The EMBO Journal Vol.17 No.14 pp.3990–4003, 1998

A cyclin-dependent kinase family member (PHOA) is required to link developmental fate to environmental conditions in *Aspergillus nidulans*

Henk-Jan Bussink[1] and Stephen A.Osmani[2]

Henry Hood Research Program, Weis Center for Research, Pennsylvania State University College of Medicine, Danville, PA 17822, USA

[1]Present address, Carlsberg Laboratory, Department of Physiology, GI, Carlsbergvej 10, DK-2500 Copenhagen Valby, Denmark

[2]Corresponding author
e-mail: sosmani@psghs.edu

We addressed the question of whether *Aspergillus nidulans* has more than one cyclin-dependent kinase gene and identified such a gene, *phoA*, encoding two PSTAIRE-containing kinases ($PHOA^{M1}$ and $PHOA^{M47}$) that probably result from alternative pre-mRNA splicing. $PHOA^{M47}$ is 66% identical to *Saccharomyces cerevisiae* Pho85. The function of this gene was studied using *phoA* null mutants. It functions in a developmental response to phosphorus-limited growth but has no effect on the regulation of enzymes involved in phosphorus acquisition. *Aspergillus nidulans* shows both asexual and sexual reproduction involving temporal elaboration of different specific cell types. We demonstrate that developmental decisions in confluent cultures depend upon both the initial phosphorus concentration and the inoculation density and that these factors influence development through *phoA*. In the most impressive cases, absence of *phoA* resulted in a switch from asexual to sexual development (at pH 8), or the absence of development altogether (at pH 6). The phenotype of *phoA* deletion strains appears to be specific for phosphorus limitation. We propose that PHOA functions to help integrate environmental signals with developmental decisions to allow ordered differentiation of specific cell types in *A.nidulans* under varying growth conditions. The results implicate a putative cyclin-dependent kinase in the control of development.

Keywords: *Aspergillus*/cyclin-dependent-kinase (CDK)/development/PHOA/Pho85

Introduction

Cyclin-dependent kinases (CDKs) were first identified during genetic screens aimed at isolating cell cycle-specific genes from yeast (Nasmyth and Reed, 1980; Beach *et al*., 1982; Nurse, 1990; Nasmyth, 1993). Since their original isolation, numerous other protein kinases have been isolated (Uesono *et al*., 1987; Toh-e *et al*., 1988; Meyerson *et al*., 1992) which are structurally similar to the founding members Cdc28 and Cdc2. These kinases display the unifying feature of requiring a bound cyclin partner for function (Peeper *et al*., 1993; Kaffman *et al*., 1994; Jeffrey *et al*., 1995).

In the yeast *Saccharomyces cerevisiae*, *CDC28* encodes the major cell cycle-specific CDK that promotes cell cycle transitions when bound to different cyclin partners (Nasmyth, 1993). The nearest relative to Cdc28 in *S.cerevisiae* is the Pho85 (Toh-e *et al*., 1988) protein kinase (51% identity). *PHO85* was first identified as a negative regulator of phosphate-repressible genes involved in the acquisition of phosphate (Hirst *et al*., 1994; Kaffman *et al*., 1994; Schneider *et al*., 1994; O'Neill *et al*., 1996; for review, see Lenburg and O'Shea, 1996). *PHO85* subsequently has been implicated not only in the regulation of acid phosphatase expression but also in cell cycle regulation (Espinoza *et al*., 1994; Measday *et al*., 1994), regulation of carbon metabolism (Gilliquet and Berben, 1993; Huang *et al*., 1996; Timblin *et al*., 1996), repression of stress response genes (Timblin and Bergman, 1997) and vacuole inheritance (Nicolson *et al*., 1995).

Consistent with these apparently disparate functions, Pho85 interacts with numerous different cyclin partners. To regulate acid phosphatase expression, Pho85 binds to the Pho80 cyclin-like molecule (Kaffman *et al*., 1994; Schneider *et al*., 1994; O'Neill *et al*., 1996). To contribute to the regulation of Start, Pho85 interacts with the G_1-specific cyclins Pcl1 and Pcl2 (Espinoza *et al*., 1994; Measday *et al*., 1994). Recently, Pho85 has been shown to interact with a further seven cyclin-like proteins (Measday *et al*., 1997), bringing the total estimate to 10 potential cyclin partners for Pho85. Thus Pho85 interacts with multiple cyclins which presumably direct it to fulfill numerous non-essential functions in the life cycle of *S.cerevisiae*.

One CDK gene ($nimX^{cdc2}$) has been isolated from *Aspergillus nidulans*, which encodes $NIMX^{cdc2}$, a protein kinase essential to promote transitions through the cell cycle (Osmani *et al*., 1994). To study the role of other CDKs in a genetically facile multicellular microorganism, we sought to isolate other potential CDK genes using a PCR approach in *A.nidulans*. This filamentous fungus produces 10 different cell types (Champe and Simon, 1992; Champe *et al*., 1994), some of which are genetically programmed for elaboration only during asexual or sexual spore formation (Timberlake, 1990). In addition, this fungus has a wide and diverse biosynthetic capacity and is able to grow from completely inorganic components when supplied with a suitable carbon source. Our studies identify a gene with high identity to the *S.cerevisiae PHO85* gene which, unlike *S.cerevisiae PHO85*, is not required for regulation of scavenging phosphatase production. The *A.nidulans* gene, *phoA*, is instead essential at the interphase between environmental conditions and the developmental programs that allow switching from vegetative growth and commitment to either asexual or sexual spore formation. The data implicate a putative CDK in the modulation of the developmental program of a multicellular eukaryote.

Fig. 1. Structure of the *phoA* gene. Intron sequences are in lower case, the PHOA-encoding sequence is separated in codons, and alternative start methionines are indicated in the three-letter code. The putative translation termination codon (end) and the start of the poly(A)$^+$ tail (A+) of transcripts derived from an immediately upstream gene are indicated below the sequence. The sequence between the underlined *Dra*I and *Eco*RV sites has been replaced in the *phoA1* allele. The closed triangles indicate the start of *phoA* sequence in expression plasmids pPAP(Met1) and pPAP(Met47). DDBJ/EMBL/GenBank accession No. U59215.

Results

Molecular cloning of the phoA gene

Preliminary attempts to identify *cdc2*-related genes by hybridization, using a *nimX*^{cdc2} cDNA as the probe and employing hybridization conditions of moderate stringency, did not yield indications of a gene highly related to *nimX*^{cdc2}. Therefore, a PCR-based approach was adopted using degenerate primers that had been designed on the basis of sequences of kinase domains I (Hanks *et al*., 1988) [oligo ERK1(F)], VIII [ERK3(R)] and VII [ERK4(R)] of both functional cdc2 homologs and more distantly related members of the cdc2 protein kinase family (Simon *et al*., 1986; Toh-e *et al*., 1988; Boulton *et al*., 1991; Meyerson *et al*., 1992). PCR products derived from six putative *A.nidulans* protein kinase genes, including those of the previously cloned *nimX*^{cdc2} (Osmani *et al*., 1994) and *crkA* (M.Mischke and N.R.Morris, personal communication) genes, were identified as detailed in Materials and methods. One of the newly isolated sequences showed a single open reading frame that has the capacity to encode a protein sequence 60% identical to that of the C-terminal domain kinase (Cismowski *et al*., 1995) encoded by the *KIN28* gene (Simon *et al*., 1986) in the region specified by, but not including, primers ERK1(F) and ERK4(R). In the other three cases, the presence of intron sequences in the PCR fragments recovered was assumed on the basis of alignments of the conceptual translation products and protein kinases of known structure, and also the introns tentatively identified showed sequences identical or similar to those in other filamentous fungal introns (Gurr *et al*., 1987). Two of the derived amino acid sequences showed highest similarity to MAP kinases and the other one to Pho85. The latter was the only one showing a sequence highly similar to the so-called PSTAIRE motif (Pines and Hunter, 1991), characteristic of functional cdc2 homologs, and the corresponding gene was therefore selected for further analysis. It was designated *phoA*, on the basis of the phenotype of *A.nidulans* strains lacking this gene.

