Dissection of a circadian oscillation into discrete domains

(frequency/Neurospora/reconstruction/clock/quinate)

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Abstract The circadian oscillator in Neurospora is a negative feedback loop involving as principal players the products of the frequency (frq) locus. frq encodes multiple forms of its protein product FRQ, which act to depress the amounts of frq transcript. In this scheme there are two discrete and separable steps to the circadian cycle, negative feedback itself (repression) in which FRQ acts to decrease the levels of its own transcript, and recovery from repression (derepression) in which frq transcript levels return to peak amounts. By introducing an exogenously regulatable frq transgene into a frq loss-of-function strain (frq0), we created an artificial system in which the two separate steps in the circadian cycle can be initiated and followed separately for purposes of observing their kinetics. Under these conditions the frq-FRQ cycle occupies the time scale of a full circadian cycle. During this time, the process of negative feedback of FRQ on frq transcript levels is rapid and efficient; it requires only 3 to 6 h and can be mediated by on the order of 10 molecules of FRQ per nucleus, a level even less than that seen in the normal oscillation. In contrast, recovery from negative feedback requires 14 to 18 h, most of the circadian cycle, during which time de novo FRQ synthesis has stopped, and existing FRQ is progressively posttranslationally modified. Altogether the time required to complete both of these steps is in good agreement with the 22-h observed period length of the normal circadian cycle.

Circadian rhythms regulate the timing of a multitude of biological processes ranging from development to photosynthesis to vertebrate behavior (1). By definition their observable characteristics occur once per 24 h in typical environmental conditions, and approximately that frequently when organisms are held in nonchanging conditions. Circadian rhythms have been documented in many eukaryotes and more recently in prokaryotic species (2, 3). Despite their widespread occurrence, the precise mechanisms that result in sustained overt rhythms in constant conditions are just beginning to be resolved. To this end, circadian rhythms and their characteristics have been extensively analyzed to identify properties of an endogenous intracellular oscillator. Recent advances in describing the molecular hallmarks of circadian oscillations in Neurospora and Drosophila (4–6) make possible the design of new molecular-based experimental approaches for solving these problems.

In Neurospora crassa the circadian rhythm is most often characterized by following the accumulation of asexual spores, or conidia, into so-called conidial bands (7). Band formation persists in constant darkness (DD) with a periodicity of about 22 h. The appearance of the first band after the transfer from constant light (LL) to DD appears at a time corresponding to subjective dawn (circadian time 0 or CT0) at approximately 12 h into the DD incubation. Two genes that control the period length of the conidiation rhythm have been molecularly isolated, frequency (frq) and period-4 (prd-4) (3, 8), and there are seven distinct frq alleles giving rise to long-period, short-period, and arrhythmic phenotypes (2). The molecular characteristics of frq expression have been documented and allow classification of the abundance of frq products as state variables of the Neurospora circadian oscillator. Namely, the frq transcript is expressed rhythmically with a period length reflecting that of the overt rhythm (5), and this rhythmic expression is essential for the overt rhythm. Induced expression of frq at a constant elevated level results in disruption of rhythmicity in a normally rhythmic (frq0) strain and fails to support rhythmicity in a conditionally arrhythmic (frq0) strain. In addition, induction of the frq gene from a heterologous promoter results in depression of the amount of transcript arising from the endogenous frq locus, thereby placing frq RNA(s) and FRQ protein(s) within a negative feedback loop. An additional and universal characteristic of a circadian program is its responsiveness to light, and recent experiments indicate that frq is intimately connected to the signal transduction pathway that regulates entrainment of the circadian rhythm by light (9). Specifically, frq mRNA rises dramatically in abundance after exposure of mycelia to light, providing a mechanism for light-induced resetting of the clock (9). Induction of frq by this method, as well as by induced expression (5), sets the phase of the clock to a specific time of day. Collectively, these data place frq at the heart of the Neurospora circadian program and make it a good candidate for use in studying the kinetics of various aspects of a circadian oscillation.

