Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 8249–8253, September 1993
Genetics

Day/night and circadian rhythm control of *con* gene expression in *Neurospora*

(light regulation/day/night control/conidiation/circadian periodicity)

FRANK-ROMAN LAUTER AND CHARLES YANOFSKY

Department of Biological Sciences, Stanford University, Stanford, CA 94305

Contributed by Charles Yanofsky, May 5, 1993

ABSTRACT **In the filamentous fungus *Neurospora crassa*, several events in the process of conidiation are influenced by light. Two genes, *con-6* and *con-10*, which were previously shown to be transcriptionally activated during conidiation and by exposure to light, were found to be unexpressed in mycelium maintained in constant darkness or in constant light. However, when mycelium was shifted from darkness to light, transcripts of both genes appeared and were abundant. Upon further illumination both transcripts disappeared—i.e., their continued production was light repressed. When dark-grown mycelium was exposed to a light pulse and reincubated in the dark, expression of *con-6* and *con-10* exhibited a 20-hr circadian periodicity. Both genes were photoinducible throughout the stages of the circadian cycle. In the mutant strains *bd* and *bd;frq*^{9}, *con-6* and *con-10* were light inducible but were not normally light repressible. Mutant genes such as *acon-2*, *acon-3*, and *fl* that block developmental expression of *con-6* and/or *con-10* did not prevent their photoinduction.**

The ascomycete *Neurospora crassa* produces three different types of spores: macroconidia* and microconidia, formed during the asexual cycle, and ascospores, formed during the sexual cycle. Light has significant effects on the process of conidiation. In the light, conidiophores orient toward the light, they develop faster, and they yield greater numbers of conidia (refs. 1–3; F.-R.L., C. Yamashiro, and C.Y., unpublished data). The developmental program directing conidia formation is controlled by a light-influenced endogenous "clock" (4–6). When cultured in the dark in the absence of external signals, the fungus switches periodically (every 21.5 hr) from mycelial growth to conidiation (7). However, a 5-min light pulse given at 24-hr intervals during conidiation is sufficient to "entrain" conidiation to a 24-hr period (8, 9). At the molecular level, it has been shown that expression of *eas*, the gene encoding the major rodlet protein of the conidium (10, 11), is in fact light and clock responsive (12, 13).

The *con* genes of *N. crassa* were cloned on the basis of their preferential expression during conidiation (14). Several of these genes, including *con-6* and *con-10*, have been shown to be transcriptionally activated at the mycelial stage by exposure to light (15). In view of the varied effects of light on conidiation, it was of interest to examine the light and dark behavior of mycelial *con* gene expression. In this report, we show that expression of *con-6* and *con-10* is subject to what might be considered day/night control. We also show that expression of these genes can exhibit circadian periodicity. These genes were observed to be photoinducible in the conidiation-defective mutants *acon-2, fld, acon-3*, and *fl*.

MATERIALS AND METHODS

Strains and Plasmids. Wild-type *N. crassa* strain 74-OR23-IVA (FGSC 987) was used throughout. Mutants examined (16) include *acon-2* (FGSC 3262), *acon-3* (FGSC 3286), *fl* (FGSC 4317), *fld* (FGSC 7022), *bd* (FGSC 1858), and *bd;frq*^{9} (kindly provided by M. Lewis, University of California, Santa Cruz). pBT3 was the source of *tub-2* DNA (17). pCOXV was the source of *cox-5* DNA (18). pCon-6/6-6 (19), pBW100 (19), and pJYC-opd (20) were used to prepare *con-6, con-10*, and *cpc-1*-specific antisense RNA probes.

Light Experiments. Cultures were grown and mycelia were photoinduced as described (15) using Sylvania Energy Saver lamps (6 W/m^2 in the blue light region). In day/night experiments (see Figs. 1–3 and 5), $\approx 10^7$ conidia were inoculated into 75 ml of Vogel's liquid minimal medium (21) containing 2% sucrose as carbon source and 0.2% Tween 80 as wetting agent. Logarithmic phase cultures were grown at 34°C in 250-ml Erlenmeyer flasks with agitation (200 rpm) for 24 hr; the mycelial dry weight per flask was typically 200–250 mg. The mycelia in each flask were collected by filtration on a Büchner funnel and the resulting mycelial pads were cut in half. All mycelial pads were wetted with 0.5 ml of prewarmed (25°C) growth medium initially and every 30 min thereafter. This treatment prevented aerial hyphae formation and arrested growth in the vegetative phase. After filtration, all manipulations were performed at 25°C. For subsequent steps, see Figs. 1–3 and 5.