Structure of the phoA gene

Most of the protein-coding region of the *phoA* gene (Figure 1) could be assigned on the basis of homology of the encoded protein, PHOA, with other protein kinases. The presence of the two downstream introns was confirmed by showing their absence in a PCR fragment amplified from an *A.nidulans* λgt10 cDNA library. The gene has two possible in-frame translation initiation codons, the more upstream one being located in a 66 bp sequence that

shows all the sequence characteristics of an *Aspergillus* intron. Preliminary attempts to amplify fragments from high-quality cDNA libraries using forward primers located before this putative intron were unsuccessful. Therefore, a first-strand cDNA synthesis was carried out using a *phoA*-specific primer (RACE1), its products were used directly in PCR reactions employing a forward primer (IP1) that has its 3′-end three bases before the putative intron, and the two *phoA*-specific products produced were cloned and sequenced. The shorter product showed the absence of all the three introns as depicted in Figure 1, establishing that the upstream translation initiation codon is present in an intron. However, this intron had not been removed in the longer product, although the two downstream introns had been spliced out. The longer product was the slightly more abundant product, in duplicate PCR reactions, suggesting that inefficient splicing of the first intron might be of physiological relevance, resulting in two forms of PHOA that differ by the presence or absence of an N-terminal amino acid sequence of 46 amino acids.

To investigate the occurrence of these two forms of PHOA *in vivo*, and to study gene function (see below), strains were generated in which the major portion of the *phoA* gene (between the *Dra*I and *Eco*RV sites indicated in Figure 1) had been replaced by the *A.nidulans pyrG* gene (Oakley *et al*., 1987). This deletion allele was designated *phoA1*. A *phoA1* strain was transformed with plasmids that contained the *A.nidulans pyroA* gene as the selectable marker and one of two versions of the *phoA* structural gene under the control of the promoter of the alcohol dehydrogenase-encoding *alcA* gene (Waring *et al*., 1989). The *phoA* sequence present in plasmid pPAP(M1) has the capacity to encode the larger form of PHOA starting at amino acid position 1 (Figure 1), whereas plasmid pPAP(M47) can only encode the smaller form of PHOA starting at position 47. The wild-type *A.nidulans* strain R153, the *phoA1* mutant and complemented strains obtained with plasmids pPAP(M1) and pPAP(M47) were grown for 18 h on minimal medium containing glucose as the carbon source and extracts were then prepared for Western blot analysis (Figure 2). The antibody raised against *Escherichia coli*-expressed PHOA is not entirely PHOA-specific as it recognizes some other proteins in the extract from the *phoA1* mutant of a mobility that can be expected for a CDK. However, it is quite clear that deletion of the *phoA* gene results in the absence of two immunoreactive proteins of apparent mol. masses of 32 and ~40 kDa found in the wild-type strain. The larger form of PHOA, $PHOA^{M1}$, is expressed from plasmid pPAP(M1), while plasmid pPAP(M47) only directs expression of the smaller form, $PHOA^{M47}$. These results show that both possible translation initiation codons in the *phoA* gene are indeed used. It can be seen that $PHOA^{M47}$ is also expressed at a relatively low level from plasmid pPAP(M1), possibly resulting from read-through of the first translation codon or from initiation of transcription downstream of this codon, which could be due to the use of the *alcA* promoter. As expected, much higher levels of PHOA were observed when its synthesis in the transformants was induced with ethanol, but it was also much more degraded compared with glucose-grown cells (data not shown). Preliminary experiments gave no indications of regulation of the ratio of the two PHOA forms.

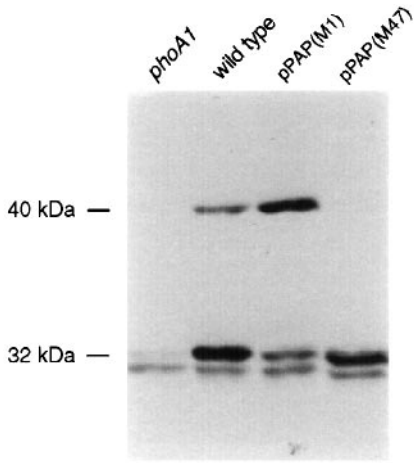


Fig. 2. The *phoA* gene encodes two polypeptides, $PHOA^{M1}$ and $PHOA^{M47}$, that are expressed from different translation initiation codons. The *phoA* deletion strain PHOΔ17 (lane 1), the wild-type strain R153 (lane 2) and strain PHOΔ17 complemented with plasmid pPAP(M1) (lane 3) or pPAP(M47) (lane 4) were grown for 18 h in minimal medium and mycelial extracts were analyzed by Western blotting using a polyclonal antibody raised against *E.coli*-expressed PHOA.

The sequence of *phoA* is preceded by a long open reading frame, from position 2 to 604 and in the same direction of transcription as *phoA*, that is likely to extend much further upstream on the basis of sequence obtained from only one strand of DNA not included in Figure 1. Putative transcripts having a poly(A) tail following the bases at positions 689 and 697 were identified using a 3′-RACE procedure. The partial conceptual translation product shows low, but probably significant, similarity to Sir2-related proteins (Brachmann *et al*., 1995).

Homology of PHOA to other protein kinases

The sequence of PHOA shows all the invariant residues found in protein kinases (Hanks *et al*., 1988), except that an aspartic acid (D224) rather than a glutamic acid is present in subdomain VIII, as has been observed recently for several protein kinases (Figure 3). PHOA is most similar to Pho85 (Toh-e *et al*., 1988), showing 66.3% identity to $PHOA^{M47}$ (Figure 3). It is also highly similar (64.7% identical) to the *Dictyostelium discoidum* protein tentatively designated Crp kinase (Michaelis and Weeks, 1993). While the function of this protein is unknown, the amount of *crp* mRNA is very low in vegetative cells and increases dramatically during development. The vertebrate protein kinases showing highest similarity to PHOA found in databases were human cdk5 (Meyerson *et al*., 1992) and *Xenopus laevis* cdk2 (Paris *et al*., 1991).

