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The *frequency* Gene Is Required for Temperature-Dependent Regulation of Many Clock-Controlled Genes in *Neurospora crassa*

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ABSTRACT

The circadian clock of Neurospora broadly regulates gene expression and is synchronized with the environment through molecular responses to changes in ambient light and temperature. It is generally understood that light entrainment of the clock depends on a functional circadian oscillator comprising the products of the *wc-1* and *wc-2* genes as well as those of the *frq* gene (the FRQ/WCC oscillator). However, various models have been advanced to explain temperature regulation. In nature, light and temperature cues reinforce one another such that transitions from dark to light and/or cold to warm set the clock to subjective morning. In some models, the FRQ/WCC circadian oscillator is seen as essential for temperature-entrained clock-controlled output; alternatively, this oscillator is seen exclusively as part of the light pathway mediating entrainment of a cryptic "driving oscillator" that mediates all temperature-entrained rhythmicity, in addition to providing the impetus for circadian oscillations in general. To identify novel clock-controlled genes and to examine these models, we have analyzed gene expression on a broad scale using cDNA microarrays. Between 2.7 and 5.9% of genes were rhythmically expressed with peak expression in the subjective morning. A total of 1.4–1.8% of genes responded consistently to temperature entrainment; all are clock controlled and all required the *frq* gene for this clock-regulated expression even under temperature-entrainment conditions. These data are consistent with a role for *frq* in the control of temperature-regulated gene expression in *N. crassa* and suggest that the circadian feedback loop may also serve as a sensor for small changes in ambient temperature.

THE ascomycete *Neurospora crassa* has a long standing as a model organism for the investigation of circadian rhythms (Dunlap 1999). The circadian system of *N. crassa* is composed of several interdependent molecular feedback loops (Aronson *et al.* 1994a; Crosthwaite *et al.* 1997; Lee *et al.* 2000; Cheng *et al.* 2001; Denault *et al.* 2001; Goerl *et al.* 2001; Heintzen *et al.* 2001) that regulate the time-of-day-specific expression of a number of output genes, thereby generating distinct phenotypes, *e.g.*, the clock-dependent rhythm of macroconidiation. The clock integrates input signals, including light and temperature changes, to keep the organism in tune with the environment. Both light and temperature have similar entrainment effects on the circadian clock in that 12 hr/12 hr cycles of light/dark or warm/cold result in a 24-hr rhythm of conidiation with the peak of conidiation occurring just prior to the transition from dark to light or from cold to warm, the time point defined as subjective dawn. Known players involved in circadian and light regulation of these processes include the products of the *frq*, *wc-1*, *wc-2*, and *vvd* genes (Loros and Dunlap 2001). However, there is evidence that other genes also contribute to the circadian system in Neurospora and might comprise independent, if not circadian, feedback loops (*e.g.*, Loros and Feldman 1986; Merrow *et al.* 1999; Lakin-Thomas and Brody 2000; Ramsdale and Lakin-Thomas 2000).

Part of clock-controlled gene expression in Neurospora is exercised at the level of transcription (Loros and Dunlap 1991). Over the last decade, clock-controlled genes (*ccgs*), which exhibit cycling mRNA levels, have been identified by subtractive hybridization, differential screening of cDNA libraries, and expressed sequence tag (EST) sequencing (Loros *et al.* 1989; Bell-Pedersen *et al.* 1996b; Zhu *et al.* 2001). Another method for the identification of genes that are differentially regulated is microarray technology. In recent years, microarrays have been widely used to analyze gene expression in a growing number of organisms, including plants, mammals, bacteria, and yeast (Schena *et al.* 1995; DeRisi *et al.* 1997; Wilson *et al.* 1999; Harmer *et al.* 2000; Perou *et al.* 2000). For filamentous fungi, cDNA microarrays have been established for *Trichoderma reesei* (Chambergo *et al.* 2002) and for *N. crassa* (Lewis *et al.* 2002).

Here we report the use of microarrays for the identification of novel clock-controlled genes in *N. crassa* and for a comparison of temperature control of gene expression in the wild-type and a *frq* null mutant strain. This analysis allowed us to evaluate existing models for tem-

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perature-influenced clock-regulated gene expression. One recent model (Merrow *et al.* 1999) states that FRQ/WCC are required only for light input, with temperature input and output not requiring FRQ. To be consistent with this model, temperature regulation of clock-controlled genes should be largely independent of *frq* and therefore similar in the knockout strain frq^{10} and in the wild type. However, we found that clock-controlled genes that are also temperature-regulated lose both temperature and clock regulation in the frq^{10} strain. This demonstrates that at least part of temperature-regulated gene expression in Neurospora depends on *frq* and on a fully functional circadian clock.

MATERIALS AND METHODS

Strains and growth conditions: The following *N. crassa* strains were used: frq^{+} (wild type) strain 87-3 (*bd; a*), long period mutant 585-7 (*bd;* frq^{7} *a*), and *frq* knockout strain 86-1 (*bd;* frq^{10} *A*; Aronson *et al.* 1994b). Neurospora media (Vogel's) were as described (Davis and deSerres 1970). Culture conditions for rhythmic RNA analysis and light-induction experiments were similar to published methods (Heintzen *et al.* 2001) with the following modifications: For analysis under free-running conditions in constant darkness (DD), strains were grown in 100-ml Erlenmeyer flasks in 50 ml of liquid culture medium with 1× Vogel's salts, 2% glucose, 0.5% arginine, and 50 μg/liter biotin at 25°. For time courses, the following times in constant darkness were used for array experiments: DD12, DD16, DD20, DD24, and DD28 for strain 87-3 and DD17, DD22, DD27, DD32, and DD37 for strain 585-7. Samples were kept in constant light prior to transfer to constant darkness, and transfer to DD was staggered to ensure that samples were of similar developmental age at the time of harvest (Loros and Dunlap 1991). Neurospora does not respond to light with wavelengths >550 nm (red light), so for temperature-entrainment experiments mycelial mats were grown in constant darkness or red light (DD) for 48 hr at 22°. Mycelial disks (1 cm in diameter) were cut from mats in DD and transferred to liquid medium. These samples were then transferred to entrainment conditions (cycles of 12 hr at 22°/12 hr at 27°) and harvested after at least 44 hr under entrainment (for a representation of the temperature entrainment, see Figure S1 in supplementary material at http://www.genetics.org/supplemental/). For array experiments, the following five time points were used (time in hours given after the last change of temperature from high to low or low to high, respectively; for exact times, see individual experiments): 10–11.5 hr at 27°, 2–6 hr at 22°, 10–11.5 hr at 22°, 2 hr at 27°, and 4–6 hr at 27°.

