Multiple oscillators regulate circadian gene expression in *Neurospora*

Alejandro Correa*†, Zachary A. Lewis*†, Andrew V. Greene*, Irene J. March*, Richard H. Gomer‡, and Deborah Bell-Pedersen*§

*Center for Biological Clocks Research, Department of Biology, Texas A&M University, College Station, TX 77843; and ‡Howard Hughes Medical Institute and Department of Biochemistry and Cell Biology, Rice University, Houston, TX 77005-1892

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High-density microarrays were used to profile circadian gene expression in *Neurospora crassa* cultures grown in constant darkness. We identified 145 clock-controlled genes (ccgs). The ccgs peaked in mRNA accumulation at all phases of the day, with the majority peaking in the late night to early morning. The predicted or known functions of the ccgs demonstrate that the clock contributes to a wide range of cellular processes, including cell signaling, development, metabolism, and stress responses. Although the period of rhythm of most of the ccgs was found to depend on the well characterized FREQUENCY (FRQ)-based oscillator, three ccgs appeared to have a rhythm that was significantly short in the long period (29-h) *frq*^{7} mutant strain. These ccgs accumulate mRNA rhythmically with a circadian period in a *frq*-null strain, confirming the existence of a second oscillator in *N. crassa*.

clock-controlled gene | microarray | frequency

Circadian rhythms are endogenous self-sustaining oscillations that are regulated by a central pacemaker composed of one or more biochemical oscillators (1, 2). These rhythms are observed in a wide variety of organisms, ranging from daily rhythms in photosynthesis in cyanobacteria and plants to activity and sleep–wake cycles in rodents and humans. An important aspect of rhythmicity involves control of specific target genes by oscillators; however, this remains one of the least understood areas in chronobiology.

In *Neurospora crassa*, the well characterized FREQUENCY (FRQ)-based oscillator consists of an interlocked autoregulatory molecular feedback loop containing positive PAS domain-containing elements WHITE COLLAR-1 (WC-1) and -2 and a negative element (FRQ), which depresses the activity of the positive elements (3). WC-1 is a blue-light photoreceptor that links the circadian oscillator to the external environment (4, 5). Using subtractive hybridization and differential screens of time-of-day-specific libraries, eight clock-controlled genes (ccgs) have been identified in *N. crassa* (3). All of the ccgs peak in expression in the late night/early morning, and the associated proteins function in intermediary metabolism, stress responses, and development. Mutation of the *frq* gene affects the rhythmic expression of all of the known ccgs, suggesting that they are controlled by the FRQ-based oscillator. However, circumstantial evidence suggests that the *N. crassa* clock is built by using more than one oscillator (6–8), and these other FRQ-less oscillators (FLOs) may participate in the regulation of ccgs.

In this study, we used microarray technology to search for ccgs in *N. crassa*. Our data reveal the importance of the clock in the life of the fungus and provide molecular evidence for the existence of the FLO.

Materials and Methods

Strains and Growth Conditions. The *frq*$^{+}$ strain 87-3 (*bd; a*), the long-period mutant strain 585–70 (*bd; frq7; a*), and the *frq*$^{-}$ strain (*bd; frq*^{10}*; a*) were obtained from Jay Dunlap (Dartmouth Medical School, Hanover, NH). The *frq*^{10} mutation was produced by gene replacement resulting in a null allele. The *band* mutation (*bd*) enhances the circadian rhythm of conidiation (9) but does not affect the clock itself. Growth media (Vogel's and Fries' minimal media), vegetative growth conditions, and crossing protocols are described (10).

Culture Harvesting Conditions. For rhythmic RNA analyses, the clock was synchronized by a light-to-dark transition in mycelial mats grown in shaking (100 rpm) liquid culture (Fries' minimal media containing 0.03% glucose and 0.05% arginine) at 25°C (11, 12). The light-to-dark transfer sets the clock to circadian time 12 (CT12). Light-to-dark transfer times were such that the ages of the cultures at harvest were approximately the same, but the CTs varied (13). Specifically, the mycelia were grown in liquid shaking cultures in the light on day 1 and transferred to the dark on day 1 [for collection at hours in the dark (DD) 36, 38, 44, 51] or day 2 (for collection at DD 12, 16, 22, 28, 29, 32) and harvested either at 9:00 a.m. (DD 12, 16, 22, 36, 38, 44) or 5:00 p.m. (DD 28, 32, 29, 51) on day 3. Tissue for RNA extraction was harvested after the indicated times in the dark for each experiment.

