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Distinct *cis*-Acting Elements Mediate Clock, Light, and Developmental Regulation of the *Neurospora crassa eas* (*ccg-2*) Gene

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The *Neurospora crassa eas* (*ccg-2*) gene, which encodes a fungal hydrophobin, is transcriptionally regulated by the circadian clock. In addition, *eas* (*ccg-2*) is positively regulated by light and transcripts accumulate during asexual development. To sort out the basis of this complex regulation, deletion analyses of the *eas* (*ccg-2*) promoter were carried out to localize the *cis*-acting elements mediating clock, light, and developmental control. The primary sequence determinants of a positive activating clock element (ACE) were found to reside in a 45-bp region, just upstream from the TATA box. Using a novel unregulated promoter/reporter system developed for this study, we show that a 68-bp sequence encompassing the ACE is sufficient to confer clock regulation on the *eas* (*ccg-2*) gene. Electrophoretic mobility shift assays using the ACE reveal factors present in *N. crassa* protein extracts that recognize and bind specifically to DNA containing this element. Separate regions of the *eas* (*ccg-2*) promoter involved in light induction and developmental control are identified and shown not to be required for clock-regulated expression of *eas* (*ccg-2*). The distinct nature of the ACE validates its use as a tool for the identification of upstream regulatory factors involved in clock control of gene expression.

Circadian biological clocks have been identified in photosynthetic prokaryotes and in eukaryotes ranging in complexity from unicellular organisms to mammals. They function to anticipate the daily needs of the organism and supply timing information for various biochemical, cellular, and physiological processes. These clock-driven output rhythms result from regulation of specific target genes and/or gene products (reviewed in reference 23), presumably through signal transduction pathways originating from the circadian oscillator. As a first approach in understanding clock output pathways, the focus has been on identifying the genetic elements involved in clock regulation of the target genes; however, these elements have proven difficult to localize.

One of the most extensively studied circadian clocks resides in the filamentous fungus *Neurospora crassa*. In *N. crassa*, an oscillator acts to control different rhythmic events, including the time of asexual spore (conidium) development, providing a convenient means to observe and measure daily rhythmic output from the clock (for reviews, see references 18 and 33). In addition, the availability of mutant alleles in the frequency (*frq*) locus (for a review, see reference 19), a pivotal component of the *N. crassa* clock mechanism (5), and the isolation of clock-regulated output genes (9, 34) together provide a unique system with which to address questions central to our understanding of the biological clock.

In the molecular analysis of circadian systems, three sets of questions are generally considered salient. (i) What are the components of the oscillator, and how are they assembled? (ii) How are environmental time cues perceived and used to reset the clock? (iii) How is time information, generated by the oscillator, transduced within the cell and used to control metabolism and behavior? One strategy for studying output signaling involves tracing an output pathway from a clock-controlled gene (*ccg*) (34, 36) backwards to the central oscillator. This can be accomplished by first identifying *cis*-acting clock regulatory sequences and subsequently using these sequences as probes to isolate *trans*-acting factors either genetically or biochemically. However, this approach has been limited to very few systems, including plants (26) and *N. crassa* (34, 36), in which bona fide output *ccg* genes have been identified and cloned. A recurring theme in the analysis of these *ccg* genes has been that they are photoinduced (26, 33), and so far it has not been possible to dissociate the *cis*-acting element(s) conferring clock and light regulation.

One of the most highly characterized *ccg* genes is the *N. crassa eas* (*ccg-2*) gene (10, 28, 34). The *eas* (*ccg-2*) locus was originally identified through mutation, resulting in a morphologically abnormal strain with easily-wettable conidiospores (8, 45). The *eas* gene was independently isolated on the basis of rhythmic abundance of the transcript as *ccg-2* (34) and as a blue-light-inducible gene, *bli-7* (46). The abundantly expressed *eas* (*ccg-2*) gene meets all of the criteria for being a true clock-controlled output gene. First, the circadian rhythm in RNA abundance persists under constant conditions with a period that reflects the genotype of the strain (22 h in wild-type clock strains and 29 h in strains with the long-period clock mutant allele frq^7) (34). Second, the dramatic reduction in expression of *eas* (*ccg-2*) in the original *eas* mutant strain UCLA191 (45) was found to have no effect on the normal operation of the circadian clock, confirming that *eas* (*ccg-2*) is a true output gene that does not feed back on the oscillator (10). Nuclear run-on experiments performed on *eas* (*ccg-2*) indicate that rhythmic expression results from clock control of transcription (35). This finding suggests that clock regulation of *eas* (*ccg-2*) is mediated, at least in part, by *cis*-acting regulatory elements. These genetic elements provide the requisite tools for isolating upstream factors involved in clock control of *eas* (*ccg-2*).

We and others have previously shown that *eas* (*ccg-2*) encodes a fungal hydrophobin that is the major constituent of the

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hydrophobic, proteinaceous, rodlet coating on the surface of maturing asexual conidiospores (10, 28). Thus, the EAS protein is likely of importance to the success of the organism in the wild (10). Reflecting this importance, the gene is subject to complex regulation. Not only is *eas* (*ccg-2*) controlled by the circadian clock in a time-of-day-specific manner, but as noted above, it is positively regulated by blue light (6, 28, 46) and transcripts accumulate during asexual development (28).

To begin to describe the components of the *N. crassa* clock output pathways, deletion analyses of the *eas* (*ccg-2*) promoter were carried out to localize *cis*-acting sequence elements mediating clock control. We report here a distinct activating clock element (ACE) residing within a 45-bp region, just upstream of the putative TATA box. Using a novel unregulated promoter/*lacZ* reporter system, we demonstrate that the ACE not only is necessary but is sufficient to confer clock-regulated expression. Factors present in *N. crassa* extracts that specifically recognize and bind to ACE-containing DNA fragments are identified by using electrophoretic mobility shift analysis (EMSA). Finally, we have localized diverse *cis*-acting elements mediating both photoinduction and developmental regulation of *eas* (*ccg-2*) and have established that the ACE is spacially distinct from other elements governing *eas* (*ccg-2*) expression.

MATERIALS AND METHODS

***N. crassa* strains, growth conditions, and transformation.** Strains of *N. crassa* used in this study include 30-7 (*bd*; *A*), in which the band mutation enhances the circadian rhythm of conidiation (43); 87-12 (*his3*; *bd*; *A*) (5); and 95-3 (*his-3*; *bd*; *frq*^{7}; *A*) (this study). The two *eas* (*ccg-2*)-deficient strains used to monitor expression of the deletions were 104-4 (*his-3*; *eas* [UCLA191]; *A*) (this study) and TDP200-2 (*his-3*; *eas*^{RIP}; *bd*) (10). TDP200-2 was shown by Northern (RNA) analysis not to express *eas* (*ccg-2*) mRNA (data not shown). Growth media (Vogel's and Fries minimal media), vegetative growth conditions, and crossing protocols were as described previously (17). To examine the different *ccg-2* (*eas*) constructs in a constant chromosomal context, each plasmid (2 μg) was targeted to the *his-3* locus by homologous recombination (20, 40), using a standard transformation protocol (48). Transformants were grown on minimal Vogel's medium to select for histidine prototrophy, and genomic DNA isolated from the transformants was analyzed by Southern blotting to verify proper integration of a single plasmid into the *his-3* locus and to select homokaryons. Because of the low levels of *eas* (*ccg-2*) transcripts synthesized from plasmids lacking the upstream activator, all pKX plasmids were examined in strain TDP200-2 to rule out any background expression from the endogenous locus in the original *eas* mutant UCLA191. Rescue of the *eas* mutant phenotype of dark, wetted, nonairborne conidia was monitored by inspection of conidia on agar slants, where full rescue produces light orange conidia that are readily dispersed in air (10).

