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Martha W. Merrow
Dartmouth College

Norman Y. Garceau
Dartmouth College

Jay C. Dunlap
Dartmouth College

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Genetics

Dissection of a circadian oscillation into discrete domains

(***frequency*/*Neurospora*/reconstruction/clock/quinate**)

MARTHA W. MERROW, NORMAN Y. GARCEAU, AND JAY C. DUNLAP*

Department of Biochemistry, Dartmouth Medical School, Hanover, NH 03755

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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 (*frq*^{9}), 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 mechanics 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 (*frq*$^+$) strain and fails to support rhythmicity in a conditionally arrhythmic (*frq*^{9}) 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. 1*A*).

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,

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Abbreviations: *frq*, *frequency*; DD, constant darkness; LL, constant light; UTR, untranslated region; QA, quinic acid.

*To whom reprint requests should be addressed at: Department of Biochemistry, Dartmouth Medical School, 7200 Vail, Room 413, Hanover, NH 03755. e-mail: jay.c.dunlap@dartmouth.edu.

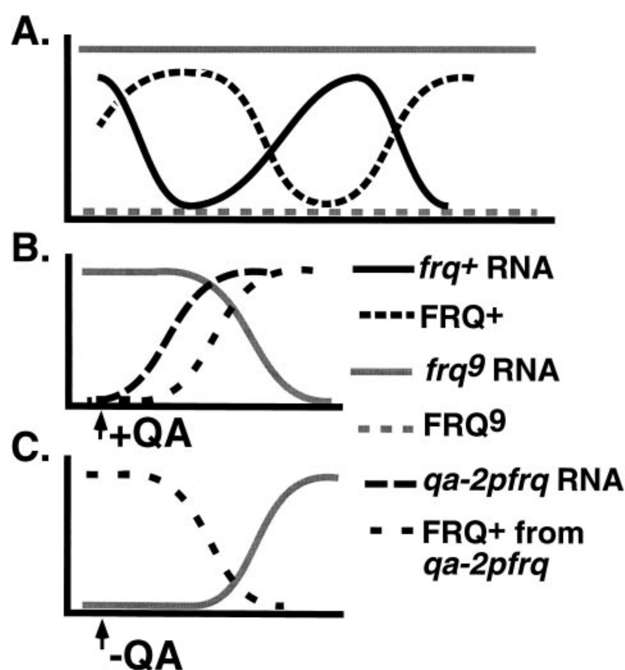


FIG. 1. Schematic representation of a linearized circadian oscillation. (*A*) When held in DD, frq^{+} mRNA and FRQ protein levels oscillate, completing 1 full cycle in about 22 h. In contrast, frq^{9} mRNA generally fluctuates around high levels, failing to demonstrate rhythmicity, and the truncated protein does not accumulate. (*B*) Experimental induction of negative feedback. A $frq^{9}qa$-$2pfrq$ strain is induced with QA, such that *qa-2pfrq* mRNA and functional FRQ protein accumulate. Endogenous frq^{9} mRNA levels decrease over time, mimicking the negative feedback aspect of a frq^{+} mRNA oscillation. (*C*) Release from negative feedback, or derepression. A long-term repression culture (characterized by lack of frq^{9} mRNA and induced FRQ protein) is generated by incubation of $frq^{9}qa$-$2pfrq$ with QA for 12–21 h. Mycelia are washed extensively to remove QA, and cultures are continued in fresh media. The disappearance of FRQ protein (derived from *qa-2pfrq*) is followed, as is the reappearance of endogenous frq^{9} mRNA.

