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Nuclear localization is required for function of the essential clock protein FRQ

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The *frequency* (*frq*) gene in *Neurospora* encodes central components of a circadian oscillator, a negative feedback loop involving *frq* mRNA and two forms of FRQ protein. Here we report that FRQ is a nuclear protein and nuclear localization is essential for its function. Deletion of the nuclear localization signal (NLS) renders FRQ unable to enter into the nucleus and abolishes overt circadian rhythmicity, while reinsertion of the NLS at a novel site near the N-terminus of FRQ restores its function. Each form of FRQ enters the nucleus soon after its synthesis in the early subjective day; there is no evidence for regulated sequestration in the cytoplasm prior to nuclear entry. The kinetics of the nuclear entry are consistent with previous data showing rapid depression of *frq* transcript levels following the synthesis of FRQ, and suggest that early in each circadian cycle, when FRQ is synthesized, it enters the nucleus and depresses the level of its own transcript.

Keywords: circadian rhythm/frequency/*Neurospora*/nuclear localization

Introduction

Circadian rhythmicity is a general aspect of regulation existing in a wide variety of organisms ranging from prokaryotes to eukaryotes (Dunlap, 1993, 1996). The cellular machinery that generates this capacity is known as the biological clock. Such clocks are intrinsic to the cell, are endogenous and self-sustaining under constant conditions, and respond to environmental cues such as light and temperature (Crosthwaite *et al.*, 1995; Hunter-Ensor *et al.*, 1996; Lee *et al.*, 1996; Zeng *et al.*, 1996; Liu *et al.*, 1997).

The products of the *frequency* (*frq*) gene are central components of the *Neurospora* oscillator (Dunlap, 1993, 1996; Loros, 1995), and aspects of their regulation have pointed to roles for transcription and translation in the clock. *frq* mRNA is expressed rhythmically with a period reflecting that of the overt rhythm. Constantly elevated expression of *frq* message from a heterologous inducible promoter eliminates overt rhythmicity in a wild-type background, and is unable to support rhythmicity in a loss-of-function strain (frq^9). Step reduction of *frq* mRNA resets the clock to a predicable phase. Elevated expression of *frq* mRNA from an inducible promoter rapidly depresses the level of the endogenous *frq* message (Aronson *et al.*, 1994a; Merrow *et al.*, 1997). These data place *frq* mRNA and FRQ protein in a negative feedback loop defining the circadian oscillator. *frq* mRNA accumulates very quickly (within 2 min) in response to short light pulses, and the threshold and kinetics of the response are similar to those of light-induced resetting (Crosthwaite *et al.*, 1995). Two FRQ polypeptides arise from the *frq* transcript, resulting from alternative initiation of FRQ translation from two in-frame initiation codons: codon #1 and codon #100 (Garceau *et al.*, 1997). Both FRQ forms are immediately and progressively phosphorylated after being synthesized (Garceau *et al.*, 1997). Temperature regulates the ratio of the two different FRQ forms by favoring different translation initiation sites at different temperatures and thus sets the physiological temperature limits for rhythmicity (Liu *et al.*, 1997).

The mechanism by which FRQ acts to depress the level of its own transcript or more generally contributes to clock function is unknown. This negative action may be achieved transcriptionally, post-transcriptionally or both. In the *Drosophila* system, a complex composed of the clock proteins PER and TIM is thought to regulate *per* and *tim* transcription through a negative feedback loop (Gekakis *et al.*, 1995; Myers *et al.*, 1996; Zeng *et al.*, 1996) to generate the circadian oscillation. Both PER and TIM are predominantly nuclear proteins in wild-type flies (Liu *et al*., 1992), and nuclear translocation is regulated by the dimerization between the two proteins (Saez and Young, 1995). Studies done in *Drosophila* cell cultures have shown that each protein contains sequences that confer cytoplasmic localization, and dimerization between them suppresses the cytoplasmic localization function (Saez and Young, 1995). Thus, both proteins accumulate in the cytoplasm following their translation, then enter into the nucleus in a concerted manner after this delay to execute their effect on transcription (Curtin *et al.*, 1995; Saez and Young, 1995). On the other hand, in the silkmoth *Antheraea pernyi*, the PER protein is nuclear in eye photoreceptor cells but is predominantly cytoplasmic in the brain cells thought to drive behavioral rhythm (Sauman and Reppert, 1995). *per* mRNA and PER protein oscillate in both cell types, and the predominantly cytoplasmic PER in the brain cells has suggested that the silkmoth may use a different mechanism to construct the molecular oscillator. In *Neurospora*, FRQ has several characteristics consistent with an involvement in transcription, including a putative helix–turn–helix DNA-binding domain, a nuclear localization signal (NLS) and highly charged regions (Aronson *et al.*, 1994b; Merrow and Dunlap, 1994; Lewis *et al.*, 1997). Both *frq* mRNA and FRQ oscillate, the peak of FRQ lags the peak of *frq* mRNA by 4–6 h, and *frq* mRNA levels begin to fall before the level of FRQ reaches

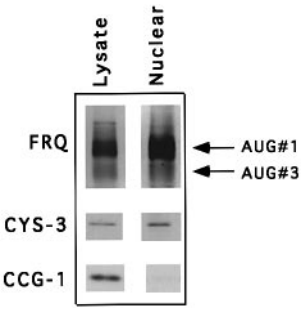

Fig. 1. Both forms of FRQ are found inside nuclei in *Neurospora*. Western blots show FRQ and two control proteins (CYS-3 and CCG-1) in total cell lysate and nuclear fractions. The two arrows point to the two different forms of FRQ resulting from two in-frame translational initiation sites in the same message. Both FRQ forms are detected in the nuclear fraction. There is no CCG-1 signal in the nuclear fraction, indicating that the isolated nuclei are free from cytosolic contamination.

its peak (Garceau *et al.*, 1997), suggesting that FRQ might execute its role quickly, probably very soon after being synthesized.

