^+$(*qa*-2pFRQ*his*-3) uninduced (lane 7).

complementation of the *frq*^{9} phenotype by *qa*-2pFRQ was the result of expression that is not subject to feedback regulation; these data confirm the second prediction.

Setting the phase of the rhythm with step changes of inducer. The third prediction of the feedback model was that, in a *frq*$^+$ strain bearing an inducible copy of *qa*-2pFRQ, a step from constitutive induced *frq* expression to the normal, feedback-regulated amounts, should reset the phase of the clock. The new phase established at the time of inducer withdrawal should correspond to the low point in the normal *frq* mRNA oscillation (CT 12, subjective evening). Additionally, it was important to eliminate the possibility that the elevated expression of *frq* was simply inducing conidiation, thereby masking the normal expression of the clock. To test the third prediction and to rule out the masking possibility, liquid culture experiments (*26*) were done. This enabled the addition and subsequent withdrawal of the inducer from the culture under conditions in which the clock could be monitored (Fig. 4). The experimental genotype and condition was *frq*$^+$(*qa*-2pFRQ) grown in medium containing quinic acid. The controls were *frq*$^+$ grown in the presence or absence of quinic acid and *frq*$^+$(*qa*-2pFRQ) grown in the absence of quinic acid. Groups of all four cultures were transferred from light to dark at successive 5-hour intervals, and then all of the cultures were washed in fresh medium to remove the inducer and transferred to race tubes to measure the phase of the rhythm (*26*, *27*). The phase of control cultures [*frq*$^+$ strain with and without inducer as well as the *frq*$^+$(*qa*-2pFRQ) strain with no inducer] was set by the light to dark transition, as shown by the 5-hour staggered phasing of the banding of the successive light-dark cultures (Fig. 4A). The final phase of the inducible *frq* strain, however, was not set by light. Instead, all of these cultures, regardless of the time of light to dark transfer, recovered from the quinic acid treatment in synchrony at an identical phase, the inducer release–driven phase (Fig. 4B). This shows that the withdrawal of inducer from the media, not the light to dark transition, dictated the phase of the rhythm in the experimental strain; this would not be the case if elevated *frq* was simply acting to mask the normal rhythm. Because the center of a conidial band occurs at approximately CT 0 (*28*), the phase of the rhythm at the time of quinic acid release could be calculated in independent experiments to fall between CT 9 and CT 11.5 (subjective evening), close to the observed minimum in the normal *frq* oscillation, and at the phase predicted by a feedback model. Thus, induction of the *qa*-2pFRQ gene fusion strongly affected the phase of the rhythm; the phase was fully set by the withdrawal of the inducer, rather than the light-dark transfer. The phase was set to the early evening hours, similar to the phase established by a light to dark transfer. These data confirm the third prediction.

