***cot-1*, a gene required for hyphal elongation in *Neurospora crassa*, encodes a protein kinase**

**Oded Yarden^{1,2}, Michael Plamann³,
Daniel J. Ebbole^{3,4} and Charles Yanofsky^{1,5}**

¹Department of Biological Sciences, Stanford University, Stanford, CA 94305 and ³Department of Biology, Texas A&M University, College Station, TX 77843, USA

²Present address: Department of Plant Pathology and Microbiology, Faculty of Agriculture, The Hebrew University of Jerusalem, Rehovot 76100, Israel

⁴Present address: Department of Plant Pathology and Microbiology, Texas A&M University, College Station, TX 77843, USA

⁵Corresponding author

Communicated by U. Henning

***Neurospora crassa* is a filamentous fungus that grows on semisolid media by forming spreading colonies. Mutations at several loci prevent this spreading growth. *cot-1* is a temperature sensitive mutant of *N. crassa* that exhibits restricted colonial growth. At temperatures above 32°C colonies are compact while at lower temperatures growth is indistinguishable from that of the wild type. Restricted colonial growth is due to a defect in hyphal tip elongation and a concomitant increase in hyphal branching. We have isolated a genomic cosmid clone containing the wild type allele of *cot-1* by complementation. Sequence analyses suggested that *cot-1* encodes a member of the cAMP-dependent protein kinase family. Strains in which we disrupted *cot-1* are viable but display restricted colonial growth. Duplication, by ectopic integration of a promoter-containing fragment which includes the first one-third (209 codons) of the structural gene, unexpectedly resulted in restricted colonial growth. Our results suggest that an active COT1 kinase is required for one or more events essential for hyphal elongation. Key words: hyphal growth/cAMP-dependent protein kinase/fungal development**

Introduction

In most filamentous fungi, newly formed hyphae elongate and branch to generate the mycelial mat which is typical of the growth of these organisms. Under standard laboratory growth conditions, the biomass of the filamentous ascomycete *Neurospora crassa* is mainly comprised of hyphae. Hyphae first appear upon germination of macroconidia, the predominant asexual propagules of *N. crassa*. The hyphal germ tube is characteristically tubular in shape and has an average diameter of 4–10 µm (Schmit and Brody, 1976). Hyphae typically branch every 15–150 µm. Continuous hyphal elongation and branching results in the formation of spreading radial colonies on solid media and spherical cellular aggregates during submerged aerated growth in liquid media. Appropriate environmental stimuli (i.e. nutrient deprivation, desiccation and light etc.) induce sexual or asexual development by promoting the formation

of specialized hyphae. The frequency of hyphal branching is markedly influenced by compounds such as tergitol (Springer, 1991) or L-sorbose (Tatum *et al.*, 1949). Exposure to these substances results in the formation of dense, restricted growth characterized by extensive branching. Colonial growth on L-sorbose has been utilized extensively in screening procedures with *N. crassa*.

Many 'colonial' genes have been identified which, when mutated, result in compact colonial growth under conditions favoring spreading growth in wild type (Perkins *et al.*, 1982). One of these mutant genes, *colonial temperature sensitive-1* (*cot-1*), causes colonial growth at 32°C and above, but normal growth, morphology and fertility at or below 25°C (Mitchell and Mitchell, 1954; Galsworthy, 1966). When cultured at temperatures above 32°C, *cot-1* colonies grow slowly, their hyphae branch excessively and they do not conidiate. These cultures rapidly resume normal growth when shifted to a permissive temperature. Because of its high viability and compact growth at restrictive temperatures and normal growth and development at 25°C, *cot-1* strains have been used widely in procedures requiring compact colonial growth (Perkins *et al.*, 1969, 1982).

We have cloned, sequenced and characterized the wild type *cot-1* gene in order to understand the molecular basis of hyphal elongation and branching in filamentous fungi. *cot-1* was disrupted and shown not to be essential for viability, but required for normal hyphal elongation. The predicted sequence of COT1 is homologous to polypeptides in the cAMP-dependent protein kinase family. The results of this study suggest that the COT1 protein kinase is essential for normal hyphal elongation. Our studies provided an unexpected result; ectopic duplication of the initial segment of the *cot-1* clone, encoding the N-terminal 209 residues of COT1, was negative complementing.

