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Light and Development Regulate the Expression of the *Albino-3* Gene in *Neurospora crassa*

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The *albino-3* gene of *Neurospora crassa* codes for geranylgeranyl pyrophosphate synthase, an enzyme involved in the biosynthesis of carotenoids. The *albino-3* locus encodes two overlapping transcription units that give rise to two mRNAs of 2.2 kb (*al-3*(c)) and 1.6 kb (*al-3*(*m*)), with the promoter of the latter residing in the transcribed region of the former. The 1.6-kb transcript was transiently expressed in the mycelium after light induction, while the 2.2-kb mRNA appeared in conidiating cultures only, independently of light. *Al-3*(c) mRNA was enhanced in the conidiophores and was impaired in mutants blocked at the early stages of conidiation; *al-3*(c) therefore represents a conidiation-specific transcript. The *al-3*(c) mRNA level increased upon illumination with blue light and fluctuated according to the circadian cycle. © 1995 Academic Press, Inc.

INTRODUCTION

In *Neurospora crassa* the products of the three *albino* genes, *al-3*, *al-2*, and *al-1*, are required for biosynthesis of carotenoids.

The *albino 3* (*al-3*) gene encodes geranylgeranyl pyrophosphate synthase (GGPPS) (Sandmann *et al.*, 1993). The enzyme is a member of the prenyltransferase family that catalyzes the *trans* addition of three molecules of IPP onto DMAPP to form geranylgeranyl pyrophosphate, essential for the biosynthesis of carotenoids and of prenylated compounds.

Exposure to blue light induces carotenoid biosynthesis in *Neurospora* mycelia. *Al-3*(*m*) mRNA is not detectable in dark-grown mycelia, but is readily induced following illumination (Baima *et al.*, 1991). Deletion analysis of the *al-3*(*m*) promoter region established that 226 bp in the region upstream of the *al-3*(*m*) mRNA start site were necessary and sufficient to drive light-regulated *al-3* gene expression in mycelia. One element controlling expression in response to light, the APE element, was identified in the *al-3* promoter. This element is conserved through the *albino* genes and, among the others, in the promoter of *ccg-2* and *con-10* genes (Carattoli *et al.*, 1994).

Environmental signals such as starvation and desiccation, together with general developmental cues, stimulate, during the vegetative cycle, hyphal differentiation into two kinds of dormant asexual spores, macroconidia and microconidia. In macroconidia (henceforth called conidia) the accumulation of carotenoid is constitutive (De Fabo *et al.*, 1976; Harding and Shropshire, 1980). "Blind" mutant strains, *wc-1* and *wc-2*, present orange conidia and a white mycelium, indicating that different mechanisms regulate carotenoid production at different stages.

Many genes other than those involved in carotenoid biosynthesis are also regulated in response to either light, developmental stage, or circadian periodicity. In many instances, genes appear to respond to all three stimuli, as in the case of *ccg-2*(*eas*) (Arpaia *et al.*, 1993; Lauter *et al.*, 1992), *ccg-1* (Arpaia *et al.*, 1995; J. J. Loros and J. C. Dunlap, personal communication), *con-6*, and *con-10* (Lauter and Yanofsky, 1993).

Cis sites controlling these expression patterns include, in addition to the APE element that controls light response, the CRS-B box that binds a cellular factor acting in *trans*, required for activation of the conidia developmental pathway (White and Yanofsky, 1993).

In this paper we show that the *al-3* locus encodes two overlapping but distinct transcripts, *al-3*(*m*) (i.e., mycelial) and *al-3*(*c*) (i.e., conidial), that respond to different regulatory stimuli acting on different promoter regions. We determined the patterns in which these transcripts respond to light, to conidiation, and to circadian rhythm. The expression of the *al-3*(*c*) unit appeared to be specifically triggered by conidiation and *al-3*(*c*) mRNA was greatly reduced in mutants that are blocked in the early stages of conidiation. Conidiation induced the expression of *al-3*(*c*) independently of the presence of light, although illumination with blue light did exert a stimulatory effect on transcriptional activity. This expression pattern sharply contrasted with the behavior

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of the mycelial transcript (*al-3(m)*), which was tightly controlled by light (Baima *et al.*, 1991). The expression of the *al-3(c)* transcript was also under control of the circadian cycle, while in the same experimental conditions the mycelial unit was never observed to cycle.

MATERIALS AND METHODS

Strains of N. crassa

The *Neurospora crassa* wild-type and mutant strains used in this work (*fl*, fluffy; *acon-2*, aconidial; *frq-7*, frequency; *wc-1*, *wc-2*, white collar) were obtained from the Fungal Genetic Stock Center (FGSC; University of Kansas, Kansas City, KS): Oak Ridge wild-type 74-OR23A (FGSC 987), *fl* (FGSC 4317), *acon-2* (FGSC 3262), *frq;bd* (FGSC 4898), *wc-1* (FGSC 4398), and *wc-2* (FGSC 4408).

Nucleic Acid Sequencing

A *Hin*dIII fragment of pNC39 (Nelson *et al.*, 1989) was used to screen a λ J1.74A genomic DNA library kindly provided by M. Orbach. A clone was isolated that extended 5′ to the pNC39, digested with *Hin*dIII, and cloned into pNC39 to give pNC16. For sequencing, the *Cla*I-*Nae*I fragment of pNC16 was subcloned in a pGem 7zf(−) Promega vector, obtaining the pGA1200 plasmid clone. *Nsi*I, *Sac*I and *Bam*HI restriction digestions and religations of pGA1200 plasmid gave rise, respectively, to pGA*Nsi*I, pGA*Sac*I, and PGA*Bam*HI plasmid clones containing progressive deletions of the original DNA insert. All the clones were sequenced on both strands by the dideoxy chain termination method of Sanger *et al.* (1980) with universal and homemade primers, using a Sequenase kit (United States Biochemical Corp.).

