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Jennifer J. Loros
Dartmouth College

Jay C. Dunlap
Dartmouth College

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Neurospora crassa Clock-Controlled Genes Are Regulated at the Level of Transcription

JENNIFER J. LOROS* AND JAY C. DUNLAP

Department of Biochemistry and Molecular Genetics Center, Dartmouth Medical School, Hanover, New Hampshire 03756

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Although an extensive number of biological processes are under the daily control of the circadian biological clock, little is known about how the clock maintains its regulatory networks within a cell. An important aspect of this temporal control is the daily control of gene expression. Previously we identified two morning-specific genes that are regulated by the clock through daily control of gene expression (J. Loros, S. Denome, and J. C. Dunlap, Science 243:385–388, 1989). We have now introduced a method for transcriptional analysis in *Neurospora crassa* and used this nuclear run-on procedure to show that regulation of mRNA abundance for these two morning-specific genes occurs at the level of transcription. This transcriptional regulation by the circadian clock provides a basis for isolating circadian rhythm mutants.

Circadian rhythms are extremely widespread phylogenetically, representing a type of cellular metabolic control common to most species of eucaryotes (24) and possibly even some procaryotes (29). All of these rhythms share some common characteristics, including entrainment to daily environmental cycles such as light intensity and temperature and, under constant environmental conditions, a manifestation of continuing endogenous rhythmicity with a period length close to (but not exactly equal to) 24 h that displays little dependence on the ambient temperature (11). The oscillators giving rise to these rhythms are commonly known as biological clocks. These clocks typically have a focus in the nervous tissues of animals and insects, although the existence of functionally similar clocks in plants, fungi, and unicells (13) indicates that the metabolic and genetic complexity of nervous tissue is not a requirement for the generation of circadian rhythmicity.

All of the wide variety of processes controlled by the clock are predicated on the clock's ability either accurately to measure elapsed time or reliably to recognize local time. Each of these uses in turn, whether observed in a cellular or a multicellular organism, ultimately requires the communication of temporal information within a rhythmic cell's metabolic network so as to bring about changes in the cell's behavior. One important aspect of this temporal control is the daily control of gene expression. We are interested in the mechanisms that regulate and coordinate the temporal expression of genes under control of the biological clock. Our initial studies resulted in the isolation of two morning-specific genes, *ccg-1* and *ccg-2*, that are strongly regulated by the clock at the level of RNA abundance (17). To achieve this regulation, the clock must be exerting control on transcriptional or posttranscriptional processes.

Transcription is one of the main control points in the regulation of differential gene expression, and nuclear run-on transcription studies (3, 9, 19) have been extensively used to determine the relative amounts of RNA polymerase activity associated with specific genes at the time of nuclear isolation. For experiments described here, we adapted a method developed for nuclear run-on transcriptional analysis in plant tissues (2) for use with the *Neurospora crassa* fungal system to examine transcription of the *ccg-1* and *ccg-2* genes through time. We found that the gene-specific rates of transcription for both genes varied with time, the greatest rates of transcription being seen in the early subjective morning.

Production of rhythmic liquid cultures of mycelia and assay of the biological clock. *N. crassa* wild-type 74A carrying the *bd* mutation was used in all experiments (27). Media used for the growth of rhythmic liquid cultures, and manipulations associated with the production of mycelial disks for RNA preparation, were as previously reported (17, 21). All cultures were grown at 25°C.

Neurospora mycelial disks held in starvation conditions in liquid culture display, between 24 and 60 h, no growth and a uniformly low respiratory output (21). Additionally, the circadian clock of such a culture has a normal light phase response curve and is not reset by transfer of the disks from liquid starvation medium to solid nonstarvation medium. Although we have previously shown the levels of both the *ccg-1* and *ccg-2* mRNAs to be under circadian control in these conditions (17), this liquid culture method for clock studies relies on the simultaneous variation of subjective clock time (as determined by the number of hours between the constant light [LL]-to-constant dark [DD] transition and harvest) and developmental age (as determined by the number of hours between the time of transfer of disks to starvation conditions and harvest). Preliminary run-on data generated by using nuclei isolated from such cultures revealed a large decrease in transcription rate with increasing developmental age that obscured our estimates of circadian clock time-specific variations (data not shown). In addition, the method used here to isolate nuclei requires freshly harvested mycelia. For these reasons, the previously used culture regimen was modified such that at the time of nuclear isolation, all cultures were approximately developmentally synchronous while at the same time circadianly varied. The timing of the LL-to-DD shifts was thus arranged so that harvests fell within the age range at which growth arrest was complete (24 to 60 h) while avoiding as much as possible the

* Corresponding author.