Results

***cot-1* morphology**

In order to determine the extent of the morphological changes conferred by the *cot-1* mutation, we conducted a microscopic analysis of a *cot-1* mutant grown under permissive and restrictive temperatures. When grown at 25°C, no distinct differences (either changes in rate of hyphal elongation or frequency of hyphal branching) were observed between hyphae of *cot-1* and wild type strains (Figure 1a and b). Within 2 h of transferring *cot-1* cultures from 25°C to a non-permissive temperature, 37°C, there was a rapid reduction in the rate of hyphal elongation accompanied by the appearance of numerous branches (Figure 1c). The temperature shift had no apparent effect on growth of wild type cultures. In *cot-1* cultures, cessation of hyphal elongation was evident both at the tips of pre-existing hyphae (which had grown prior to the temperature shift) and at the tips of newly formed branches. The initial branching that occurred when cultures were shifted at 37°C was not

Fig. 1. (a) Wild type *N. crassa* grown in Vogel's minimal salts medium for 18 h at 25°C (shaking liquid culture). (b) *cot-1* grown in Vogel's minimal salts medium for 18 h at 25°C (the permissive temperature for this temperature sensitive mutant). (c) *cot-1* grown for 16 h at 25°C, followed by 2 h of growth at 37°C (the non-permissive temperature). Note the uniform initiation of multiple branches along the hyphal filaments, resulting in a 'barbed-wire' morphology. (d) A 30 h culture of *cot-1* grown in liquid on a microscope slide at 37°C. In time, hyphal tips originating from the newly formed branches (shown in picture c) will cease to elongate. In some cases, secondary and tertiary branching occurs prior to arrest of hyphal tip growth. (e) A pMP30 transformant grown for 20 h at 20°C in the presence of hygromycin. *cot-1*-like structures and hyphae resembling those of wild type are present. Bars indicate 100 μ M.

terminal, i.e. secondary and tertiary branches appeared close to primary branches (Figure 1d). Bulb-like structures at branch junctions and deterioration of hyphal tips were common morphological abnormalities in *cot-1* cultures grown for long periods at a restrictive temperature.

Cloning of the *N. crassa cot-1* gene

The wild type *cot-1* gene was cloned by complementation of the *N. crassa cot-1* mutant. Spheroplasts of a *cot-1* strain (prepared from conidia produced at 25°C) were transformed with 10 pools of a *N. crassa* genomic cosmid library (Orbach/Sachs, Fungal Genetics Stock Center), plated onto sucrose medium containing hygromycin and incubated overnight at 37°C. Four of the cosmid pools yielded hygromycin resistant, *cot-1*⁺ transformants, i.e. colonies that displayed rapid, spreading growth. One of these cosmid pools was used to obtain sub-pools in *Escherichia coli*, which were used in a second round of *cot-1* complementation analyses. Following a third round, with cosmids isolated from individual *E. coli* transformants, two cosmids containing *cot-1* complementing DNA were identified (pCT1A and pCT1B). These cosmids were determined to be identical by restriction enzyme analysis. *cot-1* was co-transformed with aliquots of one of these clones, pCT1A, which had been digested separately with a number of endonucleases and pMP6, a plasmid containing a *cpc-1*–*hph* gene fusion which confers hygromycin resistance on *N. crassa* (Plamann, M. and Yanofsky, C., unpublished results). *Bam*HI, *Eco*RI, *Xho*I and *Sma*I digested pCT1A complemented the *cot-1* mutation, suggesting that these endonucleases do not cleave within the complementing genetic segment. A 4.2 kb *Eco*RI–*Sma*I fragment of pCT1A which complements *cot-1* was subcloned (pOY18; Figure 2).

Fig. 2. Molecular organization of the *N. crassa cot-1* gene. Sm (= *Sma*I) and E (= *Eco*RI) mark the boundaries of the genomic clone containing *cot-1* (pOY18). The predicted initiation codon, stop codon and polyadenylation signal sequence are marked, as are the boundaries of coding and non-coding sequences. pOY311 and pOY411 are *cot-1* cDNA clones used to verify the intron boundaries of the *cot-1* gene. Intervening sequences absent from the cDNA clones are marked.