S1 Nuclease Mapping and Primer Extension

To perform the S1 nuclease mapping experiments to map the 5′ end and the *al-3(c)* intron boundaries (Fig. 2), the synthetic oligonucleotides M 5′-ACGGCCATGGTGACGTGTTCCATTTCC-3′ (complementary to nucleotides +1057 to +1084 of Fig. 1) and P 5′-AACCAAGGATTGAGCG-3′ (+170 to +185) were end-labeled by T4 polynucleotide kinase with [γ-^{32}P]dATP according to standard methods (Sambrook *et al.*, 1989). The oligonucleotides were then annealed to the PCN16 plasmid, which was digested with *Pst*I, and elongation by *Taq* polymerase was performed in the presence or absence of [α-^{32}P]dATP in the nucleotide mix to obtain, respectively, uniformly labeled or end-labeled probes after elution from a strand-separation gel. To map the 3′ end of *al-3(c)* mRNA, an *Eco*RI-*Nar*I uniformly labeled single-strand probe was utilized. Probes were hybridized with 10-20 μg of total RNA in 0.8 *M* NaCl, 40 m*M* Pipes, pH 6.4, 4 m*M* EDTA at 42°C for 3 hr. S1 nuclease digestions were carried out in S1 digestion mix (0.3 *M* NaCl, 30 m*M* NaAcetate, pH 4.6, 1 m*M* $ZnSO_4$) containing 300 U/ml of S1 nuclease. The digestion products were analyzed by 6% acrylamide/bis-acrylamide urea gel electrophoresis.

The end-labeled oligonucleotide P was used in primer extension experiments for 5′ end determination. It was annealed to 5 μg of total RNA and elongation was performed with AMV reverse transcriptase by standard methods (Sambrook *et al.*, 1989).

To map intron boundaries we also sequenced a RT/PCR-derived DNA fragment. Reverse transcription utilized the oligo 5′-CCAATCGACAGCAAT-3′ (+829 to +844). The product was then amplified by PCR using the same oligonucleotide and the oligo 5′-CAATCCTTGGTTATTCT-3′ (+174 to +193).

Media and Culture Conditions

To induce conidiation by nitrogen deprivation, cultures were grown in Vogel's minimal medium (Davis and De Serres, 1970; Vogel, 1964), supplemented with 1.5% (wt/vol) sucrose as the carbon source, for 24 hr in the dark. Mycelia were then filtered onto filter paper disks under red safelight illumination and transferred to a modified Vogel minimal medium in which the concentration of the nitrogen source (NH_4NO_3) was lowered to 1/100 of the normal. Growth was continued in the dark for between 2 and 24 hr before harvesting. The liquid culture system for the production of conidiating cultures by glucose deprivation used the high-concentration glucose medium and the low-concentration glucose medium described in Loros *et al.* (1989) and Nakashima (1981). Conidia were germinated in high glucose Fries' medium with shaking in the dark. After 24 hr mycelia were filtered as above and transferred into low-glucose Fries' medium for between 2 and 28 hr, with shaking, in the dark. Mycelia were harvested by rapid filtration onto filter paper disks and frozen in liquid nitrogen under red safelight illumination. At each time point pieces of mycelia were taken immediately before harvesting and the morphology of the cultures was monitored under a Leitz optical microscope.

Conidia (1×10^7) of all strains except *fl* and *acon-2* were inoculated in 100 ml of medium in 250-ml flasks and shaken at 150 rpm at 25°C. For the *fluffy* mutant we inoculated hyphal fragments, while the *acon-2* strain was grown in the inductive medium at the nonpermissive temperature of 34°C.

To separate the conidiating portion of the mycelia we used the method of Berlin and Yanofsky (1985) with minor modifications. Briefly, 1×10^6 conidia/ml were germinated in 200 ml of minimal medium and grown with shaking for 24 hr at room temperature. The culture was then filtered onto sterile filter paper and placed on a layer of glass beads in a petri dish containing 10 ml of medium. A second filter paper moistened with the me-

dium was placed on the mycelium and the culture was left to grow in a sterile hood. After an additional 24 hr the portion growing above the upper filter was scraped with a rubber scraper and frozen in liquid nitrogen (conidiating portion). Microscopic analysis revealed that it consisted of conidiophores, conidial chains, and free conidia. Part of the underlying mycelium was frozen at the same time (mycelial portion).

Photoinduction

For photoinduction studies 5×10^6 conidia were inoculated into 100 ml of Vogel's minimal medium in 250-ml flasks and shaken at 150 rpm in the dark at 25°C. Aged cultures were grown for 2 days. Cultures were then divided in two, one-half only illuminated and samples of both were taken after 20 min from the onset of the illumination. Photoinduction was elicited by treatment of the cultures with saturating light (energy fluency rate $>130\ J/m^2$ in the blue region).

In experiments to determine the kinetics of the light response in conidiating cultures, growth was in Vogel's minimal medium as described above. Cultures were then divided, when necessary, and transferred to the low-nitrogen medium for 5 hr, after which cultures were illuminated for 5 min and immediately harvested or transferred to darkness and additional samples taken at various intervals. At the time of harvesting, mycelia were removed from the growth medium by filtration onto filter paper disks and were frozen in liquid nitrogen.

Production of Rhythmic Mycelia

Production of rhythmic mycelia was carried out using a liquid culture system according to a modification of the protocol of Lauter and Yanofsky (1993). Mycelial disks of wild-type or *bd;frq-7* strains were transferred to the low-glucose medium and subjected to a complete 12-hr light/12-hr dark period to entrain the cycle. After an additional 1-hr light pulse, the cultures were placed in the dark and harvested every 4 hr for the following 40–44 hr. For the *bd;frq-7* strain samples were harvested at 27 and 29 hr after the light pulse instead of at 28 hr.

