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The Interplay of Light and the Circadian Clock[1]

Independent Dual Regulation of Clock-Controlled Gene *ccg-2(eas)*

Giuseppina Arpaia, Jennifer J. Loros, Jay C. Dunlap, Giorgio Morelli, and Giuseppe Macino*

Dipartimento di Biopatologia Umana, Sezione di Biologia Cellulare Policlinico Umberto I, Universita' di Roma La Sapienza, 00161 Roma, Italy (G.A., G. Macino); Department of Biochemistry, Dartmouth Medical School, Hanover, New Hampshire 03755–3844 (J.J.L., J.C.D.); and Unita' di Nutrizione Sperimentale, Istituto Nazionale della Nutrizione, via Ardeatina 546, 00178 Roma, Italy (G.M.)

Ambient light is the major agent mediating entrainment of circadian rhythms and is also a major factor influencing development and morphogenesis. We show that in *Neurospora crassa* the expression of clock-controlled gene 2 (*ccg-2*), a gene under the control of the circadian clock and allelic to the developmental gene easy wettable (eas), is regulated by light in wild-type strains. Light elicits a direct and important physiological effect on *ccg-2(eas)* expression as demonstrated using several mutant *Neurospora* strains. In white collar mutants (*wc-1* and *wc-2*) that are "blind" to blue light, *ccg-2(eas)* mRNA shows no variation following illumination with saturating light. By contrast, *ccg-2(eas)* mRNA is photoinduced in clock-null strains such as frequency^9 (*bd;frq*^{9}). The results in the clock mutants show that an intact circadian oscillator is not required for light induction of *ccg-2(eas)*. Thus, *ccg-2(eas)* is subject to a dual regulation that involves separable regulation by light and circadian rhythm.

Light influences many processes in biological systems. In the Ascomycetes *Neurospora crassa*, known to respond only to blue light (Gressel and Rau, 1983), the effects of light are exerted on many different biological aspects of the organism. Primary effects include promotion of carotenogenesis and the induction of differentiated structures such as conidia and protoperithecia. Light can also exert its effect secondarily by effecting changes in other regulatory processes. For example, light acts on the entrainment of the circadian rhythm in developmental processes (e.g. conidiation). In these ways, light carries out its role as one of the major environmental variables governing the life of the organism.

Many light-induced changes such as those mentioned above, whether primary or secondary, require the expression of new genes. Thus, in recent years much effort has been directed toward understanding the mechanisms by which light can act to regulate genes, and in many organisms a part of this mechanism appears to reflect input from the circadian clock. In plants, light and the clock are involved in the regulation of Cab, a part of the nuclear-encoded light-harvesting Chl *a/b*-binding protein complex of chloroplasts (Nagy et al., 1988), and expression of a number of other plant genes varies with either circadian or diurnal regulation (Giuliano et al., 1988). In vertebrate systems, both the rate of synthesis and total amount of several retinal photoreceptor components, including opsin and transducin, oscillate in a circadian or diurnal manner (Korenbrot and Fernald, 1989). Finally, in the vertebrate suprachiasmatic nucleus wherein lies the heart of the mammalian circadian pacemaker, light induces the expression of a number of immediate early genes, including c-*fos*, *jun-B*, *jun-D*, and *NGF-IA*, in a dose-dependent and time-of-day-specific manner (Aronin et al., 1990; Earnest et al., 1990; Kornhauser et al., 1990, Rusak et al., 1990; Kornhauser et al., 1992; reviewed by Taylor, 1989). Data such as these demonstrate a close correlation between the two kinds of control and has suggested that the regulation of genes by light and the biological "clock" may actually be interdependent rather than simply coincident. This interdependence, however, can make it difficult to identify the primary effectors of gene expression, because in any given case it may not be clear whether light responses are a primary or secondary effect.

An understanding of these two interconnected aspects of gene regulation can be advanced by experimentation in a system in which the individual elements of control, regulation by light and regulation by the clock, are to some extent already understood. Whereas light responses have been quite well studied in plants, the groundwork for genetic studies of plant circadian rhythms (Millar et al., 1992) has only recently appeared. For this reason we have pursued these questions in the paradigmatic model system *Neurospora crassa* (Feldman and Dunlap, 1983; Dunlap, 1990), in which mutations affecting both light and circadian regulation have been identified and characterized. Using these tools, we have investigated the effect of light on the expression of a well-known clock-regulated gene, *ccg-2*. This gene is one of the two clock-controlled genes identified in *Neurospora* (Loros et al., 1989) that are specifically turned on in the subjective morning. It has recently been shown by us and others (Bell-Pedersen et al., 1992; Lauter et al., 1992) to be allelic to two other genes,

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* Corresponding author; fax 39–6–446–2891.

bli-7 and *eas*, the first developmental gene to be isolated in *Neurospora*.

