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The *Neurospora crassa* colonial temperature-sensitive 3 (*cot-3*) gene encodes protein elongation factor 2

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Abstract At elevated temperatures, the *Neurospora crassa* mutant colonial, temperature-sensitive 3 (*cot-3*) forms compact, highly branched colonies. Growth of the *cot-3* strain under these conditions also results in the loss of the lower molecular weight (LMW) isoform of the Ser/Thr protein kinase encoded by the unlinked *cot-1* gene, whose function is also involved in hyphal elongation. The unique *cot-3* gene has been cloned by complementation and shown to encode translation elongation factor 2 (EF-2). As expected for a gene with a general role in protein synthesis, *cot-3* mRNA is abundantly expressed throughout all asexual phases of the *N. crassa* life cycle. The molecular basis of the *cot-3* mutation was determined to be an ATT to AAT transversion, which causes an Ile to Asn substitution at residue 278. Treatment with fusidic acid (a specific inhibitor of EF-2) inhibits hyphal elongation and induces hyperbranching in a manner which mimics the *cot-3* phenotype, and also leads to a decrease in the abundance of the LMW isoform of COT1. This supports our conclusion that the mutation in *cot-3* which results in abnormal hyphal elongation/branching impairs EF-2 function and confirms that the abundance of a LMW isoform of COT1 kinase is dependent on the function of this general translation factor.

Key words Elongation factor 2 · *Neurospora crassa* · Fusidic acid · *cot-3* · Hyphal branching

Introduction

Neurospora crassa is a filamentous fungus that grows on semisolid media by forming spreading colonies. Like most filamentous fungi, *N. crassa* forms hyphae that elongate and branch to generate a mycelial mat. Continuous hyphal elongation and branching results in the formation of spreading radial colonies on solid media and of submerged spherical cellular aggregates during aerated growth in liquid media.

In *N. crassa*, many “colonial” genes have been identified which, when mutated, result in compact colonial growth under conditions that favor spreading growth in the wild type (Perkins et al. 1982). Some of these genes have been designated colonial temperature-sensitive (*cot-1*, -2, -3, -4 and -5), since their mutant alleles confer colonial growth at 32 °C and above, but normal or near-normal growth, morphology and fertility at or below 25 °C (Perkins et al. 1982).

The best characterized of these conditional colonial strains is *cot-1*. *cot-1* forms very small, slow-growing colonies at elevated temperatures, as the result of a virtual cessation of hyphal tip elongation and a concomitant increase in hyphal branching. These cultures rapidly resume normal growth when shifted to a permissive temperature (Mitchell and Mitchell 1954; Collinge et al. 1978; Yarden et al. 1992). The *cot-1* gene has been shown to encode a Ser/Thr protein kinase, which could be involved in the transduction of signals that govern cell-shape determination (Yarden et al. 1992; Lauter et al. 1998).

In a recent study, Gorovits et al. (1999) analyzed the expression pattern of the COT1 polypeptide in *N. crassa*. Two main polypeptides were detected in the wild type: a predominant one of 73 ± 2 kDa and a less abundant one of 67 ± 2 kDa, indicating the presence of two COT1 isoforms. The 73-kDa COT1 isoform was present in the

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five *cot* mutants analyzed (*cot-1*, *cot-2*, *cot-3*, *cot-4*, *cot-5*), irrespective of whether they were grown at the permissive or the restrictive growth temperature. However, the abundance of the 67-kDa isoform was significantly lower in *cot-3* and *cot-1* strains grown at the restrictive temperature. Since the abundance of the 67-kDa isoform is much lower in both *cot-1* and *cot-3* mutants at the restrictive temperature, it was proposed that the products of these two genes might be functionally linked in the control of hyphal elongation/branching (Gorovits et al. 1999).

We cloned and characterized the *N. crassa cot-3* gene and show here that it encodes the elongation factor 2 (EF-2) protein. Based on these results, we suggest that proper hyphal elongation, and the abundance of the 67-kDa COT1 isoform, both depend on EF-2 function.

Materials and methods

N. crassa strains, media, growth and transformation conditions

N. crassa wild-type (74-OR23-1A; FGSC987), *cot-3* (FGSC 1517) and *cot-1* (FGSC 4065) strains were used in all experiments. Procedures used for fungal growth studies are described in Davis and de Serres (1970). Strains were grown in either liquid or solid (containing 1.5% agar) Vogel's medium with 2% (w/v) sucrose or 1.5% (w/v) L-sorbose.

