Microbiology (1997), **143**, 1211–1220 Printed in Great Britain

Characterization and overexpression of the *Aspergillus niger* gene encoding the cAMP-dependent protein kinase catalytic subunit

Mojca Bencina,[1,2] Henk Panneman,[1] George J. G. Ruijter,[1] Matic Legiša[2] and Jaap Visser[1]

Author for correspondence: Jaap Visser. Tel: +31 317 484439. Fax: +31 317 484011.
e-mail: office@algemeen.mgim.wau.nl

[1] Wageningen Agricultural University, Section Molecular Genetics of Industrial Microorganisms, Dreijenlaan 2, 6703 HA Wageningen, The Netherlands

[2] National Institute of Chemistry, Hajdrihova 19, SLO-61115 Ljubljana, Slovenia

The gene *pkaC* encoding the catalytic subunit of cAMP-dependent protein kinase has been isolated from the industrially important filamentous fungus *Aspergillus niger*. A probe for screening *A. niger* phage libraries was generated by a polymerase chain reaction using degenerate primers. cDNA and genomic DNA clones were isolated and sequenced. An open reading frame of 1440 bp, interrupted by three short introns, encodes a polypeptide of 480 amino acids with a calculated molecular mass of 53813 Da. The cAMP-dependent protein kinase catalytic subunit (PKA-C) from *A. niger* has a 126 amino acid extension at the N-terminus compared to the PKA-C of higher eukaryotes that – except for the first 15 amino acids, which are homologous to the *Magnaporthe grisea* PKA-C – shows no significant similarity to the N-terminal extension of PKA-C of other lower eukaryotes. The catalytic core of PKA-C of *A. niger* shows extensive homology with the PKA-C isolated from all other eukaryotes. Low-stringency hybridization did not reveal any other *pkaC* homologue in *A. niger*. The cloned *pkaC* was used for transformation of *A. niger*, leading to increased levels of *pkaC* mRNA and PKA-C activity. Transformants overexpressing *pkaC* were phenotypically different with respect to growth, showing a more compact colony morphology, accompanied by a more dense sporulation, especially on media containing trehalose and glycerol. A number of transformants also showed a strongly reduced or complete absence of sporulation. This phenotype was quickly lost upon propagation of the strains.

Keywords: cAMP-dependent protein kinase catalytic subunit, *Aspergillus niger*, PKA-C overexpression, defective sporulation

INTRODUCTION

cAMP-dependent protein kinase (PKA) plays a crucial role in the regulation of metabolic pathways by means of enzyme phosphorylation (Walsh & van Patten, 1994). Furthermore, it modulates the function of nuclear factors that bind to DNA sequences present in the promoter regions of cAMP-inducible genes (Lalli & Sassone-Corsi, 1994). In fungi, PKA appears to be involved in the control of the activity of a large number of enzymes. PKA has a role in aerobic germination of the dimorphic fungus *Mucor rouxii* (Rossi & Moreno, 1994) and in differentiation processes of the slime mould *Dictyostelium discoideum* (Mann & Firtel, 1993). The most detailed investigation on the function of PKA in signal transduction has been carried out with *Saccharomyces cerevisiae* (Thevelein, 1994). In *S. cerevisiae*, PKA activity is essential for growth, cell cycle progression, sporulation and sensitivity to various forms of stress (Toda *et al.*, 1987b). PKA mediates the expression of cytosolic catalase T (*CTT1*) (Marchler *et al.*, 1993), an HSP70 gene (*SSA3*) (Boorstein & Craig, 1990) and trehalose phosphate phosphatase (*TPS2*) (Gounalaki & Thireos, 1994).

In the filamentous fungus *Aspergillus niger*, 6-phosphofructo-1-kinase seems to be phosphorylated by

Abbreviations: PKA, cAMP-dependent protein kinase; PKA-C and PKA-R, catalytic and regulatory subunit of PKA, respectively; RT-PCR, reverse transcription PCR.

The EMBL accession number for the nucleotide sequence reported in this paper is X94399.

0002-1196 © 1997 SGM

PKA (Legiša & Bencina, 1994), possibly influencing the rate of glycolysis. PKA might also be involved in morphology changes in *A. niger* during an early stage of citric acid fermentation, because the morphology changes coincide with changes in cAMP concentration (Legiša & Grapulin-Gradišnik, 1995).

According to its mechanism of activation PKA is one of the simplest members of a large protein kinase family. In most cases the inactive form of PKA is a tetrameric protein composed of two regulatory and two catalytic subunits. Upon binding of cAMP, inactive PKA dissociates into two active catalytic subunits and a dimer of regulatory subunits. Several isoforms of PKA subunits, encoded by distinct genes, have been identified in higher eukaryotes (Døskeland *et al.*, 1993). In mammals three different genes encoding distinct catalytic subunits (Cα, Cβ, Cγ) have been found, whereas two types of regulatory subunits have been isolated and four different genes encoding PKC-R have now been identified (Taylor *et al.*, 1992). It seems that the presence of only one type of regulatory subunit is the rule in lower eukaryotes (Mutzel *et al.*, 1987; Toda *et al.*, 1987b). Genes encoding the catalytic subunit of PKA have been isolated from a number of fungi including *Magnaporthe grisea* (Mitchell & Dean, 1995), *S. cerevisiae* (Toda *et al.*, 1987a), *Blastocladiella emersonii* (de Oliveira *et al.*, 1994), *Schizosaccharomyces pombe* (Maeda *et al.*, 1994) and *D. discoideum* (Mann & Firtel, 1991). In *S. cerevisiae* three isoenzymes of the catalytic subunit have been found (Toda *et al.*, 1987a). PKA was purified from the fungi *Neurospora crassa* (Powers & Pall, 1980) and *Mucor rouxii* (Pastori *et al.*, 1985), and more recently also from *A. niger* (Legiša & Bencina, 1994). PKA from *D. discoideum* differs in structure from the others: it is a dimeric protein composed of one catalytic and one regulatory subunit (Mutzel *et al.*, 1987).

In order to analyse the *A. niger* PKA in more detail and to study its role in the regulation of glycolysis and morphology, cDNA and genomic DNA clones of the catalytic subunit of PKA have been isolated and sequenced and *A. niger* strains overexpressing the cloned gene constructed.

METHODS

Strains and plasmids. The *A. niger* strains used were: *A. niger* wild-type N400 (CBS 120.49) and *A. niger* NW219 [*cspA1 nicA1 leuA1 pyrA6*], which was used for transformation. *Escherichia coli* DH5α [F⁻/*endA1 hsdR17* (r_k^- m_k^+) *supE44 thi-1 recA1 gyrA* (Nal^r) *relA1* Δ(*lacIZYA–argF*)*U169* *deoR* (φ80 d*lac* Δ(*lacZ*) M15)] (New England Biolabs) was used for propagation of plasmid DNA and *E. coli* LE392 [Δ(*lacIZY*)*6 galK2 galT22 metB1 trpR55* λ⁻] (Promega) and BB4 [LE392, Δ(*argF–lacZ*)*U169* F′ *lacI*^{q}ZΔM15 *proAB* Tn*10* (Tet^r)] (Stratagene) were used for phage amplification and purification. Phage R408 (Stratagene) was used as a helper phage for phagemid excision. Plasmid vector pGEM-T (Promega) was used for cloning PCR fragments, pBluescript KS (Stratagene) was used for subcloning and plasmid pGW635 containing the *A. niger* orotidine-5-phosphate decarboxylase (*pyrA*) gene (Goosen *et al.*, 1987) was used as selection plasmid in transformation of *A. niger*.