PHOA contains a sequence highly similar to the 16 amino acid PSTAIRE motif present in functional cdc2 homologs (Pines and Hunter, 1991) that has been identified as a key element in the activation of cdk2 by cyclin A (Jeffrey *et al*., 1995). The two amino acid substitutions in PHOA with respect to this PSTAIRE motif are also found in Pho85. Cdc2 and cdk2 are activated further by phosphorylation of a specific threonine residue by CDK-activating kinase. A serine, rather than a threonine, is found at the corresponding positions in PHOA (S212) and

A

```
 PHOA MTSQRPTSSSSSLLDIVANLASPKSLLFNAHHTNPPPSLPSQRAPTMDKS 50
Pho85                                                -SS-
  crp                                                -E-.
cdk5hs                                               -Q-.
cdk2xl                                               -EN.

 PHOA QQPSSFQQLEKLGEGTYATVFKGRNRQTGELVALKEIHLDSE.EGTPSTA 99
Pho85 S-...-K------N------Y--L-KT--VY-----VK----.-------
  crp .....YSKI--------GI-N-AK--E---I----R-R----D--V-C--
cdk5hs .....YEK---I-----G----AK--E-H-I----RVR--DDD--V--S-
cdk2xl .......KV--I-----GV-Y-A---E---I----K-R--T-T--V----

 PHOA IREISLMKELKHESIVSLYDVIHTENKLMLVFEYMDKDLKKYMDTR...G 146
Pho85 ------------N--R----------T----F--N-------S-TVAN
  crp ------L-----PN--R-H------R--T-----L-Q-----L-E....C
cdk5hs L---C-L-----KN--R-H--L-SDK--T----FC-Q-----F-S....C
cdk2xl ------L---N-PN--K-L---------Y----FLNQ----F--G....S

 PHOA DRGQLDQATIKSFMHQLMSGIAFCHDNRVLHRDLKPQNLLINKKGQLKLG 196
Pho85 TPRG-ELNLV-Y-QW--LQ-L----E-KI-------------R------
  crp G.-EISKP------Y--LK-V-----H--------------R--E---A
cdk5hs N.-D--PEIV---LF--LK-LG---SRN-------------RN-E---A
cdk2xl NISGISL-LV--YLF--LQ-L----SH--------------SD-AI--A
                    ♦           ◊
 PHOA DFGLARAFGIPVNTFSNEVVTLWYRAPDVLLGSRTYNTSIDIWSAGCIMA 246
Pho85 ---------------S------------M-----S-------C---L-
  crp ------------R-Y-H------------M---K-S-P------L--F-
cdk5hs ------------RCY-A--------P----F-AKL-S----M------F-
cdk2xl ---------V--R--TH----------EI---CKF-S-AV----L---F-

 PHOA ELY.TGRPLFPGTTNEDQLQKIFRLMGTPSERSWPGISQLPEYRANFHVY 295
Pho85 -MI.--K------ND-E--KL--DI----N-SL--SVTK--K-NP-IQQR
  crp -MA.S-------SGTS---FR--KIL---N-E---S-TE----KTD-P-H
cdk5hs --ANA-------NDVD---KR----L---T-EQ--SMTK--D-KP.YPM-
cdk2xl -MI.-R-A----DSEI---FR---TL---D-V----VTTM-D-KST-PKW

 PHOA AT.QDLGLILP.....QIDPLGLDLLNRMLQLRPEMRIDAHGALQHPWFH 339
Pho85 PP.R--RQV-QPHTKEPL-GNLM-F-HGL---N-D--LS-KQ--H----A
  crp PA.HQ-SS-VH.....GL-EK--N--SK---YD-NQ--T-AA--K--Y-D
cdk5hs PATTS-VNVV-.....KLNAT-R---QNL-KCN-VQ--S-EE-----Y-S
cdk2xl IR.--FSKVV-.....PL-ED-R---AQ---YDSNK--S-KV--T--F-R

 PHOA DLPQLQAQLQQQQMAGYGGMMPPQQAY 366
Pho85 EYYHHAS                          66.3% identity
  crp G-EPIN                           64.7%
cdk5hs -FCPP                           58.4%
cdk2xl -VSRPTPHLI                      58.4%
```

B

```
 PHOA  MTSQRPTSSSSSLLDIVANLASPKSLLFNA...HHTNPPPSLPSQR 43
Mkk1p  H-PEPL---T-T-FSQTRLHP-DS-MTL-TMKKRP..A------LS 93
Mkk2p  NN-TSA--TT--MASNSTL-YNRS-TT..TIKNRP..V--P--PLV 95
NUC-1  L-PLI-------TAGVTDSENG-ISPENL-DVVLPVEM---P--KP- 442
           S/T rich                          P rich
```

Fig. 3. Similarity between PHOA and other proteins. (**A**) Alignment of PHOA^{M1} and related protein kinases. Conserved amino acids are indicated by dashes, and where alignment has been improved by the introduction of gaps, this is marked by points. The perfect PSTAIRE motif in the cdk2Xl sequence is underlined twice and Ser(212) and Asp(224) in PHOA are indicated by closed and open diamonds, respectively. Similarities were calculated on the basis of PHOA^{M47} sequence. (**B**) Similarity between the N-terminal extension of PHOA^{M1}, sequences in the N-terminal non-catalytic regions of yeast MAP kinase kinases and *Neurospora crassa* NUC-1. See text for details.

some other cdc2-related protein kinases. While mutagenesis of this serine to alanine results in a non-functional Pho85 kinase (Santos *et al*., 1995), the activity of cdk5 does not depend on phosphorylation (Qu *et al*., 1995).

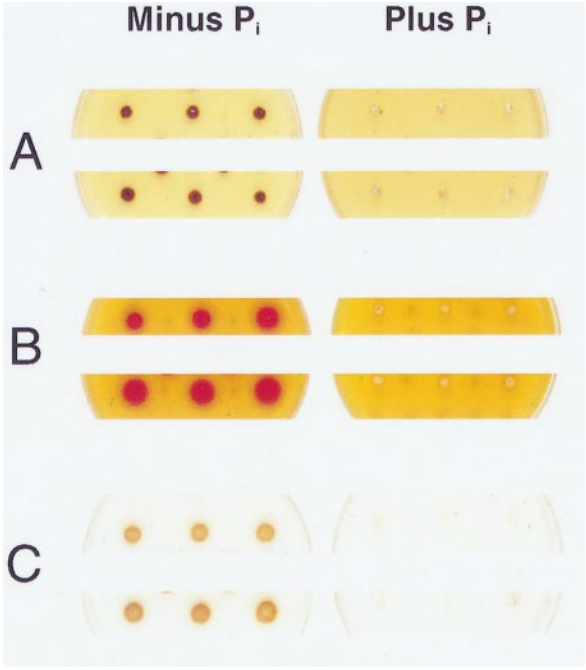


Fig. 4. Normal regulation of excreted phosphatase activities in the absence of *phoA*. Colonies were stained for (**A**) alkaline phosphatase, (**B**) phosphodiesterase and (**C**) acid phosphatase. In each panel, three strains containing the *phoA1* mutation (top) and non-deleted control strains (lower) were grown on agar plates containing 11 mM P_i or no added P_i as indicated for 22 h prior to activity staining.

The 46 amino acid N-terminal extension of PHOA^{M1} is rich in serine, proline and leucine, together comprising 50% of the residues. The similarity of the PHOA^{M1} extension to regions of other proteins (Figure 3B), essentially consisting of a serine/threonine-rich region of six amino acids and a proline-rich region separated by ~20 amino acids, may suggest a regulatory function for the extension (see Discussion).

