

# Photoregulation of *cot-1*, a Kinase-Encoding Gene Involved in Hyphal Growth in *Neurospora crassa*

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Accepted for publication February 12, 1998

Lauter, F.-R., Marchfelder, U., Russo, V. E. A., Yamashiro, C. T., Yatzkan, E., and Yarden, O. 1998. Photoregulation of *cot-1*, a kinase-encoding gene involved in hyphal growth in *Neurospora crassa*. *Fungal Genetics and Biology* 8:300–310. Blue light plays a key role as an environmental signal in the regulation of growth and development of fungi and plants. Here we demonstrate that in *Neurospora crassa* hyphae branch more frequently in cultures grown in light. Previous studies had identified *cot-1* as a gene that controls apical hyphal cell elongation. In the *cot-1* mutant, cessation of elongation is accompanied by hyperbranching. Here we demonstrate that the *cot-1* gene encodes two transcript species of about 2100 nt (*cot-1* (s)) and about 2400 nt (*cot-1* (l)) in length and that the ratio of both transcript species abundance is photoregulated. The origin of the difference between *cot-1* (l) and *cot-1* (s) was localized to the 5' end of the *cot-1* transcripts, suggesting that two COT1 isoforms with different activities may be formed. Both light effects, on branching and on *cot-1* expression, were dependent on functional *wc-1* and *wc-2* gene products. In addition to light, L-sorbose comprises another environmental cue that controls hyphal branching in *N. crassa*. In the presence of L-sorbose, photoregulation of *cot-1* was blocked, suggesting the involvement of alternative and

potentially interdependent signaling pathways for the regulation of hyphal elongation/branching. © 1998 Academic Press

**Index Descriptors:** hyphal branching; alternative transcripts; photomorphogenesis; *Neurospora crassa*.

Blue light is an important cue controlling different developmental programs in fungi and plants. Photomorphogenesis has been studied in a wide variety of these organisms (Perkins, 1969; Corrochano and Cerdà-Olmedo, 1992; Chory, 1993; Wessels, 1993; Lauter, 1996). In the ascomycete *Neurospora crassa* blue light controls different phases of sexual and asexual differentiation. Illumination stimulates the production of both protoperithecia (initials of sexual fruiting bodies) and conidia (asexual spores) (Klemm and Ninnemann, 1978; Degli-Innocenti and Russo, 1984; Lauter *et al.*, 1997). Consequently, expression of the conidiation-specific *Neurospora* genes *con-5*, *con-6*, *con-8*, *con-10*, and *eas* was found to be photoregulated (Lauter, 1996; Lauter and Russo, 1991; Lauter *et al.*, 1992; Lauter and Yanofsky, 1993), and it is reasonable to predict that some photoperithecium-specific genes which are photoregulated will be identified in the future.

In this paper we show that the vegetative phase of the *N. crassa* life cycle is also under the control of light. During this phase, *Neurospora* grows as a mycelium composed of branching hyphal cells. We demonstrate that hyphal branching frequency is increased by light, as has been shown for the basidiomycete *Schizophyllum commune* (Raudaskoski and Viitanen, 1982). Previous studies have identified

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genetic, as well as environmental factors, in addition to light, that control branching frequency in *N. crassa*. One genetic factor is the colonial temperature sensitive-1 (*cot-1*) gene. On solid media, continuous hyphal elongation and branching results in the formation of spreading radial colonies. Mutants have been isolated that display compact colonial growth on solid agar medium under conditions favoring spreading growth in the wild type (Perkins *et al.*, 1982). Mutations in *cot-1* cause colonial growth at or above 32°C, but exhibit normal spreading radial growth at or below 25°C (Mitchell and Mitchell, 1954). When cultured at temperatures above 32°C, *cot-1* colonies grow slowly and hyphae branch extensively. *cot-1* colonial growth morphology can be phenocopied by the wild type when grown on solid media containing 1.5% dehydrated ox gall (Littmann, 1947) or 1.0% L-sorbose (Tatum *et al.*, 1949). The toxic effect of sorbose is due, at least in part, to a direct effect on cell wall biosynthesis, as the sugar has been shown to partially inhibit glycogen synthetase and glucan synthetase *in vivo* and *in vitro* (Mishra and Tatum, 1972).

The *cot-1* gene has been isolated and on the basis of the deduced COT1 amino acid sequence, it encodes a serine/threonine-specific protein kinase (Yarden *et al.*, 1992; Justice *et al.*, 1995). Other close members of the serine/threonine kinase family are encoded by the *Drosophila warts/lats* gene and the human *DM* gene. The *warts/lats* gene is required for the control of the amount and direction of cell proliferation as well as for normal morphogenesis (Justice *et al.*, 1995; Xu *et al.*, 1995). Mutations in *DM* result in myotonic dystrophy (Mahadevan *et al.*, 1993). Thus, functional expression of those genes is required for normal cell differentiation in different organisms. Inactivation of *Neurospora cot-1* results in increased hyphal branching frequency (Yarden *et al.*, 1992). Plamann *et al.* (1994) analyzed some of the *N. crassa ro* mutants, which suppress the *cot-1* phenotype. They have demonstrated that at least some of the *ro* genes (*ro-1*, *ro-3*, and *ro-4*) encode structural and regulatory proteins belonging to the cytoplasmic dyadine motor complex. They suggested that the strains harboring mutations in *ro* partially bypass the requirement for COT1 kinase, rather than the possibility that COT1 is a direct regulator of the *ro* gene products. This hypothesis is supported by the fact that *ro-2* apparently encodes yet another regulatory protein (Vierula, 1996). Since COT1 function is required for normal hyphal elongation/branching, and since hyphal branching frequency is influenced by illumination, we set out to study the influence of light on *cot-1* expression. Two key players in *N. crassa* blue light signal transduction are encoded by the

