

Carbon source affects PKA-dependent polarity of *Neurospora crassa* in a CRE-1-dependent and independent manner

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Abstract

A defect in *mcb*, encoding the cAMP-dependent protein kinase A (PKA) regulatory subunit in *Neurospora crassa*, which confers an apolar growth phenotype, is accompanied by an increase in PKA activity levels. Both PKA and CRE-1 [a key carbon catabolite repression (CCR) regulator] mediate the cellular response to carbon-source availability. Inactivation of the *cre-1* gene resulted in reduced growth rate, abnormal hyphal morphology and altered CCR. Both PKA and CRE-1 affected morphology in a carbon-dependent manner, as fructose suppressed the apolar morphology of the *mcb* strain and enabled faster growth of the $\Delta cre-1$ mutant. An increase in *cre-1* transcript abundance was observed in *mcb* and a reduction in PKA activity levels was measured in $\Delta cre-1$. CRE-1 is involved in determining PKA-dependent polarity, as an *mcb*; $\Delta cre-1$ strain displayed partial reestablishment of hyphal polarity. Taken together, our results demonstrate regulatory interactions between PKA and CRE-1 that affect cell polarity in a filamentous fungus.

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1. Introduction

Filamentous fungi are defined by their ability to form highly polarized hyphae, which is a prerequisite for efficient colonization of growth niches and substrate utilization (reviewed by Harris, 2006). During vegetative growth, the establishment of polarity is an important initiation step for primary germ-tube emergence from the spore/conidium on the one hand, and branch emergence from existing hyphae on the other. Once established, polarity must be maintained during hyphal extension. Structural components of the cytoskeleton (especially actin) play a pivotal role in establishing and maintaining fungal polar growth (reviewed by Momany, 2002; Harris and Momany, 2004; Harris, 2006).

One of the key regulators of polarity in fungi as well as in higher eukaryotic cells is cAMP-dependent protein

kinase A (PKA)¹ (Harris, 2006). In its inactive form, PKA is a tetramer composed of two regulatory subunits bound to two catalytic subunits. In response to signals that increase intracellular cAMP levels, cAMP binds to the regulatory subunit and triggers conformational changes that release the active catalytic subunit (Lengeler et al., 2000). PKA has been shown to control a number of developmental events, such as germination and growth polarity, in different fungi, including *Neurospora crassa* (Bruno et al., 1996), *Aspergillus niger* (Saudohar et al., 2002) and *A. fumigatus* (Zhao et al., 2006).

The *N. crassa mcb* temperature-sensitive (t.s.) mutant, which is defective in the gene encoding the regulatory subunit of PKA, shows complete loss of growth polarity during both conidial germination and hyphal elongation when incubated at restrictive temperatures (32 °C and above). In the *mcb* mutant, actin patches have been shown to be

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¹ Abbreviations used: CCR, carbon catabolite repression; PKA, cAMP-dependent protein kinase A.

distributed uniformly throughout hyphae whose growth has become apolar or bulbous (Bruno et al., 1996), in contrast to their typical localization to hyphal tips (Tinsley et al., 1998). Thus, PKA has been suggested to be involved in the regulation of actin microfilament organization. Although the genetic nature of the *mcb* t.s. mutation has not been investigated, it was proposed that at restrictive temperatures the defective regulatory subunit is released from the catalytic subunit, resulting in increased PKA activity (Bruno et al., 1996).

More than four decades ago, morphogenesis was proposed to be dependent on the rate of intracellular catabolic and biosynthetic reactions (Brody and Tatum, 1966), and this has been recently supported by genetic studies demonstrating that primary metabolic processes are determinants of hyphal morphology (Oshero and May, 2001; Momany, 2002; Seiler and Plamann, 2003; Harris, 2006). The observation that mutations in both regulatory elements and metabolism-related and structural-element-encoding ('housekeeping') genes can result in phenotypically similar polarity defects suggests that metabolic input might regulate polar growth.

A clear link between the PKA pathway and carbon-source sensing has been established in *N. crassa* (Li and Borkovich, 2006) and *Aspergillus* (Oliver et al., 2002), and has been studied in depth in *Saccharomyces cerevisiae* (Rolland et al., 2002). Addition of glucose to de-repressed cells of *S. cerevisiae* was shown to cause a rapid, transient, increase in cAMP levels (Beullens et al., 1988). The cAMP signal induces a protein-phosphorylation cascade which is mediated, in part, by the activation of PKA (reviewed by D'Souza and Heitman, 2001; Santangelo, 2006). When cAMP levels increase, the PKA catalytic subunits are rendered enzymatically active and can phosphorylate target substrates that include metabolic enzymes as well as transcription factors (D'Souza and Heitman, 2001). Among the PKA-regulated DNA-binding proteins are transcription factors that have been found to be involved in cell morphogenesis and filamentation (Pan and Heitman, 1999; Tebarth et al., 2003), as well as growth regulation, stress response and carbohydrate-store accumulation (Smith et al., 1998).

One of the most studied transcriptional regulation mechanisms in response to glucose is carbon catabolite repression (CCR). Even though they are sometimes considered synonymous, glucose repression is only one specific case of a general carbon repression phenomenon (Ruijter and Visser, 1997). In filamentous ascomycetes, glucose transcriptional repression is mediated by CreA, a zinc-finger protein related to Mig1p from *S. cerevisiae* (Ronne, 1995; Ruijter and Visser, 1997). The *creA* genes of several filamentous fungi have been cloned and sequenced (Dowzer and Kelly, 1991; Drysdale et al., 1993; Strauss et al., 1995; de la Serna et al., 1999; Vautard et al., 1999; Tudzynski et al., 2000). However, little is known about the biological activity of CreA in the various systems, mainly due to a lack of available mutants. Although the aforementioned

CreA-related DNA-binding proteins exhibit a strong similarity to the yeast Mig1p, they are probably not fully functionally interchangeable between uni- and multicellular fungi as, for example, the *Sclerotinia sclerotiorum cre1* gene does not complement yeast $\Delta mig1$ mutants (Vautard et al., 1999). However, functional interchangeability among filamentous fungi has been demonstrated in several instances, including complementation of the *A. nidulans creA* loss-of-function mutant with the *S. sclerotiorum cre1* gene (Vautard et al., 1999), as well as with the *creA* gene from *Gibberella fujikuroi* or *Botrytis cinerea* (Tudzynski et al., 2000).

Functional regulation of the CreA/Cre1 transcription factor in filamentous fungi is not yet fully understood. In *A. nidulans*, *S. sclerotiorum* and *Hypocrea jecorina*, *creA/cre1* expression is regulated at the transcriptional level and has been shown to be repressed in the presence of glucose as a result of auto-regulation (Ilmen et al., 1996; Strauss et al., 1999; Vautard-Mey and Fevre, 2003). In contrast, expression of *G. fujikuroi* and *B. cinerea creA* was demonstrated to be continuously high in the presence of all carbon sources tested (Tudzynski et al., 2000). However, the transcript-abundance pattern of the *creA/cre1* gene did not correlate with the repressing activity of the protein (Strauss et al., 1999; Vautard-Mey et al., 1999; Tudzynski et al., 2000). Thus, a post-translational mechanism is probably also involved in the regulation of Cre-mediated repression activity. In fact, CreA/Cre1 has been shown to be capable of undergoing several modes of post-translational modification, including phosphorylation (Vautard-Mey and Fevre, 2000; Cziferszky et al., 2002) and ubiquitination (Lockington and Kelly, 2001; Boase and Kelly, 2004), which were found to affect transcription factor abundance and activity.

A long history of genetic and biochemical studies of metabolism in *N. crassa* has shown that many genes are subject to CCR. However, the molecular basis of CCR in this fungus has yet to be elucidated. Some processes known to be regulated by CCR in *N. crassa* are repressed to a similar extent by glucose and fructose, while others are subjected exclusively to glucose repression. This suggests that there are at least two forms of CCR in *N. crassa* and that one of these is glucose-specific (Ebbbole, 1998). For example, several extracellular hydrolytic enzymes are repressed by glucose (Ebbbole, 1998). Moreover, fructose uptake, which is completely distinct from glucose uptake, is repressed by glucose (Schneider and Wiley, 1971a,b; Rand and Tatum, 1980).