Circadian Rhythm Analyses. Circadian rhythm experiments were performed as described by Loros *et al.* (22) with two modifications: wild type was used instead of the band (*bd*) mutant and a different light regime was followed. For details see Fig. 4.

RNA Analysis. Total RNA was extracted from each mycelial sample as described (23). Northern blot analyses with DNA or RNA probes were performed by standard procedures (14, 24). Eight micrograms of total RNA from each sample was separated by electrophoresis through a 1.5% agarose/formaldehyde gel and transferred to GeneScreen. RNA was probed with radiolabeled *tub-2, con-6, con-10, cox-5*, and *cpc-1* probes. Radiolabeled *tub-2* and *cox-5* DNA probes were prepared by hexamer-primed labeling (25). Radiolabeled *con-6, con-10*, and *cpc-1* RNA probes were prepared by generating ^{32}P-labeled antisense RNA (24). Less than 10% of each radiolabeled probe hybridized to the RNA immobilized on the nylon membrane. All of the Northern blot analyses presented were repeated with RNA samples obtained in at least two independent experiments.

RESULTS

Constant Light Represses *con* Gene Expression. In previous studies, it was shown that exposure of wild-type mycelium to light activated transcription of several *con* genes (15). It was also shown that mutations in two genes that mediate light responses in *N. crassa*, white collar-1 (*wc-1*) and white

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*Since macroconidia comprise the majority of asexual spores in *N. crassa*, we use the terms macroconidia and conidia interchangeably.

collar-2 (*wc-2*), prevented *con* gene light induction (15). In the present study, our initial objective was to determine the dark/light requirements for this light induction. No detectable *con-6* or *con-10* mRNA was observed in mycelia that had been maintained in constant light or constant darkness (Fig. 1). However, transcripts of both genes were detectable when a 30-min light pulse was given after 1 hr or more of incubation in the dark. The response to the light pulse was most pronounced after 5 hr of incubation in the dark. Thus, *N. crassa* mycelium requires prior growth in the dark for transcription of *con-6* and *con-10* to become responsive to light. The levels of *tub-2* mRNA (encoding β-tubulin of *N. crassa* (17) were measured as controls. Similar levels of *tub-2* RNA were detected in each pair of RNA samples (Fig. 1).

Constant Light Represses *con* Gene Induction. Transcripts of the *con* genes are not detectable in mycelium maintained in constant light. However, as shown in Fig. 1, these transcripts are detected when mycelium maintained in the dark is briefly exposed to light. To determine the length of the period of illumination required for repression of *con-6* and *con-10* expression, we examined *con* gene transcript levels as a function of the length of the period of exposure of mycelium to light (Fig. 2). It is evident that light has a transient induction effect on *con-6* and *con-10* expression. Both mRNAs were readily detected after a half hour exposure of mycelium to light. These mRNAs reached their maximum levels after 1 hr in the light, but they were undetectable after 4 hr of exposure (Fig. 2). Apparently, after growth in the dark, several hours in constant light is sufficient to eliminate detectable levels of the transcripts of *con-6* and *con-10*. The *tub-2* mRNA levels were essentially identical in paired RNA samples (Fig. 2).

Light Inducibility in Developmental Mutants. In the experiment described in Fig. 1, we established that exposure to darkness is required for light-induced expression of *con-6* and *con-10*. We next determined whether the light response of *con-6* and *con-10* was dependent on the presence of unaltered components of the conidiation pathway. Transcriptional activation of *con-6* and/or *con-10* is known to be blocked in several morphological mutants that are defective in conidia formation (26, 27). We measured photoinducibility in *acon-2, fld, acon-3*, and *fl* following their growth in liquid medium in total darkness for 24 hr. *acon-2* is temperature sensitive for conidia production (28); it was examined at both permissive (25°C) and nonpermissive (34°C) temperatures. Other strains were grown at 34°C. After exposure to light, mycelia of each of the mutant strains displayed elevated levels of *con-6* and *con-10* mRNA comparable to that observed in wild type (Fig. 3). Therefore, the *acon-2, fld, acon-3*, and *fl* gene products are not required for light induction of *con-6* and *con-10* expression, although *fl* is required for developmental expression of either gene and *acon-2* and *acon-3* block *con-10* but not *con-6* expression. The control *tub-2* mRNA levels were essentially identical in each pair of dark and light half pads (Fig. 3).