Plasmids. Parental plasmids for *his-3* targeting include pDE3 of ca. 9.5 kb (20, 40) and derivatives thereof. An *Eco*RI deletion (3,018 bp) of pDE3 yielded vector pDE3E, and a *Bam*HI-*Hin*dIII deletion (3,811 bp) yielded vector pDE3BH (24). The *eas* (*ccg-2*) gene used to generate the deletions described in this study (Fig. 1) originated from a genomic clone of ca. 13 kb (7C1) (34). Plasmid pDP3600 harbors a 5-kb *eas* (*ccg-2*) *Xba*I fragment isolated from 7C1 (shown in Fig. 1A) inserted into the *Xba*I site of pDE3E. Plasmid pDP1900 contains a 3.2-kb *Eco*RI-*Xba*I *eas* (*ccg-2*) fragment derived from pDP3600 inserted into the *Eco*RI-*Xba*I interval of pDE3E. Plasmid pDP625 contains a 1.9-kb *eas* (*ccg-2*) fragment isolated from a *Pvu*II digest of pLW1K (10) inserted into the *Stu*I site of pDE3BH. The pEX and pKX series of deletions were constructed by using the indicated restriction enzymes (Fig. 1B) in parental plasmids pDP1900 and pDP625, respectively. The deletion endpoints were verified by DNA sequencing (data not shown). Plasmid pDP103 contains a ca. 2-kb *Eco*RV-*Nae*I *eas* (*ccg-2*) fragment isolated from pDP1900 inserted into the *Sma*I site of vector pDE3 to create a translational fusion to the *lacZ* gene at amino acid 52 of EAS. Plasmid pSYN1 was constructed by replacing an *Eco*RI fragment containing the *lacZ* gene from pDE3 into the *Eco*RI site of pDE3BH. The start sites of transcription for the synthetic promoter located upstream of the *lacZ* gene in pSYN1 were mapped by primer extension (data not shown) and found to occur 8 and 11 bp upstream of the *Eco*RI site in vector pDE3. Insertion of the *eas* (*ccg-2*) ACE element from bp −118 to −50 (generated by standard PCR protocols) into the *Bgl*II site of pSYN1 yielded plasmid pDP110. Altogether, we have examined at least 10 *N. crassa* promoters (including the *sod-1*, *cpc-1*, *rps-1*, *cox-5*, *atp-1*, β-tubulin, and *ccg-1* [*grg-1*] promoters) and 1 *Aspergillus nidulans* promoter (*trpC*), without success, for constant expression in our rhythmic culture regime (9, 24). All plasmids were engineered by using standard techniques (41) and were propagated in *Escherichia coli* XL1Blue or RR1. Restriction and DNA-modifying enzymes were obtained from New England Biolabs and used according to the manufacturer's instructions.

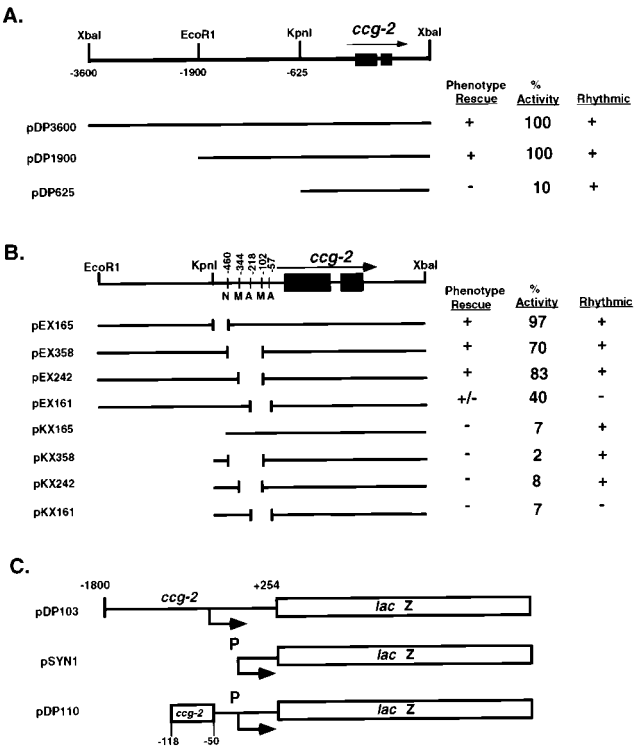

FIG. 1. Promoter deletion constructs of the *eas* (*ccg-2*) gene. (A) The 5-kb *Xba*I DNA fragment containing the entire *eas* (*ccg-2*) locus is shown on the top (10). The coding region of the *eas* (*ccg-2*) gene is indicated by black boxes, and the transcript is depicted as an arrow. Schematic diagrams of 5′-end deletions of *eas* (*ccg-2*) are shown below. Plasmids harboring the deletion variants were transformed into *eas* (*ccg-2*)-inactivated strains and assayed for rescue of the *eas* mutant conidial phenotype, where + refers to full rescue (light orange air-dispersed conidia), − refers to incomplete rescue (dark, wetted, non-air-dispersed conidia), and +/− indicates partial rescue (conidia are light orange but are not air dispersible). The levels of expression of the various *eas* (*ccg-2*) deletion constructs are summarized (percent activity) and are based on densitometric analysis with identical probes and exposures of equal duration; 100% reflects *eas* (*ccg-2*) message levels observed from the endogenous locus. The ability of the deletion plasmid to direct rhythmic *eas* (*ccg-2*) transcription is shown (+ indicates rhythmic *eas* [*ccg-2*] mRNA; − indicates no obvious circadian rhythm). The results are summarized to the right of each construct. (B) The schematic of the *eas* (*ccg-2*) locus, from *Eco*RI to *Xba*I (3.2 kb), at the top represents the starting DNA for the pEX series of internal promoter deletions (shown below). The pKX series of deletions were made from a plasmid containing a 1.9-kb *Kpn*I-*Xba*I fragment of the *eas* (*ccg-2*) locus. For each deletion of *eas* (*ccg-2*) indicated, the line represents DNA that is present in the construct and the empty space designates the deleted region. Phenotypic rescue, transcriptional activity (percent activity), and rhythmicity are shown as described for panel A. N, *Nde*I; M, *Msc*I; A, *Ava*II. (C) *lacZ* fusion constructs used in this study. Transcripts containing the *E. coli lacZ* gene arise either from the start site of transcription contained in the *N. crassa eas* (*ccg-2*) promoter in pDP103 or from the *E. coli*-derived synthetic promoter (P) in pSyn1 and pDP110.

Culture harvesting conditions. For rhythmic RNA analysis, the clock was synchronized in mycelial mats, grown in shaking (100 rpm) liquid culture (1× Vogel's minimal medium containing 0.03% glucose and 0.05% arginine) at 25°C, by a light-to-dark transition (34, 35). This light-to-dark transfer sets the oscillator to CT12 (dusk). (Circadian time [CT] is used to normalize biological time in strains or organisms with different endogenous period lengths to 24 circadian h per cycle. By convention, CT0 represents subjective dawn and CT12 represents subjective dusk.) Light-to-dark transfer times were such that the ages of the cultures at harvest were approximately the same, but the circadian times varied (10). All liquid cultures were maintained in constant light for at least 2 h prior to transfer to the dark. Tissue for RNA extraction was harvested after the indicated times in the dark for each experiment. Induction of conidiation was performed as previously described (11), and RNA was isolated from tissue harvested immediately after filtration of mycelia (time zero) and at 2, 4, and 8 h after desiccation. Light induction was carried out by a previously reported method (6).

