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A Circadian Oscillator in *Aspergillus* spp. Regulates Daily Development and Gene Expression

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We have established the presence of a circadian clock in *Aspergillus flavus* and *Aspergillus nidulans* by morphological and molecular assays, respectively. In *A. flavus*, the clock regulates an easily assayable rhythm in the development of sclerotia, which are large survival structures produced by many fungi. This developmental rhythm exhibits all of the principal clock properties. The rhythm is maintained in constant environmental conditions with a period of 33 h at 30°C, it can be entrained by environmental signals, and it is temperature compensated. This endogenous 33-h period is one of the longest natural circadian rhythms reported for any organism, and this likely contributes to some unique responses of the clock to environmental signals. In *A. nidulans*, no obvious rhythms in development are apparent. However, a free running and entrainable rhythm in the accumulation of *gpdA* mRNA (encoding glyceraldehyde-3-phosphate dehydrogenase) is observed, suggesting the presence of a circadian clock in this species. We are unable to identify an *Aspergillus* ortholog of *frequency*, a gene required for normal circadian rhythmicity in *Neurospora crassa*. Together, our data indicate the existence of an *Aspergillus* circadian clock, which has properties that differ from that of the well-described clock of *N. crassa*.

Demonstrations of circadian rhythms are widespread. Among fungi, the ascomycete *Neurospora crassa* is the only model system in which the molecular mechanisms of circadian rhythms have been examined. Thus, we know essentially nothing about the mechanism of the clock in other fungal species. To compare the components of circadian clocks in related fungi, we have begun an investigation of circadian clocks in the medically and agriculturally important ascomycetes, *Aspergillus nidulans* and *Aspergillus flavus.*

Circadian clocks (composed of one or more oscillators) provide organisms with the ability to keep in synchrony with the external world (13). The clock generates a program with a duration of ca. 24 h, allowing anticipation of cyclic changes in the environment, particularly light and temperature changes. The formal properties defining circadian rhythms are nearly identical in all organisms studied thus far and provide the diagnostic criteria for identifying an endogenous circadian clock (13). By definition, a rhythm is circadian if it persists in the absence of temporal signals (e.g., constant temperature and darkness) with a period of ca. 24 h. A rhythm that persists under constant conditions is called a free-running rhythm, and its period is referred to as the free-running period (FRP).

A second fundamental characteristic of circadian rhythms is that they can be reset (a process termed entrainment) by external signals, such as the daily light and temperature cycles (2, 7). Entrainment results from perception of external time cues (zeitgebers) by one or more clock components and shifts the circadian clock to an appropriate phase. The magnitude and direction of the phase change is dependent on the intensity and duration of the zeitgeber and the phase of the internal circadian clock when the pulse is given. Organisms can also entrain to environmental cycles of greater or less than 24 h with species-specific limits (7). In this case, the circadian clock will systematically adopt a stable phase relationship to the imposed cycles and the rhythm will have the same period as the entraining cycle.

Another defining characteristic of circadian rhythms is that their FRPs are temperature compensated (16). This means that over a physiologically defined range of temperatures, the FRP of a circadian rhythm remains relatively unchanged with a Q_{10} (the ratio of the rate at a given temperature to the rate at a temperature that is 10° lower) ranging from 0.8 to 1.4 (16). This is in contrast to most biochemical reactions, which typically increase in rate as the temperature increases (Q_{10} ~2 to 3). Yet, because circadian rhythms can be entrained by temperature transitions (pulses or cycles), the system is not entirely temperature independent.

A simple model of a circadian clock includes three central components: a self-sustained oscillator(s), input pathways that convey environmental information and synchronize the oscillator(s) to the external world, and output pathways leading from the oscillator(s) to the expressed rhythms. In *N. crassa*, the circadian clock directs the daily production of asexual spores (conidiospores) with an FRP of 22 h (34). Several proteins involved in rhythmicity have been identified in *N. crassa* (22). These include the FREQUENCY (FRQ), WHITE COLLAR-1 (WC-1), and WC-2 proteins. These clock components form a feedback loop in which the negative element FRQ feeds back to shut down its own expression presumably by repressing

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the activity of the positive-acting elements, WC-1 and WC-2 (22). There are functional similarities between the proteins of the *N. crassa* clock and those of other kingdoms. The *N. crassa* WC-1 and WC-2 proteins each contain PAS domains involved in protein-protein interactions that are similar to those found in the mammalian clock proteins PERIOD, CLOCK, and BMAL1 (12). WC-1 was recently shown to be a blue-light photoreceptor, linking the environment through an input pathway to the circadian oscillator (15, 17). The output pathways are less well characterized in *N. crassa*, although several output clock-controlled genes are known that are involved in general stress responses, cellular metabolism, and development (3).

While *N. crassa* has greatly contributed to a molecular understanding of clock function, this has not been extended to other fungal genera. One genus of immense medical, industrial, and agricultural importance is *Aspergillus. A. flavus* is a toxigenic fungus that contaminates plants such as corn and peanuts with the carcinogenic metabolite aflatoxin and is responsible for billions of dollars of crop losses each year. Furthermore, infection by *Aspergillus* spp. (aspergillosis), including *A. flavus*, can lead to allergic, superficial, saprophytic, or invasive disease, particularly in immunocompromised patients. This genus also includes the model eukaryote *A. nidulans*, which has been used to address several fundamental questions of biology (24). The asexual *Aspergillus* spp., such as *A. flavus*, produce two types of reproductive structures: conidiospores, which are small dispersal spores, and larger survival structures called sclerotia (5). Both conidiospore and sclerotium production are photoinducible (9), and recent studies have shown a link between sclerotium production and aflatoxin biosynthesis (11). The sexual *Aspergillus* spp., including *A. nidulans*, generally do not produce sclerotia but produce asexual conidiospores and sexual ascospores (1).

Several observations prompted our initial investigation of circadian rhythmicity in *Aspergillus*. First, we noticed that *A. flavus* exhibits a rhythm in sclerotial development; however, it was not known whether this rhythm is endogenously generated. Second, although both *A. flavus* and *A. nidulans* possess orthologs of the *N. crassa wc-1* and *wc-2* genes, no ortholog of *frq* appears to be present in either species. These data suggested that an *Aspergillus* clock might be constructed of some components that are different from those of *N. crassa* and indicated that investigation of the circadian system in *Aspergillus* could provide important information regarding clock mechanisms and their evolution.

