The *Neurospora* circadian clock-controlled gene, *ccg-2*, is allelic to *eas* and encodes a fungal hydrophobin required for formation of the conidial rodlet layer

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The *Neurospora crassa* clock-controlled gene (*ccg-2*) is transcriptionally activated by the circadian clock in a time-of-day-specific manner. Transcript and sequence analyses of *ccg-2* reveal that the predicted Ccg-2 polypeptide bears significant similarity to a class of low-molecular-weight, cysteine-rich, hydrophobic proteins (hydrophobins), first identified in *Schizophyllum*, and including the product of the developmentally regulated *Aspergillus* gene, *rodletless*, required for spore surface rodlets. Allelism between *ccg-2* and *easily wettable* (*eas*) (one of the first developmental genetic loci identified in *Neurospora*) was predicted on the basis of this similarity, their close genetic linkage, and previous findings demonstrating that *eas* mutants lack rodlets. In this study allelism is confirmed experimentally by showing that (1) transformation of an *eas* mutant strain with *ccg-2* DNA results in phenotypic complementation, including restoration of surface rodlets, (2) inactivation of the *ccg-2* gene, by RIP, results in an *eas* phenotype including loss of rodlet fascicles, and (3) the original *eas* strain has dramatically reduced levels of *ccg-2* mRNA. Thus, the clock-controlled *ccg-2* gene encodes an integral component of fungal asexual spores important for spore dispersal. The dramatic reduction of *ccg-2* expression in the *eas* mutant has no apparent effect on the normal operation of the circadian clock, confirming that there is no feedback of this clock output on the oscillator itself. These data, in conjunction with the previous observation that *ccg-2* is light induced, serve to focus attention on the dual interacting role of light and the circadian clock in the regulation of fungal spore development.

[Key Words: Clock-controlled gene; *ccg-2*; *easily wettable*; hydrophobins; rodlets; circadian clock]

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Circadian rhythms are biological rhythms that are present in most, if not all, eukaryotes (Dunlap 1990). Daily fluctuations in cellular and organismal activities are controlled by an endogenous oscillator, commonly referred to as the biological clock. The clock exerts its effects on a wide variety of processes ranging from asexual spore formation in the ascomycete *Neurospora crassa* and the control of cell division in the marine protist *Gonyaulax* to photosynthesis in plants and cognitive functions in people (for review, see Edmunds 1988). However, at all phylogenetic levels, clocks appear to be assembled at the level of intracellular regulation and not intercellular communication. Thus, simple eukaryotes provide appropriate and powerful systems for molecular genetic analysis of this phenomenon.

Of the eukaryotes known to possess a well-defined circadian clock, the filamentous fungus *N. crassa* provides an ideal model system for clock analysis (for review, see Feldman and Dunlap 1983; Dunlap 1990; Lakin-Thomas

et al. 1990). Advantages include an easily measurable circadian rhythm in developmental potential (rhythmic formation of aerial hyphae and asexual spores, called conidia), the ability to manipulate and synchronize the clock with light, temperature, or drugs, and the availability of strains with genetically altered clocks. With regard to clock mutants, one locus, *frequency* (*frq*), is of particular interest because independently isolated mutants mapping to the *frq* locus are able to alter one or more clock parameters, including period length and temperature compensation (for review, see Dunlap 1990; Lakin-Thomas et al. 1990). For these reasons, we have chosen *N. crassa* as a model organism for investigating the pathways by which the clock functions to relay time information throughout the cell.

One approach to understanding the mechanisms by which the clock controls cellular metabolism is through the identification and analysis of genes or proteins that are expressed in a circadianly regulated fashion, in the same manner in which one might identify and study developmentally regulated genes. In a systematic comparison of mRNAs isolated from synchronous *N. crassa*

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cultures representing subjective early morning [circadian time 1 (CT1)] and early evening (CT13), Loros et al. (1989) isolated two morning-specific circadianly regulated clock-controlled genes [*cgc-1* and *cgc-2*]. (Circadian time is a formalism whereby biological time in strains or organisms with different endogenous period lengths is normalized to 24 circadian hours per cycle. By convention, CT0 represents subjective dawn, and CT12 represents subjective dusk.) The circadian nature of the regulation of these genes was discerned by examining the pattern of their RNA accumulation in both *frq*⁺ (21.5 hr period length) and a long period mutant strain, *frq*⁷ (29 hr period length), where transcripts arising from the clock-controlled genes cycled with period lengths reflecting the genotype (i.e., 21.5 or 29 hr). Both genes are abundantly expressed at CT0–CT1, display an ~10- to 15-fold decrease in expression at the opposite phase of the cycle (CT12–CT13), and both appear to be light inducible (Dunlap 1990; G. Arpaia, J. Loros, J.C. Dunlap, and G. Macino, in prep.). Nuclear run-on experiments performed on both *cgc-1* and *cgc-2* indicate that rhythmic expression results from clock control of transcription (Loros and Dunlap 1991). This regulation suggests that clock control of gene expression for *cgc-1* and *cgc-2* is mediated by *cis*-acting regulatory elements located within or near these genes.

The biological function of clock-controlled genes is of considerable interest. The study of rhythmic genes will ultimately provide information about the mechanism by which time information is transmitted within the cell. Additionally, determination of the identity of the *cgc*s will provide insight into the rationale for clock regulation of development and the means by which this regulation is effected. In this study we describe our analysis of the *N. crassa* *cgc-2* gene. Molecular studies of *cgc-2* reveal that the gene product is involved in the formation of the thin, proteinaceous, hydrophobic rodlet layer present on conidial surfaces (Hawker and Madelin 1976; Beever and Dempsey 1978; Beever et al. 1979; Dempsey and Beever 1979). Complementation of an *N. crassa* rodlet-deficient mutant [*easily wettable* (*eas*)]; Selitrennikoff 1976] with *cgc-2* DNA, and generation of the *eas* phenotype by inactivation of the *cgc-2* gene provides evidence that *cgc-2* and *eas* are allelic. These data demonstrate a role for circadian regulation in the development of filamentous fungi and their selective adaptation to the environment in which they grow.