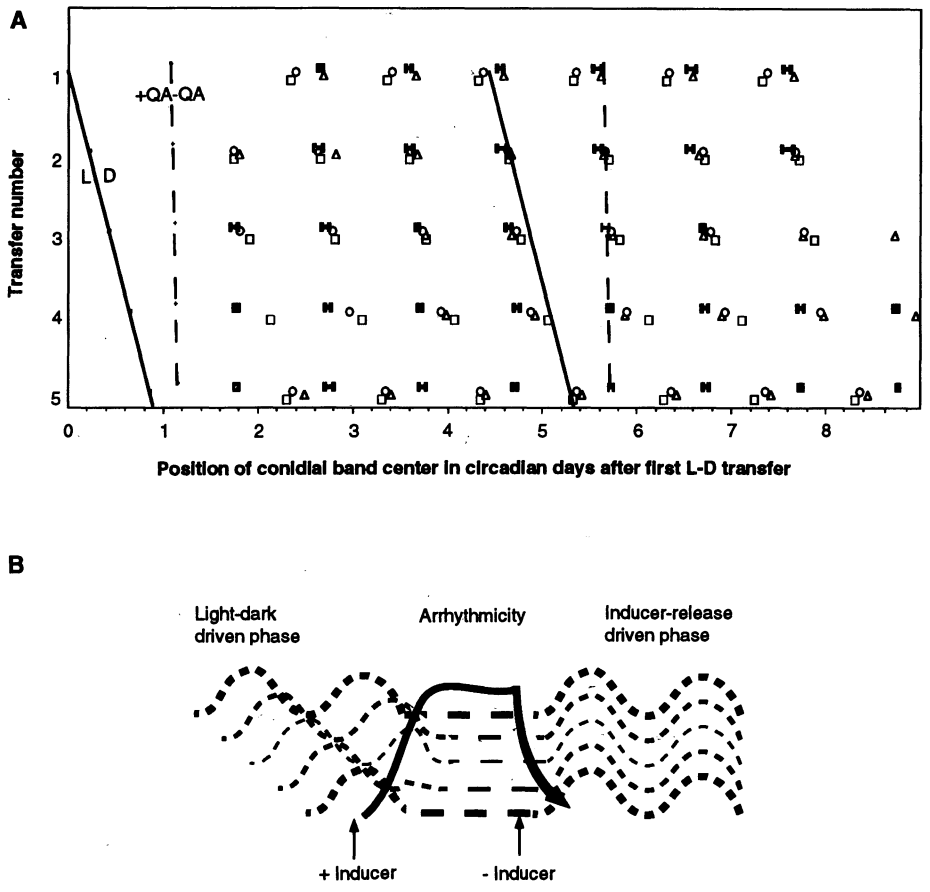

Fig. 4. Step decreases in the concentration of *qa*-2pFRQ inducer completely reset the phase of the rhythm. (**A**) After growth in the presence or absence of inducer as appropriate, mycelial pads were transferred from light to dark (L to D) to set the clock and then inoculated onto race tubes to monitor the expression of the circadian conidial banding rhythm (*49*). The times (in circadian days) of the centers of the conidial bands are plotted against the transfer number where the transfer number describes a set of cultures transferred from L to D at the same time (transfer 1 = first L to D transfer, transfer 5 = last L to D transfer). The negatively sloped solid line plots the expected center of the conidial bands of the cultures if the L to D transfer is setting the phase to subjective dusk (CT 12) as expected (below). The dashed line marks the time of quinic acid withdrawal (+QA, −QA) and parallels the expected phase of banding if the step-down of QA has reset the phase of the rhythm. Strains and conditions used were: □, *frq*$^+$ with no QA; △, *frq*$^+$(*qa*-2pFRQ) with no QA; ○, *frq*$^+$ with 10^{-4} M QA; and ■, *frq*$^+$(*qa*-2pFRQ) with 10^{-4} M QA, where the distance between the bars of the ■ represents ±1 standard deviation. Error bars are not shown for the control strains for the sake of clarity, but in all cases were less than or equal to those of the experimental strain. (**B**) Schematic of the phase setting effects of a step decrease in FRQ inducer. Because the L to D transition sets the phase of the rhythm, the five groups of light to dark transfers will have 5-hour staggered phases. However, the addition of inducer results in an increase in ectopic FRQ-encoding transcript (solid black line). This acts to repress the endogenous *frq* rhythm and maintain constitutive and low amounts of endogenous *frq* mRNA. Release from the inducer allows re-initiation of the oscillation in the amount of *frq* transcript, beginning from the low point in the endogenous *frq* cycle, which corresponds to subjective evening (Fig. 1).

Clock components, feedback cycles, and circadian rhythms. We have established that *frq* RNA and, by extension, FRQ lie within the feedback loop comprising the circadian oscillator. The amount of *frq* transcript in the cell is regulated by the clock, *frq* mRNA encodes FRQ, and point mutations in FRQ set the period of the clock; *frq* must therefore determine the timing of its own expression through regulation of transcript synthesis or turnover. The characteristic loss of stable rhythmicity in *frq* loss-of-function mutations shows that this gene is critical for circadian rhythmicity, and the observation of high amounts of transcript in *frq*^{9} suggests autoregulation by negative feedback. We found that elevated expression of *qa*-2pFRQ RNA depressed the amount of *frq* transcript, consistent with the operation of negative feedback. These