Culture media and growth conditions. For the preparation of conidiospores *A. niger* strains were grown at 30 °C for 3–4 d on complete medium, originally described for *Aspergillus nidulans* (Pontecorvo *et al.*, 1953), using 2% (w/v) glucose as a carbon source unless stated otherwise, with appropriate supplements and solidified with 1·5% (w/v) agar. In liquid cultures the mycelium was grown by inoculating 10^6 conidiospores ml^{-1} in medium containing, per litre: 6 g $NaNO_3$, 1·5 g KH_2PO_4, 0·5 g KCl, 0·5 g $MgSO_4.7H_2O$, pH 6·0, 0·2 ml trace metal solution (Visniac & Santer, 1957), 1 g yeast extract and 1 g Casamino acids, using 2% (w/v) glucose as a carbon source and appropriate supplements, which are, for *A. niger* NW219, 1 mg nicotinamide, 200 mg leucine and 1200 mg uridine. The mycelium was grown at 30 °C in a rotary shaker (New Brunswick) at 250 r.p.m.

Chemicals and enzymes. Restriction enzymes, DNA polymerase I Klenow fragment, M-MuLV reverse transcriptase, bacteriophage T4 DNA ligase and T4 DNA polymerase were obtained from Life Technologies; bacteriophage T7 DNA polymerase and *Taq* DNA polymerase from Pharmacia; [α-^{32}P]dATP and [^{35}S]dATPγS, from Amersham; dNTPs from Boehringer. All other chemicals used were of analytical grade.

DNA manipulation. *A. niger* chromosomal DNA was isolated as described by de Graaff *et al.* (1988). Propagation of plasmid DNA, isolation of plasmid DNA, Southern blot analysis and other DNA manipulations were essentially done as described by Sambrook *et al.* (1989). [α-^{32}P]dATP-labelled probes were synthesized using random hexamer primers (Sambrook *et al.*, 1989). Sequencing was done using the dideoxy chain-termination procedure (Sanger *et al.*, 1977) with the T7 DNA polymerase sequencing kit (Pharmacia). For sequence determination, DNA restriction fragments were subcloned into pBluescript vectors and sequenced with the M13 universal, reverse and gene-specific oligonucleotide primers. Sequence analysis was done using the PC/GENE (Intelligenetics) program and the GCG suite. Digital images of autoradiograms were acquired using a CCD camera. The composite image of the Northern blot analysis was created using the CorelDraw 4.0 software package.

RNA manipulation. For isolation of *A. niger* total RNA, TRIZOL (Life Technologies) was used. Poly(A)$^+$ mRNA was isolated by oligo(dT) column chromatography (Sambrook *et al.*, 1989). For Northern blot analysis, 15 μg RNA aliquots were separated on formaldehyde agarose gels, blotted to nylon membranes (Hybond-N, Amersham) and hybridized with [α-^{32}P]dATP-labelled probes. Hybridization was performed at 42 °C in 0·9 M NaCl, 0·09 M disodium citrate, 0·5% Ficoll 400, 0·5% polyvinylpyrrolidone, 0·5% BSA fraction V, 100 μg denatured herring sperm DNA ml^{-1}, 0·5% SDS and 50% formamide. The blots were washed under conditions of high stringency at 65 °C with 0·3 M NaCl, 0·03 M disodium citrate, 0·5% SDS, pH 7·0 (homologous conditions) or under conditions of low stringency at 56 °C with 0·6 M NaCl, 0·06 M disodium citrate, 0·5% SDS, pH 7·0 (heterologous conditions).

Transformation of *A. niger*. Mycelium was prepared by growing *A. niger* NW219 in liquid culture on complete medium with appropriate supplements for 16–18 h. The preparation of protoplasts and subsequent transformation of *A. niger* NW219 were performed essentially as described by Kusters-van Someren *et al.* (1991). The *A. niger pyrA* gene was used as a selection marker. For transformation, 1 μg pGW635 DNA and 20 μg of the co-transforming plasmid pPKAC1 were added to 2×10^7 protoplasts.

Isolation of the genomic and cDNA clones. The *A. niger pkaC*

and the corresponding cDNA clone were isolated by homologous hybridization, using a PCR-generated fragment (see below) as a probe, from an *A. niger* N400 genomic library in the λ replacement vector EMBL4 (Promega) (Harmsen *et al.*, 1990) and a cDNA library from *A. niger* N400 glucose-oxidase-induced mycelium in λZAP II (Stratagene) (Witteveen *et al.*, 1993), respectively. Plasmids containing the cloned cDNAs were obtained from the λZAP II phages by *in vivo* excision according to the manufacturer's instructions.

Polymerase chain reaction (PCR). Phage DNA (200 ng), isolated from the amplified glucose-oxidase-induced cDNA library (see above) was used as a template in the PCR. The 100 µl reaction volume contained 50 mM KCl, 1·5 mM $MgCl_2$, 10 mM Tris/HCl pH 9·0, 200 µM dNTPs, 2·5 U *Taq* DNA polymerase and degenerate primers PA (0·264 nmol; 5′-YANAARCARRTNGARCA-3′) and PB (0·132 nmol; 5′-CCACCARTCNACNGMYTT-3′). Conditions for PCR were: 3 min at 95 °C; three cycles of 95 °C/1 min, 35 °C/1·5 min and 72 °C/1 min; 30 cycles of 95 °C, 43 °C and 72 °C, each of 1 min. The final elongation step was done at 72 °C/5 min.

Reverse transcription PCR (RT-PCR). Poly(A)$^+$ mRNA (100 ng) was denatured for 10 min at 68 °C in the presence of 1 µM primer P9 (5′-CAGAGGTATGATGGGCA-3′) (Fig. 2). Subsequently the reverse transcription buffer (50 mM Tris/HCl pH 8·3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 500 mM dNTPs) and 200 U M-MuLV reverse transcriptase were added and the mixture was incubated at 42°C for 1 h. One-fourth of this reaction was used in a 100 µl PCR reaction with 300 nM of primers P9 and P11 (5′-GTTTAGGAGGTTTGCTG-3′) (Fig. 2), 50 mM KCl, 1·5 mM $MgCl_2$, 10 mM Tris/HCl pH 9·0, 500 µM dNTPs and 2·5 U *Taq* polymerase. The empirically determined conditions were: denaturation step 95 °C/3 min; 5 cycles of 95 °C/10 s, 56 °C/40 s, 72 °C/1 min; 30 cycles of 95 °C/10 s, 52 °C/40 s, 72 °C/1 min; and a final elongation step at 72 °C for 5 min. The products were cloned and sequenced.

Enzyme assay. Mycelium grown in liquid culture for 20 h was harvested by filtration, washed briefly with ice-cold 0·8% NaCl, and frozen in liquid nitrogen. The frozen mycelium (0·5 g) was ground and suspended in extraction buffer (25 mM Tris pH 7·4, 10 mM $MgCl_2$, 0·15 M NaCl, 1 mM DTT, 1 mM EDTA). After 30 min extraction the homogenate was centrifuged at 10000 ***g*** for 10 min. The clear supernatant was used for the enzyme assay. Two- to tenfold dilutions of cell extracts (the protein concentration of cell extracts was around 2 mg ml^{-1}) were used. The enzyme activity was measured by the nonradioactive Spinzyme (Pierce) method with dye-labelled Kemptide as a substrate according to the manufacturer's protocol. The incubation time of an assay was 30 min at 30 °C. A unit of enzyme activity is defined as the amount of enzyme required to transfer 1 pmol phosphate from ATP to substrate (Kemptide) min^{-1} at 30 °C. PKA-C enzyme (bovine heart, Pierce) with known activity was used as a standard.

