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Alternative Initiation of Translation and Time-Specific Phosphorylation Yield Multiple Forms of the Essential Clock Protein FREQUENCY

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Summary

The *frequency* (*frq*) gene encodes central components of the transcription/translation-based negative-feedback loop comprising the core of the Neurospora circadian oscillator; posttranscriptional regulation associated with FRQ is surprisingly complex. Alternative use of translation initiation sites gives rise to two forms of FRQ whose levels peak 4–6 hr following the peak of *frq* transcript. Each form of FRQ is progressively phosphorylated over the course of the day, thus providing a number of temporally distinct FRQ products. The kinetics of these regulatory processes suggest a view of the clock where relatively rapid events involving translational regulation in the synthesis of FRQ and negative feedback of FRQ on *frq* transcript levels are followed by slower posttranslational regulation, ultimately driving the turnover of FRQ and reactivation of the *frq* gene.

Introduction

The ability of organisms to measure time on a 24 hr scale in the absence of environmental stimuli is a common phenomenon in nature. The regulatory machineries that generate this temporal information are known collectively as circadian clocks, and they control a variety of rhythms in biochemistry, physiology, and behavior. Circadian clocks can be reset by environmental stimuli such as light (Crosthwaite et al., 1995; Reppert and Sauman, 1995) and temperature (Liu et al., 1997 [this issue of *Cell*]), and the rhythms they generate maintain a constant period over a range of physiological temperatures (referred to as ''temperature compensation''). It is now clear that circadian clocks are a cell-autonomous phenomenon and are subject to complex regulation (Dunlap, 1993, 1996; Michel et al., 1993; Welsh et al., 1995).

Although the biochemical basis of circadian rhythmicity is fully understood in no organism, identification of clock mutations in a series of organisms (Konopka and Benzer, 1971; Feldman and Hoyle, 1973; Hotz-Vitaterna et al., 1994; Kondo et al., 1994; Millar et al., 1995; Dunlap, 1996) has provided an entrée to the problem and resulted in the identification of actual components of the clock in Neurospora (FRQ) (Aronson et al., 1994a, 1994b; Merrow et al., 1997; reviewed in Loros, 1995; Dunlap, 1996) and in flies (PER/TIM) (Sehgal et al., 1995; Myers et al., 1996; Zeng et al., 1996; reviewed in Hall, 1995; Hardin and Siwicki, 1995; Sehgal, 1995). The *frequency* (*frq*) gene in Neurospora encodes a product(s) that is a central component of a presumptive transcription/translation-based negative-feedback loop that comprises the core of the circadian oscillator in this organism (Aronson et al., 1994a). Analysis of the transcriptional regulation of *frq* has begun to explain in broad outline both how the feedback loop comprising the clock is assembled and how it is reset by light (Aronson et al., 1994a; Crosthwaite et al., 1995). Specifically, the products of *frq* satisfy all of the following criteria proposed for circadian clock components (Aronson et al., 1994a): (i) continual expression of mRNA encoding the *frq* protein (FRQ) under control of an inducible promoter abolishes overt rhythmicity in a strain that harbors a wild-type *frq* gene and is unable to support rhythmicity when expressed in a *frq* loss-of-function strain; (ii) step reductions in expression of FRQ-encoding mRNA set the phase of the clock to a consistent and predictable phase; (iii) elevated expression of FRQ-encoding mRNA depresses levels of the endogenous *frq* mRNA; (iv) deletion of *frq* results in loss of normal overt rhythmicity; and most importantly, (v) the abundance of the *frq* mRNAs oscillates with a period that reflects the overt rhythm of the strain analyzed (reviewed in Loros, 1995; Dunlap, 1996). These and other data have established the *frq*/FRQ autoregulatory negative-feedback loop as the basis for a circadian oscillator and induction of *frq* as the basis for light resetting. The data predicted that a single protein encoded by the 989 codon *frq* ORF would be the *trans*-active factor mediating rhythmicity (Aronson et al., 1994b) and that, as required by its role as a clock component, its level would oscillate over the course of the cycle with a period reflecting the overt periodicity of the clock.

To understand the role of *frq* transcript and FRQ in the assembly of the circadian oscillator, it is critical to describe and understand the regulation of FRQ. To this end, we carried out a series of molecular and biochemical analyses of FRQ and have found it is subject to several distinct aspects of regulation. FRQ cycles in abundance over the course of the day displaying a ca. 4 hr lag between *frq* RNA and protein similar to that seen for clock genes in Drosophila (reviewed in Reppert and Sauman, 1995). Next, alternative initiation of translation results in the production of two distinct FRQ polypeptides from the single FRQ open reading frame, providing evidence for translational regulation in the synthesis of clock proteins. Furthermore, each form of FRQ is subject to extensive and time-of-day specific phosphorylation (similar to the Drosophila protein PER [Edery et al., 1994]), modifications that may govern the turnover of FRQ. Finally, by fractionating undenatured cell extracts we find that FRQ exists chiefly as a monomer in solution but possibly one with a highly asymmetric shape. The temporal profiles of the amounts of *frq* mRNA and the two forms of FRQ, along with other recent data explicitly describing the kinetics of FRQ feedback (Merrow et al., 1997), suggest that distinct regulatory processes are associated with different parts of the cycle. Following the appearance of *frq* transcript, translational control governs the synthesis of the two forms of FRQ that rapidly feed back to reduce *frq* transcript levels, events that are complete within much less than half

the cycle. Most of the circadian day involves the slower posttranslational processing of both FRQ forms through a series of phosphorylation stages, resulting finally in the rapid reaccumulation of *frq* transcript and the turnover of FRQ.