For complementation of *cot-3*, the Orbach/Sachs *N. crassa* genomic DNA cosmid library (Fungal Genetics Stock Center) was used. Transformation of *cot-3* was performed as described by Orbach et al. (1986). When appropriate, the medium was supplemented with 100 µg/ml hygromycin B (Calbiochem or Boehringer Mannheim). Fusidic acid (Sigma) was used to study the effects of inhibition of EF-2 on morphology and COT1 kinase expression pattern. Fusidic acid was added (from a 300 mM stock concentration in ethanol) to either liquid or molten solid medium at the appropriate concentrations. The effect of fusidic acid on fungal growth was monitored by determining fungal colony growth rates and observing the growth pattern by microscopy. The effect of fusidic acid on the expression pattern of COT1 kinase was determined by Western analyses as described by Gorovits et al. (1999).

To produce synchronized conidiating cultures, conidia (10^7 per ml) were rehydrated overnight (at 4 °C) in Vogel's sucrose medium, transferred to an orbital shaker (120 rpm) and grown for 1.5–19 h at 25 °C or 37 °C, as required. The cultures were filtered through a 90 mm No. 1 Whatman filter disc on a Büchner funnel and the mycelium was transferred to a petri dish containing glass beads (5 mm diameter) submerged in liquid medium which just covered the beads. The cultures were exposed to air for either 7 h or 19 h and harvested from the paper filters at various times.

Nucleic acid isolation and DNA sequencing

Genomic DNA and RNA were isolated from *N. crassa* as previously described (Yatzkan et al. 1998). The mutant *cot-3* allele was amplified by PCR using the primers c3+272 (5'-CCACACCG-cACGTCTAAACACC-3'), c3+1561 (5'-CAGCGTACCGTCCT-CATGAT-3'), c3+1082 (5'-GGTCGATCGTGCTCTTCTCG-3'), c3-1561 (5'-ATCATGAGGACGGTACGCTGA-3') and c3-3216 (5'-TGAGAACGGTGCCATACGC-3'). PCR products were isolated using Gene Clean resin (Bio 101) and sequenced without subcloning.

Bidirectional DNA sequencing was performed at Canadian Molecular Research Services using an automated, IR2 system (Li-Cor). A T7 Sequenase system (Amersham) was used to manually

confirm some ambiguous regions. Sequence analysis was performed using the GCG (v10.0) software package maintained at www.cbr.nrc.ca.

Northern analyses

RNA samples were transferred to Magnacharge NT Nylon membranes (MSI). The blots were probed with a [α -³²P]dCTP hexamer-labeled DNA probe (Prime-A-Gene, Promega) prepared from the 5-kb *Bam*HI-*Not*I insert isolated from pB1 (harboring the *cot-3* gene) as template. Hybridization was performed at 44 °C in the presence of 50% formamide, 5× SSC, 2× Denhardt's solution, 1% (w/v) SDS, 50 mM sodium phosphate (pH 6.5), 100 µg/ml yeast tRNA and 100 µg/ml salmon sperm DNA. The most stringent washes were carried out at 68 °C with 2× SSC and 1% SDS.

tub-2 RNA levels were determined by probing Northern blots with a hexamer-labeled 1.2-kb *Sac*I fragment isolated from pBT3 (Orbach et al. 1986).

cot-3 was mapped by restriction fragment length polymorphism (RFLP) analysis of the "small cross", according to the procedure of Metznerberg et al. (1985).

Light and electron microscopy

For light microscopy, samples were viewed with a Zeiss Axioscope microscope. Photographs were taken with Fujichrome 100 ASA film.

For scanning electron microscopy, colonies were grown on cellophane membrane overlays, on Vogel's minimal medium plates, for 48 h at 22 °C and then shifted to 37 °C for 7 h. Small, 5-cm² sections of cellophane were removed, mounted and rapidly frozen by plunging into liquid N₂. Specimens were viewed without coating, using a JEOL JSM scanning electron microscope.

Results and discussion

cot-3 morphology

In order to determine the extent of the morphological changes caused by the *cot-3* mutation, we conducted a microscopic analysis of *cot-3* grown at permissive and restrictive temperatures. When grown at 25 °C, no clear differences in the rate of hyphal elongation or the frequency of hyphal branching were observed between mature cultures of *cot-3* and wild-type strains. Nonetheless, at temperatures below 32 °C (regarded as permissive for the growth of *cot-3*), we noticed a clear difference in morphology between *cot-3* and the wild type at the early stages of conidial germination on solid media. When conidia were germinated at 25 °C, a lag period of approximately 8 h was observed prior to the formation of normal germ tubes. Even 36 h post-germination, it was evident that the hyphae which initially emerged from the *cot-3* conidia were curled rather than straight as is characteristic of the wild type (Fig. 1A and E). However, once growth had progressed, the linear extension and branching frequency of *cot-3* were very similar to those observed in the wild type (Fig. 1B). Once growing cultures were shifted to the restrictive temperature, formation of curled hyphae was once again observed in the young areas of the colony (Fig. 1C). This growth form was maintained for as long as the culture