Results

cgc-2 encodes a clock-regulated hydrophobin

Circadian control of *cgc-2* mRNA abundance and mapping of the *cgc-2* transcription unit. Clock control of *cgc-2* expression is observed in RNA isolated at different times of the day from mycelia grown in constant darkness (Loros et al. 1989). The *cgc-2* message (776 nucleotides) is present at minimal levels in the evening (CT12) and increases in abundance ~10- to 15-fold in the morning (CT0) (Fig. 1A and Loros et al. 1989).

The gene encoding *cgc-2* was first isolated from a λ J1 genomic library (clone 7C1; Loros et al. 1989). An ~6-kb *Xba*I fragment containing the *cgc-2* transcriptional unit was subcloned from 7C1 and inserted into pBluescript + (pBSK+, Stratagene). The resulting plasmid, pS7C1, was used as a template to further define the *cgc-2* transcription unit. Rhythmic RNA was probed with various length restriction fragments generated from pS7C1 (Fig. 1B). Circadianly regulated *cgc-2* mRNA was detected using a 1.9-kb *Kpn*I–*Xba*I DNA fragment as a probe but was not detected using sequences upstream of the *Kpn*I site or downstream of the *Xba*I site (data not shown). This indicated that the *cgc-2* transcriptional unit was contained within the 1.9-kb *Kpn*I–*Xba*I fragment.

Sequence and transcriptional analysis of *cgc-2*. The 1.9-kb *Kpn*I–*Xba*I genomic fragment and several *cgc-2* cDNA clones were sequenced (Fig. 2). The ends of the transcript were mapped by primer extension and S1 nuclease protection assays, with the 5' end indicated as nucleotide 1 and the 3' end as nucleotide 869 (the last nucleotide of the *cgc-2* sequence; Fig. 2B). The poly(A) addition site was corroborated by sequencing the 3' end of *cgc-2* cDNAs. Sequence comparisons of cDNA to genomic DNA revealed an intron of 94 nucleotides, which when spliced yields a mature message of 776 nucleotides. The splice site consensus sequences are typical of *N. crassa* intervening sequences (Gurr et al. 1987) and are underscored in Figure 2B. A putative TATA box (at –39) and a putative CAAT box (at –254) were identified and are indicated in Figure 2, A and B.

The *cgc-2* gene is predicted to encode a polypeptide of 108 amino acids (Fig. 2B), with an estimated *M_r* of 11,000. The codon bias of the *cgc-2* open reading frame (ORF) is consistent with genes that are highly expressed in *N. crassa* (Gurr et al. 1987). Overall, hydrophobic amino acids predominate in Ccg-2 (45% hydrophobic residues: A, F, I, L, M, P, V), with the protein containing a strong hydrophobic amino terminus [characteristic of a signal sequence (Boyd and Beckwith 1990)] and a central hydrophobic domain as shown in Figure 3. In addition, the ORF specifies an unusually high number of cysteine residues (8). The polypeptide contains potential sites of phosphorylation by casein kinase 2 (amino acids SIDD following residue 36) and protein kinase C (amino acids SVK following residue 83), and three overlapping potential myristylation sites (amino acids GCVVGV at residue 70, GVIGSQ at residue 74, and GSQCGA at residue 77) occur near the end of the ORF in a relatively more conserved region of the protein (see below; Fig. 3A).

Comparison of the Ccg-2 amino acid sequence with protein data bases (SWISS-PROT and NBRF-PIR protein) revealed that Ccg-2 protein shares significant sequence similarity to several low-molecular-weight, cysteine-rich fungal proteins involved in cell wall hydrophobicity (called hydrophobins). Three hydrophobin genes, *Sc1*, *Sc3*, and *Sc4* from *Schizophyllum commune* are abundantly expressed in fruiting dikaryons (as well as in non-fruiting monokaryons and dikaryons for *Sc3*) (Schuren and Wessels 1990). Each protein shares extensive se-

Figure 1. Clock regulation and subcloning of *cgc-2*. (A) Rhythmic expression of *cgc-2*. Mycelia from control strain *bdA* was grown in liquid culture (Materials and methods) in constant dark and harvested after 32, 36, 18, 22, 25, 8, and 12 hr in the dark representing ~ CT0, CT4, CT8, CT12, CT16, CT21, and CT1, respectively. In wild-type clock strains this represents one full circadian cycle. Total RNA was extracted, and 10 μ g of RNA was loaded on a denaturing gel. The RNA was transferred to a nylon membrane and hybridized to a radiolabeled *cgc-2* KpnI (K)–XbaI (X) DNA probe (shown in B). Following autoradiography, the relative amount of *cgc-2* message was quantitated by densitometry and plotted as relative band intensity vs. circadian time. Constant loading was verified by examination of the amount of rRNA in each lane (data not shown), levels of which have been shown to remain constant over time under these growth conditions (Loros et al. 1989). Clock-controlled morning-enhanced expression of *cgc-2* mRNA is evident from the observed 14-fold increase of *cgc-2* message at CT0 and CT1, as compared with the opposite phase of the cycle at CT12. A more detailed description of the clock-regulated expression of *cgc-2* mRNA can be found in Loros et al. (1989). (B) Localization of the *cgc-2* gene. Genomic clone 7C1 (Loros et al. 1989) was subcloned as a 6-kb XbaI fragment into pBSK⁺ yielding plasmid pS7C1. This 6-kb XbaI fragment (top) hybridizes to the 776-nucleotide morning-specific *cgc-2* RNA. The restriction fragments shown below were used to locate the *cgc-2* transcription unit. Plus signs (+) indicate the ability of the fragment to hybridize to *cgc-2* mRNA, whereas the minus sign (–) designates a lack of detection of *cgc-2* message.