Absence of PHOA does not lead to constitutive expression of phosphatases

Because deletion of *PHO85* leads to constitutive expression of the phosphatase genes under its control, the *A.nidulans phoA1* strains were also tested for the activity of three phosphate-regulated extracellular phosphatases, alkaline phosphatase, acid phosphatase and phosphodiesterase, by activity staining of colonies grown in the presence or absence of inorganic phosphate (P_i). Agar contains sufficient phosphate to sustain growth. Deletion of the *phoA* gene did not result in constitutive synthesis of any of the three activities on P_i-rich medium (11 mM), while the induced activities on medium without P_i were comparable with those of the wild-type strain (Figure 4). Measurement of acid phosphatase activity produced in liquid culture using phosphate-depleted rich media that had been fully reconstituted with phosphate, or with only 0.1 mM phosphate (low-P_i), confirmed that the regulation of acid phosphatase gene expression in response to phosphorus availability was not affected by the *phoA1* deletion. The *phoA1* strain also grew at the same rate as the wild-type under different P_i concentration growth conditions as revealed by dry weight measurements (Table I). While yeast *pho85* null mutants show reduced growth on acetate, ethanol and glycerol (Gilliquet and Berben, 1993), no

Table I. PHOA is not required to repress the synthesis of extracellular acid phosphatase under phosphate-sufficient growth conditions

Strain	Acid phosphatase[a]	Biomass[b]
R153 (wild-type)		
11 mM P_i	0.13	1.56
0.1 mM P_i	32.0	0.90
PHOΔ17 (*phoA1*)		
11 mM P_i	0.10	1.69
0.1 mM P_i	21.5	0.84

[a]Acid phosphatase activity of culture filtrates expressed as nmol of *p*-nitrophenol liberated/mg of mycelial dry weight/min.
[b]Grams of mycelial dry weight/l after 14 h.

obvious phenotype was observed when the *phoA1* strain was grown on these carbon sources (data not shown).

A temperature-sensitive *phoA1–nimX3*^{cdc2} double mutant was obtained to investigate if the absence of PHOA enhances the effect of impaired NIMX^{cdc2} function as may be expected if PHOA functioned redundantly with *nimX*^{cdc2}. However, no significant effect of the presence of the *phoA1* allele was observed when the *nimX3*^{cdc2} mutant and the double mutant were grown at 37°C, at which temperature *nimX3*^{cdc2} strains grow poorly compared with wild-type strains. *phoA1* strains appeared to form conidia (asexual spores) normally and could be crossed to each other to yield viable recombined progeny. The *phoA* gene is therefore apparently not essential for any part of the *A.nidulans* life cycle under standard laboratory growth conditions.

PHOA functions in the cellular response to phosphorus limitation

When the *phoA1* mutant was grown at pH 8 in the presence of normal P_i (11 mM) levels, it grew and differentiated like the wild-type strain, and both strains produced similar amounts of a brown pigment (Figure 5). However, on low-P_i medium, only very little pigment was formed in the wild-type strain, whereas the mutant overproduced the brown pigment. The contrast in pigment production between the *phoA1* and wild-type strains was greatly diminished when tested at pH 6.5, because the wild-type strain produced more of the brown pigment at this pH, while the *phoA1* strain produced less than at pH 8.

The production of the pigment started around 2 days after inoculation, after a colony of considerable size had been formed, and it appeared to be secreted into the agar medium. All *phoA* deletion strains isolated showed this phenomenon, and in a cross of strains PHOΔ17 and HB23, this trait and uridine prototrophy, i.e. the *phoA1* allele, co-segregated as a single Mendelian marker. While we have not analyzed this pigment, it may be a phenolic compound (Hermann *et al*., 1983; Champe and Simon, 1992; Champe *et al*., 1994). An *in situ* staining method for sexual development (Hermann *et al*., 1983) depends on the activity of laccase II, a developmentally regulated phenoloxidase, and coupling of the reaction product to DMA (3,5-dimethylaniline). *phoA1* colonies (carrying the *yA2* allele to inactivate laccase I) producing the pigment showed evenly distributed staining upon application of this method, even when the laccase substrate was omitted from the reaction mixture (data not shown) presumably due to reaction of the pigment and DMA.

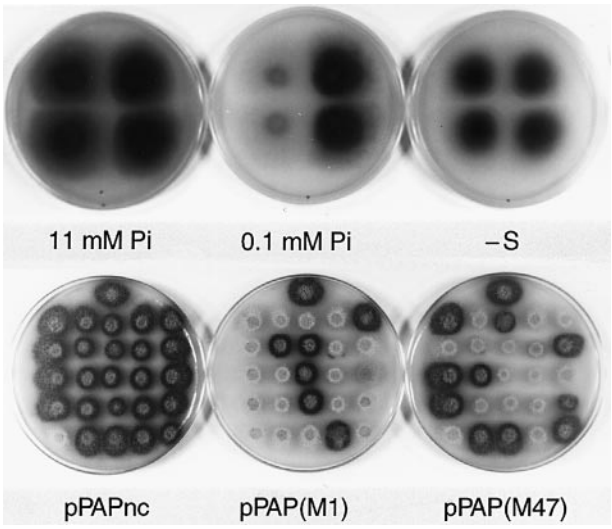


Fig. 5. PHOA is required to repress the synthesis of a pigment during phosphate-limited growth at pH 8. Upper panel: two colonies each of the wild-type strain R153 (on the left) and the *phoA* deletion strain PHOΔ17 (on the right) were grown for 5 days on regular pH 8 medium, or on media with only 0.1 mM P_i or no added sulfate. The back of the plates is shown. Lower panel: complementation of strain PHOΔ17 with plasmids pPAPnc, pPAP(M1) and pPAP(M47). Colonies were grown for 5 days in the presence of 0.1 mM P_i. Strain PHOΔ17 was inoculated at the top of the plates and strain R153 in the lower left corner. The upper face of the plates is shown.

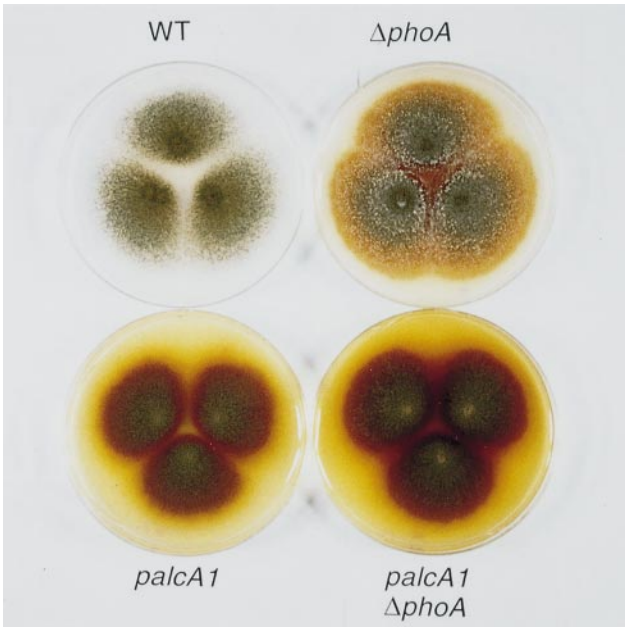


Fig. 6. Morphology of wild-type and mutant strains grown on 0.1 mM P_i medium for 7 days. Strains HB38 (WT), HB9 (Δ*phoA*), HB37 (*palcA1*) and HB34 (Δ*phoA*, *palcA1*) are shown.