characteristics, however, could equally apply to a gene or gene product that is feedback-regulated independent of the clock and acts to supply a critical substrate for the (separate) circadian oscillator, or to maintain an intracellular milieu compatible with rhythmicity (*29*). To resolve this issue, it was additionally determined that rhythmic expression of *frq*, rather than simple constitutive expression, was essential for rhythmicity; this result is inconsistent with *frq* playing a support role. Finally, it was shown that step reductions in *qa*-2pFRQ expression were sufficient to set the phase of the circadian clock to a unique point corresponding to subjective dusk, the point predicted for a circadian oscillator bearing *frq* transcript as a component. Collectively these data satisfy the essential requirements needed to establish that a putative clock factor is a bona fide component, a state variable, of a circadian oscillator.

Increasing amounts of *frq* mRNA in the frq^+, frq^7, and frq^9 allelic series is consistent with the proposal that long period length mutants are hypomorphic (*9*). Thus, one function of *frq* is to regulate its own expression. Loss of this function results in runaway expression of *frq* mRNA as shown in the frq^9 case. From this perspective, the frq^7 mutation would result in a protein with partial loss of this autoregulatory function. More mRNA must accumulate before the requisite amount of protein activity is produced for autoregulation, resulting in a longer cycle (29 hours instead of 21.5 hours).

The phase of peak *frq* mRNA occurs at CT 0 to 5, which corresponds to subjective early morning. This coincides with peak mRNA amounts of the morning-specific clock-controlled genes targeted for regulation by the clock: *ccg*-1, *ccg*-2 (*18, 30*), *ccg*-4 (*31*), and *ccg*-7 (*32*). It is not clear whether FRQ is acting directly or indirectly (through the clock) to regulate these clock-controlled genes. The similarity of peak times of mRNA accumulation is consistent with rapid translation of *frq* mRNA and the subsequent direct action of FRQ upon expression of the clock-controlled genes. Because the products of several morning-specific *ccg*'s have been associated with the conidiation process (*31, 33*), a developmental pattern regulated by the clock, the observation that constitutive elevated expression of *frq* tended to promote more conidiation (Fig. 2) is consistent with a direct role of FRQ in activating these genes. Alternatively, the near coincidence of the peaks of *frq* and *ccg* expression could reflect the simultaneous action of other factors that promote the expression of both *frq* and the clock-controlled genes.

The time of day at which steady-state amounts of *frq* mRNA reach a peak coincides with the phase at which the *N. crassa* clock shows maximum sensitivity to inhibitors of transcription and translation (*27, 34, 35*). This suggests that it may be the RNA and protein products of *frq* that are the direct targets of these clock-resetting agents and that the generalized resetting effects of chemicals such as these (*36*) may be visualized in terms of their effects in disrupting a feedback loop. Two effects seen at low *frq* induction in the frq^+ background, the gradual loss of rhythmicity observed during continuous exposure to low concentrations of inducer and the lack of period effects, remain unexplained. It may be that FRQ is quite unstable and requires more than a day to reach an effective concentration. Although effects on the period might have been expected based on *per* gene dosage studies in *Drosophila* (*37*), *N. crassa* heterokaryons carrying a nuclear ratio of 10 percent frq^+ and 90 percent frq^9 have normal period lengths (*19*). Generally, dosage effects on period length are not required by all multicomponent mathematical models describing temperature-compensated oscillators.