These findings prompted us to further investigate the differential effects of glucose and fructose on polarity during vegetative growth and to examine the possible involvement of the CRE-1 transcription factor, in addition to PKA, in the regulation of polarity.

In this paper, we show that fructose can suppress the apolar morphological consequence of elevated PKA levels occurring in the *mcb* strain (whose genetic defect is also analyzed). Our results demonstrate a regulatory role for fructose in determining PKA-dependent polarity, thereby

establishing a link between carbon-related regulation and polarity. In addition, we describe the phenotypic consequence of deleting *cre-1*, a key CCR regulator in *N. crassa*, which resulted in altered CCR and abnormal hyphal morphology, and we show that (i) CRE-1 is involved in determining PKA-dependent hyphal polarity, (ii) both PKA and CRE-1 independently control germination rate and (iii) high PKA activity levels enhance fructose-related derepression of the CRE-1-regulated genes *inv* and *cre-1*.

2. Materials and methods

2.1. Strains, media and growth conditions

General procedures and media used for handling *N. crassa* have been described previously (Davis, 2000) or are available through the FGSC (Fungal Genetic Stock Center, www.fgsc.net). The *N. crassa* strains used in this study are listed in Table 1 (see also McCluskey, 2003). Strains were grown in either liquid or solid (supplemented with 1.5% agar) Vogel's minimal medium with 1.5% (w/v) sucrose, unless otherwise stated. When required, the medium was supplemented with 100 µg/ml hygromycin B (Calbiochem, Riverside, CA).

2.2. Nucleic acid extraction and analysis

Recombinant DNA methods were performed according to standard protocols (Sambrook et al., 1989). For *N. crassa* genomic DNA isolation, approximately 20 mg of lyophilized and ground 1- to 2-day-old mycelial culture was resuspended in 500 µl DNA extraction buffer (0.15 M Tris-base, pH 7.5, 0.15 M sorbitol, 5 mM EDTA, 1 M NaCl, 1% w/v CTAB, 1% w/v sarkosyl, 4 mM sodium bisulfite). After incubation for 10 min at 60 °C, 700 µl of chloroform–octanol (24:1, v/v) was added and vigorously mixed. The samples were centrifuged (12,000g) at room temperature for 5 min. The aqueous layer of each sample (~400 µl) was transferred to a new microfuge tube and was gently mixed with 400 µl cold isopropanol and 40 µl sodium acetate (3 M, pH 5.2). The samples were incubated for 10 min at –20 °C and then centrifuged (5 min, 20,000g, 4 °C). The supernatant was removed and the pellet was washed with cold ethanol (75%, v/v) and recentrifuged.

After centrifugation, the ethanol was removed and the pellet was resuspended in 100 µl TE or nuclease-free water (Ambion, Foster City, CA). For *N. crassa* total RNA isolation, cultures were grown for 20–24 h and then filtered and immediately frozen in liquid nitrogen. The samples (0.5 g) were ground (twice, 30 s each time, at 4600 rpm) with 0.5 mm glass beads in a FastPrep FP120 bead beater (Savant, Farmingdale, NY) in the presence of 1.25 ml Tri-reagent (Sigma–Aldrich, St. Louis, MO). Chloroform (250 µl) was added to the extracts and the samples were ground again for 15 s (4600 rpm). The homogenates were centrifuged for 10 min at 12,000g at 4 °C, and the aqueous layer (~600 µl) was collected in a new microfuge tube and gently mixed with 600 µl isopropanol. The samples were incubated for 10 min at room temperature and then centrifuged (10 min, 20,000g, 4 °C). The supernatant was removed and the pellet was washed with cold ethanol (75%, v/v) and recentrifuged (5 min, 9000g, 4 °C). After centrifugation, the ethanol was removed and the pellet was resuspended in 300 µl nuclease-free water. PCR was performed according to standard protocols (Sambrook et al., 1989), using SuperTerm JMR801 Polymerase (JMR Holdings, Saint Louis, MO). Primers used in this study are listed in Table 2. PCR amplicons were first cloned into pDrive (QIAGEN, Hilden, Germany) and then sequenced using the T7 and SP6 primers, unless otherwise indicated. Sequencing was performed at the Center for Genomic Technologies, the Hebrew University of Jerusalem, Israel.

For RT-PCR, RNA samples were treated with RQ1 RNase free DNase (Promega, Madison, WI) and then purified with the RNeasy kit (QIAGEN) according to the manufacturer's protocol. The RT reaction was performed using SuperScript II RNase H-reverse transcriptase (Invitrogen, Carlsbad, CA).

For Northern blot analysis, total RNA samples (15 µg) were transferred to Hybond-XL nylon membranes (Amersham Biosciences, UK). Hybridization was performed at 65 °C in the presence of ULTRAhyb solution (Ambion, Austin, TX). The most stringent washes were carried out at 65 °C with 0.1× SSC and 0.1% (w/v) SDS. The blots were probed with [α -³²P]UTP-labeled RNA probes corresponding to 500–700 bp of the open-reading frame (ORF) of the gene of interest and were prepared by *in-vitro* transcription system using the T7/SP6 MAXIscript kit

Table 1
Neurospora crassa strains used in this study

Strain	Genotype	Source
Wild type	74-OR23-1 A	FGSC 987
Wild type	ORS-SL6 a	FGSC 4200
<i>mcb</i> (RM110-9)	<i>mcb</i> (RM110-9) A	FGSC 7454
<i>mcb</i> (RM110-5)	<i>mcb</i> (RM110-5) a	FGSC 7453
<i>mcb</i> (14-4)	<i>mcb</i> (14-4) a	Seiler and Plamann (2003)
Δ <i>mus-51</i>	Δ <i>mus-51::bar</i> ⁺ ; <i>his-3</i> A	FGSC 9717
Δ <i>cre-1</i>	Δ <i>cre-1::hph</i> a	This study
Δ <i>cre-1::mcb</i> (21)	Δ <i>cre-1::hph::mcb</i> (RM110-9) a	This study
Δ <i>cre-1::mcb</i> (22)	Δ <i>cre-1::hph::mcb</i> (RM110-9) A	This study

Table 2
Primers used in this study

Name	Sequence
mcbF	GCTCGACACCTCACAAGA
mcbR	CAATTCAATTTGCACTCTGGTC
mcb590F	TCTAACGCTTTTTGGCAGGT
mcb204F	ACCGTCCAGCACCTTGAGTT
mcb1678R	TGGGGAGTTGTAGGCCAAGT
mcb1509R	GTGAGGTGTGTCGAGCTGAA
mcb705F	CAGCCTTGTCTGCTCTGCTT
cre-1-778F	CAGGCCATACATGTCCAG
cre-1-1271R	GCTCCAGAGAGGCTGGTAGA
cre-1-386F	GGATGTGGAAGATTTTCTGG
5R-hph	ATCCACTTAACGTTACTGAAATCTCCAAC
KO-F	GTAACGCCAGGGTTTTCCAGTCACGACG
KO-R	GCGGATAACAATTTACACAGGAAACAGC
invF	GAACACGACCAAGTGGCCTA
invR	CCATCGCTGGTGATCCTAAT
actF	GGCCGTGATCTTACCGACTA
actR	GCTCTCGTCTACTCTCTGCT

(Ambion, Austin, TX), according to the manufacturer's protocol. rRNA staining was used as a loading control.

Invertase (NCU04265.3) expression was determined by Northern blot analysis using a 519-bp fragment of the *inv* ORF as a probe. The PCR product obtained with primers *invF* and *invR* was ligated into pDrive and the resulting plasmid was digested with XbaI. The RNA probe was synthesized using T7 RNA polymerase.

Actin (NCU04173.3) expression was determined by Northern blot analysis using a 616-bp fragment of the *act* ORF as a probe. The PCR product obtained with primers *actF* and *actR* was ligated into pDrive and the resulting plasmid was digested with HindIII. The RNA probe was synthesized using T7 RNA polymerase.