Nucleic Acid Isolation, Hybridization, and Sequencing. Total RNA isolation and Northern blotting were as described (12, 14). Riboprobes were generated by *in vitro* transcription (Maxiscript, Ambion, Austin, TX) in the presence of [α-^{32}P]UTP [3,000 Ci mmol^{-1} (1 Ci = 37 GBq), Perkin–Elmer] from pLW1K for *ccg-2* (12). DNA probes for clones identified in microarray experiments were generated by random primed DNA synthesis (DECAprime, Ambion) in the presence of [α-^{32}P]dCTP (3,000 Ci mmol^{-1}, Perkin–Elmer) by using the appropriate plasmid as a template. The EST clones that corresponded to the ccgs (Table 3, which is published as supporting information on the PNAS web site) were sequenced by using the Prism dideoxy sequencing kit (Applied Biosystems) to confirm their identity.

Microarray Construction and Hybridization. EST libraries corresponding to three stages of the *N. crassa* life cycle (conidial, mycelial, and sexual) were generously provided by the *Neurospora* Genome Project at the University of New Mexico (15) (http://biology.unm.edu/biology/ngp/home.html). From this collection, a set of 1,343 unique genes was used to construct cDNA microarrays as described (16). Together, the ESTs and negative control spots yielded 1,778 probe spots per array. Using the 3DNA Submicro EX Expression array detection kit (Genisphere, Newark, NJ), 5–7 μg of total RNA was reverse transcribed, labeled with Cy3 and/or Cy5, and hybridized to the microarrays according to the manufacturer's instructions. After

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Abbreviations: CT, circadian time; ccg, clock-controlled gene; FRQ, FREQUENCY; FLO, FRQ-less oscillator; DD, hours in the dark; LN, late subjective night.

†A.C. and Z.A.L. contributed equally to this work.

§To whom correspondence should be addressed. E-mail: dpedersen@mail.bio.tamu.edu.

hybridization at 62°C for 24 h, the slides were washed and scanned by using a Scanarray 5000 scanner (GSI Luminomics, Ottowa).

Data Analysis. Fluorescence data from each microarray were collected by using SCANALYZE 2.44 (written by Michael Eisen and available online at http://rana.lbl.gov/EisenSoftware.htm). Data normalization and qualitative clustering were performed by using GENESPRING software (Silicon Genetics, Redwood City, CA). Values for phase were determined by fitting a cosine function to the normalized microarray data by using nonlinear regression with the PRISM software package (GraphPad, San Diego). Initially, the background fluorescence from the negative controls was subtracted from the experimental spots. Each spot was divided by the control channel data (Cy5-generated data). The value for each spot was then divided by the 50th percentile of all measurements for that slide to account for differences in overall intensity between slides. To permit comparisons of the genes on the same scale, each gene was normalized to itself. The average normalized ratio for each gene at each time point was divided by the median value for that gene, resulting in at least one time point value being equal to 1. We imposed a minimum raw intensity value to eliminate DNA spots with low signal intensities from further analysis. These minimum intensity values were chosen to be higher than values observed for the average of negative control spots; the higher the background signal, the greater the minimum signal intensity required. The raw data are available at http://plpa2linux.tamu.edu/Microarray.html.

Results

Circadian Clock Regulation of mRNA Abundance. To investigate regulation of gene expression by the circadian clock on a global scale in *N. crassa*, cDNA microarrays were generated from EST libraries corresponding to the major stages of the *N. crassa* life cycle (conidial, mycelial, and sexual) (15). The libraries are predicted to represent a comprehensive view of expressed *N. crassa* genes; however, rare transcripts are likely underrepresented in the libraries. The microarrays contained 1,778 probe spots representing 1,343 unique genes [≈14% of the total number of *N. crassa* genes (17)]; 401 of these were represented by more than one EST. These repeated sequences provided one level of control for the reproducibility of the microarray hybridizations.