Preparation and analysis of RNA and proteins: RNA was prepared as described previously (Yarden *et al.* 1992; Heintzen *et al.* 2001) and poly(A) RNA was extracted from total RNA with a poly(A) tract kit according to the manufacturer's protocol (Promega, Madison, WI). Integrity of all RNAs were verified by agarose gel and Northern blot analysis using genes with known expression patterns as probes prior to poly(A) RNA extraction. Northern blots were prepared and hybridized according to standard techniques (Maniatis *et al.* 1982) using DNA probes or riboprobes synthesized with DECAprime II kit or Strip-EZ RNA T3/T7 kit (Ambion, Austin, TX). Preparation of protein extracts, SDS-PAGE, and Western blot analysis were performed as described before (Denault *et al.* 2001).

Generation of a unigene library and microarray preparation: The generation of two time-of-day-specific *N. crassa* cDNA libraries and the sequencing and analysis of 13,000 clones from these libraries have been described (Zhu *et al.* 2001). A unigene library was established by choosing a single clone representing each gene in the libraries. The unigene library consists of ∼1100 clones in 12 microtiter plates (plates UA–UL). As it was found that there were errors in the original annotation of the morning and evening libraries (a common problem in EST libraries, Knight 2001), most of the clones of the unigene library have been resequenced at the Advanced Center for Genome Technology (University of Oklahoma, Norman, OK) as described previously (Zhu *et al.* 2001). A copy of the unigene library was deposited with the Fungal Genetics Stock Center (University of Kansas Medical Center, Kansas City). Sequence information can be found in the online supplementary material at http://www.genetics.org/supplemental/. Amplification of the cDNA inserts from the library was done according to Hedge *et al.* (2000) using the universal forward primer (5′ GACGTTGTAAAACGACGGCC) and the universal reverse primer (5′ CACAGGAAACAGCTATGACC). PCR results were verified by agarose gel electrophoresis and the PCR products were purified using a QIAquick multiwell PCR purification kit according to the manufacturer's protocol (QIAGEN, Hilden, Germany). PCR products were then dissolved in 3× SSC buffer and spotted onto CLONTECH (Palo Alto, CA) DNA-ready type II slides or dissolved in 50% DMSO and spotted onto GAPSII slides (Corning, Acton, MA) with a GMS 417 arrayer (Affymetrix, Santa Clara, CA). Each PCR product was spotted twice on each slide. Postprinting slide procedures were performed according to the manufacturer's recommendations.

Microarray target preparation, hybridization, and scanning: For time course experiments in DD, microarray targets were made using a CLONTECH Atlas glass fluorescent labeling kit and FluoroLink Cy3 or Cy5 monofunctional dyes (Amersham Pharmacia, Piscataway, NJ) according to the CLONTECH protocol. A total of 3–5 μg of DNaseI-treated poly(A) RNA was used as starting material for each target, and a mixture of 2 μg oligo(dT) and 2 μg random hexamer oligonucleotides (both from GIBCO Invitrogen, Carlsbad, CA) was used as a primer for reverse transcription. For time course experiments in DD, each slide was hybridized with two targets, one "experimental target," which was made from the RNA of interest and varied for each slide, and one "reference RNA," which consisted of a combination of RNAs from different growth conditions and was the same for each slide used in a given experiment. (RNAs used as references were extracted from mycelia grown as described above and harvested at different DD times, with some of them being given a 5-min light pulse 15 min to 1 hr prior to harvest.) "Experimental" and "reference" RNAs were labeled with Cy3 and Cy5 dyes, respectively, and the dyes were switched in one of the biologically independent replicas of each experiment. Hybridization was done for 16 hr at 50° in CLONTECH GlassHyb hybridization solution using a CLONTECH hybridization chamber, and slides were washed in wash solution according to the manufacturer's recommendations (CLONTECH).

For temperature-entrainment experiments, microarray targets were made from DNase-treated total RNA or poly(A) RNA (7 or 1 μg, respectively). Reverse transcription was done in the presence of aminoallyl-dUTP (Sigma, St. Louis), 2 μg oligo(dT), 5 μg random hexamer oligonucleotides, and 600 units Superscript II reverse transcriptase (GIBCO, Gaithersburg, MD) in a 30-μl reaction at 42°. Cy3 or Cy5 dyes were coupled in a second step in 0.5 m sodium bicarbonate buffer (pH 9.0). Targets were cleaned with QIAquick PCR purification kit (QIAGEN) and vacuum dried. For hybridization, slides were prehybridized in 5× SSC, 0.1% SDS, 1% BSA for 45 min at 42°. Targets were resuspended in 14-μl hybridization

solution (50% formamide, 5× SSC, 0.1% SDS) and hybridized in the presence of 0.1 μg/μl ssDNA and 0.2 μg/μl tRNA (both Sigma) for 16 hr at 42°. For each temperature experiment, five or six samples corresponding to five or six different time points for *frq* wild type and *frq*[10], respectively, were labeled with Cy3 and Cy5, respectively, and hybridized to five different slides so that the corresponding time points of the two different strains were hybridized to the same slide. In repeat experiments, dyes were exchanged between the strains. Slides were washed twice (1 min and 5 min) in 2× SSC, 0.1% SDS, 10 min in 0.1× SSC, 0.1% SDS, and 1 min in 0.1× SSC. They were briefly rinsed first in distilled water and then in 95% ethanol and dried by a brief centrifugation step. All washes were done at room temperature except the first one (42°). Slides were scanned using a GMS 418 Scanner (Affymetrix) and stored as tiff files.