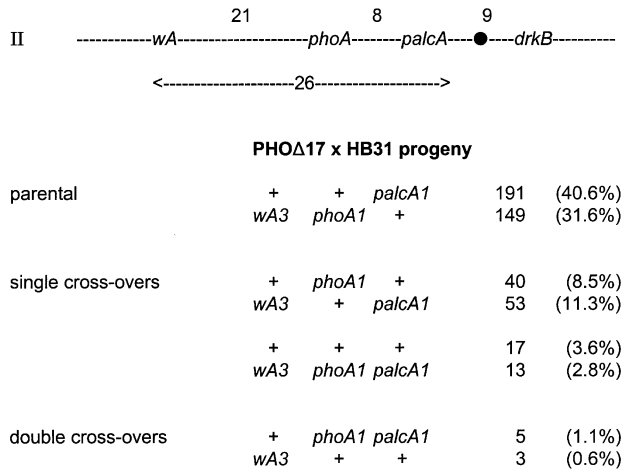
Wild-type strains produced only few cleistothecia (sexual fruiting bodies) on low-P_i medium, whereas the *phoA1* strain showed abundant sexual development under these conditions. This can be seen in Figure 6 where the green color is caused by conidia and the yellow/tan structures on the surface of the colonies indicate sexual development. This yellow color stems from so-called hulle cells whose appearance is the first morphological manifestation of sexual development, and sexual spores

are formed in developing cleistothecia that are surrounded by these hulle cells. On complex MAG medium, the *phoA1* strain produced typical large clusters of cleistothecia that were, in most experiments, absent in the wild-type strain R153, although some of these clusters were also seen in R153 in an experiment where they were particularly abundant in the *phoA1* strain.

The *phoA1* mutation was complemented with the cloned gene, as described above using plasmids pPAP(M1) and pPAP(M47). These plasmids do not contain any of the gene upstream of *phoA*, eliminating the possibility that these plasmids could integrate in the upstream gene by homologous recombination and thus complement a conceivable phenotype of the *phoA1* mutant that might be due to truncation of the 3′-non-coding region of the upstream gene, rather than the absence of PHOA. In addition, a frameshift mutation was introduced in the PHOA-encoding region of pPAP(Met47), and this plasmid, pPAPnc, was used as a negative control. The *pyroA*$^+$ transformants obtained with these plasmids were first grown on low-P_i medium with glucose as the carbon source and scored for pigmentation (Figure 5). Plasmid pPAPnc was not able to complement the *phoA1* mutation, and plasmids pPAP(M1) and pPAP(M47) gave similar complementation frequencies of 65 and 61%, respectively. Essentially identical results were obtained when the transformants were retested on low-P_i medium with the *alcA*-inducing carbon source ethanol. It thus follows that low-level expression of PHOA from the *alcA* promoter during growth on the 'repressing' carbon source, glucose, is sufficient to complement the *phoA1* mutant. This low level of expression from the *alcA* promoter in the presence of glucose previously has been observed by others (Som and Kolaparthi, 1994). The *pyroA*$^+$ transformants that were not complemented with respect to the *phoA1* mutation are likely to result from plasmid-dependent repair of the *pyroA4* mutation or from plasmid integrations in which the *phoA* gene has been inactivated. The transformants were tested further on MAG medium for the formation of the large clusters of cleistothecia. Plasmids pPAP(M1) and pPAP(M47) both also complemented this sexual differentiation characteristic of the *phoA1* mutant at high frequency; ~95% of the transformants obtained with these plasmids, that did not produce the brown pigment, showed normal sexual differentiation on MAG medium, whereas all strains tested that did produce the brown pigment, including the transformants constructed using plasmid pPAPnc, showed the hypersexual phenotype of the *phoA1* mutant (data not shown).

To test the nutritional specificity of the phenotype of the *phoA1* strain, several other nutrients were made limiting for growth. The colonies of the *phoA1* strain and the wild-type strain formed under conditions of carbon, nitrogen and sulfur limitation, respectively, were similar. The response to the individual limitations was, however, quite different. Upon sulfur limitation (Figure 5), for example, unlike the case of phosphorus limitation, strong pigmentation was seen in both the wild-type and *phoA1* strains, and both strains also showed strong sexual development.

These data demonstrate that there is a mechanism that represses sexual development and pigmentation specifically during phosphorus-limited growth which requires PHOA's function.



PHOΔ17 x HB31 progeny

parental	+	+	*palcA1*	191	(40.6%)
	wA3	*phoA1*	+	149	(31.6%)
single cross-overs	+	*phoA1*	+	40	(8.5%)
	wA3	+	*palcA1*	53	(11.3%)
	+	+	+	17	(3.6%)
	wA3	*phoA1*	*palcA1*	13	(2.8%)
double cross-overs	+	*phoA1*	*palcA1*	5	(1.1%)
	wA3	+	+	3	(0.6%)

Fig. 7. Map position of *phoA* on the left arm of linkage group II, linked to *palcA*. The position of the centromere (●) and the distance between *palcA* and *drkB* are from Clutterbuck (1993), and other distances are from the cross shown. Distances are expressed as uncorrected recombination frequencies. There appears to be a slight selection against the *phoA1* allele (43.9% recovery) which was also observed in a cross between strains PHOΔ17 and HB23 (44.3% recovery of *phoA1*; 23.6% recombination between *wA* and *phoA*; 512 progeny analyzed).

Interaction between phoA and palcA

The positive-acting regulatory *palcA* gene controls the syntheses of several P_i-repressible phosphatases and probably also a phosphate permease (Caddick *et al*., 1986). Loss-of-function *palcA* mutations (*palcA1*, *palcA40*) also lead to strong pigmentation (Figure 6), suggesting that the *phoA* and *palcA* gene products may function in a common pathway that represses pigmentation. While a strong correlation was observed between the synthesis of the pigment and sexual development, no cleistothecia were observed in the *palcA1* strain on low-P_i medium. However, morphologically abnormal hyphae were observed in the later stages of colony development, suggesting that the absence of sexual development might be due to low intracellular phosphorus concentrations caused by the *palcA1* mutation. This was tested by constructing a *phoA1/palcA1* double mutant strain, in which process the *phoA* gene was also mapped onto the left arm of chromosome II, linked (8% recombination) to *palcA* (Figure 7). The double mutant did not show sexual development and, thus, sexual development under these conditions requires the presence of a functional *palcA* gene. This result also indicates that while pigmentation may be coordinated with sexual development, it is not caused by advanced stages of sexual development. This notion is supported by the observation that pigment production also occurred when *palcA1* and *phoA1* strains, but not wild-type strains, were grown under specific P_i-limiting conditions in shaken liquid cultures where differentiation was not observed (data not shown).

The phoA deletion affects development in confluent plate cultures depending on inoculation density as well as phosphate concentration

While the phenotype of the *phoA* deletion strain depends on the P_i concentration of the growth medium, the differ-

ences with respect to the yeast *PHO85* system raise the question of whether PHOA is involved specifically in transmitting a phosphorus-related signal (see Discussion). The possibility that another factor might play an important role was suggested initially by the fact that sexual development in the *phoA* deletion strain is particularly strong at the border of a colony (Figure 6), either in the case of a single large colony or where colonies meet each other. In wild-type strains, sexual development occurs typically in the older part of a colony, near the center (Champe *et al*., 1987). In order to eliminate gradients of the type that may exist in colonies, in further experiments spores were plated in a top-agar layer.