white collar genes, *wc-1* and *wc-2* (Harding and Turner, 1981; Degli-Innocenti and Russo, 1984; Ballario *et al.*, 1996; Linden and Macino, 1997). Previous studies have identified *wc*-dependent and *wc*-independent photoeffects in this fungus. Functional *wc* products were found to be required for photocarotenogenesis (Harding and Turner, 1981), photoconidiation (Lauter *et al.*, 1997), and photoinduced protoperithecia formation (Degli-Innocenti and Russo, 1984). At the molecular level, photoinduced expression of *al-1* (Schmidhauser *et al.*, 1990), *al-2* (Schmidhauser *et al.*, 1994), *al-3* (Nelson *et al.*, 1989), *bli* genes (Sommer *et al.*, 1989), *con-5* and *con-10* (Lauter and Russo, 1991), *con-6* (Lauter and Yanofsky, 1993), *eas* (Lauter *et al.*, 1992), and *wc-1* (Ballario *et al.*, 1996) were shown to be *wc-1*- and *wc-2*-dependent. On the other hand, photoregulation of the genes *fr-9* and *wc-2* was found to be independent of a functional *wc-2* gene product (Crothwaite *et al.*, 1997; Linden and Macino, 1997). To analyze *wc*-dependence of photoinduced branching and *cot-1* expression, both phenomena were analyzed in *wc-1* and *wc-2* mutant strains.

## MATERIALS AND METHODS

### Strains and Plasmids

Wild-type *N. crassa* strain 74-OR23-IVA (FGSC 987) was used throughout. Mutants examined included *cot-1* (FGSC 4065) (Perkins *et al.*, 1982), *wc-1* (FGSC 4395), and *wc-2* (FGSC 4407) (Degli-Innocenti and Russo, 1984). The plasmids pOY311 (Yarden *et al.*, 1992), PCTY21 (Yamashiro *et al.*, 1996), and pBW100 (Lauter and Yanofsky, 1993) were used to generate *cot-1*, *rco-1*, and *con-10* antisense RNA probes, respectively. pOY18 (Yarden *et al.*, 1992) was used as template for generating *cot-1* fragments in PCR experiments.

### Light Experiments

Cultures were grown and mycelia photoinduced as previously described (Lauter and Russo, 1991), using Sylvania Energy Saver lamps (6 W/m<sup>2</sup> in the blue light region). Approximately 10<sup>7</sup> conidia were inoculated into 75 ml of Vogel's liquid minimal medium (Davis and de Serres, 1970) containing 2% (w/v) sucrose as a carbon source. When required, the sucrose was replaced with 2% (w/v) L-sorbose, 0.05% (w/v) glucose, and 0.05% (w/v) fructose. Cultures were grown at 34°C in 250-ml Erlenmeyer flasks with agitation (200 rpm) for 24–36 h. The mycelium (typically 100–250 mg) in each flask was harvested by filtration through a Büchner funnel and the resulting

mycelial pads were cut in half. All mycelial pads were wetted with 0.5 ml prewarmed (34°C) growth medium, which was reapplied every 30 min thereafter. This treatment prevented the formation of the aerial hyphae and arrested growth in the vegetative phase. After filtration, all manipulations were performed at 34°C.

### Light Microscopy

Vogel's minimal medium (100 µl) with 2% sucrose as the carbon source (Davis and de Serres, 1970) was inoculated with 10<sup>3</sup> conidia, applied to sterile microscope depression slides, and cover slips were placed over the medium. The slides were incubated in plexiglass moist-chambers placed in a lighted incubator. As a dark control, chambers were covered with aluminum foil to prevent light from reaching the growing cultures. In all experiments, 95% relative humidity and 25°C were maintained within the moist chambers. Samples were viewed with a Zeiss Axioscope microscope. Photographs were taken either with Fujichrome 100 ASA film or via an Applitech CCD camera coupled to NIH Image software.

### Northern Analyses

Total RNA was extracted from each mycelial sample as described previously (Sachs and Yanofsky, 1991). Northern analyses with DNA or RNA probes were performed using standard procedures (Berlin and Yanofsky, 1985; Zinn *et al.*, 1983). Total RNA (8 µg) from each sample was resolved by electrophoresis through a 1.5% agarose-formaldehyde gel and transferred to nylon membranes (Gene Screen, DuPont or Magnacharge, MSI). RNA was probed with radiolabeled *cot-1* and *rco-1* probes, which were prepared by generating <sup>32</sup>P-labeled antisense RNA (Zinn *et al.*, 1983). The pOY311 *cot-1* cDNA clone (Yarden *et al.*, 1992) was used to prepare *cot-1*-specific antisense RNA probes. This clone contains the entire sequenced region of the 3' untranslated end of *cot-1* (Yarden *et al.*, 1992) and extends to 107 bp downstream of the presumed translational start point (ATG) at the 5' end of (*cot-1(l)*). *cot-1*-specific antisense RNA was generated using the T7 promoter (present in the Bluescript vector) from pOY311 linearized with *Bam*HI endonuclease. *rco-1*-specific antisense RNA was generated as described (Lauter *et al.*, 1997). *rco-1* encodes a nonphotocontrolled regulator of conidiation (Lauter *et al.*, 1997; Yamashiro *et al.*, 1996). *con-10*-specific antisense RNA was generated as described (Lauter and Yanofsky, 1993). All of the Northern analyses were repeated with RNA samples obtained in at least three independent experiments.

### RT-PCR and RACE Experiments

RT-PCR was carried out with DNA-free total RNA, isolated from 60-min illuminated and dark (control) mycelia, following the GIBCO BRL protocol provided with the Superscript II RNase H Reverse Transcriptase kit (Life Technology Inc., Gaithersburg, MD, U.S.A.).

For 5' and 3' RACE experiments, the Marathon cDNA Amplification kit (CLONTECH, Palo Alto, CA, U.S.A.) was used with total RNA and in accordance with the manufacturer's instructions. Primer 3010 and primer 3021 (Table 1) were used as the 5' and 3' end gene-specific primers, respectively.