Protein concentrations were determined by the bicinchoninic acid protein assay kit (Sigma) according to the supplier's instructions. Bovine serum albumin (fraction V) (Boehringer) was used as a standard.

Primer extension mapping. Poly(A)$^+$ mRNA was isolated from mycelium grown for 20 h. Poly(A)$^+$ mRNA (6 µg) and primer P12 (5′-TTCTTCAGCAAACCTCC-3′) (Fig. 2) (5 µg) were used for primer extension mapping, which was performed according to Calzone *et al.* (1987) with minor modifications. The reaction products were analysed on a denaturing polyacrylamide gel parallel to a dideoxy sequence reaction of genomic pPKAC1 clone using primer P12.

RESULTS

Cloning of the *pkaC* gene

The primers used for preparation of a probe by PCR were based on well-conserved regions of eukaryotic PKA-C proteins. To avoid possible introns interfering with proper primer annealing, cDNA from *A. niger* N400 was chosen as a template. Under the empirically determined conditions, as described in Methods, a product of approximately 420 bp was found (data not shown). Sequence analysis of this fragment revealed high similarity with the *pkaC* of other organisms.

The *A. niger* N400 genomic and cDNA libraries were screened by homologous hybridization using the 420 bp PCR product as a probe. Screening of 10^5 p.f.u. of the genomic and the cDNA library resulted in 50 positive genomic clones and two positive cDNA clones. Five genomic phages and both cDNA phages were purified. The genomic phages were characterized by restriction analysis and a 5 kbp *Eco*RI fragment was isolated and cloned in pBluescript, resulting in plasmid pPKAC1 (Fig. 1). The cDNA clones were found to be identical and were isolated by *in vivo* excision, resulting in plasmid pPKAC2, for which a detailed restriction map is also given (Fig. 1).

Although isoforms of PKA-C exist in several organisms, low-stringency hybridization did not reveal the existence of any other *pkaC* homologue in *A. niger* N400.

Sequence of the *pkaC* gene and primary structure of its deduced protein

The nucleotide sequence of the 3245 bp *Eco*RI–*Xba*I fragment of pPKAC1 was determined from both strands (Fig. 2). The cDNA clone was sequenced to establish

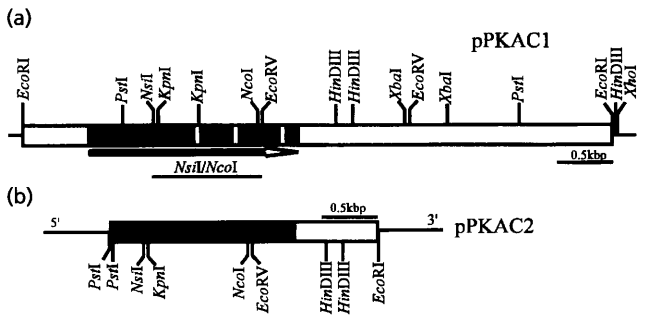

Fig. 1. (a) Restriction map of clone pPKAC1 isolated from the genomic *A. niger* N400 library. The line below the restriction map indicates the *Nsi*I–*Nco*I fragment that was used as a probe for Southern and Northern hybridization analysis. (b) Restriction map of cDNA clone pPKAC2. The coding region is presented as a filled box; open boxes indicate non-coding regions and introns. The open arrow indicates the direction of transcription.

```
      gaattcacgccaatccaaccccgcctcttctctcccttgctttccctttttccatttttccactccctctctccatcaaac -625

taatcaaaaataattataatgaaataggataaataattgcggtgcttgtgctcgtagtgtttttcagctagttggtttagtagtagtagt -535
                                                C4T                                C4T
agtggtggtgctggtcacagcaagcatctctttccctcccctctcatctcttctcctctctcctctctttctcccccctcaccatcagcat -445

catcagcatcatcatcgatcctcccgcacaccagccatactctcaccacccaccccccggtccaagctatcatcccacgagattcccgca -355
 C4T
ctcccccgcgttacatattctcccttcttatccggacatcctcacggtcgcccgtctgtccaattgagtttccgttgaccgccgttcggg -265

accagtgttctgttctgtttgggggctccgctgtatccgcgcccgaacccgtcccaagagaaaaagctcgccgctttccctctccatatt -175
                                C4T                        C4T
attcttactgtgttgcttcctttcccaccccccctctctgagagcatctcttaaagaggggtcgcgttgatctgctatcgcacgcatcct -85
                                                *
ttttcctccctttttccacgtggataataccatcgtgggctgctctatcattttaattttactgcccacagcagcgtgttctccATGCCT 6
                                                                                        M  P  2
                P12
AGTTTAGGAGGTTTGCTGAAGAAACGGCGAACGAAAGATTCGCAGACCCTCTCCAAAGAGCTTGAAGCCGGTTCGGCCCAGACGCAGACG 96
          P11
S  L  G  G  L  L  K  K  R  R  T  K  D  S  Q  T  L  S  K  E  L  E  A  G  S  A  Q  T  Q  T  32
TCACCAAACGCTGCCGAAGACCACCACAACCACAACCACCACCAACACCACCACCACCTCTTCCACCACCACCATCAACCCCAACCCGCC 186
S  P  N  A  A  E  D  H  H  N  H  N  H  H  Q  H  H  H  H  L  F  H  H  H  H  Q  P  Q  P  A  62
ACCAATTCTGGCTCTGCAGCAAACACCCCCCCTCAGCCTCAAGATTCCGTGCCTCAACAGTCCAATCGGTCTTCGGGAGCAGAAAAATCC 276
T  N  S  G  S  A  A  N  T  P  P  Q  P  Q  D  S  V  P  Q  Q  S  N  R  S  S  G  A  E  K  S  92
TCCGACGGTCAGGTAGCCTCCATGCAATCCGCTGTGACGCAAGCCTCGCCGTCTGCCCATCATACCTCTGGCCTCCCGCAGCCCAATGCC 366
                                                                          P9
S  D  G  Q  V  A  S  M  Q  S  A  V  T  Q  A  S  P  S  A  H  H  T  S  G  L  P  Q  P  N  A  122
AATGCGGCTAGTATACAGAATATAATTAACCCCTCCCAGCAAGGCGCCATGCATTCGGCTTCCAGTGGTCATACGCAATCTCACCATGCC 456
N  A  A  S  I  Q  N  I  I  N  P  S  Q  Q  G  A  M  H  S  A  S  S  G  H  T  Q  S  H  H  A  152
GGTCGCAGTGATGCCCGCACCACCAAAGGGAAATACTCCTTGGATGACTTCAGCCTTCAGCGCACCCTGGGTACCGGTAGCTTCGGTCGC 546
G  R  S  D  A  R  T  T  K  G  K  Y  S  L  D  D  F  S  L  Q  R  T  L  G  T  G  S  F  G  R  182
GTGCACTTGGTGCAGTCGAAGCACAACCATCGCTTCTACGCTGTCAAGGTGCTGAAGAAGGCGCAAGTGGTCAAGATGAAACAGATTGAG 636
                                                                          PA
V  H  L  V  Q  S  K  H  N  H  R  F  Y  A  V  K  V  L  K  K  A  Q  V  V  K  M  K  Q  I  E  212
CACACCAACGATGAGCGCCGAATGCTGAATCGCGTCAGACATCCATTCCTAATCACATTATGGGGTACATGGCAGGATTCCCGGAACTTG 726
H  T  N  D  E  R  R  M  L  N  R  V  R  H  P  F  L  I  T  L  W  G  T  W  Q  D  S  R  N  L  242
TACATGGTCATGGACTTCGTAGAGGGTGGTGAACTCTTCAGTCTCGTCCGCAAGTCACAGgtgggttattctgacttggcccgcccgacc 816
Y  M  V  M  D  F  V  E  G  G  E  L  F  S  L  V  R  K  S  Q                    intron A 262
aacgtaaagctgatgtctggtacctcttttcatgtagCGGTTCCCCAACCCCGTCGCCAAGTTCTATGCGGCGGAAGTCACTCTTGCCCT 906
                                    R  F  P  N  P  V  A  K  F  Y  A  A  E  V  T  L  A  L  280
TGAGTACTTGCACACGCAGAACATCATCTACCGGGATCTCAAGCCCGAGAACTTGCTGCTAGACCGCCACGGCCATCTGAAGATCACCGA 996
 E  Y  L  H  T  Q  N  I  I  Y  R  D  L  K  P  E  N  L  L  L  D  R  H  G  H  L  K  I  T  D  310
TTTCGGTTTTGCCAAGGAGGTGCCTGACATCACATGGACCCTTTGTGGTACTCCCGATTATCTCGCCCCGGAGGTGGTTTCCTCTAAGGG 1086
 F  G  F  A  K  E  V  P  D  I  T  W  T  L  C  G  T  P  D  Y  L  A  P  E  V  V  S  S  K  G  340
TTATAACAAGTCGGTTGACTGgtaagggttgccattattgatgttcatgtccctttggcgcaggttactaatgatgcgaaagGTGGTCTC 1176
                                                                                    PB
 Y  N  K  S  V  D  W                    intron B                                W  S  L  350
TGGGGATTTTGATCTTCGAGATGCTGTGTGGTTTCACGCCTTTCTGGGACAGTGGCTCCCCAGTCAAGATCTACGAGAACATCCTGCGTG 1266
  G  I  L  I  F  E  M  L  C  G  F  T  P  F  W  D  S  G  S  P  V  K  I  Y  E  N  I  L  R  G 380
GCAGGGTAAAATACCCGCCCTACCTGCACCCGGATGCTGTTGATCTGCTGTCGCAACTGATCACGGCTGATCTGACCAAACGCCTTGGAA 1356
  R  V  K  Y  P  P  Y  L  H  P  D  A  V  D  L  L  S  Q  L  I  T  A  D  L  T  K  R  L  G  N 410
ACCTCCATGGCGGTTCGGATGATGTCAAGAATCATCCTTGGTTTGCCGAGGTCACCTGGGACCGTTTGGCCCGCAAGGATATCGACGCTC 1446
  L  H  G  G  S  D  D  V  K  N  H  P  W  F  A  E  V  T  W  D  R  L  A  R  K  D  I  D  A  P 440
CTTACGTGCCTCCCATCCGTGGTGGCCAAGGAGATGCGAGTCAGTATGACCGCTATCCGGAAGAAACTGAACAGTATGGCATGGCTGGTG 1536
  Y  V  P  P  I  R  G  G  Q  G  D  A  S  Q  Y  D  R  Y  P  E  E  T  E  Q  Y  G  M  A  G  E 470
AAGACCCgtatgtgctatctttcccatttcctaataacgcgtggtgggtcacattactgacgctgcttgcagACACGGCCATTTGTTCCC 1626
  D  P                    intron C                                H  G  H  L  F  P  478
AGACTTCTAGtgtgtgctacgagttatgatggaatgattgtctcgctaccgagatggcatcgtgtcatccgatgatattgcgagccctgt 1716
 D  F                                                                                      480
aagacaggactatctcgctaatccttaccctctgtgctaccaaaggtagcaccacacccatcattcgttgagttccccttgacgtcggtt 1806
tgtcttcgttatttgatcgctggccgacttgctttttggctacctttgacgtagagcatatatctaagagctctgtcattctcagcgtat 1896
gcctttcaccatctttcaagtatacgctatggtcttatctatctgcttttccggtatccggactgttggtggtctgcatataagcttacc 1986
cttatatgagctctggggagatctgatgagtggatgattttcccggtgatctttatctaggtgggcattgtctatatttcctcgcgttat 2076
gccaggcaggagggtgtttgcggtggtgtttgtgccggaagcttcgggggtttctcgtgattatgccgaccgttgactggtgaatggtca 2166
ccagcggcacccgtttctttcttcaatgtttcctgcttattctatagtcatattgtgcaaatactggggagctactctgggtcattcttg 2256
atgtcatctttccgtttatgcctttccctgtccctgtggcggcagttgtataatgtacatattcgatttggccattgcaataaaagtagt 2346
cttggataagtggacgctggtcccccaactgtatcataagcatagcctgccattcaaggctcttgtcttttcaaaagccaccggctgccc 2436
gaaccaaaaccgtccgtccgtaatatacacaaagatcatcacacaccaatggccactgcctcatatcacaccaaggtataccggtcaaaa 2526
acacctctaga 2537
```