On the basis of our data on the regulation of *frq*, we propose a model of the feedback oscillator generating the *N. crassa* circadian rhythm (Fig. 5) in which *frq* transcript and its product are envisioned as components, state variables, of the clock. The clock is functional in single germinated conidiospores on both solid and liquid media (*38*) and is thus a product of intracellular regulation. The *frq* gene and product have multiple roles; although autoregulation of *frq* transcript is seen as central to the clock mechanism and our data confirm the importance of transcript cycling for the rhythm, they do not yet prove that this cycling is derived solely through regulation of the *frq* promoter as suggested in Fig. 5. In no case is the actual number of regulatory steps within the clock or from the clock to a controlled process known; specifically the existence of other state variables within the clock is anticipated (*39*). Although all transcriptionally regulated clock-controlled genes will possess circadian clock responsive elements (CCRE's) (Fig. 5) (*2, 5*), these DNA elements may be different for different genes activated at different times. Several of the clock-regulated genes are also regulated by light, both acutely and indirectly through light regulation of the clock (*4, 31, 33, 40*).

The feedback loop defined here is distinguishable from one involving the *Drosophila* gene *period* (*15, 41*). First, although *per* mRNA oscillates with a periodicity regulated by the clock, its peak in expression is in the early evening, roughly antiphase to that seen here for *frq*. Second, the negative feedback of clock protein-encoding RNA on the amount of clock gene transcript that was shown explicitly here for *frq* and FRQ has only been inferred for *per* (*41*), whereas the clock protein rhythm known for PER (*14*) has only been inferred for FRQ. Third, the feedback regulation of FRQ (either transcriptional or post-transcriptional) that results in the cycling of *frq* transcript is seen here as central to the oscillator. Production of PER from a heat shock promoter under constant elevated temperature is sufficient to complement a *per* null mutation (*42*); these and other data have suggested that rhythmicity in transcript concentration may not be required for rhythmicity. However, the protein coding region of the *per* gene may carry DNA elements sufficient to direct rhythmicity in its transcript (*43*). If so, it may be that expression of PER from a heat shock promoter is not constitutive, but rhythmic after all, a finding that would serve to reemphasize the importance of the RNA transcript oscillations here shown to be essential for the *Neurospora* clock. Last, because rhythms in *N. crassa* are seen in dilute freshly germinated spore cultures and in undifferentiated syncytia (*2, 38*), the *frq* feedback loop is intracellular, whereas it has not yet been possible to determine whether the proposed *per*-associated loop is completely contained within individual cells.

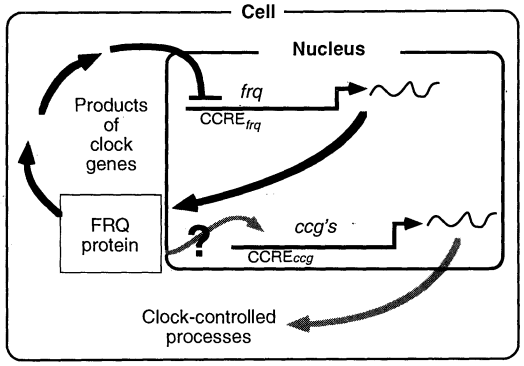

Fig. 5. A schematic diagram of a cell expressing a circadian rhythm. The *frq* gene encodes the FRQ protein that has multiple roles, of which the best established is to regulate, probably through intermediates, the amount of *frq* mRNA as a part of the negative feedback loop that constitutes the circadian oscillator. The other role is to activate (perhaps directly or more probably indirectly through other clock components) genes such as the clock-controlled genes (*ccg*'s) that are transcriptionally regulated (*30*), and other processes required for the overt expression of the rhythm. CCRE, the circadian clock responsive element or "clock box" (*2, 5*), is the DNA element that mediates the action of clock-regulated transcription factors on clock-controlled genes; these are associated with the *ccg*'s, inferred for *frq*, and may be different for different genes.

Despite these distinctions, however, several important common features describing circadian rhythms and clock genes remain. Both of the genetically pertinent clock genes, *frq* and *per*, are self-regulating in terms of their transcripts, and in the case of *frq* we have shown that this self-regulation naturally follows from the position of FRQ within the loop comprising the clock. In *frq* and *per*, these rhythms in transcript cycling are associated with, and at least for *frq*, essential for, the overt rhythms. Other regulation, post-transcriptional and translational (*44*), will surely be required for the assembly of a compensated, entrainable clock, as these transcripts are turned into trans-active proteins that are, in time, turned over. These transcript and protein cycles provide a ready explanation for the universal effectiveness of transcriptional and translational inhibitors in clock resetting. This universality, in turn, suggests that the clock-gene transcript and protein cycling seen and implied here may be a universal feature of circadian oscillators.