A single Neurospora circadian cycle as described here consists of at least two separable events. The first is “repression,” wherein the frq gene product exerts negative feedback, either directly or indirectly, and frq mRNA concentrations fall to the low levels typically seen at night. The second set of events describe “derepression,” or release from negative feedback, and represent the reaccumulation of frq mRNA to levels characteristic of day time. The sequential effect of these two events can be schematically represented in graphic form as approximating the circadian cycle (Fig. 1A).

To begin to understand and eventually to model a single cycle it is important to know whether the frq-FRQ cycle occupies a circadian time frame or whether undescribed elements must be present to fill out the circadian day. It is further essential to be able to assign meaningful kinetic constraints to both the repression and derepression steps; that is, specifically, to see how long it takes for frq mRNA to yield FRQ protein(s) and to lower frq transcript levels, and independently, when frq expression is turned off and FRQ decays away, how long it takes for frq levels once again to return to their peak. These steps can be best described independently.
accumulate. Endogenous frq from h. Mycelia are washed extensively to remove QA, and cultures are defined point in time, as indicated by time frame of a wild-type circadian period, with either method we have reconstituted a cycle that approximates the should represent an isolated single cycle of an oscillation and not be followed. Addition of these two aspects of an oscillation—negative feedback and release from negative feedback—be similar induced from qa-2frq, but in a frq+ background: frq+qa-2frq [bd;frq+ A; his-3(his-3 qa-2frq)hom pBA50#6], frq+ [bd;frq+ A] was used as a control strain. For race tube experiments, where the effect of induced frq expression on overt rhythmicity was assayed, frq+ was similarly induced from qa-2frq, but in a frq+ background: frq+qa-2frq [bd;frq+ A; his-3(his-3 qa-2frq)hom pBA50#1] was induced with QA. The frq+ pDE3ΔBH control strain for these experiments [bd;frq+ A; his-3(his-3’DBH)hom pDE3ΔBH#1] contained the related but non-FRQ-expressing plasmid pDE3ΔBH (5) similarly integrated at the his-3 locus.

Culture Methods. In experiments where mycelial pads were grown in liquid culture (repression and derepression), approximately 5 × 10⁶ conidia/ml from 7- to 10-day-old slants were germinated in 2% glucose in 1× Vogel’s salts (13), 0.5% L-arginine, and 5 mg/liter biotin. After incubation at 30°C for 36 to 48 h the mycelial mats were cut into disks 1 cm in diameter, distributed to flasks, and shaken at 100 rpm at 25°C. For experiments in which the process of repression was followed, 4–5 disks were suspended in 50 ml of 0% glucose in 1× Vogel’s salts, 0.17% L-arginine, and 5 mg/liter biotin, kept in LL for 2 h and transferred to DD for 12 h. QA was added at staggered intervals such that when all of the disks were harvested at DD12, QA had been present in the cultures for various amounts of time. Four flasks (16–20 disks) were collected for each time point. For derepression experiments, 25–50 disks were suspended in 0.3% glucose plus 1.5 × 10⁻² M QA in 1× Vogel’s salts, with 0.17% arginine, and 5 mg/liter biotin in a single flask. Cultures were incubated in LL for 2 h, at which time they were transferred to DD for 12–21 h, an incubation duration that insures steady-state repression and loss of frq mRNA (5). After this interval of repression, derepression was initiated by the removal of 3–4 disks from the bulk repression cultures at regular intervals. Disks were washed extensively to remove residual QA, and suspended in 25–50 ml of QA-deficient (inducer-free) derepression media (in different experiments either 2% glucose/0.5% L-arginine or 0.03% glucose/0.17% L-arginine, both in 1× Vogel’s and 5 mg/liter biotin). Approximately 1 day into this process, all samples were harvested simultaneously, such that frq+ or frq+qa-2frq disks had been in derepression medium for various amounts of time. The 0 h point remained in repression conditions for the entire incubation before the time of harvest with the other samples (approximately 34 or 43 h).