If FRQ functions at a transcriptional level in the feedback inhibition loop, it must be in the nucleus at least at certain times during a circadian cycle. To examine this, we adapted a cellular fractionation technique and determined the intracellular compartmentation of FRQ as a function of time of day. Our data demonstrate that FRQ is indeed a nuclear protein, and its nuclear location is essential for its function in the circadian clock. A single NLS located at amino acids 195–200 is necessary and sufficient for FRQ nuclear entry. This NLS is still functional when moved to a different site near the N-terminus of FRQ; it allows nuclear localization of the large form of FRQ alone and is sufficient to restore clock function. Both forms of FRQ move into the nucleus very soon after being synthesized and well before levels of FRQ peak or phosphorylation of FRQ has progressed very far. The absence of a noticeable delay prior to nuclear entry suggests that delay prior to nuclear localization contributes little to the long-term control of the circadian cycle, nor is it required to generate the long time constant of the circadian oscillator.

Results

Both forms of FRQ are found inside the nucleus

In order to study the subcellular localization of FRQ, we have utilized a biochemical method to isolate nuclei from *Neurospora*, resolved the nuclear fractions by SDS–PAGE, and detected FRQ by Western blot analysis. Two proteins were used as control markers in this method: CYS-3 and CCG-1. CYS-3 is used as the nuclear protein marker. It is a transcription factor involved in regulating structural proteins in the sulfur metabolism pathway in *Neurospora* (Fu *et al.*, 1989; Paietta, 1992; Kanaan and Marzluf, 1993). CCG-1 is the product of *clock-controlled gene-1* (McNally and Free, 1988; Loros *et al.*, 1989). It is an abundant and exclusively cytoplasmic protein (Loros *et al.*, 1995; Garceau, 1996), and it is used as a cytoplasmic protein marker in this study. The two protein markers were used to show the quality of the isolated nuclei, specifically to verify the absence of cytoplasmic contamination in the nuclear preparation (Figure 1).

It is known that two different forms of FRQ arise from two in-frame AUGs in the *frq* transcript generating ~135 and ~145 kDa proteins. Each form of FRQ is phosphorylated progressively after being synthesized (Garceau *et al.*, 1997). Biochemically isolating nuclei and detecting where FRQ is has the advantage of providing information on how these different FRQ forms behave. Figure 1 shows the results of Western analysis of FRQ, CYS-3 and CCG-1 from a mid-subjective morning sample (DD15, see Materials and methods) in the total cell lysate and nuclear fractions. CYS-3 was present in both lysate and nuclear fractions, and its signal was enriched ~3- to 4-fold in the nuclear fraction compared with that in the lysate fraction. This is consistent with incomplete nuclear localization combined with breakage of nuclei upon isolation. CCG-1 was present exclusively in the lysate fraction. The results from the two protein markers confirm that the isolated nuclear fraction has no cytoplasmic contamination. FRQ, like CYS-3, was found in the nuclear fraction (FRQ nuclear panel), suggesting it is a nuclear protein. Its signal is enriched in the nuclear fraction, and the fold enrichment is similar to that of the nuclear marker protein CYS-3. In addition, both large and small FRQ forms are found in the nuclear fraction (as denoted by the two arrows in Figure 1).

Deletion of the NLS from FRQ eliminates nuclear localization and abolishes overt circadian rhythmicity

To determine whether the nuclear localization of FRQ is required for its function, we identified and deleted the NLS of FRQ. Two putative SV40 large T antigen-type NLSs located at amino acids 194–199 and 564–568 are predicted from computer analysis of FRQ's amino acid sequence (Aronson *et al.*, 1994b; Merrow and Dunlap, 1994; Lewis *et al.*, 1997). The first one is phylogenetically more conserved among *frq* homologs (Lewis *et al.*, 1997). Constructs bearing deletions of each NLS separately and a double deletion of both were made (Figure 2A), and targeted by transformation to the *his-3* locus in the frq^{10} null strain (strain #93-4) (Aronson *et al.*, 1994b). A putative NLS (RKKRK) starting at amino acid 564 was deleted in construct pCL7; the frq^{10} transformants bearing this construct still had apparently normal nuclear localization of FRQ and retained the overt circadian conidial banding rhythm (data not shown). In construct pCL10, a putative NLS (PRRKKR) beginning at amino acid 194 was deleted. Transformants bearing this construct produced a normal amount of FRQ, but almost no FRQ was detected in the nuclear fraction (Figure 2B and C), suggesting that this region is the important NLS directing FRQ into the nucleus. To assess the functional significance of this mutation on the action of FRQ in the oscillator, the overt circadian rhythm in these transformant strains was examined in race tubes (Figure 3A). If the nuclear localization of FRQ is important for its function, we would expect that this construct could not rescue the loss of circadian rhythmicity in the frq^{10} (null) strain. As expected, this NLS-deleted *frq* failed to support the overt conidiation rhythm (compare pCL10 with pKAJ120 race tubes). Aside from the loss of rhythmicity, the growth rate and general morphology were not altered in the pCL10 transformants, suggesting that no other major defects were associated