## RESULTS

### Effect of Light on Hyphal Elongation and Branching Frequency

To determine whether the presence of light influences the rate of linear hyphal branching, we microscopically

TABLE 1  
Oligonucleotides Listed were Used as Primers in RT-PCR and RACE Experiments

Primer	Sequence	Position	Comments
		5' end	
2066	GTCTGGGAGCTCGACTCTGAT	965-985	
2063	CACCCGGAATCCACTGTTTAC	1050-1070	
2091	GATAGGTACGATCGTTTGGAGC	1168-1147	
2090	GATAGTAGTTGCCGGACTGGG	1270-1250	
2093	GTATGCCAGCCACTACGC	1281-1298	
2094	GCTAGTACGGTCAATGTTAG	1359-1340	
3001	CTCTGGCCAGCAGCAAACC	1482-1500	
3010	CTGGGTATTGGAAGGCATCGGG	1542-1521	5'-RACE
3002	CAGATCGATTCCGACGGC	1809-1791	
3020	GGCCGACGGCAAGGTTTACGC	1939-1959	
3021	CAGCTTGCCACGTCAGGCA	2000-2020	3'-RACE
3011	GTTCCGATCCTGGAAGGTGGTG	2092-2071	
2064	TCACCACGGGCAAGAAGTCC	2130-2110	
2070	GCTCCAGGGCAAATCCAAC	2418-2436	
2071	CAATAGCAACCGAGTTGCG	2470-2452	
2072	GAGAACCGTCTCGGCCG	2855-2871	
2073	GATACAGTTCAGCGTTCGAC	3114-3136	
2061	AGACAGCGAGAAGACGTAAC	3175-3156	
2219	AACCAACGCTCATTATTC	3193-3176	
2077	CTTGGCTTTGACGCTTTGCTAG	3285-3307	
2074	GTCTCTGCGGCTTTTATTCTCC	3485-3463	
		3' end	

Note. Positions given refer to primer position within the *cot-1* sequence in Fig. 7. Oligonucleotides 3010 and 3021 were used as gene-specific primers in 5'-RACE and 3'-RACE experiments, respectively.

examined the apical extension zones in microcolonies of wild-type and white collar (*wc*) mutant strains grown on depression slides. The *wc* mutant strains, *wc-1* and *wc-2*, are blind for the majority of photoeffects in *N. crassa* (Harding and Turner, 1981; Degli-Innocenti and Russo, 1984; Lauter and Russo, 1990; Lauter, 1996; Linden and Macino, 1997) and, therefore, were chosen as controls. Sufficient medium under the coverslip of the depression slides allowed fungal biomass accumulation, while at the shallow ends of the depression, colony edges were mostly mono-cell layered (and thus more convenient to view). Strains were grown for 24 h at 34°C in Vogel's minimal liquid medium with 2% sucrose in the presence or absence of light. In the wild type, branching frequency of the apical hyphal cells was higher in cultures grown in the light- vs dark-grown cultures (Fig. 1). Thus, in contrast to an average branching frequency of once in  $120 \pm 40 \mu\text{M}$  measured (in several fields of view) at edges of the wild-type culture grown in constant light, branching frequency of the same strain grown in the dark was only once every  $220 \pm 60 \mu\text{M}$ . No photoeffect on hyphal branching frequency was observed in the white collar mutant strains *wc-1* and *wc-2* (Fig. 1), which in the dark and in the light exhibited a branching event at an average of once every

$230 \pm 60 \mu\text{M}$ , a frequency which was comparable to that of the dark-grown wild type. All microscopic examinations were restricted to apical hyphal cells at the periphery of microcolonies. In all other areas, hyphal layers were highly intertwined (data not shown), making it impossible to determine hyphal branching frequency.

Results established that illumination increases apical hyphal cell branching frequency and that functional *wc-1* and *wc-2* gene products are required for this photoeffect.

### *Effects of Light on cot-1 Transcription in Wild-Type and White Collar Mutants*

Since light affected branching frequency (Fig. 1) and because the *cot-1* product has been shown to be involved in hyphal elongation and branching (Yarden *et al.*, 1992), we set out to determine the effect of light on *cot-1* expression. RNA was extracted from mycelia that had been exposed to light for different periods of time, and *cot-1* transcript abundance was analyzed in Northern experiments (Fig. 2). Results showed that *cot-1* encodes two transcripts. From their migration in formaldehyde/agarose gels, the size of the longer *cot-1* mRNA species (*cot-1(l)*)

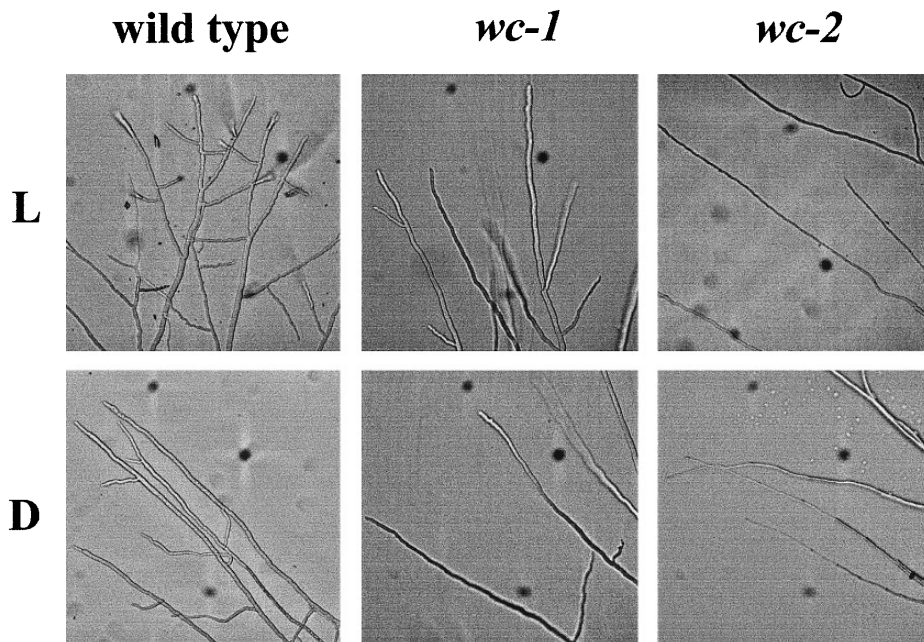


FIG. 1. Photoeffect on hyphal morphology in the wild type and *wc-1* and *wc-2* mutant strains of *Neurospora crassa*. Strains were grown in constant light (L) or in complete darkness (D) for 24 h at 34°C on depression slides containing Vogel's minimal medium with 2% sucrose. Periphery of young colonies was examined by light microscopy. Colony edges are shown.