RNA Extraction and Northern Hybridization Blot

Frozen mycelia were powdered in a Waring blender under liquid nitrogen. Total RNA was extracted with a mini-prep extraction method that utilizes phenol extraction and LiCl precipitation, essentially as described in Sokolowsky *et al.* (1990). RNA, denatured in formaldehyde, was electrophoresed on 1.5% agarose gels containing formaldehyde (Lehrach *et al.*, 1977) and transferred to Hybond N membranes (Amersham). The filters were hybridized and autoradiographed with Kodak X-OMAT films and intensifying screens. RNA was hybridized with two specific *al-3* probes: Probe M was a 5′ end-labeled oligonucleotide (see Fig. 1); probe C was a PCR product corresponding to the +764, +947 region of the *al-3(m)* promoter, ^{32}P-labeled using the random-primer method (Feinberg and Vogelstein, 1983) (see Fig. 1). Other hybridization probes were a 5′ end-labeled *con-10*-specific oligo, an *Xba*I-*Cla*I *al-1* fragment, an *Xba*I DNA fragment specific for the *ccg-2* gene, a DNA probe for IF2, a cDNA known not to be photoinduced (Baima *et al.*, 1991), and a cDNA probe for a *Neurospora* ribosomal protein (Tarawneh *et al.*, 1990). The latter probes were ^{32}P-labeled by the random-primer method. In order to rehybridize filter blots for the purposes of normalization or rehybridization, probes were removed by washing the blots in 0.005 *M* Tris-HCl, pH 8.0, 0.002 *M* Na_2EDTA, 0.1× Denhardt's at 65°C for 2 hr.

RESULTS

Al-3 Consists of Two Overlapping Transcription Units

We previously reported that the *Neurospora al-3* gene (encoding the enzyme geranylgeranyl pyrophosphate synthase) is transcribed as a 1.6-kb mRNA without intervening sequences in the undifferentiated mycelium upon blue light exposure (Nelson *et al.*, 1989). More recently we demonstrated that the light-dependent expression of the 1.6-kb mRNA is fully controlled by promoter signals located in a 226-nucleotide stretch immediately upstream of the transcriptional start site. This 226-nt upstream fragment of the *al-3* gene, when placed in an appropriate construct, confers light inducibility to a light-independent gene, thus showing that this region indeed contains all of the essential elements of an independent, light-responsive promoter (Carattoli *et al.*, 1994). Northern blot analysis of the RNA extracted from 2-day-old *Neurospora* cultures with an oligonucleotide probe complementary to the 1.6-kb transcript (probe M, Fig. 1) occasionally revealed the presence of an additional 2.2-kb mRNA band. When hybridization was carried out with a probe spanning the previously characterized *al-3(m)* promoter region (probe C, Fig. 1) only the 2.2-kb mRNA was detected.

To characterize this second transcript, we sequenced 1240 nucleotides upstream of the AUG initiator codon of the *al-3* reading frame (Fig. 1). We also determined the sequences of the mature mRNAs by reverse transcription and PCR amplification and defined their 5′ and 3′ extremities by primer extension and S1 mapping techniques (Fig. 2). No mRNAs other than the 1.6-kb and 2.2-kb transcripts were identified. The small mRNA was identical to the known light-inducible mycelial transcript. The large mRNA included the 1.6-kb mRNA, with 500 additional nucleotides at the 5′ end, including RNA corresponding to promoter regions of the small transcript. Comparison of the cDNA with the genomic se-

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                                   CRS-B
-139
CACTTTATGCACTCGGCGCTTTCCTGTGTCAAGTCTTGCATCGCCGACAGACGTGCACAAAGT
                                     APE
          CRS-B
-75
CGCCGCGTGTGTGCTTGAATCCGTTGCCATCGAGTGCTATATAAAGTGTCCAGACGTCCCCTT
                      APE
                          5'
-11
TCCTAAATCGTCTTGAATCTCCCCAATATGCATGGATGAAGTGGACTGAACTCGCACGTTCG
           +1
+52
ACCGCTTTTCTGGAATACCCCTAATTTGCGCAAGGTCCACAACAGCCGACCTCGTTCAACAT

+115
CTCTTACCACCTACAGTTACCTACCCATTTTTGACGAGCCCCTTACCAGCAACAACGCT

+174
CAATCCTTGGTTATTCTCTGTTTGTTCATGTCGGTCTGAAGCCCTGACAAGAAGAGACAACA

+236
TCACAGCAGAAATTTGAACGCTTTTCCACAAACTACAAGgtgagaaaccctccccagttttcatccc

+303
tagaagatgccggtttcagcagggagctcccgataaagagcgcgagacgggatcgcccgttcgatcttcagttg

+379
tgaagctcttttgtccccctgtgagagctcccgccccgcatctgaaaccccaccacgctactttcaggtgtcgtcg

+455
gggtttcattcttgcaaacatgtcctccgtcggccgcccaagaagtctccgcacttgcaatcaccttgcatccact

+531
gccaggggaaaggaaaggacaggcaggtcagatctgaggaaagcgctacggcagcttgatccgatttgtccgccc

+607
ctagtttcccttcctgatcgcttccgaggatccagtgacgatggctggcatgtgacaagatcgggggatgcaatct

+683
cgagttttgctggaacctagcagaaagaggccttctttccattcggcgtgattctttgctgaccccagatacaGAT

+759
AGATCTCTTGGCCTTTGTTCACTCAGCAGGCAAGCAGGGCAAATCCCCCTTCTCATAGCAAA

+821
GTGAGGTCGATTGCTGTCGATTGGCACACGACCTGTCAAGCGGTATTATCGTCATAGCGTGCG
                              probe C
+884
GGTATCGAATATTGCCCCCGAGACCGTGAAGCTTGCCTCCGGTTGTCACACAGCACGTCAAGT
        APE                   APE
                                                  5'
+947
AATTATAAGAAGCCAGCCAGAGCGCCGGCCACTTTGGATCAGACGACGCACGGGGTTAGCAT