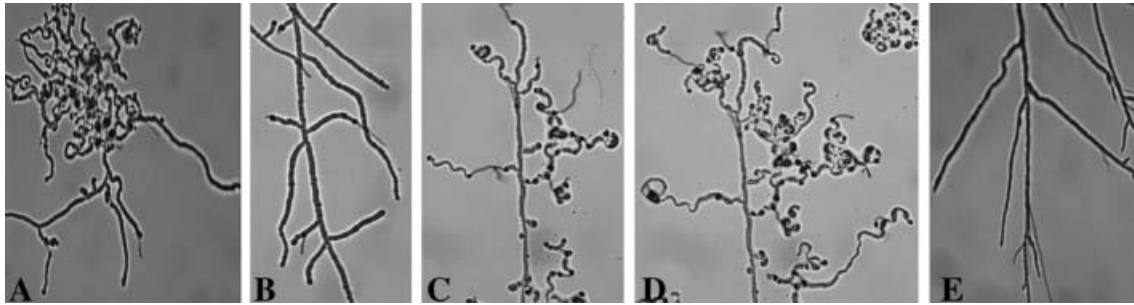


Fig. 1A–E Morphology of *cot-3*. **A** A *cot-3* colony, 36 h after germination at 25°C on solid Vogel's minimal medium. **B** The colony edge 60 h after germination. **C, D** Edges of a *cot-3* colony germinated at 25°C and shifted to 34°C, 4 (**C**) and 10 h (**D**) after the temperature shift. **E** The edge of a wild-type colony germinated at 25°C and shifted to 34°C, 6.5 h after the temperature shift

was kept at the restrictive temperature (Fig. 1D). When germinated at the restrictive temperature, the *cot-3* mutant produced abnormal microcolonies with bud-like, hyperbranching hyphae (Fig. 2).

Within 7 h of transferring *cot-3* cultures from 22 °C to 37 °C, there was a rapid reduction in the rate of hyphal elongation, accompanied by the appearance of numerous branches. Hyperbranching occurred predominantly in the newly formed tips, while the pre-existing hyphae (which had grown prior to the temperature shift) did not produce multiple branches (Fig. 2C). This is in sharp contrast to the morphological change that occurs in *cot-1* cultures which, following a shift to the restrictive temperature, produce multiple branch initials along the entire mycelium (Collinge et al. 1978; Yarden et al. 1992).

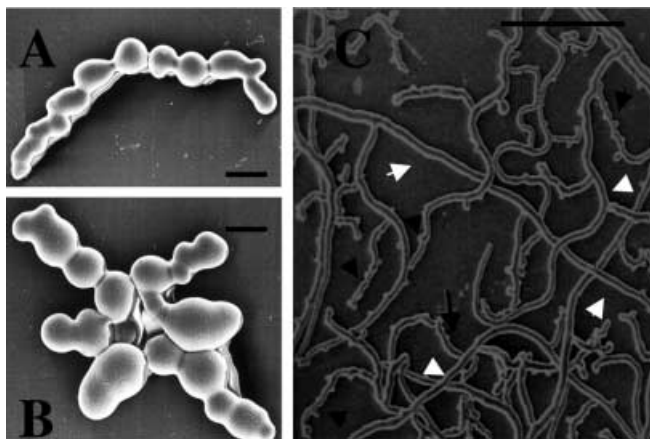


Fig. 2A–C Morphology of *cot-3*. Scanning electron microscopy was used to analyze *cot-3* morphology. **A, B** Microcolonies of *cot-3* grown at 37°C. **C** A segment of a *cot-3* colony grown at 22°C for 48 h and shifted to 37°C for 7 h prior to fixation. Note the normal branching pattern in the older mycelium (white arrows) and the increased, abnormal, branching frequency and hyphal morphology in younger hyphae (black arrows). The scale bars indicate 10 µM in **A** and **C** and 100 µM in **B**