MATERIALS AND METHODS

Strains and growth conditions. The strain of *A. flavus* used in the present study was 12S, a field isolate obtained from P. Cotty. Strains of *A. nidulans* include A4 (ve^+) and PW1 (*argB2 methG1 biA1 veA1*) (Fungal Genetic Stock Center, Kansas City, Mo.). Unless otherwise indicated, strains were grown at 30°C on race tubes (34) containing 14 ml of *Aspergillus* complete medium (CM), comprised of 2% glucose, 1× *Aspergillus* salts plus trace elements, 0.2% peptone, 0.1% yeast extract, 0.1% Casamino Acids, and 1.5% agar at pH 6.5 (www.fgsc.net/methods/anidmed.html). Race tubes were 30-cm-long glass tubes bent up at both ends with an inner diameter of 1.5 cm. Conidia ($\sim 5 \times 10^6$) from 3- to 14-day-old plate cultures were suspended in 1 ml of water, and 2 μl of this spore suspension was used to inoculate the race tubes. Race tube cultures were incubated in a 24-h light-dark cycle (12:12 LD) for 3 to 7 days prior to transfer to constant darkness (DD) or constant light (LL) in controlled environmental chambers (Percival Scientific, Inc.) containing broad-spectrum fluorescent lights (Phillips F20T12 CW 20W). The light intensity was 45 μmol of photons/m^2/s (ca. 2,300 lx). Previous studies have shown that *Aspergillus* is responsive to both red and blue light but not to green light (28, 38). When cultures were grown in DD, a green safe light was used to observe and mark the growth front at the same time each day. The green safe light consisted of a green light bulb (Sylvania, 25 W) in a Kodak adjustable Safelight lamp covered with 20 layers of green cellophane. The emission spectrum of the green light bulb was measured by using a USB2000 spectroradiometer (Ocean Optics, Inc.) in which a single peak was observed at 540 nm. In 12:12 LD cycles with the green light as the light source, the cultures were not entrained, and the growth rate of the cultures was not affected (data not shown). Digital photographs of race tubes were analyzed with the CHRONO program (33) generously provided by Till Roenneberg, and traces were generated by using NIH Image 1.61.

The ratio of the frequency of the average FRP at 40°C (28.4 h) to that at 30°C (33.4 h) was used to calculate the Q_{10} for the sclerotial rhythm between these temperatures. The estimated Q_{10} for the sclerotial rhythm at between 28 and 30°C was calculated as the ratio of the frequency of the average FRP at 30°C (33.4 h) to that at 28°C (51.4 h) multiplied by 5.

Environmental entrainment. For LD entrainment assays, *A. flavus* 12S was grown on CM in a 10:10 LD cycle, a 12:12 LD cycle, or an 18:18 LD cycle at 30°C. For each cycle length, the race tubes were marked at the mycelial growth front at the dark-to-light transition for reference. For temperature entrainment assays, the cultures were inoculated onto CM race tubes and incubated in DD with 24-h cycles of 30°C (12 h) and 38°C (12 h). The race tubes were marked at the transition from 30 to 38°C.

To examine the phase response of *A. flavus* to external signals, strain 12S was cultured on race tubes and transferred to DD at 30°C after 3 days of growth in 12:12 LD at 30°C at the end of the last dark phase in the LD cycle. Cultures of *A. flavus* strain 12S were given a light pulse of 45 μmol of photons/m^2/s (ca. 2,300 lx) for 1 h before transfer back to DD 30°C. If we assume a 33-h FRP (see Results for details), the pulses were given starting at circadian time zero (CT0) of circadian day 3. Circadian time is a way to normalize biological time in strains or organisms with different endogenous period lengths to 24 circadian hours per cycle. By definition, CT0 is subjective dawn and CT12 is subjective dusk. Light pulses were given to a subset of race tubes at intervals of 4 circadian hours (every 5.5 local hours) over one circadian day (33 h). Thus, one set of tubes remained in the dark for the entire time, and other sets of tubes were pulsed at CT0, CT4, etc. After the pulse, the race tubes were returned to DD at 30°C for 4 days. The change in phase of the sclerotial rhythm after the light pulse was determined 3 days after the pulse compared to the nonpulsed control, and the data are plotted as a phase-response curve (PRC) (30).

Rhythmic RNA analysis. *A. nidulans* strain A4 was inoculated into petri dish cultures of minimal medium, comprised of 1× *Aspergillus* salts plus 1% glucose and trace elements (www.fgsc.net/methods/anidmed.html). After 16 to 20 h of growth at 37°C in DD, sections of the resulting mycelial mat were cut with a 7-mm cork borer. Individual sections were transferred to liquid shaking cultures of low-glucose medium (0.1% glucose and 1× *Aspergillus* salts plus trace elements) to inhibit development (21) and then incubated at 30°C in LL for 24 h. For free-running experiments, cultures were transferred from LL to DD at 4-h intervals so that, at the subsequent time of harvest, the cultures were all of the same age but represented different phases in the circadian cycle. For entrainment experiments, cultures were transferred to alternating 12-h light at 38°C and 12-h dark at 30°C cycles and harvested after one full cycle every 4 h for 2 days. At harvest, the mycelia were frozen in liquid nitrogen. To extract RNA, frozen mycelia were ground with a mortar and pestle in RNA extraction buffer (0.1 M sodium acetate, 1 mM EDTA, and 4% sodium dodecyl sulfate [SDS] at pH 8). After four phenol extractions, total RNA was precipitated with ethanol. Northern analysis (10 μg of RNA/lane) was performed as described previously (4). Hybridization was to a probe made from a 500-bp exon of the *Aspergillus gpdA* gene, which was amplified from *A. nidulans* genomic DNA by using the upstream PCR primer 5′-GCCTTAAGCACCATTGAGACCTACGACGA-3′ and the downstream primer 5′-GCCCTAGGTCAACTAGTTAGTCGAAGATG-3′. The upstream primer was designed to add an *Eco*RI restriction site (underlined), whereas the downstream primer was designed to add a *Bam*HI site (underlined) to facilitate cloning into a vector for sequence verification and maintenance. The PCR cycles were 94°C for 5 min, 50°C for 2 min, and 72°C for 3 min for a total of 30 cycles. The probe was labeled with [^{32}P]dCTP (6,000 Ci/mmol) by using the DECAprime II kit (Ambion, Austin, Tex.). Ethidium bromide-stained rRNA was used as a control for RNA loading.