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16. The standard clock wild-type genetic background used in all experiments includes the *band* (*bd*) mutation that permits clear observation of the conidial banding phenotype. This mutation has no effect on the clock itself.
17. The concept of circadian time (abbreviated CT) has been formulated to allow circadian pacemakers with different endogenous periodicities to be compared. Biological time is thus normalized by dividing the circadian cycle into 24 equal parts, each a circadian hour. By convention, CT 0 is subjective dawn and CT 12 corresponds to the end of the light period in a 12:12 light:dark cycle or to the time of transfer from constant light to constant darkness. CT 6 is thus always mid–circadian day and CT 18 is midnight.
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22. An exact fusion between the *qa*-2 promoter and the *frq* open reading frame was created by polymerase chain reaction (PCR) [R. M. Horton, H. D. Hunt, S. N. Ho, J. K. Pullen, L. R. Pease, *Gene* **77**, 61 (1989)] such that the nucleotide sequence that was downstream of the initiating methionine residue was from *frq* and the sequences upstream belonged to *qa*-2. Oligonucleotides were used to amplify the *qa*-2 promoter and 5′ untranslated region: BDA14, **CAT**TGTGTTTGGTACCTCTG (*qa*-2 ATG, coordinates +3 to −16); and BDA15, GCTCTAGAGTCCTTGACAAGCAGT, *qa*-2 upstream, coordinates −1131 to −1112, and a small region of the *frq* open reading frame; BDA16, GTACCAAACACA**ATG**GCGGATAGTGGGGATAA, *frq* ATG with *qa*-2 overlap, coordinates +1 to +20; and BDA17, CTCCTCTGGGATGTCGATTC, *frq* COOH-terminal, coordinates 641 to 660. The bold sequence corresponds to the initiating methionine of either *qa*-2 (BDA14) or *frq* (BDA16). All coordinates are listed using the A of the initiating ATG as +1. The primer at the *frq* ATG was designed to include 12 bp of the *qa*-2 promoter region, providing overlap for the second round of PCR. The spliced product from the second round of PCR was then subcloned into pSKII⁻ (Stratagene) and further subcloned into a vector carrying the entire *frq* open reading frame. This clone, designated pBA40, has the entire *frq* open reading frame under *qa*-2 promoter control. The pBA40 insert was further subcloned into vector pDE3 (*23*), compatible with targeted transformations at the *his*-3 locus, and designated pBA50. All regions of pBA40 and pBA50 that had been subjected to PCR were sequenced. A single base pair alteration (C to T at coordinate 2249) in the *qa*-2 promoter was detected; this coincided with the most distal and weakest of the four *qa*-1F binding sites. This alteration is predicted to have no effect on quinic acid induction because in the wild-type case this position is nonconsensus. The inducer, quinic acid, is a well characterized utilizable carbon source for wild-type *N. crassa*. In the presence of glucose, quinic acid can be considered nutritionally gratuitous and at the concentrations used here its only effects should be submaximal induction of the elements of the quinic acid metabolic pathway as well as the *qa*-2pFRQ. The *qa*-2 promoter can be induced up to 500 times by growth in the presence of quinic acid (*21*); however, because the promoter is catabolite-repressed, induction of only 25 to 50 times basal amounts are achieved when both glucose and quinic acid are present (*21*). All experiments were done in the presence of glucose unless otherwise noted.
23. In *N. crassa*, transforming DNA can be introduced at random ectopic sites in the genome [S. J. Vollmer and C. Yanofsky, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 4869 (1986)] or targeted to the *his*-3 locus [D. Ebbole, *Fungal Genet. Newsl.* **37**, 15 (1990)] in order to eliminate chromosomal positioning effects. Transformants of both types were used here. The formal strain designations for transformants in this study are: *bd*; (*qa*-2pFRQ)$^{ec}_{pBA40}$ for random ectopic insertions of the *qa*-2pFRQ construct into a *frq*$^+$ strain, or *bd*;*his*-3(*his*-3$^+$ *qa*-2pFRQ)$^{hom\ pBA50}$ for *his*-3–targeted insertions. For convenience, a shorthand notation is used. The ectopic strains are designated *frq*$^+$(*qa*-2pFRQ^{ec}) and *his*-3–targeted strains are designated *frq*$^+$(*qa*-2pFRQ*his*-3). Similarly the formal *frq*^{9} designations are: *bd*;*frq*^{9};(*qa*-2pFRQ)$^{ec}_{pBA40}$ and *bd*;*frq*^{9};*his*-3(*his*-3$^+$ *qa*-2pFRQ)$^{hom\ pBA50}$ but the shorthand notation is *frq*^{9}(*qa*-2pFRQ^{ec}) and *frq* ^{9}(*qa*-2pFRQ*his*-3) respectively. Where appropriate, individual transformant strains are identified by an isolate number that follows the name.