for instance, by triggering FRQ expression through the use of a regulatable promoter and then after the effects of its expression on endogenous, feedback-regulated *frq* RNA levels. If this is done in a clock-wild-type (frq^{+}) strain, then the endogenous FRQ and the natural momentum of the circadian cycle would affect the apparent rates of repression or derepression. For this reason, to follow each part of the clock cycle at a single defined phase rather than from multiple, multiphasic initiation points, we analyzed each set of events independently in a strain bearing the frq^{9} allele and thus devoid of a *frq*-based oscillator (10, 11). frq^{9} is a loss-of-function mutation that results in the production of a full-length *frq* transcript but a truncated FRQ protein that does not accumulate (see Fig. 1*A*). Therefore, under typical culture conditions, levels of frq^{9} mRNA fluctuate nonrhythmically and expression is generally high at all times, but the gene is still subject to feedback regulation by FRQ induction (5). frq^{9} is thought to be held at a defined point in time, as indicated by *frq* mRNA levels, and so this strain provides a clean background in which to track the rate of disappearance of frq^{9} mRNA resulting from negative feedback after FRQ expression from an inducible promoter. Similarly, release from negative feedback (derepression) can be followed. Addition of these two aspects of an oscillation—negative feedback and release from negative feedback—should represent an isolated single cycle of an oscillation and allow dissection of the component parts within. Using this method we have reconstituted a cycle that approximates the time frame of a wild-type circadian period, with either *frq* mRNA or FRQ protein detectable virtually throughout. Ultimately these data can be used, together with the collection of data representing *frq* mRNA and FRQ protein expression under various environmental conditions, for constructing a cohesive model of a self-sustaining circadian oscillator.

MATERIALS AND METHODS

Strains. For repression and derepression experiments the loss-of-function mutant frq^{9} was used (5). Regulatable, induced expression of a functional *frq* gene product was driven from an integrated plasmid containing the *Neurospora qa-2* promoter and 5′ untranslated region (UTR) (12) fused in frame with the first ATG of the *frq* ORF (*qa-2pfrq*) (5). The *qa-2pfrq*-containing plasmid, pBA50, was targeted to the *his-3* locus by standard methods and has been described previously (5). *frq* was induced by the addition of quinic acid (QA) to cultures of $frq^{9}qa$-$2pfrq$ [$bd;frq^{9}A$; his-$3(his$-$3^{+}qa$-$2pfrq)^{\mathrm{hom}}_{\mathrm{pBA50\#6}}$]. frq^{9} [$bd;frq^{9}A$] 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-2pfrq*, but in a frq^{+} background: $frq^{+}qa$-$2pfrq$ [$bd;frq^{+}A$; his-$3(his$-$3^{+}qa$-$2pfrq)^{\mathrm{hom\ pBA50\#1}}$] was induced with QA. The $frq^{+}pDE3\Delta BH$ control strain for these experiments [$bd;frq^{+}A$; his-$3(his$-$3^{+}DBH)^{\mathrm{hom\ pDE3\Delta BH\#1}}$] 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 5×10^{4} 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^{9} 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^{9} or $frq^{9}qa$-$2pfrq$ disks had been in derepression medium for various amounts of time. The 0 time 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 p*qa-25'*UTR were used to make riboprobes for the 5' UTRs of the endogenous *frq* and the *qa-2pfrq* transcripts, respectively. p*qa-25'*UTR was constructed by ligation of the 0.5-kbp *Kpn*I 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, *Kpn*I-digested SK-II plasmid vector (Stratagene). Riboprobes for pCRM129ΔBB were made as described (5) and for p*qa-25'*UTR by *in vitro* transcription with T7 polymerase (Promega). Ribosomal RNA was quantitated in one of three ways. DNA probes were made from a *Bam*HI-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 *Bam*HI-digested rDNA (cosmid 20:11G) with [^{32}P]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% SDS-polyacrylamide gel. Duplicate gels were either blotted or stained with Coomassie blue for correction of loading differences. For detection of FRQ, gels were electroblotted onto poly(vinylidene difluoride) membrane (Millipore), and probed with preadsorbed polyclonal rabbit anti-FRQ (N. Y. G, unpublished work). A second antibody (horseradish peroxidase conjugated goat anti-rabbit IgG; Southern Biotechnology Associates) was detected using the ECL system (Amersham). A standard curve was run on each gel, consisting of dilutions of *in vitro* translated FRQ (TnT, Promega). The absolute number of molecules of FRQ per mg of protein was determined by comparison with [^{35}S]methionine-labeled *in vitro* translated FRQ. By using knowledge of the specific activity of the [^{35}S]methionine (about 1,000 Ci/mmol), the fact that the FRQ polypeptide contains 26 methionines, and the size of the FRQ polypeptide (about 108 kDa by sequence), the number of FRQ polypeptides in an *in vitro* translated sample could be calculated and then used to produce a standard curve for calibrating the amount of FRQ detected in Western blots. By applying knowledge of the amount of DNA and protein in a given sample used for Western analysis, and the genome size of *Neurospora* [about 47 million kb (16)], the number of molecules of FRQ per *Neurospora* nucleus-equivalent could be determined. The determination of copies per nucleus (rather than copies per cell) was used because of *Neurospora*'s syncytial morphology.