+1010
CCTCTACAGTACCGACGGGTTTCCAATAATAAAACATAAAAAAAAGGAAATGGAACACGTC
                                                    probe M
+1072
ACCATG
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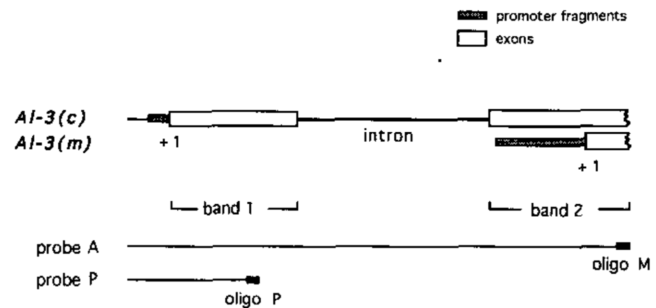
FIG. 1. Nucleotide sequence of the *al-3* genomic region upstream from the *al-3(m)* AUG initiator codon. Nucleotide numbers are given on the left. Nucleotide numbering begins at the first transcriptional start site (+1); upstream nucleotides have negative numbers. Multiple transcription start sites for *al-3*(c) and *al-3*(*m*) are indicated by arrows and intronic sequences are in lowercase. Potential transcriptional regulatory sequences in the 5' upstream region are indicated: TATA boxes and four small ORFs are underlined with continuous and dashed lines, respectively. CRS-B boxes and APE elements are indicated. The probes M and C used in this study are highlighted with a gray background. GeneBank sequence Accession Number: U20940.

quence revealed the presence of an intervening sequence of 481 nucleotides (lower case in Fig. 1) located upstream of the −220 mycelial promoter sequence (see also S1 mapping experiment in Fig. 2B, lanes 3 and 4). The splice site sequences and the internal element probably involved in lariat formation closely match the *Neurospora crassa* consensus sequences (Bruchez *et al.*, 1993). The S1 mapping and primer extension experiments detected some heterogeneity in the 5' end of the *al-3*(*c*) mRNA, possibly due to the presence of multiple transcription start sites (Fig. 2). Reverse transcription from oligonucleotide primer P gave four elongation products of 162, 172, 177, and 186 nucleotides. Consistent with these start sites, S1 mapping with a DNA probe P, obtained by elongation with oligo P, revealed two main protected fragments of 162 and 177 nucleotides (data not shown). The multiple start sites are evidenced by arrows in Fig. 1. At the 3' end of the mRNA no differences between the two transcripts were found by the S1 nuclease assay.

The putative promoter region upstream of the *al-3*(*c*)



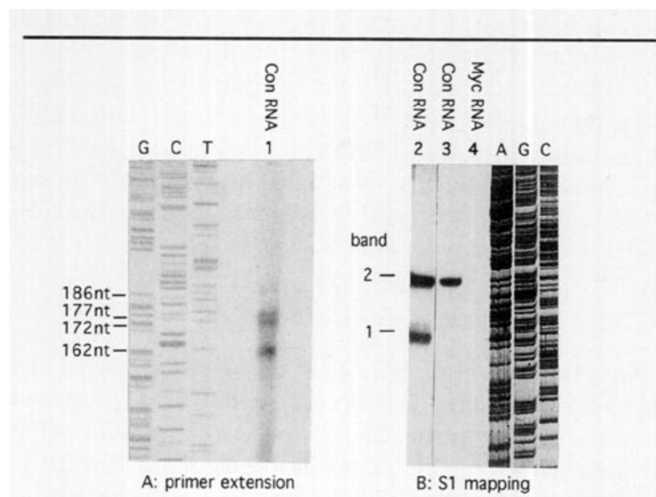


FIG. 2. Mapping of the *al-3*(*c*) mRNA start site. (Upper) Map of the *albino-3* transcripts and probes used in this experiment. (Lower, A) Primer extension analysis. Lane 1 shows the four products obtained by elongation from oligo P. The length of fragments is indicated on the left near the standard sequence used as a ladder. S1 nuclease digestion of probe P gave two products of 162 and 177 nucleotides (data not shown). (B) S1 nuclease-protected products of uniformly labeled probe A (lanes 2 and 4) or 5' end-labeled probe A (lane 3). In lane 4 digestion was of total RNA extracted from an uninduced mycelium; in lanes 1, 2, and 3 the RNA was extracted from cultures grown in low-nitrogen medium for 24 hr. Band 1 and band 2 are 324 and 259 nt long, respectively. A standard sequence used as a ladder is on the right.

TABLE 1
CHANGES IN THE DEVELOPMENT STAGE AND IN THE MORPHOLOGY OF THE CULTURES AFTER TRANSFER TO LOW-NITROGEN OR LOW-GLUCOSE MEDIA

Hours from induction	Stage/morphology
0	Vegetative mycelium
4	Formation of conidiophores
8 12	Septation
16	Separation of poconidial chains, release of long conidial chains in the medium
20	Conidial chains in the medium (mainly 2–3 conidial units)
24 28	Single conidia plus conidial chains in the medium

Note. Observations were made with a Leitz optical microscope.

transcript start site contained potential regulatory sequences (Fig. 1). In addition to a TATA box at nucleotide −35, there are two sequences that resembled the *Al-3* proximal element (APE), identified as a light-responsive element in the promoter of *al-3(m)* (Carattoli *et al.*, 1994), one at nucleotide −58 and a more degenerated one at nucleotide −110. Interestingly, both these regions also share homology with the CRS-B box (White and Yanofsky, 1993; Corrochano *et al.*, 1995). Sequence data suggest that the *al-3(m)* and *al-3(c)* mRNAs encode the same polypeptide, since no long open reading frames (ORFs) were found in the novel transcript other than the one previously described and characterized in the *al-3(m)* transcript (Carattoli *et al.*, 1991; Sandmann *et al.*, 1993). Only a number of small ORFs (underlined in the Fig. 1 with a dotted line) are detectable immediately downstream of the *al-3(c)* initiation site and in the untranslated 5′ region of the *al-3(c)* mRNA.