2.3. Identifying the *mcb* mutation

To sequence the *mcb* ORF, primers *mcbF* and *mcbR* were used for PCR and the resulting product was sequenced directly using primer *mcb590F*. To sequence the genomic 5'UTR of *mcb*, primers *mcb204F* and *mcb1678R* were used for PCR and the resulting product was sequenced directly using primer *mcb1509R*. To sequence the *mcb* mRNA 5'UTR, primers *mcb705F* and *mcb1678R* were used for PCR and the resulting product was sequenced directly using primer *mcb1678R*.

To detect the presence of the t.s. allele of *mcb* without sequencing, the PCR product obtained with primers *mcb204F* and *mcb1678R* was digested with BglII and the products resolved on a 10% TBE-acrylamide gel (1 mm, 8 V/cm, 2.5 h). HyperLadder V (Biolone, London, UK) was used as a DNA size marker. The digested PCR products were stained with ethidium bromide.

2.4. Deletion of *cre-1*

Deletion of *cre-1* was performed using the *Neurospora* genome project Gene Knockout Kit (obtained from the

FGSC) according to Colot et al. (2006). Briefly, the construct for homologous recombination of NCU08807.3 was provided by the *Neurospora* knockout project. The cassette was PCR-amplified using the KO-F and KO-R primers and was transformed into *N. crassa* strain Δ *mus-51*.

Several transformants were obtained and screened for *cre-1* gene replacement by PCR using primers *cre-1-778F* and *cre-1-1271R* (for *cre-1* ORF) and *cre-1-386F* and 5R-hph (to identify homologous recombination events) and were verified by Southern blot analysis. One of the candidate *cre-1* deletion transformants was crossed with *N. crassa* wild type (FGSC #4200) to obtain the *cre-1* deletion in a wild-type background. For Northern blot analysis, a 494-bp fragment corresponding to the *cre-1* ORF was PCR-amplified using primers *cre-1-778F* and *cre-1-1271R*, ligated into pDrive and the plasmid was linearized with BamHI to enable T7 RNA polymerase *in-vitro* transcription.

2.5. Determining growth rate, germination rate and biomass accumulation

For growth-rate measurements, 10 μ l of a conidial suspension (2×10^6 conidia/ml) were inoculated in race tubes containing Vogel's minimal medium supplemented with either glucose or fructose as carbon source. The race tubes were incubated for several days at 34 °C and the radial growth was measured twice daily.

To determine the germination rate of the different strains, pre-warmed Vogel's minimal medium supplemented with either glucose or fructose as carbon source was inoculated with a conidial suspension (final concentration of 10^7 conidia/ml) and incubated (34 °C) at 150 rpm for several hours. Cultures were sampled every 1–2 h for 10 h and examined by light microscopy (see further on).

To determine biomass accumulation, 20 ml of Vogel's minimal medium supplemented with either glucose or fructose as carbon source were inoculated with a conidial suspension (final concentration of 2×10^5 conidia/ml) and incubated (34 °C) at 150 rpm for 12, 16 or 20 h. At each time point, triplicate samples of each strain's cultures were filtered on a pre-weighed filter paper. The filter papers with the fungal culture were then dried at 65 °C for 24 h and weighed again. The net weight of the fungal dry biomass was then calculated.

2.6. Enzyme assays

Endogenous invertase activity was measured by a colorimetric test for glucose concentration (Herwig et al., 2001), based on the ability of invertase to disassociate the glucose and fructose components of sucrose.

For amylase-secretion assays, Petri dishes with Vogel's minimal medium were supplemented with 0.5% (w/v) starch and either 1.5% (w/v) glucose or fructose. The media dishes were inoculated with culture discs and incubated overnight at 34 °C. The dishes were then stained with

Lugol's solution (5% w/v iodine and 10% w/v potassium iodide in distilled water). Lugol is yellow-brown and when it reacts with starch, a blue-black iodide starch is produced.

For PKA activity assays during mycelial growth, strains were grown for 20 h in Vogel's sucrose medium at the permissive temperature (25 °C). They were then filtered, washed with Vogel's minimal medium and transferred to pre-warmed fresh Vogel's medium containing either glucose, fructose or no carbon source (starvation) and were further incubated at the restrictive temperature (36 °C) for 4 h. After morphological inspection, the culture samples were frozen in liquid nitrogen and immediately assayed for PKA activity. For determining PKA activity during germination, 10^6 conidia/ml were incubated for 3–6 h in pre-warmed (36 °C) Vogel's minimal medium supplemented with either glucose or fructose. Germinating conidia were harvested when most of the germ tubes were twice as long as the conidium. After morphological inspection, the germinating conidia were collected by centrifugation (10 min, 3000g, 4 °C) and immediately assayed for PKA activity. To extract protein, fungal samples were ground (twice, for 30 s each time, at 4600 rpm) with 0.5 mm glass beads in a FastPrep FP120 bead beater (Savant, Farmingdale, NY) in the presence of cold PKA extraction buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 5 mM EGTA, 10 mM NaF, 0.1 M KCl, 0.5% w/v Triton X-100, 0.2% w/v SDS). The homogenates were centrifuged for 40 min at 10,000g, 4 °C, and the supernatants were recovered for PKA activity assays. Protein concentration was determined using the Bradford protein assay kit (Bio-Rad, Hercules, CA). PKA activity was immediately assayed, on the basis of Kemptide phosphorylation, as measured by using a PepTag kit (Promega) according to the manufacturer's instructions, with minor modifications. Cell extracts containing 2 µg protein were incubated with 0.4 µg PepTag[®] A1 peptide (Kemptide) without the PKA activator at 36 °C.

2.7. Microscopy

Light microscopy was performed with a Zeiss Axioscope microscope equipped with a Nikon DXM1200F digital camera. Conidia of the different *N. crassa* strains were inoculated on glass slides covered with Vogel's minimal medium supplemented with either glucose or fructose as carbon source and incubated at 34 °C at high humidity. The germinating conidia and, at a later stage, the edge of the growing colonies were observed directly at different time points.

For scanning electron microscopy (SEM), samples were fixed for 4 h with 5% (v/v) glutaraldehyde in 0.1 M phosphate buffer, pH 7.2. The samples were washed five times with the same buffer and then dehydrated in a series of 25–100% ethanol washes. The fixed samples were dried for 1 h in a CPD750 drier (Bio-Rad) and gold-coated in a E5150 Polaron SEM coating system apparatus (Bio-

Rad). The samples were observed under a Jeol (Tokyo, Japan) JSM 35 microscope.

2.8. Computation

To quantify the Northern blot analysis results and PKA activity assay gels, Gel-Pro-Analyzer 3.0 (Media Cybernetics, Silver Spring, MD) was used. ImageJ 1.37V (Rasband, W.S., US National Institutes of Health, Bethesda, MD, <http://rsb.info.nih.gov/ij/>, 1997–2006) was used to analyze the microscopic documentation (to measure lengths between branches and calculate conidial germination rate). The Spidey utility (NCBI) was used for mRNA-to-genomic alignment (Wheelan et al., 2001).

3. Results

3.1. Altered carbon source affects *mcb* morphology

PKA plays an important role in signal-transduction pathways that are involved in carbon-source sensing and in polarity establishment and maintenance. To assess the possibility that carbon-related regulation of PKA activity may also affect polarity in *N. crassa*, the conditional *mcb* mutant was cultured at the restrictive temperature (36 °C) in the presence of different sugars (known to support *N. crassa* growth at different efficiencies) as sole carbon sources. Glucose, sucrose and fructose are metabolic sugars that support *N. crassa* biomass accumulation to a similar extent, while xylose, galactose, glycerol and lactose are substantially less efficient (Davis, 2000). When cultured in standard media containing either glucose (Fig. 1) or sucrose (Supplementary data S1), *mcb* exhibited apolar morphology. However, surprisingly, *mcb* exhibited complete suppression of its morphological defect at restrictive temperature when grown in fructose-containing medium (Fig. 1). In the presence of the less metabolic sugars (i.e. xylose, galactose, glycerol and lactose), partial suppression of the *mcb* morphological defect (characterized by the clear presence of filaments along with the bulbous cells) was observed (Supplementary data S1), similar to that observed when *mcb* was grown under carbon starvation (0.15%

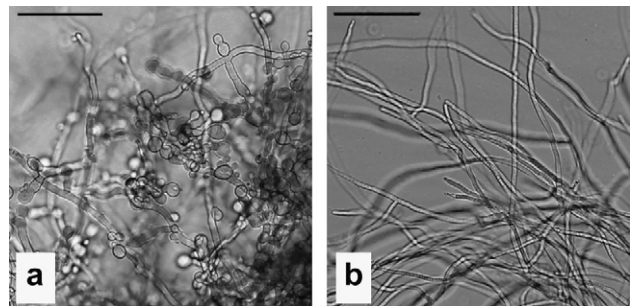


Fig. 1. *mcb* morphology at the restrictive temperature (36 °C) in Vogel's minimal medium supplemented with either glucose (a) or fructose (b) as a sole carbon source. Bar = 100 µm.

glucose or less). The fact that these sugars partially suppressed *mcb* morphology implies that cellular availability of the carbon source may be involved in the reestablishment of polar growth in the presence of fructose. To determine if this is indeed the case, *mcb* was grown in Vogel's minimal medium with different concentrations of fructose or glucose, ranging from 0.09% to 1.8%. The different concentrations did not alter the general effect of the two sugars on *mcb* morphology, with the exception of 0.09% glucose in which partially elongated hyphae were visible, as expected in the case of carbon starvation.