RESULTS

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 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 *frq*^{9} strain: in constant conditions, strains bearing *frq*^{9} express elevated levels of *frq*^{9} mRNA, in an unregulated pattern [i.e., devoid of rhythmicity, see Fig. 1*A* for schematic representation; (5)]. Addition of QA to a *frq*^{9} strain containing a copy of the *frq* ORF fused to the inducible QA promoter (*frq*^{9}*qa-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 *frq*^{9} RNA arising from the endogenous *frq* promoter (Fig. 1*B*). 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 *frq*^{9} RNA expression levels (see Fig. 1*C*). 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 *frq*^{9} mRNA. Collectively these experiments effectively reconstitute a single circadian oscillation, albeit dissected into two parts.

FRQ Rapidly Depresses *frq* mRNA Levels. Figs. 2 and 3 show the results of experiments designed to determine the kinetics of FRQ-mediated negative feedback and the dependence of the kinetics on the concentration of *frq* mRNA and FRQ. Incubation of the *frq*^{9}*qa-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 *frq*^{9} 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 *frq*^{9} mRNA (data not shown). The overall decrease in *frq*^{9} 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 *frq*^{9} mRNA levels of more than 80%.

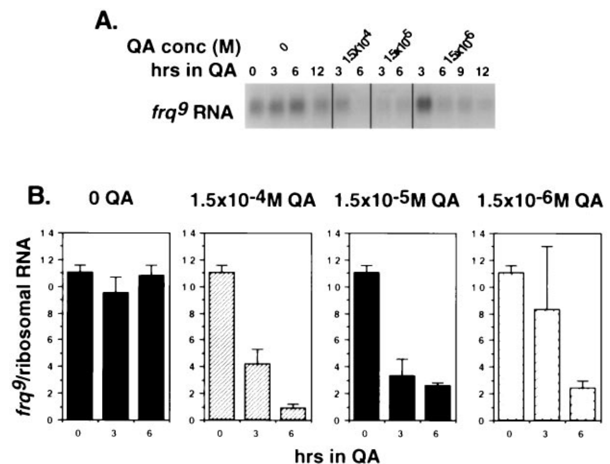


FIG. 2. Repression of *frq*^{9} mRNA accumulation by induction of FRQ protein is complete within a quarter of the circadian cycle. (*A*) Northern blot analysis of mRNA derived from *frq*^{9}*qa-2pfrq* cultures incubated in 0, 1.5×10^{-4}, 1.5×10^{-5}, or 1.5×10^{-6} M QA for the times shown. mRNA was probed with a pCRM129ΔBB riboprobe, which recognizes the 5'UTR of the endogenous *frq*^{9} 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.

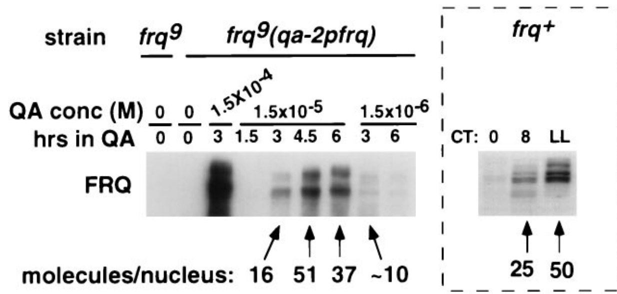
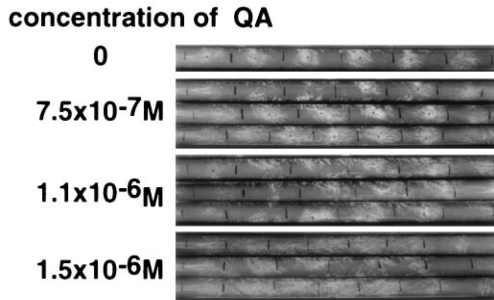