Al-3(c) Is Expressed during Conidiation

Liquid dark-grown 2-day-old cultures expressed the 2.2-kb transcript and produced differentiated structures such as conidiophores. Therefore, it was possible that the appearance of the longer *al-3* mRNA was linked to the conidiation process. To test this hypothesis, we induced conidiation in liquid cultures by nutrient deprivation (see Materials and Methods). Microscopic observation of such cultures revealed sequential changes in the morphology of the hyphae occurring following transfer to either low-nitrogen or low-glucose medium (Table I). Conidial chains appeared 16 hr after the transfer; after 24 hr the conidiation process was complete and many mature single conidia were found in the culture medium. We refer to the moment of transfer to the starvation medium as the starting point for induction of the conidiation process. Total RNA samples extracted from dark-grown cultures harvested at various times after start were subjected to Northern blot analysis with probe M (Fig. 3). The *al-3(c)* mRNA began to accumulate at 8 or from 8 to 12 hr after transfer to starvation media. The transcript levels remained high for the following 8 hr and then decreased. Under these conditions, *al-3(c)* expression was thus triggered by a light-independent stimulus linked to the developmental process leading to conidiation.

In order to establish a temporal scale of expression of *al-3(c)* with respect to other conidiation-specific genes, we monitored the expression of *ccg-2(eas)* and *con-10*. In our experimental conditions, the *ccg-2(eas)* mRNA appeared after 8 hr in low-glucose medium and was abundantly expressed during the following 20 hr (Fig. 3B). Accumulation of *con-10* mRNA began 16 hr after start and the gene continued to be expressed for a further 12 hr (Fig. 3B). Therefore *al-3(c)* mRNA is expressed almost at the same stage as *ccg-2(eas)*, but considerably earlier than *con-10*.

Al-3(c) expression appeared to be controlled by genetic factors regulating conidiation. Two mutants, *acon-2* and *fl*, defective in different stages of conidiation (Springer and Yanofsky, 1989) failed to express *al-3(c)*. The *acon-2* mutant produces normal aerial hyphae, but conidiation is blocked before the minor constriction stage at the restrictive temperature. The *fluffy* mutant is blocked before the major constriction stage; therefore, after a few cycles, budding growth of conidiophores stops and septa do not thicken and separate. The *al-3(c)* transcript was almost undetectable in either strain during a period of 24 hr from the transfer in starvation medium (Fig. 3C). Therefore, *al-3(c)* depends upon the normal conidiation process to be correctly expressed.

Al-3(c) Expression Is Restricted to the Conidiating Structures

To differentiate between gene expression in the conidiophores and in the mycelium, we separated them and extracted mRNA from each. *Al-3(c)* mRNA appeared only in the conidiating part (Fig. 4, lane 2); the transcript was not detectable in the mycelial part (Fig. 4, lane 3) or in a noninduced vegetative mycelium (Fig. 4, lane 1). A similar pattern of expression was observed for the *ccg-2/eas* gene, while a conidiation-independent gene coding for a ribosomal protein (Tarawneh *et al.*, 1990) was maximally expressed in the uninduced mycelium.

Carotenoid biosynthesis needs the coordinated expression of at least two other genes involved in the carotenoid biosynthetic pathway: *albino-2* and *albino-1* (Harding and Turner, 1981). The expression of the

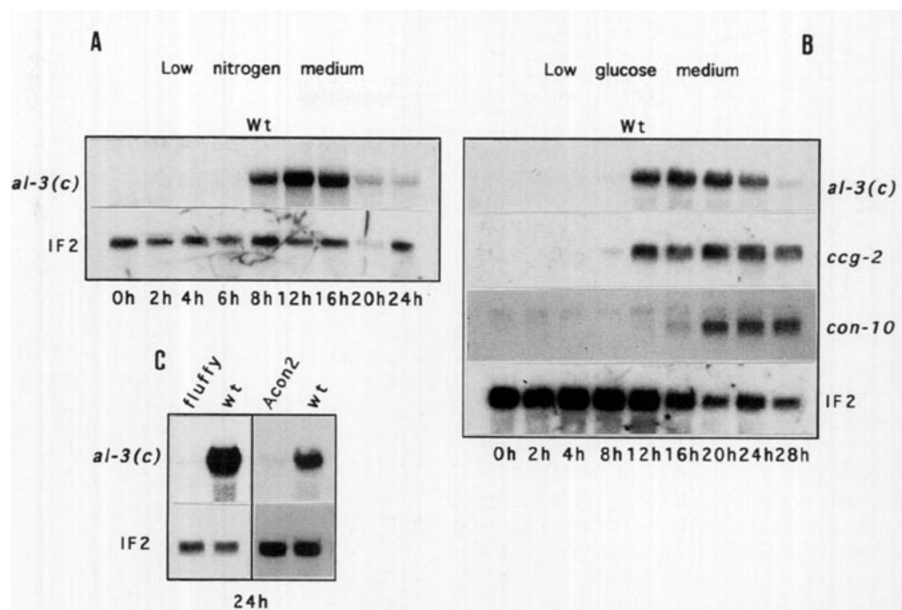


FIG. 3. Expression of *al-3(c)* is induced by the conidiation process and is impaired in conidiation mutants. Conidiation was induced in the dark by transferring wild-type cultures to a low-nitrogen medium (A) or a low-glucose medium (B). Cultures were harvested at different times from transfer, as indicated, and frozen in liquid nitrogen. *fluffy* and *acon-2* strains (C) were transferred to a low-nitrogen induction medium and harvested after 24 hr. Ten micrograms of total RNA extracted from these cultures was loaded in each lane. The blots in A and B were hybridized with an *al-3*-specific probe (probe M) and, in the case of B, with *con-10*- and *ccg-2*-specific probes. In C hybridization was performed using probe C. An IF2 probe was used for normalization (Baima *et al.*, 1991).

al-1 gene was indeed enhanced in the conidiating portion of the culture with respect to the mycelial part (Fig. 4) and its expression pattern, monitored at different times following induction of conidiation in our experimental conditions, closely resembled that of the *al-3* gene (data not shown). The expression of the *al-2* gene was also shown to be developmentally regulated (T. J. Schmidhauser, personal communication).