Results

Circadian Oscillations of *frq* RNA and Protein Are Not Mirror Images

To characterize FRQ, antisera were generated, first against the N-terminal 70 amino acids of FRQ produced in bacteria and later against recombinant full-length FRQ produced in a baculovirus-expression system. We were able to visualize FRQ only with the antiserum to full-length FRQ. When it was used to probe Western blots of N. crassa cell homogenates prepared from cultures harvested at ca. 4 hr intervals after a light-to-dark transfer (Figure 1), several bands of differing mobility could be observed in the wild-type strain (frq^+, period = ca. 22 hr; Figure 1A) and in the long-period mutant, frq^7 (period = ca. 29 hr; Figure 1D). The bands display a circadian oscillation in abundance and mobility consistent with the periods of the two strains; they are absent both in extracts from the frq^{10}-null (*frq*-deletion) strain (Figure 1A) (Aronson et al., 1994b) and when Western blots are probed with preimmune serum (data not shown). FRQ displays maximal abundance in the mid-subjective day around circadian time (CT) 8, while the *frq* mRNA displays maximal abundance in the early subjective morning (CT0–CT6; Figures 1B and 1E).

Several aspects of the shapes of these curves are noteworthy. First, the peak in FRQ protein levels lags behind the peak in *frq* transcript by about 4–6 hr, suggesting that synthesis of FRQ does not follow immediately upon the appearance of *frq* transcript. Second, *frq* transcript levels begin to fall before FRQ levels are at their highest point, suggesting that FRQ is effective in executing its autoregulatory role at less than maximal levels and that the maximal level of FRQ is determined at least in part through a posttranscriptional mechanism. Third, *frq* RNA appears to rise and fall relatively rapidly in time. However, the reverse is true for FRQ, whose levels remain greater than half of peak height for most of the day. These data suggest that *frq* and FRQ are being regulated at a variety of points in the daily cycle.

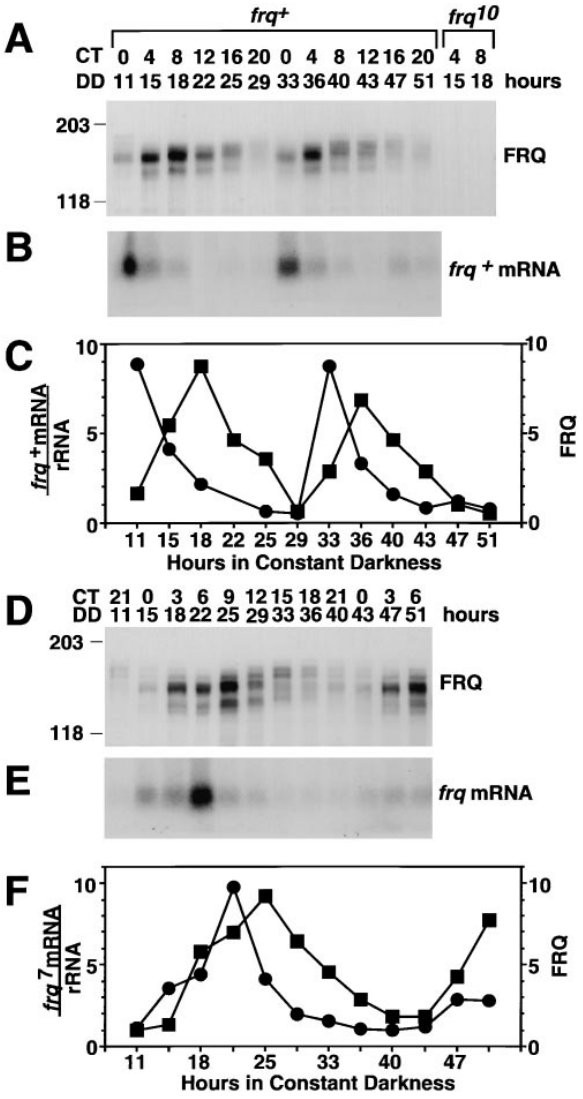

Figure 1. Circadian Clock-Dependent Accumulation of FRQ

Total RNA and protein were isolated from separate halves of tissue harvested at 4 hr intervals for 48 hr after a light-to-dark transfer.

(A and D) Western blot of extracts (100 μg per lane) from frq^+ (A) and frq^7 (D) probed with anti-FRQ. Size markers are shown in kilodaltons on the left of the blot.

(B and E) Northern blots of total RNA from frq^+ (B) and frq^7 (E). Equal amounts of total RNA were subjected to electrophoresis, transferred to nylon membrane, and probed with an *frq*-specific riboprobe.

(C and F) Densitometry of *frq* mRNA (closed circles) and protein (closed squares) for the frq^+ (C) and frq^7 (F) strains. This experiment is representative of five (for FRQ) or more (for *frq* mRNA) experiments.