The microarrays were used to determine the levels of *N. crassa* mRNAs over 1.5 consecutive days. Longer cycle times were not used because in liquid cultures, the amplitude of the rhythm begins to damp after 1.5 cycles (11). To verify that a given gene was expressed rhythmically, we examined gene expression in both a frq^+ strain (87–3) that has a period of 22 h and a clock mutant strain, frq^7 (585–70), that has a period of 29 h in constant darkness at 25°C. The use of these two strains aids in establishing circadian regulation of transcript abundance (11, 13, 18); the levels of authentic clock controlled transcripts would cycle in both strains, but due to the difference in circadian period lengths, the same mRNA would be expected to cycle at different rates. The microarrays were hybridized with Cy3-labeled probes derived from RNA isolated from cultures kept in constant darkness and harvested at subjective dawn (CT0), noon (CT6), dusk (CT12), midnight (CT18), and CT0 and CT6 of the next subjective day. CT is used to normalize biological time in strains with different endogenous period lengths to 24 circadian hours per cycle. Thus, the two strains were in the dark for different lengths of time to achieve the same CT at time of harvest (see *Materials and Methods*) (11). The quality of the RNA used to generate the microarray probes and the circadian properties of the cultures were confirmed by Northern blots by using the previously characterized late-night- to morning-specific clock-controlled *ccg-2* gene as a probe (Fig. 1*a*) (11, 12). For each time point, the arrays were cohybridized with a reference sample (CT12) labeled with Cy5. Each slide contains two copies of the array, and two different slides were probed for each time point. Thus, four spots representing each gene were analyzed for each time point. The overall cross correlation coefficient between replicate samples in both frq^+ and frq^7 strains was ≥0.89, indicating a high degree of reproducibility between experiments (see Fig. 4, which is published as supporting information on the PNAS web site). After the application of minimum intensity requirements (16), 970 of the 1,778 probe sets from frq^+ and 1,090 of the 1,778 probe sets from frq^7 were considered reliable. Seven hundred sixty probe sets passed the restrictions for both strains and were further analyzed. Our filtering parameters excluded genes that were poorly expressed under our growth conditions; thus, it is likely that some genes may be scored circadian by independent methods. For example, *frq* mRNA accumulates rhythmically (19). Although there is sufficient *frq* mRNA for the clock to function normally in our growth conditions (11), *frq* mRNA is expressed at low levels and therefore did not pass our selection criteria.

To determine with confidence which of the 760 genes were expressed under control of the circadian clock, we imposed three requirements. First, the expression profile had to have at least one peak in both frq^+ and frq^7. Second, when two peaks were observed [note that genes that peak in the night have only one peak (Fig. 1*b*)], a period between 18 and 30 h in frq^+ was required. Third, we required nonoverlapping standard errors between the highest and the lowest of the six time points. Using these criteria, 145 genes were classified as being clock-controlled, representing ≈19% of the 760 genes that passed our restrictions (Table 3 and Table 4, which is published as supporting information on the PNAS web site). A minimum 1.5-fold increase from the trough to the peak was observed for all selected genes, and several of these were independently verified by Northern assays (Table 3). Genes previously identified as ccgs, including *ccg-1 -2*, -6, -7, and -*12* (11, 13), were scored as rhythmic in the appropriate phase in our data set, further validating our selection criteria (Table 3).

The rhythmically expressed genes were organized into clusters by peak expression time (Table 4). All possible phases of peak gene expression were represented in the clusters (Fig. 1*b*). For convenience, each gene was classified as early subjective day (ED), late subjective day, early subjective night (EN), or late subjective night (LN), representing genes whose expression peaked between DD12–14 and DD31–36 (CT22 to 3.99), DD15–19 (CT4 to 9.99), DD20–26 (CT10 to 15.99), or DD27–31 (CT16 to 21.99), respectively, in frq^+ cells. In those cases where ccgs fell into a different class in the frq^7 strain (Table 4), the frq^+ classification was used. Class ED and LN genes are the most highly represented and contain all of the previously characterized ccgs. No genes were known in *N. crassa* to cycle with a peak time in the EN. Therefore, to further verify the microarray data, we chose candidates from each class and performed Northern blot analysis by using RNA isolated every 4 h over 2 days from frq^+ cells. Four representative Northern blots are shown in Fig. 1*c*. Each of the chosen representatives provided a good test of gene discovery using the microarrays, because none of the chosen genes would likely have been predicted to be rhythmically expressed by other means. In all cases, we observed that the array profile closely matched the Northern data; however, the amplitudes were typically lower in the array data. In all, we have examined 27 of the candidate genes by Northern assay (Table 3). Rhyhmicity was confirmed in 26 of 27 genes; the one gene that was not confirmed (W13B7) was difficult to detect by Northern assay.