quence homology, with 8 conserved cysteines and a hydrophobic amino terminus (likely to include a signal sequence), and an internal hydrophobic domain, similar to Cgc-2. Recently, sequence analysis of the developmentally regulated *rodletless* (*rodA*) locus from *Aspergillus nidulans* revealed the same arrangement of 8 cysteine residues and hydrophobic regions observed in the *S. commune* genes (Stringer et al. 1991), as observed for Cgc-2. The Cgc-2, RodA, and Sc3 gene products are shown aligned in Figure 3A. Overall, the three sequences show 16% identity, with 52% conserved amino acid changes. Two-way pairing shows even greater similarity, and the positions of the *cgc-2* intron and the 3'-most *rodA* and Sc3 introns are similar, suggesting that all three gene products (as well as Sc1 and Sc4) originated from a common ancestor. The hydrophobic amino termini of these related proteins indicates that they are secreted, as has been demonstrated for the Sc1, Sc3, and Sc4 hydrophobins (Wessels et al. 1991).

Direct inactivation of the *rodA* locus results in strains that lack the parallel bundles of rodlets typically found on the outer surface of the conidia (Stringer et al. 1991), a characteristic noted to be reminiscent of the previously identified *N. crassa* mutant *eas* allele UCLA191;

Selitretnnikoff 1976] (Beever and Dempsey 1978). Additionally, the asexual spores of both *eas* and *rodA* have a darkened, wetted appearance, and are not readily dispersed in air.

cgc-2 is allelic to the developmental locus *eas*

The *N. crassa eas* mutation has been genetically mapped to linkage group II (Selitretnnikoff 1976), very close to the location of *cgc-2* as defined by restriction fragment length polymorphism (RFLP) mapping (Loros et al. 1989). Together, the map location of *eas*, the striking homology of Cgc-2 to RodA protein, and the similarity of developmental phenotype between *eas* and *rodA* suggested that *cgc-2* might be allelic to *eas*. This was supported further by subsequent experiments (see below).

DNA-encoding *cgc-2* rescues the *eas* mutant phenotype. We first tested the ability of *cgc-2* DNA to rescue the *eas* mutant phenotype. A *cgc-2* construct (pLW1), containing a 3-kb *EcoRI*–*XbaI* *cgc-2* genomic DNA fragment (with ~1.7 kb of upstream sequences) was linearized at the unique *XbaI* site, and the *Aspergillus trpC* promoter-driven hygromycin phosphotransferase gene

To demonstrate that the wild-type conidial phenotype observed in the rescued transformants (TDP176–180) was the result of ectopic integration and subsequent expression of the introduced copy of *cgg-2*, Southern analysis was performed on one of the *eas*-rescued transformants (TDP176) (Fig. 4B). Genomic DNAs from control strain *bdA*, rescued transformant TDP176, and *easA* (lanes 1–3, respectively) were digested with *KpnI* or *NdeI*. Following transfer to a nylon membrane, the DNA was hybridized to a labeled *cgg-2* DNA probe. For both the *KpnI* and *NdeI* digests, a DNA fragment of the expected size from the endogenous copy of *cgg-2* was observed in all three strains (5.2 and 7.2 kb, respectively). Consistent with ectopic integration of a single copy of pLWIH, an additional single *cgg-2*-specific fragment was detected for both digests in the TDP176 rescued strain, which was absent in the control strains (3.5 kb for *KpnI*

and 9.8 kb for *NdeI*). The sizes of the additional bands are compatible with an ectopic integration event that did not interrupt the region of pLW1H containing *cgc-2* and the selectable *hph* marker. These data are consistent with expression of *cgc-2* from the integrated pLW1H plasmid complementing the defect in the *easA* strain and restoring surface rodlets.

The presence of the rodlet layer in TDP176 was verified by transmission electron microscopy, performed on 13-day-old conidiating cultures, as described by Beever and Dempsey (1978). As shown, rodlet bundles are present in the control *bdA* strain (Fig. 5A), but absent (or in some replicas, greatly reduced to <90% of *bdA*) in *easA* (Fig. 5B). The rodlet layer was restored in TDP176 (Fig. 5C) but not in strains transformed with the vector alone (data not shown). Although these data suggest that *cgc-2* and *eas* are the same gene and that Ccg-2 encodes a necessary component of the rodlet layer, inactivation of *cgc-2* was accomplished, as described below, to rule out the possibility that phenotype rescue resulted from *cgc-2* encoding a closely linked suppressor of *eas*.

Inactivation of *cgc-2* by RIP results in an *eas* phenotype
During the sexual phase of the *N. crassa* life cycle, duplicated genes are recognized and modified at frequencies ranging from about 10 to 100% by a process termed RIP [repeat induced point mutations] (Selker 1990). This mutational process provides a unique means of generat-

ing a gene-specific null strain and was used to inactivate the *cgc-2* locus as follows. The 1.9-kb *cgc-2* *KpnI*–*XbaI* fragment (Fig. 2) was inserted into vector pCSN43 (Staben et al. 1989) conferring hygromycin resistance, to generate plasmid pLW1KH. *N. crassa bdA* was transformed with pLW1KH, and the transformants were isolated on medium containing 200 µg/ml of hygromycin B. A putative homokaryotic transformant (TDP200) was obtained by conidial plating and was subsequently crossed to a strain of the opposite mating type, *bdA*.