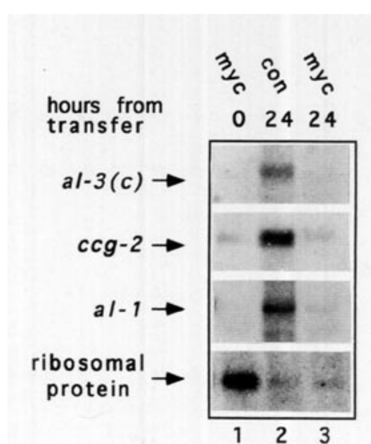


FIG. 4. *Al-3(c)* is induced in the conidiating part of a *Neurospora* culture. Total RNA was extracted from a wild-type mycelium grown in Vogel's minimal medium for 24 hr (lane 1) and, separately, from the conidiating (conidiophores and free conidia; lane 2) and mycelial (lane 3) portion of the same culture, after 24 hr from the induction of conidiation (see Materials and Methods). The blot was hybridized with *al-3* and, after stripping, with specific probes for *ccg-2*, *al-1*, and a *Neurospora* ribosomal protein (Tarawneh *et al.*, 1990).

Al-3(c) Expression Is Enhanced by Light in Conidiating Cultures

Although conidiation can occur in the dark, it is affected by light in several ways: illuminated cultures develop conidia faster, produce larger numbers of conidia, and orient conidiophores toward the light (Siegel *et al.*, 1968; Turian, 1977; Lauter and Yanofsky, 1993). Accordingly, the expression of conidiation-specific genes such as *con-6*, *con-10*, and *ccg-2* has been shown to be influenced by light (Arpaia *et al.*, 1993; Lauter and Yanofsky, 1993).

Northern blots of total RNA extracted from dark or illuminated cultures were probed with probe M (Fig. 1) that recognizes both the *al-3(m)* and the *al-3(c)* transcripts. *Al-3(m)* mRNA accumulated in light-exposed cultures only, regardless of conidiation (Fig. 5). By contrast, the expression pattern of the *al-3(c)* mRNA remained principally dependent upon the stage of differentiation, as no transcription was observed until late after the onset of conidiation; once started, however, conidiation-dependent transcription was significantly stimulated by light (Fig. 5, lanes 5–8).

Mutations in the *wc-1* and *wc-2* genes eliminate light responsiveness in *Neurospora* (Degli Innocenti and Russo, 1984; Harding and Turner, 1981). Transcription

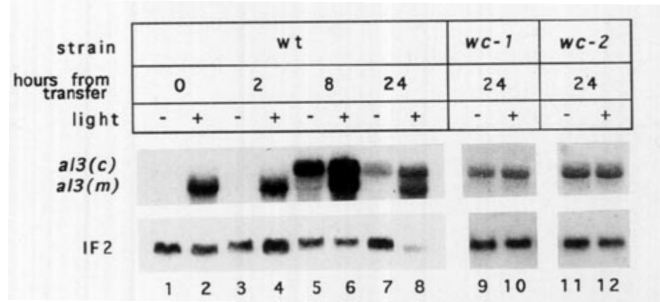


FIG. 5. *Al-3(c)* is induced by light in conidiating cultures; *wc* genes are required for light induction. Wild-type conidia germinated in minimal medium for 24 hr, were divided in two halves, and were transferred to the low-nitrogen medium. One of the two halves was illuminated for 20 minutes with saturating blue light 0, 2, 8, and 24 hr from the transfer. *wc-1* and *wc-2* cultures were illuminated at 24 hr from the transfer. Both halves of the same culture were then immediately frozen in liquid nitrogen. The *al-3*-specific transcript was detected by using probe M that recognizes both the mycelial and conidial transcripts. The IF2-specific probe was used for normalization. Ethidium bromide staining revealed comparable RNA amounts in all lanes.

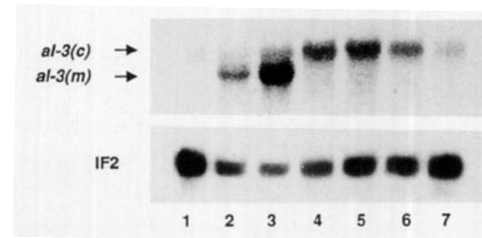


FIG. 6. The kinetics of the *al-3(c)* response to light is compared with that of *al-3(m)*. After 5 hr from transfer to a low-nitrogen medium, wild-type *Neurospora* cultures were illuminated with a 5-min pulse of saturating blue light. Cultures were harvested either immediately (t_0, lane 2) or after additional periods of growth in the dark: 10 min (lane 3); 25 min (lane 4); 55 min (lane 5); and 95 min (lanes 6–7). Lanes 1 and 7 represent dark-grown control cultures harvested at t_0 and 100 min, respectively. Total RNA was extracted, loaded on denaturing gels (10 μg per lane), and blotted. Probe M was used for *al-3*-specific hybridization since it recognizes both transcripts, and an IF2 probe was used for normalization.

of all light-induced genes tested up to now, at least in single *wc-1* or *wc-2* mutants, is not increased by illumination (Nelson *et al.*, 1989; Arpaia *et al.*, 1993, 1995; Lauter and Russo, 1991; Sommer *et al.*, 1989; Schmidhauser *et al.*, 1990, 1994). Although *wc* mutants do not express the *al-3(m)* transcript in mycelia upon light exposure, they develop orange conidia even in the dark. The two *wc* genes affect light regulation but not developmental regulation of *al-3(c)* expression. In *wc-1* and *wc-2* mutants light did not superinduce the expression of *al-3(c)* during conidiation (Fig. 5). Developmentally regulated expression of the gene was not impaired in these strains. The pattern of expression of *al-3(c)* closely resembled that in a dark-grown wild-type strain (data not shown).