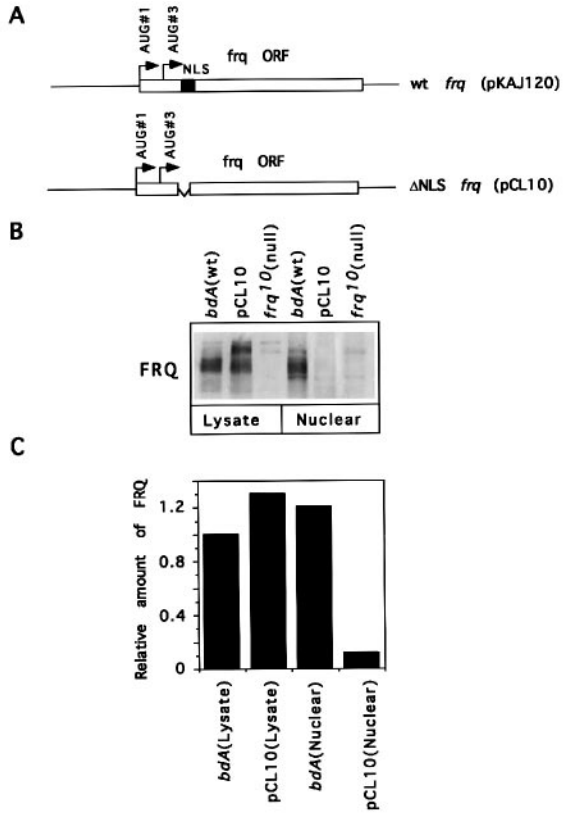

Fig. 2. An NLS located between amino acids 194 and 199 is required for FRQ nuclear localization. (**A**) A schematic diagram of the ΔNLS construct. The open box represents the *frq* ORF, and arrows mark the positions of the methionines initiating the large and small FRQ translation products. The black box marked NLS corresponds to amino acids 194–199. (**B**) Western blot of FRQ in total lysate and nuclear fractions of three different strains: *bdA* (wild-type, frq^{+}), pCL10 transformant (ΔNLS construct) and frq^{10} (*frq* null). frq^{10} served as a negative control. In the nuclear fraction in the pCL10 transformant, the amount of FRQ is reduced significantly. (**C**) Densitometry of the amount of FRQ in different fractions in each strain shown in (B). Levels of FRQ are normalized against the FRQ level in the lysate fraction in the *bdA* (frq^{+}) strain at DD15.

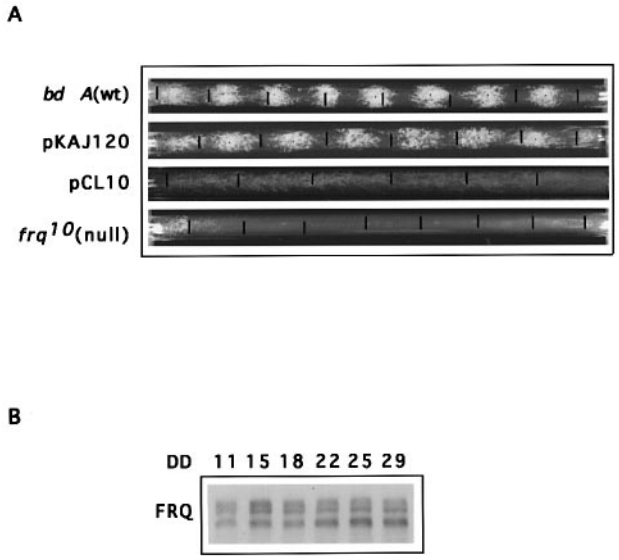

Fig. 3. Deletion of the NLS in FRQ abolishes overt circadian rhythmicity. (**A**) Race tube analysis showing the banding rhythm in different strains at 25°C: wild-type (frq^{+}), a transformant bearing an intact *frq* locus (pKAJ 120), a transformant bearing the NLS deletion construct (pCL10) and the frq^{10} strain (null). No conidial banding rhythm is found in the pCL10 transformant. Cultures were inoculated at the left ends of race tubes, incubated in constant light for 1 day and transferred into darkness at 25°C. The growth front (vertical lines) was marked at 24 h intervals thereafter. (**B**) Western blot of FRQ from ΔNLS strain pCL10 over time points spanning a circadian cycle. Both the amount and the phosphorylation states of FRQ remain unchanged over time, indicating an absence of rhythmicity.

with this mutation. In confirmation of the race tube data, neither *frq* mRNA nor FRQ showed any rhythmicity in pCL10 transformants, as shown in Figure 3B. Interestingly, both the amount and the phosphorylation states of FRQ remained relatively unchanged across different time points spanning a circadian cycle in the pCL10 transformants. The observation that the phosphorylation states remain the same over time is similar to the behavior of wild-type FRQ under constant light conditions (N.Y.Garceau, J.J.Loros and J.C.Dunlap, in preparation). In both cases, there is no circadian oscillation in the organism. This suggests that the phosphorylation of FRQ is coordinated with the circadian cycle. Together, these data show that it is necessary for FRQ to enter into the nucleus to generate both molecular and overt rhythmicity.