Photoinduction studies reported here show that *ccg-2(eas)* is inducible by light in wild-type strains. The kinetic analysis of this response confirms and extends estimates previously made for light induction of *bli-7* (Sommer et al., 1989), because it is now clear that light first induces, and later depresses at least to some extent, *ccg-2(eas)* expression. As expected (Sommer et al., 1989), light induction is blocked in *wc-1* and *wc-2* mutants, which are defective in the typical blue-light responses seen in *Neurospora* (Harding and Shropshire, 1980). Importantly, however, light induction remains intact in strain *bd,frq*^{9}, in which the circadian clock does not function normally; therefore, an intact circadian oscillator is not required for light induction. Surprisingly, the regulation of *ccg-2(eas)* expression appears to be altered in the band (*bd*) strain, previously noted for its role in clarifying expression of the circadian rhythm in conidial banding (Perkins et al., 1982). This is also evident in the *bd,frq*^{9} strain but not in another light-inducible gene, *albino 3*. Expression of *ccg-2(eas)* is thus governed by several interdependent factors. However, because the clock-mutant strain is still fully photoinducible and the circadian clock can induce time-specific expression in the absence of light, regulation of expression involving responses to ambient light and the clock appear to involve independent pathways.

MATERIALS AND METHODS

Strains of *Neurospora crassa*

The Oak Ridge wild-type 74-OR23A strain and mutant strains *wc-1* ER53A, *wc-2* ER33a, and *bd* no#A were obtained from the Fungal Genetics Stock Center (Nos. 987, 4397, 4408, and 1858, respectively). *bd;frq*^{9} A (strain 94–43) is a mutant of the circadian clock gene frequency (*frq*).

Media and Culture Conditions

For routine *Neurospora* cultivation, cultures were grown in Vogel's minimal medium (Vogel, 1964; Davis and de Serres, 1970) supplemented with 2% Suc, a medium that supports vigorous growth.

The liquid culture system utilized for the production of rhythmic mycelia cultures used the high-concentration Glc medium and the low-concentration Glc medium described elsewhere (Nakashima, 1981; Loros et al., 1989). This culture regimen was originally designed for long periods of extremely slow growth in the absence of overt differentiation so that changes due solely to the circadian clock could be monitored. Mycelial discs were transferred to and grown in low-concentration Glc medium for between 48 and 68 h, and the light/dark transfer times were adjusted so that at the time of harvest cultures had been in the dark between 4 and 68 h. With this culture regimen, mycelia could be harvested at times spanning several circadian cycles while being relatively developmentally synchronous (Loros and Dunlap, 1991).

Photoinduction

For induction studies of growing cultures, 5×10^6 conidia were inoculated into 100 mL of medium in 250-mL flasks and shaken at 150 rpm in the dark for 18 to 24 h at 25°C. Cultures were then divided in two, one of which was illuminated, and samples were taken at different times from the onset of the illumination. Photoinduction was elicited by treatment of the cultures with saturating white light (energy fluence rate >130 J m^{-2} in the blue region). At the time of harvest, mycelia were immediately removed from the growth medium and frozen in liquid nitrogen.

RNA Extraction and Northern Hybridization Blot

Frozen mycelia were powdered in a Waring Blendor under liquid nitrogen. Total RNA was extracted essentially as described by Sokolowsky et al. (1990) with a miniprep extraction method that utilizes phenol extraction and LiCl precipitation. In the RNA hybridization (northern) blots, RNA was first denatured in formaldehyde, then electrophoresed on 1.5% agarose gels containing formaldehyde (Lehrach et al., 1977), and transferred to Hybond-N membranes (Amersham). The filters were hybridized with ^{32}P-labeled probes prepared using the random-primer method (Feinberg and Vogelstain, 1983) at 50°C in 5× SSC, 5× Denhardt's solution, 0.5% SDS, 50% formamide, 50 μg mL^{-1} of denatured salmon sperm DNA, and 1.5×10^6 cpm mL^{-1} of probe for 15 to 18 h. Washes were at 60°C in 1× SSC, 0.1% SDS and in 0.1× SSC, 0.1% SDS for 15 min each. Filters were autoradiographed with Kodak X-OMAT films in the presence of intensifying screens. To rehybridize filter blots for the purposes of normalization, probes were removed by washing the blots in 0.005 M Tris-HCl (pH 8.0), 0.002 M Na_2EDTA, and 0.1× Denhardt's solution at 65°C for 2 h.