Sequence of *cot-1*

A 3.3 kb segment of pOY18 containing the *cot-1* gene was completely sequenced on both strands. The nucleotide sequence of *cot-1* and the predicted amino acid sequence of the COT1 polypeptide are presented in Figure 3. The predicted *cot-1* open reading frame showed a distinct bias for codons preferred by *N. crassa*. The sequence was also analyzed for the presence of additional hallmarks of *N. crassa* genes. The context of the presumed *cot-1* translation initiation codon (ACCAAGATGG) is similar to the *N. crassa* consensus (^A_GTCA^A/_CAATGG) compiled for 26 genes (Paluh *et al.*, 1988). Three tentative intervening sequences were identified on the basis of consensus 5' and 3' splice junction sequences (Orbach *et al.*, 1986; Hager and Yanofsky, 1990). The existence of these introns was verified

2161

by isolation and characterization of two *cot-1* cDNA clones (obtained from a *N.crassa* cDNA library provided by Exley and Garrett). Sequence analysis of these cDNA clones (pOY311, pOY411; Figure 2) confirmed the location and length of the intervening sequences.

Fig. 4. (A) Northern analysis of *cot-1* transcript production in *cot-1* and wild type strains of *N.crassa*. Duplicate shake cultures of each strain were grown for 16 h at 25°C. Flasks were either maintained for an additional 4 h at 25°C or transferred to 37°C for various time periods (lanes 2 and 4) prior to RNA extraction. Ten μ g of RNA were loaded in each lane. pOY311 was used as a template for preparing the RNA probe. (B) Northern analysis of *cot-1* transcript presence during development of *N.crassa*. Ten μ g of RNA, isolated from germinating or conidiating cultures taken at times indicated, were loaded in each lane and probed with a labeled pOY311 RNA probe.

Northern analyses showed that the *cot-1* transcript is 2.4 kb in length (Figure 4A). The site of transcription initiation was not determined. *cot-1* transcripts were detected in RNA prepared from *cot-1* and wild type strains, regardless of growth temperature (25°C or 37°C), suggesting that the colonial phenotype of the mutant was not due to the absence of the *cot-1* transcript (Figure 4A). While almost undetectable in conidia, high levels of *cot-1* transcript were observed in mycelial samples from early phases of germination and throughout growth (Figure 4B). During conidiation, a stage in which hyphal elongation is significantly reduced, *cot-1* transcript levels were markedly reduced. This finding is consistent with the observed reduction in transcript levels of many other biosynthetic genes during conidiation (Sachs and Yanofsky, 1991).

COT1 amino acid sequence homology to cAMP-dependent protein kinases

cot-1 is predicted to encode a 622 residue polypeptide with a calculated mass of 70 kDa and a relatively high isoelectric point, 8.6. A database search revealed that COT1 was homologous to protein kinases. The greatest extent of amino acid sequence identity was observed between COT1, the catalytic subunit of the mouse cAMP-dependent protein kinase and the *Saccharomyces cerevisiae* DBF2 (Johnston et al., 1990) gene product (34% and 32%, respectively; Figure 5). Similar sequence identities (data not shown)

Fig. 5. Multiple sequence alignment of COT1, *S.cerevisiae* DBF2 and the catalytic domain of the mouse cAMP-dependent protein kinase (MPKA) predicted polypeptides. Yeast and mouse amino acid residues identical in the *N.crassa* polypeptide are boxed. Hyphens mark gaps.

existed between COT1 and the deduced product of the DBF2 homologue *DBF20* (Toyn *et al.*, 1991). The sites in the catalytic core which are believed to be essential for kinase activity (Hanks *et al.*, 1988; Taylor, 1989) are conserved in residues 196–570 of COT1 (Figure 5). The conserved functional sites include the glycine-rich loop containing the MgATP binding site (Gly243–Lys265), the catalytic loop (Arg358–Asn364) and carboxyl groups that participate in substrate recognition (Asp377–Gly379). A 47 residue segment (residues 383–429) is located between conserved presumed catalytic subdomains VII and VIII (Hanks *et al.*, 1988) of the COT1 polypeptide. This segment is similar in length (though not in sequence) to comparable segments in *S.cerevisiae* *DBF2*, *DBF20* and *CDC7* (Patterson *et al.*, 1986), but is absent from other cAMP-dependent protein kinases characterized to date. The overall similarity between COT1 and CDC7 is not high (25% homology).