Insertion of the NLS near to the N-terminal region of FRQ restores its function in the clock

To test if the nuclear localization of FRQ could be restored and be sufficient for its function in the clock, the NLS and its surrounding region (amino acids 193–208, including the six amino acids extending from 194 to 199) was inserted into the N-terminal region of ΔNLS construct pCL10 (Figure 4A). A putative casein kinase II phosphorylation site (T^{204}LRD) was included in the 16 amino acid sequence, since it has been reported that phosphorylation at a CKII site close to an NLS can enhance the rate of nuclear translocation (Jans and Jans, 1994). This construct (pCL11) was transformed into the frq^{10} null strain (strain #93-4) at the *his-3* locus, and the transformants were examined for nuclear localization of FRQ and for overt rhythmicity. Theoretically, NLSs can be located anywhere in a protein's primary structure, provided that after protein folding, the NLS is on the surface of the protein (reviewed by Garcia-Bustos *et al.*, 1991; Jans and Hubner, 1996). The insertion was made into an *Sph*I site near the 5′ end of *frq*'s open reading frame (ORF), just 11 amino acids after AUG#1 (Garceau *et al.*, 1997). We chose to insert the putative NLS near to the N-terminus of FRQ since structural predictions place this on the surface of the protein and this insertion also provides a convenient internal control in the subsequent analysis. Specifically, insertion of the NLS at residue 11 should support nuclear localization of the large form of FRQ but not the small form which arises from alternative translation initiation at amino acid 100. This insertion is not likely to destroy any essential functional domain(s) in FRQ since small FRQ alone has been shown to be sufficient for establishing rhythmicity (Liu *et al.*, 1997).

Reinsertion of the NLS restored nuclear localization of the large form of FRQ. Figure 4B shows the Western blot results of total cell lysate and nuclear fractions of strains *bdA* (frq^{+}), pCL11 (N-terminal NLS insertion construct) and pCL10 (NLS deletion construct) at DD15. All three

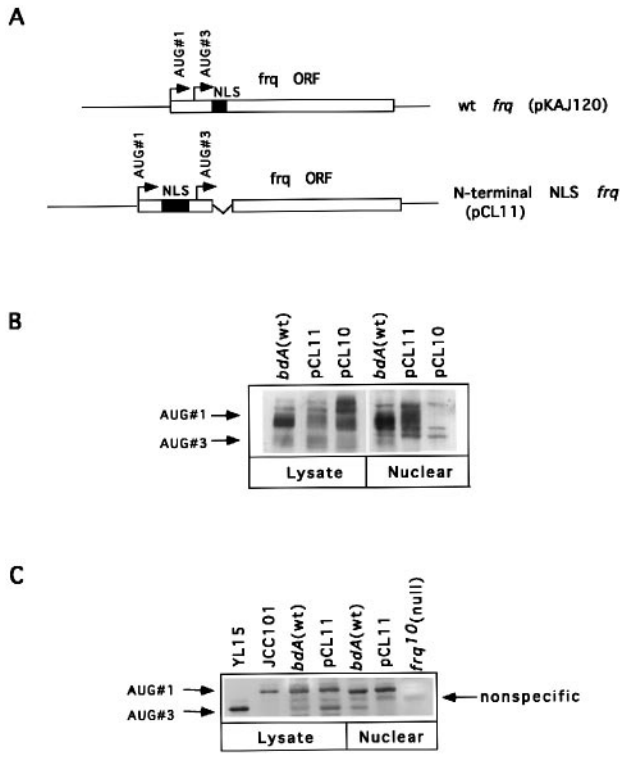

Fig. 4. Insertion of the NLS at a different position in ΔNLS FRQ directs the FRQ protein to enter the nucleus. (**A**) Schematic diagram of the constructs (see Figure 2 and text for details). (**B**) Western blot of FRQ in the total cell lysate and nuclear fractions of different strains: *bdA* (wild-type, frq^{+}), pCL10 (ΔNLS) transformant and pCL11 (NLS insertion) transformant. Both forms of FRQ shown by the two arrowheads were found in the total lysate in all three strains. In the nuclear fractions, both FRQ forms are detected in the wild-type strain but only the large FRQ form is detected in the pCL11 transformant, and no FRQ is detected in the pCL10 transformant. (**C**) Western blot of total cell lysate fractions and nuclear fractions together with controls, subjected to λ phosphatase treatment. JCC101 bears a disruption at the third AUG and YL15 bears a disruption at the first AUG; they were used as controls for strains making large and small FRQ, respectively. Both FRQ forms were detected in the wild-type and pCL11 transformants (wt, pCL11 lysate), while only large FRQ is found in the nuclear fraction from the pCL11 transformant.

strains synthesized both FRQ forms (Figure 4B lysate panel, as pointed out by the arrowheads). Both FRQ forms were inside the nucleus in the *bdA* (frq^{+}) strain. FRQ was found inside the nucleus of strains bearing construct pCL11 (pCL11 nuclear panel), confirming that the NLS can direct FRQ into the nucleus despite the altered position. In addition, only the large FRQ form was found in the nucleus; this is in agreement with our prediction, since only the large FRQ has the reinserted NLS. The progressive phosphorylation of FRQ described above results in a series of FRQ bands that tend to obscure the appearance of protein bands on the gels. In order to generate a visually clearer result, total cell lysate and the nuclear fraction were treated with λ phosphatase (Garceau *et al.*, 1997) to convert all different FRQ forms (arising from different levels of phosphorylation) into just the two bands corresponding to large and small FRQ. The result is shown in Figure 4C. JCC101 and YL15 are strains that make only large and small FRQ, respectively, since they bear a genetically engineered knock-out of the third AUG (JCC101) and first AUG (YL15) (Liu *et al.*, 1997). In