RESULTS

***A. flavus* displays an endogenous rhythm in sclerotium production.** Preliminary experiments with petri dish cultures of *A. flavus* 12S indicated that the organism exhibits rings of sclero-

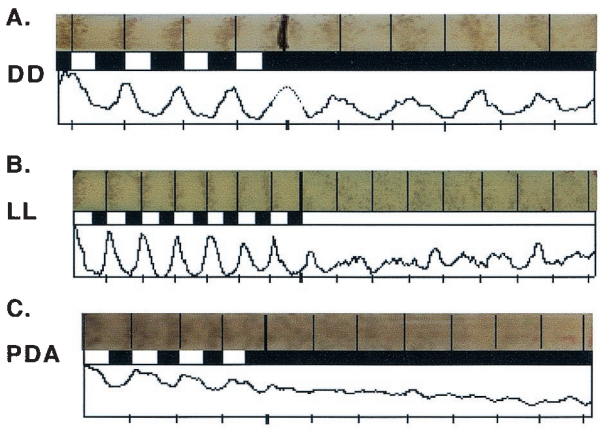

FIG. 1. *A. flavus* forms bands of sclerotia in constant darkness. *A. flavus* strain 12S was inoculated onto race tubes containing CM and grown in 12:12 LD cycles for 5 days at 30°C before transfer into DD (A) or LL (B). The time of transfer is marked with a heavy black line. Bars below the race tubes represent the times that the cultures were in the light (white bars) or dark (black bars). Representative race tubes are shown. The direction of growth is from left to right. The mycelial growth front was marked at the same time each day (black lines) for reference. (C) *A. flavus* strain 12S was inoculated onto race tubes containing PDA medium and grown in 12:12 LD cycles for 7 days at 30°C before transfer into DD. In each panel, an intensity tracing of the race tubes is shown below, with the lines on the *x* axis representing the 24-h growth front marks.

tia when a spore suspension is point inoculated and allowed to grow in the absence of temporal cues. To investigate whether this developmental behavior was under control of the circadian clock, *A. flavus* 12S was grown on race tubes half-filled with CM (see Materials and Methods) in constant environmental conditions. We found that 12S cultures that were synchronized to 12:12 LD at 30°C and then transferred to DD consistently exhibited a free-running rhythm of sclerotium development with a period of 33.4 ± 0.2 h (mean ± the standard error of the mean [SEM]; $n = 56$) (Fig. 1A). The peak in sclerotium production occurred in the early evening, at ca. CT14. A stable 33-h free-running rhythm began immediately after transfer into DD; thus, there is no transient behavior. In addition, the rhythm persisted for up to 3 weeks in DD, until the time at which the cultures reached the end of the race tube (data not shown). When cultures were transferred from 12:12 LD cycles to LL at 30°C they formed sclerotia continuously for several days (Fig. 1B); however, we observed that ca. 20% cultures showed broad bands of sclerotia in LL after 5 days (data not shown).

In *N. crassa*, the period of the conidiation rhythm is essentially unchanged when cultures are grown on different media (35). We examined *A. flavus* sclerotial rhythms when strain 12S was grown on potato dextrose agar (PDA), Champe's medium (2% glucose–0.5% yeast extract), and minimal medium (http://www.fgsc.net/methods/anidmed.html). *A. flavus* grew more slowly on these media (0.58 ± 0.04 cm/day [$n = 11$], 0.51± 0.03 cm/day [$n = 12$], and 0.33 ± 0.02 cm/day [$n = 6$] on PDA, Champe's medium, and minimal medium, respectively) compared to CM (0.93 ± 0.02 cm/day [$n = 33$]). Sclerotial rhythms were observed on minimal medium in DD; however, no free-running rhythms in development were detected on PDA or Champe's medium (Fig. 1C and data not shown). These data indicated that the circadian rhythm in sclerotium development, unlike the *N. crassa* conidiation rhythm, is not well nutritionally compensated.

***A. flavus* developmental rhythms are entrained by environmental cycles.** A defining property of circadian rhythms is that they are able to synchronize, or entrain, in response to cyclical cues in the environment (2). We examined *A. flavus* strain 12S to determine whether the sclerotial rhythm could be entrained by light or temperature cycles. Cultures were grown on race tubes in LD cycles that were 20, 24, or 36 h in duration. In each case, the period of the rhythm matched the entraining cycle (Fig. 2A). For example, the 12:12 LD cycle resulted in shortening the 33-h FRP to 24 h. Importantly, the phasing of the peak of sclerotium formation differed with cycle length (Fig. 2A). In the 10:10 LD cycle, the peaks of sclerotia occurred ca. 3 h (2.7 ± 0.1 h [$n = 38$]) prior to the dark-to-light transition. In the 12:12 LD cycle the peaks of sclerotia occurred slightly after the time of the dark-to-light transition (−0.75 ± 0.2 h [$n = 23$]), whereas in the 18:18 LD cycle the peaks of sclerotia occurred 3 h (−2.9 ± 1.64 h [$n = 38$]) after the dark-to-light transition. These observations demonstrated that the clock which controls the developmental rhythm is synchronized by the LD cycle and is not merely driven by the exogenous time cues since the phase of a driven rhythm would not change with respect to the length of the cycle.

The developmental rhythm was also entrained to temperature cycles. In a 12:12 temperature cycle of 30 and 38°C, the period of the rhythm was 24 h (Fig. 2B). The sclerotial bands occurred ca. 3 h (−2.6 ± 0.1 h [$n = 20$]) after the transition from low to high temperature. When race tubes were placed in DD at 30°C after a few days in the temperature cycles, the cultures immediately returned to a 33-h period (Fig. 2B).