Two Forms of the FRQ Protein Arise as a Result of Alternative Initiation of Translation

In Figures 1A and 1D, two series of bands representing FRQ can be observed in extracts prepared from both the frq^+ and frq^7 strains. The difference in mobility between the two series of bands is consistent with a difference in molecular mass of ca. 10 kDa, with the smaller series of bands centered at ca. 135 kDa and the larger at ca. 145 kDa. At least two hypotheses could explain this. First, FRQ protein could be proteolytically cleaved to generate a product that is 10 kDa shorter than the full-length protein. Second, translation of *frq* mRNA could initiate at methionine codons located at two or three sites within the *frq* ORF: at codon #1 ($FRQ^{1–989}$), codon #11 ($FRQ^{11–989}$), or codon #100 ($FRQ^{100–989}$).

To determine if the clusters of FRQ bands are due to choice of translational initiation sites, we selectively eliminated some or all of the potential initiation sites (Figure 2A). The constructs were introduced into the frq^{10} (null) strain and assayed for expression of FRQ by Western blot (Figure 2B). In the wild-type strain, both series of bands are observed. In $FRQ^{\Delta AUG\#1and\#2}$ (lacking the first two AUGs in the FRQ ORF), only the lower molecular weight series of bands are produced; in

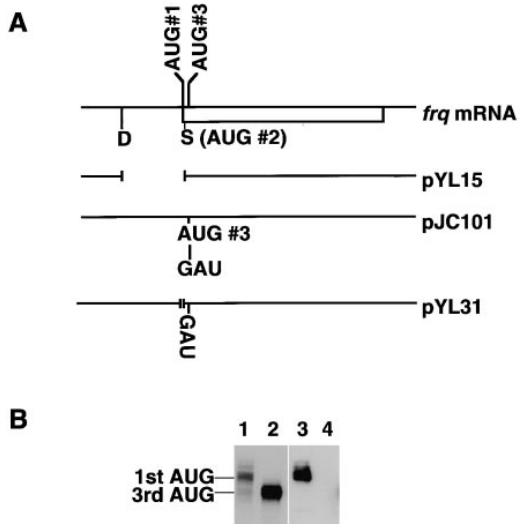

Figure 2. Alternative Translational Initiation Results in the Synthesis of Multiple Forms of FRQ

(A) AUG deletion constructs.

(B) Western blot analysis of FRQ in the AUG deletion mutants using extracts prepared from cultures grown in light for 4 hr. Lane 1, *bd;frq*$^{+}$ *A*; Lane 2, *bd;frq*^{10}; *his-3(his-3*$^{+}$, *frq*$^{\Delta AUG\#1and\#2}$)$^{hom\ pYL15}$; Lane 3, *bd;frq*^{10}; *his-3(his-3*$^{+}$, *frq*$^{AUG\#3\rightarrow GAT}$)$^{hom\ pJC101}$; Lane 4, *bd;frq*^{10}; *his-3 (his-3*$^{+}$, *frq*$^{\Delta AUG\#1,\#2,and\#3\rightarrow GAT}$)$^{hom\ pYL31}$. Proteins produced from the 1st and 3rd AUGs are indicated on the left of the blot.

FRQ$^{AUG\#3\rightarrow GAU}$ (lacking the third AUG), only the higher molecular weight series of bands are produced; and in FRQ$^{\Delta AUG\#1,\#2,and\#3\rightarrow GAU}$, both series of bands are eliminated. These results clearly demonstrate that translational initiation in the *frq* ORF can occur at more than one position. The predicted difference in size for the proteins initiated at AUG#1 or AUG#2 and AUG#3 is ~10 kDa, consistent with the differences in sizes observed by Western blot. Also, the AUGs at codons #1 and #100 have good consensus sequences for translational initiation (while the AUG at codon #11 does not) (Edelmann and Staben, 1994), and the AUGs at codons #1 and #100 (but not codon #11) are conserved in the *frq* ORF from two evolutionarily divergent fungal species, Chromocrea spinulosa and Leptosphaeria australiensis (Lewis et al., 1997). Altogether, this is strong evidence that two proteins are translated from the *frq* ORF beginning at codon #1 and codon #100 and that they differ at the N-terminus by 100 amino acids (FRQ$^{1–989}$ and FRQ$^{100–989}$). The unexpected internal initiation of FRQ may explain in part our inability to detect FRQ with our original anti-N-terminal antisera.