Cloning of the *N. crassa cot-3* gene and mutation analysis

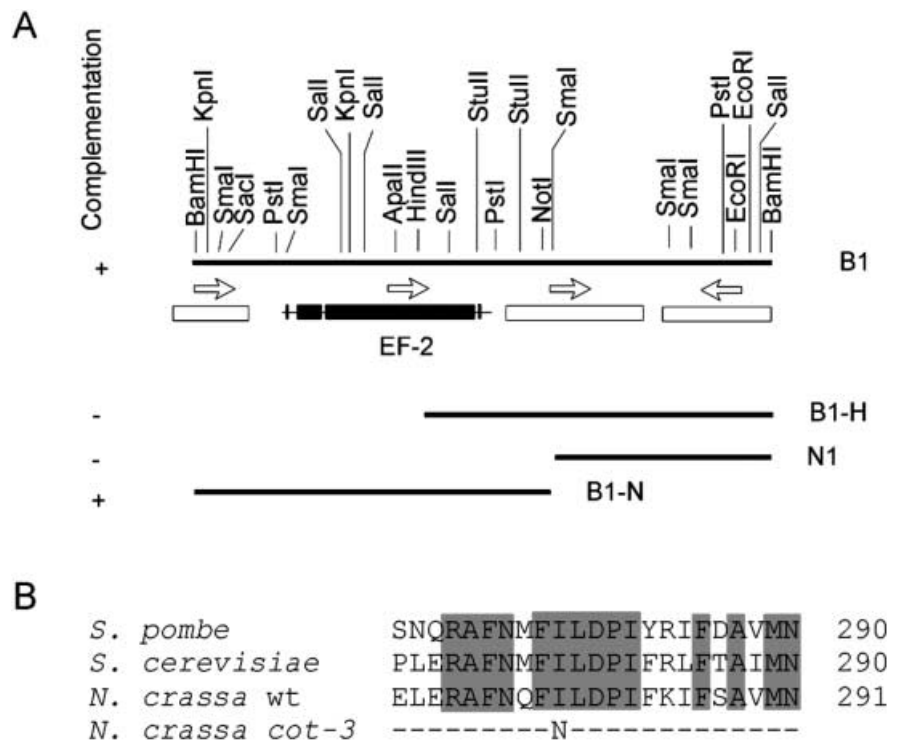
The *cot-3* gene was cloned by complementation of the *N. crassa cot-3* mutant. The fact that *cot-3* had previously been mapped to linkage group IV (Perkins et al. 1982) enabled us to reduce the number of screened cosmids to those which had been assigned to linkage group IV (<http://www.fgsc.net/walkdata/walkdata2.html>). Thus, protoplasts of the *cot-3* strain (prepared from germinating conidia produced at 25 °C) were transformed with two subpools (each consisting of 96 cosmids) of the *N. crassa* genomic cosmid library which are specific for linkage group IV.

The transformed protoplasts were plated on sucrose-containing regeneration medium supplemented with hygromycin, and incubated at 37 °C for 48 h. One of the cosmid pools yielded hygromycin-resistant colonies that displayed rapid, spreading growth and were thus regarded as *cot-3*⁺ transformants. A strategy for the identification of a single complementing clone from a standard microtiter dish described by Metzberg and Kang (1987) was used to isolate one cosmid (G15:E3), containing *cot-3*-complementing DNA. A 10-kb *Bam*HI fragment of G15:E3, which complements *cot-3*, was subcloned. This subclone was designated pB1. Further experiments showed that a 5.4-kb *Bam*HI-*Not*I fragment of pB1 was sufficient to complement *cot-3* (Fig. 3).

Sequence of *cot-3*

Both strands of pB1 were sequenced completely and analyzed for the presence of hallmarks of *N. crassa* genes. A nucleotide sequence resembling a putative CAATT box was identified 301 nt upstream from the tentative translation start site. No TATA box or consensus transcription start (TCATCAANC) sites (Bruchez et al. 1993b) were detected upstream of *cot-3*. However, the 5'-terminal sequences of three different *cot-3* cDNA clones, NM9B6-T3, SC2D3-T3 (<http://biology.unm.edu/~ngp>) and e3a03ne.f1 (<http://www.genome.ou.edu>) were all within 50 bp of the translation start site, suggesting that transcription of *cot-3* initiates in this region. The putative translation start site is located at nt 329. The CGCCAAAATGGT sequence is in good agreement with the conserved *N. crassa* translation initiation sequence (Bruchez et al. 1993a). A putative polyadenylation signal

Fig. 3A, B Physical map of the *cot-3* locus and mutant analysis. **A** Partial restriction map and complementation data for the 10-kb *Bam*HI fragment containing the *cot-3* (EF-2) gene. Complementation by the B1-N fragment is abolished by prior digestion with *Hind*III or *Sal*I, both of which cut within the EF-2 coding sequences only. Transcribed sequences are shown as a thin line, with exons represented by the shaded boxes. The sequence data reported in this paper have been deposited in the GenBank database under Accession No. AF258620. Additional ORFs have been identified within the sequenced region (J. Vierula, unpublished data) and are marked by the white arrows. **B** Amino acid sequence comparison illustrating the C-terminal end of the G domain of EF-2, and the location of the Ile → Asp substitution in the *cot-3* mutant



(ACAAAAT) at nt 3371 is located 20 bp from the presumed polyadenylation site GACC (Bruchez et al. 1993b).