Approximately 25% of the 145 ccgs have no predictable function. Many of the identified genes function in previously defined clock-regulated processes, including development, stress response, and intermediary metabolism (Tables 1 and 3). In

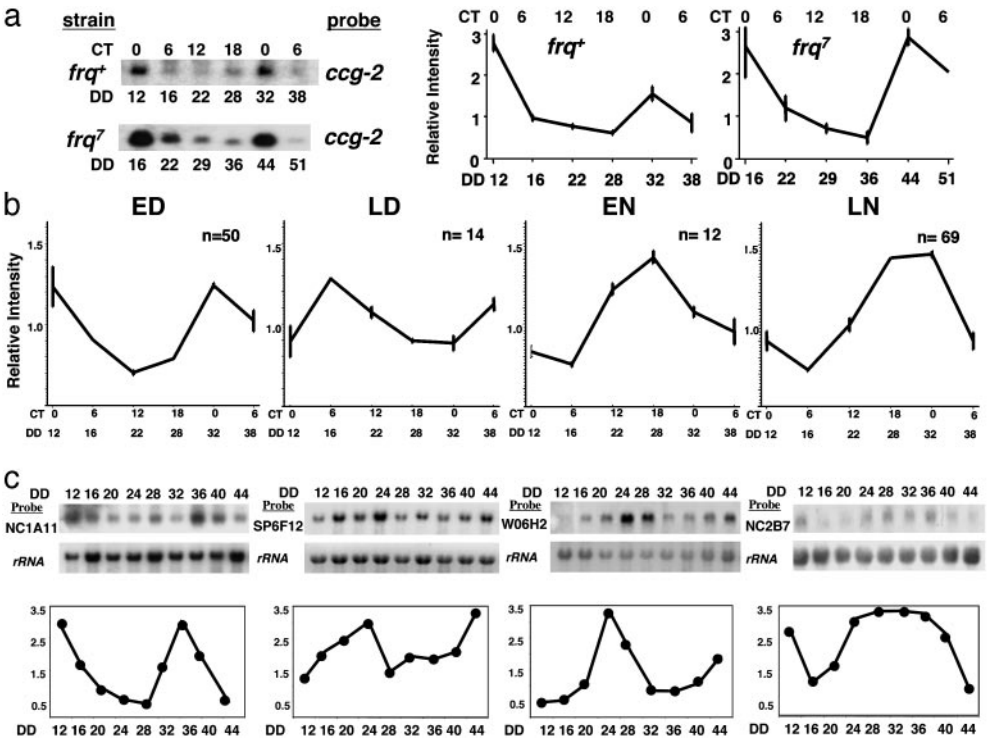

Fig. 1. (*a*) Total RNA was harvested from dark-grown clock *frq*$^+$ and *frq*^{7} cultures after the indicated times in the dark (DD) and probed with *ccg-2* to verify rhythmicity of the cultures. The corresponding CT are designated at the top. The microarray tracings of *ccg-2* are shown (*Right*) for both strains. The values (means ± SEMs; $n = 4$) for each time point are shown. CT (top) and DD (bottom) times are indicated. (*b*) Circadian clock-regulated genes were grouped into four classes based on their peak time of mRNA accumulation in *frq*$^+$ cells. The values (means ± SEMs) for each time point represent the average of all genes (*n*) in that class. DD and CT times are indicated below the tracings. (*c*) Total RNA was isolated from cultures after the indicated times in the dark, and Northern blots were probed with the designated EST clone. rRNA was used as a loading control for each experiment. Relative mRNA levels are plotted as relative band intensity vs. time (below).

addition, several genes are predicted to be involved in transcriptional control and signaling. There was some correlation between function and time-of-day expression; 30 of the 145 genes (21%) are involved in translation, and 25 of these belong to the LN class (Tables 1 and 3). Twenty-eight genes encode putative ribosomal proteins; another encodes an elongation factor-1α, and another is NOP58, which is involved in ribosome biosynthesis. Northern analysis of several of the putative ribosomal protein genes confirmed their rhythmic RNA accumulation profile (Fig. 1*c*, NC2B7, and Table 3).