Time-of-Day Specific Changes in the Apparent Sizes of both FRQ Forms Are the Result of a Circadian Rhythm in Phosphorylation

The mobilities of both FRQ$^{1–989}$ and FRQ$^{100–989}$ show striking changes over the circadian day, with the minimum mobility immediately preceding the disappearance of FRQ (Figure 3). Furthermore, the estimated sizes of the two series of bands range from 140–160 kDa for FRQ$^{1–989}$ and 125–140 kDa for FRQ$^{100–989}$, and deviate greatly from the predicted sizes of 108 and 97 kDa. To determine if the size discrepancy is due to phosphorylation, FRQ

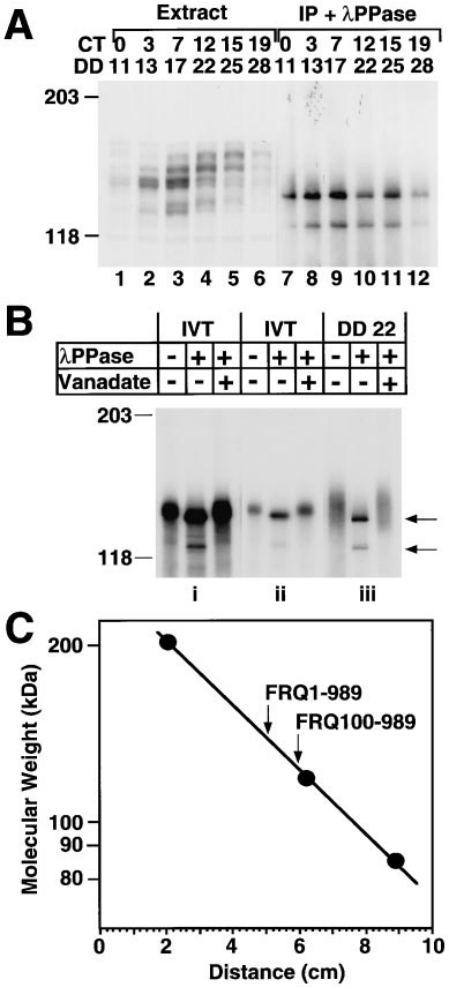

Figure 3. FRQ Is Immediately Phosphorylated Following Translation and Multiply Rephosphorylated in a Time-of-Day Specific Manner

(A) Lanes 1–6: Western blot of total cell extract prepared from cultures harvested at 4 hr intervals over one day. Lanes 7–12: Western blot of FRQ from extracts (same as shown in lanes 1–6) subjected to immunoprecipitation and treatment with λPPase; lane 10 was slightly underloaded.

(B) Western blot of FRQ produced by in vitro translation (IVTFRQ) or immunoprecipitated from an extract prepared from a culture grown in 22 hr in darkness. The immunoprecipitation complexes were either treated with λPPase (+) or buffer (−) in the presence (+) or absence (−) of vanadate. Two exposures of IVTFRQ are shown.

(C) Estimate of the molecular masses of unmodified FRQ polypeptides. From an SDS–polyacrylamide gel, a standard curve was generated by plotting mobility (cm) versus molecular mass for protein standards (myosin, 205 kDa; β-galactosidase, 116 kDa; albumin, 86 kDa). The sizes of FRQ were extrapolated from the standard curve.

from circadianly timed extracts was subjected to immunoprecipitation followed by treatment with lambda protein phosphatase (λPPase; New England Biolabs) and Western blot analysis (Figure 3A). λPPase removes phosphate residues on serine, threonine, tyrosine, and histidine residues and is fully inhibited by vanadate. In samples not subjected to immunoprecipitation and incubation with λPPase (Figure 3A, lanes 1–6), at least five bands were resolved for the upper FRQ series and three bands for the lower FRQ series. Upon incubation

with λPPase, FRQ is converted to two distinct forms at all times of the day, with the lower mobility band likely corresponding to $FRQ^{1–989}$ and the higher mobility band to $FRQ^{100–989}$. The increased mobility of FRQ is fully inhibited by sodium vanadate, indicating that the change in mobility is not due to the activity of contaminating proteases (Figure 3B). Treatment of in vitro-translated FRQ with phosphatase also generates two bands with mobilities indistinguishable from the two bands observed for endogenous phosphatase-treated FRQ. This indicates that the upper FRQ band represents the full-length protein with a mobility indistinguishable from full-length in vitro-translated FRQ (IVTFRQ; Figure 3B), in addition to showing that the alternative initiation described above does not require Neurospora-specific factors. The estimated sizes of both IVTFRQ and both forms of endogenous FRQ after λPPase treatment are 135 and 125 kDa (Figure 3C), still appreciably larger than the predicted sizes of 108 and 97 kDa, suggesting that aspects of the FRQ primary (Aronson et al., 1994b) or secondary structure (see below) may influence the polypeptides' mobilities. Nevertheless, these results demonstrate that all of the striking time-dependent decreases in mobility of FRQ are due to phosphorylation. Furthermore, at no time in the circadian cycle is the mobility of FRQ the same as that seen in the phosphatase-treated samples, suggesting that newly synthesized FRQ is almost immediately phosphorylated and becomes highly and processively phosphorylated thereafter. We have followed the time course of appearance and disappearance of seven resolvable forms of phosphorylated FRQ (data not shown) and find no evidence for dephosphorylation and little support for a change in the rate of phosphorylation over the course of the day. Maximum phosphorylation invariably precedes turnover of FRQ (Figures 1 and 3; see also Discussion).

