in the apical membrane of renal IMCD cells. These data suggest that the natriuretic activity of ANP is related in part to cGMP mediated-inhibition of electrogenic Na^+ absorption by the IMCD.

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Molecular Cloning of Genes Under Control of the Circadian Clock in *Neurospora*

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To investigate the regulation of messenger RNA abundance by circadian clocks, genomic and complementary DNA libraries were screened with complementary DNA probes enriched, by means of sequential rounds of subtractive hybridization, for sequences complementary to transcripts specific to either early morning or early evening cultures of *Neurospora*. Only two morning-specific genes were identified through this protocol. RNA blot analysis verified that the abundance of the transcripts arising from these genes oscillates with a period of 21.5 hours in a clock wild-type strain and 29 hours in the long-period clock mutant strain *frq*^{7}. Genetic mapping through the use of restriction fragment length polymorphisms shows the two genes, *ccg-1* and *ccg-2*, to be unlinked. These data provide a view of the extent of clock control of gene expression.

CIRCADIAN RHYTHMS ARE GENERALly thought to be the output of an intracellular metabolic network (*1*). However, these clocks must also act to regulate cellular metabolism, one salient aspect of which is clock control of mRNA abundance. Historically, a great deal of effort has gone into attempts to elucidate the molecular mechanism of the biological clock (*2*); however, in general, little is known about the nature of temporal information within cells (*3*). Thus, in order to begin to understand and identify the pathways and molecular components involved in the transfer of temporal information within a cell, we have undertaken the systematic isolation of clock-controlled genes (morning- and evening-specific genes) from the ascomycete *Neurospora crassa*. These studies have identified only two genes that are strongly regulated by the clock as measured by mRNA abundance.

The protocol we used for the isolation of genes under the control of the clock is based on a liquid culture system developed for biochemical studies on the *Neurospora* clock (Fig. 1A) (*4*). Disks are cut from a mycelial mat of a single cell type growing in a rich medium. The mycelia of the mat, and of each disk cut from it, contain identical and synchronous clocks. To prevent production of conidia (*5*) and subsequent clock desynchronization, the disks are transferred en masse to a nutritionally poor medium in which they will remain alive but not grow rapidly or form conidia. When such disks are transferred from light to constant darkness, the clocks are all reset to subjective dusk circadian time 12 (CT12) (*6*) and will run from that point at their endogenous rate. In wild-type *Neurospora* the circadian cycle is 21.5 hours. We also used a mutant of the *Neurospora* clock, *frq*^{7}, with an endogenous period of 29 hours at 25°C. The only known phenotypes associated with the semidominant mutations at the *frq* locus, including *frq*^{7}, are those affecting aspects of the circadian clock (*7, 8*).

Our initial isolation of clock-controlled genes relied on the use of subtractive hybridization (*9*). Polyadenylated [poly(A)$^+$] RNA (*10*) was isolated from cultures held 23 hours and 34 hours in the dark, representing subjective evening and morning, respectively. These RNAs were used as templates for the synthesis of radiolabeled cDNAs (*11*), which were then hybridized and subtracted twice with an excess of RNA from the opposite time to remove cDNAs complementary to RNAs present at both phases of the clock cycle (*12*). The subtracted cDNAs resulting from this protocol thus represented putative time-specific probes and were used to probe both a cDNA and a genomic library.

As a result of the culture and subtractive hybridization protocols, we expected to identify (i) developmentally regulated genes displaying activity changes resulting from the interruption in the natural progression toward conidiation (*5*), (ii) environmentally responsive genes with mRNAs regulated in response to either starvation or abrupt shifts in the ambient light level (*13*), and (iii) clock-controlled genes with activity that cycled according to circadian clock phase. These three types of genes were distinguished in the following way (Fig. 1): Total cellular RNA was isolated every 4 hours for 2 days from wild-type [*frq*$^+$ (*14*)] and *frq*^{7} mycelial disks held in liquid culture in total darkness. In wild-type *Neurospora* this time period corresponds to approximately 2.5 circadian cycles, whereas in the long-period

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frq^7 strain this is less than two full cycles. Thus, the amount of mRNA arising from true clock-controlled genes will cycle from maximum to minimum more than once during this time, and the same mRNAs should cycle at different rates in the wild-type and clock mutant strains. In these respects, clock-controlled genes will differ from environmentally, nutritionally, and developmentally controlled genes. In this way then, RNA blot analysis of the timed RNA samples (Fig. 1B) was used to establish the type of control exhibited by the subtractive hybridization–selected genes.