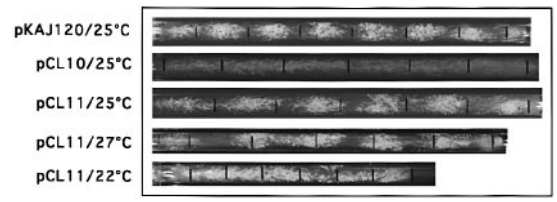

Fig. 5. Insertion of the NLS into the N-terminal region of ΔNLS FRQ pCL10 restores a functional clock. Race tube analysis showing the overt conidial banding rhythm of transformants bearing a wild-type *frq* locus (pKAJ120), the NLS deletion (pCL10) and the NLS insertion (pCL11) constructs. Race tubes were inoculated and incubated in constant light for a day before being transferred into constant darkness at different temperatures. The rhythm was restored in the pCL11 transformant; rhythmicity was apparent at 25°C and at a higher temperature (27°C), but the rhythm was lost at a lower temperature (22°C).

Table I. Period length in different strains

Strains	Period (h ± SD)
bdA/25°C	21.2 ± 1.3
pKAJ120/25°C	23.6 ± 1.1
pCL10/25°C	ND
pCL11/25°C	24.8 ± 1.5
pCL11/27°C	24.6 ± 0.7
pCL11/22°C	ND
frq^{10}/25°C	ND

Figure 4C, both FRQ forms were in wild-type and pCL11 lysate fractions, but only the large form of FRQ was found in the pCL11 nuclear fraction. The faint nuclear-enriched band sandwiched between the two FRQ bands is nonspecific, since it is recognized by the antiserum in the frq^{10} (null) strain also.

Reinsertion of the NLS also restores the function of the large form of FRQ. Transformation of pCL11 into a *frq* null strain rescued the clock null phenotype (Figure 5; Table I), suggesting that once FRQ gets into nuclei it can function normally. It has been shown that the small FRQ form is important for the clock to function at the lower end of the physiological temperature range (Liu *et al.*, 1997). Thus, a reasonable prediction is that pCL11 should support rhythmicity at higher temperatures but not at lower temperatures since only the large FRQ enters the nucleus in this strain. This prediction was proved to be correct: at 27°C the clock was functional in pCL11 transformants while at 22°C the clock no longer operated (Figure 5, pCL11 race tubes at 27°C versus 22°C). Data shown in Table I confirm that the period lengths of the rescued strains were comparable with those rescued by wild-type *frq*, again confirming normal function of large FRQ with a displaced NLS.

The amount of FRQ in nuclei cycles with a peak amount at CT4

In order to address the behavior of FRQ in terms of cellular localization during a whole circadian cycle, a quantitative analysis of FRQ in the nuclear extract was performed. Liquid cultures were grown in constant light at room temperature before being transferred into constant darkness at 25°C. The cultures were harvested at 4 h intervals after the light to dark transfer. Nuclei were isolated from samples at different circadian time points

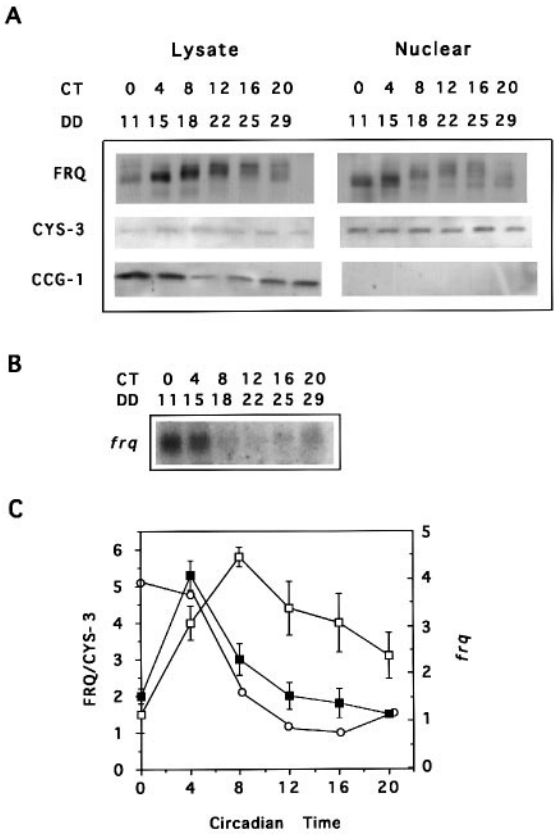

Fig. 6. Nuclear cycling of the FRQ protein. (**A**) Western blots of FRQ, CYS-3 and CCG-1 during a circadian cycle. In the total cell lysate (left panel) and nuclear fractions (right panel), CYS-3 protein levels are constant throughout the circadian cycle. CCG-1 is a clock-controlled protein, peaking in the subjective morning (CT0). No CCG-1 is detected in the nuclear fraction at any time. FRQ cycles in both fractions, but the phases are different. The amount of FRQ in the nuclear fraction peaks at DD15 (CT4) while the amount of FRQ in the total cell lysate fraction peaks at DD18 (CT8). These data are representative of five experiments. (**B**) Northern blot of *frq* mRNA in a wild-type (frq^+) strain over a circadian cycle. (**C**) Densitometric analysis plotting the amount of FRQ normalized against the time-invariant protein CYS-3 in total cell lysate (□), nuclear fraction (■) and *frq* mRNA (○) over a circadian cycle. The nuclear cycle is phase-advanced with respect to that of the total cycle by ~4 h, and it has a similar phase to that of the mRNA cycle. The data are plotted as mean ± 2 SEM (n=5).