As no differences in germination rate, growth rate or biomass accumulation were observed when the wild-type strain was cultured on solid or liquid Vogel's medium containing glucose or fructose as a sole carbon source (Table 3), we concluded that fructose and glucose have equal abilities to support fungal growth. Furthermore, no morphological differences were observed in wild-type hyphal growth in the presence of either sugar, and branching frequencies of colonies grown on fructose vs. glucose as carbon source were similar (Table 3). These results support the possibility that fructose plays a regulatory rather than metabolic role in determining *mcb* morphology.

3.2. Aberrant splicing of the *mcb* transcript in the *t.s.* mutant

To further assess the effect of fructose on *mcb* morphology and to determine whether this sugar directly affects *mcb* expression or activity, the *mcb* mutation was characterized. The *mcb* loci in the wild type and in three different *t.s.* alleles (RM110-9, RM110-5 and 14-4) were sequenced. An identical deletion of a 19-bp segment at the end of first *mcb* intron (309 bp upstream of the start codon) was evident in the two mutant alleles RM110-9 and RM110-5 (Fig. 2a, GenBank Accession No. EF495262), while a very similar, but shorter, deletion (17 bp) was detected in the third allele (14-4). As the DNA deletion was mapped to the end of an intron, we analyzed the mRNA of *mcb* allele RM110-9 to determine if any aberrations were conferred by the mutation and determined that, indeed, this was the case. In contrast to the wild type, where RT-PCR (using primers *mcb*705F and *mcb*1678R, Table 2) yielded a single ~600-bp product (as expected), the *mcb* RNA template yielded multiple products of different sizes (Fig. 2b). The sequences of the cloned RT-PCR products provided clear evidence for the occurrence of aberrant *mcb* splicing in the analyzed RM110-9 allele of *mcb* (Supplementary data S2).

Table 3
The effect of fructose and glucose on *N. crassa* wild-type growth

	Glucose	Fructose
Growth rate (mm/h) ($n = 3$)	4.8 ± 0.1	4.6 ± 0.1
Biomass accumulation after 20 h (mg) ($n = 3$)	52 ± 7	58 ± 10
Distance between branch points (μm) ($n = 400$)	161 ± 26	140 ± 35
Germination rate (%) after 5 h ($n = 300$)	93 ± 2.1	95 ± 1.4

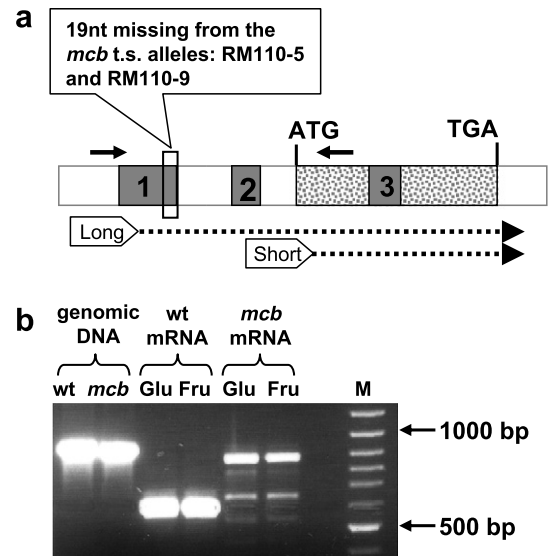


Fig. 2. Identifying the *mcb* mutation. (a) Schematic diagram (not to scale) of the *mcb* gene and the deletion identified in the two identical alleles *mcb*(RM110-5) and *mcb*(RM110-9). Dark gray and stippled boxes represent introns (numbered) and coding sequences, respectively. Dotted arrows represent mRNA and small arrows represent primers used for RT-PCR. (b) PCR and RT-PCR analyses of wild type (wt) and *mcb*(RM110-9) (*mcb*) genomic DNA and cDNA, respectively. M, DNA marker; Glu, glucose; Fru, fructose.

The length and abundance of the different splicing products were not altered in *mcb* cultures grown in either glucose or fructose media and were also not influenced by the incubation temperature (25 or 36 °C). The lack of influence of the different growth conditions on *mcb* mRNA processing suggests that alterations in downstream processes, such as translation rate, abundance and/or localization of the translated products, may contribute to the severe mutant phenotype.

3.3. Fructose does not affect PKA activity in *mcb*

To further characterize the effect of fructose on the *mcb* mutant, relative PKA activity levels were measured in protein extracts of both wild type and *mcb* grown in fructose vs. glucose, as well as in extracts from cultures grown under carbon starvation. The results (Fig. 3) demonstrated markedly higher (2- to 2.5-fold) PKA activity levels in the *mcb* extract relative to the wild type and supported the presence of an increase in PKA activity in the *mcb* mutant, as previously proposed (Bruno et al., 1996). Moreover, even though PKA activity levels in both wild type and *mcb* grown in the presence of fructose were slightly lower than those measured in the presence of glucose, PKA activity levels of *mcb* grown in fructose were still twice as high as those measured in the wild type. Thus, the phenotypic suppression of *mcb* morphology conferred by fructose cannot be attributed to a reduction in PKA activity levels. In contrast, PKA activity levels in *mcb* incubated in the absence of a carbon source were comparable to those measured in

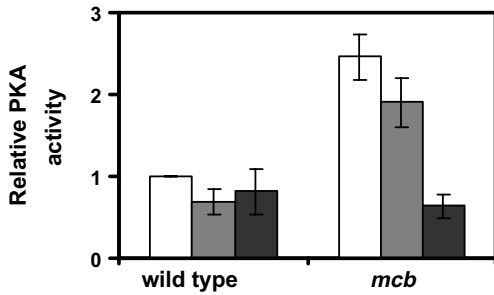


Fig. 3. Relative PKA activity in extracts prepared from cultures, 4 h after being shifted to 36 °C. PKA activity levels were normalized to those of the wild type grown in glucose-containing medium. Cultures were incubated in Vogel's minimal medium supplemented with either glucose (white bars), fructose (gray bars), or without any carbon source (black bars) and were assayed for PKA activity. Data are means of three independent experiments with two replicates each. Standard errors are shown.

the wild type, suggesting that in this case, the partial morphological suppression observed in *mcb* cultures under conditions of carbon starvation could be the result of the observed near-normal PKA activity levels.

3.4. Fructose is a fermentable, non-repressing carbon source

To further investigate the regulatory effect of glucose and fructose on gene expression, the expression of *inv* (encoding invertase; NCU04265.3) was examined. This enzyme hydrolyses extracellular sucrose into glucose and fructose. Expression of the invertase-encoding gene as a marker for CCR has been previously demonstrated in *Schizosaccharomyces pombe* (Tanaka et al., 1998), *S. cerevisiae* (reviewed by Trumbly, 1992) and *N. crassa* (Allen et al., 1989). *inv* transcription levels and activity were assessed in the wild type and *mcb* mutant grown in medium containing either glucose or fructose. *inv* expression was almost completely absent in both strains grown with glucose (Fig. 4a). However, in fructose medium, *inv* transcript levels, as well as invertase activity levels, were high in both strains, indicative of a de-repressing event. Moreover, the level of *inv* transcript in the de-repressing fructose-containing medium was approximately 2-fold higher in the *mcb* mutant than in the wild type.