The ORF is interrupted by three introns which are 216-, 54- and 60-bp long. All introns show the usual characteristics of *N. crassa* introns: they contain the consensus 5' splice-donor site (GTA/GA/CGT/C), the consensus 3' splice-acceptor site (C/TAG) and the lariat or internal element (G/ACTA/GAC) (Bruchez et al. 1993b). Intron positions were confirmed by comparing the genomic sequence with EST sequences NM3E7-T7, NC4G1-T7 and NM9B6-T3 from the *Neurospora* genome project (Nelson et al. 1997; <http://biology.unm.edu/~ngp/sequencedata>).

COT3 amino acid sequence: homology to elongation factor 2

The *cot-3* gene encodes a predicted 844-residue polypeptide with a calculated mass of 93 kDa and a pI of 6.26. A database search revealed that COT3 is homologous to the eukaryotic EF-2 protein (EF-G in prokaryotes). EF-2, formerly known as aminoacyltransferase II or transferase II, is a highly conserved protein comprised of a single polypeptide chain with an apparent molecular mass of 95–110 kDa. The predicted *N. crassa* EF-2 polypeptide is highly similar to EF-2 proteins identified in other organisms. The degree of predicted amino acid sequence identity ranges from 64% relative to EF-2 polypeptides found in humans (Rapp et al. 1989), *Drosophila* (Grinblat et al. 1989) and *C. elegans* (Ofulue and Candido 1991) to over 73% identity to EF-2 poly-

peptides found in fungal species (Perentesis et al. 1992; Mita et al. 1997; Mendoza et al. 1999). This predicted *Neurospora* translation product contains the highly conserved E-X-X-R-X-I-T-I effector motif, as well as the C-terminal diphthamide domain (Perentesis et al. 1992).

EF-2 catalyzes the translocation of peptidyl-tRNA from the A site to the P site on the ribosome (Rapp et al. 1989). This translocation facilitates the movement of the ribosome relative to the mRNA by a distance of three bases, to reach the next codon (Moazed and Noller 1989) and to eject the deacylated tRNA from the P site. During the translocation cycle, GTP is bound by the EF-2-ribosome complex, followed by extremely rapid hydrolysis to EF-2-GDP, and a conformational change that releases EF-2 for the next round of translocation (Justice et al. 1998).

EF-2 is a five-domain protein whose N-terminal portion is a G nucleotide-binding domain (G domain) that is responsible for EF-2 GTPase activity. The other domains are designated by the numbers 2 to 5, from the N-terminal regions to the C-terminus, respectively (Czworkowski et al. 1994). The G domain itself includes five regions, designated G-1 to G-5, which are critical for GDP/GTP exchange, GTP-induced conformational changes and GTP hydrolysis. These regions are well conserved in other members of GTPase families among eukaryotes and prokaryotes (Bourne et al. 1991). The G domain has also been shown to be involved in the interaction of EF-2 with the ribosome (Agrawal et al. 1998).

A variety of mutations in EF-2 have been recovered, and their effects on the in vitro function of the protein have been analyzed (Chen and Bodley 1988; Omura

et al. 1989; Riis et al. 1990). Some of these mutations have been shown to affect post-translational modification and activation of the protein. During the course of this work, we determined the genetic basis of the mutation in *cot-3*.

Sequence analysis of the R2006 mutant allele of *cot-3* revealed a transversion mutation (ATT to AAT) that results in an Ile to Asn substitution at residue 278 (Fig. 3B). The TT/AT transversion was confirmed for two independently generated PCR products. This mutation occurs within a short subdomain, located in the C-terminus of the G domain, which is conserved in the EF-2 and absent from the EF-G polypeptide. This short region has been predicted to form an α -helix or coil that is likely to be responsible for the interaction with, and stabilization of EF-2 by, the eukaryotic ribosome. The Asn residue is hypothesized to increase the likelihood of forming a turn in this helical/coiled domain. Interference with proper folding of the protein may affect the EF-2-ribosome interactions mediated by the G domain.