The phenotype of the potential RIP *cgc-2* progeny was examined, where 29% (63/214 progeny) appeared by several criteria to be identical to that of *eas*. First, conidia displayed the typical wetted dark orange appearance. Second, the conidia did not become airborne when agar slants were tapped. Both features are indicative of a lack of outer conidial rodlet fascicles. To confirm this, electron microscopy was performed on one of the *cgc-2*-inactivated progeny, TDP200-1. The results are shown in Figure 5D where, similar to most spores from *easA*, the outer conidial rodlet layer is reduced to undetectable levels in the *cgc-2* RIP strain.

cgc-2 transcript levels are greatly reduced in *easA* In addition to finding tight linkage between two mutations, similarities in mutant phenotypes, and complementation of the mutation in *eas* by a normal copy of *cgc-2*, an assertion of allelism between *eas* and *cgc-2* would be

Figure 5. Transmission electron microscopy of conidial cell surface morphology in wild-type, mutant, transformed, and inactivated strains. Pictured are conidial replicas from 13-day-old slants of control strain *bdA* (A), *easA* (B), TDP176 (C), TDP200-1 (D), all examined by transmission electron microscopy and magnified at 25,000 $\times$; each panel is ~ 3.4 µm on a side. Parallel bundles of rodlets are evident in both *bdA* and TDP176 (A, C), present but greatly diminished in *easA*, and altogether absent in the *cgc-2*-inactivated strain TDP200-1 (B,D).

supported by showing that the canonical *eas* mutation lies within the *cgc-2* transcription unit or its *cis*-acting regulatory sequences. Partial DNA sequence analysis of the *cgc-2* protein-coding region from *eas* (UCLA191), however, revealed no changes in the sequence (data not shown), suggesting that the defect lies in the elements regulating transcript abundance. To confirm this, total RNA was isolated from mycelia grown in liquid culture in constant darkness. Tissue from control *bdA*, *easA*, and TDP176 strains was harvested at times representing CT0, CT6, CT12, and CT18. RNA was transferred to nylon and probed with a *cgc-2*-specific RNA riboprobe (Fig. 6). Rhythmic expression of the 776 nucleotide *cgc-2* message is observed in both *bdA* (Fig. 6A) and in the rescued TDP176 strain (Fig. 6C) (although observed to be slightly phase-shifted in TDP176) but reduced to barely detectable levels in *easA* (Fig. 6B). Low levels of *cgc-2* mRNA are evident at CT0 and CT18 only after extended exposure of the autoradiograph. As a control for circadian-regulated expression of RNA, the same membrane was probed with a different clock-regulated gene identified in *N. crassa* (D. Bell-Pedersen, unpubl.). This gene, *cgc-4*, encodes a message of ~1.2 kb, which accumulates at high levels at CT18–CT0. The *cgc-4* transcript is present and correctly regulated in all three strains. The extremely low expression levels of *cgc-2* mRNA in *eas*, together with the close genetic map positions, the complementation of *eas* with *cgc-2* DNA, and the loss of rodlets in a *cgc-2* RIP strain, provide additional evidence that *cgc-2* and *eas* are allelic.

Identification of the *eas* defect In the course of examining *eas* DNA by Southern analysis, we observed that an *EcoRV* fragment generated from the *cgc-2* locus was altered in size (data not shown). Double digestion of

Figure 6. *cgc-2* expression in mutant and rescued strains. Total RNA was isolated from mycelia harvested at CT0, CT6, CT12, and CT18 in control *bdA* (A), *easA* (B), and TDP176 (C) strains. The RNA was transferred to nylon and probed with a [³²P]UTP-labeled *cgc-2* riboprobe. The membrane was subsequently hybridized with a riboprobe generated from *cgc-4*, a clock-regulated gene control (D. Bell-Pedersen, unpubl.). Both the *cgc-2* (0.8 kb) and *cgc-4* (1.2 kb) transcripts are evident and under clock control in A and C, whereas the *cgc-2* transcript is absent in the *eas* background (B). Limited amounts of *cgc-2* message are, however, detectable at CT0 and CT18 after extended exposure.

easA, TDP176, and control *bdA* DNA with *EcoRV* and *SalI* was performed to determine which area of the *cgc-2* locus was altered, as two *EcoRV* sites are present in this region. A 1.8-kb upstream fragment present in both *bdA* and *cgc-2*-transformed TDP176 is absent in *eas*, whereas the downstream 0.5-kb band is present in all three lanes, particularly after longer exposure times (Fig. 7A). This missing 1.8-kb fragment from *eas* coincides with the appearance of a larger ~5-kb band in both *easA* and TDP176, consistent with an altered *EcoRV* site or the presence of an insertion upstream of the *KpnI* site. However, if the *EcoRV* was specifically mutated, digestion of DNA with *EcoRV* and *SalI* should generate a fragment of ~2.8 kb that utilizes an upstream *SalI* site; this 2.8-kb fragment is not observed in either *easA* or TDP176. Also, in work reported previously elsewhere, when a strain carrying the *eas* mutation (UCLA191) is crossed, ~20% of the progeny are observed to be deficient in cytochrome *aa₁*, the product of the subsequently identified unlinked *cya-8* locus (Selker 1990; D. Perkins, pers. comm.). This observation led to the prediction that the *eas* mutation is caused by an insertion that includes *cya-8* sequences. Strains carrying the putative *eas* insertion mutation, when crossed, would be subject to inactivation of *cya-8* by RIP. Thus, both the molecular and genetic data are consistent with the presence of an insertion.