Chromosomal localization of *cot-1*

cot-1 has been mapped to the right arm of linkage group IV (Mitchell and Mitchell, 1954). To establish that the gene we cloned was *cot-1*, we mapped its location by RFLP procedures. We probed a *Pst*I digest of genomic DNA isolated from 18 random progeny (Metzenberg *et al.*, 1985) with hexamer-labeled fragments of the *Eco*RI–*Sma*I insert in pOY18. The polymorphism pattern (data not shown) placed the cloned DNA on the right arm of linkage group IV, in full agreement with the segregation of *cot-1* progeny from the cross (one of the parents used in the cross was *cot-1*[−]).

Disruption of *cot-1*

In *N.crassa*, as in other filamentous fungi, stable transformants mainly result from ectopic integration of trans-

forming DNA is observed (for review see Rambossek and Leach, 1987; Fincham, 1989). Since *N.crassa* is a coenocytic haploid organism, it is possible to disrupt an essential gene and have the mutated nucleus survive in a heterokaryon. If the disrupted gene is essential it should not be possible to obtain homokaryons containing only the transformed nuclei.

We constructed two plasmids for disruption of *cot-1*, pMP30 and pMP41 (Figure 6A). pMP30 has the DNA segment from the *Nru*I site to the distal *Sph*I site removed and replaced by the *trpC*–*hph* construct (Materials and methods). In pMP41 the DNA segment extending from the *Kpn*I site, just proximal to the ATG initiation codon to the distal *Sph*I site, was removed and replaced by *trpC*–*hph* DNA.

Wild type *N.crassa* was transformed with a *Sma*I–*Eco*RV DNA fragment from pMP30 (containing the intact DNA segment encoding the N-terminal region of COT1). Unexpectedly, ~95% of the stable Hyg^r pMP30 transformants showed varying degrees of colonial growth. Some transformants had a severe colonial phenotype that was evident on the primary selection plates. This result was unexpected since primary transformants should be heterokaryons. Southern analysis of a number of these transformants revealed that they had one or more copies of the truncated *cot-1* gene integrated at ectopic sites and they contained intact *cot-1* DNA at its normal chromosomal location. These findings suggest that the truncated *cot-1* gene acted *in trans* to confer a colonial phenotype. It is possible that production of a truncated polypeptide containing the first 209 amino acids of COT1, encoded by pMP30 DNA, inhibits the function of wild type COT1. Alternatively, the *cot-1* DNA segments of pMP30 which flank the *trpC*–*hph* gene contains sequences that can titrate limiting factors that are required for *cot-1* expression.

A photograph of a representative transformant containing

Fig. 6. Southern blot analysis of *cot-1::trpC-hph* transformants. (A) Restriction maps of pOY18 and two plasmids, pMP30 and pMP41, used in *cot-1* disruption experiments. Relevant restriction sites are indicated: E = *Eco*RI, EV = *Eco*RV, K = *Kpn*I, N = *Nru*I, Sm = *Sma*I, S = *Sph*I and X = *Xho*I. The sizes of the diagnostic fragments identified in the Southern blot (B) are indicated. (B) Total DNA was isolated from a wild type strain (lane A), two heterokaryons containing both wild type nuclei and nuclei containing *cot-1* disrupted by homologous integration of the *Xho*I–*Eco*RV fragment of pMP41 (lanes B and C) and a homokaryon containing one or more copies of the *Sma*I–*Eco*RV fragment of pMP30 ectopically integrated into the *N.crassa* genome. All DNA samples were digested with *Sma*I and *Eco*RV prior to electrophoresis. The probe used was the entire pMP41 plasmid. Note the presence of both the 2.0 kb band, characteristic of the wild type *cot-1* gene and the 4.1 kb and 1.1 kb bands, diagnostic of the *cot-1::trpC-hph* homologous replacement allele, in DNA from the heterokaryons shown in lanes B and C.

ectopically integrated pMP30 DNA is shown in Figure 1e. This transformant is a homokaryon and has a phenotype intermediate between wild type and the pMP41 *cot-1* disruptant (see below). Southern analyses with the transformant revealed the two 2.0 kb *SmaI*–*EcoRV* and *EcoRV*–*EcoRV* fragments characteristic of the wild type *cot-1* allele and a larger *SmaI*–*EcoRV* fragment containing ectopically integrated pMP30 sequences (Figure 6B, lane D).