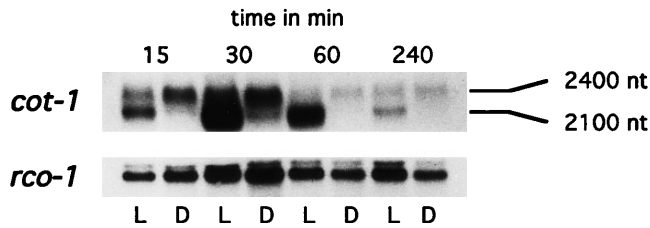


FIG. 2. Photocontrol of *cot-1* transcription. Wild-type mycelium, grown for 24 h at 34°C in the dark in Vogel's minimal medium with 2% sucrose, was harvested by filtration; the filtered pads were halved, and either illuminated (lanes L) for 15, 30, 60, or 240 min, or kept in the dark as controls (lanes D). Light and dark exposures were terminated by quick-freezing the half-pads in liquid nitrogen. Total RNA was extracted from each mycelial half-pad. RNA (8 µg per lane) was resolved by formaldehyde/agarose gel electrophoresis and transferred to a nylon membrane. Membranes were probed with <sup>32</sup>P-labeled antisense *cot-1*- and *rco-1*-specific RNA and visualized by autoradiography. Probes are indicated on the left. Size estimations in nucleotides (nt) of the two *cot-1* mRNA species are indicated on the right.

was estimated to be approximately 2400 nt, and that of the shorter *cot-1* mRNA species (*cot-1(s)*) approximately 2100 nt (Fig. 2). Following a 15-min exposure to white light, *cot-1(s)* mRNA accumulated, and transcript levels peaked after 30 min of illumination. *cot-1(s)* mRNA levels decreased after 60 min of illumination and had further diminished by the end of the 4-h illumination period. Illumination of the mycelial pad was a prerequisite for *cot-1(s)* transcript accumulation, as when analyzed immediately following harvesting (time = 0), only the *cot-1(l)* transcript was observed (data not shown), at an abundance similar to that found in extracts from cultures maintained in the dark, 15 min after harvesting (see Fig. 2, left lane).

Photoinduced increase of *cot-1(s)* mRNA abundance was accompanied by a photoinduced decrease of *cot-1(l)* mRNA levels (Fig. 2; compare also Figs. 3 and 4). This light-induced decrease was observed over the time course of the experiment, [a shorter exposure of the Northern blot presented in Fig. 2 indicated the presence of *cot-1(l)* transcript at a level which was intermediate between time = 15 and time = 60 (data not shown)]. As a second phenomenon, the decrease of the *cot-1(l)* mRNA species was observed upon prolonged and photoindependent exposure (60 min and 240 min, respectively) of the mycelium to aerial conditions (Fig. 2).

Transcript abundance of *rco-1*, encoding a regulator of conidiation (Yamashiro *et al.*, 1996), was measured as a control (Fig. 2). Previous studies have shown that *rco-1* transcript accumulation is not influenced by light

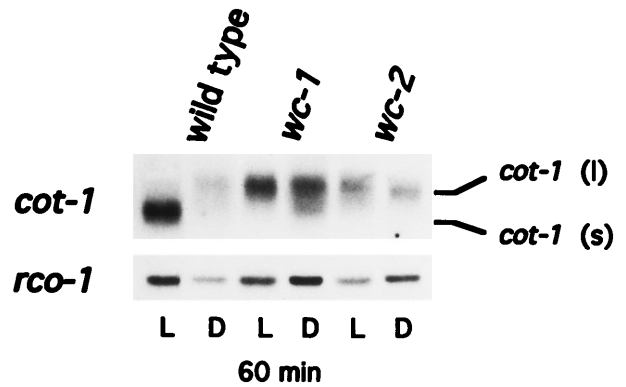


FIG. 3. Photocontrol of *cot-1* in a white collar mutant background. Wild-type, *wc-1*, and *wc-2* mutant mycelia were grown for 24 h at 34°C in the dark in Vogel's minimal medium with 2% sucrose. The mycelia were harvested by filtration, the filter pads were halved, and half-pads were either exposed to light (lanes L) for 60 min or kept in the dark (lanes D). Incubation was terminated by freezing in liquid nitrogen. RNA was extracted from mycelial samples and total RNA (8 µg) was loaded per lane and analyzed as described in the legend of Fig. 2. The two *cot-1* mRNA species, the 2400-bp longer transcript (*cot-1(l)*) and the 2100-bp shorter transcript (*cot-1(s)*), are indicated on the right.

(Lauter *et al.*, 1997). In addition to the wild type, light-inducibility of *cot-1* was also analyzed in the white collar mutant strains *wc-1* and *wc-2* (Fig. 3). No significant difference could be detected in *cot-1(s)* and *cot-1(l)* transcript accumulation between illuminated and dark-



FIG. 4. Photocontrol of *cot-1* transcription in the presence of L-sorbose. Wild-type mycelia, grown for 24 h in the dark at 34°C in Vogel's minimal medium with either 2% sucrose or 2% L-sorbose (with the addition of 0.05% glucose and 0.05% fructose) were harvested by filtration. The filter pads were halved, and half-pads were either illuminated (lanes L) for 30 min or kept in the dark (lanes D). After light and dark exposures, samples were treated as described in the legend of Fig. 2. Membranes were probed with <sup>32</sup>P-labeled antisense *cot-1*-, *con-10*-, and *rco-1*-specific RNA. Probes are indicated on the left.

grown cultures (Fig. 3). These results established that photoregulation of *cot-1* is dependent on functional *wc-1* and *wc-2* gene products.

### Influence of L-Sorbose on *cot-1* Transcript Size

In addition to light, L-sorbose comprises another environmental cue that controls hyphal branching frequency in *Neurospora* (Tatum *et al.*, 1949). In the presence of sorbose, independent of light, hyphal branching frequency is greatly enhanced resulting in a colonial growth morphology in liquid and on solid media. To determine whether light influences the effect of L-sorbose on *cot-1* transcription, wild type was grown in liquid shaking cultures at 34°C in Vogel's minimal media with either 2% sucrose or 2% L-sorbose. After 24 h in constant darkness, mycelia were illuminated for 30 min as described (see Fig. 2). RNA was extracted from the different samples and subjected to Northern analysis (Fig. 4). In the presence of L-sorbose, only the *cot-1(l)* transcript species was detectable, and the *cot-1(s)* transcript species was not photoinducible. Thus, despite its influence on hyphal branching frequency, L-sorbose did not stimulate expression of the *cot-1(s)* transcript. Transcript accumulation of another photoregulated gene, *con-10* (Lauter and Russo, 1991; Lauter and Yanofsky, 1993), was analyzed as a control (Fig. 4, center panel). In contrast to *cot-1*, photoregulation of *con-10*

remained functional under the described growth conditions.