Fig. 2. For legend see facing page.

intron borders. Since the isolated cDNA clone pPKAC2 was shorter than the coding region of *pkaC*, RT-PCR was used to determine the presence of possible introns in the region upstream of the cloned cDNA. It was found that the part of *pkaC* lacking in pPKAC2 was not interrupted by introns.

The structural region is interrupted by three short introns, 67 bp, 61 bp and 65 bp in length (Fig. 2). The positions of all three introns found in *pkaC* of *A. niger* are conserved between *A. niger* and *Magnaporthe grisea* (Mitchell & Dean, 1995), suggesting that the *pkaC* genes of these fungi are closely related. The presence of introns has also been reported for *pkaC* genes of *Caenorhabditis elegans* (Gross *et al.*, 1990) and *Blastocladiella emersonii* (de Oliveira *et al.*, 1994). The borders of intron B (5′ GTAAGG and 3′ AAG) do not fit to the consensus sequence as proposed by Unkles (1992), whereas they do in introns A and C. Similar aberrant 3′ and 5′ splice sites were found in the *A. niger* var. *awamori glaA* gene and in the *pelA* gene of *A. niger* (Unkles, 1992).

Primer extension analysis (data not shown) revealed that the major transcription start point is located 41 nt upstream from the deduced AUG start codon. A putative TATA box (ATAATA) is found 20 nt upstream from the transcription start point (−61 nt from the start codon) and a putative CCAAT box is found upstream at position −292. The promoter region has long stretches of CT-rich regions, five CCCCT elements (C_4T or inverse complement) and an AT-rich region between positions −580 and −630 (Fig. 2).

Downstream of the proposed stop codon, the mRNA still contains 727 nt of non-coding area as indicated by the cDNA sequence. A putative polyadenylation signal AATAAA is found 702 bp downstream from the stop codon (Fig. 2).

The deduced length of the mRNA is 2204 nt, containing a 1440 nt open reading frame encoding a 480 aa protein (Fig. 2). The calculated molecular mass of the protein is 53813 Da and the theoretical isoelectric point is 7·8.

Statistical analysis of codon usage showed that a wide range of codons is used for *pkaC*. A relatively high frequency of codons using A in the third position was found. The most striking difference from most fungal genes was found in the case of Lys and Gln, for which 36% and 41% of the codons were AAA and CAA, respectively. These codons are hardly ever used in the currently known *A. niger* genes.

The 480 aa *A. niger* PKA-C protein has a 126 aa N-terminal extension compared to PKA-C of higher eukaryotes. Analysis of the gene using the GCG program Testcode indicated that the larger part of this area is possibly coding (data not shown). A strong argument in favour of the proposed AUG start codon is the high similarity (over 70%) of the first 15 aa of the *A. niger* and *Magnaporthe grisea* proteins. The *A. niger* PKA-C exhibits a sequence identity of over 50% with the PKA-C of higher eukaryotes and over 60% with the PKA-C of lower eukaryotes when only the catalytic core is considered (Fig. 3). The N-terminal extension of PKA-C of *A. niger* shows no significant homology with the N-terminal extension of PKA-C of lower eukaryotes except for the extreme N-terminal part of the ascomycete *M. grisea* PKA-C (underlined in Fig. 3).