Briefly, mycelia were grown in shaking (100 rpm) liquid cultures (1× Fries minimal medium with 0.03% glucose and 0.05% arginine) in the dark for 18 h. The cultures were either held in the dark for an additional 30 min or transferred to fluorescent light (3,000 lux; 35 $\mu mols^{-1}\ m^{-2}$) and harvested after 30, 60, and 120 min.

Nucleic acid isolation, radioactive probes, and hybridization. RNA was isolated from tissue disrupted in 0.1 M sodium acetate (pH 5.3)–0.001 M EDTA–4% sodium dodecyl sulfate (51). Total RNA (10 μg unless otherwise indicated) was separated on a 1% agarose-formaldehyde gel (31), transferred to nitrocellulose membranes (Nitropure; Micron Separations, Inc.), and probed with antisense RNA probes (riboprobes). Riboprobes were synthesized by using the appropriate polymerase (Promega) in the presence of [α-^{32}P]UTP (6,000 Ci/mmol; New England Nuclear) as described by Promega. For *eas* (*ccg-2*) riboprobes, plasmid pLW1K was used as the template with T3 RNA polymerase (Promega). For *ccg-1* riboprobes, plasmid pKL119 (32) was used as the template with T3 RNA polymerase. For *lacZ* riboprobes, an *Eco*RI fragment of the *lacZ* gene from pDE3 was cloned into the *Eco*RI site of plasmid pSP70 (Promega), and SP6 polymerase was used. DNA probes specific for rRNA were prepared by the random-primer method (21). Hybridization and washing were performed by using standard techniques (41) for DNA probes or as recommended by Promega (37a) for RNA probes. RNA loading was normalized to rRNA for each sample, which remains at constant levels under the conditions used (34). For all experiments, multiple exposures of autoradiograms were generated for purposes of densitometry. This allowed quantitation and visualization of band intensities when the band density fell within the linear range. For comparisons between gels, identical exposure times with a probe of the same specific activity were used. Densitometry was performed with an Apple Color One scanner, and the image data were analyzed with the Macintosh Image program, version 1.52. Each experiment was performed at least twice with identical results.

EMSA. *N. crassa* nuclear proteins were isolated from 30-7 cells grown in the light for 18 h as previously described (49). Briefly, nuclei from disrupted cells were isolated by differential centrifugation and lysed in buffer containing 10 mM KCl, 15 mM *N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES; pH 7), 5 mM $MgCl_2$, and 1 mM dithiothreitol. Following precipitation in 80% ammonium sulfate, the samples were dialyzed three times in nuclear extraction buffer containing 40 mM KCl, 25 mM HEPES (pH 7), 0.1 mM EDTA, 0.1 mM dithiothreitol, and 5% glycerol. The samples were divided into small aliquots and frozen in liquid nitrogen. For EMSA, 2 ng of end-labeled PCR fragments spanning the *eas* (*ccg-2*) gene from −50 to −118 (10) was incubated at 25°C for 15 min with or without protein extract (20 μg) in the presence of 5 μg of poly(dI-dC) (Pharmacia). Nonradioactive competitors include the −50 to −118 *eas* (*ccg-2*) PCR fragment and a nonspecific *eas* (*ccg-2*) PCR fragment spanning bp +75 to +135 (for the *eas* [*ccg-2*] DNA sequence, see reference 10). Following incubation, the products were resolved on a 5% acrylamide (19:1 ratio of acrylamide to bisacrylamide) gel in 0.5× Tris-borate-EDTA buffer. Electrophoresis was carried out at 150 V for 4 h at 4°C, and the dried gel was exposed for autoradiography.

RESULTS

Localization of *eas* (*ccg-2*) clock-responsive elements by deletion analyses. (i) 5′ deletions define a region of clock regulation. To determine the location of the genetic element(s) within the *eas* (*ccg-2*) promoter responsible for circadian clock regulation, a series of plasmids containing 5′-end deletions of the *eas* (*ccg-2*) gene were constructed (Fig. 1A and B), transformed into *eas* (*ccg-2*)-deficient strains, and analyzed for clock-regulated expression.

A 5.5-kb *Xba*I DNA fragment of the *eas* (*ccg-2*) gene (pDP3600) containing ca. 3,600 bp of upstream promoter sequence and 355 bp of 3′ untranslated sequences (Fig. 1A) retains the required circadian clock control genetic elements, as evidenced by the rhythm in *eas* (*ccg-2*) transcripts (Fig. 2A). A peak in RNA accumulation is observed after 12 h in constant darkness (DD12), representing CT1, and between 36 and 40 h (DD36 and DD40), representing CT4 to CT9, in the first (DD4 to DD24) and second (DD28 to DD44) cycles, respectively. The amplitude of the rhythm is approximately 10-fold, and expression levels are identical (100% transcriptional activity; summarized in Fig. 1A) to those observed for the endogenous *eas* (*ccg-2*) gene (10, 34). The percent transcriptional activity data are not shown for the deletion constructs but are summarized in Fig. 1A and B. Exposure times of the autoradiograms shown in Fig. 2 and in subsequent figures are varied for purposes of densitometry and visualization. Therefore, while these