Examples of two different morning-specific clock-controlled genes are seen in Fig. 1B. Clones 6C1 and 3a6 [clock-controlled gene 1 (*ccg-1*)] (Fig. 1B, part a) detect an approximately 600-nucleotide (nt) mRNA with an increased concentration just after early subjective dawn (about CT2). In this experiment, mRNAs from *ccg-1* cycle 2.5 times in wild-type cultures, whereas RNAs from the same gene in the long-period clock mutant *frq*^{7} cycled less than twice. Moreover, by 40 to 44 hours in the dark, the transcript concentrations in *frq*^{7} are maximal, whereas those in *frq*$^+$, due to the shorter cycle time, are minimal.

Figure 1B, part b, shows analogous data for morning-specific gene *ccg-2* (genomic clones 7C1, 7C5, and 6C3), which encodes an approximately 800-nt mRNA with a peak concentration at an earlier time than *ccg-1*, in the late subjective night to early morning (between CT18 and CT2) for both strains. As with the *ccg-1* gene, the cycle time of the mRNA is clearly different between the wild-type strain and the long-period mutant, and the period lengths of these cycles are as predicted from the strain genotype, 21.5 hours for *frq*$^+$ and 29 hours for *frq*^{7}. Clones were also isolated in which the mRNA levels changed monotonically with time and in which the kinetics of the change were identical in the two strains used, reflecting an absence of circadian input into the mechanism of control of these genes.

Because the ribosomal RNA (rRNA) genes were largely excluded from our initial subtractive procedure and subsequently from selection in our library screens, we would not have picked them up if they were regulated in a circadian fashion. Additionally, circadian fluctuations in total RNA concentration have been reported in *Neurospora* and other systems (*15*). In the present study, although RNA content was found to decrease as the disks aged, the proportion of cellular RNA present as rRNA was time-invariant. Figure 1B, part c, shows the same RNA blot probed with *ccg-1* as in Fig. 1B, part a, stripped, and reprobed with *Neurospora* rDNA sequences. The 17*S* and 26*S* rRNA levels do not change in a circadian fashion in liquid culture. Results similar to these were seen in RNA blots probed with the *oli* gene that encodes subunit 9 of the *Neurospora* mitochondrial adenosine triphosphatase (*16*).

Absorbance measurements from light densitometry were calculated from the RNA blots in Fig. 1B, a and c. A plot of the ratio of *ccg-1* to 17*S* rRNA content over time illustrates the circadian character of the regulation of this transcript in the wild-type versus the long-period strain (Fig. 2). The *frq*$^+$ and *frq*^{7} strains are initially close in phase after 12 hours in darkness, thereafter becoming progressively out of phase, until at 44 hours after light-to-dark transfer the *frq*$^+$ transcript is at minimum abundance while the *frq*^{7} transcript is high.

Eight CT1 and six CT13 plaques were purified from two screenings, each with an independently generated, subtracted, time-specific probe. These 14 isolates were then subjected to RNA analysis to determine circadian regulation, and hybridization and restriction analysis were used to determine