Transformation of *A. niger* with the *pkaC* gene

Co-transformation of *A. niger* with pGW635 and pPKAC1 and subsequent expression analysis was performed to prove the functionality of the cloned gene. From 80 transformants, initially selected for uridine prototrophy, over 50% were phenotypically different from the parental strain, NW219. Some of the transformants lacked or had retarded sporulation; others had dense sporulation. Sporulation defects were accompanied by slow growth. During preservation the transformants showing no or retarded sporulation turned out to be very unstable and reverted to the phenotype of the parental strain (Fig. 4). We were not able to maintain these phenotypes. Four apparently stable transformants were chosen for further analysis. Southern analysis revealed single-copy ectopic integrations for transformants w1, 3 and 13 and multicopy ectopic integration for transformant 53. In contrast to strain 53, no obvious rearrangements of integrated *pkaC* copies were present in transformants w1, 3 and 13 (data not shown).

Expression of *pkaC*

Northern blot analysis of total RNA of the transformant and wild-type strains using the *NsiI–NcoI* fragment of *pkaC* as a probe (Fig. 1) showed two- to fourfold increased levels of mRNA in transformed strains w1, 3 and 13. Strain 53 showed a truncated mRNA and an increased level of mRNA of the size of the wild-type (Fig. 5). The results of the Northern analysis were also confirmed by dot-blot analysis (data not shown). The level of overexpression is slightly higher, as might be expected from the single copy integrations. This discrepancy might be caused by the genomic environment of the integration site, or a specific regulatory mechanism such as titration of a specific repressor protein.

In order to prove that the transcribed *pkaC* was also translated, PKA-C activities were measured in cell extracts of *A. niger* NW219 and transformants. Elevated levels of PKA-C activity were found in the transformed

Fig. 2. Nucleic acid sequence and deduced amino acid sequence of the *A. niger pkaC* gene. The coding part of the gene is typed in upper-case. The ultimate 5′ and 3′ bases of the cDNA insert from pPKAC2 are marked with filled triangles. The positions of primers PA and PB used for generating a probe for screening the libraries and the primers used in RT-PCR and primer extension experiments are indicated by arrows. Intron B splits primer PB. The putative TATA motif and CCAAT sequence are boxed. The transcription start point is marked by an asterisk. Putative C_4T elements are underlined. The AT-rich region is indicated by a dotted line. The putative polyadenylation signal is underlined.

```
Mg                                   mpslgflkkkrtrdgnndnssqpaspvtptaaqsfeqaqvlgapsain   49
Sp                                                                  mdttavaskgstnvgsstdtl   21

An                 mpslggllkkrrtkdsqtlskeleagsaqtqtspnaaedhhnhnhhqhhhhlfhhhhqp   59
Mg  nshahtqqqsylvpqpgysvgtevqaqqpqmnsisqqqqqafappahtpspgtidpqqslpsisnlmnpa   119
Sp  stsaslhpsmnagsvneyseqqrhgtnsfngkpsvhdsvgsdasvsnghnnhnesslwtsgipkaleeat   91
Be                                                                      mtlidk   6

An  qpatnsgsaantppqpqdsvpqqsnrssgaekssdgqvasmqsavtqaspsahhtsglpqpnanaasiqn   129
Mg  vqqqnsqpsanfqpqsqsqsqsqsqsqfplppshgngdqsqqqnfqvqqqiqsqqdamdiqpsqvqdqshsq   189
Sp  kskkpdslvststsgcasahsvgyqnidnlipsplpesasrsssqsshqrhsrdgrgelgsehgerrsam   161
Be  lmektkkvvgssdkdapapaspsspstaagagsasstasstttaaasgnlsipsplvagsttsssishaq   76
Sc                          myvepmnnneirklsitaktettpdnvgqdipvnahsvheecssntpaaa   50
Bo                                                                      gna   3

An  iinpsqqgamhsassghtqshhagrsdarttkgkyslddFslqrTLGTGSFGRVhLVqskhnhrfYAvKv   199
Mg  qaqpqhqpqhhvqhhvnhahqgsqdqqtrvtkgkysltdFeilrTLGTGSFGRVhLVqsrhnqrfYAvKv   259
Sp  dglrdrhirkvrvsqlldlqrrrirpadhttkdrygiqdFnflqTLGTGSFGRVhLVqsnhnrlyYAiKv   231
Be  kmataahtnsdyspspaatpsapldavaerrrrkttladLelrqTLGTGSFGRVhLVrlrstgkyYAmKv   146
Sc  kkgseqesvkeflasagiclvkkpmlqyrdtsgkyslsdFqilrTLGTGSFGRVhLIrsnhngrfYAlKt   118
Bo  aaakkgseqesvkeflakakedflkkwenpaqntahldqFerikTLGTGSFGRVmLVkhmetgnhYAmKi   73

                                                                    ↓
An  LkKaqVVkmKQIEHTndErrmLnrVrhPFlitLwgtWqDsrnlYMVMDFveGGELFslvRksqRFpnpvA   269
Mg  LkKaqVVkmKQVEHTndErkmLgeVknPFlitLwgtFqDcrnlYMVMDFveGGELFsllRksgRFpnpvA   329
Sp  LeKkkIVdmKQIEHTcdEryiLsrVqhPFitiLwgtFqDaknlFMVMDFaeGGELFsllRrigRFsephA   301
Be  LkKaeVVkhKQVEHTlnEkgiLeqIdhPFlvaLhssFqDsanlYMVMEYvtGGELFtylRrsqRFsnnvA   216
Sc  LkKhtIVklKQVEHTndErrmLsiVshPFiirMwgtFqDsqqvFMVMDYieGGELFsllRksqRFpnpvA   188
Bo                                                                          143

                                                        *
An  kFYAAeVtLaLEYLHtqniiYRDLKPENlLlDrhGhlkItDFGFAKeVp.diTWTLCGTPDYlAPEVVss   338
Mg  kFYAAeVtLaLEYLHakniiYRDLKPENlLlDrhGhlkItDFGFAKrVp.dkTWTLCGTPDYlAPEVVsn   398
Sp  kFYAAeViLaLDYLHhnqivYRDLKPENlLlDrfGhlkIvDFGFAKrVstdiTWTLCGTPDYlAPEIIqs   371
Be  kFYAAeVvLaFEYLHskdiiYRDLKPENlLlDaqGhvkItDFGFAKhVp.diTWTLCGTPDYlAPEIIqs   285
Sc  kFYAAeVcLaLEYLHskditYRDLKPENiLlDknGhikItDFGFAKyVp.dvTYTLCGTPDYiAPEVVst   257
Bo  rFYAAqIvLtFEYLHsldliYRDLKPENlLiDqqGyiqVtDFGFAKrVk.grTWTLCGTPEYlAPEIIls   212

                  ↓
An  kgYnksvDWWsLGILIFEMlcGFtPFWdsgspvkiYEnIlrgrvkYPpylhpdavDLLsqLitaDltkRL   408
Mg  kgYnksvDWWsLGILIYEMlcGYpPFWdsgspmkiYEnIlkgkvrYPayinpdaqDLLqrLitaDltkRL   468
Sp  kpYnkaaDWWsLGILIFEMlaGYpPFY.senpmklYEnIlegkvnYPsyfspasiDLLshLlqrDitcRY   440
Be  rgYgravDWYaLGVLIFEMlaGYpPFYd.edhvrmYEkIlqgkvkWPshfdpaakDLLkrLlttDltkRY   354
Sc  kpYnksvDWWsFGVLIYEMlaGYtPFYnsn.tmktYEnIlnaelkFPpffhpdaqDLLkkLitrDlseRL   326
Bo  kgYnkavDWWaLGVLIYEMaaGYpPFFa.dqpiqiYEkIvsgkvrFPshfssdlkDLLrnLlqvDltkRF   281

                *                                                       ↓
An  GNLhgGsdDVknHpWFaevtWdrlarkdIdaPYvPpirgGqGDaSqYDrYpEeteqygmagedphghlfpdF   480
Mg  GNLygGsqDVrnHpWFaevtWdrlarkdIdaPYtPpvkaGaGDaSqFDrYpEeterygqtghdeygnlfpgF   534
Sp  GNLkdGsmDIimHpWFrdisWdkiltrkIevPYvPpiqaGmGDsSqFDaYaDvatdygtsedpeftsifkdF   512
Be  GNLkgGskDIkmHkWFagldWtklfnkqIppPYtPpnr.GdGDtSnFDaYpEetepygkvqpdpyaqlfkdF   425
Sc  GNLqnGseDVknHpWFneviWekllaryIetPYePpiqqGqGDtSqFDrYpEeefnygiqgedpymdlmkeF   398
Bo  GNLkdGvnDIknHkWFattdWiaiyqrkVeaPFiPkfk.GpGDtSnFDdYeEeeirvsi..nekcgkefseF   350
```