FIG. 3. Physiological and subphysiological levels of FRQ protein are sufficient to effect repression of *frq* mRNA levels. (*Left*) Western blot analysis of FRQ protein induced by incubation of *frq*^{9}*qa-2pfrq* with QA over time. The number of molecules of FRQ protein per nucleus was calculated relative to a standard curve determined with *in vitro* translated FRQ (see *Materials and Methods*). (*Dashed box*) Analysis of FRQ protein levels seen during the course of a typical circadian cycle in constant conditions (DD 11.5 and 19, respectively, for CT0 and CT8) or in LL in a *frq*$^{+}$ strain.

Fig. 3 documents the amount of FRQ protein that results in negative feedback and compares it with the levels of FRQ observed in a *frq*$^{+}$ strain in either DD or LL. The induction profile of FRQ protein at 1.5×10^{-5} M QA for 3 h indicates approximately 16 molecules of FRQ per nucleus. This is about half of that found at peak levels in a wild-type FRQ oscillation, and one-third of what is detected in LL. With even weaker induction (1.5×10^{-6} M QA) on the order of 10 molecules of FRQ accumulate per nucleus, a level that is close enough to the limit of detection that it is hard to quantify, but that nonetheless is at or below what is typically seen in the course of a circadian oscillation. Despite this, repression of *frq*^{9} mRNA occurs (Fig. 2).



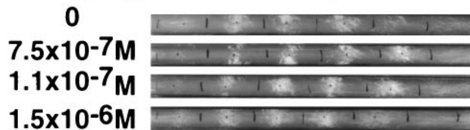


FIG. 4. Continual expression of even very low levels of FRQ results in severe disruption of the overt rhythm. *frq*$^{+}$ *Neurospora* cultures bearing an integrated copy of *qa-2pfrq* were grown on race tubes with different concentrations of QA under conditions for assaying rhythmicity. Race tube cultures were germinated in LL then transferred to DD; thereafter, the mycelial growth front was marked every 24 h (7). Discernible conidial bands are marked with single dots, and the effects of induction of FRQ from *qa-2pfrq* at inducer concentrations equal to or below those used in Figs. 2 and 3 were noted. (*A*) Effects of QA addition in the *frq*-inducible *qa-2pfrq* strain. (*B*) Effects of QA addition in the control noninducible strain. No rhythm is apparent at 10^{-6}M QA, which induces FRQ to barely physiological levels (Fig. 3), and induction of FRQ to even lower levels disrupts expression of the rhythm and lengthens the period length (see text).

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*$^{+}$*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*^{9}*qa-2pfrq* strain (i.e., 0% glucose, but with 1.5% agar). At 1.5×10^{-6} M inducer the overt rhythm is either disrupted or masked (Fig. 4). Comparison of these tubes with either the control strain (*frq*$^{+}$pDE3Δ*BH*) at the same inducer concentration, or the experimental strain with no QA shows conidial banding with periods of 23.6 h $\pm$ 0.32 (mean $\pm$ 2 SEM) and 23.4 h $\pm$ 0.62, respectively. Interestingly, induction of FRQ to levels that fail to disrupt rhythmicity can have effects on period length. At 7.5×10^{-7} M QA the period of *frq*$^{+}$*qa-2pfrq* lengthens to 24.8 h $\pm$ 0.57, and at 1.1×10^{-6} M QA the period of lengthens to 25.6 h $\pm$ 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 oscillation is recovery from negative feedback, or the reaccumulation of *frq* mRNA. To follow the kinetics of release from repression (derepression), cultures of *frq*^{9}*qa2pfrq* were first induced in QA for at least 12 h. Mycelia from these long-term repression cultures express very low levels of *frq*^{9} RNA (see Figs. 1*C* 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*^{9} 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*^{9} mRNA is present in amounts that are not significantly different from the *frq*^{9} control strain.