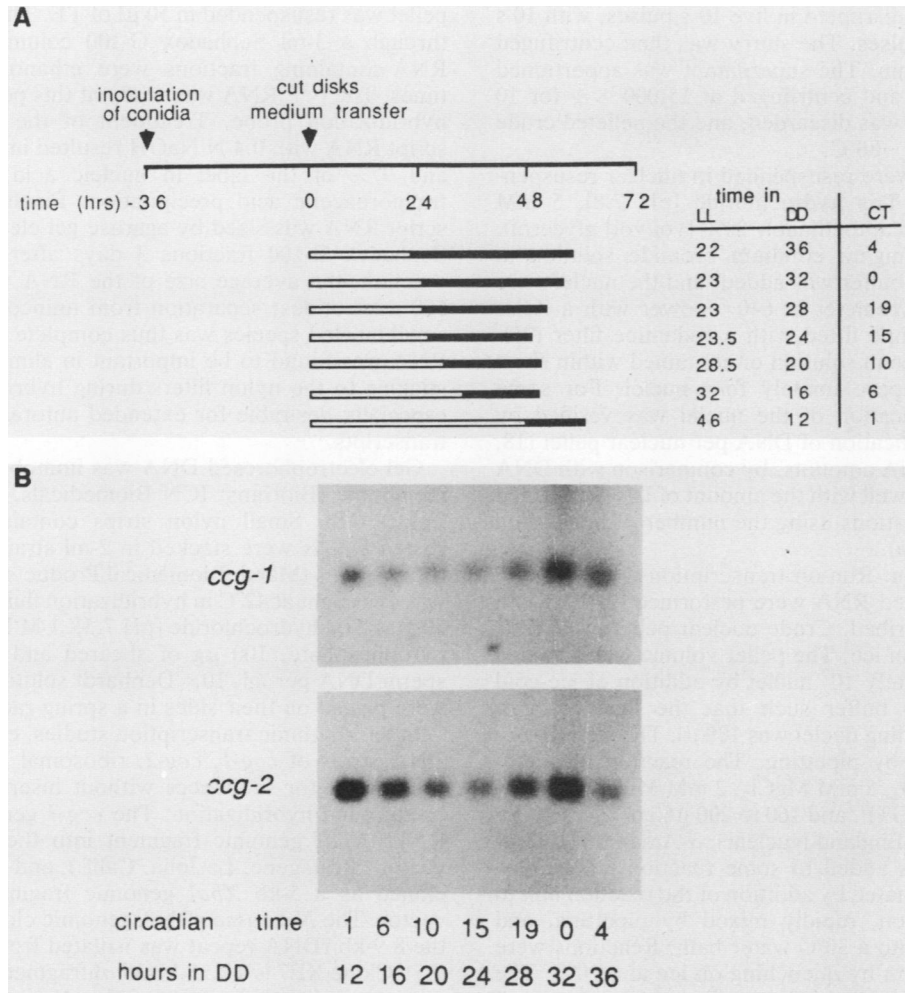

FIG. 1. (A) Mycelial harvest protocol for the preparation of rhythmic cultures for isolation of nuclei and total RNA. At the top is shown the time line for inoculation of conidia and subsequent hours of growth. Below this, time for each shaking culture is represented by a bar, the times in LL and DD being represented by open and filled areas, respectively. To the right is shown the hours for which each sample was held in LL and DD and the corresponding circadian time. (B) Comparison of mRNA abundance of the *ccg-1* and *ccg-2* genes over a 24-h cycle, showing circadian regulation. Shown are autoradiographs of Northern blots from mycelial disk samples harvested as described in the text. RNA (20 μg per lane) was transferred to nylon and probed with randomly primed (6) DNA corresponding to either a 6.5-kb *Xba*I fragment containing *ccg-1* or a 5-kb *Xba*I fragment containing *ccg-2*. Below are shown the number of hours for which each mycelial sample was held in the dark and the corresponding circadian time.

sequential aging of cultures that affected the efficiency of transcription.

At the time of a mycelial harvest, the presumed biological time (or circadian time, abbreviated CT) of a liquid culture was verified directly by placement of mycelial disks from each sample flask onto sets of individual race tubes (CT0 corresponds to subjective dawn, and CT12 corresponds to subjective dusk [5, 17]). The circadian time of the liquid culture was then calculated from the subsequent phase of the rhythm seen on the race tubes (4, 23).