spanning a whole cycle, and the total cell lysate and nuclear fractions were subjected to Western blot analysis. Western blot results for FRQ, CYS-3 and CCG-1 are shown in Figure 6A. CYS-3 is a constitutively expressed protein; the amount of CYS-3 at different circadian times remained unchanged in both lysate and nuclear fractions (Figure 6A, CYS-3 lysate and nuclear panel). For the clock-controlled protein CCG-1, the amount of protein cycled over the circadian cycle with a peak at subjective morning, confirming that the cultures are normally rhythmic (Figure 6A, CCG-1 lysate panel). There was no CCG-1 signal detected in the nuclear fractions at any time point (Figure 6A, CCG-1 nuclear panel). In the total lysate fractions, FRQ showed the characteristic mobility shifts resulting from progressive phosphorylation over the circadian cycle, and the amount of the protein peaked at CT8 as previously reported (Garceau *et al.*, 1997; Figure 6A, FRQ lysate panel). In the nuclear fractions, FRQ was present at all time points and, especially at early stages of the circadian cycle (CT0), significant amounts of FRQ were detected in the nuclear fraction (nuclear panel CT0). This suggests that once FRQ is synthesized, it rapidly enters the nucleus with essentially no delay. Consistent with this, the maximal amount of FRQ inside nuclei is at CT4, earlier than that in the total cell lysate, and, at CT0, when there is little FRQ detected in the total lysate, FRQ accumulates to a significant level inside the nucleus. Although the nuclear FRQ distribution profile is phase-advanced with respect to the total cell lysate, it is very similar to that of *frq* mRNA, which normally peaks at CT4 (Crosthwaite *et al.*, 1995); this is apparent comparing Figure 6A, nuclear panel, with Figure 6B (see also Garceau *et al.*, 1997), although the *frq* RNA level at CT0 was abberantly high in this series. Since it has been reported that different phosphorylation forms of the same protein may be extracted differently during nuclei isolation (Mittnacht and Weinberg, 1991; Templeton *et al.*, 1991), we examined the effect of salt concentration on the apparent cellular localization of FRQ. Nuclei were isolated under different salt concentrations (0 and 200 mM NaCl), and the nuclear FRQ profiles were the same (data not shown), indicating that different FRQ phosphorylation forms are not artificially producing an apparent change in localization. Figure 6C plots the amount of FRQ normalized against the time-invariant protein CYS-3 and the amount of *frq* mRNA over a circadian cycle. It is clear that the amount of FRQ oscillates in a circadian manner in both lysate and nuclear fractions, although the phase of each cycle is different: the peak amount of FRQ inside the nucleus occurs at CT4 and has a similar phase to that of the *frq* mRNA cycle, peaking ~4 h before the total amount of FRQ peaks in the cell (CT8).

Discussion

It has been well established that *frq* mRNA and FRQ protein, the central components of a negative feedback loop, are defining elements of the *Neurospora* circadian clock. When FRQ is induced from a heterologous inducible promoter, the level of endogenous *frq* mRNA is depressed (Aronson *et al.*, 1994a; Merrow *et al.*, 1997). Nevertheless, many details of how this negative feedback loop works are poorly understood. Data presented here clearly demonstrate that both the large and small forms of FRQ, arising from different translation initiation sites in the same message, are nuclear proteins. This result is consistent with FRQ acting transcriptionally to down-regulate its own message. The same NLS exists for both FRQ forms and, in the context of the rest of FRQ, this signal is necessary and sufficient to direct the FRQ protein into the nuclei. Previous results showed that rhythmic expression of *frq*, rather than simple constitutive expression, was essential for a functional clock (Aronson *et al.*, 1994a). We have demonstrated further here that in order to generate the rhythmic expression of *frq* mRNA and protein, FRQ must be transferred into the nucleus. When the NLS is deleted, FRQ does not enter the nucleus and both molecular and overt rhythms are abolished; when the NLS is put back at a different position in the protein sequence, the rhythm of the clock is restored.

At all the time points tested during a circadian cycle, some FRQ is always found inside the nucleus. Particularly

at early stages of the circadian cycle such as CT0 and CT4, a significant amount of FRQ has accumulated in the nucleus, suggesting that FRQ enters the nucleus very quickly after being synthesized. If FRQ executes its negative effect on its own transcription while inside the nucleus, then it could repress transcription and reduce transcript levels within just a few hours, consistent with previous data (Merrow *et al.*, 1997). In this study, we showed that the amount of FRQ not only cycles in the total cell lysate but also cycles within the nucleus. The nuclear cycling is phase-advanced with respect to that of the total cell lysate, but has a similar phase to that of *frq* mRNA. This is consistent with a model in which once FRQ is made, it enters the nucleus and represses its own transcription, resulting in a significant decline in *frq* mRNA before maximum FRQ levels are reached. This early negative effect executed by FRQ would help to generate the phase lag between total FRQ and *frq* message previously observed (Garceau *et al.*, 1997).

