Glyceraldehyde-3-phosphate Dehydrogenase Is Regulated on a Daily Basis by the Circadian Clock*

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Circadian clocks function to govern a wide range of rhythmic activities in organisms. An integral part of rhythmicity is the daily control of target genes by the clock. Here we describe the sequence and analysis of a novel clock-controlled gene, ccg-7, showing similarity to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a glycolytic enzyme widely used as a constitutive control in a variety of systems. That ccg-7 encodes GAPDH was confirmed by demonstrating that in vitro synthesized CCG-7 possesses GAPDH activity. Rhythms in both ccg-7 mRNA accumulation and CCG-7 (GAPDH) activity are observed in a clock wild-type strain where the peak in GAPDH activity lags several hours behind the peak in ccg-7 mRNA accumulation in the late night. Together with our previous observation that ccg-7 mRNA is not developmentally regulated, we show that ccg-7 is not induced by environmental stresses such as glucose or nitrogen deprivation (which also trigger development), heat shock, or osmotic stress. Thus, the finding that GAPDH is clock-regulated points to a specific role for the circadian clock in controlling aspects of general metabolism and provides evidence for circadian regulation of a gene found in most living organisms.

Circadian rhythms are daily rhythms that are generated and controlled by endogenous, self-sustaining, temperature-compensated biological clocks that can be reset by environmental cues (reviewed in Ref. 1). These rhythms are observed in a wide variety of organisms ranging from bacteria to mammals and are coordinated with exogenous environmental cycles to limit activities at particular times of the day. One important aspect of rhythmicity involves control of specific target genes within an output pathway on a daily basis by the circadian clock. The Neurospora circadian clock controls the timing of asexual spore (conidium) development, where conidiation is initiated once conidiation takes place (5–7), it has been widely assumed that the ccg genes are directly and uniquely associated with this asexual developmental pathway. This clock-regulated developmental association was viewed to be distinct from that imagined for “housekeeping genes” whose expression was expected to be relatively time-invariant; this expectation of time-invariant expression underlies the use of genes such as GAPDH as loading or normalization controls. Given this, it was of particular interest in the analyses of a recent set of novel ccg genes (7) to find three ccg genes that are neither light- nor developmentally regulated. This suggested that the circadian clock could be regulating cellular metabolism at a much more basic level than had been anticipated.

Here we show this to be the case. One of the clock-controlled genes, ccg-7, encodes N. crassa GAPDH, the first energy-harvesting enzyme in the glycolytic pathway and the homolog of an mRNA often used as a “constitutively expressed control” in different tissues and cell types. Although we find circadian regulation to be apparent at the level of both mRNA and enzyme activities, ccg-7 mRNA levels are refractory to changes in many common environmental stimuli, reinforcing the unique importance of this circadian influence. Through GAPDH regulation, the circadian clock may be controlling the glycolytic pathway and thereby influencing the organisms’ fundamental metabolic activities. ccg-7 is a clock-regulated gene encoding a product participating in basic metabolism, a function found in most living organisms. The regulation and identity of ccg-7 suggest that circadian influences may be more pervasive at the cellular level than previously anticipated.

EXPERIMENTAL PROCEDURES

Strains, Plasmids, and Culture Conditions—All experiments described in this report were carried out using strain bd; A (30-7, laboratory stock). Growth conditions and media of Neurospora cultures were as described (8). The plasmid pCCG7 includes ccg-7 cDNA in pBluescript SK+ (Stratagene) and was isolated from differential screening of morning versus evening XZapII cDNA libraries (7). A 4.5-kilobase PstI fragment and a 3.0-kilobase BamHI-XhoI fragment from ccg-7 genomic DNA were subcloned into pBluescript SK+, resulting in...
plasmids pMLS701 and pMLS702, respectively. The plasmid pKL119 (9) containing ccg-1 genomic DNA was used as a positive control for glucose deprivation (10) and for osmotic stress.2 As positive controls for the nitrogen deprivation experiments (11) and heat-shock experiments, eas (ccg-2), in the plasmid pLW1 (12) and hap30 (13) cDNA were used, respectively. The sexual strain XLI-Blu (Stratagene) was used for all plasmid manipulations.