Figure 1A shows the time course of the culture conditions used to prepare rhythmic mycelial cultures for isolation of nuclei and RNA. Because of the change in culture conditions compared with those used in previous work, it was necessary to confirm the transcript abundance rhythmicity of *ccg-1* and *ccg-2*. Total RNA was isolated and size fractionated on 1% agarose-formaldehyde denaturing gels for RNA blot hybridizations (Northern analyses) (16, 17). Figure 1B shows Northern blots of total RNA from an aliquot of the mycelia harvested for preparation of nuclei. Individual blots were probed with the *ccg-1* and *ccg-2* genes. Below each lane are shown the circadian time and total number of hours for which each sample was held in the dark. As expected, both genes were clearly under circadian control, with peaks of RNA abundance shown between CT0 and CT1.

Isolation and quantification of nuclei. Crude nuclei were isolated (12, 30), with modifications as described. All steps up to freezing of crude pellets were carried out at 4°C. Forty 9-mm disks of mycelia grown in 200 ml of medium were collected in the dark, using a red safe light when necessary, rapidly blotted dry, and placed on ice. The cooled mycelial samples were placed with cold glass beads in a bead beater (Biospec Products, Bartlesville, Okla.) with an ice jacket and enough ice cold buffer A (1 M sorbitol, 7% [wt/vol] Ficoll, 20% [vol/vol] glycerol, 5 mM $MgCl_2$, 10 mM $CaCl_2$, 1% [vol/vol] Triton X-100, pH 7.5) to exclude air (approximately

40 ml). Mycelia were disrupted in five 10-s pulses, with 10 s of cooling between pulses. The slurry was then centrifuged at 1,500 × *g* for 10 min. The supernatant was apportioned into microfuge tubes and centrifuged at 15,000 × *g* for 10 min. The supernatant was discarded, and the pelleted crude nuclei were frozen at −80°C.

Samples of nuclei were resuspended in nuclear resuspension buffer (50 mM Tris hydrochloride [pH 7.8], 5 mM $MgCl_2$, 10 mM β-mercaptoethanol, 20% [vol/vol] glycerol), 1/10 volume of a 1-mg/ml ethidium bromide solution in nuclear resuspension buffer was added, and the nuclei were counted on a hemacytometer at 640× power with a Zeiss fluorescence microscope fitted with a rodamine filter (25). Nuclei were either free in solution or contained within short hyphal sections of approximately four nuclei. For some preparations, quantification of the nuclei was verified by extraction and quantification of DNA per nuclear pellet (18, 28a). Estimates of DNA amounts, by comparison with DNA standards, correlated well with the amount of DNA expected on the basis of calculations using the number of nuclei in a pellet (data not shown).

Run-on transcription. Run-on transcription reactions and isolations of ^{32}P-labeled RNA were performed (2, 19), with modifications as described. Crude nuclear pellets at −80°C were thawed briefly on ice. The pellet volume was adjusted to contain approximately 10^7 nuclei by addition of ice-cold nuclear resuspension buffer such that the final reaction mixture volume including nuclei was 100 μl. The nuclei were quickly resuspended by pipetting. The reaction mix contained 75 mM NH_4SO_4, 5 mM $MgCl_2$, 2 mM $MnCl_2$, 0.5 mM each ATP, CTP, and GTP, and 160 to 200 μCi of [α-^{32}P]UTP (3,000 Ci/mmol; New England Nuclear). α-Amanitin (Boehringer Mannheim) was added to some reaction mixes. The run-on reaction as initiated by addition of the reaction mix to the nuclear suspension, rapidly mixed by pipetting, and placed immediately into a 30°C water bath. Reactions were terminated after 30 min by quenching on ice at −20°C. The reaction time course was initially monitored by removal of several samples from the reaction mix between 0 and 70 min, and incorporation of [^{32}P]UMP into trichloroacetic acid-precipitable product was determined (18). Thereafter, samples were removed at the beginning and end of each reaction for trichloroacetic acid precipitation. The reaction was always complete by 30 min, with no evidence of reinitiation of transcription. Under conditions of saturating substrate, approximately 1 dpm was incorporated per four nuclei. This level is lower than those given in some reports from other systems (19) and may reflect several factors, including the crudeness of the nuclear preparation, the age of the mycelia at harvest, the starvation growth conditions required to attenuate conidium formation and thus necessary for production of a clock-synchronous culture, or the inherent activity of isolated fungal nuclei.