To identify possible promoter elements that confer circadian rhythmicity on gene expression, we scanned 1 kb of sequence upstream of the predicted transcriptional start site of each of the cycling genes for common sequences within each class and within the entire rhythmic gene set. By using BIOPROSPECTOR (http://bioprospector.stanford.edu), we identified an 8-nt element (TCTTGGCA) occurring 42 times in 29 of 69 LN class genes and five times in three of 50 early subjective day (ED) class genes (Table 3). These sequences match the core of the previously characterized activating clock element (AACTTGGCCAAGTT) shown to be necessary and sufficient for cycling of the ED class *ccg-2* transcript (20). We found four genes that contain two WC-1-binding sites and 13 genes that contain one WC-1-binding site (5) in the promoter regions, suggesting that WC-1 may directly regulate some *N. crassa* ccgs (Table 3). However, these data suggested that relatively few ccgs are directly regulated by WC-1 and pointed to the vast majority of ccgs being controlled indirectly by a cascade involving several output pathways. Similarly, by using *Drosophila* microarray analyses, only a small number of genes were identified as being direct targets of the circadian clock transcription factor CLOCK (21).

Table 1. Classification of ccgs

Category*	Number
Cell division	1
Signaling/communication	15
Cell structure/cytoskeleton	8
Cell defense	4
Development	7
Gene regulation	5
Metabolism	31
Protein processing	7
Protein synthesis	30
Unclassified	37

*Genes were classified according to their known or predicted functions from the Whitehead Institute *Neurospora* Sequencing Project http://www-genome.wi.mit.edu/annotation/fungi/neurospora.

Molecular Rhythms in the Absence of the FRQ-Based Oscillator. Oscillators that are either coupled to or distinct from the well described FRQ-based oscillator may exist in *N. crassa* (6, 7) and may regulate some *N. crassa* ccgs. Consistent with this idea, we identified three genes in the microarrays that appeared to have a short period in constant darkness in the *frq*^{7} strain, suggesting that cycling of these gene transcripts might be independent of the FRQ-based oscillator (Fig. 2 and Table 2). For example, in *frq*^{7}, W01D5 mRNA peaked at DD22 (CT6) during the first day and DD44 (CT0) in the second day, whereas *ccg-2*, which is controlled by the FRQ-based oscillator (11), peaked at DD16 (CT0)

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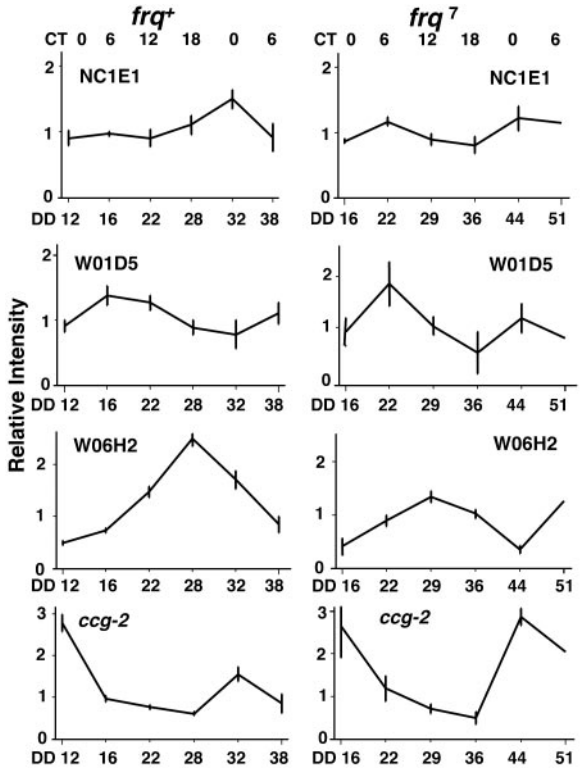