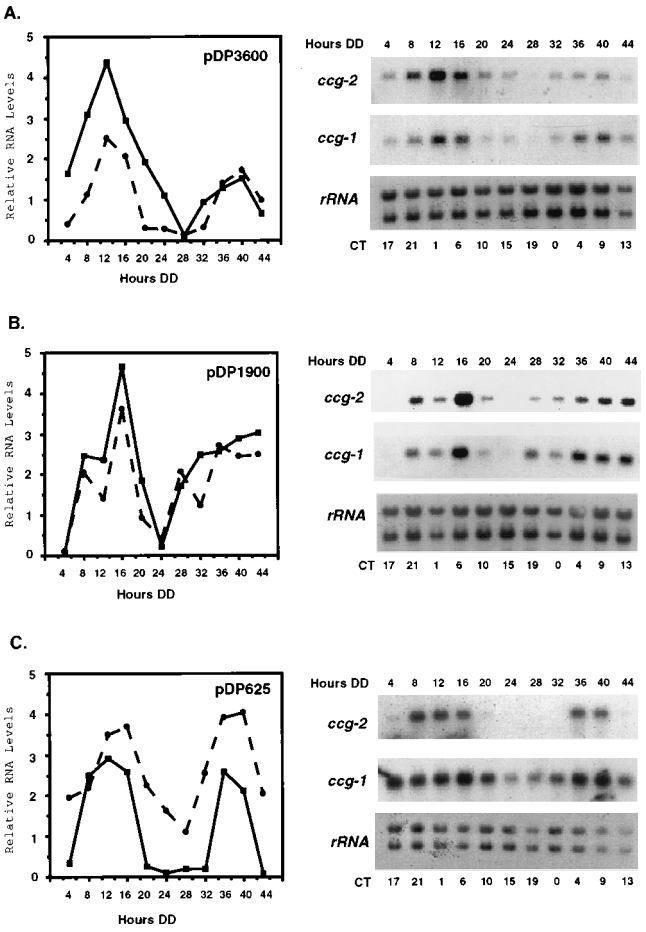

FIG. 2. Resection of the *eas* (*ccg-2*) promoter separates distal elements governing amplitude from proximal elements governing circadian regulation. Levels of rhythmic expression of *eas* (*ccg-2*) mRNAs from plasmids pDP3600 (A), pDP1900 (B), and pDP625 (C) were assayed by Northern analysis from mycelia grown in liquid culture in constant darkness and harvested after the indicated times in the dark (Hours DD) representing the approximate circadian times (CT) shown below the autoradiograms. In wild-type clock strains, this represents two full circadian cycles. Total RNA was hybridized sequentially with probes specific for *eas* (*ccg-2*), *ccg-1*, and rRNA. The exposure times of the autoradiograms were varied, and therefore the optical densities of the images shown do not directly reflect transcriptional activity. Following autoradiography, mRNA was quantitated by densitometry, normalized to rRNA, and plotted as relative band intensity (mRNA/rRNA) versus times in the dark (Hours DD) for both *eas* (*ccg-2*) (solid line) and *ccg-1* (dotted line).

data reflect the actual transcriptional activities of the deletion constructs, the observed optical density of one Northern blot cannot be readily compared with the optical densities of others on an absolute scale. The period and time of peak expression of the rhythm for *eas* (*ccg-2*) fluctuate to some extent between experiments, as is typical for an output rhythm in *N. crassa* (9, 32, 34). However, we controlled for experimental differences by comparing rhythmic expression of the similarly regulated *N. crassa* morning-specific clock-controlled *ccg-1* gene (34, 35).

A similar rhythm in *eas* (*ccg-2*) expression is observed for strains harboring plasmid pDP1900 (with 1,900 bp of upstream promoter sequences) and plasmid pDP625 (with 625 bp of upstream promoter sequences), each with a typical amplitude of ca. 10-fold (Fig. 2B and C, respectively). However, in pDP625, the overall levels of *eas* (*ccg-2*) transcript are markedly lower than those in pDP3600 and pDP1900 (Fig. 1A). This 10-fold reduction in message accumulation is reflected in the

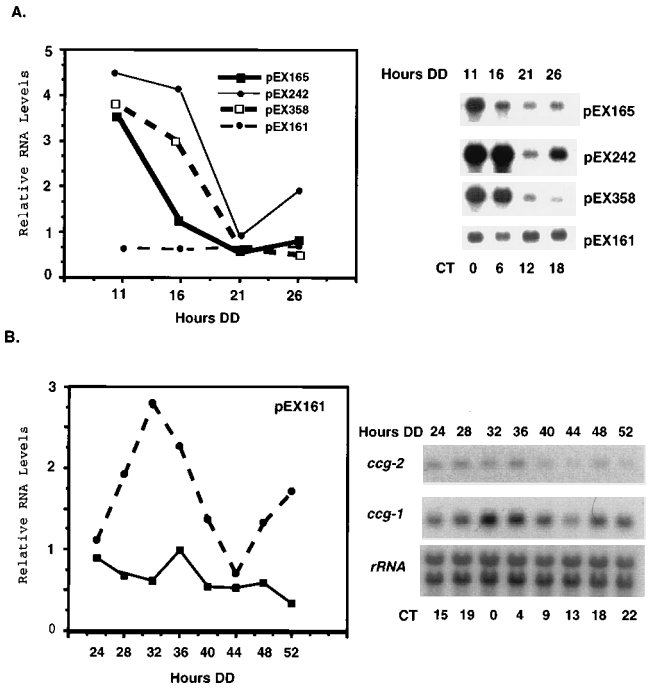

FIG. 3. Sequence elements required for circadian regulation lie between 57 and 102 bp from the start of *eas* (*ccg-2*) transcription. (A) Northern analysis of each pEX deletion construct is shown on the right with RNA isolated after the indicated times in the dark (Hours DD), representing the approximate circadian times (CT) shown at the bottom. Total RNA was hybridized to an *eas* (*ccg-2*)-specific RNA probe. The amount of mRNA for each time point was quantified by densitometry, normalized to rRNA, and plotted as in Fig. 2. (B) Northern analysis of RNA isolated from pEX161 after the indicated times in the dark (Hours DD), representing the approximate circadian times (CT) shown at the bottom. RNA was sequentially hybridized to *eas* (*ccg-2*), *ccg-1*, and rRNA probes. Densitometric analysis is shown on the left for *eas* (*ccg-2*) (solid line) and *ccg-1* (dashed line). For both panels, exposure times of the autoradiograms were varied.

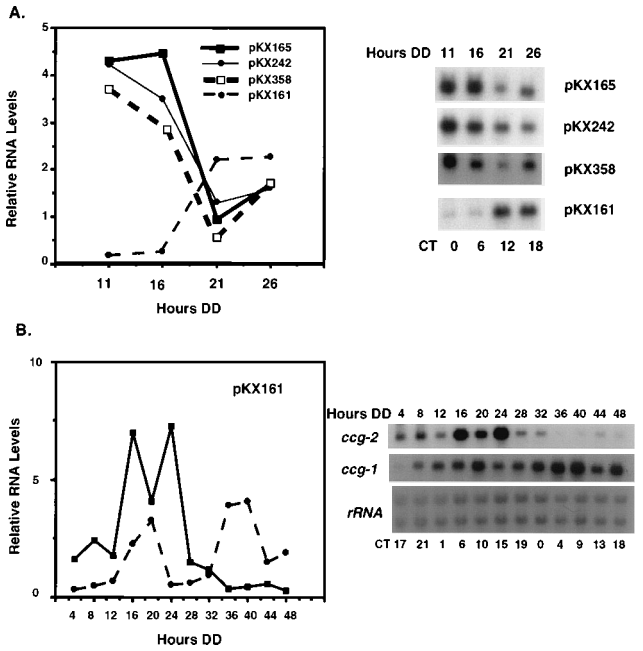

FIG. 4. Circadian clock regulatory sequences do not require upstream amplitude elements in the *eas* (*ccg-2*) promoter. (A) RNA was isolated from the pKX deletion constructs at the indicated times (Hours DD), representing the approximate circadian times (CT) depicted at the bottom, and hybridized to an *eas* (*ccg-2*)-specific RNA probe (right). Densitometric plots on the left are as described in the legend to Fig. 2. (B) For Northern analysis of pKX161 (right), RNA was hybridized sequentially to *eas* (*ccg-2*), *ccg-1*, and rRNA probes. RNA was isolated at the times indicated on the top (Hours DD), representing the approximate circadian times (CT) shown at the bottom. Densitometric analysis is shown on the left; the solid line represents *eas* (*ccg-2*), and the dashed line represents *ccg-1*. For both panels, exposure times of the autoradiograms were varied.

inability of pDP625 to completely rescue the mutant *eas* (*ccg-2*) phenotype of darkened, wetted conidia that are not readily dispersed in air (Fig. 1A) (10, 45). Loss of high-level expression, resulting from the removal of promoter sequences from −625 to −1900, is consistent with the previous identification of a positive *cis*-acting element, located between bp −1000 and −1500, that is disrupted by the insertional event that results in *eas* allele UCLA191 (10, 25, 28). This element, however, does not play a role in clock-regulated rhythmic expression of the *eas* (*ccg-2*) gene, as evidenced by normal rhythmic expression of *eas* (*ccg-2*) from pDP625. Consistent with this finding, while the levels of *eas* (*ccg-2*) arising from UCLA191 are extremely low, the mRNA is still rhythmic (9).