To test for an upstream insertion, polymerase chain reaction (PCR) amplification was performed on genomic DNA isolated from *easA*, TDP176, and control strain *bdA*, using primers that span the *cgc-2* locus (Fig. 7B). The results from three different amplifications are shown using oligodeoxynucleotide pairs: (I) oligos 01 and 010; (II) oligos 05 and 011; and (III) oligos 013 and 016 (for I, II, III, see Fig. 7B). As predicted, by using oligodeoxynucleotides that encompass the *cgc-2* transcriptional unit (I and II) the sizes of the PCR products appear to be identical in all three strains. However, PCR products generated from the upstream region (III) of *eas* DNA are absent, whereas a 1.7-kb band is synthesized from control *bdA* and in *cgc-2*-transformed TDP176 DNA templates. Although we expected to observe a larger band from *eas* DNA and TDP176 as the result of an insertion, we were unable to detect this fragment directly or by probing the PCR products with upstream *cgc-2* sequences under any conditions tested (possibly owing to the large size of the amplicon or disruption of the oligodeoxynucleotide-binding site). The lack of the 1.7-kb band in *eas* is, however, consistent with the Southern analysis described above and, together, point to the presence of an insertion located upstream of the *cgc-2* transcription unit.

The circadian clock is unaffected in strains lacking *cgc-2* The circadian clock is thought to be comprised of a feedback loop that allows for coordinate regulation of metabolic behavior and time perception (Dunlap 1990). Because in more complex systems it is known that clock-driven output can feed back to modulate the clock itself (e.g., Mrosovsky and Salmon 1987), it was of inter-

A

Figure 7. Molecular analysis of the *eas* defect. (A) Southern analysis of genomic DNA isolated from control strain *bdA* (lane 1), *easA* (lane 2), and TDP176 (lane 3). DNAs were double-digested with *EcoRV* (RV) and *SalI* (S), transferred to nylon, and probed with a 2.2-kb radiolabeled *ccg-2* *EcoRV* fragment (shown below). The approximate sizes of the expected fragments are 1.8 and 0.5 kb. The lack of detection of the 1.8-kb band in *easA* (lane 2) indicates the presence of a sequence aberration upstream of the *ccg-2* transcription unit generating a larger fragment of ~5.0 kb. This 5.0-kb band is also observed in TDP176. The 0.5-kb fragment is evident in each lane after long exposure times (data not shown). The origin of the 2.5-kb band observed in each lane is attributable to incomplete digestion with *SalI*. (B) PCR products were generated in the *ccg-2* region using a combination of oligodeoxynucleotides 01/010 (I), 05/011 (II), or 013/016 (III) (Materials and methods). Together, these pairs of oligodeoxynucleotides encompass all of the *ccg-2*-coding region and the 5' upstream sequences as indicated on the map below. (K) *KpnI*; (X) *XbaI*; ORF (*ccg-2* ORF). The DNA templates for PCR were *bdA*, *easA*, TDP176, and a no DNA control (lanes 1–4, respectively). Fragment sizes are indicated (in kb) at left.

B

est to determine whether lesions of *ccg-2* could affect the clock circuit. Hence, we investigated clock function in a *ccg-2* mutant background by analyzing clock-regulated conidial banding (Feldman and Hoyle 1976; Dunlap 1990). *easA* was first crossed to *bdA*, which enhances assessment of the banding pattern on race tubes. Figure 8

shows the results of the clock assay for *bdA*, and *bd; easA* strains. We found that *bd; easA* conidia display an *eas* phenotype, that the period lengths for *bdA* (top) and *bd; easA* (bottom) strains are identical (~21.5 hr), and that the phase of the rhythm is unaffected by the *eas* mutation.

Figure 8. Clock control of conidiation. Race tube assay of clock control in *bdA* (top), and *bd; easA* (bottom). The leftward-most vertical black bar on each tube corresponds to the position of the growth front at the time of the light-to-dark transfer. Subsequent vertical black bars correspond to the hyphal growth front marked at 24-hr intervals. Because linear growth rate is essentially constant throughout the day, horizontal distance is equivalent to hours in constant darkness following the light-to-dark transition. (●) The center of the conidial band on each day.

Discussion

The mechanism by which the circadian clock controls cellular metabolism, one important feature of which is clock control of mRNA abundance, constitutes one of the least studied aspects of circadian biology. In attempts to begin characterization of clock output pathways, clock-regulated genes were targeted for isolation by subtractive hybridization (Loros et al. 1989), and two genes, *cgc-1* and *cgc-2*, were identified. Both are abundantly expressed and transcriptionally activated specifically in the early subjective morning through a mechanism ultimately directed by the circadian clock (Loros and Dunlap 1991). Our initial focus in the analysis of these genes revolved around determining their sequence, structure, and function. To this end, *cgc-2* has been shown here to encode a mature message of 776 nucleotides, which specifies an ORF of 108 amino acids (Figs. 1B and 2).