Isolation and hybridization of the ^{32}P-labeled transcripts. Twenty-five micrograms of yeast tRNA and 1 to 2 μg of RNase-free DNase (Worthington Biochemicals) were added to the quenched reaction mix and incubated for 5 min at room temperature. Then 10 μg of proteinase K (Sigma) and 10 μl of 10× TES (100 mM Tris hydrochloride [pH 7.5], 5 mM EDTA, 10% sodium dodecyl sulfate [SDS]) was added for an additional 15 min at room temperature. TE (10 mM Tris hydrochloride [pH 7.5], 1 mM EDTA) was then added to a final volume of 280 μl. The nucleic acids were extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1) and twice with chloroform. The aqueous phase was then ethanol precipitated with sodium acetate and centrifuged. The RNA pellet was resuspended in 50 μl of TE. The RNA was passed through a 3-ml Sephadex G-100 column in TE, and the RNA-containing fractions were ethanol precipitated two times. The [^{32}P]RNA was ready at this point to be used as a hybridization probe. Treatment of the radiolabeled transcript RNA with 0.4 N NaOH resulted in loss of between 95 and 97% of the label in nucleic acid, as measured by trichloroacetic acid precipitation. Finally, when the transcript RNA was sized by agarose gel electrophoresis of the Sephadex G-100 fractions 3 days after the initial run-on reaction, the average size of the RNA was approximately 500 nucleotides; separation from unincorporated label and small labeled species was thus complete. Purification of the RNA was found to be important in eliminating nonspecific sticking to the nylon filters during hybridization. This was especially desirable for extended autoradiography of some transcripts.

Gel-electrophoresed DNA was immobilized onto a nylon membrane (Biotrans; ICN Biomedicals, Inc., Costa Mesa, Calif.) (18). Small nylon strips containing restriction-digested DNAs were stacked in 2-ml straight-wall microcentrifuge tubes (Marsh Biomedical Products). Prehybridization was overnight at 42°C in hybridization fluid (50% formamide, 50 mM Tris hydrochloride [pH 7.5], 1 M NaCl, 0.1% sodium pyrophosphate, 100 μg of sheared and denatured herring sperm DNA per ml, 10× Denhardt solution [18]). The tubes were placed on their sides in a spring rack.

In the rhythmic transcription studies, each tube contained DNA strips of *ccg-1*, *ccg-2*, ribosomal DNA (rDNA), *oli*, and the vector sequences without inserts as a control for background hybridization. The *ccg-1* gene was cloned as a 6.5-kb *Xba*I genomic fragment into the Bluescript (SK^+) vector (Stratagene, La Jolla, Calif.), and the *ccg-2* gene was cloned as a 5-kb *Xba*I genomic fragment into the same vector. The *N. crassa* rDNA genomic clone 7:5F containing the 8.9-kb rDNA repeat was isolated from a cosmid library (35). Clone XE7 is a 2.35-kb *Xho*I fragment containing the *oli* gene (encoding subunit 9 of the *N. crassa* mitochondrial ATPase) cloned into pBR322 (34) and was the gift of Walter Sebald. Order in the stacking of the DNA-containing nylon strips was found to make no difference.

The [^{32}P]RNA was dissolved in a small volume of glass-distilled H_2O and heated briefly at 45°C, hybridization fluid was added to make 0.5 ml, and disintegrations per minute per volume was determined by scintillation counting of a small aliquot. After removal of prehybridization solution, hybridization solution was added to the filters with [^{32}P]RNA to bring the volume to 1.0 ml and the disintegrations per minute of [^{32}P]RNA to a minimum of 10^6/ml. All tubes had the same volume and number of disintegrations per minute in a given experiment. Tubes were placed in a shaking bath at 65°C, the temperature was lowered to 42°C, and the tubes were incubated for approximately 60 h. The nylon filters were washed two times for 5 to 15 min at room temperature in 2× SSPE (18), two times for 30 min at 65°C in 2× SSPE–0.1% SDS, and two times for 30 min at room temperature in 0.1× SSPE. Filters were Cerenkov scintillation counted overnight at 50 min per sample. The filters were then exposed to XAR-5 film for periods ranging from 1 h (some rDNA hybridizations) to 25 days (some polymerase II transcript hybridizations), with or without intensifying screens as needed. No corrections were made for efficiency of hybridization versus size of transcripts.