To disrupt the initial segment of the *cot-1* coding region and avoid the complication introduced by expression of the initial portion of the *cot-1* gene, we constructed pMP41 and performed a homologous replacement analysis. In pMP41 the *cot-1* structural gene segment upstream of the distal *SphI* site is removed (Figure 6A). Transformation of *N.crassa* with linear pMP41 sequences resulted in the production of many transformants, most of which displayed wild type phenotype. Among ~50 stable Hyg^r transformants that were isolated, two displayed a phenotype that was intermediate between *cot-1* strains at the restrictive temperature and wild type. When conidia from these two transformants were plated we observed large colonies with wild type characteristics and very small colonies that resembled *cot-1* strains growing at the restrictive temperature. The small colonies presumably were homokaryons in which there had been homologous replacement of *cot-1* by *cot-1::trpC-hph*.

It was difficult to obtain sufficient mycelium for DNA isolation from the slowly growing putative disrupted homokaryons. Accordingly, DNA was isolated from the two heterokaryons and from wild type, the DNA was cut with *SmaI* and *EcoRV*, and analyzed by Southern analysis using the entire pMP41 plasmid as a probe. As shown in Figure 6B lane A, a single 2.0 kb band was observed with wild type DNA since the *SmaI*–*EcoRV* and *EcoRV*–*EcoRV* fragments are both 2.0 kb in size (Figure 6A). With the two heterokaryotic strains we observed the 2.0 kb fragments characteristic of wild type nuclei as well as the 4.1 kb and 1.1 kb fragments expected from nuclei containing the homologous *cot-1* replacement (Figure 6). When the *XhoI*–*EcoRV* fragment of pMP41 integrates ectopically the 4.1 kb *SmaI*–*EcoRV* and 1.1 kb *SmaI*–*SmaI* fragments are not observed. Southern analyses employing additional restriction endonuclease digestions of the DNA of heterokaryotic strains verified further that homologous replacement had occurred in these two transformants (data not shown).

We could obtain homokaryons from primary transformants containing the homologously integrated pMP41 DNA either by plating conidia from the primary transformants or by crossing the transformants with a wild type strain. *cot-1::trpC-hph* homokaryons grew as very small dense colonies in a temperature independent manner. These results establish that *cot-1* function is not essential for viability or for germination of asexually or sexually derived spores.

Discussion

cot-1 of *N.crassa* encodes a polypeptide that is homologous to members of the cAMP-dependent protein kinase family. Gene disruption experiments were used to show that *cot-1* is dispensible for viability but essential for normal hyphal growth. This finding implies that the restricted colonial growth of the *cot-1* mutant at the non-permissive temperature

is due to loss of COT1 function. The presence of *cot-1* transcript in extracts prepared from the *cot-1* mutant grown at the non-permissive temperature suggests that the *cot-1* phenotype is a consequence of a post-transcriptional defect, most likely temperature sensitivity of the COT1 polypeptide.

Normal growth of filamentous fungi proceeds by apical extension of hyphal tips. It is conceivable that once the biosynthetic capacity or volume of the cytoplasm in a hyphal tip has reached a level exceeding the ability of the tip to respond simply by elongation, a signal is generated promoting hyphal branching and the concomitant formation of a new growing tip. Therefore in *cot-1* strains growing at the restrictive temperature, cessation of hyphal tip elongation would result in excessive induction of hyphal branching. The formation of new hyphal tips presumably accommodates the increased volume or metabolic activity. However, since the new tips of *cot-1* strains cannot elongate normally, tip inhibition causes an abnormal increase in hyphal branching (Figure 1b and c). COT1 may function as a regulator that couples cell volume or biosynthetic capacity with hyphal tip elongation. Following a shift to the non-permissive temperature, branching of *cot-1* hyphae proceeds uniformly throughout the mycelium (Figure 1c). This rapid morphological change associated with a halt of hyphal elongation raises the possibility that elongation and branching are interrelated processes. Nonetheless, extensive branching does not always accompany the inhibition of hyphal tip growth; deterioration of hyphal tips has been shown to occur in a *N.crassa* strain in which the chitin synthase 1 gene has been inactivated, yet in this *chs-1*^{RIP} strain no increase in the rate of branching was detected (Yarden and Yanofsky, 1991).