Race Tube Analysis. Race tube experiments were executed as described previously (5), except that medium completely lacking glucose was used. Determination of period length and standard deviation was accomplished with the aid of the computer program CHRONO II (v. 9.3, T. Roenneberg, University of Munich).

RNA Preparation and Analysis. RNA was prepared as described previously (9). Twenty to 40 mg was loaded onto gels containing 1× Mops, 5% formaldehyde, and 1.5% agarose. The gels were blotted onto nitrocellulose (NitroPure, Micron.
Separations, Westboro, MA) and probed. Plasmids pCRM129ΔBB (5) and qa-2p'UTR were used to make riboprobes for the 5' UTRs of the endogenous frq and the qa-2pfrq transcripts, respectively. qa-2p'-UTR was constructed by ligating of the 0.5-kbp KpnI fragment from pBA50 (5) containing the 86-bp qa-2 5' UTR and an additional ∼400 untranscribed bases from the qa-2 promoter, into a phosphatase-treated, KpnI-digested SK-II plasmid vector (Stratagene). Riboprobes for pCRM129ΔBB were made as described (5) and for qa-2p'-UTR by in vitro transcription with T7 polymerase (Promega). Ribosomal RNA was quantitated in one of three ways. DNA probes were made from a BamHI-digested rDNA cosmID, 7:D4 (9), by randomly priming with DIG-11-dUTP (Boehringer–Mannheim), and the hybridized probe was detected according to manufacturer's specifications. Alternatively, DNA probes were made by random priming of BamHI-digested rDNA (cosmid 20:11G) with [32P]dCTP. Methylene blue staining also was used (14).

Protein Preparation and Detection. Mycelia were ground in liquid nitrogen, added to 500 μl of 2× sample buffer (15) and held at 100°C for 5 min. DTT was added to the supernatant to a final concentration of 0.1 M, and the solution was reheated for 5 min. Fifty to 100 mg of protein was run on a 7.5% Kpn digestion of the 0.5-kbp transcripts, respectively. pCRM129ΔBB was detected using the ECL system (Amersham). A standard curve was run on each gel, consisting of dilutions of[35S]methionine (about 1,000 Ci/mmol). Ribosomal RNA was quantitated in one of three ways. DNA probes were made from a BamHI-digested rDNA cosmID, 7:D4 (9), by randomly priming with DIG-11-dUTP (Boehringer–Mannheim), and the hybridized probe was detected according to manufacturer's specifications. Alternatively, DNA probes were made by random priming of BamHI-digested rDNA (cosmid 20:11G) with [32P]dCTP. Methylene blue staining also was used (14).

Experimental Design. The frq gene encodes integral components of a circadian oscillator that is characterized by a progressive accumulation and subsequent loss of both frq RNA and FRQ protein over the course of a day. Using previously characterized tools (5), component parts of a single oscillation were generated in vivo. Specifically, negative feedback was created by inducing frq expression from a heterologous promoter. Release from negative feedback was demonstrated by removal of inducer, allowing the induced frq and FRQ to decay and the endogenous frq RNA to reaccumulate. The time required for each of these two processes is distinct and can be measured separately; together they add up to a single circadian cycle.

In these experiments the isolated aspects (negative feedback and release from negative feedback) of a single circadian oscillation were measured. The first event in a single oscillation is repression or negative feedback. An approximation of this repression process can be artificially induced in the frq9 strain: in constant conditions, strains bearing frq9 express elevated levels of frq9 mRNA, in an unregulated pattern [i.e., devoid of rhythmicity, see Fig. 1A for schematic representation; (5)]. Addition of QA to a frq9 strain containing a copy of the frq ORF fused to the inducible QA promoter (frq9qa-2pfrq) leads to accumulation of qa-2pfrq mRNA and production of FRQ protein(s). The induced FRQ protein(s) act through negative feedback to decrease the level of frq9 RNA arising from the endogenous frq promoter (Fig. 1B). The kinetics of this autoregulation and the dependency of the kinetics on the dosage of FRQ can be experimentally determined by adding different amounts of the inducer QA and by collecting samples that were cultured in QA-containing media for various amounts of time. The subsequent defining event in an oscillation is release from repression, as characterized by the return to normal, elevated frq9 RNA expression levels (see Fig. 1C). In the absence of functional FRQ from the endogenous frq locus, the disappearance of the induced FRQ protein can be monitored and correlated with the reappearance of frq9 mRNA. Collectively, these experiments effectively reconstitute a single circadian oscillation, albeit dissected into two parts.