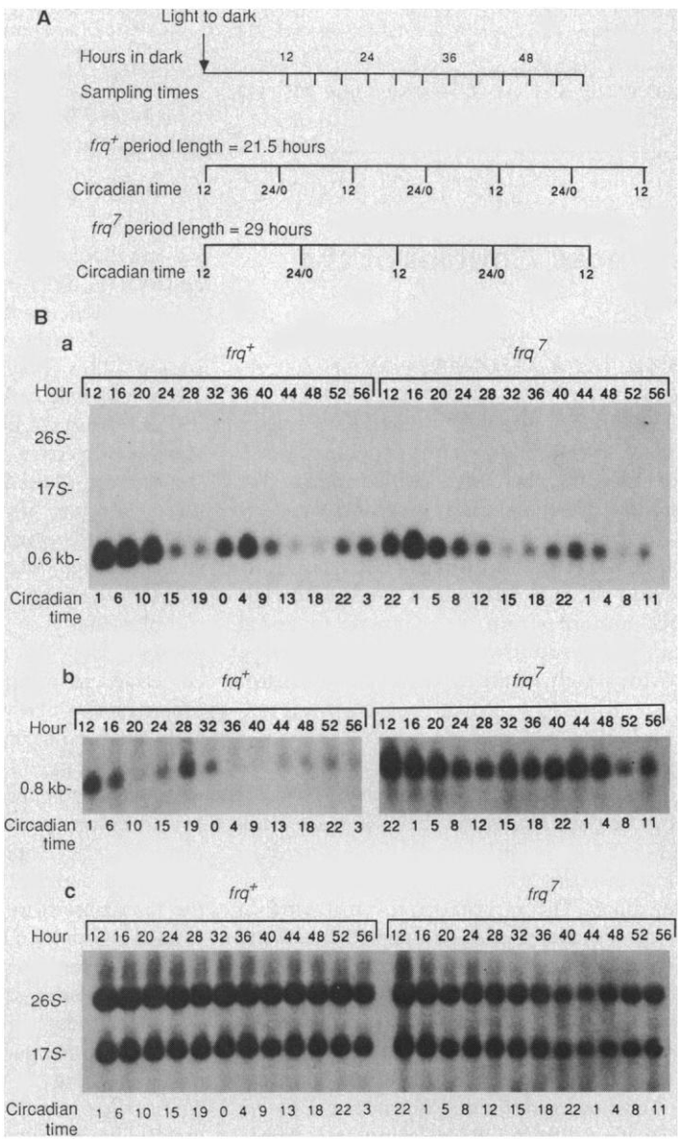


Fig. 1. (**A**) Mycelial harvest protocol for clock-controlled genes. Filtered conidia were inoculated into a high-concentration glucose-arginine liquid medium at approximately 6×10^7 conidia per milliliter. The conidia were allowed to germinate and grow in constant light (LL) at 22°C until a uniform mycelial mat was formed but before conidiation had begun. Disks were cut with a cork borer and transferred to a low-concentration glucose-arginine shaking culture in constant darkness (DD). Growth and differentiation, including formation of conidia, were thus arrested. In some experiments *pan*$^-$ auxotrophs were cultured and transferred to pantothenate-limited, glucose-rich medium as an alternative method to slow growth and arrest of conidiation (*4*). In either case, beginning 12 hours after the LL to DD transition, disks were harvested every 4 hours for 56 hours in DD. The circadian time of the disks was monitored at the time of harvest by race tube analysis (*24*). Between 40 and 44 hours in DD the *frq*$^+$ and *frq*^{7} cultures should be approximately 180° out of phase. (**B**) RNA blot analysis of transcripts arising from morning-specific and non–clock-controlled sequences. Total RNA from both *frq*$^+$ and *frq*^{7} cultures was isolated (*10*) from harvested mycelial disks. From each sample, 20 μg of total RNA were loaded into a lane and fractionated electrophoretically through 0.8% agarose-formaldehyde gels (*25*), transferred to nylon membranes, and probed with DNA that had been randomly labeled with ^{32}P (*26*). The total number of hours in darkness before harvest is shown above each blot, and the approximate circadian time of the sample is shown below. (a) A CT1 cDNA phage, λgt11-3a6, corresponding to the *ccg-1* gene, was used to probe RNA isolated from *frq*$^+$ or *frq*^{7}. (b) A 3-kb Xba I–Eco RI fragment from genomic clone 7C1, corresponding to *ccg-2*, was used to probe RNA from *frq*$^+$ or *frq*^{7}. (c) The same RNA blot as in (a) was stripped and probed with DNA from cosmid clone 7:5F (*27*) corresponding to the *N. crassa* rDNA repeat, thus demonstrating time-invariant expression. Sizes were determined by interpolation with RNA size marker standards.

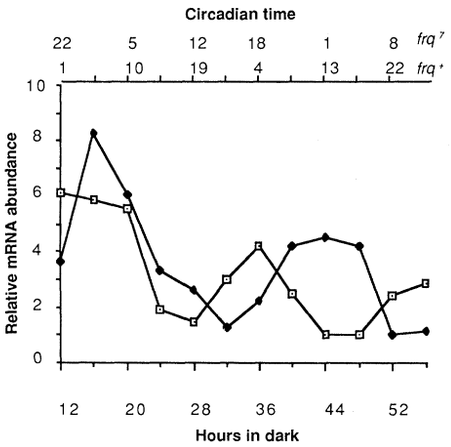
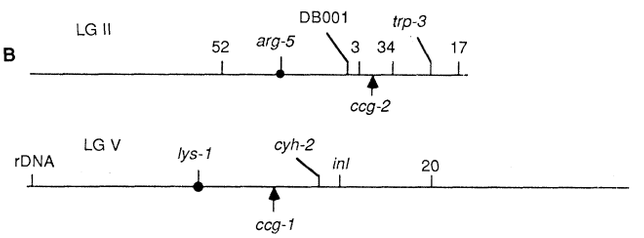