Primary microarray data analysis: Analysis of tiff files from arrays was done with ScanAlyze (written by Michael Eisen, Stanford University, http://rana.lbl.gov/EisenSoftware.htm). Grids were predefined and manually adjusted to ensure optimal spot recognition. Spots with dust or locally high background were discarded. The resulting data files were further analyzed in Excel (Microsoft, Redmond, WA) or by using Cluster and Treeview (Eisen *et al.* 1998). Thresholds for CH1GTB1 and CH2GTB1 values calculated by ScanAlyze were set to ≥0.55 or ≥0.65 to eliminate spots that had signals not significantly above background levels. For the final data analysis, data points were averaged from two replicates for each PCR product on each slide. To correct for differences between slides or for uneven loss of samples during target preparation, one of the following normalization methods was employed: For time courses in DD, the "experimental RNA" value (for each spot on the slide) was divided by the "reference RNA" value for each spot. Alternatively, for normalization within time courses, the average fluorescence value for the whole slide was determined for each slide within an experimental series and a normalization factor was determined. The resulting expression patterns after normalization for previously characterized control genes were similar to the expected behavior, thereby verifying that our microarray hybridizations could reproducibly detect changes in gene expression patterns.

For the time course experiments, the corrected value for each cDNA clone was divided by the value of DD24 [24 hr in darkness, equal to circadian time (CT) 15 for strain 87-3] or the value of DD32 (equal to CT15 for strain 585-7), thereby setting the CT15 value for each clone at 1. The CT15 value is typically the trough of the rhythm. The resulting values give the amplitude of change for each cDNA clone within a time course relative to CT15. CT is a formalism whereby the endogenous circadian biological day (22 hr in the wild type, 29 hr in *frq*[7]) is divided into 24 equal parts so that equivalent phases in the cycle can be compared in strains having different period length. By convention, CT0 corresponds to subjective dawn on a 12 hr/12 hr light/dark cycle, so CT15 corresponds roughly to 3 hr after subjective dusk. Normalization of the temperature-entrainment experiments was done similarly to the experiments under free-running conditions (DD) in that the expression value for each gene at one of the five or six time points (usually 10 hr or 11.5 hr at 27°) was set to 1. Expression values at the other four time points are then given in relation to this time point. Alternatively, expression levels were compared between the wild type and the *frq*[10] mutant strain by dividing the wild-type value for each cDNA clone by the *frq*[10] value. Using this method of normalization allowed us to focus on changes within the expression of a given gene by eliminating information about absolute expression levels. Normalized expression values for all microarray experiments can be found in the supplemental Table S1 at http://www.genetics.org/supplemental/.

Microarray data analysis to identify clock-controlled and temperature-regulated genes: After normalization as described above, microarray data from free-running and temperature-entrainment time courses were subjected to the following analysis to identify cycling genes: The resulting data files were further analyzed in Excel (Microsoft) and using Cluster and Treeview (Eisen *et al.* 1998) to find clones that fulfilled the following criteria: (1) Expression patterns are consistent with clock control (one peak and one trough ∼12 hr apart in wild type connected by increasing or decreasing values, respectively, within the observed time span) in at least three out of the four experiments; (2) amplitudes are at least 2-fold in one and at least 1.8-fold and 1.5-fold in the other two experiments; and (3) peaks and troughs of expression for the individual experiments are in phase (no more than 4 hr apart). Data files from free-running experiments were additionally analyzed using the CORRCOS algorithm (M. Straume, University of Virginia Center for Biomathematical Technology, Charlottesville, VA) as initially described in Harmer *et al.* (2000) and also as used by Duffield *et al.* (2002) to identify clock-controlled genes. CORRCOS empirically tests for statistically significant ($P < 0.05$) cross-correlation between the time series arising from hybridization to each microarray probe and cosine waves of specific period and phase. A significant correlation with a cosine wave of an optimal period between 18 and 28 hr is reported by the algorithm. The analysis is independent of signal strength and amplitude of change. The CORRCOS algorithm was used to identify rhythmic expression patterns in the four free-running time course experiments. Genes that were identified in at least two out of the four experiments were carefully reexamined to determine whether they were consistent with the criteria described under 1–3. For the free-running experiments, both the CORRCOS analysis, which is amplitude independent, and the analysis using Excel, Cluster, and Treeview, identified several genes that generally had cycling expression patterns but did not meet the amplitude criteria or were rhythmic in only two out of four experiments. These genes, which might comprise a set of weak clock-controlled genes, can be found in supplemental Table S2 at http://www.genetics.org/supplemental/.

Supplementary data and tables: Primary data and analyses too extensive to be printed in the journal have been deposited in a publicly accessible form at http://www.genetics.org/supplemental/.

RESULTS

We have assembled an *N. crassa* unigene library from two existing cDNA libraries (Zhu *et al.* 2001). The unigene library represents ∼1000 different genes and was used to generate cDNA microarrays. With these arrays, we examined the changes that occur in the abundance of transcripts during the course of the circadian day and during temperature-entrainment conditions. A table with hybridization results can be found in the supplementary material (supplemental Table S1 at http://www.genetics.org/supplemental/). In general, we found that microarrays accurately reported qualitative changes in gene expression known to occur from previous work, but quantitatively revealed smaller responses than prior Northern blot or real time-PCR approaches did, as had also been

found by other studies in our own and other laboratories (Duffield *et al.* 2002).

Identification of clock-controlled genes: For time courses in DD, five different time points spanning a circadian cycle were investigated in each experiment. Three independent experiments were carried out with tissues from a clock wild-type strain (strain 87-3, *frq*$^+$, period length 21.6 hr) and one experiment was performed using a *frq*^{7} mutant (strain 585-7). This mutant has a period length (29 hr) longer than that of the wild type and is used to verify that gene expression patterns that appear to be rhythmic are truly under control of the circadian clock rather than of developmental regulation (Loros *et al.* 1989; Bell-Pedersen *et al.* 1996b). For the experiments with the wild-type and the *frq*^{7} strains, DD times were chosen to correspond to similar CTs, thereby allowing a direct comparison between the wild-type and the *frq*^{7} samples. The time courses used for array hybridization experiments were chosen to cover almost an entire circadian day (CT1–CT19) to allow observation of at least one peak and trough of transcript abundance for each clock-regulated gene. Genes with expression patterns consistent with clock control were identified using two different data analysis methods: In one approach, genes were analyzed according to their expression patterns and amplitudes using Excel, Cluster, and Treeview (see materials and methods for details); in the second approach, we used the CORRCOS algorithm to identify cycling genes. With both methods, several previously known clock-controlled genes, *e.g.*, *ccg-1*, *eas (ccg-2)*, *ccg-13*, and *lyz* (Loros *et al.* 1989; Zhu *et al.* 2001) were among the genes identified. In general, CORRCOS identified fewer rhythmic genes than the other approach, probably reflecting the relatively short time courses that were used for the four experiments.