The close genetic linkage of a previously identified *Neurospora* developmental locus, *eas*, with *cgc-2* (linkage group II R; Perkins 1990), the similarity of phenotype between alleles of *eas* and the *A. nidulans* developmental gene *rodA*, and the homology of Cgc-2 to the *Aspergillus* rodlet protein RodA suggested that *cgc-2* and *eas* were allelic. This was verified experimentally by showing that *cgc-2* DNA complements the *eas* mutant strain and restores the rodlet layer on conidial surfaces (Fig. 5C). Additionally, we have demonstrated here that inactivation of the *cgc-2* gene by RIP results in strains with an *eas* phenotype, including the lack of the conidial rodlet fascicles (Fig. 5D). Although it was reported originally that the *eas* mutant strain, allele UCLA191, completely lacked rodlet bundles (Beever and Dempsey 1978), we observed that some conidia had bundles present at low levels (<10% of wild-type levels, e.g., see Fig. 5B). This is consistent with the small amount of *cgc-2* mRNA produced from the *eas* mutant allele, indicating that the *eas* defect is leaky. Preliminary investigations into the molecular defect in the original *eas* mutant strain points to a large insertion occurring upstream of the *cgc-2* transcription unit. It was speculated previously (Selker 1990; D. Perkins, pers. comm.) that this insertion contained sequences of the *cya-8* gene, as evidenced by inactivation of the *cya-8* gene in progeny from crosses with *eas* (UCLA191). Although the exact location or nature of the insertion was not determined in this study, we believe it occurs at least 625 nucleotides upstream of the *cgc-2* transcriptional unit based on Southern and PCR analysis of *eas* DNA (Fig. 7). These results indicate the presence of a *cis*-acting sequence element required for *cgc-2* expression located far upstream of the *cgc-2*-coding region. Two additional pieces of evidence support this hypothesis. First, we were unable to detect rescue of *eas* with just the 1.9-kb *cgc-2* *KpnI*-*XbaI* fragment, even though this DNA fragment encodes the entire *cgc-2* transcriptional unit and contains 625 nucleotides of 5' sequences. Second, in initial experiments aimed at dissecting the *cgc-2* *cis*-acting control elements, we observed that sequences upstream of the *KpnI* site are required for expression of *cgc-2* mRNA (D. Bell-

Pedersen, unpubl.). Together, this information points to sequences upstream of *cgc-2* being essential for normal levels of transcription. It appears, though, that *cgc-2* expression is simply a driven output of the clock, because defects in expression have no apparent effect on the operation of the pacemaker itself (Fig. 8).

The clock-regulated *cgc-2* gene encodes a characteristic fungal hydrophobin, a class of low-molecular-weight, hydrophobic proteins essential for establishment of the rodlet structures that are a critical element contributing to spore wall hydrophobicity and, therefore, ultimately spore dispersal. Typically, members of this class of developmentally regulated fungal proteins share a number of common characteristics including eight conserved cysteines, a putative signal sequence for secretion, and a strong hydrophobic amino terminus and central domain, characteristics shared by the polypeptides encoded by *cgc-2*, *rodA*, *Sc1*, *Sc3*, and *Sc4* (Fig. 3A; Schuren and Wessels 1990; Stringer et al. 1991). Rodlets are present on the surface of aerial cells in many filamentous fungal (Cole et al. 1979) and bacterial species that elaborate dry spores (Holt and Leadbetter 1969; Williams et al. 1972). In *Neurospora*, rodlets are arranged in bundles, or fascicles, where individual chains are laid down in a parallel fashion within a single fascicle. Interdigitation of adjacent fascicles on the cell surface results in an interwoven appearance (Fig. 5). The conserved cysteine residues may be involved in the formation of disulfide bonds necessary for cross-linking the individual components.

We have noted previously that the coincidence of light and clock regulation in the *cgc* genes may be illustrative of an emerging theme in this area of research (Dunlap 1990; G. Arpaia, J. Loros, J.C. Dunlap, and G. Macino, in prep.). In plants, a number of genes are known to be light regulated and circadianly regulated (e.g., Giuliano et al. 1988; for review, see Kay and Millar 1992). In vertebrate systems, several retinal photoreceptor components oscillate (e.g., Korenbrot and Fernald 1989), and in the vertebrate suprachiasmatic nucleus, light induces the expression of a number of immediate early genes in a time-of-day-specific manner (Aronin et al. 1990; Earnest et al. 1990; Kornhauser et al. 1990; Rusak et al. 1990; for review, see Taylor 1990). In fungi, a number of processes are known to be dependent on or influenced by blue light (for review, see Degli-Innocenti and Russo 1984a), and this represents a focus of work in *Neurospora*. For instance, in the context of sexual reproduction, light enhances the production of protoperithecia (Degli-Innocenti and Russo 1984b), which themselves display a positive phototropism (Harding and Melles 1983), and ascospore release from the mature perithecium follows a clearly defined circadian rhythm in both *N. crassa* (S. Brody, unpubl. in Lakin-Thomas et al. 1990) and *Neurospora tetrasperma* (R. Brambl, pers. comm.). This interplay between light regulation and clock regulation may provide some insight into the adaptation of the fungus to its ecological niche.

Neurospora species are typically found in warm temperate or semitropical environments, where the breezes necessary for efficient spore dispersal occur most de-

pendably at the times of maximum temperature differential between air and land—in the morning and evening. Conidia elaborated in the middle of the day for dispersal in the evening would be subject to desiccation and UV mutation for hours before the evening. However, if conidial development were to be initiated in the late night in anticipation of dawn, initially triggered by an endogenous clock and further enhanced by light at dawn, then conidia might be present to be carried off by morning winds before the heat of midday. Clock control of this event may therefore represent a practical evolutionary adaptation to ensure that spores are prepared for dissemination at the proper time of day in the wild. It is reasonable to assume that genes involved in the conidiation process, including conidial maturation (such as *cgc-2*) are under the control of the circadian clock to allow for coordinate expression at the proper time of day. In this regard, it is of interest to note that the upstream region of *cgc-2* was found to share extensive sequence homology to a conidiation gene, *con10*, from *N. crassa* (Roberts et al. 1988) as well as to regions of the *cgc-1* gene (data not shown). The functions of both *con10* and *cgc-1* are not yet known.