As actin has been suggested to play a role in the apolar defect of *mcb* (Bruno et al., 1996), we examined *act* expression in the different strains under the different growth conditions. Although *act* expression was not affected by the change in carbon source in either the wild type or the *mcb* mutant, a clear difference in *act* transcript levels was evident between the two strains, with expression being significantly lower in *mcb* (Fig. 4a).

As fructose was found to suppress the defect in *mcb* morphology without directly affecting *mcb* expression or PKA activity in the t.s. mutant, and it was found to de-repress the expression of the carbon-regulated gene *inv*, we analyzed one of the key regulatory CCR factors in *Neurospora*. *N. crassa* CRE-1 is the homolog of CreA

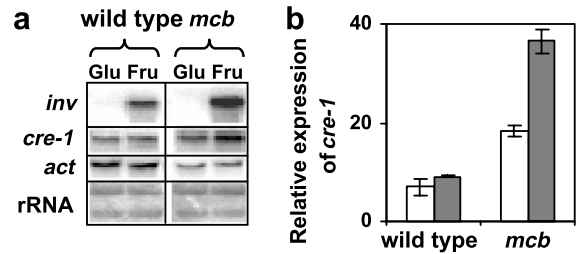


Fig. 4. Altered regulation by glucose and fructose in wild type and *mcb*. (a) Northern blot analysis of RNA samples from cultures incubated in Vogel's minimal medium supplemented with either glucose (Glu) or fructose (Fru) 4 h after being shifted to 36 °C. RNA samples were tested for the expression of invertase (*inv*), the transcription factor CRE-1 (*cre-1*), and actin (*act*). rRNA is presented as a loading control. (b) Quantification of *cre-1* expression, as detected by Northern blot analysis of RNA samples from wild type and *mcb* cultures incubated in Vogel's minimal medium supplemented with glucose (white bars) or fructose (gray bars) after 4 h shift to 36 °C. Values are means of three independent experiments. Standard errors are shown.

(de la Serna et al., 1999), a well-known CCR regulatory factor in other filamentous fungi. We characterized the expression pattern of *cre-1* in the *N. crassa* wild type and *mcb* strains grown with either fructose or glucose as the carbon source. Northern blot analysis (Fig. 4a and b) indicated that *cre-1* expression in the wild type is similar in the presence of either of the two sugars (albeit a bit lower in glucose). However, *cre-1* expression was higher in the *mcb* mutant and resulted in a 2.6- and 4-fold increase in transcription levels in glucose and in fructose, respectively.

These results indicate that growth with glucose and fructose can result in different levels of gene expression (as in the case of *inv*). In addition, the *mcb* mutation affects the expression levels of both *inv* and *cre-1* under the de-repressing conditions conferred by fructose, by increasing their transcription levels. Moreover, the *mcb* mutation affects the transcription levels of *act*, a key determinant in polarity.

3.5. CRE-1 affects growth rate and morphogenesis in *N. crassa*

To further analyze the role of CRE-1 in *N. crassa* CCR and its possible interaction with PKA, a *cre-1* deletion strain was produced using a cassette designed for *cre-1* homologous recombination (i.e. harboring the bacterial *hph* gene between the 5'- and 3'-flanking regions of *cre-1*). Although the cassette used for the deletion of *cre-1* included the *hph* gene, transformant strains exhibited only mild resistance to hygromycin. The deletion was verified by PCR, Southern blot analysis and RT-PCR. In addition, Northern blot analyses verified that the *cre-1* transcript is absent in the $\Delta cre-1$ strain under all conditions tested (Fig. 5).

When cultured in Vogel's glucose medium the $\Delta cre-1$ strain grows more slowly than the wild type and produces a dense colony (Fig. 6a). Microscopic examination of the

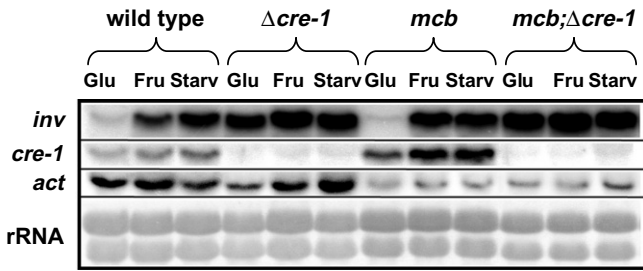


Fig. 5. The *mcb;Δcre-1* strain exhibits altered CCR. Northern blot analysis of RNA samples from cultures incubated in Vogel's minimal medium supplemented with 1.5% glucose (Glu), 1.5% fructose (Fru) or 0.1% glucose (Starv) for 4 h at 36 °C. RNA samples were tested for the expression of invertase (*inv*), CRE-1 (*cre-1*) and actin (*act*). rRNA is presented as a loading control.

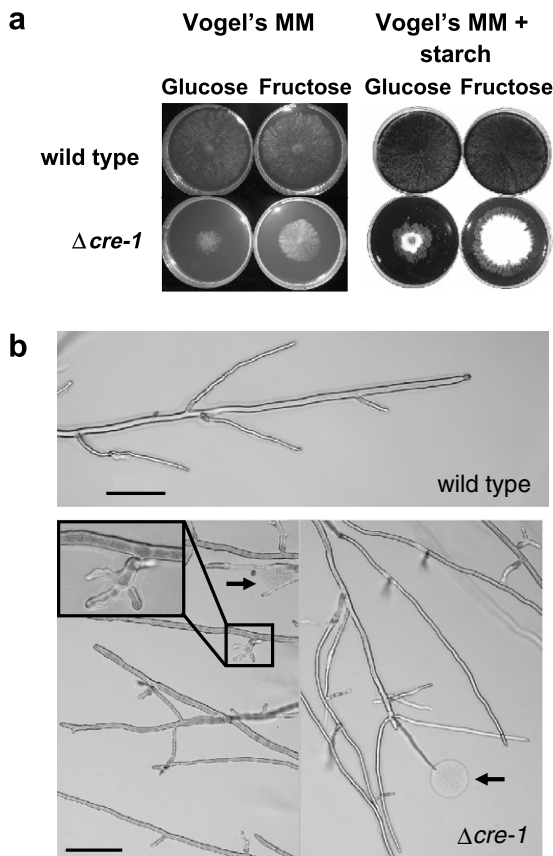


Fig. 6. The $\Delta cre-1$ strain grows poorly on glucose. (a) Left panel: growth of wild type and $\Delta cre-1$ on Vogel's minimal medium (MM) plates supplemented with either 1.5% glucose or 1.5% fructose as carbon source. Right panel: changes in amylase activity as a result of altered CCR in the $\Delta cre-1$ strain. The growth medium was supplemented with 0.5% starch in addition to either 1.5% glucose or 1.5% fructose as carbon source. Cultures of wild type and $\Delta cre-1$ were grown overnight at 34 °C and were subsequently dyed with Lugol (see M&M). Dark areas indicate the presence of starch. White halos represent areas where starch was degraded as a result of secreted amylase. (b) Hyphal morphology of wild type (upper panel) and $\Delta cre-1$ (lower panels) cultured on 1.5% glucose. Arrows mark areas of hyphal tip lysis. Bar, 100 μ m.

mutant revealed hyphal morphology that differs from the typical tubular hyphae of the wild type. The $\Delta cre-1$ hyphae

were swollen, irregular in diameter and displayed an abnormal branching pattern, with multiple branches emerging from single branching points (Figs. 6b and 7). In addition, when grown on solid medium, apical lysis at the hyphal tips was a common phenomenon (Fig. 6b), characteristic of mutants affected in cell-wall integrity (Bowman et al., 2006; Upadhyay and Shaw, 2006), thus suggesting a role for CRE-1 in controlling cell-wall biosynthesis. Similar phenotypic characteristics were observed when glucose was substituted with sucrose (data not shown). A complementation experiment, in which a full length *cre-1* gene was introduced into the mutant, verified that the phenotype associated with the $\Delta cre-1$ strain can be attributed to the deletion of *cre-1* (data not shown).