The surprising degree of polymorphism in the protein products of FRQ combined with the precedent of PER–TIM interaction in Drosophila (reviewed in Gekakis et al., 1995; Sehgal, 1995; Hunter-Ensor et al., 1996; Lee et al., 1996; Myers et al., 1996; Zeng et al., 1996) prompted us to examine nondenatured extracts to determine whether evidence of a clock protein complex involving one or both of the FRQ forms could be found. When extracts made at CT8 (the time of the peak in FRQ amount) were subjected to sucrose-gradient analysis, FRQ sedimented as a symmetrical peak displaying a size slightly smaller than amylase (200 kDa), with only part of the tail being detected in higher molecular weight fractions. However, when extracts from several times of day (or in vitro-translated FRQ) were analyzed by gel-filtration chromatography, FRQ was observed only in a single symmetrical peak at ca. 1 million daltons eluting well within the included volume, suggesting that the large size is unlikely to be due to nonspecific protein aggregation. In both methods, β-galactosidase (540 kDa) was used as an internal control; FRQ always eluted from the column ahead of β-galactosidase while in the sucrose gradient, the size of FRQ appeared smaller than β-galactosidase. To clarify this discrepancy, sucrose-gradient fractions containing FRQ were collected and immediately subjected to gel filtration; FRQ again displayed a column fractionation behavior consistent with a large stokes radius (data not shown). These results suggest that the elution profile of FRQ in gel filtration is probably due to a distinctly nonglobular shape and that FRQ is probably not typically associated with a large protein complex, but rather exists as a monomer in the cell.

Discussion

Previous work on *frq* has focused on transcriptional control in the oscillator feedback loop. This has established that *frq* encodes components of the circadian oscillator, that the rhythmic expression of *frq* (as distinct from simple constitutive expression) is essential for overt organismal rhythmicity (Aronson et al., 1994a), and that light resetting of this and potentially all ''day-phase'' oscillators is mediated by light-induced transcriptional activation of a central clock component (Crosthwaite et al., 1995; Hastings, 1995). Here, we have identified novel aspects of posttranscriptional regulation of *frq* and its products, both translational regulation in determining when and what type of FRQ is made, and posttranslational processing of both forms of FRQ through phosphorylation. Consistent with data from a reconstructed cycle (Merrow et al., 1997), negative feedback of FRQ to reduce its own transcript level appears rapid and efficient, so that most of the circadian day is devoted to posttranscriptional processing of FRQ prior to its turnover (Figure 4). That *frq* mRNA levels do not rise substantially until around CT22 (when FRQ disappears) suggests that all through the day the various phosphorylated forms of FRQ are effective at maintaining depression of *frq* transcript levels, in addition to executing any other functions they may have. There is solid evidence for posttranslational regulation of clock components in other systems (including protein–protein association and phosphorylation) (reviewed in Hardin and Siwicki, 1995; Loros, 1995; Sehgal, 1995), and these data combined with recent advances in understanding the regulation of *frq*—the generally low levels of *frq* transcript

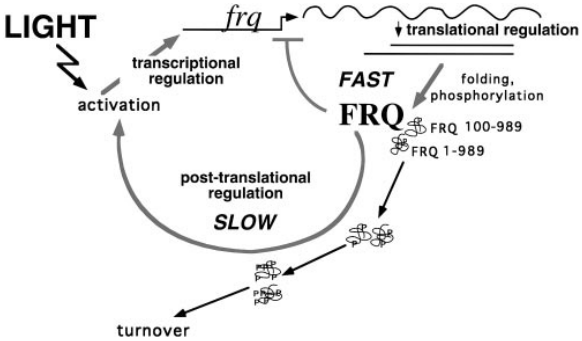

Figure 4. Diverse Aspects of Regulation Contribute to the Neurospora Circadian Cycle

frq transcript is made and, subject to regulation, translated to yield two forms of FRQ protein (Liu et al., 1997); negative feedback of FRQ to reduce its own transcript level is then rapid and efficient. This is followed by posttranslational processing of FRQ to yield multiple forms having potentially different activities, which occupies most of the day and ends in the turnover of both forms of FRQ and the reaccumulation of *frq* transcript. Accumulation of *frq* transcript can be independently promoted by light, thereby resetting the cycle (Crosthwaite et al., 1995).

throughout most of the day, the speed and effectiveness of the negative feedback exerted by FRQ (Figure 1) (Merrow et al., 1997), the possibility that phosphorylation may control FRQ turnover (see also below), and the demonstrated importance of posttranscriptional processes to at least some temperature responses (Liu et al., 1997)—suggest that the actual kinetics of the circadian oscillator feedback loop and part of its interface with the environment may be largely determined posttranscriptionally.

In its role in the clock, FRQ negatively regulates, either directly or indirectly, the level of *frq* mRNA (Aronson et al., 1994a), and in this light the profiles for *frq* mRNA, FRQ protein, and FRQ phosphorylation are noteworthy. First, and most obvious, is the fact that both RNA and protein cycle. That clock gene products must cycle for the clock to run has recently been questioned, based on the observation of noncyclic expression of the clock genes *per* and *tim* in the embryonic silkmoth brain (Hall, 1996; Sauman et al., 1996). However, given the robust cyclic expression of *frq* RNA (Aronson et al., 1994a) (and of *per* and *tim* in the fly and adult silkmoth [reviewed in Hall, 1996]), the cyclic expression of FRQ seen here is not a surprise. Altogether, it seems likely that cyclic expression of clock components will indeed be the rule.