### Mapping the Origin of the Difference between *cot-1(l)* and *cot-1(s)* Transcripts

To determine the structural difference between the two *cot-1* mRNA species, a series of RT-PCR, 5' RACE, and 3' RACE experiments were performed. A set of *cot-1*-specific oligonucleotides was synthesized (Table 1) for use as primers in PCR experiments and to scan *cot-1* mRNA for differences between *cot-1(l)* and *cot-1(s)* (Fig. 5). All RT-PCR and 3' RACE experiments resulted in a single amplicon (PCR amplification product), independent of whether RNA from illuminated (60 min) or dark control mycelia was used as the template. To ensure that the obtained PCR products were *cot-1*-specific, amplicons were cloned and sequenced. This procedure enabled us to sequence a set of *cot-1* cDNA clones and resulted in the identification of additional sequence information from the 3' end of *cot-1*, and the identification of an additional intron and a frame shift (Fig. 6) that had escaped detection in the original *cot-1* sequence (Yarden *et al.*, 1992). Results from 3' RACE experiments enabled us to identify an additional 109 bp at the untranslated 3' end of *cot-1* (Fig. 6, position 3376–3485). RT-PCR results also led to the identification of an additional intron, designated intron II

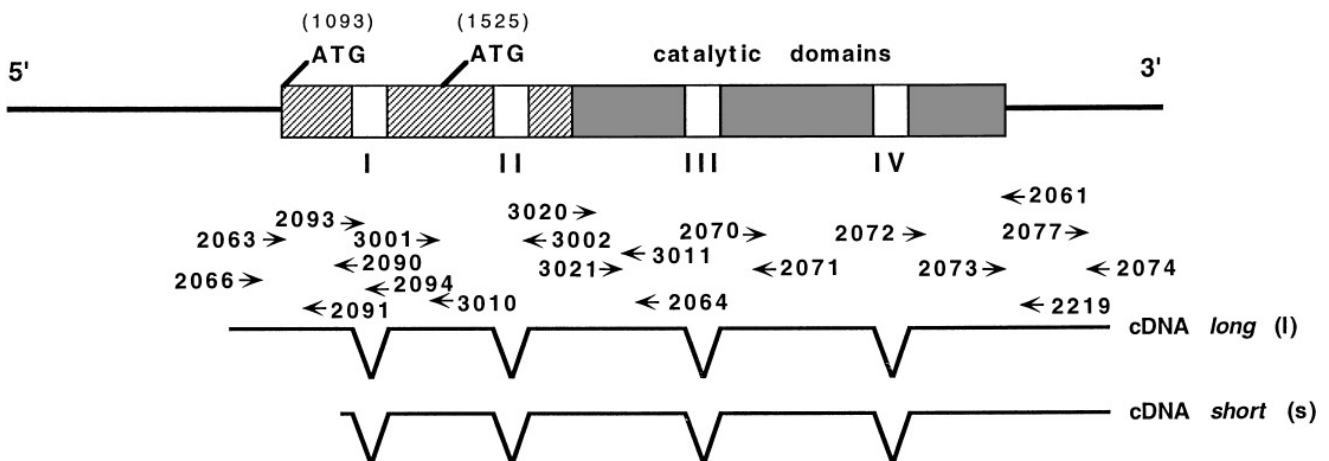


FIG. 5. Organization of the *cot-1* gene. The four introns of *cot-1* (I–IV) are presented as white boxes. The catalytic domains of the encoded kinase are presented as dark-shaded boxes. The putative regulatory domains are presented as light-gray shaded boxes. The putative translational start codons (ATG) for the COT1 (*l*) and COT1 (*s*) polypeptide are indicated together with their positions within the *cot-1* sequence presented in Fig. 6. The two *cot-1* cDNA species, *cot-1(l)* and *cot-1(s)*, are shown. Positions of the introns within the cDNAs are indicated as gaps. The arrows indicate the position of the primers, listed in Table 1, used for the RT-PCR, 5' RACE, and 3' RACE experiments.

CCGGGAGTGCCCGTCCAGCCAGTAACAAATCCCGGACCAGAGTCTCGATCTCTCGAGGTCCAATGCCGTTGCGTCGAGCTTCAACACTCGGGGA 100  
 CTGGTGGTTTACCTATAGTGTGCTTGTGAGGCACGAGAAGATGGCAGCAGTGGCGAACAGACAGCAACGAGTTGACGCTGACGATGAGGTTGGAGTCAG 200  
 GGTTTTACAGTTGACCAGGGCACCCCATAGTTAGGTGGTCTGTTTCGGCCTGGAGCTCACCACTGGACCTGGGCATGTCATCCAAACATGGAACCCC 300  
 TCTTTCTACGACCTCTTATCTCTGGGCATCGTGTACTAGATTACATCGAGGCCAGCTTCTCTGTGGCAACAGAAAACGGAACGTAAGTCCATTGTTG 400  
 TCTGTTGTTATTCAAGGTGCTCTTGAATGCGCCCGTGGCTGTCTCATATCATCACAGTCTATGTCTTGGTCACTTATAGATGGGATCTGTTAGGTTCCA 500  
 TGTACCTCTATACGTCTAGGCTTTCCTTTTATACTGATACCGCAGGCACCCCTGGCCCACTGCTTGGCCTTGGCCTTGGCTGCTGATTCGTGGCCCTA 600  
 GGTTCGAAATTACAGAGGCCCTGAATCGCCTCCAGCCAGTCTCTCTGTGAGACTCTTCTGTAGGTAGGCTGGAGAGGCTCACGTCGGTCACGCTT 700  
 CCACTTTGCTTCCACTTCCACACTGCGCCTATCGACTGCACGTTCCAGCGGGACACTTCCACTTCGGACGGTGCCACTCAACCACCTGCCCCCAGCCT 800  
 GGCCGCCCTCGATTCCCATCCCTACCATCTACAGTAGCCAACCGTCTGTTCCGATCTCTGTTGGACCTGGTCCCAAGAAAAGCAACCTCTTCCCTCCCG 900