The predicted COT1 polypeptide contains a segment with appreciable sequence identity to the catalytic domain of cAMP-dependent protein kinases. The three-dimensional structures of the catalytic domain of the mouse cAMP-dependent protein kinase (Knighton *et al.*, 1991a) and its complex with a substrate analog (Knighton *et al.*, 1991b) have confirmed the importance of this domain which is highly conserved in the various protein kinases and in COT1. Unlike many cAMP-dependent kinases, COT1 contains a relatively long amino-terminal segment as well as an additional sequence of 47 residues within its presumed catalytic domain. Knighton *et al.* (1991a) suggest that these non-conserved regions may determine substrate specificity. This suggestion is supported by the observation that the similar segment of the *S.cerevisiae* CDC7 polypeptide is necessary for *in vivo* function but not for protein kinase activity *in vitro* (Bahman *et al.*, 1988).

The observation that ectopic integration of pMP30 DNA confers a colonial phenotype was unexpected. We presume that the additional copy of the *cot-1* promoter and first 209 codons of the structural gene interferes with the normal function of COT1 specified by the intact resident copy of *cot-1*. This interference could be attributed to competition for factors involved in transcription of *cot-1* or more likely, the truncated COT1 polypeptide produced in pMP30 transformants interferes with COT1 function. The latter hypothesis is supported by the observation that ectopic transformants obtained with pMP41 (a construct containing the *cot-1* promoter but lacking the first 209 codons of COT1) display a wild type phenotype. It is possible that the putative truncated COT1 product of pMP30 DNA contains a

regulatory domain that inactivates COT1 kinase. However, the predicted amino acid sequence of the N-terminal segment of COT1 does not resemble any known kinase regulatory domain. Another possibility is that COT1 activity is dependent on dimerization or complex formation with other proteins, in which case the putative truncated COT1 polypeptide might act by titrating binding surfaces essential for COT1 activity. Since *cot-1* disruption halts hyphal elongation predominantly, it is possible that COT1 and the COT1 substrate are localized at hyphal tips, perhaps as components of a complex responsible for cell wall biogenesis (Grove and Bracker, 1970).

Many mutants of *N.crassa* have been identified, so called gulliver mutants, which contain suppressors of the *cot-1* mutation (Perkins *et al.*, 1982). Analysis of these mutants may elucidate the functions and regulation of *cot-1*. Further studies of colonial mutants should increase our understanding of the factors and events responsible for hyphal elongation and branching.

We have learned recently that M.Robb and J.P.Vierula have also cloned the *N.crassa cot-1* gene and are studying its function (J.P.Vierula, personal communication).

Materials and methods

Strains and media

Wild type *N.crassa* strains 74-OR23-1A (FGSC 987), 74-ORS-6a (FGSC 4200) and *cot-1* (FGSC 4065) were used throughout. Procedures used in growth studies, crosses etc. have been described in Davis and de Serres (1970). Where appropriate, the medium was supplemented with hygromycin B (Calbiochem) at 150 µg/ml. Induction of conidiation was performed as described by Berlin and Yanofsky (1985). DNA mediated transformation was carried out as described by Vollmer and Yanofsky (1986).

Isolation and analysis of nucleic acids from *N.crassa*

Genomic DNA was isolated as follows: two day-old mycelial cultures grown in 25 ml of Vogel's N medium (Vogel, 1956) were collected by filtration on Whatman No. 2 filter paper on a Buchner funnel. Samples were quickly frozen in liquid nitrogen and lyophilized. The dry samples were powdered by grinding and were suspended in an equal volume of lysis buffer (50 mM Tris-HCl pH 8.0, 50 mM EDTA, 2% SDS, 1% β-mercaptoethanol) containing 25 µg/ml RNase A. Following a 30 min incubation at 37°C, 100 µg/ml proteinase K was added to the solution and incubation was continued for 1 h at 65°C. Two phenol:chloroform (1:1) extractions were performed; these were followed by a single chloroform extraction, an ethanol precipitation and a 75% ethanol wash. The DNA pellet was dried and dissolved in TE buffer (10 mM Tris-HCl pH 7.4, 1 mM EDTA pH 8.0).