Second, a significant decline in *frq* mRNA occurs before maximum FRQ levels are reached, resulting in a lag of ca. 4 hr between the peaks of *frq* mRNA and FRQ and suggesting that FRQ fulfills one biochemical function early in the subjective day before maximal FRQ protein accumulates and well before FRQ phosphorylation has peaked. The lag between *frq* RNA and protein expression is similar to that seen with *per* and *tim* in the fly (reviewed in Reppert and Sauman, 1995). Here again, the silkmoth data is the exception in that even during colocalized expression of *per* and *tim* in the adult moth brain, the rise in *per* mRNA does not phase lead the expression of either PER or TIM (Sauman and Reppert, 1996). These silkmoth data prompted the suggestion (Hall, 1996) that when there is no lag in expression, PER and/or TIM might be functioning in output rather than in the oscillator itself. In our data, the timing of FRQ autoregulatory activity is coincident with the decline in mRNA amounts of several morning-specific clock-controlled genes, including *ccg-1* and *ccg-4* (Bell-Pedersen et al., 1996), consistent with FRQ directly or indirectly regulating the expression of these clock-output genes in addition to functioning in the clock.

A third aspect of the cyclic expression of *frq* is its phase: FRQ is made in the late night through midday (between about CT22 and CT10), unlike PER and TIM, which are made in the early night (betwen ca. CT10 and CT18) (Siwicki et al., 1988; Edery et al., 1994; Hunter-Ensor et al., 1996; Lee et al., 1996; Myers et al., 1996; Zeng et al., 1996). These phases are of interest in the context of the RNA and protein synthesis inhibitor studies that represent one of the most universally similar characteristics of rhythms physiology from unicells through mammals; virtually all of the common pharmacological evidence from microbial, invertebrate neuronal, and vertebrate (including mammalian) pacemakers is consistent with an oscillator in which clock-critical protein synthesis is confined to a period between CT21 and CT9 (reviewed in Takahashi et al., 1989; Takahashi, 1995; Dunlap, 1996). Finally, it is of note that *frq* mRNA levels rise and fall relatively rapidly (Figures 1B and 1D), whereas FRQ is present throughout most of the day. This is unlike the case for the proteins associated with the best-understood cellular cycle, the cell cycle, where stage-specific cyclins appear and disappear during specific parts of the cycle (Nasmyth, 1996). Had FRQ been seen rapidly to turn over such that both *frq* transcript and FRQ were absent for most of the cycle, one could have predicted with certainty the existence of additional components in the clock loop. Instead, the data suggest that other components may be required only in as much as they participate in the cyclic events associated with FRQ synthesis and turnover.

One theme to emerge from this study is that several aspects of posttranscriptional regulation contribute to the formation of temporally distinct, biochemically distinguishable, and potentially functionally distinct FRQ proteins. Indeed, time of day can be read from the amount, apparent size, and extent of modification of FRQ. First, there is alternative initiation of translation (Figure 2) that results in the production of two distinct FRQ polypeptides. As precedents, differential use of in-frame initiation codons is known to result in the differential synthesis of transcriptional-activator versus -inhibitor proteins in the vertebrate brain (Descombes and Schibler, 1991), and choice of alternate translational start sites can yield differences in stability or subcellular localization of fibroblast growth factor (e.g., Bugler et al., 1991). The mRNAs encoding FRQ have unusually long 5′ untranslated regions ($\sim$1.5 knt) that contain six upstream open reading frames (uORFs), one of which has an excellent consensus for translational initiation. Thus, it seems possible that the choice of the form of FRQ could be governed by translational control and that the regulation of this choice is important for the operation of the clock under some environmental conditions (Liu et al., 1997). Interestingly, however, data suggest that regulation mediated by the the uORFs is not required for the generation of the rhythm itself, since deletion of all 6 uORFs eliminates neither the 4 hr lag between *frq* transcript and FRQ peaks nor overt rhythmicity in the organism (N. Y. G. and J. C. D., unpublished data). Although there are no precedents for translational regulation in the context of other clock proteins, it is possibile that this is more widespread in the regulation of circadian cycles. For instance, the principle transcript of the *per* gene contains a long 5′ untranslated region arising in part from an untranslated first exon (Citri et al., 1987) that contains uORFs, and in different Drosophilids the clock protein TIM utilizes alternative initiating methionines (Rosata et al., 1997). In the context of clock output, alternative initiation of translation results in the production of alternative forms of the circadianly regulated LBP protein in Gonyaulax (Mittag and Hastings, 1996).