Chromosomal localization of *cot-3*

Garnjobst and Tatum (1967) mapped *cot-3* to the right arm of linkage group IV, left of *pan-1* and linked to *arg-2*. In order to confirm that the G15:E3 cosmid originated from the same chromosomal region, we mapped its location by RFLP procedures. Genomic DNA of two parents and their 18 random progeny (FGSC strains 4411–4430) were digested with *EcoRI*. Subsequent probing with a hexamer-labeled G15:E3 cosmid clone localized *cot-3* next to *chs-5* on the right arm of linkage group IV (Fig. 4).

cot-3 is expressed throughout all asexual phases of the life cycle in wild-type and *cot-3* *N. crassa* strains

During the asexual stage of its life cycle, *N. crassa* grows as filamentous hyphae. Upon proper environmental induction, aerial hyphae are formed. To determine the pattern of *cot-3* expression throughout the asexual phases of the life cycle, the abundance of the *cot-3* transcript was analyzed in RNA samples extracted from wild-type conidia, from 1.5-, 7-, and 15-h-old mycelial cultures, as well as from a culture in which aerial hyphal formation had been induced 7 and 19 h prior to har-

vesting. Northern analyses indicated that *cot-3* is expressed throughout all asexual phases of the *N. crassa* life cycle (Fig. 5). This pattern of expression would be expected for a key component in protein production. This analysis of *cot-3* expression during development was repeated with RNA isolated from a *cot-3* mutant, grown at the permissive temperature, and yielded similar results (data not shown). The detection of the *cot-3* transcript in conidia was not surprising, as it is highly likely that EF-2-dependent protein production is also required during conidial maintenance and/or that the transcript must be available for early phases of germination.

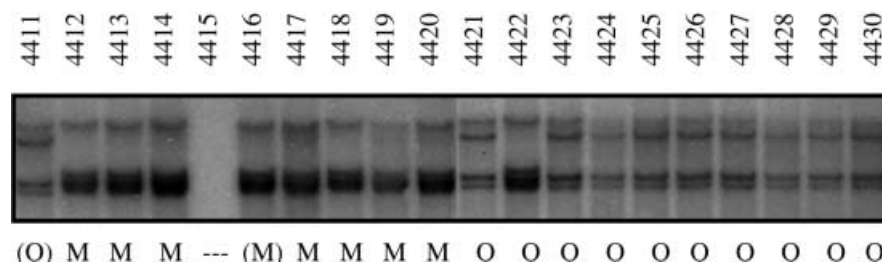
Since no significant changes in *cot-3* expression were detected in the *cot-3* mutant grown at the permissive temperature (even during the early germination phase in which a lag in growth and the production of abnormal hyphae were observed), we also analyzed *cot-3* transcript levels in the mutant grown at the restrictive temperatures. The *cot-3* and wild-type strains were grown at 25 °C for 24 h before shifting to 34 °C for 4 h prior to harvesting and RNA extraction. Similar levels of *cot-3* transcript were observed in RNA samples from *cot-3* and wild-type strains grown at permissive or restrictive temperature (Fig. 5B). We therefore concluded that the *cot-3* mutation has no discernible effect on *cot-3* transcription or mRNA stability; this is compatible with the single nucleotide substitution found in the coding region of the EF-2 gene.

Fusidic acid, an inhibitor of EF-2, affects hyphal growth and branching in *N. crassa*

As an apparent defect in the gene encoding EF-2 results in a slow-growing and highly branched phenotype, we performed experiments to determine whether the genetic defect could be mimicked by a chemical inhibitor of EF-2, fusidic acid.

Fusidic acid is a universal inhibitor of EF-2 and EF-G (elongation factor G-common to prokaryotes) that inhibits translocation by stabilizing the EF-2-GDP-ribosome complex (Justice et al. 1998). This ability has been suggested to be related to some detergent characteristics displayed by the antibiotic, which optimize and stabilize the nucleotide-amino acid interaction in the active center of the protein (Arias et al. 1991). Fusidic acid inhibits the release of EF-2-GDP

Fig. 4 RFLP analysis of the *cot-3* gene. Southern analysis of 18 progeny of the standard cross of Mauriceville-1c-A (FGSC 4416) (M) with Oak-Ridge (FGSC 4411) (O). The segregation pattern of the *cot-3* gene is indicated below the blot