As homologs of CRE-1 are known to be involved in mediating cellular responses to carbon availability in filamentous fungi (Ronne, 1995; Ruijter and Visser, 1997), we determined the effect of deleting *cre-1* on growth rate and morphology in the presence of different carbon sources. Both wild type and $\Delta cre-1$ were grown on Vogel's minimal medium supplemented with different carbon sources. While the $\Delta cre-1$ strain grew poorly on rich media (e.g. PDA, YPD, Joham's, SD, 2% peptone, 1.5% glucose or 1.5% sucrose), it's colony diameter was increased when grown on poor media (0.1% glucose, 0.3% QA, 1.5% xylose, 2% glycerol or 0.1 M sorbitol). Interestingly, while fructose and glucose supported *N. crassa* wild-type growth to a similar extent (Table 3), the $\Delta cre-1$ strain grew much better on 1.5% fructose than on glucose (Fig. 6a). Nevertheless, $\Delta cre-1$ grew more slowly than the wild type in the presence of either of these sugars (as was also determined by race tube experiments, data not shown). In liquid medium, biomass accumulation of the deletion strain was similar in the presence of either sugar, but still markedly reduced relative to the wild type.

Interestingly, deletion of *cre-1* also affected the expression level of cytoskeletal genes in a sugar-dependent manner, as *act* expression was reduced in the $\Delta cre-1$ strain grown in glucose-containing medium (Fig. 5). The reduced level of *act* transcript may be the cause for the aberrant morphology observed in the $\Delta cre-1$ strain, but further investigation is needed to establish this proposed connection.

Based on our results, we concluded that CRE-1 participates in the regulation of hyphal growth in a carbon-source-dependent manner. To investigate the involvement of CRE-1 in the fructose effect on *mcb* morphology, we crossed *mcb* with $\Delta cre-1$. The presence of the 19-bp deletion corresponding to the *mcb* t.s. mutant allele was used to identify the *mcb* mutation in *mcb*(t.s) \times $\Delta cre-1$ progeny. Briefly, when digesting the 5'UTR genomic *mcb* amplicon with BglII, a typical 78-bp band was present in the wild type whereas a shorter 59-bp band was obtained in the *mcb* mutant (Supplementary data S3). Potential double mutants were also tested for distribution of *cre-1* by Southern (not shown) and Northern blot analyses (Fig. 5). Two of the identified double mutants (designated 21 and 22)

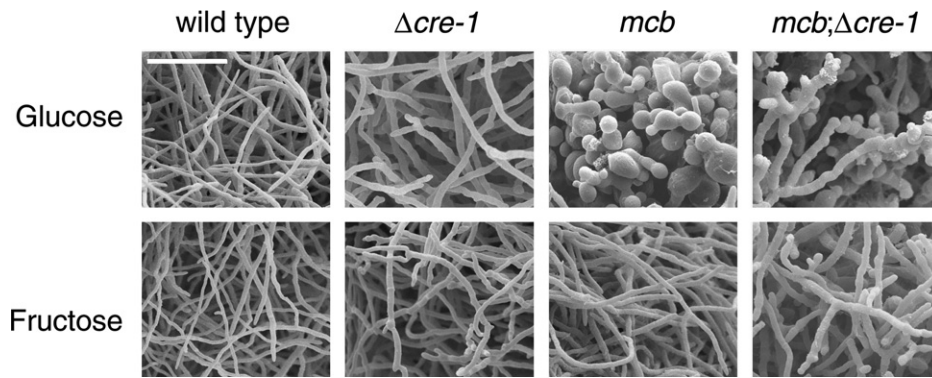


Fig. 7. Morphology of wild type, $\Delta cre-1$, *mcb* and *mcb*; $\Delta cre-1$ after a 4 h shift to restrictive temperature in glucose- or fructose-containing media. Bar, 50 μm .

were used for further analysis. As a final verification step, both *mcb*; $\Delta cre-1$ 21 and 22 were backcrossed to the wild type. In both cases, the parental strains, *mcb* and $\Delta cre-1$, were recovered.

Initial characterization of the *mcb*; $\Delta cre-1$ strains demonstrated improved growth rate and morphology when the double mutants were grown on fructose vs. glucose, a characteristic of the parental strains. Their growth rate, both in race tubes and on Petri dishes, was found to be very similar to that of *mcb*.

To investigate the involvement of CRE-1 in the fructose vs. glucose effect on *mcb* polarity in more detail, we examined the hyphal morphology of the *mcb*; $\Delta cre-1$ double mutants in liquid and on solid media. The deletion of *cre-1* did not completely abolish the fructose-dependent suppression of *mcb* morphology (Fig. 7). Thus, we concluded that the fructose-based suppression of the apolar defect in *mcb* during filamentous growth is, at least in part, independent of CRE-1. Nevertheless, partial suppression of the *mcb* morphology defect in glucose was clearly evident (Fig. 7).

3.6. Deletion of *cre-1* suppresses *mcb* morphology during conidial germination

As reduced growth rate and reduced biomass accumulation can be attributed, in part, to a slow germination rate, we also investigated the germination characteristics of the *mcb*, $\Delta cre-1$ and *mcb*; $\Delta cre-1$ strains in detail. The germination kinetics of both single mutants was different than that of wild type and was similar in both glucose and fructose-containing media (Fig. 8a). Following a lag period of several hours, conidial germination rate of $\Delta cre-1$ was similar to that of the wild type. *mcb*, on the other hand, exhibited a slower germination pattern consisting of both a delay in the onset of germination as well as a slower germination rate once the process had started. The *mcb*; $\Delta cre-1$ double mutant germinated even slower than each of the single mutants. Nonetheless, more than 85% of the double mutant conidia germinated within 10 h, indicating that no severe effect on conidial viability was conferred. These

results support the hypothesis that the two mutations affect germination independently.

While germination rates were reduced, the polarity characteristics of the germinating double mutant was markedly improved when compared to *mcb* germination on glucose (Fig. 8b). More specifically, *mcb*; $\Delta cre-1$ germ tubes regained polar growth and could be easily distinguished from the bulbous apolar morphology of *mcb*, suggesting that a CRE-1-mediated cellular response to carbon source is involved in determining PKA-dependent polar growth during the germination of *N. crassa* conidia.

To further characterize the effect of the *cre-1* deletion on germination morphology of *mcb*, PKA activity levels were assayed in protein extracts of the wild type, *mcb*, $\Delta cre-1$ and the double mutants during germination. PKA activity levels were about 2-fold higher in *mcb* germlings than in the wild type, in the presence of either glucose or fructose (Fig. 8c). Interestingly, although PKA activity levels of $\Delta cre-1$ in glucose were very similar to those of the wild type, a marked reduction in these levels ($\sim 50\%$) was measured in the mutant germinated in fructose. Furthermore, a reduction in PKA activity levels (relative to *mcb*) was measured in extracts of the *mcb*; $\Delta cre-1$ strains, indicating a reduction in PKA activity had occurred as a result of inactivating CRE1 (Fig. 8c). Although deletion of *cre-1* resulted in a reduction in PKA activity levels in the double mutants when germinated in glucose, it was still 1.5-fold higher than in the wild type and probably cannot fully account for the observed morphological suppression of the *mcb* defect. These results, along with the fact that elevated PKA levels affect *cre-1* expression, demonstrate a functional link between PKA and CRE-1 in determining carbon-source-affected morphology.