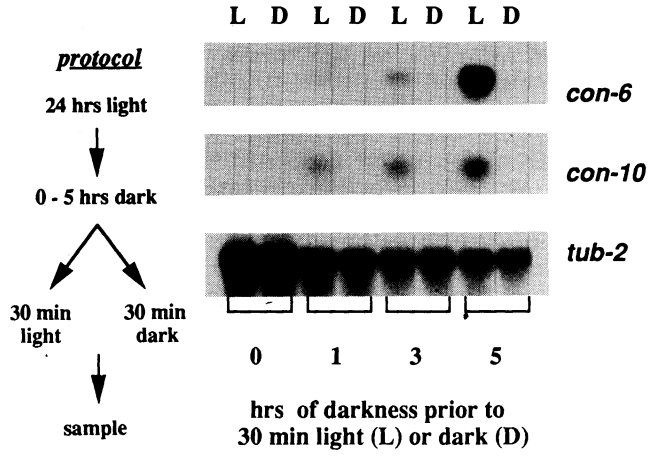

FIG. 1. Dark dependence of photoinduced accumulation of *con-6* and *con-10* mRNA in vegetative mycelia. Wild-type mycelia, grown for 24 hr in the light, were harvested by filtration, the filter pads were cut in half, and half pads were either incubated in darkness (25°C) for 0–5 hr prior to 30-min illumination (lanes L) (25°C) or kept in darkness as controls (lanes D) (25°C). The protocol followed is indicated on the left. Light and dark exposures were terminated by quick freezing half pads in liquid nitrogen. Total RNA was extracted from each mycelial half pad. RNA (8 μg per lane) was resolved by formaldehyde/agarose gel electrophoresis and transferred to a nylon membrane. Membranes were probed with ^{32}P-labeled antisense *con-6*- and *con-10*-specific RNAs and visualized by autoradiography. The membrane used for the *con-10* probe was stripped and rehybridized with a *tub-2* DNA probe. Probes are indicated on the right.

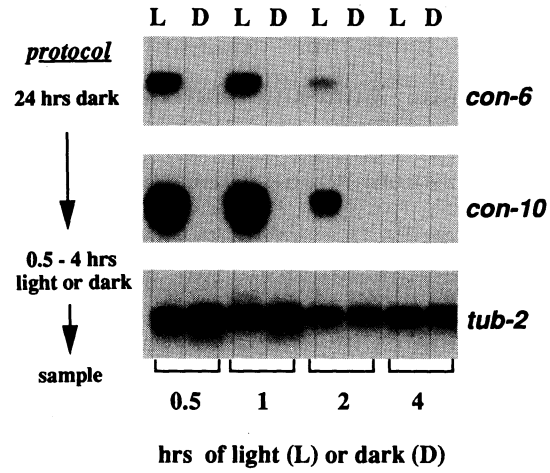

FIG. 2. Light inhibition of accumulation of *con-6* and *con-10* mRNA in vegetative mycelia grown in the dark. Wild-type mycelia, grown for 24 hr in darkness, were harvested by filtration, the filter pads were cut in half, and half pads were either exposed to light for 0.5–4 hr (lanes L) or kept in the dark (lanes D). Incubation was terminated by freezing in liquid nitrogen. RNA was extracted from mycelial samples and equal amounts of total RNA (8 μg) were loaded in each lane and analyzed as in Fig. 1.