24. Nine *frq*$^+$(*qa*-2pFRQ) isolates were extensively studied; five of these were ectopic insertions and four were *his*-3–targeted. The pBA40 construct was introduced into *N. crassa* by cotransformation with a Bml^R plasmid pSV50. These ectopic Bml^R primary pBA40 transformants were analyzed on race tubes in the absence of inducer (QA) as well as the presence of 10^{-2} M QA. In the case of *frq*$^+$ recipients, the conditional loss of rhythmicity was apparent and occurred in 11 of 80 Bml^R transformants. For targeted insertions, the pBA50 construct was introduced into the *his*-3 *N. crassa* recipient and transformants were selected for histidine prototrophy. Of 11 *frq*$^+$ recipients that were transformed to His$^+$, six had the conditional arrhythmic phenotype, and all six were shown by Southern (DNA) analysis to carry a single copy of pBA50, appropriately targeted by insertion at *his*-3. One His$^+$ transformant examined that did not display a conditional phenotype was shown by Southern analysis not to carry transforming DNA, and the other five transformants that did not display the conditional phenotype were not further characterized. The *frq*^{9}(*qa*-2pFRQ^{ec}) candidates were screened by PCR for the presence of the *qa*-2pFRQ gene. His$^+$ *frq*^{9} transformants were directly tested by Southern analysis and 6 of 10 showed appropriately targeted transforming DNA; four correctly targeted isolates were chosen for extensive phenotypic analysis. Specifically, all four of the *his*-3–targeted *frq*^{9} strains used in attempts to rescue rhythmicity through constitutive expression of a FRQ-encoding RNA were shown to have a properly targeted *qa*-2pFRQ construct, and of the three examined by Northern analysis, all produced high amounts of inducible *qa*-2pFRQ mRNA.
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the point predicted if *frq* and FRQ were the only state variables, these data do not support a contention that feedback between *frq* and FRQ exclusively comprises the clock. Rather we expect that there are additional state variables yet to be discovered, but this raises the issue of whether the inducer release–driven phase was predictable a priori, because the final phase of the clock after release would also depend on the values of the other state variables at the time the oscillation was reinitiated. Despite this caveat, the fact that the phase was predictable is noted, and we suggest the following interpretation for our data. The extremely strong clock resetting stimulus represented by the high concentration of FRQ may artificially drive not only *frq* and FRQ, but the other state variables as well, to unique phase points they might assume when *frq* is fixed at its low point in the normal cycle. The analogy can be drawn to resetting by light. A short pulse of light minutes in length will reset (advance or delay) the clock toward the daytime part of the cycle, no matter where in the cycle the clock is when light is perceived. However, the clock is driven not to any one point in the day, but rather to phases lying broadly within the day—a span of some hours—the final phase depending on where in the cycle the clock was before the pulse. A long pulse of light (over 12 hours), though, will appear to drive the clock to a unique phase point, so that upon transfer to darkness, the clock will begin from subjective dusk (a span of about 1 hour), regardless of where the clock was when the long pulse of light began. In the present case, the "pulse" of FRQ was more than 12 hours long so it may be that FRQ is driving all of the state variables rather than just *frq*, in a manner analogous to the action of light.