Fig. 2. Microarray tracings of RNA accumulation data from three EST clones in clock frq^{+} and frq^{7} strains. The control *ccg-2* tracing is shown for comparison. The values (means ± SEMs; $n = 4$) for each time point are shown. The times in the dark (DD) are shown below each graph, and the corresponding CT for each strain is indicated at the top.

and DD 44 (CT0). However, some level of influence of the FRQ-based oscillator on these genes is evidenced by the differences in phasing of the peaks in frq^{+} vs. frq^{7} strains after the shift from light to dark, which is particularly visible for W06H2 (Fig. 2).

To determine whether the rhythmic expression of these genes requires the FRQ-based oscillator, we used Northern blots to assay rhythmicity of the same candidates in a FRQ^{-} strain (19), in conditions where conidiation is arrhythmic (Fig. 3). In each case, a rhythm in mRNA accumulation with a period of 20–24 h was observed in cells that were synchronized by a light-to-dark transfer (Fig. 3). Because the cultures were close to the same age at the time of harvest (see *Materials and Methods*), we can rule out the possibility that the change in mRNA accumulation is due to age or nutritional or developmental state. These data provide molecular evidence of a light-responsive FLO that functions with a 20- to 24-h period in constant conditions.

The products of the three genes regulated by the FLO include CPC-1, a bZIP transcription factor of the GCN4 subfamily that regulates the expression of amino acid biosynthetic genes (22); a predicted 1,4 α glucan involved in glycogen synthesis (23, 24); and an unknown protein. The transcripts from these genes peak at different times of day in frq^{+} strains (Fig. 3 and Table 2), suggesting that they might be regulated by different output pathways from the FLO.

Discussion

We used high-density microarrays to identify rhythmically expressed genes in *N. crassa.* We have found examples of *N. crassa* genes that are expressed at times other than the late night/early morning, as well as a class of genes that are rhythmically expressed in a *frq*-null strain.

Using microarrays containing a subset of the genome, 145 genes were shown to be under the control of the circadian clock. The percentage of ccgs identified in this study is high compared with the 1–9% observed in microarray studies in other eukaryotes (21, 25–35), which may partly reflect the use of the customized EST library and the inclusion of low-amplitude rhythms. If we imposed a cutoff of a 2-fold increase from the trough to the peak, 113 genes would still be classified as cycling representing 15% of the 760 genes that passed our restrictions. However, Northern confirmation of genes with peak-to-trough differences of <2-fold (Tables 3 and 4) supports the inclusion of low-amplitude cycling genes. On the other hand, it is likely that several ccgs were missed in these analyses. Genes that are poorly expressed under the growth conditions used, and transcripts that cycle with a <1.5 fold change from peak-to-trough levels, would not have been detected. Furthermore, a transcript with a circadian rhythm in abundance does not necessarily give rise to a protein with a rhythm and, conversely, a nonrhythmic RNA does not necessarily mean that the protein is not rhythmic. In summary, although the microarrays provide an effective tool for the discovery of ccgs, they can be used only as a starting point for the analysis of clock control of gene expression. More detailed investigation is needed to validate the involvement of these genes in the clock.

The biochemical functions of the proteins encoded by the ccgs are yielding insights into the diversity of processes that are clock regulated. Several genes encoding enzymes involved in carbon and nitrogen metabolism show circadian rhythms in mRNA accumulation, with peaks occurring in the late night to early morning (Table 3). In addition, the genes encoding glycogen phosphorylase and mannitol-1-phosphate dehydrogenase peak in the early night, suggesting that flux into the glycolytic pathway may increase at this time of day. The largest group of coordinately cycling transcripts encodes ribosomal proteins. We found that 36% of the genes in the LN class are involved in protein synthesis. Assuming that the levels of the ribosomal proteins are also rhythmic, these data suggest that the number of ribosomes increases in the late night to prepare for the times of day when