Under conditions of normal P_i concentration and low inoculum level (3×10^5 conidia per plate), both the wild-type and *phoA1* strains produced abundant dark green asexual spores leading to a uniform, dark green colony (Figure 8). Much to our surprise, when either wild-type or *phoA1* spores were plated on low-P_i medium at a density of 3×10^5 per plate, the resulting colony showed very early and strong sexual development. This can be seen as a mixture of yellow-looking patches, caused by hulle cell development, on a dark green background resulting from asexually produced conidia (Figure 8A). However, sexual development became repressed when the number of spores per plate was raised to 2×10^6 for the wild-type, but this response was totally lacking in the *phoA1* strain. When the inoculum level was increased further to 10^8 spores per plate, there was almost a total lack of sexual development in the wild-type culture but, under these conditions, very vigorous early sexual development continued to occur in the *phoA1* strain. At a higher level of magnification, this contrast in developmental fate between the wild-type and the *phoA1* strain can be seen very clearly by the predominant cell types produced (Figure 8B). Examples of developing conidiophores as produced by the wild-type (Figure 8B1) can be contrasted to the hulle cells produced by the *phoA1* strain (Figure 8B2) when both are grown at high inoculum levels and low P_i concentration. When plated at high density on P_i-rich growth medium, both strains showed similar abundant conidiation.

The influence of initial cell density on development during P_i-limited growth was also investigated at pH 6, a pH more commonly used to grow *Aspergillus* in the laboratory than pH 8, which was used for the preceding experiments. On P_i-rich medium, conidiation for both the wild-type and *phoA1* strains was largely independent of the inoculum in the range of 3×10^5–3×10^6 spores per plate, and then an ~3-fold increase in conidiation (Figure 9A) and a concomitant reduction in sexual development was seen upon further increasing the inoculum. This effect was also seen when the wild-type strain was grown on low-P_i medium, where good conidiation required a high inoculation density. In this experiment, *A.nidulans* was grown for 4.5 days and thus the low spore counts at the lower inoculum density are most probably not caused by slower asexual development. Rather, strong sexual development, which commences after conidiation ceases in confluent plate cultures (Champe *et al*., 1987), was observed at the lower densities and was repressed at higher densities ($>10^7$ spores/plate) as previously observed at pH 8. The ratio of asexual to sexual sporulation in the wild-type control strain therefore depends on the inoculation density.

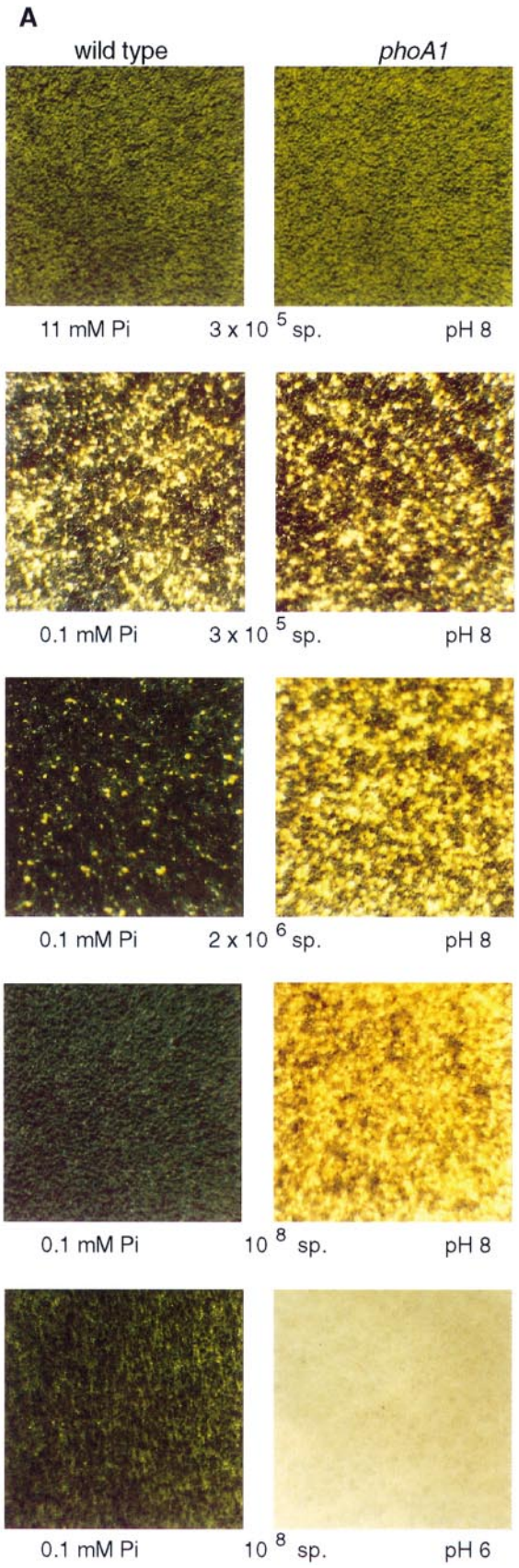
At low density, the *phoA* deletion strain looked very similar to the wild-type, showing strong sexual development and giving essentially identical asexual spore counts. However, it failed to repress sexual development and to enhance conidiation at intermediate inoculation densities. At higher inoculation densities, the development, both sexual and asexual, of the *phoA1* strain was greatly affected, resulting in a spore count several hundred fold lower than that of the wild-type strain. Vegetative growth was, however, not affected, as the *phoA* deletion strain showed abundant undifferentiated aerial mycelia (Figure 8), a so-called 'fluffy' phenotype, that was also observed when a *fluG* deletion mutant (TTA127.4) was plated under these conditions. This effect required a low P_i concentration as well as a high inoculation density (Figure 9B). At an intermediate P_i concentration of 0.3 mM at high inoculum density, *phoA1* and wild-type both showed reduced conidiation and much stronger sexual development than at higher P_i concentrations. Reducing the P_i concentration to 0.1 mM repressed sexual development in the wild-type strain, but had no effect on conidiation. Conidiation only became reduced at lower P_i concentrations that were clearly growth limiting, whereas the fluffy phenotype of the *phoA1* strain depends on continued growth in the absence of development, indicating that it is not due to an effect of the *phoA* deletion on phosphorus acquisition. Moreover, it was tested to see if inoculum density had an effect on acid phosphatase production in liquid culture, but at the highest density tested (3×10^7 spores/ml) the *phoA1* strain still produced 72% of wild-type specific activity, similar to the value found at low inoculum size (Table I). These small differences in acid phosphatase activities strongly contrast with the ~10-fold reduction in P_i concentration needed to reduce conidiation in the wild-type strain to the level of the *phoA1* strain at 0.1 mM P_i, further supporting the notion that the phenotype of the *phoA1* mutation is not caused by effects on phosphate assimilation.