E long

TCTCCCATACCTATCCCGTCTGTACACAAACCCCTTCTGTGCGCATAGATCCCATCGATCGGCTGGGAGCTCGACTCTGATCGTTTCTCCATCTGC 1000  
 CGTCCCTCGGAATCTCGTCTGAGCTCGCTTGGCCCTTCCCGCTACATACACCCGGAATCCAAGTGTTCACCGTTTAGAGACAAGTACCAGATGGACAA 1100  
M D N

CACCAACCGCCCCATCTCAACCTGGGCACCAACGATACCCGCATGGCTCCAAACGATCGTACCTATCCACCACCCCGTCCACCTTCCCCAACCCGTC 1200  
 T N R P H L N L G T N D T R M A P N D R T Y P T T P S T F P Q P V

E short

TTCCAGGTCAACAAGCCGGTGGTTCCAGCAGTACAACCAGGCCCTACGCCAGTCCGGCAACTACTATCAACAGAACCAGTATGCCAGCCACTACGCC 1300  
 F P G Q Q A G G S Q Q Y N Q A Y A Q S G N Y Y Q Q N H

CTCCTGTTGCCCGGACTACGGCTTCGTTCAAGCTCATTTCAACATTGACGGTACTAGCAACGACCCCAACTGGTCTCGCCACCAGTTCGCCCATCA 1400  
N D P N T G L A H Q F A H Q

GAACATTGGCAGCGCCGAAGAGCCTCTCCCTACGGTCTCTGTTGGCCATCTCCCGTCAACGCCCTCGCACATCCGAAACTCTGGCCAGCAGCAAAC 1500  
 N I G S A G R A S P Y G S R G P S P A Q R P R T S G N S G Q Q Q T

TAGGGCAACTATCTGAGCGCCGATGCTTTCGAATACCCAGACCGAGTTGCTTCCCGCTCCGAGCGGAACCCGACAAATATGGCCCAATGCCAACA 1600  
 Y G N Y L S A P M P S N T Q T E F A P A P E R N P D K Y G P N A N N

ACAACCAGAAGAAGTGTCTACAGCTGGCCTGACTTCTTTAAGGACAGCGTCAAGCGCCAGGAGCGCAACCAGAGGTGAATGCTCGACGATCGCC 1700  
 N Q K K C S Q L A S D F F K D S V K R A R E R N Q R

TTTTGGCCCTACATCCCGCGTATCGGTGCTGACACCCTTTTCAGACAGAGCGAGATGGAGCAGAAGCTCGGTGAGACCAACGATGCCCGCGCTCGCGA 1800  
Q S E M E Q K L G E T N D A R R R E

ATCGATCTGGTCAACCGCTGGCCGGAAGGAGGGCCAGTATTTGCGCTTCTGAGAACCAAGGACAAGCCCGAGAACTACCAGACCATCAAGATCATCGGC 1900  
 S I W S T A G R K E G Q Y L R F L R T K D K P E N Y Q T I K I I G

AAGGGCCTTTTCGGCAGGTTCAAGCTCGTGCAGAAGAAGGCCAGCGCAAGGTTTACGCCATGAAGTTCGCTTATCAAGACGGAAATGTTCAAGAAGGACC 2000  
 K G A F G E V K L V Q K K A D G K V Y A M K S L I K T E M F K K D Q

AGCTTGCCACGTCGGAGCAGAAAGAGATACTTGGCCGAGTCCGACAGCCATGGGTTGTCAAGCTCTACACCACCTTCCAGGATCGCAACTTCTTTA 2100  
 L A H V R A E R D I L A E S D S P W V V K L Y T T F Q D A N F L Y

CATGCTCATGGAGTTCTTGGCCGGTGGTACTTGTATGACCATGCTGATCAAATACGAAATCTTCTCGGAGGATATTACCCGTTTCTACATTGCGGAAATC 2200  
 M L M E F L P G G D L M T M L I K Y E I F S E D I T R F Y I A E I

GTCTTGGCTATCGACGCGGTCATAAGCTGGGCTTCATTCACAGGTAAGCTTCGCCGGAAGCAATGCTCTGGAAGAGATACTGACACGGTTGTGCATG 2300  
 V L A I D A V H K L G F I H R

CAGAGATATTAAGCCAGACAACATCTTCTTGTATAGGGTGGTCAATGTCAGGCTTACAGATTTCGGTCTCTTACCAGCTTCCACAAGCTCCATGATAAC 2400  
 D I K P D N I L L D R G G H V K L T D F G L S T G F H K L H D N

AACTACTACACAACCTGCTCCAGGGCAAATCCAACAAGCCGCGGACAACCCGCAACTCGGTTGCTATTGACCAAAATCAACCTGACGGTCAGCAACCGTG 2500  
 N Y Y T Q L L Q G K S N K P R D N R N S V A I D Q I N L T V S N R A

CACAGATCAATGACTGGAGACGCTCCAGACGTTTGTATGGCCTACTCGACCGTTCGGTACACCAGATATATTTGCTCCGAAATCTTCACTGGCCATGGTTA 2600  
 Q I N D W R R S R R L M A Y S T V G T P D Y I A P E I F T G H G Y

CTCGTTTGTATGCGATTTGGTGGTCTTTGGGTACCATCATGTTCCAGTGTCTTGGTTCGGCTGGCCTCTTCTGCGCCGAGGATAGCCACGACACTTACCGC 2700  
 S F D C D W W S L G T I M F E C L V G W P P F C A E D S H D T Y R

AAGATCGTGAACCTGGAGGCACTCGCTTACTTCCCGGATGACATCACCTTGGTGTAGATGCCGAGAACCTTATCAGAAGGTATGCATGCTTTTACTTTC 2800  
 K I V N W R H S L Y F P D D I T L G V A D A E N L I R S