Using a combination of these two approaches, 27 genes were identified as robustly clock controlled (Figure 1, Table 1). [Table 1 associates unigene/EST clone names with corresponding unique locus identifier (NCU) numbers found in the annotated Neurospora genomic sequence at http://www-genome.wi.mit.edu/annotation/fungi/neurospora/.] Six of these were found to be strongly rhythmically expressed in all four experiments; among these are the known robustly clock-controlled genes *ccg-1*, *eas (ccg-2)*, *ccg-13*, and *ccg-14* (Loros *et al.* 1989; Zhu *et al.* 2001). The other two strongly rhythmic genes (unigene clones UBh05 and ULd10, neither of which has significant homology to genes previously associated with a phenotype) had not previously been identified. Some other genes that have been identified previously as clock controlled did not meet the threshold criteria, but we found in a previous study that *ccgs* vary greatly with respect to the level of clock control (Zhu *et al.* 2001), and our use of stringent thresholds might lead to exclusion of more weakly clock-controlled genes. Also, tissues used in this investigation were grown in medium with 2% glucose, a condition that increases the amplitudes of several known clock-controlled genes but decreases amplitudes of others (Bell-Pedersen *et al.* 1996b). All the putative clock-controlled genes that were identified show peaks in expression late in the subjective night or early in the subjective morning (CT19–CT6). These data establish a broad baseline characterization by identifying ~2.7% of the genes as being regulated by circadian rhythms at the level of RNA abundance. An additional 32 genes that showed rhythmic expression patterns but did not meet the threshold criteria with respect to amplitude, or showed cycling expression in only two of four experiments, might comprise a set of weakly clock-controlled genes (see the supplemental Table S2 at http://www.genetics.org/supplemental/).

To verify our array results, we tested six genes by Northern blot analysis (Figure 1C; due to space constraints, only two time courses are shown). For all genes, at least two Northern blots with RNAs from independent time courses were investigated; one time course originated from the wild type and one from a *frq*^{7} mutant (strain 585-7). The Northern analyses confirmed our array data and demonstrated that amplitudes of 1.5- to 2-fold on arrays, which could reproducibly be followed

Figure 1.—Clock-controlled and temperature-regulated gene expression. Cluster and Northern blot analysis of clock-controlled and temperature-dependent gene expression of a subset of genes from the microarrays. (A) Cluster analysis and visualization were done with Cluster and Treeview (Eisen *et al.* 1998). The graph shows three out of four array experiments carried out under free-running conditions, or temperature-entrainment conditions, and the ratios for *frq*$^+$/*frq*^{10} at the end of the warm temperature period (first time point in each temperature-entrainment experiment) for all four experiments. Growth conditions are indicated above each experiment. The cluster is divided into three sections, the top section containing genes that are both clock controlled and temperature regulated in the wild type, the middle section containing genes that are clock controlled but not robustly temperature regulated, and the bottom section contains a selection of genes that are not regulated under either condition. Unigene clone numbers are given on the right, and their identities, when known, are given in Table 1 along with their unique genome annotation locus identifiers. (B) Representation of one of the genes from each subset from A; labeling of the *x*-axis indicates hours in the respective temperature for temperature-entrainment experiments or CT times for free-running experiments. (C) Northern blot verification of two newly identified clock-controlled genes. Total RNA (30 μg/lane) was extracted from the *frq*^{7} strain after growth in darkness for the indicated times in DD. The corresponding CT is given below the DD values. Below each blot, values (normalized to ethidium-bromide-stained rRNAs on the gels) are plotted. The lowest value was set to 1. Riboprobes used are indicated in the graph. For each gene, two independent RNA sets were hybridized; only one is shown in this figure.

A

free-running exp.#1 frq^+ | free-running exp.#2 frq^+ | free-running exp.#4 frq^7 | ratio frq^+/ frq^{10} | temp. entrainment experiment #1 | temp. entrainment experiment #2 | temp. entrainment experiment #3

CT15 CT19 CT2 CT6 CT10 CT15 CT19 CT2 CT6 CT10 CT15 CT19 CT2 CT6 CT10

exp #1 exp #2 exp #3 exp #4

frq^+: 27oC 11.5 h, 22oC 2 h, 22oC 11.5 h, 27oC 2 h, 27oC 4 h; frq^{10}: 27oC 11.5 h, 22oC 2 h, 22oC 11.5 h, 27oC 2 h, 27oC 4 h

frq^+: 27oC 10 h, 22oC 6 h, 22oC 10 h, 27oC 2 h, 27oC 6 h; frq^{10}: 27oC 10 h, 22oC 6 h, 22oC 10 h, 27oC 2 h, 27oC 6 h

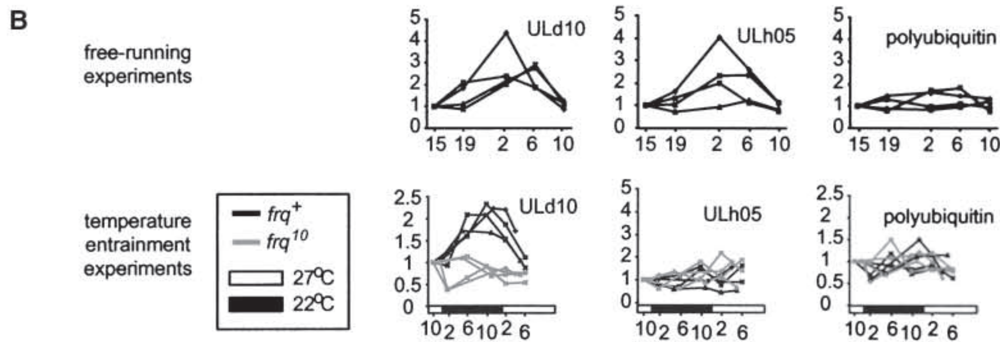
frq^+: 27oC 11 h, 22oC 5 h, 22oC 11 h, 27oC 2 h, 27oC 5 h; frq^{10}: 27oC 11 h, 22oC 5 h, 22oC 11 h, 27oC 2 h, 27oC 5 h