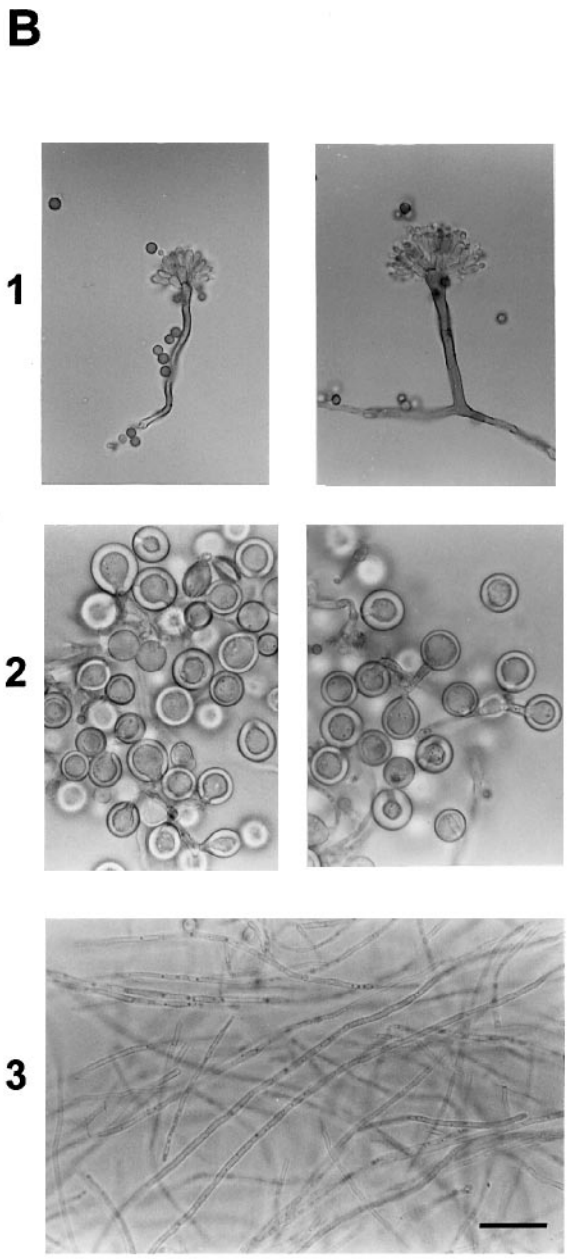
The fluffy phenotype of the *phoA1* mutant observed with high inoculum levels and low P_i concentrations could be fully complemented by plasmids encoding $PHOA^{M1}$ or $PHOA^{M47}$. The pPAP(M1) and pPAP(M47) strains shown in Figure 2 produced 211% and 112%, respectively, of the number of conidia observed with the wild-type strain R153 after 4 days. No significant effect on conidiation in

Fig. 8. PHOA is required for developmental decisions in P_i-limited confluent cultures initiated at high spore densities. (**A**) Conidia of strains HB38 (wild-type) and HB9 (*phoA1*) were plated in top agar at densities (spores per plate) and on media (with 0.1 mM or 11 mM P_i, at pH 8 or pH 6) as indicated and the plates were photographed at low magnification after 4 days of growth. Asexual development results in formation of conidiophores carrying chains of conidia that cause the green color, whereas sexual development is indicated by the yellow color of hulle cells occurring in clusters which surround nascent cleistothecia. In the lower right panel, focusing was on a single conidiophore in the middle but individual aerial hyphae (see B3) are not resolved. (**B**) Examples of the different multicellular structures and cells formed. (1) Conidiophores and asexual spores. Note the two rows of specialized cells on top of the stalk (metulae and phialides) and the nascent spores. (2) Clusters of hulle cells. (3) Unbranched aerial hyphae. The scale bar is ~30 μm and applies to 1–3.

the *phoA* deletion strain was observed when plated at high density on nitrogen (1 mM nitrate) or sulfur-limiting (no sulfate added) growth media (not shown).

Regulation of development under conditions of phosphorus limitation is therefore very complex, depending on inoculation density as well as P_i concentration. While the absence of PHOA has no significant effect at low inoculation density, its effect becomes apparent at higher





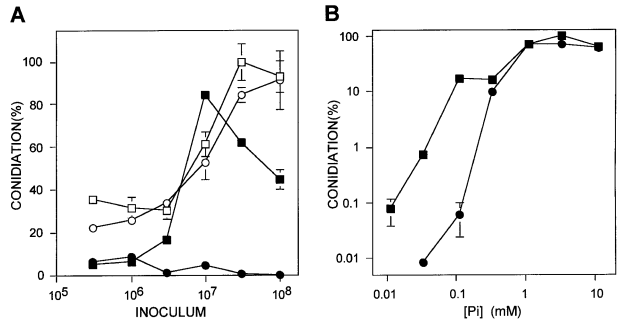


Fig. 9. Dependence of asexual sporulation on PHOA. Conidia of the *phoA1* strain HB9 (circles) and the wild-type strain HB38 (squares) were plated on pH 6 media. The numbers of conidia formed after 4.5 days are expressed relative to the highest values observed, note the different scales in (A) and (B). (**A**) Conidiation as a function of inoculum size (spores per plate) on P_i-rich (open symbols) and P_i-limiting (closed symbols) media; 100% conidiation = 3.6×10^{11} spores/m^2. (**B**) Conidiation as a function of P_i concentration with a fixed inoculum size of 10^8 conidia per plate; 100% conidiation = 5.6×10^{11} spores/m^2.

densities, correlating with inoculation density-related changes of development in the wild-type strain, namely an increase of conidiation and repression of sexual development.

Discussion

The developmental schedule of *A.nidulans* is genetically driven and is responsive to one major environmental stimulus, exposure to an air surface. Development is not a response to starvation or stress and occurs under most nutrient conditions (Timberlake, 1990). Growth of a single uninucleate asexual spore on a solid nutrient surface leads to a well-defined program of colony formation and differentiation when exposed to air (Champe *et al*., 1994). The spore germinates to form a vegetative hypha which invades and utilizes the growth medium. Next, at ~24 h, aerial structures called conidiophores are elaborated which contain five different cell types that function to produce dormant asexual spores called conidia (Champe and Simon, 1992). After conidiation ceases, growth of a fruiting body called a cleistothecia ensues in which sexual spores (ascospores) are produced (*A.nidulans* is homothallic, i.e. it is self-fertile). During ascospore formation, a further four developmentally specific cell types are formed. Growth in liquid media, in the absence of an air surface, suppresses elaboration of all nine developmentally determined cell types (Champe *et al*., 1994). The studies reported here demonstrate that a CDK family member plays a role in the elaboration of developmentally determined cell types in *A.nidulans* under defined environmental conditions involving P_i-limited growth.

Our studies of the *phoA* gene, which encodes a CDK-like kinase with 66.3% identity to *S.cerevisiae Pho85*, show that a null mutation of *phoA* (termed *phoA1*) does not affect the regulation of inducible phosphate acquisition genes that are regulated by available phosphate concentrations. Therefore, unlike *S.cerevisiae PHO85*, the *A.nidulans phoA* gene is not required for negative regulation of phosphate acquisition genes. The *phoA1* null mutation did, however, cause two striking phenotypes when colonies were point inoculated in P_i-limiting conditions. Unlike wild-type, *phoA1* strains constitutively produced a dark brown pigment that was excreted into the agar growth media at pH 8.0. This phenotype co-segregated in crosses as a single Mendelian marker linked to *pyrG*, demonstrating it to be caused specifically by lack of *phoA*. Secondly, we found that P_i-limiting conditions promoted sexual differentiation of the *phoA1* strain. Both the hypersexual phenotype and the pigmentation phenotype were completely repaired upon reintroduction of the *phoA* gene into a null allele strain. In addition, pigment production was not a secondary result of sexual development because pigment production could be induced in the *phoA1* strains when sexual development was repressed. Therefore, PHOA functions in a regulatory process that represses sexual development and pigmentation specifically during phosphorus-limited growth.