The kinetics of light induction of *al-3(m)* and *al-3(c)* differ. Wild-type conidia were germinated in a rich medium, transferred to a starvation medium, and illuminated with a 5-min pulse of blue light 5 hr after transfer. At this stage the *al-3(c)* mRNA starts to be transcribed. The 1.6-kb *al-3(m)* transcript reached a peak 10 min after the light-pulse (Fig. 6, lane 3) and decreased immediately afterward to become undetectable after 25 min in the dark (Fig. 6, lane 4). In contrast, the large 2.2-kb transcript reached a maximum between 25 and 60 min (Fig. 6, lanes 4–5) and 95 min after the onset of illumination its level was still higher in photoinduced cultures than in dark-grown controls (Fig. 6, lanes 6–7).

Al-3(c) Is Under Control of the Circadian Clock

The expression of *al-3(c)* fluctuates with a period resembling that characteristic of other conidiation-specific genes in wild-type *Neurospora* (Loros *et al.*, 1989; Lauter and Yanofsky, 1993) (Fig. 7B).

The period length of the circadian rhythm reflects the genotype of the strain used. To confirm that the oscillation depended on circadian regulation, we exploited therefore a long period mutant (*bd;frq-7*) in which tran-

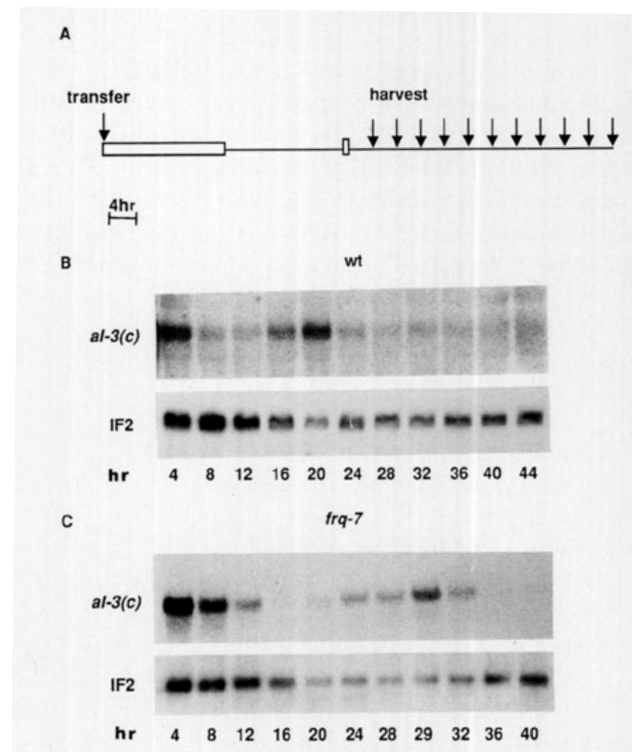


FIG. 7. *Al-3(c)* expression exhibits circadian periodicity. (A) Schedule of growth conditions used to generate synchronized mycelia to assay circadian periodicity of *al-3(c)* expression. Open bars represent light periods; arrows are placed at the moment of harvesting every 4 hr (see text). (B) Northern blot of total RNA (10 μg/lane) extracted from a wild-type strain. (C) Northern blot of RNA extracted from a long period *bd;frq-7* mutant strain. Hybridization was carried out with an *al-3*-specific probe M and an IF2 probe was used for normalization.

scripts arising from clock-controlled genes cycle with a period of 29 hr. In such a mutant *al-3(c)* mRNA accumulated under the circadian stimulus with a peak at 29 hr (Fig. 7C). In these experiments, although the oligo probe M recognizes both *al-3* transcripts, clock-directed *al-3(m)* expression was not detected. Clock control therefore seems to act specifically on the conidiation-linked *al-3(c)* transcript.

DISCUSSION

The *Neurospora crassa al-3* gene consists of two overlapping transcription units. One of these, *al-3(m)*, was previously characterized as a mycelial transcript, specifically and transiently induced by blue light (Baima *et al.*, 1991; Carattoli *et al.*, 1991). Expression of *al-3(m)* is controlled by promoter signals located in the region −30/−226 from the transcription start site. A light-responsive element, APE, is crucial to this regulation (Carattoli *et al.*, 1994). The *albino-3* locus contains a second, longer transcriptional unit, partially overlapping *al-3(m)*, that we term *al-3(c)*. This unit was not expressed in undifferentiated mycelia, but was specifically induced during conidiation. Comparison between the genomic and the cDNA sequences revealed that *al-3(c)* is transcribed as a precursor which, after after splicing of a 481-nt intron, originates a mature mRNA comprising the entire *al-3(m)* transcript as well as an additional 500 nt upstream of the *al-3(m)* transcription start site, including RNA corresponding to the *al-3(m)* promoter. The possibility that the *al-3(c)* and the *al-3(m)* mRNAs represent the result of alternative splicing of a single primary transcript must be discarded, in view of our previous demonstration that an independent, light-regulated promoter exists immediately upstream of the *al-3(m)* transcript (Carattoli *et al.*, 1994) and also in view of the sharp difference in the time and mode of expression of the two transcriptional units.

The *al-3(c)* mRNA contains no extended ORFs other than that encoding geranylgeranyl pyrophosphate synthase. Thus, the two transcriptional units most likely specify the same protein, the only difference being that with respect to *al-3(m)*, the *al-3(c)* mRNA has a long untranslated leader sequence. Such a feature frequently indicates translational regulation, especially if the long untranslated leader contains one or more small ORFs, as in *al-3(c)* (Fig. 1) (Hinnebusch, 1984; Werner *et al.*, 1987). For instance the *Aspergillus nidulans brlA* and *stuA* genes, involved in the coordination of temporal and spatial patterns of cell differentiation in the conidiophore, are transcribed as two independent mRNAs, containing in the leader sequence uORFs that regulate their translation (Han *et al.*, 1993; Miller *et al.*, 1992; Prade and Timberlake, 1993). The possibility that the *al-3(c)* transcript is translationally regulated is currently under scrutiny.