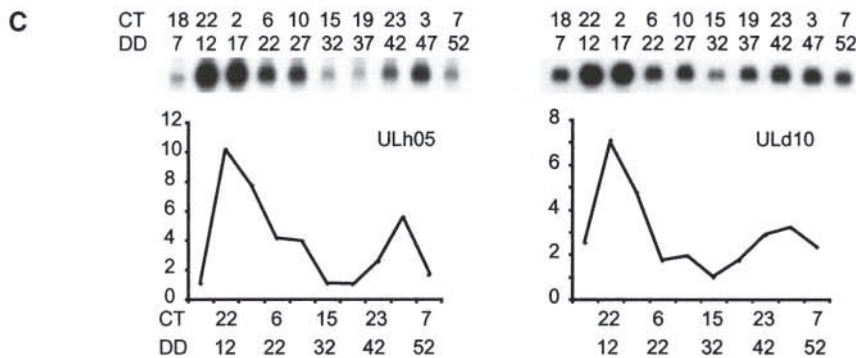
UAb05 UDe07 UBh05 UAa08 ULa04 UAa10 UEg05 UCc08 UCa09 ULd10 UAg12 UKg07 UBg12 UJc02 — clock-controlled and temperature-regulated

UDa01 ULh05 UBe11 UCh03 UCa02 UAa05 ULd09 UIf04 ULf06 UKh04 UEh12 UFd06 UKc03 — clock-controlled but not temperature-regulated

UAb06 UAa02 UAa03 UAb07 UAa07 UAb09 — neither clock- nor temperature-regulated

fold change: <0.3 0.5 0.7 1.0 1.6 2.0 >3.0 n. s.





when found in repeated independent array experiments, correspond to 3- to 10-fold amplitudes when assayed by alternative means.

Temperature entrainment of many clock-controlled genes is dependent on *frq*: Temperature is one of the main signals that keep the circadian clock in synchrony with the environment (Liu *et al.* 1998). In this study, we investigated the effects of a 12 hr at 22°/12 hr at 27° temperature regime on gene expression. This is a temperature change capable of yielding robust circadian entrainment but well below that eliciting a heat-shock response. Five to six time points covering changes from high to low temperature and vice versa within a subjective day were investigated in each of four independent array experiments. The strains we used for these experiments were a frq^+ strain and a *frq* knockout mutant (frq^{10} allele) that lacks normal circadian rhythmicity in constant darkness (Aronson *et al.* 1994a,b). Normalization of the temperature-entrainment experiments was similar to the experiments under free-running conditions (DD) in that the expression value for each gene at one of the five or six time points was set to 1. The time point chosen as a reference is the end of the warm period (Figure 1, 10–11.5 hr at 27°) within each time course. This time point corresponds to subjective evening and marks the trough expression levels for most of the clock-controlled genes. Expression values at the other four or five time points are then given in relation to this time point. Additionally, we compared expression levels of the wild type *vs.* frq^{10} at the end of the warm period when FRQ protein in the wild type is at its peak (Figure 2 and Figure 3). For this analysis, wild-type transcript levels for each gene were divided by frq^{10} transcript levels, thereby setting frq^{10} levels to 1.

The temperature-entrainment experiments led to the identification of 14 genes that respond to temperature changes in the wild type and have expression patterns consistent with circadian regulation under entrained conditions in at least three out of four experiments with the same thresholds that were used for the experiments under free-running conditions (Figure 1). We confirmed the expression of five of the genes by Northern analysis and found the same patterns as with the array analysis. Three of the genes tested were among the temperature-regulated genes and two genes were among the nonregulated genes (Figure 3; only blots for *ccg-1* and the gene for ribosomal protein L6 are shown).

All 14 temperature-regulated genes show peak expression at the end of the 12 hr at 22° period (cold period), which would represent late night in the subjective circadian day and coincides with the trough of FRQ protein under temperature-entrainment conditions (Figure 2). The group of genes consists solely of genes already identified as clock controlled, and among them are all the genes seen to be most robustly clock controlled, that is, those identified as rhythmic in all array experiments carried out under free-running conditions. Surprisingly, then, no genes that were not already identified as clock regulated were identified as temperature regulated. From the 32 genes that were identified as weakly rhythmic under free-running conditions, only four were temperature controlled (supplemental Table S2 at http://www.genetics.org/supplemental/). Another group of ~22 genes peaked 2–6 hr after the onset of the 27° (warm) period, but these genes showed regulation in only two out of four experiments, although not all of them were regulated in the same experiments. None of these genes are clock controlled under free-running conditions, but as they did not show consistent temperature-dependent expression patterns, we did not investigate them further.

Overall, this means that of the 27 clock-controlled genes comprising ~2.7% of the genes on the arrays, 52% (14 of 27) respond dependably to temperature regulation, whereas none of the non-clock-controlled genes do so consistently even though they comprise 98% of the genes examined. Interestingly, the 14 clock-controlled genes that are also temperature regulated completely lost temperature-regulated expression in frq^{10} and displayed uniform or randomly changing expression levels over the time course (Figure 1). The same was true for the four weakly clock-controlled genes that were temperature regulated in the wild type (supplemental Table S2 at http://www.genetics.org/supplemental/). Thus, among the genes on our arrays, the clock-controlled genes that are also temperature controlled invariably lost their temperature responses in frq^{10}, a strain in which the clock gene *frq* is deleted.

To see whether there was any diversity in the regulation of the temperature-responsive genes, we further analyzed these genes by comparing their transcript levels in wild type to those seen in frq^{10} at the end of the warm period (Figure 3). At this time point, FRQ protein in the wild type reaches its peak (Figure 2) and transcript levels for all the rhythmic genes investigated reach their troughs (Figure 1 and Figure 2). By choosing this time point, we thereby compare basal levels of gene expression and also compare at a stage where large amounts of FRQ protein are present in the wild type. Genes were sorted into groups depending on whether they were downregulated, upregulated, or similar compared to frq^{10} (Figure 3). The results were also verified by Northern blot analysis (Figure 3). Ten of the genes were downregulated from 1.5- to 6.5-fold in the wild type compared to frq^{10}. One, *eas (ccg-2)*, was significantly upregulated, and three did not show any difference. Implications of these findings are discussed below.