Light pulses reset the sclerotium developmental rhythm. A fundamental property of circadian oscillators is that short pulses of light and/or temperature can advance or delay the observed rhythm, depending on the phase of the oscillator at the time of the pulse. We examined the *A. flavus* sclerotial rhythm growing at 30°C in DD for phase shifting by using a 1-h light pulse given every 5.5 h for 33 h. The rhythm was responsive to the light pulses at all of the times tested and, as expected for a circadian rhythm, the magnitude of response varied over the course of the day. The data are plotted as a PRC (Fig. 3). A light pulse given to cultures from mid-day to the early evening resulted in a phase advance of the rhythm compared to the nonpulsed control, with the largest advances occurring at subjective dusk (CT12). Phase delays were observed from subjective late night to dawn (CT20 to CT0), but only small changes in the phase were observed at these times. These data demonstrate that phase resetting occurs primarily by phase advances in *A. flavus*, since phase delays were very weak and infrequently observed.

The rhythms of sclerotium development are temperature compensated. Another defining feature of circadian rhythms, but one that is poorly understood, is that the FRP remains relatively constant (compensated) over a range of physiologically relevant temperatures; thus, we sought to determine whether the rhythm in sclerotium production is temperature compensated. The optimal growth temperature for *A. flavus* is 30 to 32°C, with a minimum of 10°C and maximum of 42°C (N.

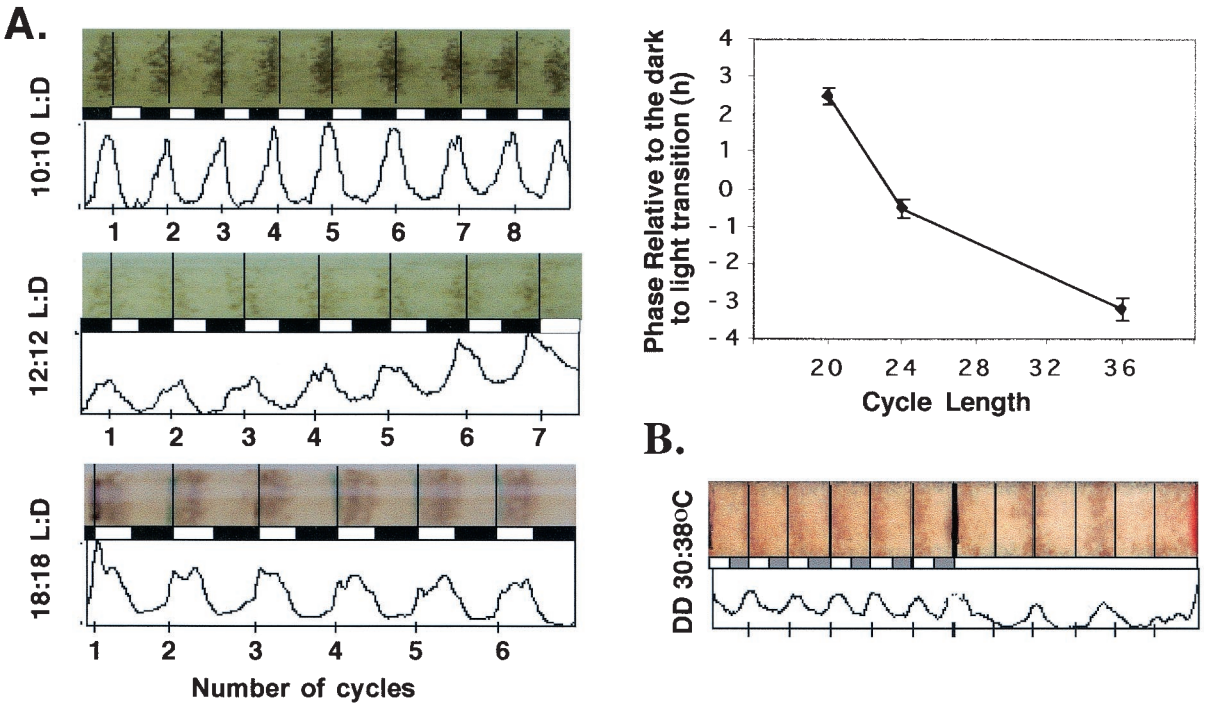

FIG. 2. *A. flavus* 12S entrains to different light and temperature cycles. (A) *A. flavus* 12S was inoculated onto CM race tubes and allowed to grow for 3 days in LL at 30°C (not shown). Race tubes were then transferred into 20-, 24-, or 36-h LD cycles at 30°C. Representative race tubes are shown on the left, with bars and intensity tracings as in Fig. 1. The mycelial growth fronts were marked daily at the dark-to-light transition (black lines). The direction of growth is from left to right. The phase of the center of the sclerotial band relative to the dark-to-light transition was measured in hours and plotted (top right side of figure). Positive numbers indicate that the band occurred prior to the dark-to-light transition, and negative numbers indicate that the band occurred after the dark-to-light transition. Values are means ± the SEM ($n \geq 23$). (B) *A. flavus* strain 12S was inoculated onto CM race tubes and grown for 3 days in 12:12 LD at 30°C (not shown) and then transferred to DD with 24-h (12:12) cycles of 30 and 38°C. The mycelial growth front was marked at the transition from low to high temperature. The heavy line indicates when the cultures were transferred to constant 30°C. The bar below the race tubes indicates the time at 30°C (white) and 38°C (gray shaded). Tracings of the image are shown below the race tube.

Keller, unpublished data). Temperatures below 28°C were not tested because *A. flavus* produces very few sclerotia below 28°C (data not shown). Cultures were grown on race tubes in DD at temperatures ranging from 28 to 40°C (Fig. 4A). Between 30 and 40°C the period and phase of the sclerotial rhythm remained essentially the same (Q_{10} 30 to 40°C = 1.2 [see Materials and Methods]), whereas the growth rate changed with culture temperature (Fig. 4B). At <30°C, the period of the sclerotial rhythm was longer than at higher temperatures (estimated Q_{10} at 28 to 30°C = 7.7), indicating that this is the lower limit of compensation.