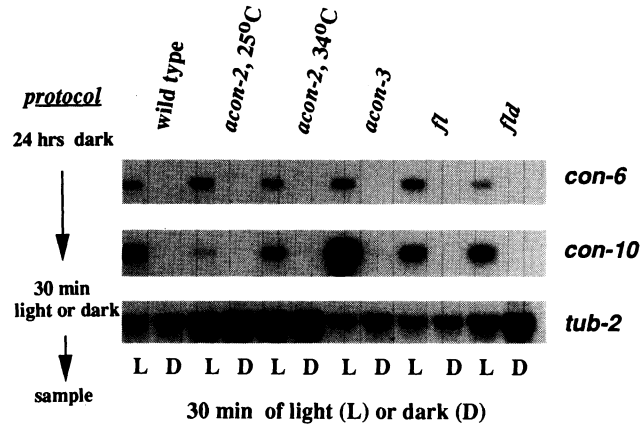

FIG. 3. Photoinduced accumulation of *con-6* and *con-10* mRNA in vegetative mycelia of developmental mutants. Mycelia of wild type, *acon-2, acon-3, fl*, and *fld* were grown for 24 hr in the dark and were harvested by filtration. Half pads were exposed to either light (lanes L) or darkness (lanes D) for 30 min. Incubation was terminated by freezing in liquid nitrogen. RNA was extracted from mycelial samples and equal amounts of total RNA (8 μg) were loaded in each lane and analyzed as in Fig. 1.

Circadian Rhythm Detection. To determine whether expression of *con-6* or *con-10* is under circadian rhythm control, we performed circadian rhythm experiments following the general protocol of Loros *et al.* (22). However, we made two modifications of their procedure: a wild-type strain was used instead of the band (*bd*) mutant, and a different light regime was followed (see legend to Fig. 4). In the dark samples, the mycelial *con-6* and *con-10* transcript levels, although low, varied in a cyclical fashion, reaching a maximum 20 and 40 hr after the mycelia were given a 60-min light pulse (Fig. 4). Thus, transcription of these genes is subject to circadian rhythm control. Identical mycelial samples were exposed to light for 1 hr just prior to sampling (Fig. 4). An appreciable increase in *con-6* and *con-10* transcript levels was observed at every stage of the circadian cycle. It was apparent that the extent of the response to light paralleled transcriptional activity in the dark. This was most noticeable during the initial 20-hr period.

As controls, we measured the transcript levels for *tub-2*, *cpc-1* (20), and *cox-5* (18). The dark *tub-2* mRNA levels showed some phased variation, mimicking that of *con-6* and *con-10* mRNA levels, but quantitatively this variation did not approach that of the *con* transcripts (Fig. 4). There was no phased variation in the level of *cpc-1* mRNA (Fig. 4) or *cox-5* mRNA (data not shown), nor was there any effect of light on *tub-2* expression (Fig. 4).

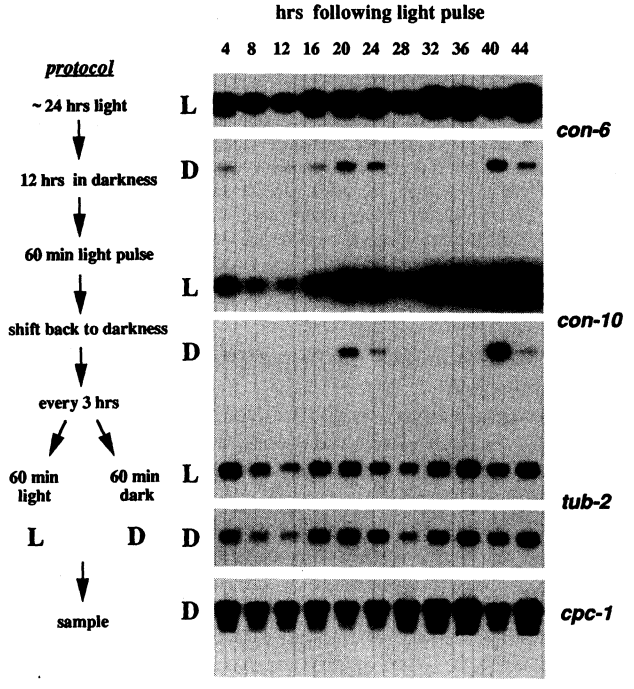

FIG. 4. Circadian oscillation of *con-6* and *con-10* transcript levels in vegetative mycelia and photoinduced accumulation of *con-6* and *con-10* transcripts as a function of the phase of the circadian cycle. The general protocol of Loros *et al.* (22) was followed. Wild-type mycelium was grown for ≈24 hr in constant light (28°C) in a high glucose/arginine medium (750 ml in a 2-liter Erlenmeyer flask) (29) and harvested by filtration. The resulting pad was cut into at least 25 pieces (20 × 25 mm each). Each mycelial rectangle was peeled off the filter paper and transferred to a low glucose/arginine medium (75 ml in 250-ml Erlenmeyer flasks) (29) and shifted to darkness (28°C). After 12 hr of growth, mycelia were exposed to light for 1 hr (28°C) and reincubated in darkness (28°C). Mycelia samples, taken every 3 hr, were filtered, the filters were cut in half, and half pads were either exposed to light for 60 min (L) or kept in darkness for an additional 60 min (D). Incubation was terminated by freezing in liquid nitrogen. RNA was extracted and equal amounts of total RNA (8 μg) were loaded in each lane and analyzed as in Fig. 1.