In a second and distinct form of posttranscriptional and posttranslational regulation, we have found FRQ to be highly and progressively phosphorylated over the day (Figure 3). A general requirement for phosphorylation of some clock proteins has been suggested based on inhibitor studies (e.g., Comolli et al., 1994), and data similar to that shown here for FRQ (albeit necessarily ca. 180° out of phase) exist for PER and TIM (Hunter-Ensor et al., 1996; Lee et al., 1996; Myers et al., 1996;

Zeng et al., 1996). Clues to the functional relevance of clock-protein phosphorylation are based on precedents from other systems and include regulation of intracellular localization (as with B-type cyclins; Pines and Hunter, 1994) perhaps through association with other proteins (as in Cactus/Dorsal or NF-κB/IkBa; Karin and Hunter, 1995), or turnover (as with Far1p; Chang and Herskowitz, 1992). In Neurospora, the only data speaking to the function of FRQ phosphorylation are correlative and point to the last of these, i.e., turnover. The amount and degree of phosphorylation of FRQ have been monitored following drug treatments or transfer from constant light to darkness, conditions that result in the turnover of FRQ. In all cases, FRQ becomes highly phosphorylated prior to its disappearance just as observed here in the normal cycle (data not shown); minimally phosphorylated forms are never seen to disappear without the concomitant appearance of highly phosphorylated forms that then disappear (Liu et al., 1997). Since FRQ acts to depress the level of *frq* transcript (Aronson et al., 1994a), it is likely that FRQ must be translocated to the nucleus as a step in each cycle, an event that could be influenced by the phosphorylation reported here. However, when the circadian cycle is reconstructed in vivo in a *frq*-null strain by induction of *frq* from a heterologous promoter, FRQ depresses *frq* mRNA levels within just a few hours of its synthesis (Merrow et al., 1997); thus, nuclear entry and repression could involve only the minimally phosphorylated forms of FRQ. In this artificial system, phosphorylation proceeds at a normal rate in a cell without a *frq*-based clock, consistent with a constitutive kinase activity acting on FRQ. Although regulation of turnover appears most likely, additional studies will be required to establish firmly the function of FRQ phosphorylation.

Circadian rhythmicity is an extremely complex, albeit common, form of cellular regulation that displays a remarkable degree of precision under constant conditions. In Neurospora, the circadian oscillations in the abundance of *frq* mRNA and FRQ proteins, together with the translational and posttranslational regulation described here, provide a variety of facets where regulatory elements might influence the operation of the clock. Altogether, these elements must contribute to the precise operation and smooth synchronization that characterize biological timekeepers operating in single cells under different environmental conditions.

Experimental Procedures

Strains and Growth Conditions

Strains used were 30–7 (*bd*; frq_{+} *A*) and 93–4 (*bd*; frq^{10} *A*; *his-3*) (Aronson et al., 1994b). All *frq* constructs were introduced into the *frq*-null strain 93–4 and were targeted to the *his-3* locus.

The method for growing liquid cultures of Neurospora was described previously (Nakashima, 1981; Aronson et al., 1994a). Liquid medium contains 1× Vogel's Salts, 2% glucose, and 50 ng/ml biotin. For circadian liquid culture, disks of mycelia were cut from mats of mycelia, transferred to 50 ml of fresh medium in 125 ml flasks, and shaken at 100 rpm in light at room temperature and then transferred into darkness and harvested at the time indicated. The light-to-dark transition synchronizes the culture to circadian time 12 (CT12). The concept of circadian time facilitates the comparison of data from circadian clocks with different periodicities. Biological time is normalized by dividing the circadian cycle into 24 equal parts, i.e., circadian hours. By convention, CT0 is subjective dawn and CT12 is subjective dusk.

Generation of Anti-FRQ Antisera

The entire *frq* ORF (Aronson et al., 1994b) was cloned into the baculovirus expression vector pVL1393 under control of the baculovirus *polh* promoter to yield pNG39. Recombinant baculovirus in the Sf9 insect cell line was made (BaculoGold Transfection Kit, PharMingen, San Diego, CA), cells were infected with virus, and recombinant FRQ (BVFRQ) was expressed and purified on preparative 6% SDS–polyacrylamide gels according to published protocols (O'Reilly et al., 1992). Antiserum was generated in New Zealand white rabbits by East-Acres Biologicals (Southbridge, MA). Before use in Western blots, the antiserum was subjected to several rounds of adsorption against formaldehyde-treated extract prepared from a *frq*-null strain (Gruber and Zingales, 1995).

In Vitro Translation of *frq*

pBA40 (Aronson et al., 1994a) was cut with BstXI and BstBI, the overhangs filled with T4 DNA polymerase, and the ends religated to yield pNG16 in which the *frq* ORF is under control of the T3 promoter. FRQ protein was synthesized using the TnT Coupled Reticulocyte Lysate System (Promega).