(ii) A clock-responsive element close to the start site of transcription is necessary for rhythmic expression. To further localize the specific clock element(s), additional deletions were engineered in the *eas* (*ccg-2*) promoter region spanning bp −625 to +1. Two series of deletion plasmids (Fig. 1B), one containing the 5′ upstream positive activating element (pEX series; Fig. 3) and the other lacking the positive activating sequence (pKX series; Fig. 4), were examined for rhythmic *eas* (*ccg-2*) expression.

Northern analyses of the pEX series of deletions are shown in Fig. 3A. All of the deletion strains display a normal *eas* (*ccg-2*) rhythm exception for pEX161, which lacks DNA between −57 and −218. In strains bearing this construct, essentially equal amounts of *eas* (*ccg-2*) mRNA are observed from RNA extracted at four time points over 1 circadian day (Fig. 3A), and *eas* (*ccg-2*) message is clearly not rhythmic in samples taken every 4 h over a 28-h period (Fig. 3B). The absence of a normal circadian rhythm for pEX161 is particularly evident in comparison with the same RNA probed with the circadianly regulated *ccg-1* gene. Furthermore, the low levels of *eas* (*ccg-2*) mRNA arising from pEX161 mirror the lower levels in a typical cycle (Fig. 3A), indicating positive regulation of *eas* (*ccg-2*) by the clock. The reduced amount of *eas* (*ccg-2*) message synthesized from pEX161 (40% of the level for pDP3600) is also reflected in the inability of this construct to fully rescue the *eas* (*ccg-2*) mutant phenotype compared with the other pEX deletions (Fig. 1B).

Northern analyses of the pKX series of deletions, lacking the upstream activator, confirm the findings for the pEX deletion constructs (Fig. 4A). In RNA isolated from tissue harvested every 4 h over a 48-h time course, *eas* (*ccg-2*) message from pKX358, pKX242, and pKX165 cycled once approximately every 22 h (data not shown to conserve space). Surprisingly, *eas* (*ccg-2*) mRNA transcribed from pKX161 is not constant (as in pEX161; Fig. 3). Rather, a 48-h time course reveals that the levels of *eas* (*ccg-2*) mRNA fluctuate greatly, albeit not with normal circadian characteristics, compared with the same RNA probed with *ccg-1* (Fig. 4B). The removal of the upstream sequences between −625 and −1900, along with the 161-bp deletion, appears to have uncovered regulation (or deregulation) from other input signals (as confirmed below).

The loss of a normal circadian rhythm in *eas* (*ccg-2*) expression for both the pEX161 and pKX161 deletions suggests the

presence of an ACE within the sequence spanning bp −57 to −218. Because *eas* (*ccg-2*) mRNA expressed from the upstream overlapping deletion in both pEX242 and pKX242 (−102 to −344) is rhythmic, the primary sequence determinants for the ACE must be located within bp −57 to −102.

The *eas* (*ccg-2*) promoter sequences containing the ACE are sufficient to confer rhythmic gene expression. To determine if the sequences between −57 to −102 of the *eas* (*ccg-2*) promoter containing the putative ACE are sufficient to confer rhythmic expression on a constitutively expressed heterologous gene, a nonregulated promoter/reporter system was needed. An extensive screen of available *Neurospora* promoters showed that while individual promoters can lack nutritional, developmental, or growth regulation, no promoters currently available were found to be genuinely unregulated at a basal level over the 2- to 4-day growth course of a typical circadian rhythm experiment (9, 24). For this reason, a nonregulated promoter/reporter system was developed.

First, the sequence between −1800 and +254 of *eas* (*ccg-2*) was fused in frame to the *E. coli lacZ* coding region in vector pDE3 (20, 40), and the construct (pDP103; Fig. 1C) was transformed into a wild-type clock *frq*$^+$ strain (87-12) containing a normal *eas* (*ccg-2*)$^+$ gene. The fusion construct is shown to express rhythmic *lacZ* mRNA in phase with the endogenous *eas* (*ccg-2*) message, with peak expression occurring at approximately CT0 and an amplitude of ca. 10-fold (Fig. 5A). These data reveal that the *lacZ* gene provides a suitable RNA reporter for rhythmic Northern assays. Attempts to assay cyclic *lacZ* enzyme levels were hindered by the presence of endogenous β-galactosidase activity in *N. crassa* (40) and by its stability in cell extracts (9). In the course of analyzing *lacZ* transcripts, it became clear that there was low-level constant expression being driven by *E. coli*-derived sequences in the vector itself that was not subject to either growth rate or developmental regulation. This synthetic *Neurospora* promoter and the transcripts derived from it were mapped by primer extension analysis (see Materials and Methods). When the *lacZ* gene is placed under control of this nonregulated basal promoter present in the pDE3 vector (pSYN1; Fig. 1C), levels of *lacZ* are extremely low but do not fluctuate (Fig. 5B). Insertion of *eas* (*ccg-2*) DNA sequences containing ACE (−50 to −118) upstream of the transcriptional start site for the constitutive promoter (pDP110; Fig. 1C) results in rhythmic *lacZ* transcript accumulation, with a fivefold amplitude (Fig. 5C). These data demonstrate that ACE is sufficient to confer clock regulation on an unregulated minimal promoter.

The period of the *lacZ* RNA rhythm arising from both pDP103 and pDP110 reflects the genotype of the wild-type clock strain used for transformation. To verify period length control on the ACE, transformants were also analyzed in a 29-h *frq*^{7} mutant allele (strain 95-3). The results demonstrate the appropriate long period regulation for the ACE (Fig. 5D to F). This is best observed by comparing the levels of RNA from these constructs at DD36; in a *frq*$^+$ strain, DD36 corresponds to CT4 (subjective morning) and *lacZ* message levels are high (Fig. 5C), whereas in the strain with the *frq*^{7} 29-h-long-period mutant allele (Fig. 5D and F), DD36 corresponds to CT18 (subjective night) and *lacZ* transcript levels are low. As expected, the levels of *lacZ* mRNA from pSYN1 remain low and constant in the *frq*^{7} strain (Fig. 5E). Together, these results along with those of the deletion experiments shown in Fig. 3 and 4 demonstrate that the sequences between −57 and −102 are necessary for clock regulation and that a 68-bp fragment containing the ACE is sufficient to confer rhythmic transcription. These data also confirm previous results showing that clock regulation of *eas* (*ccg-2*) occurs primarily at the level of transcription (35).