Fig. 3. Alignment of the PKA-C protein sequences of *A. niger* (*An*), *Magnaporthe grisea* (*Mg*; Mitchell & Dean, 1995), *Schizosaccharomyces pombe* (*Sp*; Maeda *et al.*, 1994), *Blastocladiella emersonii* (*Be*; de Oliveira *et al.*, 1994), *Saccharomyces cerevisiae* type C (*Sc*; *Toda et al.*, 1987a) and bovine type α (*Bo*; Wiemann *et al.*, 1992). Conserved amino acids are printed in upper-case. The positions of the *A. niger* introns are indicated by arrows. Asterisks indicate the conserved autophosphorylation sites. The amino acids in the N-terminal region that are conserved between *A. niger* and *M. grisea* are underlined.

strains w1, 3 and 13, with the highest activity measured in strain 13. Strain 53 had the same enzyme activity as the wild-type *A. niger* strain NW219 (Table 1). If the truncated mRNA observed for strain 53 results in a protein, this might interfere with the PKA-C assay. The low expression measured for strain 53 might also have

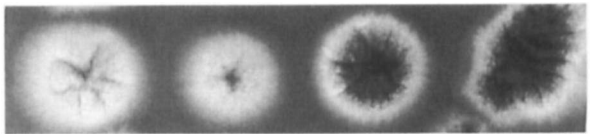

Fig. 4. Instability of an originally non-sporulating transformed strain upon propagation. The original transformant strain was propagated on minimal medium containing 1% glucose as a carbon source. Four successive generations are shown.

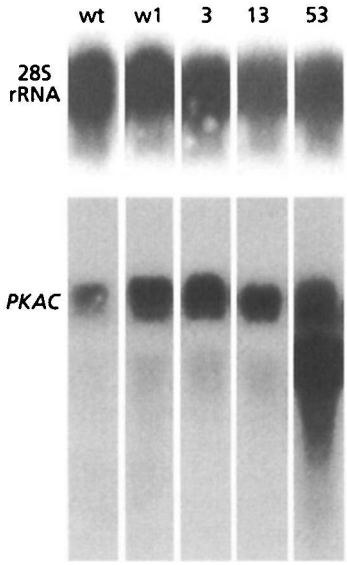

Fig. 5. Northern analysis of total RNA of the *pkaC* transformants and the wild-type (wt) of *A. niger*. RNA was quantified using a 28S rRNA probe of *Agaricus bisporus* (EMBL accession X91812).

Table 1. PKA-C activities in extracts from *A. niger* NW219 and from transformed strains

Values are the means of two independent experiments, ± the range.

Strain	Specific activity [U (mg protein)$^{-1}$]	Activity ratio transformant:NW219
NW219	65±3	1
w1	120±8	1·8
3	142±38	2·2
13	244±51	3·8
53	64±4	1

been caused by the observed instability of the non- or low-sporulating strains.

Phenotypic characterization of *pkaC*-overexpressing strains

The conidiospores of a few selected transformants were plated on minimal medium agar plates with different carbon sources. All transformants selected grew more slowly than the control, *A. niger* NW219 transformed to uridine prototrophy. Particularly on media containing 3·4% (w/v) trehalose, 10% (v/v) glycerol (Fig. 6), 1·5% (w/v) xylose, and 1% (v/v) glycerol (data not shown) as a sole carbon source, slower growth and a more dense sporulation was observed for all transformants, except for transformant 53, which showed no or almost no sporulation on any of the media tested. This is likely to be caused by the expression of a truncated PKA-C, possibly by the formation of a cAMP-insensitive complex with PKA-R or disturbance of the normal PKA-C/PKA-R stoichiometry. The more dense sporulation may be either a direct effect of *pkaC* overexpression, or an indirect effect, caused by the slower growth of the transformants on these substrates. Growth of transformants on 2% acetate was very slow, and sporulation was less dense or completely absent on this medium. On 2% glucose in the presence of 5 mM cAMP, slow growth and almost no or no (strain 53) sporulation was observed for all strains.

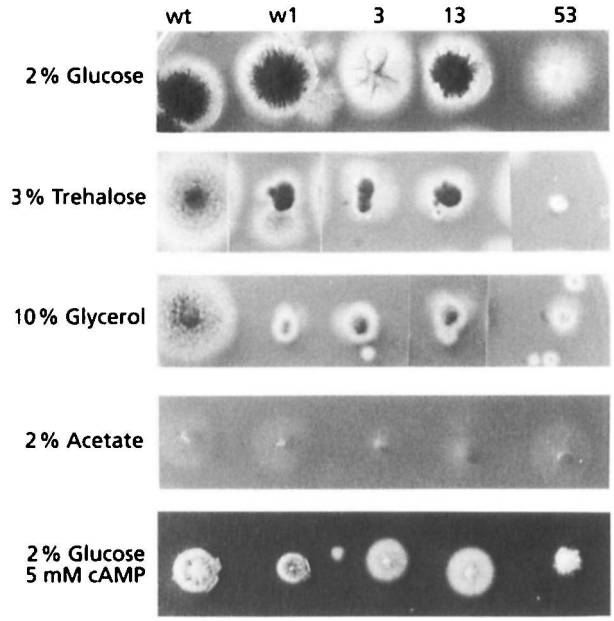

Fig. 6. Phenotypic characterization of PKA-C-overexpressing strains. Strains were grown for 5 d at 30 °C on minimal medium, containing the carbon sources indicated.

DISCUSSION

Sequence analysis

The *A. niger pkaC* gene encodes a polypeptide of 480 amino acids. The calculated molecular mass is 53813, which corresponds well with the molecular mass determined experimentally by SDS-PAGE (53 kDa) (Legiša & Bencina, 1994). Southern blot analysis indicated that the *A. niger* PKA-C is the product of a unique gene similar to the situation in *Aplysia californica*

(Beushausen *et al.*, 1988), *Caenorhabditis elegans* (Gross *et al.*, 1990), *Schizosaccharomyes pombe* (Maeda *et al.*, 1994) and *Blastocladiella emersonii* (de Oliveira *et al.*, 1994).