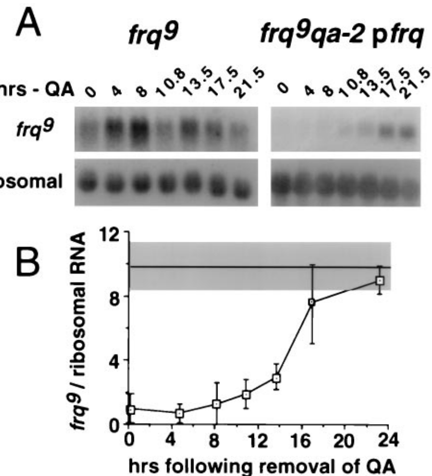


FIG. 5. Events involved in release from negative feedback and reaccumulation of *frq* mRNA occupy most of the circadian cycle. (*A*) Northern blot analysis of derepression cultures. *frq*^{9} (*Left*) or *frq*^{9}*qa-2pfrq* (*Right*) cultures were removed from a long-term repression culture, transferred to media lacking inducer, and used as a source of mRNA at the times shown. Blots were probed with pCRM129ΔBB, a probe specific for the endogenous *frq*^{9} mRNA and that does not recognize the *qa-2pfrq* transgene. (*B*) Densitometric analysis of Northern blots from two derepression experiments. *frq*^{9} RNA levels are generally high, but fluctuate over time, and are plotted as the mean of all time points from both experiments (black line) $\pm$ 2 SEM (gray box); *frq* mRNA levels in the *frq*^{9}*qa-2pfrq* strain (squares) are initially low due to repression but have returned to derepressed levels by about 18 h.

Such a long recovery of *frq*[9] 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-2pfrq* 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-2pfrq*) 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.

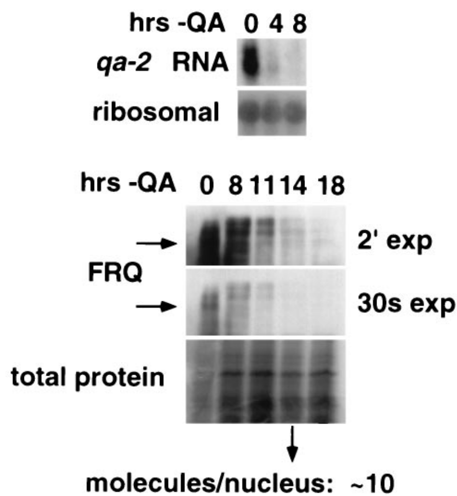


FIG. 6. Synthesis of QA-induced products stops with removal of inducer, but progressive posttranslational modification of FRQ continues. (*Upper*) *qa-2* mRNA arising from the normal sequence *qa-2* promoter in the *frq*[9]*qa-2pfrq* strain is abundant in the presence of QA but rapidly disappears after removal of the inducer. The Northern blot was probed with the p*qa-25′*UTR riboprobe that recognizes only the *qa-2* mRNA (see *Materials and Methods*). (*Lower*) Western blot analysis of corresponding *frq*[9]*qa-2pfrq* samples after removal of inducer shows FRQ to be abundant in the presence of QA and to drop slowly in concentration after the removal of inducer. Different exposures (2 min and 30 sec) of the Western blot allow the identification of the bands corresponding to the differentially modified FRQ products at different times. The position corresponding to the major (minimally modified) form of FRQ in the earliest samples is marked with an arrow. The absence of *de novo* synthesis accompanied by the progressive modification of FRQ is signaled by the gradual disappearance of predominant faster mobility (minimally modified) forms and the shift of FRQ to slower mobility forms.

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 ($t_{1/2}$ 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*[9]) 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*[9] 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*[9] that makes it attractive for these particular experiments is the persistence of *frq*[9] mRNA 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*[9] transformed with *qa-2pfrq* 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*[9] 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*/FRQ-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^9 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^9, the component parts of an oscillation can be artificially reconstituted and assessed.

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