3.7. CRE-1 is involved in the regulation of CCR in *N. crassa*

In *A. nidulans* and *H. jecorina*, CreA/Cre1 has been shown to mediate glucose repression which affects the expression of many genes, in particular those encoding enzymes that are involved in carbohydrate catabolism and extracellular hydrolytic enzymes (Ilmen et al., 1996;

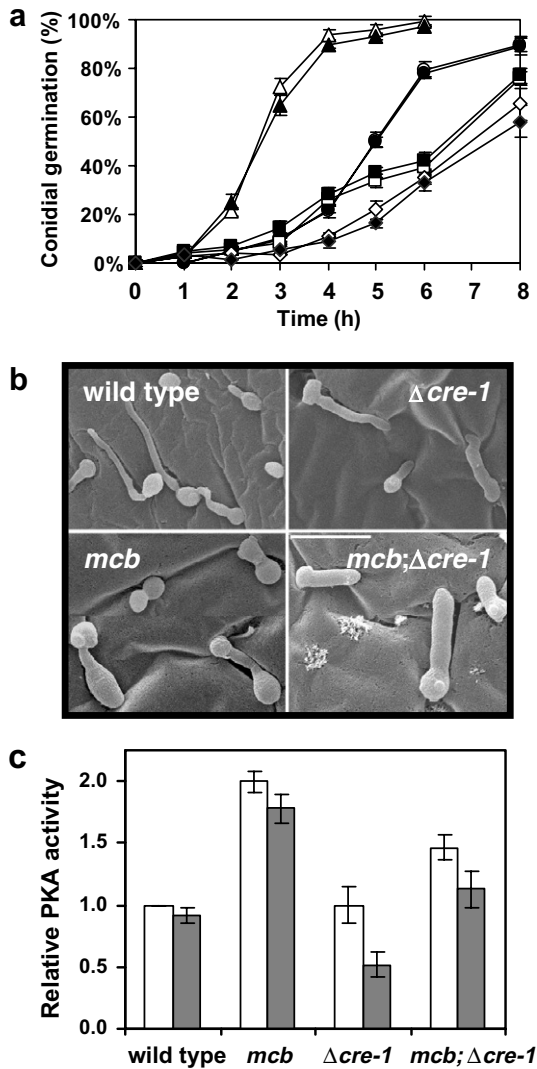


Fig. 8. The *mcb*; $\Delta cre-1$ strain exhibits reduced germination rate. The morphological defect of *mcb* during germinating is relieved by the deletion of *cre-1* and is accompanied by a reduction in the PKA activity level. (a) Germination of wild type (\triangle , \blacktriangle), *mcb* (\square , \blacksquare), $\Delta cre-1$ (\circ , \bullet) and the *mcb*; $\Delta cre-1$ double mutant (\diamond , \blacklozenge) incubated at 36 °C in Vogel's minimal medium supplemented with either 1.5% glucose (white symbols) or 1.5% fructose (black symbols) as carbon source. Standard deviations are shown. (b) Morphology of germinating conidia on Vogel's minimal medium supplemented with 1.5% glucose as the carbon source at 36 °C, 3–6 h post-inoculation. Bar, 20 μ m. (c) PKA activity of germinating conidia of wild type, *mcb*, $\Delta cre-1$ and *mcb*; $\Delta cre-1$, 3–6 h post-inoculation, relative to wild type germinated with glucose. Cultures were incubated in pre-warmed liquid Vogel's minimal medium supplemented with either 1.5% glucose (white bars) or 1.5% fructose (gray bars) as a carbon source at 36 °C and were assayed for PKA activity. Data are means of at least three independent experiments with two replicates each. Standard errors are shown.

Mach et al., 1996; Shroff et al., 1997; Mogensen et al., 2006). To assess the involvement of *N. crassa* CRE-1 in CCR, $\Delta cre-1$ was tested for the degree of expression and/or activity of genes and enzymes involved in the utilization of sucrose, ethanol, starch and lactose in the presence of either glucose or fructose. Deletion of *cre-1* resulted in mis-regulation and de-repression of the glucose-repressed

genes *inv* (Fig. 5) and *adh-1* (data not shown) and in de-repression of the secreted enzymes amylase (Fig. 6a) and β -galactosidase (data not shown) under all conditions tested. Thus, deletion of *cre-1* impairs CCR. Furthermore, the *mcb*; $\Delta cre-1$ double mutants presented an altered CCR response: *inv* expression was de-repressed in glucose-grown cultures (Fig. 5) and amylase secretion was de-repressed in the *mcb*; $\Delta cre-1$ double mutants grown on Vogel's glucose medium supplemented with 0.5% starch. Based on these results, we concluded that CRE-1-related glucose repression of the different genes is independent of PKA. Nonetheless, the fact that the *mcb* mutation affects *inv* and *cre-1* transcript levels in the presence of fructose suggests a role for PKA in de-repression of the tested transcripts.

4. Discussion

4.1. The differential effects of glucose and fructose on the morphology of *mcb*

With the exception of neurons and pollen tubes, no cell type displays the extreme degree of polarized growth seen in the fungal filament (Nelson, 2003; Harris, 2006). As polar growth of the fungal cell is such a critical component of fungal development and proliferation (Momany, 2002; Harris, 2006), understanding the mechanism contributing to its establishment and maintenance is of significant value.

The *N. crassa* *mcb* mutant was the first examined conditional mutant defective in growth polarity (Bruno et al., 1996). Here we show that the typical bulbous phenotype of *mcb* is easily recognized when grown in sucrose or glucose; however when grown with fructose as sole carbon source, the morphological phenotype of *mcb* is suppressed and near-normal hyphal elongation occurs. This result prompted us to first compare the influence of glucose vs. fructose on hyphal polarity and then examine the possible involvement of CRE-1, one of the key regulators of CCR, in mediating the effects of the different sugars on fungal growth.

Growth rate, biomass accumulation and branching frequency of the *N. crassa* wild-type strain were similar in media containing either of these metabolic sugars—fructose or glucose. Nevertheless, other studies have demonstrated that the two sugars may differ in gene-expression regulation and CCR in this fungus (Ebbole, 1998). CCR has been mostly associated with glucose, considered a highly repressing sugar. However, other sugars, including sucrose, fructose and maltose, are known to elicit CCR, albeit to a lesser extent (Ruijter and Visser, 1997; Wanke et al., 1997). In *Aspergillus*, fructose is considered a weakly repressing, fermentable carbon source relative to glucose (Oliver et al., 2002). The results presented here suggest a similar role for fructose in *N. crassa*. In the presence of fructose, *inv* and *cre-1* exhibited different expression patterns than with glucose. Interestingly, a distinct fructose effect was also evident with regard to fungal development and morphogenesis of the *mcb* and $\Delta cre-1$ mutants, as these

strains' growth rate and morphology were different in glucose vs. fructose. These results further support the hypothesis that some genes and developmental processes in *N. crassa* are similarly regulated by fructose and glucose, whereas others are not.

Taken together, the results presented here demonstrate a regulatory role for fructose in determining PKA-dependent polarity and indicate that increased PKA activity levels do not necessarily result in apolar growth. We determined that the fructose signaling pathway can bypass PKA activity regulation, as no significant reduction in PKA activity levels was observed in the *mcb* mutant in the presence of fructose. In addition, the effect of fructose on polarity was found to be partially independent of CRE-1, as the deletion of *cre-1* did not completely abolish its effect on *mcb* morphology. Thus, other factors are likely to be involved in mediating the regulatory effect of fructose. A potential candidate could be RCO-1, a repressor of conidiation related-gene expression, even though it was not found to play a role in glucose regulation of several tested genes (Lee and Ebbole, 1998).

4.2. The *mcb* mutation confers changes in PKA activity level

PKA appears to be much more important for the polarized hyphal growth typical of filamentous fungi than for yeast morphology (Harris, 2006). In this study, the defect in *mcb* resulted in elevated levels of PKA activity in the t.s. mutant. Similar changes have been reported in the *Colletotrichum lagenarium rpk1* mutant, lacking the gene encoding the regulatory subunit of PKA (Takano et al., 2001). Nevertheless, the nature of the *mcb* mutation has not yet been resolved. We analyzed three t.s. alleles of *mcb* and determined that a similar defect (a 17- or a 19-bp deletion in the 5'UTR of the long *mcb* transcript) in these mutants is responsible for the observed phenotype. Based on our results, the deletion, located at the end of the first intron, results in the accumulation of several mRNA variants which are the result of aberrant splicing. Various studies have demonstrated that UTRs have important regulatory roles in eukaryotes and can affect mRNA nuclear export, cytoplasmic localization, translational efficiency and stability (Hughes, 2006). As both short and long *mcb* transcripts have been reported to be more abundant in the *mcb* mutant than in the wild type (Bruno et al., 1996), a potential reduction of improperly spliced mRNA stability can most probably be ruled out. On the other hand, MCB protein levels were found to be reduced in the *mcb* mutant (Yi Liu, personal communication). Thus, we propose that the altered 5'UTR results in an impairment in *mcb* mRNA translation efficiency. This, in turn may lead to a reduction in the abundance (but not total depletion) of MCB protein, which subsequently results in elevated PKA activity. The presence of intracellular cAMP would result in the complete dissociation of the small amount of functional MCB protein from the catalytic subunit, rendering the latter active. Such a mechanism is also in line with

the previously reported suppression of *mcb* by *cre-1* [a mutant lacking adenylyl cyclase activity (Bruno et al., 1996; Gavric and Griffiths, 2003)]. However, it is possible that activity of the PKA-catalytic subunit is subjected to additional modes of regulation, which are independent of cAMP and/or the regulatory subunit MCB.