Light Regulation in the Mutant *bd* and a *bd;frq*^{9} Double Mutant. Strains with the mutation frequency^9 (*frq*^{9}) do not exhibit the regularity of the circadian rhythm of conidiation typical of *N. crassa* and are considered arrhythmic (9). To determine whether the *con-6* and *con-10* light responses were dependent on a normal, functional, endogenous clock, we examined light expression in a *bd;frq*^{9} double mutant and in the *bd* single mutant. Circadian conidiation is typically examined in the band (*bd*) mutant strain of *N. crassa* (30). The levels of *con-6* and *con-10* mRNA detected after a 1-hr light exposure were comparable in the three strains (Fig. 5). However, after 4 hr in constant light, transcripts of these genes were undetectable in wild-type samples but remained elevated in the *bd* and the *bd;frq*^{9} strains. Transcript levels for the control gene *tub-2* were comparable in all RNA samples (Fig. 5). These findings establish that neither the *bd* nor the *frq* gene product is required for light inducibility of *con-6* and *con-10* expression. Our observations also indicate that the *bd* mutation delays or prevents light repression of *con-6* and *con-10* mRNA synthesis. Experiments designed to measure circadian rhythm expression of *con-6* and *con-10* in the *bd* and *bd;frq*^{9} mutants remain to be done.

DISCUSSION

The *con* genes of *N. crassa* were recognized and isolated on the basis of their preferential expression during conidiation

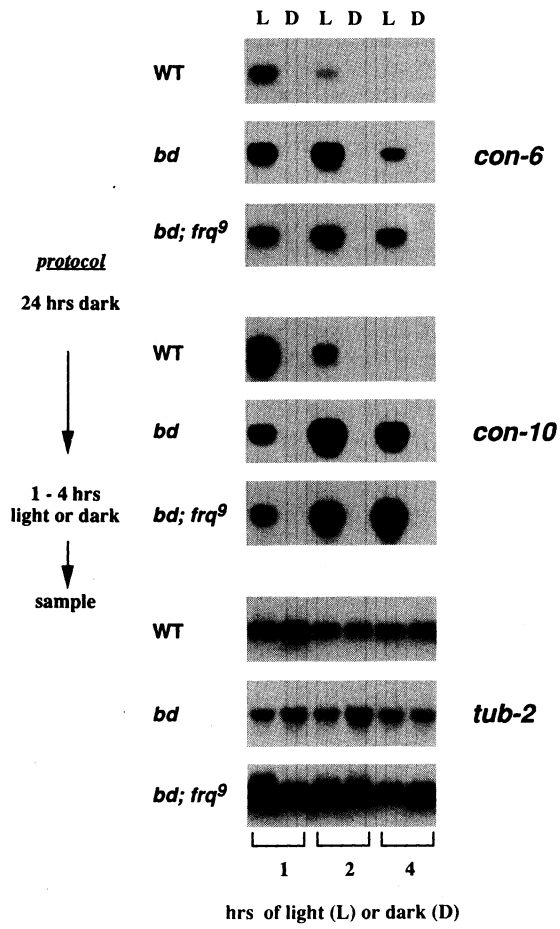

FIG. 5. Photoinduced accumulation of *con-6* and *con-10* transcripts in wild-type, *bd*, and *bd;frq*^{9} strains. The protocol described in Fig. 2 was followed. Dark-grown mycelia were harvested by filtration, the pads were cut in half, and half pads were either exposed to light for 1–4 hr (lanes L) or kept in the dark (lanes D). RNA was extracted from half pads and equal amounts of total RNA (8 μg) were loaded in each lane and analyzed as in Fig. 1.