CCCCAAAATCACGCGCTCACCATATTGACTTGTATAGCCTCATCTGCAACACTGAGAACCGCTTCGGCCGTTGGTGGTCTCACGAAATCAAGGCCAGG 2900  
L I C N T E N R L G R G G A H E I K S H A

CCTTCTTCCGCGCGTTGAGTTGACAGCTTGGTTCGCATCCGTGCGCCCTTCGAGCCCGTCTTACATCCGCCATCGATAACACATACTTCCCTACGGA 3000  
 F F R G V E F D S L R R I R A P F E P R L T S A I D T T Y F P T D

CGAGATTGACCAGCGGATAACGCCACCGTGTCAAGGCTCAGCAGGCCGTTAGGGGCGCCGCTGCCCTGCGCAGCAGGAGGAGGCCAGAACTCAGC 3100  
 E I D Q T D N A T L L K A Q A A R G A A A P A Q Q E E S P E L S

TTGCCTTTCATTGGATACGTTCAAGCGTTTCGACAACAACCTCCGATAATTGCGTTACGCTTCTCTGCTGTCTGAATAATGAGCGTTGGTTTTCTTGG 3200  
 L P F I G Y T F K R F D N N F R \*

GGGGGAGGAGGGGGCGTAATAGTGTGGCGAAAGGGAGAAGAGGTTGTGCTGCGGGGCGGCACTATCTGGGTACCCTTATACTTGGCTTGGAGCTCT 3300  
 TTGCTAGCTCGCGGTGCTGTTGAAATTTCTGATGGCAGAGACACTTTGCTGATAGTACGCAATTTCCGTTCCGCATATATGGCTGAATAAGTACAGAA 3400  
 ACATAATGTATGGACTGCATCTTCTGGCGAGGAACTGGGAAGCCCTGAAGCGGACAACGGGGGAGAATAAAAGCGGCAAGAGAC 3485

(Fig. 5) at position 1678–1747 in the *cot-1* sequence shown in Fig. 6. In addition, an extra cytosine was identified within the *cot-1* nucleotide sequence at position 1557 (Fig. 6). This cytosine insertion changed the reading frame relative to the previously reported *cot-1* sequence. This change, combined with the identification of intron II (Fig. 5), resulted in a new COT1 amino acid sequence between positions 1557 and 1680 (Fig. 6). To verify that the difference between *cot-1(l)* and *cot-1(s)* is not at the 3' end of *cot-1*, primers 2074 and 2077 (Fig. 5 and Table 1) were designed to PCR-amplify a 78-bp DNA *cot-1* fragment from the very 3' end of the gene (as determined by 3' RACE experiments). The resulting PCR product was cloned and used as a hybridization probe in Northern experiments with RNAs isolated from 60-min-illuminated and dark-grown mycelia (the same RNAs used for the experiments shown in Fig. 2). The *cot-1(l)* and the *cot-1(s)* mRNA species were detected by the 2074–2077 amplicon, establishing that the origin of the *cot-1(l)/cot-1(s)* size difference is not localized at the 3' end of *cot-1* (data not shown).

In contrast to the RT-PCR and 3' RACE experiments, 5' RACE resulted in the production of two amplicons, the lengths of which differed by about 300 bp. These 5' RACE products were cloned and sequenced. The 5' end of the longer 5' RACE product was localized to position 950 (Fig. 6) and that of the shorter 5' RACE product to position 1270, giving a difference of 320 bp (Figs. 5 and 6), in good agreement with the results obtained from the Northern experiments (Fig. 2).

To verify that the observed difference in the two 5' RACE products reflected the *in vivo* difference between the two *cot-1* mRNA species, Northern experiments were performed as controls. Based on the 5' RACE results new PCR primers were synthesized. With the primer combination 2066/2090 (Table 1), a DNA fragment was amplified from *cot-1* genomic DNA templates (pOY18; Yarden *et al.*, 1992), which was specific to the 5' end of the *cot-1(l)* mRNA species (Fig. 5). Based on the 5'-RACE results, the amplified DNA sequence was expected to be absent in the *cot-1(s)* mRNA species (Fig. 5). In addition, the primer combination 3001/3002 was used as a control to amplify a DNA sequence present in both *cot-1* mRNA species (Fig. 5). The two resulting PCR products were of identical length (320 bp) and were cloned and used as probes for

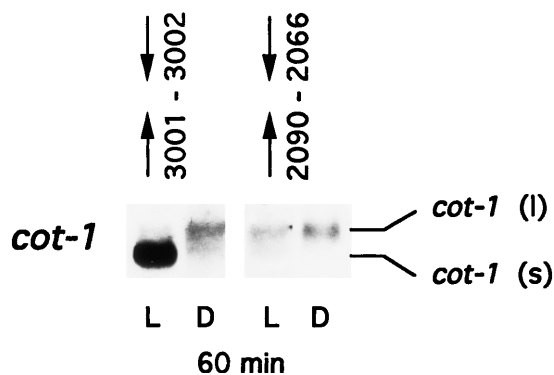


FIG. 7. The origin of the size difference between *cot-1(l)* and *cot-1(s)* is located in the 5' end of the gene. Primers 2090 and 2066 (see Fig. 5) were used to PCR-amplify DNA fragments from the very 5' end of *cot-1*. Primers 3001 and 3002 were used to amplify a control *cot-1* DNA segment present in both mRNA species (Fig. 5). Resulting PCR products were of identical length and used as hybridization probes prepared by hexamer-primed labeling. Probes (indicated at the top) were hybridized against total RNA (8  $\mu$ g per lane) from 60-min illuminated (lanes L) and dark-grown (lanes D) mycelia. *cot-1(l)* and *cot-1(s)* transcript species are indicated on the right.

Northern analyses. The PCR product 3001/3002 hybridized to both *cot-1* mRNA species, while the PCR product 2066/2090 hybridized exclusively to the *cot-1(l)* mRNA species (Fig. 7). These results established that the two mRNA species encoded by *cot-1* differ in their 5' ends (Figs. 5 and 6).

## DISCUSSION

Identifying and analyzing the genetic elements whose expression is affected by light is one way of advancing our understanding of the linkage between a major environmental cue and fungal development. In this report, we have been able to establish a connection between the expression of *cot-1*, a gene involved in hyphal elongation, and light.