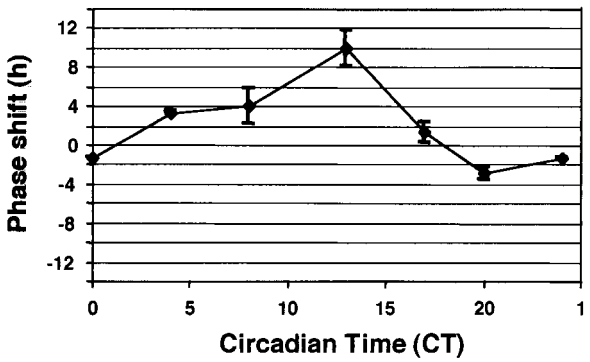

FIG. 3. The sclerotial rhythm in *A. flavus* is reset by environmental signals. A PRC was generated as described in Materials and Methods. The phase response was calculated as the difference in position of the sclerotial band after light treatment compared to the untreated controls. The *x* axis indicates the circadian time (CT) as calculated from the 33-h FRP of *A. flavus* 12S on CM medium at 30°C; the *y* axis shows the phase shift measured in hours of advance (positive numbers) or delay (negative numbers). Values are means ± the SEM ($n = 5$).

A clock in *A. nidulans* regulates gene expression. In *A. nidulans*, red light is required for conidiation during a critical period of development. Wild-type strains will conidiate in the light, but they are aconidial in the dark. Strains harboring the *veA1* mutation conidiate in the absence of light (28). Therefore, we examined wild-type and *veA1* strains of *A. nidulans* on race tubes in LD cycles, in DD, and in LL at 30°C for rhythmic conidiation. Developmental rhythms were observed only in the wild-type A4 strain in the LD cycles; however, no developmental rhythms were observed under constant conditions. These data suggested that, unlike *A. flavus*, the observed *A. nidulans* developmental rhythm does not run free and is likely controlled directly by the environmental cycle and not by an endogenous circadian clock under these conditions.

To further investigate possible circadian rhythmicity in *A. nidulans*, we isolated RNA from wild-type cultures grown in LL for 1 day and then transferred to DD and harvested at 4-h intervals over two consecutive days. Total RNA was probed

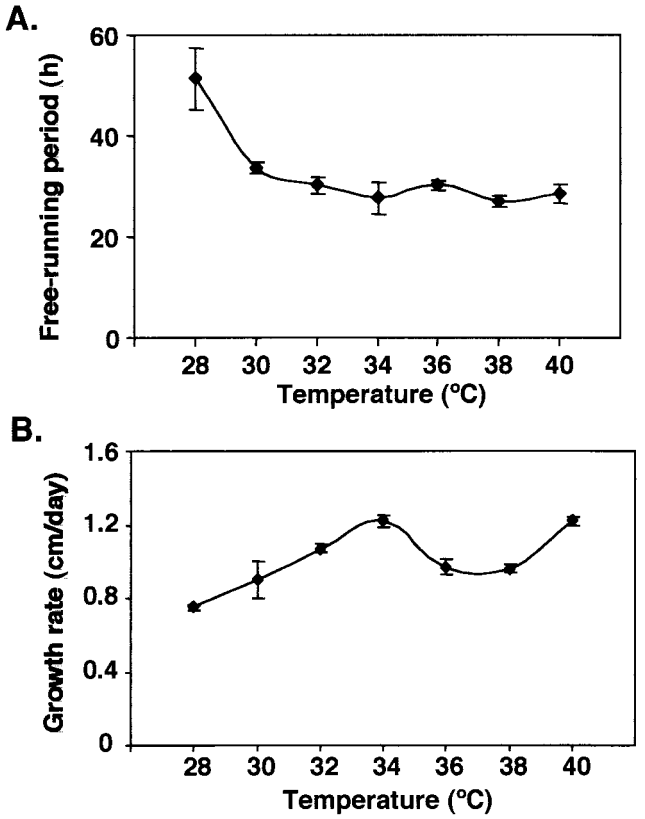

FIG. 4. The sclerotial rhythm in *A. flavus* is temperature compensated. (A) *A. flavus* strain 12S was inoculated onto CM race tubes and allowed to grow at 30°C in 12:12 LD cycles for 3 days. Cultures were then transferred to the indicated temperatures in DD for 7 days. The data are plotted as the average FRP versus temperature. (B) The growth rate of *A. flavus* is plotted versus the temperature. In panels A and B, values are means ± the standard deviation ($n \geq 14$).