RESULTS

ccg-2(eas) Is Light Inducible in Wild-Type Strains

To determine whether light exerts some influence on the expression of *ccg-2(eas)*, wild-type *Neurospora* cultures (strain 74-OR23A) were grown and photoinduced under conditions previously shown to be effective in the induction of another light-induced gene, *al-3* (Baima et al., 1991). Total RNA was extracted from dark-grown and illuminated mycelia frozen after 15, 30, 60, 90, 120, and 240 min from the onset of the illumination.

The kinetics of *ccg-2(eas)* mRNA accumulation and subsequent loss following light treatment are shown in Figure 1. The amount of *ccg-2(eas)* transcript increases relatively slowly in response to light. The effect of light is first detectable following 30 min of illumination (lanes 3–4); transcript levels reach a peak between 90 min and 2 h (lanes 7–10) and then decay (lanes 11–12). At no time following illumination do transcript levels decrease below the level of detection, a finding that is consistent with previous data showing that the gene is in fact expressed in the dark (Loros et al., 1989). The IF2 control transcript, previously shown not to be photoinduced (Baima et al., 1991), does not vary substantially among the same samples.

The kinetics of the response of *ccg-2(eas)* is different from that typical for rapidly responding light-inducible genes such as *al-1*, *al-3*, *bli-3*, and *bli-4*. For those genes, mRNA accumulation starts very quickly after the start of the illumination

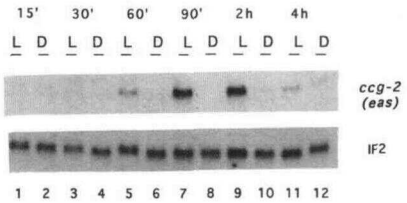

Figure 1. *ccg-2(eas)* mRNA levels are induced in photoinduced mycelia. Northern blot analysis of the kinetics of *ccg-2(eas)* mRNA in mycelia grown in dark for 18 h and then illuminated with continuous light. Times following illumination are shown. For each time, dark (D) and light (L) cultures are compared. RNA was extracted as described in "Materials and Methods." Total RNA (5 μg) was loaded per lane. The IF2 cDNA probe (Baima et al., 1991) was used for normalization.

and within 45 min decreases below the limit of detection (Sommer et al., 1989; Schmidhauser et al., 1990; Baima et al., 1991). As expected, these kinetics data confirm the light induction seen in *bli-7* with which *ccg-2(eas)* has very recently been shown to be allelic (Bell-Pedersen et al., 1992; Lauter et al., 1992).

Light Responses in *wc-1* and *wc-2*

The *Neurospora crassa* white collar genes (*wc-1* and *wc-2*) are thought to encode regulatory genes whose products act at a very early stage in the light-activated signal transduction pathway (Perkins et al., 1982). Repeated mutagenesis of *Neurospora* and screens for light response regulatory genes (Harding and Shropshire, 1980; Degli Innocenti and Russo, 1984a) have resulted in the identification of only these two *wc* genes. *wc* mutants are highly pleiotropic and exhibit alterations in all known blue-light-inducible responses. For these reasons, it was important to examine the light response of *ccg-2(eas)* in a *wc* mutant genetic background.

wc-1 and *wc-2* mutant strains were grown, the mycelia were photoinduced according to the standard photoinduction protocol, and samples were collected and processed for northern analysis (Fig. 2). The expression of the *ccg-2(eas)* gene does not vary in light-illuminated samples compared with dark-grown mycelia. Furthermore, the levels of *ccg-2(eas)* mRNA never reach the high levels seen in wild-type light-induced cultures. Thus, it appears that light induction of *ccg-2(eas)* requires the products of both *wc-1* and *wc-2*, again confirming expectations (Sommer et al., 1989).