Under most natural conditions, fungal growth rate is limited by the composition and availability of nutrients, as well as by the prevailing physical conditions in the organism's environment. The capacity of fungi to rapidly adapt to

FIG. 6. Nucleotide sequence of the *Neurospora crassa cot-1* gene and its flanking regions and the predicted amino acid sequence of COT1. 5' ends of *cot-1(l)* and *cot-1(s)* as determined by 5' RACE analyses are indicated. The putative translational start points (ATG) for COT1 (*l*) and COT1 (*s*) are underlined. The cDNA sequence of *cot-1* has been deposited in the GenBank database (Accession No. X97657).

changes in their growth niche is instrumental for successful survival and proliferation. Because one of the key forms of fungal growth is apical extension, extension rate and branching frequency are the basic parameters determining fungal biomass accumulation. To efficiently govern these processes, sensory mechanisms linked to the fungal cell growth machinery via signal transduction pathways are required. This study provides evidence of a linkage between an environmental cue—light—and the differential expression of a regulator of hyphal elongation. We demonstrate that *cot-1* encodes two transcripts and that different abundance of the two are observed in cultures grown in the light or dark. A comparison between the *cot-1(l)* and *cot-1(s)* transcripts revealed the two to be identical, except for a 320-bp truncation at the 5' end of the transcript. A putative translation initiation codon of the *cot-1(s)* transcript was identified. If the identified in-frame ATG (position 1525; Fig. 6) is, in fact, the translation start site of the light-induced transcript, the corresponding COT1(l) and COT1(s) polypeptides may be isozymes with different activities. Such a difference has been demonstrated by Kasuya *et al.* (1995) in the case of two cGMP-inhibited (cGI) cAMP phosphodiesterase (PDE) isozymes. The corresponding cGI-PDE gene was found to encode two transcripts that differed at the 5' end (one transcript was approximately 1290 nucleotides shorter) which were expressed in human placenta and cardiac tissue. Recombinant cGI-PDE genes (differing at the 5' end) were expressed in SF9 cells and exhibited differences in subcellular localization and in their binding affinity ( $K_m$ ) for cGMP. In *N. crassa*, on the other hand, the production of alternative COT1 polypeptides, as well as the structural and functional significance of the differences between the two, has yet to be determined. However, it is also possible that the alternative transcripts and corresponding polypeptides may be linked to cellular localization or substrate- and/or additional kinase subunit-specificity. We observed different branching frequencies in cultures of *N. crassa* grown under light or dark conditions. The presence of *cot-1(s)* and *cot-1(l)* transcripts was also influenced by light. To obtain additional information on the extent of the linkage between branching and *cot-1* transcript length, we grew *N. crassa* in the presence of the hyperbranching-inducing sugar sorbose. In contrast to light, no changes in the length of *cot-1* mRNA were detected in response to L-sorbose. Therefore, the toxic effect of L-sorbose does not appear to be transduced via alterations in *cot-1* transcription. Different signal transduction pathways must therefore exist influencing elongation/branching frequency in *N.*

*crassa*. The presence of converging signal transduction pathways in *Magnaporthe grisea* and *Ustilago maydis* has been suggested by Xu and Hamer (1996) and Gold *et al.* (1994), respectively. Evidence has been presented to support the involvement of cAMP signaling with other pathways (e.g., the MAP kinase cascade) in regulating fungal morphogenetic events such as hyphal growth and appressorium formation. Furthermore, Xu and Hamer (1996) suggest that proper coordination between the cAMP-dependent and MAP kinase hierarchies is essential for pathogenesis. Despite the phenotypic consequences, we have no direct evidence for linked pathways in the case of light- and sorbose-induced branching in *N. crassa*, it is conceivable that the pathways transducing light and sorbose effects also interact and/or converge at points. This view is supported by the observation that photoregulation of *cot-1* is blocked in hyphal cells that have been exposed to L-sorbose. Under the same conditions, photoinducibility of another light-regulated gene, *con-10*, was not altered. Therefore, different blue-light signal transduction pathways must exist in *N. crassa*, one being sorbose-repressible and another being sorbose-independent. Previous studies have already identified *wc-2*-dependent and *wc-2*-independent light transduction pathways (Linden and Macino, 1997; Crothwaite *et al.*, 1997).

In the C4 plant *Flaveria trinervia*, a linkage between light, alternative transcript length, and differential localization has been described (Rosche and Westhoff, 1995), involving alternative transcripts of the *pdk* gene (encoding a pyruvate orthophosphate dikinase), which are differentially expressed under varying lighting conditions and in different plant tissues. Thus, a light-inducible 3.4-kb *pdk* transcript (encoding for chloroplastic isoform of the dikinase) accumulated in leaf and stem tissues, whereas a shorter, 3.0-kb transcript, encoding a truncated *pdk* gene product lacking the plastidic transit peptide, accumulated in roots. Darkening led to a drastic depletion of the 3.4-kb transcript in both leaves and stems and the concomitant accumulation of the 3.0-kb transcript in stems and roots (but not in leaves). Similar to *cot-1*, the difference between the short and long transcripts was localized to the 5' end. Furthermore, in both cases it was suggested that the difference in the encoded polypeptide lies in an N-terminal truncation, which most likely results in the production of a highly similar gene product which lacks a signal peptide (present in the 3.4-kb translation product) and is thus predicted to be a cytosolic isoform of pyruvate orthophosphate dikinase.

Light-regulated gene transcription is a well-documented phenomenon, as is differential transcription. However, reports of light-regulated differential transcription, as demonstrated in this study, are very few. Furthermore, the existence of a linkage between light-induced transcription, fungal growth and development, and a possible linkage to other signal transduction pathways is becoming evident. Identifying additional genes and gene products interacting with *cot-1* as well as with components of the light-transducing machinery (e.g., *wc-1* and *wc-2*) will help to better understand the hierarchy of events governing hyphal elongation.

## ACKNOWLEDGMENTS

We thank Amir Sharon for his critical comments on the manuscript. This work was supported in part by BARD (U.S.-Israel Binational Research and Development Fund). F.-R.L. was supported by a fellowship from the European Molecular Biology Organization (EMBO, ASTF 7528).

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