Total RNA was isolated as follows: mycelia were harvested as described above. Following a quick freeze in liquid nitrogen, 25 mg were transferred to a 2 ml screw cap tube containing 480 µl of extraction buffer (100 mM Tris-HCl pH 7.5, 100 mM LiCl, 10 mM EDTA, 20 mM dithiothreitol), 420 µl phenol, 420 µl chloroform, 84 µl 10% SDS, 2 g zirconium beads (Biospec Products Inc.). The samples were shaken twice for 30 s in a mini bead-beater (Biospec Products Inc.). Following a 15 min centrifugation in a microfuge, the aqueous phase was transferred to a new tube and re-extracted with phenol:chloroform (1:1). After an additional chloroform extraction the RNA was precipitated, washed, dried and dissolved in 10 mM Na-HEPES pH 7.5 containing 1 mM EDTA.

Pools from the Orbach/Sachs cosmid library (Fungal Genetics Stock Center) were used to complement the *cot-1* mutant. cDNA from the Exley and Garrett library was screened as described by Benton and Davis (1977). Southern analyses were carried out as described by Sambrook *et al.* (1989) as were all other DNA modification and cloning procedures. Bluescript SK⁻ (Stratagene) and pUC119 (Vieira and Messing, 1987) were used for cloning and preparation of various constructs. Northern analyses were performed as described (Sambrook *et al.*, 1989). *cot-1* RNA probes were generated from pOY311 (Figure 2) using phage T7 RNA polymerase as described by Sambrook *et al.* (1989) with [α-³²P]CTP as the label. pDH25 was the source of the *hph* gene, encoding hygromycin phosphotransferase, which confers hygromycin resistance (Cullen *et al.*, 1987). This gene was driven by the *Aspergillus nidulans trpC* promoter region and was used as a dominant selectable marker in the isolation of *N.crassa* transformants.

Mapping of *cot-1* was carried out by restriction fragment length polymorphism (RFLP) analysis using DNA from 18 random progeny according to the procedure of Metzenberg *et al.* (1985).

DNA sequencing

DNA sequencing was performed by the dideoxy chain termination method of Sanger *et al.* (1977) and [α-³⁵S]dATP. Clones used for sequencing were generated by insertion of restriction fragments obtained from pOY18 into the Bluescript vector. Oligonucleotide primers for sequencing portions of the gene were synthesized on an Applied Biosystems DNA synthesizer.

Microscopy and computer methods

Samples were viewed with a Nikon Microphot FX epifluorescence microscope. Programs of The University of Wisconsin Genetics Group were used for analysis of nucleic acid sequences (Devereux *et al.*, 1984). Multiple sequence alignments for comparison of predicted amino acid sequences corresponding to different cAMP-dependent protein kinase genes were performed using the Tulla program (Subbiah and Harrison, 1989).

Acknowledgements

We thank G.Exley and R.Garrett for providing their cDNA library and M.Sachs and M.Orbach for their genomic library. The authors thank Lane Winkelmann for technical assistance in the disruption of *cot-1*. We thank David Perkins for helpful discussions and Ajith Kamath, Frank Lauter and Carl Yamashiro for critical reading of this manuscript. O.Y. has been supported by EMBO (European Molecular Biology Organization) and BARD (U.S.-Israel Binational Agricultural Research and Development Fund) post-doctoral fellowships for various periods of this research. D.E. was supported by a post-doctoral fellowship from the National Institutes of Health. C.Y. is a Career Investigator of the American Heart Association. These studies were supported by a grant to C.Y. from the United States Public Health Service (GM41296).

The nucleotide sequence data reported in this paper have been deposited in the EMBL, Genbank and DDBJ nucleotide sequence databases under the accession number M83093.