A Normal Circadian Clock Is Not Required for Blue-Light Induction of *ccg-2(eas)*

We have previously shown that *ccg-2(eas)* is expressed even in constant darkness. This effect is influenced in part by action of the circadian biological clock in activating the gene in the subjective morning (Loros et al., 1989). This observation suggested that a normally functioning clock might be required for normal levels of *ccg-2(eas)* expression. To test this hypothesis, we first examined expression of *ccg-2(eas)* in a strain, *bd,frq*^{9}, known to lack a functional circadian clock (Loros et al., 1986; see Table I and "Discussion").

Results from a typical experiment in which mycelial discs of *bd,frq*^{9} were transferred to and grown in low Glc medium as described in "Materials and Methods" are shown in Figure 3. Northern blots of this RNA were then probed with *ccg-2(eas)*. Under these conditions of growth, conidiation in *bd,frq*^{9} displays an uncompensated fluctuation with a period length of approximately 12 to 15 h (Loros and Feldman, 1986) instead of 21.5 h typical of a wild-type strain cycle.

Although levels of *ccg-2(eas)* are clearly not constant, neither do they fluctuate in a manner typical of a clock-regulated gene, because the rudimentary fluctuation seen in Figure 3 would have a periodicity closer to 24 h than the predicted 12 to 15 h. Additionally, because *ccg-2(eas)* is a morning-specific gene, if it were clock regulated by an oscillator with a 24-h periodicity, it would be turned on in the subjective morning and display peaks (at about 12, 36, and 60 h in darkness) and troughs (at about 24 and 48 h in darkness) in transcript abundance at appropriate times. This is not the case. Finally, the amplitude of the fluctuation is only about 3-fold, which is much less than the typical 8- to 12-fold rhythm seen in the wild type (Loros et al., 1989; Bell-Pedersen et al., 1992). Thus, by the criteria of period length, phase, and amplitude, the observed regulation in *bd,frq*^{9} does not conform to that expected for a rhythm coupled to any known oscillator. *ccg-2(eas)* is expressed, but its level fluctuates in a noncircadian manner, perhaps reflecting regulation from other inputs, the poor capacity for light entrainment displayed by *bd,frq*^{9} (Loros et al., 1986), or the highly variable phasing behavior of this strain (Loros and Feldman, 1986). Although the timing of expression of *ccg-2(eas)* is aberrant in a strain lacking a functional clock, the gene is nonetheless capable of being expressed at a normal level. Clock regulation itself is not an obligatory aspect of *ccg-2(eas)* expression.

Next, we determined whether the clock was involved in the light regulation of *ccg-2(eas)*. Because it is well known that a light pulse given at any time in constant darkness can act to reset the biological clock to the subjective morning (Sargent and Briggs, 1967), it was possible that the blue-light induction of *ccg-2(eas)* was really an indirect effect of light mediated by the action of the clock. To evaluate this possi-

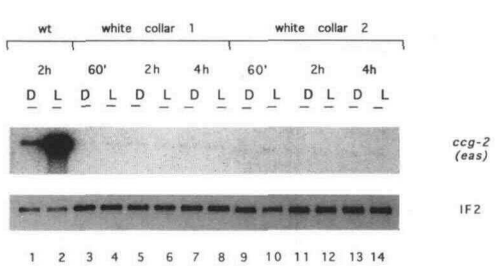

Figure 2. *ccg-2(eas)* does not respond to light in *wc* mutant strains. Northern blot analysis of total RNA extracted from *wc-1* and *wc-2* mycelial cells that were grown in the dark for 24 h, illuminated, and collected at different times as described in "Materials and Methods." For each time, dark control cultures (D) are shown; RNA extracted from wild-type cultures was used as a positive control (lanes 1–2). Filter-bound RNA samples were hybridized either to the *ccg-2(eas)*-specific probe or the IF2 cDNA (Baima et al., 1991) as a control for normalization. Total RNA (1 μg) was loaded per lane.