Rhythmic RNA and protein analyses were carried out as described previously using submerged liquid cultures, where development is curtailed (2–7, 12, 14, 15). Mycelial samples were grown in constant light for 4 h and then transferred to constant darkness to synchronize the clock to the subjective onset of night. Standard growth medium includes 1 × Vogel’s medium (8) plus 2% glucose. Analyses of the changes in RNA levels resulting from environmental changes were carried out by challenging mycelia, grown in standard growth medium, with a modified medium to apply environmental stress. These manipulations were performed in the dark, and all the samples were harvested at the same developmental age and at the same circadian time. For heat-shock analyses, mycelia were incubated in growth medium preheated to 47 °C as described previously (16). Osmotic stress was accomplished by transferring mycelia for the indicated time in growth medium containing 4% NaCl. To examine the effects of glucose and nitrogen deprivation, cultures were transferred to 1 × Vogel’s medium minus 2% glucose or 1 × modified Vogel’s medium (Vogel’s salts lacking NH₄NO₃) containing 2% glucose, respectively. Prior to transfer to the nutrient-deficient media, the mycelial pads were washed four times in nitrogen- or glucose-deficient medium.

Genomic Library Screening, Sequencing, and Computer Analyses—A ccg-7 genomic clone, GS-7H, was isolated from the Orbach/Sachs genomic cosmid library (pMOCoxX, Fungal Genetics Stock Center) by colony hybridization (17) using a ccg-7 cDNA probe radiolabeled with [α-32P]dCTP (6000 Ci/mmol; DuPont). Two subclones, pMLS701 and pMLS702, were constructed from the cosmid clone. Automated sequencing of both strands of ccg-7 cDNA and genomic DNA was accomplished using the Applied Biosystems Prism Dye deoxy sequencing kit using nested oligodeoxynucleotide primers. DNA and putative amino acid sequences were compared with other known genes and peptides using the BLAST search from the GenBank™/EMBL non-redundant data base.3 The program CLUSTALW (18) was used to align amino acid sequences. Quantitation of Northern and Western blots was performed on scanned images (Silverscanner III, LaCie) by densitometry using the ImageQuant™ software (Molecular Dynamics) and its conceptual amino acid sequence was found to have a high degree of similarity to GAPDH peptides from diverse organisms (Figs. 1 and 2). Based on RNA hybridization, cDNA size, and primer extension analysis, ccg-7 encodes a single processed transcript of 1226 nucleotides (excluding the poly(A) tail) that contains a short 5′-untranslated region (43 nucleotides) followed by a single open reading frame of 338 codons. Two introns of 567 and 76 nucleotides are processed out of the primary transcript, the former being unusually large for N. crassa genes (25). The presence and position of the shorter intron within the coding region are conserved among GAPDH genes from some filamentous ascomycetes (26–31). While this paper was in revision, the same sequence appeared in a clone identified during work in the Neurospora am gene and was identified as encoding GAPDH (32). However, the longer intron within the 5′-untranslated region was not found in previously identified GAPDH genes from other fungal species. The 3′-untranslated region contains a putative polyadenylation signal (AATAAT, nucleotides +1828 to +1833) followed by a poly(A) tail positioned after the last nucleotide at +1866 (Fig. 1).

The conceptual CCG-7 sequence possesses the universally conserved GAPDH motif located at positions 155–162 (ASCTT-NCL; underlined in Fig. 2), containing the catalytic site Cys-157. Other important residues for GAPDH enzyme activity, such as catalytic residues His-184, Thr-187, and Lys-191 and NAD+–binding residues Asp-37 and Phe-106 (30), are also perfectly conserved in CCG-7. Codon usage in ccg-7 is biased, as previously reported for other genes from N. crassa (25), where C is most preferred at the third position. Altogether, the data are wholly consistent with ccg-7 encoding the N. crassa GAPDH gene.