Light is important for conidiation in many strains of fungi, including the chestnut blight fungus *Cryphonectria parasitica*, *A. nidulans* (Mooney and Yager 1990), and for the formation of fruiting bodies in *Schizophyllum* (Perkins 1969); yet, all of these fungi grow well in the absence of light. It thus seems likely that light is being used as an overt environmental cue to stimulate development at appropriate times; hence, it seems likely that circadian rhythms may be found in these and other systems to be a covert cue, initiating aspects of growth and development in the absence of light and potentiating the developmental system to respond to light. Recalling that the overt circadian banding rhythm of *Neurospora* is obscured in wild-type strains by the levels of CO₂ built up in petri dishes or race tubes under normal culture conditions (hence, the use of the *band* mutation to relieve this CO₂ masking effect), it is possible that rhythms in other fungi may heretofore have been overlooked owing to similar masking problems. However, the present study suggests an alternate screen for rhythmicity. Because similar spore surface hydrophobins have been found in several fungi to date and appear to repre-

sent a class of proteins common to most fungi that produce aerial hyphae and spores and because a hydrophobin gene from *Neurospora*, *cgc-2*, is clearly under circadian regulation, analysis of the temporal regulation of hydrophobin genes from other fungal species might be a good place to initiate a search for a molecular correlate of the circadian clock in any novel species in which a clock has not been described previously. One would thus predict that the *Sc* genes of *Schizophyllum* and the *rodA* gene of *Aspergillus* might display a rhythm in their level of expression.

The regulation of the *cgc-2* gene appears quite complex. Not only is the gene under clock control, but expression is induced by light. Transcripts from *cgc-2* are present in germinating conidia (where levels are not constitutive, but fluctuate; K.M. Lindgren, unpubl.), and the gene is regulated along with the conidiation process. The molecular analysis of *cgc-2* described here will facilitate future studies aimed at determining how these disparate factors responsible for regulation of *cgc-2* interdigitate at the single gene level. Additionally, this gene and the other *cgc*s will provide a useful entre into the *cis*- and *trans*-acting elements responsible for clock-regulated expression in attempts to identify and define the individual components of the clock output pathway.

Materials and methods

Neurospora strains and growth conditions

Strains of *N. crassa* used in this study are listed in Table 1. Growth media (Vogel's and Fries minimal media), vegetative growth conditions, and crossing protocols are fully described in Davis and deSerres (1970). Transformation of *N. crassa* spheroplasts with plasmids containing the *hph* gene as a selectable marker was accomplished according to the standard protocol of Vollmer and Yanofsky (1986). Transformants were selected on medium containing 200 µg/ml of hygromycin B (CalBiochem).

Plasmids and oligodeoxynucleotides

The *cgc-2* gene used to generate plasmids described in this study originated from a λJ1 genomic clone of ~13 kb (7C1; Loros et al. 1989). Plasmid pS7C1 harbors a 6-kb *cgc-2* *Xba*I fragment isolated from λ7C1 (shown in Fig. 1B) and inserted into the *Xba*I site of pBluescript+ (pBSK+, Stratagene). Deletion of an upstream 3-kb *Eco*RI fragment from pS7C1 yielded plasmid pLW1

Table 1. *Neurospora* strains used in this study

Strain name	Strain no.	Genotype	Source
74-OR23-VA		St. Lawrence wild-type isolate	FGSC ^a 2489
<i>bdA</i>	30-7	<i>bandA</i>	Sargent et al. (1966)
<i>bda</i>	87-3	<i>banda</i>	Sargent et al. (1966)
<i>easA</i>	UCLA191	<i>easA</i> (UCLA191) ^b	FGSC 2960
<i>bd,easA</i>	101-4	<i>band,easA</i>	this work
TDP176 A		<i>eas</i> pLW1H transformant	this work
TDP200 A		<i>bandA</i> pLW1KH transformant	this work
TDP200-1		TDP200 A × <i>banda</i> <i>cgc-2</i> RIP progeny	this work

^aFungal Genetics Stock Center, University of Kansas Medical Center, Kansas City, Kansas.

^bUCLA191 was the only isolate of *eas* before the RIP progeny described in this study.

(containing 3 kb of the *cgc-2* locus). Insertion of the ~2-kb *trpC-hph XbaI* cassette from pCSN43 (Ståben et al. 1989) into the unique *XbaI* site of pLW1 generated plasmid pLW1H (Fig. 4). pLW1K, which contains all of the *cgc-2* transcription unit, was constructed by deletion of a 1.1-kb *KpnI* fragment from pLW1. Insertion of the *trpC-hph XbaI* cassette from pCSN43 into pLW1K yielded plasmid pLW1KH, which was used for transformation of *bdA* to inactivate *cgc-2* through RIP. All plasmids were generated using standard techniques (Sambrook et al. 1989). Restriction enzymes were obtained from New England Biolabs and used according to the manufacturer's instructions. Plasmids and M13 phages were propagated in *Escherichia coli* strains XL1 Blue or JM101.

Oligodeoxynucleotides (with map locations on the *cgc-2* sequence indicated in parentheses) used in this study were synthesized on an Applied Biosystems synthesizer and include 01, 5'-TAC CGC CTT TTG CCT-3' (1103–1089); 05, 5'-TGT CAC AAT TCT TGA TCG-3' (–262––243); 010, 5'-CTC CCA GCT TCT TTC AGC-3' (78–95); 011, 5'-TGA AGA CGC TGG TGA ACT-3' (135–118); 013, 5'-ACA CTC GTC ATT TCC AGA T-3' (–170––189); and 016, 5'-ACA TGT GGT ACG TAT GAC T-3' (located ~1.5-kb upstream of the 5' sequenced region shown in Fig. 2).