Table I. *Strains used in this study*

Strain Name	Genotype	Source	Reference
Wild type	Oak Ridge 74-OR23 A	FGSC[a]	Perkins et al., 1982
White collar-1	*wc-1* A (ER53)	FGSC	Perkins et al., 1982
White collar-2	*wc-2* a (ER33)	FGSC	Perkins et al., 1982
Frequency[9]	*bd;frq*[9] A	Lab stock	Loros et al., 1986
Band	*bd* A (no number)	FGCS	Perkins et al., 1982

[a] FGSC, Fungal Genetics Stock Center.

bility, we examined light induction of *ccg-2(eas)* in the *bd,frq*[9] genetic background and, as a control, in the *bd* background (Fig. 4). Cultures were inoculated and grown in the dark for 24 h. At this point they were illuminated and samples were taken and processed in the standard blue-light-induction protocol. The major observation is that in the clock-mutant strain, expression of *ccg-2(eas)* is still light inducible. However, the kinetics of induction are similar to those seen in the wild type only in broad detail, and there appear to be some important differences (cf. Fig. 1 and Fig. 4). First, the absolute kinetics of the up-regulation seem different; second, there are differences in the dark (uninduced) background level, which remains quite low in the wild type but increases noticeably from approximately zero to a substantial level in the mutant strain. As a result, the normal down-regulation seems to be reduced in the clock-mutant strain. The basis of the clear kinetic differences between the wild type and the *bd,frq*[9] mutant can be clarified by the observation that *bd*, a strain that is known to have a normal circadian clock, has a kinetic response to light closely matching that of the *bd,frq*[9] mutant. The dark background levels are consistently higher even in this mutant strain.

These molecular alterations are the first known to be associated with *bd*, a gene heretofore known only for its role in overcoming CO_2-induced masking of the circadian rhythm of conidiation (Perkins et al., 1982). To further prove that the light response remains in the *bd* and *bd,frq*[9] mutant strains, we tested the light inducibility of another light-regulated

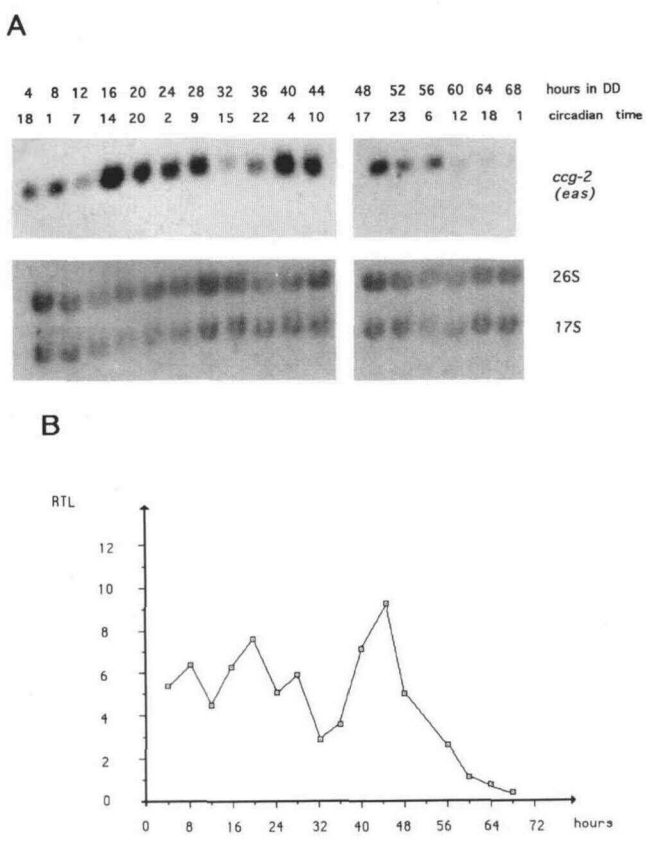

Figure 3. *ccg-2(eas)* expression in *bd,frq*[9] does not follow a circadian rhythm. A, Total RNA was collected as described in "Materials and Methods" at the times used previously (Loros and Dunlap, 1991; see "Materials and Methods"). Total RNA (20 μg) was loaded per lane. The total number of hours in darkness before harvest (DD) and the approximate circadian time (CT) is shown above the blot. The same blot was stripped and probed with a *N. crassa* rDNA probe. B, Plot of densitometry data from the blots in A. The relative transcript level (RTL) refers to the ratio of the *ccg-2(eas)* transcript to the 17S rRNA.