Sensitivity of transcript RNA to α-amanitin. Production of the putative polymerase II products was specifically sensitive to α-amanitin (Fig. 2). Figures 2A and C show electro-

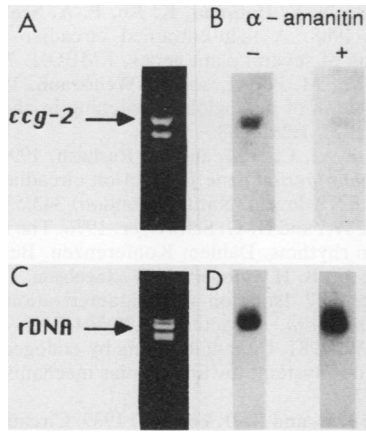

FIG. 2. Sensitivity of the nuclear run-on reaction to the presence of α-amanitin. (A) Analysis in which 1 μg of plasmid DNA containing the *ccg-2* gene was digested with *Xba*I and agarose gel electrophoresed. The 5-kb *ccg-2* band is indicated above the 2.95-kb vector band. The blots were probed with ^{32}P-labeled transcripts isolated from nuclear run-on reactions carried out in the presence or absence of 1 mg of α-amanitin per ml. (B) Autoradiographs of the blots, exposed for 17 days with intensifying screens. Careful analysis of these autoradiographs and autoradiographs of vector DNA alone (not shown) confirmed that under these hybridization and washing conditions, there was not hybridization to vector sequences. (C) Analysis in which 2 μg of the rDNA-containing cosmid 7:5F (7, 35) was digested with *Bam*HI and electrophoresed as for panel A. *Bam*HI cuts the rDNA repeat in two places, yielding a small (approximately 300 bp) and large (approximately 8.9 kb) fragment, as well as two junction fragments from each end of the repeat. *Bam*HI also cuts the vector into two fragments of 4.1 and 3.8 kb each. The 8.9-kb rDNA fragment is indicated with vector pieces below and junction fragments above; the small rDNA fragment was cut off the blots before use. The same ^{32}P-labeled run-on transcripts made in the presence or absence of 1 mg of α-amanitin per ml and used for panel B were used simultaneously to probe these rDNA blots, followed by autoradiography (D). Because of the difference in synthesis rates between *ccg-2* and rRNA, panel D needed only overnight exposure.

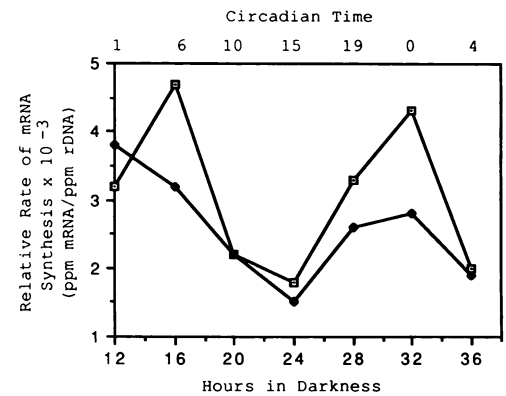

FIG. 3. Regulation of the *ccg-1* and *ccg-2* genes by the circadian clock at the level of transcription. Nuclear run-on analyses were carried out, and the ^{32}P-labeled RNA was isolated. Transcripts specifically arising from *ccg-1* and *ccg-2* were isolated by hybridization to immobilized DNA fragments containing these genes and quantified by Cerenkov scintillation spectrometry. Autoradiography of the DNA blots after counting (not shown) confirmed the rhythm in transcription in addition to verifying that there was no RNA hybridization to vector sequences. Specific hybridization to *ccg* genes is calculated as parts per million input RNA. Transcription rate is presented as a ratio between parts per million *ccg-1* (■) or *ccg-2* (⊡) and hybridization (in parts per million) to the non-clock-regulated rDNA gene (see Fig. 2). Counting error (2 standard deviations) was 5% or less.

phoresed DNA fragments from restriction digests; the arrows indicate a 5-kb *Xba*I fragment containing the *ccg-2* gene and a 8.9-kbp *Bam*HI fragment containing the rDNA repeat. Run-on reactions with CT1 nuclei were carried out in the presence and absence of 50 μg and 1 mg of α-amanitin per ml. Nylon transfers of the same or similar gel lanes were hybridized to isolated run-on RNA (Fig. 2B and D). In neither case was the amount of RNA available for hybridization to rDNA affected by the addition of α-amanitin to the reaction mix. The addition of 50 μg of α-amanitin per ml did not significantly inhibit run-on transcription of the *ccg-1* gene (data not shown), but with 1 mg/ml, *ccg-1* transcription was considerably inhibited (32).