Site-Directed Mutagenesis of AUGs in the *frq* ORF

The AUGs at positions #1 and #11 were eliminated together by digesting pKAJ120 with DraIII and SphI, filling the ends with T4 DNA polymerase, and religating with T4 DNA ligase to generate pYL15. pKAJ120 contains an 8.6 kb ClaI fragment of DNA spanning the *frq* locus in the *his-3*-targeting plasmid pDE3 (Ebbole and Sachs, 1990). The digestion removed ca. 920 nt of the coding sequence for the 5′ UTR plus 33 nt of the ORF through the second AUG at codon #11. The third AUG at position #100 was converted to an aspartate codon (GAU) using PCR to generate pJC101. One reaction was performed using oligonucleotides JCC3 (TGACGAGAGCCATATC**G AT**GAAGTCGATCCTCCTT; coordinates +282 to +316) and JCC6 (ACACCCAGCGTTCCGCATCC; coordinates +1433 to +1452). A second reaction was performed using oligonucleotides JCC4 (AAGG AGGATCGACTTC**ATC**GATAUGGCTCTCGTCA; coordinates +316 to +282 on antisense strand) and JCC5 (CGGTCTGCGGCTTCCAT AGT; coordinates −134 to −153 on antisense strand). The bold sequence corresponds to the three nucleotides that were substituted to generate a methionine to aspartate codon change. The two reactions were subjected to agarose-gel electrophoresis, and the resulting PCR products were mixed and subjected to a third round of the PCR using oligonucleotides JCC5 and JCC6. The PCR product from the third reaction was then digested with SphI and AflII and subcloned into pKAJ120 that had been digested with SphI and AflII. All three AUGs were eliminated by generating a 4 bp deletion in pJC101 to generate pYL31. The plasmid was digested with SphI, the ends of DNA were made blunt with T4 DNA polymerase, and the plasmid was ligated with T4 DNA ligase. This deletion placed the first AUG out of frame and eliminated AUG#2. Mutations in all three constructs were verified by DNA sequencing with the Taq Dyedeoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Inc.) using the ABI 373A automated sequencer. The three constructs (pJC101, pYL31, and pYL15) were introduced into the 93–4 *bd;*frq^{10}*;his-3 A* (*frq*-null) strain, and genomic DNA from the strains was analyzed by Southern blot to verify that the DNA corresponding to a single plasmid was inserted at the *his-3* locus. Extracts were prepared from cultures that were illuminated for 4 hr and analyzed by Western blot as described below.

Northern and Western Blotting Analysis

Northern blot analysis was performed as described before (Aronson et al., 1994a). For Western blot analysis, tissue was ground in liquid nitrogen with a mortar and pestle and suspended in ice-cold extraction buffer (50 mM HEPES [pH 7.4], 137 mM KCl, 10% glycerol containing 10 mM DFP, 1 mM EDTA, 1 μg/ml pepstatin A, 1 μg/ml leupeptin) at a ratio of 1 ml of buffer per 0.2 g tissue (wet mass). Equal amounts of protein (100 μg) per lane were subjected to 7.5% SDS–PAGE, transferred to PVDF membrane (Immobilon-P, Millipore) in 384 mM Glycine, 50 mM Tris (pH 8.4), 20% methanol at 400 mA

for 2.5 hr, and the membrane was then blocked with PBS, 5% milk, 0.3% Tween 20. Next, the membrane was probed sequentially with 1:3000 dilutions of anti-FRQ$^{1-989}$ and goat anti-rabbit IgG-horseradish peroxidase diluted in PBS, 5% milk, 0.3% Tween 20, and the blot was developed by chemiluminescence (ECL, Amersham). X-ray films were scanned with a Silver Scanner III equipped for transparency scanning, and densitometry was performed with NIH Image 1.59. For Northern blots, *frq* mRNA was normalized against 26S rRNA to account for loading differences between lanes as previously described (Crosthwaite et al., 1995). For Western blots, FRQ was normalized against total protein performed by densitometry of Coomassie-stained gels. The linearity of the densitometry for Coomassie-stained gels was verified with serial dilutions of extract.

Immunoprecipitation and Phosphatase Treatment

Anti-FRQ$^{1-989}$ (20 ml of a 1:3000 dilution) was incubated with protein A-agarose for 1 hr and washed 4 times with IP buffer (10 mM Tris [pH 8.0], 150 mM NaCl, 2% BSA, 1% Triton X-100). Next, 170 μg of protein per reaction was incubated with 50 μl of anti-FRQ-protein A-agarose for 1 hr at 4°C, washed once with 1 ml of ice-cold IP buffer, once with 1 ml of ice-cold IP wash (10 mM Tris [pH 8.0], 150 mM NaCl, 1% Triton X-100), and once with 50 μl of Phosphatase buffer (50 mM Tris [pH 7.8], 2 mM $MnCl^2$, 5 mM DTT). The IP complex was then incubated in 50 μl of Phosphatase buffer alone, with 1000 U λPPase (New England Biolabs), or with 1000 U λPPase plus 20 mM sodium vanadate.

Sucrose-Gradient Analysis and Gel-Filtration Chromatography

For sucrose gradients, Neurospora extracts were prepared as described above; 200 μl extract (~2 mg of protein) mixed with 50 μg β-galactosidase was layered on the top of a 4 ml 10%–35% sucrose gradient made in protein-extraction buffer (see above) lacking the glycerol. BSA, amylase, and β-galactosidase were also used as the standards in another gradient. After centrifugation at 50,000 rpm for 14 hr in an SW60Ti rotor, 200 μl fractions were collected from top to bottom. The resulting fractions were then directly analyzed by Western blot. The fractions corresponding to the peak level of FRQ were also subjected to gel filtration chromatography.

For gel filtration chromatography of FRQ, Neurospora extracts were prepared from cultures grown in constant darkness for 17, 22, and 25 hr and in constant light for 4 hr. Approximately 1.5 mg (0.2 ml) of protein was subjected to gel chromatography using Sephacryl S-400 (Pharmacia/LKB) in 50 mM Tris (pH 7.4), 150 mM KCl, and ca. 0.3 ml fractions were collected at a flow rate of 1.25 ml/min. The fractions were concentrated by precipitation in 13% trichloroacetic acid and analyzed by Western blot.

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