FRQ Rapidly Depresses frq9 mRNA Levels. Figs. 2 and 3 show the results of experiments designed to determine the kinetics of FRO-mediated negative feedback and the dependence of the kinetics on the concentration of frq mRNA and FRQ. Incubation of the frq9qa-2pfrq strain with saturating levels of inducer [1.5 × 10−5 or 1.5 × 10−4 M QA; (17)] resulted in a substantial (>50%) and rapid (within 3 h) reduction of frq9 RNA pools. After 6 h, repression is essentially complete at all concentrations of inducer that were examined, and leaving QA in the culture for longer times fails to yield an additional loss of frq9 mRNA (data not shown). The overall decrease in frq9 mRNA effected by qa-2pfrq within 6 h is comparable to that seen in a normal circadian oscillation (5). As levels of inducer drop well below saturation (compare 1.5 × 10−5 to 1.5 × 10−6 M QA), induction of frq and FRQ is less extensive (Fig. 3, and data not shown) reflecting the inducer concentration dependence of the qa-2 promoter itself (ref. 17 and data not shown). However, even at this lowest inducer concentration, 6 h of repression results in decreased frq9 mRNA levels of more than 80%.

RESULTS

Fig. 2. Repression of frq9 mRNA accumulation by induction of FRQ protein is complete within a quarter of the circadian cycle. (A) Northern blot analysis of mRNA derived from frq9qa-2pfrq cultures incubated in 0, 1.5 × 10−4, 1.5 × 10−5, or 1.5 × 10−6 M QA for the times shown. mRNA was probed with a pCRM129ΔBB riboprobe, which recognizes the 5' UTR of the endogenous frq9 mRNA transcript but not that of the qa-2pfrq transcript. (B) The autoradiographic exposures from four repression experiments of the type shown in A were scanned for densitometric quantitation of mRNA levels. The data were pooled and graphed as the mean ± 2 SEM.
We have confirmed the functional relevance of the FRQ-mediated repression seen at these very low levels of FRQ protein induction. This was accomplished by observing the effects of this amount of FRQ induction on the overt rhythm in a race tube assay using a frq<sup>-</sup> qa-2pfrq strain (Fig. 4). The composition of the solid race tube media was similar to that used in the liquid cultures (Figs. 2 and 3) with the frq<sup>-</sup> qa-2pfrq strain (i.e., 0% glucose, but with 1.5% agar). At 1.5 \times 10^{-6} \text{ M} inducer the overt rhythm is either disrupted or masked (Fig. 4).

Comparison of these tubes with either the control strain (frq<sup>-</sup> pDE3\text{ABH}) at the same inducer concentration, or the experimental strain with no QA shows conidial banding with periods of 23.6 h ± 0.32 (mean ± 2 SEM) and 23.4 h ± 0.62, respectively. Interestingly, induction of FRQ to levels that fail to disrupt rhythmicity can have effects on period length. At 7.5 \times 10^{-6} \text{ M} QA the period of frq<sup>-</sup> qa-2pfrq lengthens to 24.8 h ± 0.57, and at 1.1 \times 10^{-6} \text{ M} QA the period lengthens to 25.6 h ± 0.41. In summary, the minute amounts of FRQ that mediate negative feedback within 6 h in an artificial system are sufficient to disrupt rhythmicity in a rhythmic strain.