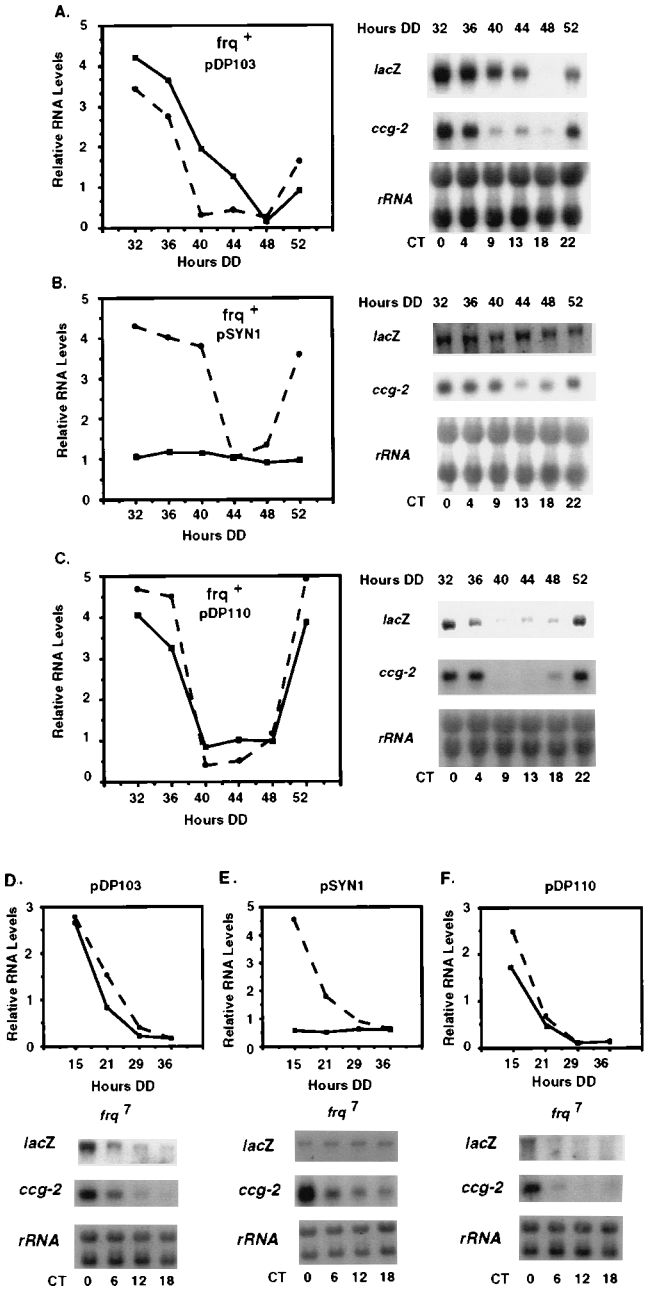

FIG. 5. A 68-bp DNA fragment containing the ACE is sufficient to confer circadian clock regulation on a basal promoter with a period length appropriate to the genetic background. (A to C) RNA was isolated from an *frq*$^+$ strain (87-12) transformed with either pDP103 (A), pSyn1 (B), or pDP110 (C) after the indicated times in the dark (Hours DD), representing the approximate circadian times (CT) shown at the bottom. Total RNA (20 μg) was sequentially hybridized to *lacZ*, *eas* (*ccg-2*), and rRNA probes. Densitometry is plotted on the left as in Fig. 2. In all panels, the solid line represents *lacZ* mRNA and the dashed line represents the *eas* (*ccg-2*) internal control mRNA. (D to F) RNAs from a long-period *frq*^{7} strain (95-3) transformed with plasmids pDP103 (D), pSyn1 (E), and pDP110 (F) isolated after the indicated times in the dark (Hours DD), representing approximate circadian times (CT) depicted at the bottom, were hybridized sequentially to *lacZ*, *eas* (*ccg-2*), and rRNA probes. The average RNA intensity for each time point is plotted as in Fig. 2.

EMSA shows a nuclear factor capable of binding to the ACE-containing promoter sequences. The finding that the

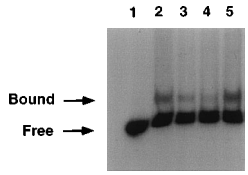

FIG. 6. Nuclear factors bind specifically to an ACE-containing DNA fragment. End-labeled DNA fragments of the *eas* (*ccg-2*) ACE-containing region from −50 to −118 were incubated in the absence (lane 1) or presence (lanes 2 to 5) of *N. crassa* nuclear protein extract with the nonspecific competitor poly(dI-dC). In lanes 3 and 4, a nonradioactive specific competitor was added at 25- and 50-fold molar excesses, respectively. In lane 5, a 50-fold molar excess of unlabeled non-ACE-containing *eas* (*ccg-2*) DNA was added to the reaction mixture. Bound, a more slowly migrating DNA-protein complex; Free, unbound DNA.

ACE confers circadian regulation on a basal constitutive promoter demands that there must exist a factor(s) capable of recognizing and binding the ACE. Hence, EMSA was used to show that a factor(s) present in *N. crassa* protein extracts recognizes and binds to the ACE-containing sequences (Fig. 6). End-labeled PCR fragments spanning bp −50 to −118 were incubated without protein (lane 1) or with nuclear protein extract (lanes 2 to 5) in the presence of the nonspecific competitor poly(dI-dC). A more slowly migrating band (bound) which persists in the presence of poly(dI-dC) is observed (lane 2). The binding of a factor(s) is specific for the ACE-containing *eas* (*ccg-2*) sequence, as evidenced by competition with a 25-fold molar excess (lane 3) and a 50-fold molar excess (lane 4) of unlabeled −50 to −118 *eas* (*ccg-2*) PCR fragment, as opposed to the lack of competition from a 50-fold molar excess of a non-ACE-containing *eas* (*ccg-2*) DNA fragment (lane 5).

Diverse genetic elements required for developmental and light regulation of *eas* (*ccg-2*) are separate and upstream from the ACE. During the natural course of asexual development, *eas* (*ccg-2*) transcript levels increase, typically rising between 4 and 8 h after induction of conidiation (28). To localize the sequences responsible for developmental control, the deletion constructs in Fig. 1 were analyzed for transcriptional regulation at 0, 2, 4, and 8 h postinduction (Fig. 7). Endogenous *eas* (*ccg-2*) levels in the control *bd* strain 30-7 are consistent with

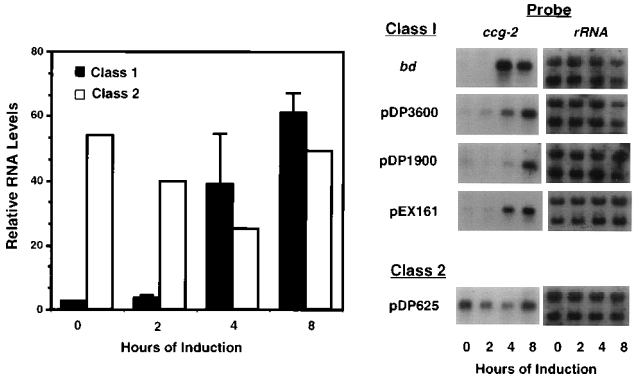

FIG. 7. Elements conferring developmental regulation lie within the promoter distal region of *eas* (*ccg-2*). RNA was isolated from *bd* cells as well as from transformants of the indicated *eas* (*ccg-2*) deletion plasmids at 0, 2, 4, and 8 h after induction of conidiation. The RNA was hybridized to an *eas* (*ccg-2*)-specific RNA probe and subsequently to an rRNA DNA probe. On the basis of phenotype, the deletions were divided into two classes. Class 1 represents deletions that show developmental expression of *eas* (*ccg-2*) similar to that of the *bd* control; class 2 represents pDP625, which displays a different developmental expression pattern. The average RNA intensity was determined for the members of each class, using autoradiograms exposed for identical times and normalized to rRNA.