DISCUSSION

Microarray analysis of *N. crassa* reveals novel clock-controlled genes: Much progress is being made toward the unraveling of the molecular mechanisms of the Neurospora circadian clock (Loros and Dunlap 2001). Nev-

TABLE 1

Clock-controlled and temperature-regulated genes identified by microarray analysis

Unigene clone	Contig	EST clone	Annotated gene	Homology	Accession no.	*P/E* value
				A. Clock-controlled and temperature-regulated genes in wild type		
UAa08	1421	a3e01nm	NCU07787.1	*ccg-14* (*N. crassa*), snodprot1 (*Phaeosphaeria nodorum*)	GenBank AF074941	$7e^{-39}$
UAa10	1432	a5f07nm	NCU08907.1	*ccg-13* (*N. crassa*), phase specific protein (*Ajellomyces dermatitides*)	GenBank AF277086	$3e^{-10}$
UAb05	1447	a8h12ne	NCU08457.1	*eas* (=*ccg-2*) (*N. crassa*)	EMBL X67339	0.0
UAg12	1446	a5b03nm	NCU03753.1	*ccg-1* (=*grg-1*) (*N. crassa*)	EMBL X14801	e^{-180}
UBg12	167	a6f05ne	NCU02289.1	Ubiquitin-conjugating enzyme (*Glomerella cingulata*)	Swissprot O74196	$2e^{-59}$
UBh05		a6h10ne	NCU00545.1			
UCa09		a7e03ne	NCU07290.1			
UCc08	47	a8g08ne	NCU02596.1			
UDe07		c1d11ne	NCU02455.1	Peptidyl-prolyl isomerase (*N. crassa*)	Swissprot O60046	$2e^{-77}$
UEg05	989	b2g06ne	NCU06977.1			
UJc02	457	a7h08nm	NCU00489.1	40S ribosomal protein S3 (*Schizosaccharomyces pombe*)	EMBL CAA19033	$2e^{-92}$
UKg07	318		NCU01555.1			
ULa04	574		NCU07569.1			
ULd10	709	f9f09ne	NCU08949.1	Hypothetical protein SPAC15E1.02c (*S. pombe*)	PIR T37717	$6e^{-18}$
				B. Clock-controlled but not temperature-regulated genes		
UAa05	1442	a2f10nm	NCU00701.1	Lysozyme (*Chalaropsis* sp.)	Swissprot P00721	$4e^{-85}$
UBe11	543		NCU06870.1	Serine palmitoyl transferase subunit (*Aspergillus nidulans*)	GenBank AAK40365	$2e^{-47}$
UCa02	1148	a7c09ne	NCU01452.1	40S ribosomal protein S3AE (*Candida albicans*)	Swissprot P40910	$5e^{-11}$
UCh03	907		NCU03038.1	40S ribosomal protein S13 (*C. maltosa*)	Swissprot P33192	$2e^{-30}$
UDa01	327	a2c07ne	NCU09864.1	Bovine 2-oxoisovalerate dehydrogenase α-subunit	Swissprot P11178	$2e^{-27}$
UEh12		b4a04ne				
UFd06	809		NCU06895.1			
UIf04	1430	a3g10nm				
UKc03	608	b9b03nm	NCU01639.1			
UKh04	600	d9g07nm	NCU09252.1			
ULd09	926	f9f06ne	NCU02820.1			
ULf06	751	g6f05ne	NCU06056.1			
ULh05	930	a7h04nm	NCU00716.1			

The first column gives the clone identification within the unigene library and the second and third columns give the contig and EST clone numbers from the original morning or evening libraries (Zhu *et al.* 2001). In the fourth column, the gene number from the *N. crassa* genome annotation (http://www-genome.wi.mit.edu/annotation/fungi/neurospora/) is given. In the last three columns, putative homologs, their accession numbers, and *P/E* values are given. The genes *ccg-1*, *eas (ccg-2)*, *ccg-13*, *ccg-14*, and lysozyme have been described as clock-controlled genes before (Loros *et al.* 1989; Zhu *et al.* 2001); for *ccg-13*, *ccg-14*, and lysozyme, the closest homologs in other fungi are given. In previous studies (Loros *et al.* 1989; Bell-Pedersen *et al.* 1996b; Zhu *et al.* 2001), novel clock-controlled genes were identified as *ccg*s once they were genetically mapped (Loros *et al.* 1989) and were given the next number in the series, the last being *ccg-15* (Zhu *et al.* 2001). With the advent of genomics tools for the identification of classes of genes such as *ccg*s, however, we believe it is time to break this precedent and instead to identify novel *ccg*s simply by their genome annotation-associated unique locus identifier (NCU number), which can then be a tag for present and future annotation. For this reason, novel *ccg*s shown here are not given *ccg* numbers.

ertheless, control of output gene expression by the clock is probably the area of clock research that is least understood. Clock-regulated genes have been identified by several different screening approaches (Loros *et al.* 1989; Bell-Pedersen *et al.* 1996b; Zhu *et al.* 2001), but the extent to which the circadian clock controls gene expression within the whole genome remains unclear. The development of microarray technologies allows in-

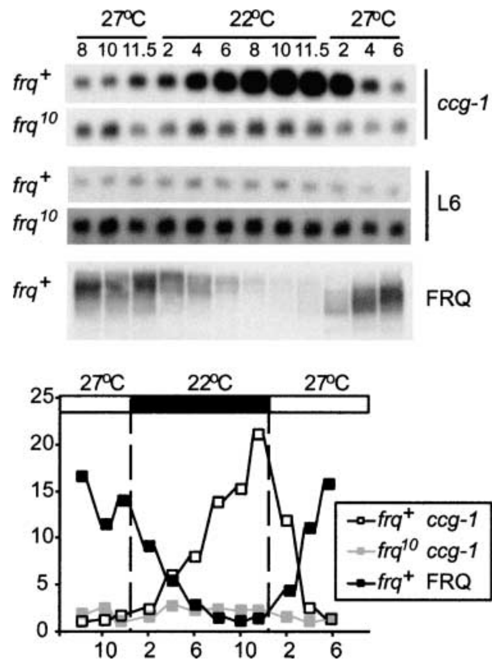


Figure 2.—Under temperature entrainment, clock-controlled genes peak when FRQ protein is at its trough. Northern analysis of total RNA (30 μg/lane) from the wild type (*frq*$^+$) and the *frq*^{10} mutant were probed with riboprobes for *ccg-1* as an example of a clock- and temperature-controlled gene (Figure 1A, top) or the gene for ribosomal protein L6 as an example of a non-clock-, non-temperature-controlled gene (Figure 1A, bottom). Western blots of protein extracts (80 μg/lane) from the wild-type strain were probed with anti-FRQ-antibody. Hours of incubation at the indicated temperature are given above each lane. Below, normalized values are plotted for *ccg-1* in the wild type and in *frq*^{10} and for FRQ protein in the wild type. Normalization was to ethidium-bromide-stained rRNAs on the gel or to Coomassie-stained proteins on the membranes. The lowest value was set to 1.