Circadianly regulated changes in the rate of transcription of the *ccg-1* and *ccg-2* genes. Nuclear run-on experiments were performed to determine transcription rates over time of the two clock-controlled genes *ccg-1* and *ccg-2*. Nuclei were isolated from mycelia timed to represent samples covering greater than one circadian cycle. The relative transcription rate of each of these timed samples is shown in Fig. 3 as the ratio of *ccg*-specific transcription to time-invariant (rDNA) transcription as measured by hybridization to probe RNAs in the same hybridization reaction. In control experiments, the number of disintegrations per minute bound to filters increased linearly with the disintegrations per minute added to the hybridization reaction and was not changed when a smaller or larger amount of driver (*ccg*) DNA was applied to the filters (data not shown); hybridizations were thus carried out in driver excess so that the disintegrations per minute hybridized accurately reflected the gene-specific incorporation in the run-on reaction. While this report was in preparation, similar uses of nuclear run-on analysis for *N. crassa* were reported elsewhere (22, 28).

Both *ccg-1* and *ccg-2* are circadianly regulated at the level of transcription. In separate experiments, the amplitude of the rhythm varied between 3- and 10-fold, with the *ccg-2* gene invariably displaying a higher rate of transcription at the maximum points. In different experiments, the time of the maximum rate varied between CT19 and CT6 but was generally near to CT0 for both genes. The minimum rate, on the other hand, was identical for both genes and consistently occurred at CT15. Similar rhythms in the rate of transcription were seen when *ccg-1* and *ccg-2* were normalized against the *oli* gene and following densitometry of autoradiographs of the time-specific transcription products arising from both genes (data not shown).

Little is known of the regulatory pathways used by the clock in the distribution of temporal information within *N. crassa* or any other organism. We therefore directed our efforts toward understanding how the clock transfers its time information from the timing mechanism out into the cell via the regulation of gene expression. The studies described here were initiated to determine the level at which the mRNA abundance rhythm of the *ccg-1* and *ccg-2* genes is controlled. The transcription rate for these genes is under control of the circadian biological clock, with changes of approximately three- to fivefold over the circadian day. From repetitions of these experiments, we have concluded that the peak of *ccg-2* transcription is always greater than that of *ccg-1*. The phases of the peaks and troughs are also

very similar for the two genes, the peaks varying around CT1 and the trough of transcription invariably occurring at the CT15 time point for both genes in all experiments. Changes in the total amount of *ccg-1* and *ccg-2* mRNAs over the circadian cycle indicate a 5- to 10-fold change in abundance (17). Therefore, the clock-controlled transcriptional changes documented here may account for the mRNA abundance rhythm, although these studies do not preclude the possibility of other means of control also, such as differing RNA stability over time.

Genes from a variety of organisms other than *N. crassa* are also known to be under control of the circadian clock. Transcriptional regulation of gene expression by the circadian clock was first shown by using reporter constructs in transgenic tobacco (20) and has subsequently been shown by using nuclear run-on analyses in other plant systems (8, 31). Several vertebrate transcripts are known to cycle in either a circadian or diurnal manner, including cerebrospinal fluid-bound vasopressin mRNA (26, 33), calcitonin mRNA (15), retinal transducin mRNA (1), and opsin mRNA (14). Most recently, the abundance of transcripts arising from a gene intimately involved in regulating the period length of the *Drosophila melanogaster* clock, the *per* gene, has been shown to be under circadian control; the gene thus appears to be involved in its own feedback regulation (10). However, in none of these vertebrate or insect systems is the mechanism of clock regulation understood.

This is the first report of transcriptional regulation by the clock in a system that is easily genetically and molecular genetically manipulable. Isolation of the *cis*-acting clock regulatory sequences from these genes, and eventually the *trans*-acting factors that interact with these sequences, should be helpful in further understanding how the clock controls developmental and environmental gene expression. Additionally, through the use of constructs in which these *cis*-acting regulatory sequences are used to control the expression of reporter genes, we are now developing a selection scheme for mutations affecting the circadian system of *N. crassa*.

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