4.3. PKA and CRE-1 affect CCR and hyphal morphology

CCR involves reduction in the expression of many fungal genes in the presence of glucose, presumably as an energy-saving response. *A. nidulans* CreA has been found to be an important regulator of CCR and it is the founding member of the Cre-related transcription factors found to be present in many saprophytic and pathogenic fungi (Ronne, 1995). Although homologs of this protein have been identified in many different fungi, only a few Cre-related mutants have been investigated. Thus, most of the functional data related to CreA is available from research conducted in yeast (Tanaka et al., 1998) as well as *Aspergillus* spp. (Ruijter and Visser, 1997) and *H. jecorina* (Ilmen et al., 1996). We characterized a *N. crassa cre-1* deletion strain— $\Delta cre-1$. This mutant displayed altered CCR (as evidenced by both expression of carbon-regulated genes and biochemical tests for secreted enzymes), similar to the reported observations in *Aspergillus* and *H. jecorina*. The diverse morphological defects evident in the *N. crassa* $\Delta cre-1$ mutant suggest that in addition to its role as a mediator of CCR, CRE-1 affects other cellular processes. Thus, we suggest that CRE-1 may also be involved in regulating the expression of genes related to fungal morphology and development.

Actin is considered a key component in determining polarity in filamentous fungi (Harris, 2006). Expression of the *N. crassa* actin-encoding gene (*act*) has been shown to be altered in response to carbon-source availability (Xie et al., 2004). In this study, Northern blot analyses demonstrated similar *act* expression levels in fructose and in glucose in the wild type and the *mcb* mutant; however, *act* transcript levels were reduced in *mcb* relative to the wild type. In contrast, *act* transcription in the $\Delta cre-1$ strain varied when cultured on the different sugars and *act* transcript levels were found to be lower in glucose than in fructose. Similar downregulation in the expression of the actin-encoding gene, as well as the tubulin alpha-2 chain-encoding gene, has been reported in transcription-profiling experiments with the *creA* deletion strain of *A. nidulans* (Mogensen et al., 2006). Thus, it appears that during transcriptional regulation of cytoskeletal-encoding genes, CreA/CRE-1 acts as an activator, rather than its traditional role as a repressor. In this context, it is important to note that glucose-dependent CreA/CRE-1-mediated regulation has been described to result in induction of gene expression (Ronne, 1995; Mogensen et al., 2006). Taken together, our results indicate that *act* expression is dependent on both PKA activity levels and CRE-1 and may

imply that other structural genes are regulated by both CRE-1 and PKA.

These results, as well as the partial suppression of *mcb* morphology by the deletion of *cre-1*, suggest an interaction between these two regulators in determining polarity. Several recent reports indicate that CreA/Cre1 affect the expression of genes involved in the utilization of alternative carbon sources by competing with other transcription-regulation factors for binding to their overlapping cognate binding sites in the promoters (Mathieu et al., 2005; Hirota et al., 2006; Rauscher et al., 2006). We propose that this counter-regulatory mode of action can also take place when CRE-1 regulates the expression of genes related to cell morphology. In this context, we suggest that CRE-1 may be involved in controlling morphology-related genes in a manner that is parallel, or opposing, the PKA-related regulatory mechanism.

4.4. The effect of PKA and CRE-1 on germination

Conidial germination is an essential developmental stage in the life cycle of all filamentous fungi (d'Enfert, 1997) and is a highly regulated process that is triggered by environmental stimuli (Osheroov and May, 2001). Conidial germination in response to carbon source has been reported to be controlled by several signaling pathways: the MAP kinase pathway, heterotrimeric G-proteins, cAMP signaling and the Ras pathway, in both saprophytic [*A. nidulans* (Fillinger et al., 2002; Lafon et al., 2005)] and pathogenic fungi [*Botrytis cinerea* (Doehlemann et al., 2006); *Colletotrichum gloeosporioides* (Barhoom and Sharon, 2004)].

Our results demonstrate a role for PKA and CRE-1 in the regulation of *N. crassa* conidial germination. We suggest that these two regulators affect gene expression in response to carbon-source availability, to control the initiation of the germination process and germ-tube polarity, in concert with multiple additional transcriptionally regulated factors (Kasuga et al., 2005). We have demonstrated that deletion of *cre-1* resulted in a delayed germination process, while the *mcb*; Δ *cre-1* mutant germinated even more slowly. Furthermore, we show that high PKA activity levels result in delayed germination, similar to results obtained in *A. niger* and *A. fumigatus* (Saudohar et al., 2002; Zhao et al., 2006). Thus, PKA and CRE-1 act as a repressor and an activator of germination, respectively, but most likely, in an independent manner. In fact, PKA has been reported to inhibit transcription activators by phosphorylation, concomitantly reducing gene expression (Higuchi et al., 2002), while CRE-1 has been proposed to function as an activator by suppressing a downstream suppressor (Mogensen et al., 2006).

At later stages of germ-tube elongation, the apolar morphology of *mcb* (Bruno et al., 1996 and this study) suggests a role for PKA in maintaining polarity, such that high PKA levels prevent polar growth. A deletion in *cre-1* was found to result in partial suppression of the typical apolar *mcb* morphology. Thus, it is possible that PKA and CRE-1

co-regulate downstream components that are involved in maintaining polarity. However, the observed changes in PKA activity levels in the Δ *cre-1* strains indicate the involvement of CRE-1 in regulating PKA activity and hence affecting polarity via the PKA pathway.

Disruption of *cre-1* had a more subtle effect on loss of the polarity defect during later stages of hyphal growth. This may reflect differences in the molecular mechanisms governing polarity during conidial germination vs. hyphal extension and is supported by the differential involvement of regulatory components such as Cdc42 and Rho GTPases in controlling polarity during germination vs. hyphal elongation (Harris, 2006).

4.5. Fructose-dependent regulation of PKA and CRE-1

Since PKA is known to play a pivotal role in determining fungal polarity and both PKA and CRE-1 are known to mediate the cellular response to carbon-source availability, we searched for a possible link between these two major regulatory factors in *N. crassa*. Our results suggest that PKA and CRE-1 can regulate each other's functions. Several lines of evidence, presented in this paper, support an interaction between PKA and CRE-1: (i) *cre-1* expression was altered in an *mcb* (high PKA) background; (ii) PKA activity levels were altered during germination in a *cre-1* deletion strain; and (iii) the *mcb*; Δ *cre-1* double mutant displayed partial reestablishment of hyphal polarity, relative to the apolar morphology typical of *mcb*.

The special nature of fructose as a fermentable and non-repressing sugar allowed to detect these interactions, as the high PKA levels in *mcb* resulted in induced expression of *cre-1*, especially in the presence of fructose, while deletion of *cre-1* resulted in a 50% reduction in PKA activity during germination of Δ *cre-1* in fructose. Thus, it seems that both factors affect each other, although most likely in an indirect manner.

To our knowledge, this is the first report on the complex regulatory interactions between PKA and CRE-1 affecting cell polarity in a filamentous fungus. The mutants produced in this study, along with the large-scale transcription-profiling platform developed for *N. crassa* (Kasuga et al., 2005) will enable further examination of the involvement of PKA and CRE-1 in regulating the expression of genes that are involved in determining polarity. These tools, along with the large collection of *N. crassa* deletion strains (Colot et al., 2006), will hopefully facilitate the identification of other factors participating in the regulatory pathways affecting polarity in general and mediating the fructose effect on *mcb* polarity in particular.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.fgb.2007.05.005](https://doi.org/10.1016/j.fgb.2007.05.005).

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