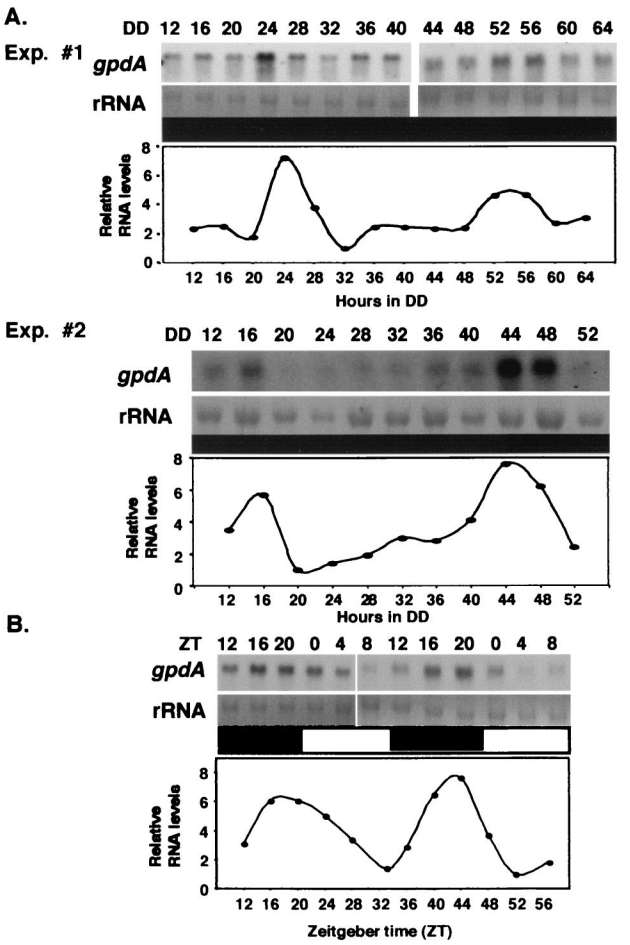

FIG. 5. *gpdA* mRNA accumulates rhythmically in *A. nidulans* strain A4. (A) Total RNA was isolated from *A. nidulans* strain A4 grown in DD (black bar) and harvested every 4 h for 2 consecutive days. Two independent experiments are shown. The Northern blots were probed with a PCR product derived from the *A. nidulans gpdA* gene to identify the 1-kb *gpdA* mRNA. Ethidium bromide-stained 25S rRNA is shown as an RNA loading control. A plot of the relative band intensities from the Northern blot is shown below. This experiment was repeated four times with similar results; however, as revealed in a comparison of the two experiments shown, the phasing of the peak of expression varied over eight circadian hours. (B) Total RNA was isolated from *A. nidulans* strain A4 grown in cycles of 12 h of light at 38°C (white bar) and 12 h of dark at 30°C (black bar) and then harvested every 4 h for 48 h. Northern blots were probed as in A. The zeitgeber time (ZT) is the time in hours from the start of a 12:12 LD cycle. The lights were turned on at ZT0 and turned off at ZT12. The relative band intensities are plotted below.

with the *A. nidulans gpdA* gene encoding GAPDH (glyceraldehyde-3-phosphate dehydrogenase). Homologs of *gpdA* have been shown to be rhythmically expressed in several organisms (14, 37), including *N. crassa* (36). We observed a rhythm in the accumulation of *gpdA* transcripts that has a period of 28 to 32 h (Fig. 5A). Similar to the variability observed between experiments in the time of peak message accumulation for *N. crassa* clock-controlled genes (4), the phase of the *A. nidulans gpdA* rhythm can vary up to 8 h in separate experiments (Fig. 5A). These data suggested the presence of a functioning circadian clock in *A. nidulans* with an FRP similar to that of *A. flavus.* To determine whether this rhythm could be entrained, we examined the *gpdA* rhythm in strains grown in cycles of 12 h of light at 38°C and 12 h of dark at 30°C (Fig. 5B). Simultaneous light and temperature cycles were used because neither light nor temperature alone was sufficient to entrain the rhythm. In the 24-h light and temperature cycle, the *gpdA* rhythm was entrained with a period of 24 h, with peaks of mRNA occurring at the end of the dark-cold phase.

Identification of clock gene orthologs in *Aspergillus*. To investigate the conservation of clock components in *Aspergillus* that are required for robust circadian rhythmicity in *N. crassa*, we cloned and sequenced *A. nidulans* orthologs of *N. crassa* *wc-1* and *wc-2* (M. Schoeser and H. Haas, unpublished data). LreA (accession no. 470313; 836 amino acids [aa]) is 37% identical and 55% similar over 758 aa to WC-1 (accession no. X9430; 1,168 aa), and LreB (accession no. 82072; 417 aa) is 35% identical and 51% similar over 333 aa to WC-2 (accession no. Y09119; 530 aa) (data not shown). In both cases, the overall similarity extends beyond the PAS domains of WC-1 and WC-2. No similar sequences were found in the current limited *A. flavus* database; however, in Southern analyses DNA hybridizing to *lreA* and *lreB* was detected (data not shown).

Several different attempts were made in the present study and

in other studies (27; M. Merrow and J. C. Dunlap, unpublished data) to identify an FRQ ortholog in *Aspergillus*. Based on sequence information, no putative ortholog was apparent in the *A. nidulans* genome sequence database (http://www-genome.wi.mit.edu), the *A. flavus* EST database, or in the newly released *A. fumigatus* database (www.tigr.org/tdb/e2k1/afu1/). Furthermore, efforts to amplify an FRQ-specific band by PCR were unsuccessful, as were attempts to identify a band by low-stringency Southern assays in both species. Based on these data, we conclude that *Aspergillus* lacks an obvious FRQ ortholog. This suggests that the *Aspergillus* circadian clock does not require a protein with strong similarity to FRQ.

DISCUSSION

Our data demonstrate that the observed rhythm in sclerotium formation in *A. flavus* strain 12S is under the control of an endogenous circadian oscillator. The rhythm persists in constant conditions, is entrained and reset by environmental signals, and is temperature compensated. However, although the canonical clock properties are present in the *A. flavus* clock, several features of the rhythm differ from those of other eukaryotes.

The FRP of the *A. flavus* developmental rhythm at 30°C is 33 h and thus is significantly longer than a typical circadian rhythm, which is usually close to 24 h. The longest documented circadian rhythms to date are 29 h in the bean plant *Phaseolus* (8) and ~30 h for the rhythm of promoter activity of the *Arabidopsis* chlorophyll a/b-binding protein (*cab2*) in DD (27). It has generally been assumed that circadian clocks have FRPs close to 24 h in order to maintain a stable phase relationship to the earth's 24-h rotational cycle; the inherent cycle cannot be too far away from the environmentally driven cycle for optimal performance (30). However, our data suggest that this assumption may require reevaluation and strongly support the importance of investigating circadian rhythms in more organisms. Thus, the long FRP in *A. flavus* suggests that the clock in this organism operates differently than clocks in other well-studied organisms that run closer to 24 h, such that it runs more slowly when environmental cues are absent.

A circadian oscillation can be phased correctly to local time by resetting (entraining) the clock every day to precisely 24 h. Thus, despite the long FRP of *A. flavus*, the sclerotial rhythm is entrained by light and temperature cycles, as would be expected to occur in the natural environment. The steady-state phase relationship between an entrained circadian rhythm and the zeitgeber has been shown to be dependent on the FRP and the period of the zeitgeber, such that the phase relationship of the entrained rhythm to the zeitgeber will change as the period of the zeitgeber is shortened or lengthened (2, 29). In all other organisms examined, when the FRP is shorter than the zeitgeber period, the peak of the entrained rhythm occurs before the time cue (positive number), whereas when the FRP is longer than the entraining cycle, the peak of the entrained rhythm occurs after the time cue (negative number). This is the case for wild-type *N. crassa* strains grown in temperature cycles, whereby conidial development occurs after a warm to cold transition when the entraining cycle is shorter than the FRP and occurs before a warm-to-cold transition when the entraining cycle is longer than the FRP (25). However, clock-controlled sclerotium development in *A. flavus* occurs after the lights are turned on (negative value), when the LD cycle is greater than the FRP, and occurs before lights are turned on (positive value), when the cycle is shorter than the FRP. The difference between the phase of the rhythm during entrainment in *A. flavus* and all other organisms examined to date may be directly related to the extremely long FRP in *A. flavus*. Furthermore, the unusual steady-state phase relationship during entrainment in *A. flavus* is close to what would be theoretically predicted based on the long FRP and the PRC to light (32).