Al-3(c) was expressed in a conidiation-specific pattern. The *al-3(c)* transcript began to accumulate 8 hr after the induction of conidiation and continued to be present at high levels for a further 8 hr. The transcript appears to accumulate selectively in the conidiophores, since essentially no *al-3(c)* mRNA was found in the mycelial portion of a differentiating culture. The conidiophores expressing the *al-3(c)* mRNA had already reached an advanced stage of differentiation when proconidial chains septated and separated. Accordingly, *al-3(c)* developmental inducibility relies on the presence of the products of the *acon-2* and *fluffy* genes. Comparison with the expression pattern of other conidiation genes indicated that the *al-3(c)* transcript starts to be accumulated at the same time as *ccg-2(eas)*, but before transcription of *con-10*.

Although conidiation is doubtless the main factor controlling the expression of *al-3(c)*, it is by no means the only one. Both light and the circadian clock had important roles as additional transcriptional modulators. The abundance of *al-3(c)* mRNA increased upon light exposure, although light alone did not activate transcription in cultures not induced to conidiate or at very early stages of differentiation. Thus, in the blind mutant strains *wc-1* and *wc-2*, *al-3(c)* transcription was no longer stimulated by light, but the developmental control remained unmodified, closely resembling that observed in a dark-grown wild-type strain. This temporal pattern contrasts sharply with the behavior of *al-3(m)*, which is rapidly and transiently activated only following illumination, although in both cases *wc-1* and *wc-2* are necessary for light stimulation. The effect of light on both transcriptional units may be mediated by similar promoter motifs. The putative promoter region of *al-3(c)* contains two elements with strong sequence homology to the APE motif that is essential to light inducibility of *al-3(m)* (Carattoli *et al.*, 1994) (see Fig. 1). In *al-3(c)*, however, the transcription-stimulating effect of the APE element would be subordinated to the presence of a developmentally induced "master" controlling factor. In this respect, it is perhaps relevant that the APE elements in the promoter region of the conidiation-controlled (and light-stimulated) *al-3(c)* gene overlap CRS-B boxes (White and Yanofsky, 1993; Corrochano *et al.*, 1995). Comparison of the *al-3(c)* promoter region at nucleotide −114 with the CRS-B boxes present in the *con-10* promoter at nucleotides −281 or −217 showed a 9/14-bp and a 11/16-bp match, respectively. This match is not shared by the APE elements in the *al-3(m)* promoter. These observations confirm the general view that considers light as a modulator of conidiation that enhances the expression of the conidiation genes and boosts the conidiation process.

As a further element of complexity, the expression of *al-3(c)* was modulated by the circadian clock. In *Neuro-*

spora crassa asexual spore formation is under the control of the circadian oscillator. The *Neurospora* circadian rhythm of conidiation is very well characterized (reviewed in Dunlap, 1990, 1993). The "clock" regulates and coordinates the temporal expression of several "clock-controlled genes," two of which, *ccg-2/eas* (Bell-Pedersen *et al.*, 1992; Lauter *et al.*, 1992) and *ccg-1* (G. Arpaia, unpublished results; and J. C. Dunlap, personal communication), turned out to also be conidiation-specific genes. On the other hand the expression of conidiation-specific genes has been shown to exhibit circadian periodicity (Lauter and Yanofsky, 1993). The possibility that this could be a general phenomenon involving all conidiation-regulated genes prompted us to determine whether the expression of *al-3(c)* was also under the control of the endogenous rhythm of conidiation. *Al-3(c)* mRNA levels fluctuate in the dark with a periodicity resembling the wild-type *Neurospora* endogenous circadian rhythm (21.5 hr). That such rhythmicity depended on clock regulation was confirmed using the *bd;frq-7* mutant whose circadian rhythm is 29 hr. It is difficult to separate circadian and developmental inputs because culture conditions that permit synchronization and detection of circadian rhythmicity of gene expression also induce conidiation. The possibility should therefore be considered that the endogenous clock may represent the underlying mechanism permitting the rhythmic activation of developmental events and consequently the turning on of development-linked genes.

Many *Neurospora* genes have been described that are induced by light and are also controlled by the conidiation process. These results suggest the convergent involvement of regulation by the circadian clock, light, and development. Nevertheless, several genes transcribed in a light-dependent fashion during vegetative growth are also constitutively turned on at some stage during conidiation. Although conventionally described as conidiation-specific, such genes are not necessarily involved in the conidiation process itself. For instance, the *con-6* gene, which is both light-inducible in the mycelia and constitutively transcribed during conidiation, apparently has no essential role in conidial differentiation (White and Yanofsky, 1993). Possibly, a number of gene products are needed in greater abundance during conidiation, even if they are not directly required for morphogenetic purposes. The peculiarity of the *al-3* locus resides in the fact that it produces two distinct transcripts in response to two separate stimuli, development and light.

Different modes of regulation of *al-3* may relate to different functions of GGPPS. GGPPS has an essential role in carotenoid biosynthesis, but may have some other essential function. *Al-3* appears to be an essential gene, and all of the *albino-3* mutants retain low levels of GGPPS activity. GGPPS activity might be essential for the prenylation of proteins that are modified by the addition of a geranylgeranyl moiety, such as the Rab proteins, involved in secretion, a process very active during conidiation for deposition of cell wall components (Peter *et al.*, 1992; Farnsworth *et al.*, 1991; Khosravi-Far *et al.*, 1991; Moores *et al.*, 1991; Kinsella and Maltese, 1992). Accordingly, flashes of light transiently induce *al-3(m)* mRNA, while a messenger RNA with the requisites of higher stability and translational regulation might be required to control the GGPPS availability during conidiation and in conidia to confer higher resistance to light and to oxidative stresses during dormant life.

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