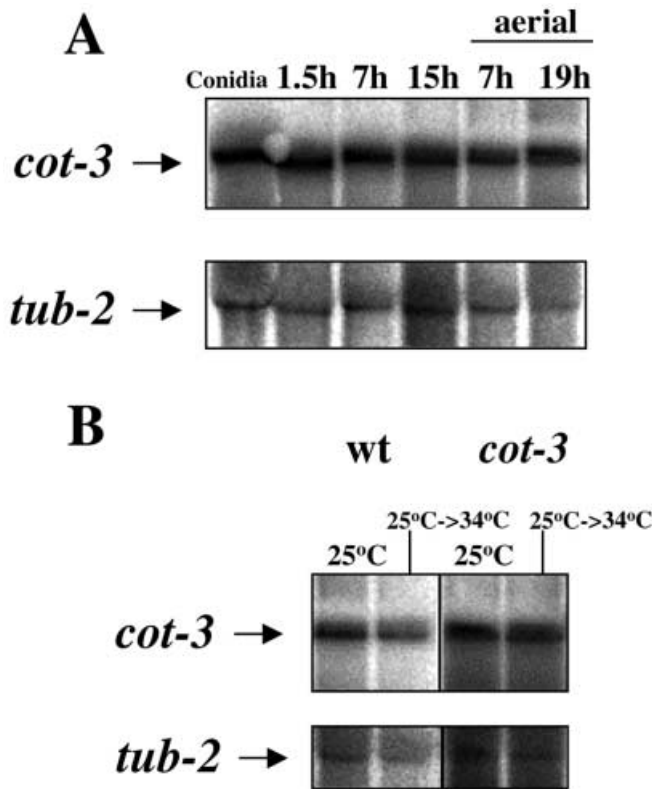


Fig. 5A, B Levels of the *cot-3* transcript. **A** Northern analysis of *cot-3* transcription during differential developmental growth of *N. crassa*. RNA was isolated from germinating or conidiating cultures at the times indicated. Equal amounts (20 μ g) of total RNA were loaded in each lane and probed with the *Bam*HI-*Not*I fragment of pB1. A *tub-2* (β -tubulin) probe was used to verify the presence of equal quantities of RNA in all lanes. **B** Northern analysis of *cot-3* transcription in *cot-3* and wild-type strains of *N. crassa*. Samples were obtained from cultures maintained for 24 h at 25°C and then transferred to 34°C for an additional 4 h prior to RNA extraction. A *Bam*HI-*Not*I fragment of pB1 was used as a DNA probe

from the ribosome (Burns and Cannon 1974). During translocation, fusidic acid can promote stable binding of EF-G, which leads to blockage of the ribosomal A-site and to subsequent inhibition of aminoacyl-tRNA binding. Fusidic acid has been shown to induce a dramatic reduction in the phosphorylation level of EF-2 in cell-free extracts. EF-2 is phosphorylated by Ca²⁺-dependent protein kinase III (Ryazanov 1987). The modification results in a reduction of the amount of functional EF-2 available for translation of other proteins. Gschwendt et al. (1989) have shown that type-2A protein phosphatase (PP2A) is capable of dephosphorylating EF-2, resulting in the reintroduction of EF-2 into the active pool without a requirement for the de novo synthesis of the factor. The *N. crassa pph-1* gene (which encodes the catalytic subunit of PP2A) has been shown to be required for proper hyphal elongation (Yatzkan and Yarden 1995; Yatzkan et al. 1998). Thus it is possible that some of the defects in hyphal elongation which occur when PP2A activity is impaired may be caused by a reduction in EF-2 activity.

The concentration-dependent inhibitory effect of fusidic acid on hyphal elongation in *N. crassa cot-3* and wild-type strains is illustrated in Fig. 6. The inhibitory effect of fusidic acid on hyphal elongation in the wild type was accompanied by hyperbranching (Fig. 6B), which is reminiscent of the *cot-3* phenotype. The apparent impairment (even if partial) of EF-2 function in *cot-3* would suggest a change in sensitivity to EF-2 inhibitors. Nonetheless, no significant differences were observed in the inhibitory effects of the antibiotic on growth between wild-type and *cot-3* strains. The effective inhibitory concentrations (150–300 μ M) of fusidic acid in *N. crassa* are significantly lower than the concentrations used for antimicrobial treatment of skin pathogens (mainly bacteria), where lotions containing 1–2% fusidic acid are administered (Sommer et al. 1997; Benfeldt and Groth 1998). The potential of EF-2 as an antifungal target has recently been demonstrated by the analysis of sorodarin, which binds directly to EF-2 (Dominguez et al. 1999).

The fact that fusidic acid inhibits hyphal elongation and induces hyperbranching in a manner that mimics the *cot-3* mutant phenotype supports our conclusion that a