Pulses of light reset the *A. flavus* sclerotial rhythm and only large advances, but not large phase delays, were observed. This finding contrasts with the remarkable similarity in PRCs to light in other organisms, in which phase advances of the rhythm occur in the late subjective night and early subjective day and phase delays occur in the late subjective day and early subjective night (30). In fact, the PRC for light in *A. flavus* most closely resembles the phase response of organisms grown in the light to a short dark pulse (18). Typically, species with FRPs longer than 24 h tend to show larger advances than delays, whereas the reverse is true for species and individuals with FRPs shorter than 24 h (7, 31). For example, if an organism has an FRP of 26 h, a daily light exposure would need to produce a 2-h advance each cycle in order for the entrained rhythm to have a period of 24 h. The *A. flavus* rhythm may be an extreme example of this phenomenon, whereby the oscillator may be primarily phase advanced by light in order to keep pace with the environment due to its naturally long FRP.

Our data demonstrating an endogenous circadian clock are not as well developed for *A. nidulans* due to a lack of an easily observable rhythm in this species. However, our experiments demonstrating a free-running and entrainable rhythm in the accumulation of *gpdA* provide support for the existence of a circadian clock. The long period of the free-running rhythm also suggests that similarities may exist between the clocks of *A. nidulans* and *A. flavus*.

The *Ascomycete* subgroup *Euascomycete* radiated into several monophyletic groups (estimated at ca. 240 million years ago), including the *Plectomycetes*, which include *Aspergillus*, and the *Pyrenomycetes*, which include *Neurospora* (6). *frq* orthologs are present in several species of *Pyrenomycetes* (19, 20, 26), but *frq* has not been found in members of the *Plectomycetes*. *Aspergillus* spp. lack a detectable *frq* gene, suggesting that the FRQ-based oscillator in *N. crassa* is not conserved among the ascomycetes. Orthologs of *N. crassa wc-1*, which encodes a blue-light photoreceptor (15, 17), and *wc-2* are present in *A. nidulans* and *A. fumigatus* and are predicted in *A. flavus*. In preliminary data, we observed that *gpdA* was rhythmic in *A. nidulans* strains lacking the *wc-1* ortholog *lreA* (data not shown). *Aspergillus* displays blue-light responses (10, 38). Together, these data imply that the primary evolutionary force for maintaining the *wc* genes in the *Ascomycetes* was to allow blue-light-sensing, rather than circadian rhythms.

The apparent absence of FRQ in *Aspergillus*, along with the unusual properties of the circadian clock in *A. flavus*, suggests that the *Aspergillus* clock differs from the well-described *N. crassa* clock. Furthermore, we have been unable to demonstrate rhythms in *gpdA* in *A. flavus*, suggesting that outputs from the clock differ in *A. nidulans* and *A. flavus*. This differ-

ence in output gene expression may reflect the different developmental programs and ecological niches of these two closely related fungal species. The difference in outputs from the clock may also be reflected in the lack of an observable developmental rhythm in *A. nidulans*. Alternatively, the conditions needed to observe the developmental rhythm in *A. nidulans* may not have been met by our experiments. In either case, our data support the notion that the lack of developmental rhythms in different fungal species or isolates does not rule out the existence of a circadian clock and is consistent with the idea that circadian rhythmicity is a general phenomenon in *Aspergillus*. A well-described case in point is in *N. crassa*. Here, the conidiation rhythm is normally obscured in wild-type strains grown in closed culture tubes due to high CO_2 concentrations. However, strains harboring the *band* mutation display conidiation rhythms under the same culture conditions (34).

Evidence now exists for a FRQ-less oscillator (FLO) in *N. crassa* that can be entrained by temperature cycles (25). It is tempting to speculate that the *Aspergillus* clock and the FLO are related and that the FLO is ancestral to the *N. crassa* FRQ-based oscillator since both oscillators display canonical clock properties and yet have several unusual features, including poor nutritional compensation (23, 25). Ultimately, comparisons between the *N. crassa* and *Aspergillus* oscillators will allow investigation of whether circadian clocks have diverse evolutionary origins or whether molecular adornments have been added to a common ancestral mechanism.