Steady-state Levels of ccg-7 mRNA and GAPDH Activity Vary Systematically over Time—When analyzed under constant environmental conditions in the dark, ccg-7 mRNA levels showed a rhythm peaking in the late night (CT 18–0) (Fig. 3, A and B). The period length of the ccg-7 mRNA abundance rhythm changed appropriately with the strain examined, 22 h in the clock wild-type frq+ strain and 29 h in the long-period clock mutated frq− (7) (Fig. 3B). The characteristics of stable rhythmicity with a period length reflecting the genotype of the strain (frq+ versus frq−) and of the constant phase (peak always in the subjective night) indicate that rhythmic expression of the ccg-7 gene is indeed controlled by the circadian oscillator. Amplitude of mRNA levels was often observed to be varied among different clock phenotypes. Although rhythmicity in ccg-7 transcript abundance is apparent, if CCG-7 protein had a long half-life under these conditions, rhythms in enzyme activity might not be evident. We first attempted to detect CCG-7 using antibodies directed against rabbit muscle GAPDH and against Trypanosoma glyoxosomal GAPDH, but CCG-7 was not detected by either. Therefore, to test the metabolic significance of the rhythm in ccg-7 mRNA, rhythmicity of GAPDH activity was assayed as a function of time (Fig. 3C). A ~1.5-fold rhythm in GAPDH activity was observed, with peak activity occurring in the early morning (CT 4–6) under conditions of constant darkness in liquid culture and in the absence of observable development. In control experiments using the same protein extracts, the clock component protein FRQ showed the characteristic rhythms in abundance and time-of-day-specific phosphorylation (Fig. 3A) (15), confirming a robust and functional clock in these cultures. On the other hand, alkaline phosphatase activity stayed constant (Fig. 3C), as previously suggested (21). As protein extracts were obtained from replicate samples from the same cultures used to isolate RNA sam-

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2 K. Lindgren, N. Garceau, and J. J. Loros, manuscript in preparation.
3 This can be accessed through the NCBI Web Server (http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST).
FIG. 1. Gene organization and nucleotide sequence of the ccg-7 gene. A, schematic representation of the ccg-7 gene. The transcribed region is indicated as a shaded box. Below are shown three exons (empty and filled boxes) and two introns. The protein coding regions are indicated by filled boxes. bp, base pairs. B, ccg-7 genomic DNA sequence and its conceptual amino acid sequence. The ccg-7 sequence, starting
ples, these data indicate that a 4–8-h time lag occurs between the peaks of ccg-7 mRNA and GAPDH enzyme activity levels. The data indicate a stable and consistent time-of-day-specific regulation of GAPDH activity that persists in the absence of environmental changes or overt developmental progression.

ccg-7 mRNA Levels Are Refractory to Changes in Most Environmental Factors—Circadian clocks are generally understood as being evolutionarily adaptive by virtue of the eligibility they confer on the organism to anticipate regularly observed changes in the environment. Thus, to understand better what role the observed GAPDH rhythm might play in the life of Neurospora, we examined the response of ccg-7 to acute environmental stimuli and agents of stress (Fig. 4).

The data indicate that ccg-7 mRNA levels are refractory to changes in most environmental factors. For example, ccg-7 mRNA levels clearly showed no increase under heat shock or osmotic stress, and occasionally, heat shock resulted in a small but real decrease in expression levels (Fig. 4, A and B).

The expression of some other ccg genes (7). All samples were harvested at the same circadian time corresponding to approximately halfway between trough and peak of ccg-7 mRNA levels. Although positive control genes (hsp30 and ccg-1) respond in a typically robust manner to these stimuli (13), ccg-7 mRNA levels clearly showed no increase under heat shock or osmotic stress, and occasionally, heat shock resulted in a small but real decrease in expression levels (Fig. 4, A and B).