Except for the 126 aa N-terminal extension the homology between *A. niger* PKA-C and PKA-C from other organisms is very high. Some parts of the protein are completely invariant, such as the glycine-rich loop (175-LGTGSFGRV), Lys-198 and Glu-217 associated with ATP binding, and the putative active site (288-IIYRDLKPENLLL) typical for Ser/Thr protein kinases. Two autophosphorylation sites, Thr-323 and Ser-415, are conserved amongst *M. grisea*, *B. emersonii*, the yeasts *Sacch. cerevisiae* and *Schiz. pombe*, and the *A. niger* PKA-C, whereas they do not contain the autophosphorylation site (Ser-338 in bovine PKA-C), which is conserved in higher eukaryotes (Fig. 3, Taylor *et al.*, 1992). The catalytic subunit of the *A. niger* PKA is rather large compared to the PKA-C of higher eukaryotes due to its N-terminal extension. The N-terminus contains a relatively high amount of polar and charged residues and a relatively high amount of rare codons like AAA and CAA (Fig. 3). Whether the N-terminus might play a role in cellular localization, subunit interaction or a translational regulatory mechanism is unknown. No N-terminal extensions have been found in organisms containing more than one *pkaC* gene. Based on the consensus for strong AUG initiation codons, as defined by Arst & Sheerins (1996), the context of the first in-frame initiation codon allows leaky scanning, possibly resulting in three different proteins, one starting at Met-1, one starting at Met-100 and one starting at Met-139 (Fig. 2). Differential transcription, splicing or post-translational modifications of the unique *pkaC* gene or its transcript might also result in isoenzymes. Transformation of gene fusions of an inducible promoter and the *A. niger pkaC* at Met-100 and Met-139 resulted in strains which showed almost no or no growth on solid media containing an inducing carbon source (M. Bencina, unpublished results). These results indicate that the truncated proteins are still active or still capable of binding PKA-R.

Characterization of the *A. niger pkaC* promoter region revealed that a putative TATA-box and CAAT-box are present in the promoter. These elements are fairly conserved among fungi both with respect to composition and in their distance to the translation start point, although some fungal genes do not contain recognizable TATA-boxes at all (Unkles, 1992). The AT-rich region present in fungal promoters might cause unwinding and prevent nucleosome formation, making the gene more accessible to the transcriptional machinery and allowing constitutive expression (Struhl, 1985).

The importance of C_4T motifs for *pkaC* expression regulation is unclear. In *Sacch. cerevisiae*, C_4T boxes were found in the promoter of the stress-response gene *DDR2* (Kobayashi & McEntee, 1993) and of the *CTT1* gene, encoding cytosolic catalase T (Marchler *et al.*, 1993). The C_4T elements of the *CTT1* gene appear to be responsible for a four- to tenfold increase in transcription upon stress. The expression of *A. niger pkaC* also increased upon osmotic shock and temperature stress (data not shown). However, whether the C_4T elements play any role in the stress response of this promoter remains to be established.

The long distance between the stop codon and the proposed polyadenylation signal (702 bp) is rather unusual for *A. niger*. Also, a rather low codon bias is found. Highly expressed genes tend to have a preferred subset of sense codons with a low percentage of A at the third position (Unkles, 1992). The Lys codon AAA, which does not appear at all in most of the published *A. niger* genes, appears nine times in PKA-C, suggesting that this might influence *pkaC* expression levels. Our data indicate that expression of *pkaC* appears to be fairly constant but can be elevated by certain stress conditions.

Phenotypic features

Co-transformants were initially selected on the basis of morphological differences. Similar to the situation in *Sacch. cerevisiae*, *A. niger* multicopy transformants grew more slowly than the parental strain. The growth tests indicated that even moderate overproduction of *pkaC* affected the growth and sporulation characteristics of *A. niger* transformants. Growth on medium containing 5 mM cAMP almost eliminates the phenotypic difference between wild-type and transformant strains that is observed on all other media tested. The overexpression of *pkaC* is thus likely to be responsible for the observed effects. A *Sacch. cerevisiae* strain in which the *BCY1* gene, encoding PKA-R, was disrupted, shows uncontrolled *pkaC* activity. This strain is unable to grow on acetate, glycerol and pyruvate, but does grow on glucose. Diploid strains homozygous for the *BCY1* disruption were also unable to sporulate. Even though PKA-R is not essential for growth of *Sacch. cerevisiae*, uncontrolled PKA-C activity does interfere with spore germination (Toda *et al.*, 1987b). A *Sacch. cerevisiae* (*tpk1 tpk2 TPK3 BCY1*) strain containing TPK3 on a high-copy-number plasmid under the control of the *GAL1* promoter even failed to grow in the presence of galactose (Mazon *et al.*, 1993). Measurements of PKA-C and PKA-R in *Mucor rouxii* clearly showed that before germination started PKA-R was present in excess relative to PKA-C. Upon germination, PKA-C slowly increased (Rossi & Moreno, 1994). In *Dictyostelium discoideum*, unrestrained activity of PKA-C leads to uncontrolled spore formation even under conditions where the wild-type does not sporulate. The same conditions also prematurely trigger terminal cell differentiation (Simoni *et al.*, 1992). Our results indicate that, as in *Sacch. cerevisiae*, *M. rouxii* and *D. discoideum*, the PKA-C of *A. niger* also influences growth and development. Some *A. niger* transformants obtained developed only a few conidia or did not sporulate at all (data not shown). Upon propagation, these phenotypes were rapidly lost, presumably due to a

reduction of the *pkaC* copy number or specific suppressor mutations. The overexpression of *pkaC* in *A. niger* did not severely affect viability but resulted in unstable transformants which were hard to maintain. The effect of disruption of the gene is currently unknown; however in *Magnaporthe grisea* no apparent effects on the vegetative growth rate or conidiation were found *in vitro* (Mitchell & Dean, 1995).

In order to be able to overexpress the PKA-C of *A. niger* for further *in vitro* protein phosphorylation studies we have adopted two strategies: expression of *pkaC* under control of an inducible promoter, and cloning and co-expression of the gene encoding the regulatory subunit.

ACKNOWLEDGEMENTS

M. Bencina was the recipient of a grant from the Slovenian government and was additionally funded by Wageningen Agricultural University.

REFERENCES

Arst, H. N., Jr & Sheerin, A. (1996). Translational initiation competence, 'leaky scanning' and translational reinitiation in *areA* of *Aspergillus nidulans*. *Mol Microbiol* **19**, 1019–1024.

Beushausen, S., Bergold, P., Sturner, S., Elste, A., Roytenberg, V., Schwartz, J. H. & Bayley, H. (1988). Two catalytic subunits of cAMP-dependent protein kinase generated by alternative splicing are expressed in *Aplysia* neurons. *Neuron* **1**, 853–864.

Boorstein, W. R. & Craig, E. A. (1990). Regulation of a yeast HSP70 gene by a cAMP responsive transcriptional control element. *EMBO J* **9**, 2543–2553.

Calzone, F. J., Britten, R. J. & Davison, E. H. (1987). Mapping of gene transcription by nuclease protection assays and cDNA primer extension. *Methods Enzymol* **152**, 611–632.

Døskeland, S. O., Maronde, E. & Gjersten, B. T. (1993). The genetic subtypes of cAMP-dependent protein kinase – functionally different or redundant? *Biochim Biophys Acta* **1178**, 249–258.

Goosen, T., Bloemheuvel, G., Gysler, C., de Bie, D. A., van den Broek, H. W. J. & Swart, K. (1987). Transformation of *Aspergillus niger* using the homologous orotidine-5′-phosphate-decarboxylase gene. *Curr Genet* **11**, 499–503.