These data are also consistent with the rapid kinetics of FRQ negative feedback as reported using a frq^9 strain, that is the negative feedback itself occurs very rapidly (Merrow *et al.*, 1997). In a frq^9 background, *frq* mRNA fluctuates around a high level and (because of a frameshift mutation) no functional FRQ is made (Aronson *et al.*, 1994a). If functional FRQ is induced from a heterologous promoter, a substantial decrease in frq^9 mRNA is observed within 3 h, and the repression is essentially complete by 6 h (Merrow *et al.*, 1997). These rapid repression kinetics are seen following induction of FRQ at or below normal physiological levels (Merrow *et al.*, 1997), so it is clear that amounts of FRQ lower than the peak are quite effective in establishing and maintaining depressed levels of *frq* mRNA. We have confirmed here that at least modest levels of FRQ remain in the nucleus throughout the subjective day and into the early evening. Depressed levels of *frq* mRNA are maintained until about the time when FRQ is turned over (Garceau *et al.*, 1997), when positive factor(s), possibly the factors encoded by the *wc* genes (Crosthwaite *et al.*, 1997), activate *frq* transcription to start the next cycle. It seems likely that all the different phosphorylation states of FRQ are effective in depressing *frq* transcript levels; all the different phosphorylation forms of FRQ are found inside the nucleus, *frq* mRNA decreases significantly when hypo-phosphorylated FRQ accumulates in the nucleus (in the early time points CT0, 4), and the message remains very low later in the circadian cycle when only more highly phosphorylated forms of FRQ are found in the nucleus. Given the nuclear localization of FRQ, its apparent action in depressing the level of its own transcript, and the reported weak similarity to a helix–turn–helix DNA-binding domain (Lewis *et al.*, 1997), it will be of interest to test FRQ's ability to bind to DNA, especially to *frq*-specific sequences.

It is noticeable that the overt rhythm rescued by construct pCL11 (NLS insertion construct) is not as tight and clean as that rescued by a wild-type *frq* construct (compare race tubes pKAJ120 and pCL11 at 25°C in Figure 6). There are two possible reasons for this: first, this NLS originally resides in a phylogenetically conserved region in the *frq* amino acid sequence (Merrow and Dunlap, 1994; Lewis *et al*., 1997), so it is possible that this deletion may have a general non-specific effect on FRQ function or stability. Additionally, we have shown previously (Liu *et al.*, 1997) that strains expressing only one of two forms of FRQ exhibit rhythms of reduced quality such as that seen here. Although both FRQ forms are expressed here, only large FRQ enters the nucleus and hence it may be that only it is active.

Among the systems used for clock research, *Neurospora* and *Drosophila* are the best characterized. A common theme arising from years of molecular research is that both oscillators are transcription–translation-based negative feedback loops, i.e. the clock proteins (FRQ in *Neurospora*, PER and TIM in *Drosophila*) enter the nucleus to down-regulate their own transcript levels. However, the mechanisms through which these proteins achieve negative feedback have seemed somewhat different: in *Neurospora* there appears to be little temporal lag between the appearance of *frq* RNA, its translation and the movement of FRQ into the nucleus in that total *frq* RNA and nuclear FRQ protein both peak around CT4. FRQ appears to execute its function in reducing *frq* mRNA levels rather quickly, beginning before peak nuclear FRQ levels are reached, with the result that *frq* mRNA levels decline significantly before total cellular FRQ levels peak at around CT8. Thus the lag between total *frq* RNA and protein levels simply reflects the well-known lag that accompanies the translation of proteins with half-lives of even a few hours (Wuarin *et al.*, 1992; Wood, 1995). Later in the cycle, some FRQ remains in the nucleus to keep *frq* transcript levels reduced till the proteins turn over completely. In contrast to *Neurospora*, in *Drosophila* there is reported to be a controlled lag between the synthesis/accumulation of PER in the cytoplasm and its translocation to the nucleus (Curtin *et al.*, 1995). In this view, the regulation of PER nuclear entry, as distinct from PER accumulation, was seen as playing an essential role in clock dynamics by providing the necessary temporal delay between PER synthesis and its effect on transcription. However, *per* mRNA levels peak at CT (or ZT) 14 (Zeng *et al.*, 1994) and have declined significantly before ZT18 when sequestered PER was seen as entering the nucleus (Curtin *et al.*, 1995); it thus seems likely that PER has executed its negative function well before ZT18. Hence, accumulation of nuclear PER–TIM after ZT14 is probably not contributing to delays in RNA suppression. Instead, delays in the *Drosophila* clock cycle probably arise earlier in the cycle perhaps as a result of the requirement for TIM to stabilize PER (Vosshall *et al.*, 1994; Price *et al.*, 1995). PER and TIM would be seen as accumulating in the cytoplasm until they form a heterodimer, an action that both stabilizes PER and masks cytoplasmic localization domains on each protein, thereby promoting translocation into the nucleus prior to the proteins' effect on transcription (Saez and Young, 1996). In this view, rising *per* RNA keeps climbing because no PER monomers accumulate because TIM levels are not yet high enough to exercise a protective function or to take PER–TIM heterodimers to the nucleus. By CT14, low levels of PER/TIM can accumulate, and they immediately enter the nucleus and are effective in turning down transcript levels. Here then, PER instability and a requirement for heterodimerization to initiate RNA suppression collaborate to delay PER accumulation in the late subjective day and to give a system that accumulates enough

protein to ensure that once shut off, the genes stay off until mid-morning.

In contrast to the *Drosophila* story, the PER protein in the giant silkmoth *A.pernyi* is a predominantly cytoplasmic protein in brain neurons. Both *per* mRNA and PER cycle in amount in the silkmoth brain, but there is no significant phase difference between the two. A TIM homolog and a *per* antisense RNA were also found in the silkmoth brain. The fact that PER is a cytoplasmic protein in *A.pernyi* suggests the possibility that it uses a different, possibly post-transcriptional mechanism to achieve the molecular oscillation, maybe through the action of the antisense *per* RNA (Sauman and Reppert, 1996; reviewed by Hall, 1996). These results demonstrate that although similar molecular components (PER, TIM and *per* mRNA) exist in insects, the mechanism used to generate rhythms may be very different. Taken together among different organisms, the data suggest that clock proteins may use different approaches to achieve a similar result: a negative feedback essential for generating a circadian rhythm in the organism. What has been found with *Neurospora* FRQ in terms of cellular biochemistry is consistent with the expected nuclear action of clock proteins while again revealing another of the ways through which circadian oscillators operate.