Recovery from FRQ-Mediated Negative Autoregulation Requires Most of the Circadian Cycle. The reciprocal aspect of a circadian oscillator is recovery from negative feedback, or the reaccumulation of frq mRNA. To follow the kinetics of release from repression (derepression), cultures of frq<sup>-</sup> qa2pfrq were first induced in QA for at least 12 h. Mycelia from these long-term repression cultures express very low levels of frq RNA (see Figs. 1C and 2). Mycelial disks were removed from the repression cultures at staggered time points and cultured in fresh medium lacking the inducer. The recovery of endogenous frq RNA pools was monitored. Fig. 5 shows that derepression requires 14–18 h, or the greater part of a day. The process is gradual, in that the reappearance starts sometime between 8 and 14 h, but the largest single increment reproducibly occurs between 14 and 18 h. By 18 h frq<sup>-</sup> mRNA is present in amounts that are not significantly different from the frq<sup>-</sup> control strain.
Such a long recovery of frq RNA could be artifactual if excess QA remained in the media. Two observations suggest that this is not the case. First, Fig. 6 shows that the level of qa-2 mRNA plummets in the cultures after removal of inducer. Because qa-2 mRNA is stable and persistent in cultures containing the low QA levels (e.g., 1.5 × 10⁻⁶ M QA, data not shown) that activate both qa-2 and qa-2frq transcription extremely weakly, we can assume, from the absence of qa-2 mRNA after 8 h, that QA is absent from the cultures, and that transcription from either of the qa-2 promoters (endogenous or qa-2frq) has ceased. Second, Western blots in Fig. 6 show FRQ protein levels from mycelia after stepwise removal of QA from cultures. Comparison of the 0 time point (long-term repression) with the 8-h time point (8 h in QA-deficient media) shows a shift in the form of the FRQ protein from the predominance of the faster mobility form (0 hrs of derepression) to exclusively the slower mobility, multiphosphorylated forms, an indication of maturation of the protein and lack of input from de novo translation after removal of inducer at time 0. This mobility shift corresponds to the shift in apparent molecular mass of FRQ over time through the circadian cycle: newly translated polypeptides are minimally modified, and with increasing time the protein decreases in mobility, apparently due in part to phosphorylation (N.Y.G., unpublished work). In the case of this experiment, repression conditions were relatively strong, thus resulting in the majority of FRQ protein (at 0 h) in the minimally processed form, and a relatively small proportion in slower mobility forms. In experiments where induction was weaker, proportionally more of the larger forms of FRQ is seen. Further, comparison of the 8-h time point with the 14-h time point reveals a precipitous decrease in overall FRQ levels. Yet, at 14 h (the turning point between repression and derepression) there are still approximately 10 copies per nucleus present.

**DISCUSSION**

In this study we have dissected the autoregulatory negative feedback loop comprising a circadian cycle into two discrete parts—repression by FRQ and recovery from repression. By using constructs that allow regulatable expression of frq from a heterologous promoter, we have estimated the amount of time required for each part of the cycle. Interestingly, we find that negative feedback by FRQ is relatively rapid, and it is complete in substantially less than half the circadian cycle (t/2, about 3 h). Derepression, however, requires most of the circadian cycle. Even in the apparent absence of de novo transcription or translation of frq, FRQ levels remain high for about 8 h resulting in stable, low levels of frq transcript, after which time FRQ protein levels fall off and frq expression rises. Importantly, however, the time required to complete both parts of the cycle in this reconstruction experiment is about 22 h, or equivalent to a typical wild-type oscillation in a frq⁺ strain.