uni. clone	homol.	avg.	sd
UAa10	*ccg-13*	0.22	0.16
UAg12	*ccg-1*	0.15	0.08
UBg12	ubi. con.	0.64	0.25
UCa09		0.45	0.09
UCc08		0.48	0.21
UEg05		0.50	0.19
UJc02	rib. prot.	0.55	0.14
UKg07		0.50	0.11
ULa04		0.50	0.14
ULd10		0.43	0.15
UAa08	*ccg-14*	0.96	0.53
UBh05		1.08	0.12
UDe07	pep. iso.	0.99	0.21
UAb05	*ccg-2*	1.89	0.92

Figure 3.—A comparison of transcript levels between *frq*$^+$ and *frq*^{10} under temperature-entrainment conditions shows opposite responses to loss of *frq* among *frq*-dependent genes. The chart at the left gives the average ratio and standard deviation (sd) of *ccg* transcript levels from four independent experiments comparing *frq*$^+$/*frq*^{10} transcript levels at the end of the warm period. Only genes that are under temperature control and also under clock control under free-running conditions are shown here. The first column gives the unigene clone designation; the second column gives significant BLAST homologies. For more information about putative homologs, see Table 1. Horizontal lines separate groups according to *frq*$^+$/*frq*^{10} ratios. On the right are Northern blot analyses of *ccg-1*, *eas* (*ccg-2*), and the gene for ribosomal protein L6, examined under two different temperature conditions (end of the warm period, 27°; end of the cold period, 22°). Northern analysis was performed with 30 μg of total or 0.1 μg of poly(A) RNA.

vestigation of gene expression patterns within large parts of genomes or within complete genomes (Duggan *et al.* 1999; Blohm and Guiseppi-Elie 2001). For Neurospora, microarrays have been used to investigate light-regulated and *wc-1*-dependent gene expression, thereby allowing analysis of light signal transduction through the circadian clock (Lewis *et al.* 2002).

In this study, we used microarrays to analyze changes in gene expression patterns in *N. crassa* over the course of the circadian day. Using microarrays providing signals for ∼1000 different genes, we were able to identify 27 genes (2.7%) that had expression profiles consistent with clock control. An additional 32 genes (3.2%) seem to be weakly clock controlled, consistent with previous findings that clock control in Neurospora can vary depending on the gene and culture conditions used for the investigation (Zhu *et al.* 2001). Our results are in a range similar to findings in Arabidopsis, where Harmer and co-workers identified 6% of genes as cycling (Harmer *et al.* 2000); in Drosophila, where 1–5% of genes are rhythmic (Claridge-Chang *et al.* 2001; McDonald and Rosbash 2001); and in mammalian cells (Grundschober *et al.* 2001; Akhtar *et al.* 2002; Duffield *et al.* 2002), in which 2–9% of genes are seen to be clock regulated. Northern blot analysis of several genes showed excellent agreement with our array results under the experimental conditions we investigated with respect to expression patterns, although the amplitude on the microarrays was generally somewhat lower than that observed on Northern blots.

Surprisingly, the genes found to be under control of the circadian clock do not seem to preferentially belong to specific pathways but instead cover a range of cellular functions (Table 1). All of the clock-controlled genes peak in the subjective late night or morning, as is the case with previously identified *ccgs* (Loros *et al.* 1989; Bell-Pedersen *et al.* 1996b; Zhu *et al.* 2001). We did not find any evening-specific genes; a possible reason for this might be that the subset of genes on our arrays does not necessarily reflect all subsets of genes found in the Neurospora genome (see below). Recent findings in Drosophila have suggested the existence of clusters of functionally related genes in the genome that are also coordinately controlled by the circadian clock (McDonald and Rosbash 2001). To find out whether this is also true for Neurospora, we determined the genomic locations of the clock-controlled genes on our arrays by comparing their sequences to the latest release

of the whole Neurospora genome sequence (http://www.genome.wi.mit.edu/annotation/fungi/neurospora/). For the genes for which linkage group and position could be determined (supplemental Table S2 at http://www.genetics.org/supplemental/), no apparent clustering of clock-controlled genes was observed.

It should be obvious on the basis of first principles, but is worth stating explicitly, that we do not believe these data are the last and final word concerning circadian-regulated gene expression in Neurospora. Our conclusions are based on a careful analysis of ∼1000 genes identified by deep EST sequencing using two libraries made from nondifferentiating vegetative tissue slowly starving in the dark. For this reason, genes involved in asexual development (spore formation, aerial hyphae extension, septation) and sexual development will be underrepresented, and light-induced genes will be severely underrepresented; these are three classes of genes known from previous work (Loros *et al.* 1989; Bell-Pedersen *et al.* 1996b; Loros and Dunlap 2001) to be highly correlated with circadian-regulated genes. It is likely that a similar study executed with an alternative set of genes would identify a different proportion of genes under clock control.