Table 2. FRQ-independent ccgs

EST*	Class frq^{+}	$frq7^{\dagger}$	ORF‡	BLAST match§	Organism	Accession no.¶
NC1E1	ED	22,44	5429.1	1,4 glucan branching enzyme	*N. crassa*	16416077
W01D5	LD	22,44	4050.1	CPC-1	*N. crassa*	11115
W06H2	EN	29,51	5495.1	None		

*The *N. crassa* ESTs identified in each class are from the *Neurospora* Genome Project.
†The peak times in mRNA accumulation in hours after the shift to constant darkness in the frq^{7} strain were determined from the microarray data and GENESPRING software.
‡The predicted ORFs are from the Whitehead Institute *Neurospora* Sequencing Project.
§The BLAST matches represent the most significant match in the databases as predicted by the Whitehead Institute (minimum e value 1×10^{-5}) and verified by us by using BLAST searches of the National Center for Biotechnology Information (NCBI) sequence databases.
¶The accession numbers for each BLAST match are from the NCBI sequence database (www.ncbi.nlm.nih.gov).

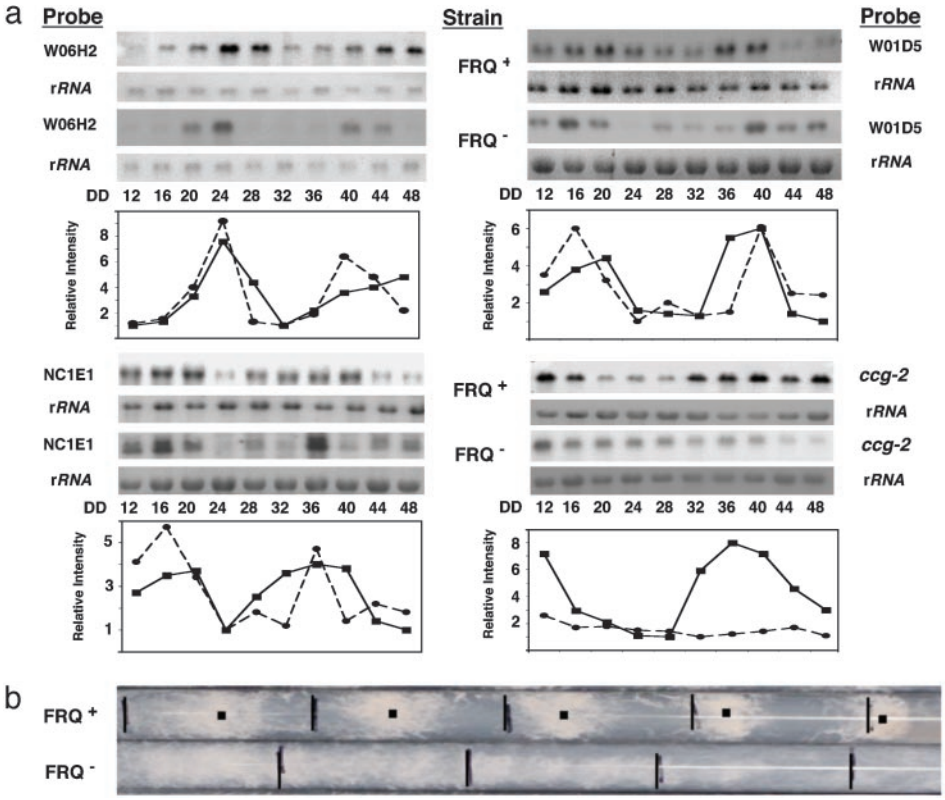

Fig. 3. (*a*) Total RNA was isolated from frq^{+} and frq^{-} strains harvested after transfer from light to dark (DD) at the indicated times, and Northern blots were probed with the EST clones. rRNA was used to verify equal loading of RNA. Relative mRNA levels are plotted as relative band intensity vs. time in the dark and are shown below the Northern blots for each gene. frq^{+} is shown as a solid line and frq^{-} as a dashed line. For all four genes, two additional Northern blots using RNA isolated from independent time courses gave similar results (not shown). (*b*) Mycelia from each culture were assayed on race tubes grown in constant darkness to verify the circadian phenotype. The growth fronts were marked every 24 h (vertical black lines). The centers of the conidial bands are marked with a black dot.

the bulk of rhythmic transcripts peak. Finally, several genes encoding transcription factors and proteins with known or suspected roles in signal transduction were found to be rhythmic in *N. crassa*. These genes provide excellent candidates for components involved in signaling time of day information from the oscillator to the output pathways.