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REFERENCES

1. **Adams, T. H., J. K. Wieser, and J. H. Yu.** 1998. Asexual sporulation in *Aspergillus nidulans*. Microbiol. Mol. Biol. Rev. **62:**35–54.
2. **Aschoff, J.** 1981. Free-running and entrained circadian rhythms, p. 81–92. *In* J. Aschoff (ed.), Handbook of behavioral neurobiology, vol. 4. Plenum Press, Inc., New York, N.Y.
3. **Bell-Pedersen, D.** 2000. Understanding circadian rhythmicity in *Neurospora crassa*: from behavior to genes and back again. Fungal Genet. Biol. **29:**1–18.
4. **Bell-Pedersen, D., M. L. Shinohara, J. J. Loros, and J. C. Dunlap.** 1996. Circadian clock-controlled genes isolated from *Neurospora crassa* are late night- to early morning-specific. Proc. Natl. Acad. Sci. USA **93:**13096–13101.
5. **Bennett, J. W., P. C. Horowitz, and L. S. Lee.** 1979. Production of sclerotia by aflatoxigenic and nonaflatoxigenic strains of *Aspergillus flavus* and *A. parasiticus*. Mycologia **71:**415–422.
6. **Berbee, M. L., and J. W. Taylor.** 2001. Fungal molecular evolution: gene trees and geologic time, p. 229–245. *In* D. J. McLaughlin (ed.), The mycota, vol. VII, part B. Springer-Verlag, Berlin, Germany.
7. **Bruce, V. G.** 1960. Environmental entrainment of circadian rhythms. Cold Spring Harbor Symp. Quant. Biol. **25:**29–48.
8. **Bunning, E.** 1960. Biological clocks. Cold Spring Harbor Symp. Quant. Biol. **25:**1–10.
9. **Calvo, A. M., L. L. Hinze, H. W. Gardner, and N. P. Keller.** 1999. Sporogenic effect of polyunsaturated fatty acids on development of *Aspergillus* spp. Appl. Environ. Microbiol. **65:**3668–3673.
10. **Champe, S. P., D. L. Nagle, and L. N. Yager.** 1994. Sexual sporulation. Prog. Ind. Microbiol. **29:**429–454.
11. **Chang, P. K., J. W. Bennett, and P. J. Cotty.** 2002. Association of aflatoxin biosynthesis and sclerotial development in *Aspergillus parasiticus.* Mycopathologia **153:**41–48.
12. **Dunlap, J. C., J. J. Loros, Y. Liu, and S. K. Crosthwaite.** 1999. Eukaryotic circadian systems: cycles in common. Genes Cells **4:**1–10.
13. **Edmunds, L. N.** 1988. Cellular and molecular bases of biological clocks. Springer-Verlag, New York, N.Y.
14. **Fagan, T., D. Morse, and J. W. Hastings.** 1999. Circadian synthesis of a nuclear-encoded chloroplast glyceraldehyde-3-phosphate dehydrogenase in the dinoflagellate *Gonyaulax polyedra* is translationally controlled. Biochemistry **38:**7689–7695.
15. **Froehlich, A. C., Y. Liu, J. J. Loros, and J. C. Dunlap.** 2002. *White Collar-1*, a circadian blue light photoreceptor, binding to the *frequency* promoter. Science **297:**815–819.
16. **Hastings, J. W., and B. M. Sweeney.** 1957. On the mechanism of temperature independence in a biological clock. Proc. Natl. Acad. Sci. USA **43:**804–811.
17. **He, Q., P. Cheng, Y. Yang, L. Wang, K. H. Gardner, and Y. Liu.** 2002. *White Collar-1*, a DNA binding transcription factor and a light sensor. Science **297:**840–843.
18. **Johnson, C. H.** 1992. Phase response curves: what can they tell us about circadian clocks?, p. 209–249. *In* T. Hiroshige and K. Honma (ed.), Circadian clocks from cell to human. Hokkaido University Press, Sapporo, Japan.
19. **Lewis, M. T., and J. F. Feldman.** 1996. Evolution of the *frequency* (*frq*) clock locus in ascomycete fungi. Mol. Biol. Evol. **13:**1233–1241.
20. **Lewis, M. T., L. W. Morgan, and J. F. Feldman.** 1997. Analysis of *frequency* (*frq*) clock gene homologs: evidence for a helix-turn-helix transcription factor. Mol. Gen. Genet. **253:**401–414.
21. **Loros, J. J., S. A. Denome, and J. C. Dunlap.** 1989. Molecular cloning of genes under control of the circadian clock in *Neurospora*. Science **243:**385–388.
22. **Loros, J. J., and J. C. Dunlap.** 2001. Genetic and molecular analysis of circadian rhythms in *Neurospora*. Annu. Rev. Physiol. **63:**757–794.
23. **Loros, J. J., and J. F. Feldman.** 1986. Loss of temperature compensation of circadian period length in the *frq-9* mutant of *Neurospora crassa*. J. Biol. Rhythms **1:**187–198.
24. **May, G. S., and T. H. Adams.** 1997. The importance of fungi to man. Genome Res. **7:**1041–1044.
25. **Merrow, M., M. Brunner, and T. Roenneberg.** 1999. Assignment of circadian function for the *Neurospora* clock gene *frequency*. Nature **399:**584–586.
26. **Merrow, M. W., and J. C. Dunlap.** 1994. Intergeneric complementation of a circadian rhythmicity defect: phylogenetic conservation of structure and function of the clock gene *frequency*. EMBO J. **13:**2257–2266.
27. **Millar, A. J., I. A. Carre, C. A. Strayer, N. H. Chua, and S. A. Kay.** 1995. Circadian clock mutants in *Arabidopsis* identified by luciferase imaging. Science **24:**1161–1163.
28. **Mooney, J. L., and L. N. Yager.** 1990. Light is required for conidiation in *Aspergillus nidulans*. Genes Dev. **4:**1473–1482.
29. **Moore-Ede, M. C., F. M. Sulzman, and C. A. Fuller.** 1982. The clocks that time us. Harvard University Press, Cambridge, Mass.
30. **Pittendrigh, C. S.** 1960. Circadian rhythms and the circadian organization of living things. Cold Spring Harbor Symp. Quant. Biol. **25:**159–184.
31. **Pittendrigh, C. S., W. T. Kyner, and T. Takamura.** 1991. The amplitude of circadian oscillations: temperature dependence, latitudinal clines, and the photoperiodic time measurement. J. Biol. Rhythms **6:**299–313.
32. **Pittendrigh, C. S., and D. H. Minis.** 1964. The entrainment of circadian oscillations by light and their role as photperiodic clocks. Am. Nat. **118:**261–285.
33. **Roenneberg, T., and W. Taylor.** 2000. Automated recordings of bioluminescence with special reference to the analysis of circadian rhythms. Methods Enzymol. **305:**104–119.
34. **Sargent, M. L., W. R. Briggs, and D. O. Woodward.** 1966. Circadian nature of a rhythm expressed by an invertaseless strain of *Neurospora crassa*. Plant Physiol. **41:**1343–1349.
35. **Sargent, M. L., and S. H. Kaltenborn.** 1972. Effects of medium composition and carbon dioxide on circadian conidiation in *Neurospora.* Plant Physiol. **50:**171–175.
36. **Shinohara, M. L., J. J. Loros, and J. C. Dunlap.** 1998. Glyceraldehyde-3-phosphate dehydrogenase is regulated on a daily basis by the circadian clock. J. Biol. Chem. **273:**446–452.
37. **Temme, A., T. Ott, T. Haberberger, O. Traub, and K. Willecke.** 2000. Acute-phase response and circadian expression of connexin26 are not altered in connexin32-deficient mouse liver. Cell Tissue Res. **300:**111–117.
38. **Yager, L. N., H. O. Lee, D. L. Nagle, and J. E. Zimmerman.** 1998. Analysis of *fluG* mutations that affect light-dependent conidiation in *Aspergillus nidulans*. Genetics **149:**1777–1786.