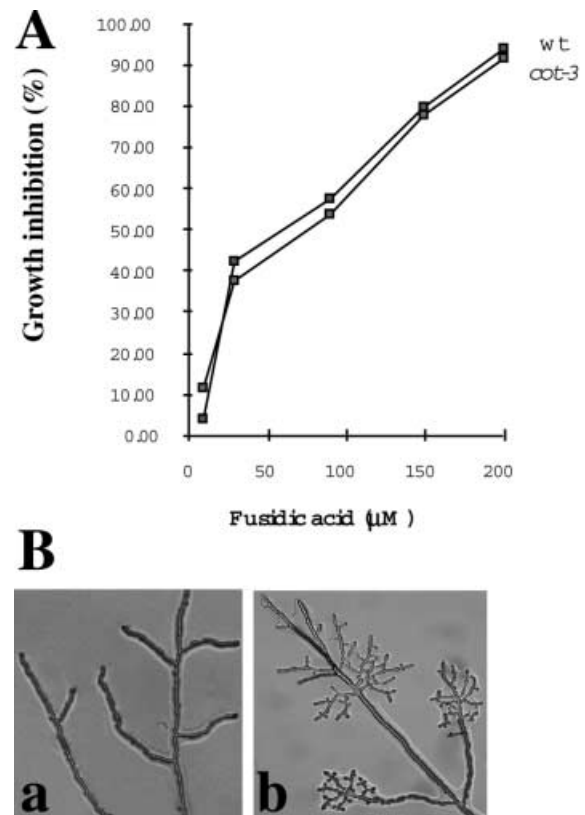


Fig. 6A, B Effect of fusidic acid on growth and morphology of *N. crassa*. **A** The inhibitory effect of fusidic acid (as determined by colony area) on *N. crassa cot-3* and wild-type strains grown (at 25°C) in the presence of the EF-2 inhibitor. Measurement of *cot-3* growth was initiated 36 h after plate inoculation. **B** Morphology of *N. crassa* grown in the absence (a) or presence (b) of 50 μ M fusidic acid

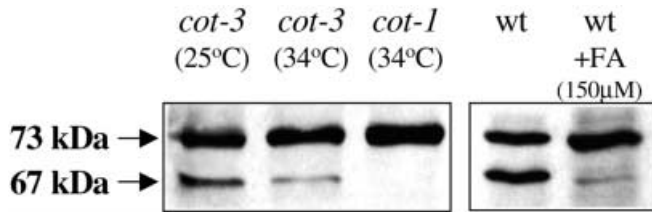


Fig. 7 Expression pattern of COT1 kinase (as detected with anti-COT60 antibodies) in various mutant *N. crassa* strains, and in the wild type grown in the presence or absence of the EF-2-inhibitor fusidic acid (FA). Cultures of *cot-1* or *cot-3* were grown at the permissive temperature for 10 h, after which they were either maintained at the same temperature for an additional 14 h (25°C) or shifted to the restrictive temperature (34°C). The wild-type strain was cultured at 34°C for the duration of the experiment

defect in EF-2 can result in abnormal hyphal elongation/branching and indicates that the defect in *cot-3* results in impaired function of the COT3 protein at non-permissive temperatures.

The phenotypic similarities observed when COT3 function was impaired by either genetic or pharmacological alteration of EF-2 activity led us to analyze the effects of fusidic acid on the abundance of the LMW isoform of COT1 in *N. crassa*. Western analyses performed with protein extracts from *cot-3* and the wild type grown in the presence of fusidic acid provided a clear indication that inhibition of EF-2 influences the abundance of the 67-kDa, but not of the 73-kDa isoform of COT1 kinase (Fig. 7). The changes in the expression pattern of the COT1 kinase induced by fusidic acid are very similar to those observed in a *cot-3* background (and as initially observed in *cot-1*). These results further support the data indicating that the 67-kDa COT1 isoform is involved in the development of normal hyphal morphology (Gorovits et al. 1999). Nonetheless, the abundance of the 67-kDa isoform should not be causally linked to general colonial growth. In fact, other *cot* mutants (*cot-2*, *cot-4*), as well as environmental factors (e.g. sorbose) that influence elongation and branching events, do not result in a detectable change in the abundance of the 67-kDa isoform of COT1.

The mechanistic role of EF-2 in the production of the LMW isoform of COT1 is still unknown. It has yet to be determined whether the LMW COT1 isoform is an independent translational product (in which case the direct involvement of EF-2 may be required) or the result of post-translational modifications in the COT1 polypeptide (in which case one or several other EF-2-dependent proteins may be involved). Thus, the functional link between COT1 and COT3 remains unclear.

Many defects in the EF-1 α and EF-G complex have been shown to be associated with changes in frameshift frequencies in both prokaryotes and eukaryotes (Hou et al. 1994; Farabaugh 1996; Dinman and Kinzy 1997). Whether or not changes in frameshift frequencies occur in a *cot-3* mutant background has not been determined. However, one mechanistic explanation for the effect of the *cot-3* mutation on COT1 may be linked with the

possibility that the abundance of different COT1 isoforms is influenced by programmed frameshifting of different cellular proteins. If that be the case, it is possible that the mutation in COT3 may influence the production (and concomitant activity) of the LMW COT1 isoform by altering the presence or abundance of proteins whose translation involves frameshifting.

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