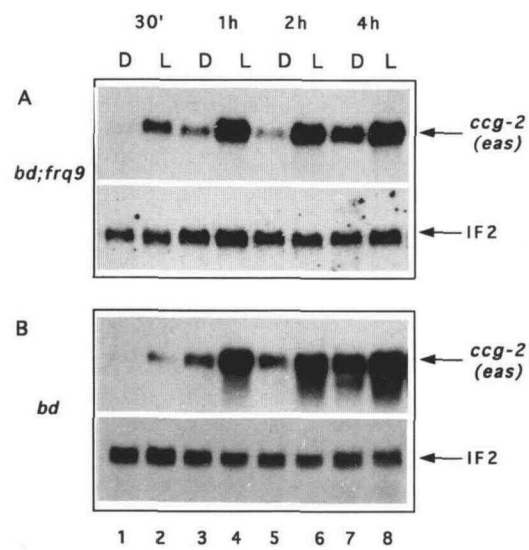

Figure 4. *ccg-2(eas)* is light-inducible in a strain lacking a functional clock. *bd,frq*[9] (A) and *bd* (B) mycelial cells were grown for 24 h in the dark, illuminated, and collected at different times (L) as described in "Materials and Methods." Controls were kept in continous darkness (D). Northern blot were hybridized to the *ccg-2(eas)*-specific probe, stripped, and rehybridized to the IF2 cDNA probe (Baima et al., 1991) as a control for normalization. Total RNA (5 μg) was loaded per lane.

gene, *albino 3*, in *bd* and *bd,frq*^{9}. It is clearly evident from Figure 5 that the kinetics of light inducibility were not altered in the mutants.

DISCUSSION

We have shown that *ccg-2(eas)*, a gene previously demonstrated to undergo a circadian oscillation of expression in constant darkness (Loros et al., 1989), is also regulated to a significant degree by changes in the ambient light intensity (Fig. 1). The rate of increase in the transcript levels induced by light treatment is not as rapid as that seen with "rapid light-inducible" genes such as *al-1*, *al-3*, *bli-3*, and *bli-4* (Sommer et al., 1989; Schmidhauser et al., 1990; Baima et al., 1991). However, the response of *ccg-2(eas)* gene expression to light is rapid enough to reflect a direct and important physiological response to light (Fig. 1). As expected, light induction of *ccg-2(eas)* requires the products of the *wc-1* and *wc-2* genes (Fig. 2), which are generally known to be critical mediators of blue-light responses in *Neurospora*. However, neither *ccg-2(eas)* expression nor light induction of the gene requires the presence of a clock (Figs. 3 and 4 and discussion below). Thus, the clock might be just one of several independent, but not obligatory, direct effectors of *ccg-2(eas)* expression, or it might act to modulate the effects of other factors (e.g. light, nutrition) that drive expression of *ccg-2(eas)*.

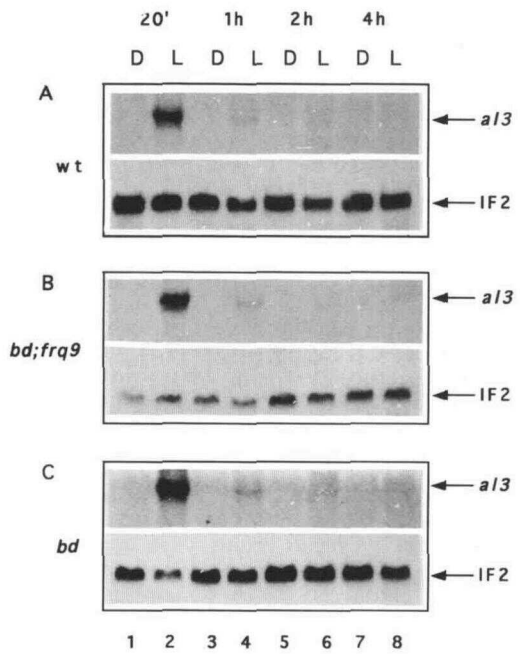

Figure 5. Light inducibility of the albino 3 gene retains normal kinetics in the *bd* and *bd,frq*^{9} mutant backgrounds. Comparison of northern blot analysis of total RNA extracted from wt (A), *bd,frq*^{9} (B), and *bd* (C) mycelia grown in the dark for 24 h, illuminated, and collected at different times as described in "Materials and Methods." For each time point, dark control cultures (D) are shown. Filter-bound RNA samples were hybridized either to the *al-3*-specific probe or to the IF2 cDNA (Baima et al., 1991) as a control for normalization. Total RNA (5 μg) was loaded per lane.