Next, since it is known that developmental induction of conidia occurs in a relatively synchronous fashion, beginning late each night and extending through the morning, we examined the response of ccg-7 to nitrogen and carbon starvation, agents known to trigger development (11, 33). Nitrogen deprivation (~1.2 kilobases downstream of the BamHI site and ending one nucleotide before the poly(A) site, is shown). The transcription start site is indicated with a dot at +1. Boldface sequences were present in the ccg-7 cDNA. Intron sequences are shown in lower-case letters. Underlined regions (motifs) designate the putative CAAT box (nucleotides −231 to −228), the CT motif (nucleotides −48 to −29) (55), and the translation start codon (nucleotide +613). The predicted amino acid sequence, starting at the first ATG codon, is indicated in single-letter code below each corresponding triplet codon. Numbers to the right of the sequence indicate nucleotide number, and numbers to the left designate amino acid number.
vation increased the mRNA levels of the control gene *eas (ccg-2)* (11), but not those of *ccg-7* (Fig. 4C). In response to glucose starvation, we consistently observed a slight decrease in *ccg-7* mRNA levels during the first 5 h and a substantial drop at 6 h following glucose deprivation (Fig. 4D). It is possible that poor availability of the GAPDH substrate, due to low glucose, may cause a decrease in *ccg-7* mRNA levels. In this experiment, the control glucose-derepressible gene *ccg-1* responded as expected (10). Two other agents known to trigger development, light and desiccation, have also been analyzed recently (7) and shown not to affect *ccg-7* mRNA levels. Thus, *ccg-7* does not respond to these classic developmental stimuli.

In Vitro Expressed CCG-7 Shows GAPDH Activity, but Not Detectable UDG Activity—GAPDH activities were compared among *in vitro* expression reaction mixtures containing the protein products of either *ccg-7* or, as controls, *cmt (ccg-12)* or lysate only. The translation mixture to which *ccg-7* was added had significant GAPDH activity compared with controls (Fig. 5A), confirming that *ccg-7* encodes *N. crassa* GAPDH. Another possible role for the *ccg-7* rhythm is suggested by the finding that a human GAPDH peptide, UDG/GAPDH, can function in DNA repair as UDG (23). This possibility is supported by the striking similarity between CCG-7 and human UDG/GAPDH (65.0%) (Fig. 2). Therefore, the same translation mixture was examined for UDG activity; however, reactions containing CCG-7 released amounts of \[^{3}H\]dUTP equivalent to the negative controls (either lysate without expressed protein or lysate

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**Fig. 3.** *ccg-7* mRNA accumulation and GAPDH activity are rhythmic. Samples were harvested every 4 h after transfer from constant light to constant dark. The corresponding circadian time is indicated. A, the 1.2-kilobase *ccg-7* mRNA was detected by Northern analysis using an antisense *ccg-7* mRNA riboprobe. Ten μg of total RNA was separated on a 1.0% formaldehyde-agarose gel. rRNA (18 S), previously shown to be time-invariant (5), was used as an internal loading control. Total protein extracts were obtained from replicate samples from the same culture used to extract total RNA. The clock component FRQ was detected as an internal control for rhythmicity: 100 μg of total protein extract was separated on a denaturing gel, and FRQ protein was detected with anti-FRQ antibody by immunoblotting (15). B, levels of relative *ccg-7* mRNA (normalized to rRNA) from a clock wild-type strain (frq \(^+\); ●) and from a long-period mutant (frq \(^-\); □) are plotted. C, levels of GAPDH activity (x), relative *ccg-7* mRNA (●), and alkaline phosphatase (ALP) activity (f) from a clock wild-type strain (frq \(^+\); □) are plotted. Enzyme activity is plotted as specific activity of either GAPDH or alkaline phosphatase measured as the number of units/total protein amount (mg). Four separate cultures were followed, and activity was measured twice at each time point from each culture. **Error bars** show mean ± 2 S.E., where n = 4.