Fig. 2. Densitometric analysis of *ccg-1* RNA abundance changes. Appropriate exposures of the RNA blot autoradiograms shown in Fig. 1B, a and c, were scanned by light densitometry. Absorbance ratios of the bands corresponding to the *ccg-1* transcript and 17*S* RNA were calculated and then normalized to the lowest ratio, that seen in frq^+ at CT 18 (lane 10). The ratio of *ccg-1* transcript to 17*S* RNA in frq^+ is shown by the open squares and in frq^7 by the filled diamonds.

Fig. 3. Chromosomal mapping of *ccg-1* and *ccg-2* by RFLPs (*17, 28*). (**A**) RFLPs were determined for genomic sequences regulated by the circadian clock. The recombination frequencies between these RFLPs and conventional and molecular genetic markers are shown. Ratios in parentheses indicate the number of recombinants per total progeny scored. (**B**) Chromosomal map positions of *ccg-1* and *ccg-2* were determined from recombination frequencies. The map positions of conventional and molecular markers are shown. All markers designated by a number only are 5*S* rRNA genes (*17*). Roman numerals refer to linkage group. Map distances were drawn to approximate scale (*29*), except in the distal-most right-hand region of LG II where map distances were not additive. Distances shown were not corrected for multiple crossover events; therefore some markers may be farther apart than shown. The centromere for each chromosome is shown as a solid circle.

A

Clone	Marker	Percentage of recombination between RFLP and markers	Enzyme revealing RFLP
ccg-1	*lys-1*	18% (7/38)	Eco RI
ccg-1	*cyh-2*	11% (4/38)	Eco RI
ccg-1	*inl*	16% (6/38)	Eco RI
ccg-2	DB001	5% (2/37)	Hind III
ccg-2	3 (5*S* RNA)	3% (1/37)	Hind III
ccg-2	34 (5*S* RNA)	5% (2/37)	Hind III



common identities between isolates. Both genes identified here were selected more than once. Genomic clones 6C3 and 7C1 have identical restriction maps and thus may be a reisolate of the same phage, whereas genomic clone 7C5 contains a different but overlapping insert that contains the same clock-regulated region corresponding to the *ccg-2* gene. The *ccg-1* gene was also isolated twice (genomic clone 6C1 and cDNA clone 3a6). Therefore, from the final eight CT1 isolates, five were represented by the two clock-controlled genes, whereas the other three isolates identified genes displaying alternate, noncircadian, modes of regulation.

The initial decision to search for CT1 genes was based on protein synthesis and inhibition studies that postulated the presence of a critical morning clock protein (*8*). These studies did not indicate evening protein synthesis to be of critical importance in clock maintenance. There were no clock-regulated evening-specific genes among the six CT13 clones arising from the subtractive hybridization protocol. Although some of these isolates identify transcripts of such low abundance that positive analysis has been difficult, in no case do they appear to be clock regulated.

The chromosomal locations of the two clock-controlled genes were determined by restriction fragment length polymorphism (RFLP) analysis (Fig. 3) (*17*). The *ccg-1* gene maps to the right arm of linkage group V, between the genetic markers *lys-1* and *cyh-2*. The *ccg-2* gene maps to the right arm of linkage group II between 5*S* rDNA markers 3 and 34 and is clearly unlinked to *ccg-1*. Thus, coordinate control of these two genes by a single local clock-controlled change in chromatin structure is not likely.

Because we isolated only two distinct clock-controlled genes, it has so far not been possible to determine the extent of clock control or to estimate the percentage of the genome that might actually be under circadian control. In general the answers to these questions will depend on the culture conditions chosen. For instance, we deliberately avoided conditions that would allow conidiation, but the clock in *Neurospora* is known to be one of the controls for the developmental switch leading to conidiation (*7*). Additionally, it is probable that we have not identified all of the clock-controlled genes present under these growth conditions. Although some of the noncircadian genes isolated were of low abundance, other messages of limited abundance could have been overlooked. Finally, in these studies we have examined only two phases of the daily circadian cycle, and clock control may be more extensive at other times of day.