Temperature entrainment of many clock-controlled genes is dependent on the *frequency* gene: Having defined a set of clock-controlled genes on our microarrays, we investigated their expression patterns under temperature-entrainment conditions, temperature being among the most important signals that reset or entrain the circadian clock (Loros and Dunlap 2001). In these experiments, we compared a frq^+ strain and a *frq* knockout mutant (frq^{10} allele). Another *frq* loss-of-function mutant, frq^9, has been shown to retain some conidial banding every 19 hr when driven under 9.5 hr 22°/9.5 hr 27° temperature-entrainment conditions (Merrow *et al.* 1999). On the basis of this and related findings, Merrow and co-workers have proposed a model in which the FRQ/WCC oscillator is involved solely in processing light signals, whereas yet-unidentified factors comprise the circadian "rhythm generator" and mediate temperature responses and circadian output from the system (Merrow *et al.* 1999, 2001a). To be consistent with this model, temperature regulation of clock-controlled genes, to a large degree, should be independent of *frq* and therefore similar in knockout strain frq^{10} and in the wild type. However, we found a significant loss of temperature-regulated gene expression in frq^{10} among the genes on our arrays: All 14 genes that are consistently temperature controlled in the wild type do not show any significant temperature regulation in frq^{10}. They are under clock control under free-running conditions in the wild type, indicating that this entire set of clock-controlled genes depends on the presence of *frq* for temperature-regulated expression. Exclusive of circadian regulatory issues, an interesting and unexpected corollary of this result is that the FRQ/WCC-based circadian regulatory system appears likely to mediate much of the organism's transcriptional responses to small temperature changes, acting as a thermal sensor in the organism. This derivative function of a circadian oscillator as a sensory module has not been previously imagined.

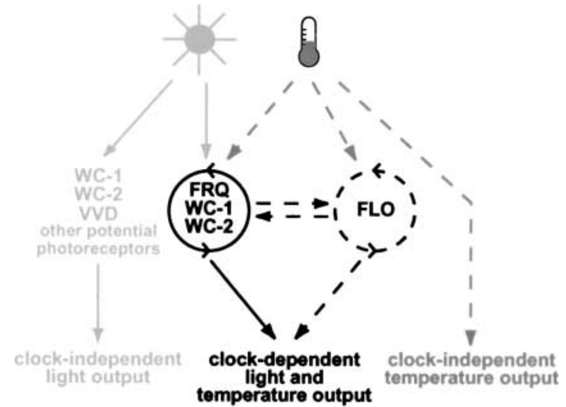


Figure 4.—Model summarizing temperature-, light-, and clock-dependent gene regulation in *N. crassa*. Clock-regulated light signal transduction is mediated by the FRQ/WCC oscillator (Crosthwaite *et al.* 1995). WC-1 is the circadian blue light photoreceptor (Froehlich *et al.* 2002; He *et al.* 2002), and light-regulated phenotypes depend on the products of the white-collar genes, WC-1 and WC-2 (Ballario *et al.* 1996; Crosthwaite *et al.* 1997; Linden and Macino 1997; Talora *et al.* 1999), VVD (Heintzen *et al.* 2001; Schwerdtfeger and Linden 2003), or possibly other uncharacterized photoreceptors (Dragovic *et al.* 2002), but not directly on FRQ. Temperature changes affect the FRQ/WCC oscillator (Liu *et al.* 1998), and in this study, we have shown that within a sample of 1000 genes, all temperature-entrained, clock-controlled gene expression depends on FRQ for relay to the output genes; an alternative model had suggested that another oscillator (FLO, FRQ-less oscillator) mediated circadian temperature regulation (Merrow *et al.* 1999, 2001a). Solid lines indicate regulatory relationships for which molecular components have been identified; dotted lines indicate parts that can be inferred from physiological experiments but for which molecular components have yet to be found, where alternative pathways cannot be distinguished, or where regulation can be imagined. Signals besides light and temperature, *e.g.*, nutritional changes, are not shown here, but have been demonstrated to influence overt rhythmicity under certain conditions (*e.g.*, Lakin-Thomas and Brody 2000).

There is now considerable evidence that other oscillatory loops in addition to the *frq*-dependent oscillator contribute to the manifestation of overt circadian rhythmicity in Neurospora (*e.g.*, Loros and Feldman 1986; Loros *et al.* 1989; Dunlap 1998; Merrow *et al.* 1999; Lakin-Thomas and Brody 2000; Ramsdale and Lakin-Thomas 2000). A model that tries to integrate these results is shown in Figure 4. The findings presented here, together with previous data (*e.g.*, Loros and Feldman 1986; Aronson *et al.* 1994b; Merrow *et al.* 1999, 2001b; Lakin-Thomas and Brody 2000), may indicate that the *frq*-dependent oscillator as well as other noncir-

cadian feedback loops may each be involved in processing a given signal but regulate a distinct portion of output genes. The loss of part of the temperature regulation may explain why the overt rhythm on a race tube, the circadian developmental phenotype, in loss-of-function mutants of *frq* is not as robust as seen in wild type under temperature-entrainment conditions (Merrow *et al.* 1999). Furthermore, previous studies (Bell-Pedersen *et al.* 2001) and this analysis indicate that *frq*-dependent regulation itself occurs through several pathways: *ccg* expression levels in the wild type at the peak time of FRQ protein can be up- or downregulated compared to frq^{10}, depending on the gene (Figure 3). Also, it is quite likely that output genes that are controlled through a certain pathway under one condition may be controlled by a different pathway under another condition; combinatorial control of gene expression by activators is well known (*e.g.*, Bell-Pedersen *et al.* 1996a), and this may also occur with circadian expression under varying conditions. A free run at a constant temperature could clearly constitute a growth condition different from that associated with a repeating 5° temperature cycle. This difference might be an explanation for the finding that some of the clock-controlled genes on our arrays do not show any significant regulation under temperature-entrainment conditions in either the wild type or the frq^{10} mutant. This seems to be especially the case for genes that are not robustly clock controlled under free-running conditions; that is, weakly rhythmic genes were often not temperature regulated, whereas genes that were strongly clock controlled in all four array experiments were also temperature regulated. This observation is further supported by the fact that among the 32 genes identified as weakly rhythmic, only 4 are temperature regulated in the wild type (and were not regulated in frq^{10}), while the other 28 do not show significant temperature-dependent expression in either the wild type or the frq^{10} mutant.

Given the appearance of even weak noncircadian developmental rhythms in a *frq* loss-of-function mutant, however, it remains plausible that some genes may be under temperature control through means other than the FRQ/WCC oscillator even though they were not identified in this screen of a thousand genes. This hypothesis is especially appealing in view of the fact that only a small portion of genes seems to be regulated at the level of transcription under temperature-entrainment conditions. This might be due to gene selection on our arrays, and additionally, parts of temperature-dependent regulation of the circadian system are known to occur at a post-transcriptional level (Liu *et al.* 1998) and would therefore not be apparent when using cDNA microarray analysis. Given the complexity of circadian regulation, a considerable overlap may exist in input integration as well as in output control among different oscillatory loops at various levels of gene expression.

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