The approach presented in this study represents a novel method for generating data for theoretical modeling of a circadian oscillator. We have used a mutant clock strain (frq⁻) whose frq expression levels at all times correspond to levels seen at a particular circadian time in wild-type strains: approximately subjective dawn, a time of substantial frq RNA accumulation, but low protein accumulation. The frq⁻ strain is typically arrhythmic and, due to the lack of FRQ protein, it cannot generate the negative feedback loop that is the basis for the sustained circadian oscillations in frq expression that characterize a circadian rhythm. By introducing functional FRQ protein (expressed from a chimeric gene with the inducible qa-2 promoter and the frq ORF), the time required for the discrete events that comprise the feedback loop can be determined on isolated aspects of a single oscillation. An additional feature of frq⁻ that makes it attractive for these particular experiments is the persistence of frq RNA in the absence of glucose-containing media. Glucose acts by inducer exclusion to modulate the strength of transcription from the qa-2 promoter (18). Thus, in frq⁻ transformed with qa-2frq and maintained in the absence of glucose, the initiation of transcription is rapid and strong except at the very lowest levels of inducer.

Negative feedback itself (repression) occurs relatively rapidly. Within 3 h of FRQ induction substantial decreases in frq RNA are observed and repression is essentially complete by 6 h. Also, transcription of the transgene is initiated in less than 15 min, and the lag between transcription and translation is less than 2 h (data not shown). This temporal progression suggests that FRQ could act directly as a transcriptional repressor or corepressor; however, this conclusion currently lacks direct biochemical support.

In addition to the rapid kinetics of frq/FRO-mediated negative feedback, the minute quantities of FRQ that are required for full function is striking. Rough estimates based on samples close to the limits of detection suggest that approximately 10–15 copies of FRQ/nucleus were sufficient to result in substantial negative feedback (Fig. 3). These levels are less than the amount of FRQ seen at peak in rhythmic cultures in DD. Further, under even weaker inducing conditions (1.5 × 10⁻⁶ M QA) the rhythmic expression of conidia in a wild-type strain (as determined by race tube assay) is disrupted (Fig. 4). This demonstrates that constitutive expression of FRQ even at levels that are low relative to the amplitude of the normal FRQ oscillation itself can disrupt the overt rhythm.

Although negative feedback occurs rapidly, the events surrounding derepression of frq occupy nearly ¾ of the circadian day. During this time FRQ protein persists such that even at...
14 h after removal of inducer it is still present in quantities that mediate negative feedback (see Figs. 2 and 3). Within 4 more h though, a further decrease in FRQ levels is seen, and derepression occurs. Had FRQ been seen rapidly to turn over during derepression such that both frq transcript and FRQ protein levels were low during much of the circadian cycle, one could have predicted the existence of additional sequential components in the feedback loop. As it is, however, these data demonstrate that the frq gene and protein are present during most of the circadian cycle, and it may be that this circadian oscillator can be largely described by the events and molecules surrounding activation and repression of frq alone.

The appearance here of relatively stable and progressively posttranslationally modified FRQ protein dovetails nicely with what is seen in a normal circadian oscillation (N.Y.G., unpublished work) where the mobility shift in FRQ is known to be at least in part the result of sequential cumulative phosphorylation. Similar phosphorylation has been observed for the PER protein of Drosophila (19), although at this time the functional significance of these phosphorylation events in relation to timekeeping, if any, is completely unknown for any clock component. Possible roles include a mechanism for gating the protein into various cellular compartments [which would have the effect of introducing time lags into the otherwise simple loop (19)], involvement in light resetting (20), or modulation of protein stability (e.g. ref. 21). In any case it is noteworthy here that the posttranslational modification of FRQ occurred at a normal rate after de novo FRQ synthesis in a system that was at all times functionally arrhythmic, consistent with the presence of a constitutive modifying activity.

Construction of a cohesive clock model that incorporates the features of frq and FRQ will include three basic premises: rapid repression, slow derepression mediated by a stable protein, and activation (in which frq is actively turned on, ref. 5; T. Roenneberg, M.W.M., and J.C.D., unpublished work). The constitutive and high levels of frq mRNA accumulation in this mutant strain suggest that in the absence of clock input (from autoregulatory feedback or from environmental factors; e.g. ref. 9) transcription of frq occurs continuously (2). Using frq<sup>−</sup>, the component parts of an oscillation can be artificially reconstituted and assessed.

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