Materials and methods

Strains, growth conditions and race tube assay

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 during transformation (Sachs and Ebbole, 1990).

Methods for growing rhythmic cultures have been published previously (Aronson *et al.*, 1994a; Crosthwaite *et al.*, 1995). Briefly, mycelial pads were inoculated into 500 ml of liquid culture and shaken at 110 r.p.m. in light at room temperature. The cultures were then transferred into darkness and harvested at the time indicated. The liquid culture contains 1× sulfate-free Vogel's salts, 2% glucose, 50 ng/ml biotin and 0.25 mM methionine. DD15 represents cultures held for 15 h in the dark prior to harvesting.

Race tube assay medium contains 1× Vogel's salts, 0.1% glucose, 0.17% arginine and 50 ng/ml biotin. Calculations of period length were done using the Chrono II version 9.3 (Dr Till Roenneberg, Ludwigs-Maximillian University, Munich).

Plasmids and Neurospora transformation

pKAJ120, which contains the entire *frq* locus, was the parental plasmid for all the constructs. pCL10 was constructed using the Transformer Site-Directed Mutagenesis kit from Clontech Laboratories, Inc. (Palo Alto, CA). The *Sph*I–*Eco*RV fragment from pKAJ120 was cloned into pUC19, and the resulting plasmid was used as the template for the *in vitro* mutagenesis. The mutagenic primer for making the NLS deletion was 5′-GAGCGTTGCCTCCAACTCTAAGCCATGTACCTTGATCTC-3′. The Trans Oligo *Nde*I–*Nco*I from the kit was used as the selection primer. The *Sph*I–*Bss*HII fragment from the resulting plasmid was then inserted into the *Sph*I–*Bss*HII-digested pKAJ120 to create pCL10. The pCL11 construct was made by cloning the PCR-amplified fragment containing the NLS into the *Sph*I site in pCL10. The primers used for PCR amplification are 5′-ACATGCATGCGCTTACCTAGAAGA-AAG-3′ and 5′-ACATGCATGCGAAAGTCGCGGAGCGTTG-3′. The resulting PCR fragment was digested with *Sph*I and then cloned into the *Sph*I-digested pCL10. This resulted in the insertion of 16 amino acids including the NLS into the N-terminal region of FRQ, 11 amino acids after the first methionine. Both constructs were confirmed by DNA sequencing.

Both constructs were transformed into strain 93-4 and targeted at the *his-3* locus as described previously (Sachs and Ebbole, 1990)

Nuclei isolation

Nuclei were isolated by a modification of the method described by Baum and Giles (1985). The mycelial pads (2–3 g, wet weight) were ground with 2 g of glass beads and 6 ml of buffer A [1 M sorbitol, 7% (w/v) ficoll, 20% (v/v) glycerol, 5 mM magnesium acetate, 5 mM EGTA, 3 mM $CaCl_2$, 3 mM dithiothreitol (DTT), 50 mM Tris–HCl, pH 7.5] for 2 min on ice. The crude homogenate was filtered through cheesecloth, and 2 vols of buffer B [10% (v/v) glycerol, 5 mM magnesium acetate, 5 mM EGTA, 25 mM Tris–HCl, pH 7.5] were slowly added to the supernatants with stirring. The diluted homogenate was layered over 15 ml of a solution consisting of a 1:1.7 mix of buffers A and B in a 50 ml tube, and centrifuged at 3000 *g* for 7 min at 4°C in a SW 28 rotor to remove cell debris. The resulting supernatants were removed (this is referred as the total cell lysate), layered onto 5 ml step gradients [1 M sucrose, 10% (v/v) glycerol, 5 mM magnesium acetate, 1 mM DTT, 25 mM Tris–HCl, pH 7.5] in a 50 ml tube and centrifuged at 9400 *g* for 15 min at 4°C in a SW 28 rotor to pellet the nuclei. The resulting nuclear pellets were gently resuspended in ice-cold storage buffer [25% (v/v) glycerol, 5 mM magnesium acetate, 3 mM DTT, 0.1 mM EDTA, 25 mM Tris–HCl, pH 7.5] and stored at –80°C. All the buffers contain 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 5 mM phenanthroline and 1 mM phenylmethylsulfonyl fluoride (PMSF).

Protein analysis

Western blot analysis was performed as previously described (Garceau, 1996; Garceau *et al.*, 1997). X-ray films of Western blots were scanned and densitometry was performed using NIH Image 1.59. FRQ was normalized against time-invariant protein CYS-3.

The exclusively cytoplasmic localization of CCG-1 was demonstrated previously by immunofluorescence (Garceau, 1996; N.Y.Garceau, K.M.Lindgren, S.J.Free, J.C.Dunlap and J.J.Loros, in preparation).

Phosphatase treatment

Total cell lysate and nuclei fractions were prepared as described above. A portion of each fraction corresponding to 40 μg of total protein was taken and put into 1× phosphatase treatment buffer (New England Biolabs), 1000 U of λPPase were added, and the reaction was incubated at 30°C for 45 min. Sample buffer was added to the resulting reaction, and the samples were subjected to SDS–PAGE and Western blot analysis.

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