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47. Race tubes were layered with glucose-arginine media (*19*) supplemented with the indicated amounts of quinic acid. Media was titrated to pH 6.0 after addition of quinic acid. Strains were inoculated at the left end (as shown) of the tubes with conidia from 4- to 7-day-old cultures. The inoculum was grown in constant light for about 24 hours and the tubes were then moved to constant dark conditions (25°C). In Fig. 2B the first hash mark represents the time at which the tubes were transferred from light to dark, and subsequent hash marks were placed at 24-hour intervals thereafter. White regions of the race tube represent areas of dense conidiation. Race tube analysis from one representative *frq*^{9}(*qa*-2pFRQ) transformant at two concentrations of inducer are shown in Fig. 2B, and results from other concentrations and other strains showed an identical lack of overt rhythmicity. Although recovery of the rhythm was not observed at any inducer concentration in any *frq*^{9}(*qa*-2pFRQ*his*-3) strain, the elevated expression of *frq* in these strains was not without effect on the pleiotropic *frq*^{9} phenotype. There appeared to be steadier and more robust conidiation in these strains (but not in nontransformed controls) when quinic acid was present. The *frq*$^{+}$ control strain showed no evidence of arrhythmicity even at inducer concentrations of 1.5×10^{-2} M.
48. Mycelial cultures from appropriate strains were grown in 2.0% glucose (except where noted), 1× Vogel's salts, plus various concentrations of inducer. After 15 hours of induction (where appropriate) and 12 hours in the dark (subjective morning, CT 1), the cultures were harvested, RNA was isolated and Northern (RNA) analysis was done as in Fig. 1. Because of catabolite repression or inducer exclusion, when cultures were grown in 2.0% glucose, induction of the *qa*-2pFRQ transcript was limited (*21, 22*). One of two different riboprobes was used for hybridizations. The riboprobe that detects endogenous *frq* mRNA, but not *qa*-2pFRQ mRNA, was derived from an Eco RI–Bgl II fragment (coordinates −18 to −1371) that lies entirely within the 5′ untranslated region and that was therefore absent from the *qa*-2pFRQ construct. The riboprobe that detected both endogenous *frq* transcript and the *qa*-2pFRQ transcript was the same probe used in Fig. 1 (*43*).
49. Conidia from the experimental *frq*$^{+}$(*qa*-2pFRQ) and control *frq*$^{+}$ or *frq*$^{+}$(*qa*-2pFRQ) strains were inoculated into 96-well microtiter plates at a concentration of 5×10^{4} conidia per milliliter in 0.1 percent glucose, 0.17 percent arginine, either with 10^{-4} M quinic acid or without quinic acid, and then grown for 36 hours in constant light. Mycelial pads (seven for each light to dark transfer) were taken from the microtiter wells and placed in 75 ml of fresh medium in a 15 by 150 petri dish, and the first transfer of samples was made from light to dark (t = 0). For each strain and experimental condition, replicate samples were then transferred at successive 5-hour intervals (a total of five light to dark transfers), thus spanning approximately one circadian cycle. Three hours after the last light to dark transfer, the pads were blotted of excess media and were washed in 75 ml of 0.1 percent glucose, 0.17 percent arginine, 1× Vogel's salts (pH 6.0) [R. Davis and D. deSerres, *Methods Enzymol.* **27A**, 79 (1970)]. After blotting excess wash media, the pads were inoculated onto race tubes containing the same media used to wash the pads plus 1.5 percent agar. The sidereal time of the center of conidial bands was determined with an automated program (*7*) and was then normalized to circadian days to adjust for small differences in period length (τ) [in this experiment, τ of *frq*$^{+}$(*qa*-2pFRQ) = 23.2 and τ of *frq*$^{+}$ strain = 23.0].

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