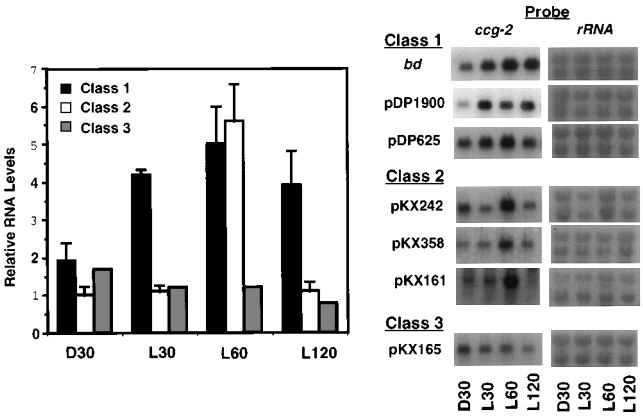

FIG. 8. Two discrete light-responsive elements lie within the proximal promoter region of *eas* (*ccg-2*), and both are distinct from the ACE. RNA was isolated from the indicated strains, either grown in the dark for 30 min (D30) or transferred to the light for 30, 60, and 120 min (L30, L60, and L120). The RNA was hybridized to an *eas* (*ccg-2*)-specific RNA probe and subsequently to an rRNA DNA probe. The strains were grouped into three classes based on similar patterns of *eas* (*ccg-2*) expression following light treatment. The average RNA intensity is graphed for the members of each class as in Fig. 7.

the previous finding that an increase in *eas* (*ccg-2*) mRNA is observed 4 to 8 h after conidial induction. Similar results are observed for both the pDP3600 and pDP1900 constructs. In addition, the developmental induction observed for ACE deletion plasmid pEX161 is identical to induction of the endogenous *eas* (*ccg-2*) message from the *bd* strain, indicating that promoter elements responsible for developmental control are distinct from the clock-regulated ACE. A change in the pattern of *eas* (*ccg-2*) mRNA levels is observed in the pDP625 construct (Fig. 7, class 2), for which a 10-fold increase in the amount of message is detected in undifferentiated mycelia (time zero) and at 2 h postinduction. These data indicate that a negative mycelial element is lost when sequences between −625 and −1900 are removed.

The *eas* (*ccg-2*) gene is also inducible by blue light (6, 25, 28). An increase in *eas* (*ccg-2*) message levels above that seen in dark-grown tissue is detectable following 30 min of illumination, and the *eas* (*ccg-2*) message peaks between 90 min and 2 h. To localize a light-responsive element(s) within the *eas* (*ccg-2*) promoter, photoinduction of the deletion constructs were tested (Fig. 8). Consistent with previous findings, in the control *bd* strain, endogenous *eas* (*ccg-2*) message levels increase and reach maximum accumulation at 60 min of continuous illumination compared with the dark control held in the dark for 30 min. The smallest of the 5′-end deletion plasmids, pDP625, displays similar kinetics (Fig. 8, class 1), indicating that important light-responsive elements are located downstream of −625. For pKX242, pKX358, and pKX161, light induction still occurs, although with kinetic properties different from those of endogenous *eas* (*ccg-2*) mRNA (Fig. 8, class 2). In this case, the induction is delayed to at least 60 min, and transcript levels return to preinduction levels by 120 min. All of these deletion constructs overlap the region between −102 and −218 (Fig. 1B), suggesting that a light-responsive element, or an element that modifies the light response, lies within these sequences. Additionally, a separate element essential for the light response is present between −460 and −625, as indicated by the complete lack of response to light of *eas* (*ccg-2*) mRNA arising from pKX165 (Fig. 8, class 3). Identical results were obtained for the pEX series of deletions (data not shown).

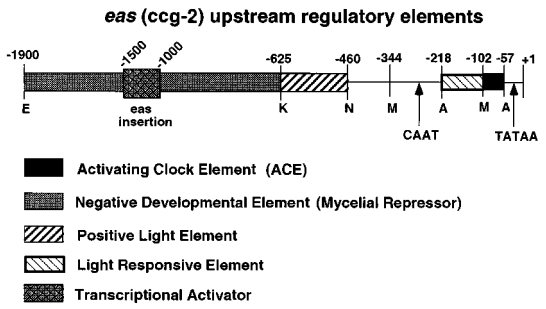

FIG. 9. Locations of *eas* (*ccg-2*) promoter regulatory elements. The putative transcriptional regulatory sequences CAAT and TATAA are also shown. E, *Eco*RI; K, *Kpn*I; N, *Nde*I; M, *Msc*I; A, *Ava*II.

DISCUSSION

Studies in a variety of different organisms have identified daily clock-controlled gene expression as a major avenue through which circadian clocks effect cellular and organismal control. To begin to understand the molecular mechanisms underlying clock-regulated gene expression, and to investigate the relationship of the endogenous clock to other external inputs, we localized the *cis*-acting genetic elements mediating clock, light, and asexual developmental control for the *N. crassa eas* (*ccg-2*) gene (Fig. 9).

In work that constituted the experimental core of this study, an ACE, localized to a 45-bp sequence extending from −57 to −102 relative to the start site of transcription, was shown to be necessary for rhythmic gene expression, and a 68-bp fragment containing this region was found to be sufficient for clock-regulated transcription (Fig. 3 to 5). Examination of *eas* (*ccg-2*) deletion strains revealed other promoter elements governing overall amplitude of transcription and both development and light responsiveness to be more diverse. Two light elements were identified between bp −102 and −218 and between bp −460 and −625 (Fig. 8), and both a negative developmental element (Fig. 7) and a general transcriptional enhancer (Fig. 2) were found to reside between bp −625 and −1900. Importantly, despite the diversity of the transcriptional control elements observed for *eas* (*ccg-2*), all of the elements are distinct from the ACE. Because of the discrete nature of the ACE, it was possible to identify binding factors within *N. crassa* nuclear protein extracts that specifically bind to ACE-containing DNA fragments (Fig. 6). These results indicate that the ACE will provide a useful and unique tool for isolating *trans*-acting regulatory factors involved in rhythmic gene expression.

During asexual development, an increase in *eas* (*ccg-2*) expression is observed 4 to 8 h after conidiation is induced (reference 28 and this study), whereas *eas* (*ccg-2*) message is barely detectable in macroconidia, microconidia, germinating conidia, mycelia (especially dark-grown mycelia), and ascospores (28). Transcriptional repression from the negative mycelial element identified in this study is likely responsible for the lack of expression of *eas* (*ccg-2*) in these tissues. Interestingly, when both the negative mycelial element and the ACE are deleted, significant, apparently unregulated fluctuations in *eas* (*ccg-2*) transcript levels are uncovered (Fig. 4), presumably as a result of loss of mycelial repression. Clock control must override or mask this effect, since deletions that remove the negative mycelial element but retain the ACE display normal rhythmicity. A similar negative developmental element was identified in the promoter of the conidiation-specific gene *con-10* (16). Like *eas* (*ccg-2*), *con-10* is also under light and clock control (11, 29). Sequence comparisons within the regions containing the negative mycelial elements for *con-10* and *eas* (*ccg-2*), as well as to the *con-8* promoter (38), suggest a possible common element with the consensus GGGAGCTTATTCCCCGCGTG beginning at bp −752 of the *eas* (*ccg-2*) sequence (16), a position consistent with the promoter deletion analysis reported here. Deletion of the region containing the negative mycelial element in *con-10* was found to have no effect on light induction (similar to *eas* [*ccg-2*]); however, circadian rhythmicity in the deletions was not examined. Two separate elements required for *con-10* conidiation induction were also identified and found to be required for normal high-level expression during asexual development (16). A positive conidiation element was not observed in this study for the *eas* (*ccg-2*) gene, suggesting that this element is located downstream of −57 or does not exist. Sequence comparisons of the 45-bp ACE fragment with the clock-regulated *ccg-1* and *con-10* gene promoters revealed an 8-nucleotide sequence (GTTGG GAT) present in all three regions. The significance, if any, of this sequence awaits further experimentation.