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**Fig. 4.** *ccg-7* expression is not induced by environmental stimuli. Shown are the results of Northern analysis of *ccg-7* mRNA following environmental changes. All cultures were harvested 24 h after transfer to darkness, a time corresponding to early subjective night or CT 15. For each experiment, genes known to be affected by the different stimuli were used as internal positive controls, and 18 S rRNA was used to verify equal loading of total RNA. A, increase in temperature from 25 to 47 °C for the times indicated; B, osmotic stress induced by a change from 0 to 4% NaCl; C, nitrogen starvation induced by a shift from 0.2 to 0% NH\(_4\)NO\(_3\); D, glucose starvation induced by a shift from 2 to 0% glucose.
Our data indicate a 4–8-fold amplitude in the ccg-7 mRNA rhythm and a low amplitude rhythm in GAPDH activity, consistent with the presence of additional post-transcriptional and/or post-translational factors affecting expression of enzyme activity. A rhythm in GAPDH activity (albeit with a different peak time) was suggested by one previous study (21) in cultures grown on solid media, where circadian development (conidiation) occurs. Although the presence of clock-controlled development in the study made it appear likely that the rhythm was reflecting the rhythm in development and mass accumulation, our data show that levels of GAPDH mRNA and enzyme activity are influenced by a circadian clock. Thus, ccg-7 appears not to be regulated by development (7); and the gene is also not induced by environmental factors such as heat shock, osmotic stress, or carbon/nitrogen starvation. Indeed, we found ccg-7 mRNA levels in general to be refractory to change except for the observed modulation by the biological clock.

Clock regulation of GAPDH is particularly interesting in view of the emerging numbers of reports regarding non-glycolytic roles for the GAPDH polypeptides. These include DNA repair (23, 37, 38); DNA, mRNA, and protein binding (39–41); a possible role in RNA export (42); transalational control (43); protein kinase activity (44); interactions with microtubules (45); and interactions with cell membranes (46). While these activities were reported mainly in mammalian GAPDH, such roles for the GAPDH protein in lower eukaryotes cannot be ruled out a priori considering the high degree of sequence conservation of GAPDH among these organisms. Of these, we examined this possibility only with UDG activity, which was not detected in CCG-7. However, it may be that the Neurospora enzyme also shows one or more of the other activities and that this is the primary reason for its rhythmic activity.

Our rhythmic ccg-7 mRNA data, together with other data on GAPDH expression, may raise questions about the use of GAPDH as a normalization control. For example, GAPDH mRNA of another filamentous fungus, Aspergillus, is osmotically induced, although this is not the case with ccg-7. In other systems, mRNA levels of GAPDH genes are responsive to pathogenic or cytotoxic agents; for instance, in potato, GAPDH mRNA levels are known to be induced by Phytophthora infestans (causing potato blight) (47). In cultured human keratinocytes, tetrachlorodibenzo-p-dioxin, known to elicit pleiotropic toxic actions, induces mRNA levels of GAPDH (48). Other reports on mammalian GAPDHs also suggest that they are not constitutively expressed (49–52). Since GAPDH plays an important role in glycolysis and a number of non-glycolytic roles in some cases as described above, changes in GAPDH activity could influence many other cellular activities and thereby facilitate adaptation to different challenges or growth conditions.

Finally, another recent report of clock-regulated GAPDH from the dinoflagellate Gonyaulax polyedra suggests that our observation here is not an isolated phenomenon and that there might be some significance in clock-regulated GAPDH. Interestingly, as distinct from ccg-7, levels of Gonyaulax GAPDH mRNA do not show rhythmity, but protein levels do (53), indicating post-transcriptional clock regulation. It may be possible that clocks influence the very core of glycolysis to regulate some aspects of general metabolism in a circadian fashion, although the exact role for the rhythm of GAPDH is still uncertain. If GAPDH oscillations exist to influence cellular activities, it may be that other enzymes in fundamental metabolic pathways are also clock-regulated in a manner coordinated with this GAPDH rhythm so as to enhance time-of-day-specific modulation of metabolism.

\(^4\) N. K. Singh, personal communication.
A number of clock-regulated genes and proteins have been identified (5–7, 34–36, 53). However, there has been a great deal of diversity seen among clock output systems reflecting different organism-specific requirements, so that no clock-controlled gene identified to date, except ccg-7, had the potential to be ubiquitous. The high degree of GAPDH sequence conservation indicates that all modern GAPDH variants arose from a single ancestral enzyme (54). Perhaps in addition to sharing a common ancestor, modern GAPDHs share the common aspect of regulation by an internal timekeeper.

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