Gounalaki, N. & Thireos, G. (1994). Yap1p, a yeast transcriptional activator that mediates multidrug resistance, regulates the metabolic stress response. *EMBO J* **13**, 4036–4041.

de Graaff, L., van den Broeck, H. & Visser, J. (1988). Isolation and expression of the *Aspergillus nidulans* pyruvate kinase gene. *Curr Genet* **13**, 315–321.

Gross, R. E., Bagchi, S., Lu, X. & Rubin, C. S. (1990). Cloning and characterisation and expression of the gene for the catalytic subunit of the cAMP-dependent protein kinase in *Caenorhabditis elegans*. *J Biol Chem* **265**, 6896–6907.

Harmsen, J. A. M., Kusters-van Someren, M. A. & Visser, J. (1990). Cloning and expression of a second *Aspergillus niger* pectin lyase gene (*pelA*): indications of a pectin lyase gene family in *Aspergillus niger*. *Curr Genet* **18**, 161–166.

Kobayashi, N. & McEntee, K. (1993). Identification of cis and trans components of a novel heat shock stress regulatory pathway in *Saccharomyces cerevisiae*. *Mol Cell Biol* **13**, 248–256.

Kusters-van Someren, M. A., Harmsen, J. A. M., Kester, H. C. M. & Visser, J. (1991). Structure of the *Aspergillus niger pelA* gene and its expression in *Aspergillus niger* and *Aspergillus nidulans*. *Curr Genet* **20**, 293–299.

Lalli, E. & Sassone-Corsi, P. (1994). Signal transduction and gene regulation: the nuclear response to cAMP. *J Biol Chem* **269**, 17359–17362.

Legiša, M. & Bencina, M. (1994). Evidence for the activation of 6-phosphofructo-1-kinase by cAMP-dependent protein kinase in *Aspergillus niger*. *FEMS Microbiol Lett* **118**, 327–334.

Legiša, M. & Grapulin-Gradišnik, M. (1995). Sudden substrate dilution induces a higher rate of citric acid production by *Aspergillus niger*. *Appl Environ Microbiol* **61**, 2732–2737.

Maeda, T., Watanabe, Y., Kunitomo, H. & Yamamoto, M. (1994). Cloning of the *pka1* gene encoding the catalytic subunit of the cAMP-dependent protein kinase in *Schizosaccharomyces pombe*. *J Biol Chem* **269**, 9632–9637.

Mann, S. K. O. & Firtel, R. A. (1991). A developmentally regulated putative serine/threonine protein kinase is essential for development in *Dictyostelium*. *Mech Dev* **35**, 89–101.

Mann, S. K. O. & Firtel, R. A. (1993). cAMP-dependent protein kinase differentially regulates prestalk and prespore differentiation during *Dictyostelium* development. *Development* **119**, 135–146.

Marchler, G., Schuller, C., Adam, G. & Ruis, H. (1993). A *Saccharomyces cerevisiae* UAS element controlled by protein kinase A activates transcription in response to a variety of stress conditions. *EMBO J* **12**, 1997–2003.

Mazon, M. J., Behrens, M. M., Morgado, E. & Portillo, F. (1993). Low activity of the yeast cAMP-dependent protein kinase catalytic subunit Tpk3 is due to the poor expression of the TPK3 gene. *Eur J Biochem* **213**, 501–506.

Mitchell, T. K. & Dean, R. A. (1995). The cAMP-dependent protein kinase catalytic subunit is required for appressorium formation and pathogenesis by the rice blast pathogen *Magnaporthe grisea*. *Plant Cell* **7**, **1869–1878.**

Mutzel, R., Lacombe, M.-L., Simon, M.-N., de Gunzburg, J. & Veron, M. (1987). Cloning and cDNA sequence of the regulatory subunit of cAMP-dependent protein kinase from *Dictyostelium discoideum*. *Proc Natl Acad Sci USA* **84**, 6–10.

de Oliveira, J. C. F., Borges, A. C. C., Marques, M. V. & Gomes, S. L. (1994). Cloning and characterisation of the gene for the catalytic subunit of cAMP-dependent protein kinase in the aquatic fungus *Blastocladiella emersonii*. *Eur J Biochem* **219**, 555–562.

Pastori, R., Moreno, S. & Passeron, S. (1985). Polymeric structure of the cyclic AMP-dependent protein kinase from the dimorphic fungus *Mucor rouxii* and purification of its catalytic subunit. *Mol Cell Biochem* **69**, 55–66.

Pontecorvo, G., Roper, J. A., Hemmons, L. J., MacDonald, K. D. & Bufton, A. W. J. (1953). The genetics of *Aspergillus nidulans*. *Adv Genet* **5**, 141–238.

Powers, P. A. & Pall, M. L. (1980). Cyclic AMP-dependent protein kinase of *Neurospora crassa*. *Biochem Biophys Res Commun* **95**, 701–706.

Rossi, S. & Moreno, S. (1994). Regulation of protein kinase A subunits during germination of *Mucor rouxii* sporangiospores. *Eur J Biochem* **222**, 501–506.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular*

Cloning: a Laboratory Manual, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Sanger, F., Nicklen, S. & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* **74**, 5463–5467.

Simoni, M. N., Pelegrini, O., Veron, M. & Kay, R. R. (1992). Mutation of protein kinase A causes heterochronic development of *Dictyostelium*. *Nature* **356**, 171–172.

Struhl, K. (1985). Naturally occurring poly(dA-dT) sequences are upstream promoter elements for constitutive transcription in yeast. *Proc Natl Acad Sci USA* **82**, 8419–8423.

Taylor, S. S., Knighton, D. R., Zheng, J., Eyck, L. F. T. & Sowadski, J. M. (1992). Structural framework for the protein kinase family. *Annu Rev Cell Biol* **8**, 429–462.

Thevelein, J. M. (1994). Signal transduction in yeast. *Yeast* **10**, 1753–1790.

Toda, T., Cameron, S., Sass, P., Zoller, M. & Wigler, M. (1987a). Three different genes in *S. cerevisiae* encode the catalytic subunit of the cAMP-dependent protein kinase. *Cell* **50**, 277–287.

Toda, T., Cameron, S., Sass, P., Zoller, M., Scott, J. D., McMullen, B., Hurwitz, M., Krebs, E. G. & Wigler, M. (1987b). Cloning and characterisation of BCY1, a locus encoding a regulatory subunit of the cyclic AMP-dependent protein kinase in *Saccharomyces cerevisiae*. *Mol Cell Biol* **7**, 1371–1377.

Unkles, S. E. (1992). Gene organisation in industrial filamentous fungi. In *Applied Molecular Genetics of Filamentous Fungi*, pp. 28–53. Edited by J. R. Kinghorn & G. Turner. London: Blackie.

Visniac, W. & Santer, M. (1957). The thiobacilli. *Bacteriol Rev* **21**, 195–213.

Walsh, D. A. & van Patten, S. M. (1994). Multiple pathway signal transduction by the cAMP-dependent protein kinase. *FASEB (Fed Am Soc Exp Biol) J* **8**, 1227–1236.

Wiemann, S., Kinzel, V. & Pyerin, W. (1992). Cloning of the Cα catalytic subunit of the bovine cAMP-dependent protein kinase. *Biochim Biophys Acta* **1171**, 93–96.

Witteveen, C. B. F., van de Vondervoort, P. J. I., van den Broeck, H. C., van Engelenburg, F. A. C., de Graaff, L. H., Hillebrand, M. H. B. C., Schaap, P. J. & Visser, J. (1993). Induction of glucose oxidase, catalase and lactonase in *Aspergillus niger*. *Curr Genet* **24**, 408–416.

Received 31 July 1996; revised 8 November 1996; accepted 21 November 1996.