References

- Bahman, M., Buck, V., White, A. and Rosamond, J. (1988) *Biochem. Biophys. Acta*, **951**, 335–343.
- Benton, W. and Davis, R. (1977) *Science*, **196**, 180–182.
- Berlin, V. and Yanofsky, C. (1985) *Mol. Cell. Biol.*, **5**, 839–848.
- Cullen, D., Leong, S.A., Wilson, L.J. and Henner, D.J. (1987) *Gene*, **57**, 21–26.
- Davis, R.H. and de Serres, F.J. (1970) *Methods Enzymol.*, **17A**, 79–143.
- Devereux, J., Haeblerli, P. and Smithies, O. (1984) *Nucleic Acid Res.*, **12**, 387–395.
- Fincham, J.R.S. (1989) *Microbiol. Rev.*, **53**, 148–170.
- Galsworthy, S.B. (1966) Ph.D. thesis, University of Wisconsin, Madison.
- Diss. Abstr.*, **26**, 6348.
- Grove, S.N. and Bracker, C.E. (1970) *J. Bacteriol.*, **104**, 989–1009.
- Hager, K.H. and Yanofsky, C. (1990) *Gene*, **96**, 153–159.
- Hanks, S.K., Quinn, A.M. and Hunter, T. (1988) *Science*, **241**, 42–52.
- Johnston, L.H., Eberly, S.L., Chapman, J.W., Araki, H. and Sugino, A. (1990) *Mol. Cell. Biol.*, **10**, 1358–1366.
- Knighton, D.R., Zheng, J., Ten Eyck, L.F., Ashford, V.A., Xuong, N., Taylor, S.S. and Sowadski, J.M. (1991a) *Science*, **253**, 407–414.
- Knighton, D.R., Zheng, J., Ten Eyck, L.F., Xuong, N., Taylor, S.S. and Sowadski, J.M. (1991b) *Science*, **253**, 414–420.
- Metzenberg, R.L., Stevens, J.N., Selker, E.U. and Morzycka-Wroblewska, E. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 2067–2071.
- Mitchell, M.B. and Mitchell, H.K. (1954) *Proc. Natl. Acad. Sci. USA*, **40**, 115–119.
- Orbach, M.J., Porro, E.B. and Yanofsky, C. (1986) *Mol. Cell. Biol.*, **6**, 2452–2461.
- Paluh, J.L., Orbach, M.J., Legerton, T.L. and Yanofsky, C. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 3728–3732.
- Patterson, M., Sclafani, R.A., Fangman, W.L. and Rosamond, J. (1986) *Mol. Cell. Biol.*, **6**, 1590–1598.
- Perkins, D.D., Newmeyer, D., Taylor, C.W. and Bennett, D.C. (1969) *Genetica*, **40**, 247–278.
- Perkins, D.D., Radford, A., Newmeyer, D. and Bjorkman, M. (1982) *Microbiol. Rev.*, **46**, 426–570.
- Rambosek, J. and Leach, J. (1987) *CRC Crit. Rev. Biotechnol.*, **6**, 357–393.
- Sachs, M. and Yanofsky, C. (1991) *Dev. Biol.*, **148**, 117–128.

- Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Sanger,F., Nicklen,S. and Coulson,A.R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5463–5467.
- Schmit,J.C. and Brody,S. (1976) *Bacteriol. Rev.*, **40**, 1–41.
- Springer,M.L. (1991) *Fungal Genetic Newsl.*, **38**, 92.
- Subbiah,S. and Harrison,S.C. (1989) *J. Mol. Biol.*, **209**, 539–548.
- Tatum,E.L., Barratt,R.W. and Cutter,V.M.J. (1949) *Science*, **109**, 509–511.
- Taylor,S.S. (1989) *J. Biol. Chem.*, **264**, 8443–8446.
- Toyn,J.H., Araki,H., Sugino,A. and Johnston,L.H. (1991) *Gene*, **104**, 63–70.
- Vieira,J. and Messing,J. (1987) *Methods Enzymol.*, **153**, 3–11.
- Vogel,H.J. (1956) *Microb. Genet. Bull.*, **13**, 42–43.
- Vollmer,S.J. and Yanofsky,C. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 4869–4873.
- Yarden,O. and Yanofsky,C. (1991) *Genes Dev.*, **5**, 2420–2430.

Received on January 9, 1992