(14). They were selected to be used as responder genes in studies on developmental regulation. Elevated levels of *con* gene transcripts and their translational products are readily detected at different stages of conidiation and in mature conidia (23, 31). In the vegetative, mycelial phase of the fungal life cycle, many *con* genes are transcriptionally inactive. However, under appropriate conditions, such as exposure to light (15), expression of these genes is induced. In this report, we have analyzed the effects of darkness and light on the pattern of expression of two of the light-responsive *con* genes, *con-6* and *con-10*. We demonstrate that their expression is subject to day/night control, and to clock (circadian rhythm) regulation.

Day/Night Control. In RNA isolated from mycelia growing in constant darkness, constant light, or during the transition from light to dark, *con-6* and *con-10* transcripts were undetected. However, transcripts of both genes appeared transiently when mycelia were transferred from dark to light. This response pattern could be described as being "morning specific" and subject to day/night control. However, *con* gene expression is not solely a function of the time of the subjective, circadian day, because the endogenous clock is readily overridden by a light stimulus. Light induction of *con-6* and *con-10* expression was found to be transient—i.e., transcripts of both genes were abundant after 0.5 or 1 hr of illumination but were undetectable after 4 hr of illumination (Fig. 2). Transient expression apparently requires a period of dark incubation during which susceptibility to light activation is acquired. This is followed by a light period during which responsiveness can become suppressed. Light activation of *con-6* and *con-10* expression was previously shown to be dependent on functional *wc-1* and *wc-2* genes (15). The latter genes mediate all known blue light responses in *Neurospora* (32, 33) and presumably mediate light activation of *con* gene expression as well. In other studies, we observed that the β-galactosidase levels of strains containing *con-6::lacZ* and *con-10::lacZ* gene fusions were increased after illumination of dark grown mycelia (unpublished data). These findings suggest that light induction of transcription of these *con* genes is followed by translation of their transcripts. In view of these findings, it seems likely that the changes we have observed reflect regulation of transcription initiation predominantly.

The light responses of *con-6* and *con-10* described in this study differ from those seen with *eas*, the gene that encodes the major rodlet protein of the conidium (12, 13). In mycelia exposed to light, *con-6* and *con-10* transcripts accumulate earlier than *eas* transcripts, and while *con-6* and *con-10* mRNAs are not detectable after 4 hr of illumination (Fig. 2), *eas* transcript levels remain high (ref. 12; unpublished results). Therefore, individual conidiation genes may show somewhat different responses to illumination.

We also observed that *con-6* and *con-10* expression was clock regulated. This finding raises the possibility that circadian rhythm modulates the light responsiveness of these genes. This was found to be the case since the extent of light activation of these genes was influenced by the stage in the circadian cycle (Fig. 4).

In studies with an arrythmic clock mutant, *frq*^{9}, it was shown that photoinducibility of *con-6* and *con-10* expression was independent of a normal *frq* locus. It was also observed that the *bd* mutation prevented normal light repression of *con* gene expression. This finding would appear to be inconsistent with the observation of Paietta and Sargent (34) that the *bd* mutation does not prevent photosuppression of the circadian conidiation rhythm in race tube cultures. However, we have not determined whether light repression of *con-6* and *con-10* transcription is merely delayed in the *bd* mutant. Paietta and Sargent (34) isolated mutants in the *bd* strain that were resistant to light repression of circadian conidiation; they called these mutants *lis* (light insensitive). These mutations have not been examined for light repression of *con-6* and *con-10* expression.

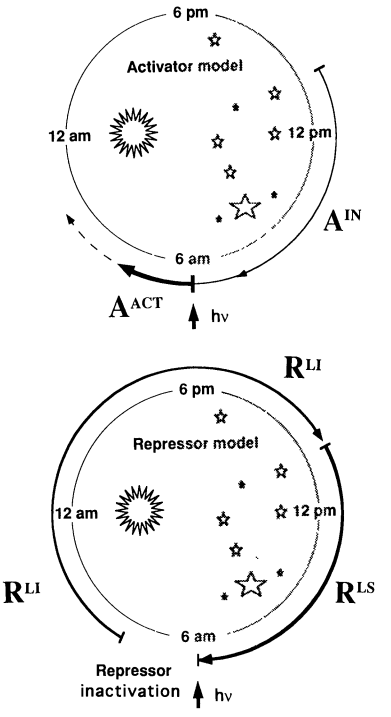

FIG. 6. Alternative models of day/night control. Activator model: an activator of conidiation gene transcription is synthesized in an inactive form (A^{IN}) in the dark. Upon exposure of mycelium to light the activator is activated (A^{ACT}), but synthesis of the activator is inhibited. If additional conidiation-related events occur, factor synthesis would continue. If these events do not occur, the activation factor would decay or be inactivated. Repressor model: a light-sensitive transcriptional repressor (R^{LS}) of conidiation gene transcription is synthesized in the dark. Exposure of mycelium to light inactivates the repressor. Upon continued exposure to light in the absence of additional conidiation-related events, a light-insensitive functional form of the repressor (R^{LI}) is produced. Upon return to darkness, this repressor species is replaced by the light-sensitive species (R^{LS}).