To understand the interaction of light and the clock in the regulation of *ccg-2(eas)*, we utilized several mutant strains. Although the "blind" *wc* mutants have been well characterized previously (Degli Innocenti and Russo, 1984a), the clock mutant (*frq*) requires additional explanation. The strain used, *bd,frq*^{9}, is a recessive null mutant of the *frq* locus, a gene complex believed to encode one of the core molecular components of the circadian clock (reviewed by Dunlap, 1990, 1993). The *frq* locus encodes two transcripts, "large" *frq* (approximately 5000 nucleotides in length) and "small" *frq* (approximately 1500 nucleotides in length). Both transcripts appear to be required for rhythmicity (McClung et al., 1989). The *bd,frq*^{9} mutant carries a frameshift mutation in the large *frq* region. The clock in *bd,frq*^{9} lacks compensation mechanisms for changes in temperature or nutrition that characterize the wild-type clock. Instead of being rhythmic, conidiation is sparse and sporadic in a *bd,frq*^{9} mutant, and a rudimentary rhythm can be driven only by long duration and full photoperiod light/dark cycles (Loros and Feldman, 1986; Loros et al., 1986). The band (*bd*) mutation, present in the *bd,frq*^{9} strain used in this work, was originally identified as a spontaneous mutation in an *inv* (invertaseless) background that reduced the growth rate and clarified the expression of the underlying circadian rhythm in conidiation (Sargent et al., 1966). During the course of mycelial growth within the closed plates or race tubes used for assaying the circadian clock in *Neurospora*, higher than normal concentrations of CO_2 would build up, and these acted to obscure the overt expression of the clock by inhibiting conidiation. It was later shown that *bd* acted to overcome this CO_2-induced masking. Until now, however, *bd* has been assumed to have little or no major effect on gene expression. We have shown here that *bd* acts to enhance overall levels of dark expression of *ccg-2(eas)*. This is the first molecular alteration known to be associated with the *bd* mutation. Because we have shown that *ccg-2(eas)* is expressed and light induced in the clock-null strain, we can deduce that light directly affects *ccg-2(eas)* gene expression and does not act indirectly via the clock.

The data presented in this communication are illustrative of an emerging research focus centering on the interaction of light and the circadian clock in the regulation of expression of some genes. At the developmental level in fungi, a number of processes are known to be dependent upon or influenced by blue light (reviewed by Degli Innocenti and Russo, 1984b). This represents a focus of work in *Neurospora* in which aspects of conidial development (Siegel et al., 1968), carotenoid biosynthesis (Nelson et al., 1989), sexual reproduction (Degli Innocenti and Russo, 1984a), and circadian rhythms (Sargent and Briggs, 1967) all show blue-light effects. Here again, there is evidence for an interaction between light and the circadian clock. For instance, in the context of sexual reproduction, light is known to enhance the production of protoperithecia induced by nutritional conditions (Degli Innocenti and Russo, 1984a). Perithecial beaks display a positive phototropism, causing spores to be shot toward the light (Harding and Melles, 1983). In constant darkness, ascospore release from the mature perithecium follows a clearly defined circadian rhythm in both *N. crassa* (S. Brody, unpublished data; Lakin-Thomas et al., 1990) and *Neurospora tetrasperma* (R. Brambl, unpublished data). This interplay between light

regulation and clock regulation may provide some insight into the adaptation of the fungus to its ecological niche.

We have shown previously (Bell-Pedersen et al., 1992) that *ccg-2* encodes a hydrophobin required for the formation of the rodlet outer layer of *Neurospora* conidia and is allelic to the *eas* gene. Additionally, through its identification as a hydrophobin, it was determined that *ccg-2* was also independently reisolated later in a screen for light-inducible genes as the gene *bli-7* (Lauter et al., 1992). Thus, in its role as a fungal spore hydrophobin, *ccg-2(eas)* may be thought of as a conidiation gene. The dual regulation of *ccg-2(eas)* by light and the clock may be understood in terms of the adaptation of the fungus to growth in the wild. Because conidia must be transported from place to place by winds that occur most dependably at the times of maximum temperature differential between the land and the air, dispersal might be expected to happen following sunrise and sunset. Thus, if conidial development, including the expression of conidiation genes such as *ccg-2(eas)*, is triggered initially by the clock in anticipation of dawn and then further enhanced by light at dawn, conidia can be developmentally ready for dispersal by winds later in the day. Hence, the dual regulation of genes by light and the clock may reflect a practical evolutionary adaptation that promotes survival and dispersal of the fungus in its natural habitat.

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