Why are there apparently so few clock-controlled genes? One answer is that the steady-state mRNA population is probably low. Although there is no estimate of the number of mRNAs present in cells grown under the conditions used here, estimates from vegetatively growing *Neurospora* suggest the presence of only about 2000 different messages, representing about 20% of the coding capacity of the genome (*18*). In our arrested growth conditions the percentage of the genome being expressed may be lower still. Both genes identified here were selected more than once. Repeated reisolation of the same clock-controlled regions suggests that there are only a few regions in the *Neurospora* genome that are under clock control at the times and in the conditions chosen.

Although *Neurospora* is the only organism in which there has been a systematic search for clock-controlled genes, the phenomenon is not limited to *Neurospora*. Hybridization experiments in *Tetrahymena* have provided evidence of time-of-day–specific transcripts (*19*). In rats, the mRNA encoding cerebrospinal fluid–bound vasopressin is under circadian control in the suprachiasmatic nucleus (*20*), and diurnal rhythms in retinal transducin mRNA levels show the kind of dawn anticipation characteristic of circadian rhythms (*21*). In peas, mRNAs encoding three proteins of the photosynthetic apparatus are regulated in a circadian fashion (*22*), and one of these, the light-harvesting chlorophyll a/b binding protein, is similarly regulated in wheat and in transgenic tobacco (*23*).

Clock control of the genes identified in this study could be exerted either by changes in initiation of gene expression or by changes in the stability of existing messages that are always transcribed at constant rates; from the present data it is not possible to distinguish between these possibilities. In either case, these data suggest that direct or indirect clock control of mRNA levels may be an important aspect of developmental and environmental control of gene expression. Finally, we hope the isolation of clock regulatory sequences from these genes will be useful in the formation of a mutant selection scheme for the *Neurospora* clock.

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Treatment with Tin Prevents the Development of Hypertension in Spontaneously Hypertensive Rats

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Cytochrome P-450–dependent metabolites of arachidonic acid (AA) increased in the kidneys of young, spontaneously hypertensive rats (SHRs) during the period of rapid elevation of blood pressure (BP) but not in adult SHRs or in Wistar Kyoto rats (WKYs) with normal BP. Treatment of SHRs and WKYs with stannous chloride ($SnCl_2$), which selectively depletes renal cytochrome P-450, restored BP to normal, coincident with a natriuresis, in young but not in adult SHRs and did not affect either BP or sodium excretion in WKYs. Depletion of renal cytochrome P-450 was associated with decreased generation of these AA metabolites only in young SHRs. The antihypertensive effect of $SnCl_2$ in young SHRs was greatly reduced by prevention of its cytochrome P-450–depleting action.

THE MECHANISMS RESPONSIBLE FOR the elevation of BP in essential hypertension are not understood (*1*). The animal model most frequently used to study essential hypertension is the SHR (*2*). In the young SHR, between ages 5 and 13 weeks, BP increases rapidly: for example, systolic BP rises from 100 to 170 mmHg, whereas in the age-matched WKY systolic BP rarely exceeds 130 mmHg (*3*). Hypertension in the SHR can be corrected by renal transplant from a normotensive donor (*4*), suggesting that abnormalities in renal function contribute to elevation of BP. Alterations in salt and water excretion, renal blood flow, and glomerular filtration rate have been described in the SHR at 4 to 8 weeks of age (*3, 5*) but are compensated for in the older SHR. It has been suggested that these alterations in renal function in the young SHR are the functional expression of abnormal renal AA metabolism through the cyclooxygenase pathway (*6, 7*). Thus, increased production of thromboxane (Tx) A_2 has been described in the SHR (*8*) and may contribute to the aforementioned abnormalities in renal hemodynamics and excretory function (*9*).

Metabolites of AA generated by cytochrome P-450 monooxygenases also have the potential to alter BP by affecting vascular tone and Na^+,K^+-adenosine triphosphatase (Na^+,K^+-ATPase) activity (*10, 11*). We have recently reported that renal cytochrome P-450 levels and metabolism of AA by cytochrome P-450–dependent monooxygenases are elevated in the SHR during the developmental phase of hypertension, ages 5 to 13 weeks, when compared to those in the WKY (*12*). When hypertension becomes

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