The recent use of microarrays to profile rhythmic genes in distinct species and tissues has revealed a wide diversity of clock-regulated functions (21, 25–35). In general, there is little overlap between cycling genes in different organisms and even among tissues (25, 32–34); however, some examples of conservation of clock-controlled processes are becoming evident. ccgs are involved in protein synthesis and processing, intermediary metabolism, chromatin modification, transcriptional regulation, and cellular signaling (21, 25–30, 33–35). The observation that several genes encoding ribosomal proteins are under clock control in *N. crassa* as well as other organisms (26, 32, 33, 36) establishes the generality of clock regulation of protein synthesis and confirms the significance of translational regulation in clock function. Similarly, the role of ubiquitin-mediated protein turnover in clock function is well established, and genes encoding components of the ubiquitin pathway are found to cycle in all organisms examined (21, 26–31, 33–36). Expression of transcription factors is found to occur at different phases of the circadian cycle, suggesting that the relay of time information from the clock to these factors coordinates the phase-specific expression of downstream ccgs. Together, these similarities furnish important clues for determining key pathways that are regulated by clocks in diverse organisms.

The observation that FRQ-null strains display conidiation rhythms in temperature cycles led to a model for the *N. crassa* circadian clock that involves multiple oscillators (6). In addition, hyphal branching in *N. crassa* is rhythmic, with a period of ≈24 h (37), whereas in the same strains, the conidiation rhythm is expressed with a 22-h rhythm (38). The branching rhythm, unlike the conidiation rhythm, is maintained in constant light, is not entrained to light–dark cycles, and is not well temperature compensated (38). These differences led to the idea that two separate clock mechanisms control the two rhythms, predicting that the oscillators that control these rhythms are desynchronized in constant conditions. In the wild, however, the two rhythms would be reset daily, which would likely result in synchronization. A multioscillator clock system has also been proposed in gonyaulax, cyanobacteria, *Drosophila*, and mice (2, 39, 40). However, to date no components of a second oscillator have been described. By using *N. crassa* microarrays, we identified three ccgs that appeared to have a short period in the long-period frq^{7} mutant strain (Fig. 2). These data suggested that under constant environmental conditions, the FRQ-based oscillator harboring the frq^{7} mutation and the oscillator driving these rhythmic genes have defective coupling and are not synchronized. FRQ-independent rhythmicity of this subset of mRNAs was verified by showing that the rhythms persisted in FRQ^{-} cultures that were synchronized by light-to-dark transfer (Fig. 3). These ccgs may themselves be components of a light-responsive FLO or may reside in output pathways from the FLO. Despite the finding that these genes are rhythmic in the absence of FRQ, some level of influence of the FRQ-based oscillator on their

expression was suggested by differences in phasing of the peaks in frq^+ vs. frq^7 strains after the shift from light to dark (Fig. 2). This may reflect an altered coupling mechanism (41) between the mutated FRQ-based oscillator and the FLO that affects phase but not period. The three genes that were found to be rhythmic in the absence of FRQ represented the most obvious genes to investigate from the microarray data; however, it is likely that other ccgs are rhythmic in the absence of FRQ. For example, some of the ccgs were found to cycle in opposite phases in frq^+ vs. frq^7 strains (Table 4), and these provide excellent candidates for additional ccgs that are outputs from the FLO. A similar microarray study in *Drosophila* heads identified 4 of 22 cycling genes that were rhythmic in a strain that lacked the central oscillator gene *period* and included the clock-associated gene *vrille* (31).

In summary, our microarray data reveal that control of outputs by the circadian clock in *N. crassa* is complex and involves regulation by more than one oscillator. The identification of ccgs that are rhythmic when the FRQ-based oscillator is absent provides powerful tools for the identification of the FLO components and for understanding how a multioscillator system functions to control rhythmic gene expression.

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