Developmental Factors. Light-induced expression of *con-6* and *con-10* was found to be independent of the ability of the organism to complete the process of conidiation. The aconidial mutants *acon-2* and *fld* form aerial hyphae but no minor constriction chains, while the aconidial mutants *acon-3* and *fl* form minor constriction chains but no major constriction chains. Functional *acon-2, acon-3*, and *fl* loci are required for developmental expression of *con-10*, while, of these, only the *fl* product is necessary for normal expression of *con-6* (27). The effect of the *fld* locus on developmental expression of the *con* genes has not been examined. We observed that light induction of *con-6* and *con-10* expression does not require the normal products of the four aconidial genes mentioned. Thus, factors other than the products of these developmental genes must mediate light activation of *con-6* and *con-10*.

Clock Control. Under circadian control in constant darkness, *con-6* and *con-10* mRNA levels increased and decreased with about a 20-hr periodicity (Fig. 4). Since the process of conidiation itself is under biological clock control in *Neurospora* (4–9), circadian appearance of transcripts of presumed conidiation-specific genes is perhaps not unexpected. However, the conditions we used to examine circadian periodicity do not allow conidiation (29). We have also examined the circadian rhythmicity of expression of the gene *eas* in wild type (F.-R.L., unpublished data). *eas* is identical to *bli-7* (12), a gene shown to be light regulated (35), and to

ccg-2 (13), a gene shown to be clock controlled and to exhibit transcriptional periodicity (13, 22). In our circadian analyses with wild type, *eas* transcript levels showed periodic cycling; however, the oscillations were not as pronounced as those observed for *con-6* and *con-10* (data not shown).

In wild type, a light pulse given at any time during the circadian cycle stimulates *con* gene transcription. This property distinguishes the behavior of these genes from certain mammalian genes that are known to be light and clock regulated—i.e., c-*fos* and *jun-B*. These genes are light-inducible in the suprachiasmatic nuclei of the hypothalamus of the golden hamster only during subjective night (36).

Preliminary Models. In proposing working models that account for *con* gene expression (Fig. 6), we have considered the relevance of light responses to the natural behavior of the organism. We assume that in *N. crassa*'s natural environment, light is one of several signals that the organism recognizes as an indication that conditions exist that are favorable for conidiation. Thus, the first light of day may trigger regulatory events that activate transcription of genes concerned with the initial stages of conidiation. Continued transcription of these genes might require the occurrence of additional events. Otherwise, transcription would be turned off upon further exposure to light. Subsequently, as daytime is replaced by nighttime, the ability of these genes to respond to light would be regained. This scenario is compatible with light having an activating and/or a repressing effect on transcription. Indeed, the same light response factor could serve as repressor and activator, if, for example, its activity were altered by a light- or dark-dependent modification. The existence of light-induced circadian rhythm could be advantageous to the organism by setting in motion a programmed sequence of events that reflect the repetition of the day/night cycle that the organism experiences. Further studies with *con-6, con-10*, and *eas* offer the prospect of identifying the regulatory sites, factors, and events that are responsible for day/night control and circadian expression.

F.-R.L. is grateful to his teacher Frank Gebhardt for his support and friendship. The authors are particularly grateful to Dr. David Perkins for his continued interest in this project and his many helpful suggestions. The helpful comments of Carl Yamashiro, Luis Corrochano, and John Royer are greatly appreciated. We thank Brian White, Matthew Sachs, and Jeff Yost for providing the *con-10, con-6*, and *cpc-1* RNA probes, respectively. We thank Mike Lewis for the *bd;frq*^{9} mutant strain. F.-R.L. was supported by a fellowship of the Deutsche Forschungsgemeinschaft (La714/1-1). C.Y. is a Career Investigator of the American Heart Association. These studies were supported by a grant from the U.S. Public Health Service (GM41296).

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