Similar to light-responsive genes in plants (13), multiple light elements appear to be involved in *eas* (*ccg-2*) regulation. Interestingly, a sequence element that is both necessary and sufficient for light responsiveness of the *N. crassa al-3* gene (15) is present at two locations in the *eas* (*ccg-2*) promoter starting at bp −110 and −565. Although deletion of the DNA containing both of these elements in the *eas* (*ccg-2*) promoter is found to affect photoinduction, a single copy of the −110 element is not sufficient to confer light inducibility (Fig. 8). The 10- to 12-bp *al-3* sequence motif is also shared by the *con-10* gene and the light-inducible *al-1* gene (15). In a separate study (25), analysis of the *eas* (*ccg-2*) promoter was carried out to examine *cis*-acting regulatory elements involved in light induction. We found no evidence supporting the involvement of two regions of the *eas* (*ccg-2*) promoter, between −1017 and −1498 and between −380 and −429, reported to be involved in light induction. However, these inferences were based on the inability to detect *eas* (*ccg-2*) transcript upon deletion of sequences, and given the complexity of *eas* (*ccg-2*) regulation, such a loss-of-expression assay does not permit an accurate assessment of promoter elements. A similar inference in the same study resulted in the placement of a glucose and nitrogen starvation element within the region of the positive transcriptional element for *eas* (*ccg-2*) expression between −1000 and −1500 (10, 28).

A significant number of *N. crassa* morning-specific clock-regulated genes have been observed to be photoinducible (6, 7, 9, 28). Dual clock and light regulation has been observed not only in fungi but also in plants (12, 26) and in mammalian genes, including the *N*-acetyltransferase (52) and retinal transducin (14) genes. Additionally, light inducibility of members of the immediate-early gene family, such as c-*fos* and *junB* is gated by the circadian clock (3, 22, 27, 39). With regard to clock-regulated genes in mammalian systems, an inducible cyclic AMP early repressor (ICER), encoding a small transcription factor made from RNA initiating within an intron of the immediate-early gene CREM, has been shown to be clock regulated (44, 47). In addition, the liver-enriched albumin D element-binding protein is transcriptionally regulated by the circadian clock (50); this transcription factor has been suggested to be involved in regulating cyclic expression of the liver enzyme cholesterol 7α-hydroxylase (30). While some of these clock-regulated promoters have been dissected and important regions have been delineated, in no prior case has it been possible to dissociate the light and clock elements. Interestingly, in addition to the endogenous circadian clock directing rhythmic transcription of target genes, recent experiments

have demonstrated clock control of translation for the *Gonyaulax* luciferin-binding protein (37). In this case, a circadianly regulated factor was shown to bind within a 22-nucleotide region of the 3′ end of the luciferin-binding protein, presumably resulting in rhythmic translational inhibition.

Other than in *N. crassa ccg* genes, the best-understood examples of light and clock regulated promoters are in plants, in which, for instance, light and the clock are involved in the regulation of the *CAB2* gene, a part of the nucleus-encoded light-harvesting chlorophyll *a*/*b*-binding protein of chloroplasts. The kinetics of the light responses in plants differs from that seen in *N. crassa*. Whereas a single pulse of red light induces *CAB2* mRNA to peak levels 4 to 8 h after the pulse in etiolated seedlings (26), *eas* (*ccg-2*) levels peak between 90 and 120 min after a light pulse and decline to preinduction levels by 4 h after the pulse (6). Promoter analysis of the *Arabidopsis CAB2* gene has identified a 78-bp region that is required for both light and clock regulation (1, 2). Sequence comparisons of the 45-bp ACE fragment to the 78-bp *CAB2* region failed to identify any significant common elements. While light-stimulated phytochrome activates *CAB2* transcription, the authors suggest that the circadian clock negatively regulates *CAB2* expression (1). As has been observed for the *N. crassa ccg* genes, the two pathways can function independently since rhythmic *CAB2* expression can be observed in plants transferred from a light/dark cycle to continuous-light conditions in which phytochrome is always active. However, it is not clear whether components of separate light and clock signaling pathways interact with the same or distinct *cis*-acting promoter elements, or if the pathways converge and mediate their responses through a single site (1). Alternatively, for *eas* (*ccg-2*), it is now clear that the light elements reside in regions that do not overlap the ACE. Deletion of the ACE does not abolish photoinducibility (Fig. 8), and deletion of light elements does not affect clock regulation of *eas* (*ccg-2*) (Fig. 3 and 4). In support of the independent nature of the clock and light in regulating *eas* (*ccg-2*) expression, it was demonstrated that photoinduction of *eas* (*ccg-2*) does not require a functional clock (6). This finding is interesting in view of the fact that light provides a primary signal for resetting the phase of the circadian clock in *N. crassa* (42) and indicates that the light response of *eas* (*ccg-2*) is a function of induced conidiation in response to light rather than a function of the clock.

Before we began to dissect the regulation of *eas* (*ccg-2*), we had anticipated that both the clock and light would regulate *eas* (*ccg-2*) in a global sense, through each one activating conidiophore development and thereby inducing expression of *eas* (*ccg-2*). However, this does not seem to be the case. Instead, at least two independent pathways appear to exist, one from the endogenous clock to control rhythmic *eas* (*ccg-2*) expression and another through an asexual developmental pathway that likely involves input from environmental factors including light, nutrient limitation, and desiccation. This observation suggests that in nature, under adequate growth conditions, asexual development is controlled by the endogenous clock and conidiation is initiated prior to sunrise in the early morning. Here, the clock controls rhythmic *eas* (*ccg-2*) gene expression so that mature conidia are produced at the appropriate time of day, possibly providing an evolutionary advantage (10). Alternatively, under suboptimal growth conditions, it may be that the organism must make lots of conidia to ensure a greater chance of survival, and one would predict that the time of day would not be critical. In this case, a separate pathway is used to achieve high-level synthesis of *eas* (*ccg-2*). This pathway probably involves the product of the *fl* gene, because *eas* (*ccg-2*) levels are greatly reduced in the aconidial mutant *fl* strain (28). The independent nature of the clock and developmental pathways for *eas* (*ccg-2*) expression is further supported by the following observations: (i) clock control of *eas* (*ccg-2*) can be observed in nondeveloping cultures (32, 34), (ii) development can proceed without a functional clock (4), (iii) robust conidiation can mask *eas* (*ccg-2*) rhythmic expression (9), and (iv) while the levels of *eas* (*ccg-2*) are markedly reduced in *fl* cells, the transcripts still oscillate (9).

The localization of an ACE within the 45-bp clock responsive element of the *eas* (*ccg-2*) gene, the best-defined clock-responsive region known, provides a focus for investigations into the mechanism through which the signals from the clock are transduced to control gene expression. The ability to separate *cis*-acting elements involved in clock control of gene expression from other control elements makes the *eas* (*ccg-2*) gene an excellent model system in which to further investigate the signal transduction pathway from the clock to a target gene.

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