Nucleic acid sequencing and protein sequence comparisons

pLW1K was used as a template for double-stranded DNA sequencing of miniprep DNA by dideoxynucleotide chain-termination techniques (Sanger et al. 1977) using a modified T7 bacteriophage DNA polymerase (Sequenase, U.S. Biochemical). The sequence of both strands was determined. Sequencing of single-stranded DNA templates isolated from M13 *cgc-2* clones was by the same method and was used to confirm the double-stranded DNA sequence results. *cgc-2* cDNA isolated from a λ gt10 library (Fu and Marzluff 1990) was used as a PCR template for sequencing. PCR products generated from λ gt10-*cgc-2* cDNA were precipitated in 0.6 volume of 20% PEG and 2.5M NaCl and were sequenced as above.

The predicted Cgc-2 protein sequence was compared with translated sequences from NBRF-PIR protein (release 29.0) and SWISS-PROT (release 19.0), using the University of Wisconsin Genetics Computer Group (GCG) FASTA sequence analysis programs (Devereux et al. 1984). Hydrophobicity plots were generated with the MacPROT/hyd program based on the Kyte-Doolittle algorithm, with a moving window of 7 residues (Kyte and Doolittle 1982). Potential sites of phosphorylation and myristylation were identified using MacPattern 1.5 (Fuchs 1991) utilizing the PROSITE data base release 8.

Nucleic acid isolation, radioactive probes, and hybridization

Genomic DNA was isolated from *N. crassa* mycelia grown in 1× Vogel's salts containing 2% glucose by the CTAB method (Zolan and Pukkila 1986; Taylor and Natvig 1987). Approximately 1 μ g of DNA was digested, fractionated on a 1% agarose gel, and transferred to nylon membrane (Hybond-N, Amersham), and the blots were processed by standard techniques (Sambrook et al. 1989). DNA probes for Southern analyses were labeled by the random priming method of Feinberg and Vogelstein (1983). The probes included a 1.2-kb *KpnI-EcoRV cgc-2* fragment (Fig. 4) or a 2.3-kb *EcoRV cgc-2* fragment (Fig. 7), both of which were derived from plasmid pLW1.

RNA was isolated by the method of Reinert et al. (1981) from clock-synchronized mycelia grown in liquid culture without shaking (Loros et al. 1989; Loros and Dunlap 1991). Light-to-dark transfer times were such that the ages of the cultures at

harvest were approximately the same, but the circadian times varied according to the following schedule. Tissue for RNA extraction was harvested after 8 (CT21), 12 (CT1), 18 (CT8), 22 (CT12), 25 (CT16), 32 (CT0), and 36 (CT4) hr in the dark (Fig. 1), or 16 (CT6), 22 (CT12), 27 (CT18), and 32 (CT0) hr in the dark (Fig. 6). Ten micrograms of total RNA was separated on a 1% agarose-formaldehyde gel (Lehrach et al. 1977), transferred to nitrocellulose membranes (Nitropure, MSI), and probed with a ³²P-labeled *cgc-2 KpnI-XbaI* DNA probe (Fig. 1) or with antisense RNA probes (riboprobes) (Fig. 6). Riboprobes were synthesized from a T3 promoter located in the pBSK⁺ vector, using T3 RNA polymerase (Promega) in the presence of [³²P]UTP as described by Promega. Hybridization and washing conditions were performed as in Sambrook et al. (1989) for DNA probes or as recommended in the Promega Protocols and Applications guide (1991) for RNA probes.

PCR amplification

λ gt10 *cgc-2* cDNA clones were amplified for dideoxynucleotide sequencing using *Taq* polymerase (Boehringer Mannheim) from a one-tenth dilution of a high titer (10¹² pfu/ml) lysate that was heated for 10 min at 70°C. One microliter of the phage dilution was used for standard PCR reactions. PCR amplification of *Neurospora* genomic DNA from *bdA*, *easA*, and TDP176 (~100 ng) was accomplished using 10 pmoles of each primer in buffer containing 300 μ M of each dNTP. Amplification was achieved by using 35 cycles of a 30-sec denaturation at 94°C, a 30-sec annealing at 45°C, a 3-min extension at 72°C, followed by a final 7-min extension at 72°C. PCR products were separated on a 1% agarose gel containing 10 μ g/ml of ethidium bromide.

Electron microscopy

Replicas of the surface of conidia from strains *bdA*, *easA*, TDP176, and TDP200-1 (Fig. 5) were prepared from 13-day-old slants as described by Beever and Dempsey (1978). Conidia were tapped onto thin glass disks, vacuum evaporated in a Balzers 301 evaporator, and shadowed at 45°C with carbon and platinum. A backing layer of carbon was then added at 90°C. The replicas were floated onto water, treated with chromic acid for 2 hr, followed by overnight washing in bleach to remove any remaining conidial material. Replicas were then washed in several changes of water, picked up on grids, and examined by transmission electron microscopy using a JEOL 100CX microscope.

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Note

During the preparation of this manuscript describing the allelism of *cgc-2* and *eas*, we became aware that a manuscript de-

scribing allelism of *eas* with another gene was ready for submission (see Lauter et al., this issue).

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The *Neurospora* circadian clock-controlled gene, *ccg-2*, is allelic to *eas* and encodes a fungal hydrophobin required for formation of the conidial rodlet layer.

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