Not only did *phoA1* strains develop cell types required for sexual reproduction under limiting phosphate conditions, these strains also demonstrated an unusual pattern of differentiation within the colony. The *A.nidulans* developmental program produces asexual spores followed later by sexual spores. Because colonies grow evenly away from the point of inoculation, it is normal first to find sexual development at the older center of *A.nidulans* colonies, not at the younger edge of the colony as was observed for *phoA1* strains. This strongly suggests that the *phoA1* mutation affects either the timing of the developmental program of *A.nidulans*, or the way in which cell–cell interactions impinge upon development. Therefore, to investigate further influences that may modulate the developmental program of *A.nidulans* through the function of *phoA*, in a manner that was independent of varying age within the colony, we investigated the effects of inoculum level and pH upon development in confluent cultures. A very strong effect on developmental differentiation was caused by the absence of PHOA in P_i-limited confluent cultures initiated at high inoculation density. The precise defect was found to be heavily influenced by the pH of the growth media (Figure 8A, bottom two panels).

At high inoculum levels, at either pH 8 or 6, the wild-type underwent normal differentiation to form abundant asexual spores. Under identical conditions, the *phoA* null mutant either failed to undergo any development (at pH 6) or showed abundant mis-scheduled sexual development (at pH 8). These effects were very striking (Figure 8) and demonstrate that the function of *phoA* is required under specific environmental conditions to regulate the developmental program of *A.nidulans*. In its normal environment, *A.nidulans* would grow and develop under varying, and often very restrictive, nutritive conditions. Additionally, *A.nidulans* is able to grow over a range of approximately eight pH units (Arst, 1994). It is therefore likely that *phoA*, although non-essential in the laboratory setting, would play an important role in the life cycle of this organism in nature.

At this time, it is unclear if *phoA* interacts directly with the pH regulatory system of *A.nidulans*. This system controls the synthesis of permeases and secreted enzymes and metabolites in response to changes in pH via the transcription factor PacC (Tilburn *et al*., 1995). Because sporulation is compromised in *pacC* mutants, the *pacC* gene has been implicated in asexual spore production.

However, in *phoA*-deleted strains, changing the ambient pH growth conditions modifies the developmental defects seen under low-P_i conditions rather than being strictly required to cause a developmental defect. Perhaps the conversion of a morphogen is influenced by changes in pH, for example the interconversion of psi factor (see below). In addition, by analogy with penicillin production, the synthesis of secondary metabolites that may influence development might be under PacC control (Tilburn *et al*., 1995) and hence cause changes in the developmental defects observed at different pHs when *phoA* is deleted.

Given the high sequence similarity between *A.nidulans phoA* and *S.cerevisiae PHO85*, it is worth considering the relationship between these two kinases and their respective regulatory roles. *PHO85* plays a well-established negative role in inducible *S.cerevisiae* phosphatase expression under conditions of sufficient phosphate. Conversely, *phoA* plays no negative role in phosphatase expression under phosphate-sufficient conditions. Instead, *phoA* plays a negative role in regulating brown pigment production during P_i-limited growth and also plays a role in promoting the normal developmental program of *A.nidulans* in response to several environmental conditions which have in common P_i-limited growth conditions. So, although both *PHO85* and *phoA* are involved in mediating a response to phosphate concentration, *PHO85* functions at high P_i concentrations and *phoA* at low concentrations, and they are involved in controlling very different cellular processes.

We had expected that *phoA* would be involved in regulation of phosphatase expression because this level of regulation is conserved between the budding yeast *S.cerevisiae* and the filamentous fungus *Neurospora crassa* (Kang and Metzenberg, 1990; Kaffman *et al*., 1994; Schneider *et al*., 1994; O'Neill *et al*., 1996; Peleg *et al*., 1996a,b). Thus, there may be a second *phoA*/*PHO85*-related gene in *A.nidulans* encoding a functional *PHO85* homolog, perhaps represented by the non-PHOA bands detected on Western blots probed with a polyclonal antibody raised against PHOA (Figure 2). This also raises the possibility of partial redundancy of PHOA function. In this regard, isolation of the gene(s) encoding the putative PHOA-related proteins observed will be of importance, to see if its deletion will extend the range of environmental conditions that influence development through this type of protein kinase, and/or effect the expression of scavenging phosphatase genes.

By what mechanism does *phoA* influence development in *A.nidulans*? One intriguing possibility is that PHOA is involved in the signaling cascades that control development in *A.nidulans*. Both asexual and sexual development in *A.nidulans* are controlled by extracellular signaling molecules that dictate developmental fate. Lee and Adams have demonstrated that the *fluG* gene is necessary for the synthesis of a small uncharacterized factor, FluG factor. This factor is required for the endogenously regulated induction of asexual sporulation which involves specific expression of a wide range of developmentally regulated genes (Timberlake, 1990; Adams, 1994). It has been hypothesized that FluG factor is constitutively produced and asexual development initiates after its concentration exceeds a fixed threshold which triggers expression of conidiation-specific genes (Lee and Adams, 1994). Furthermore, Champe and colleagues have isolated a factor, psi factor (precocious sexual inducer), from an *A.nidulans* developmental mutant that overproduces this factor and a number of phenolic metabolites. If applied to a confluent culture at the time of inoculation, it strongly inhibits asexual sporulation and induces premature sexual sporulation (Champe *et al*., 1987). Psi factor consists of a family of hydroxylated derivatives of linoleic acid, two of which are interconvertible *in vitro* and have opposing effects on development (reviewed in Champe and Simon, 1992; Champe *et al*., 1994). The PHOA kinase could be involved in integrating information from the environment, such as phosphate concentration or inoculum levels, to help control the synthesis of these extracellular morphogens. Alternatively, PHOA may be involved downstream of the morphogen receptors and modulate the cell's response to the levels of these factors during P_i-limited growth. In either case, it appears that PHOA is involved in integrating a phosphorus-related signal and at least one other signal into a controlled developmental response that allows the normal program of differentiation of *A.nidulans* to proceed under varying environmental conditions.

Two proteins, $PHOA^{M1}$ and $PHOA^{M47}$, differing by an N-terminal extension of 46 amino acids, are expressed from the *phoA* gene. Their occurrence is explained most easily by a simple version of alternative pre-mRNA splicing, splicing or non-splicing of the 5′ intron containing one of two alternative in-frame translation initiation codons, which has not been well documented in fungi. However, we cannot rule out the possibility that another mechanism also contributes to the generation of the two forms of PHOA. In any case, the existence of these two forms further suggests a complex regulatory function for PHOA. A database search revealed similarity between the $PHOA^{M1}$ extension and part of the sequence preceding the catalytic domain of the *S.cerevisiae* MAP kinase kinase (MAPKK) Mkk1p (Irie *et al*., 1993). This similarity consists essentially of a serine/threonine-rich region of six amino acids and a proline-rich region separated by ~20 amino acids, and is also present in the other yeast MAPKK, Mkk2p (Irie *et al*., 1993). The possibility that the proline-rich regions in the MAPKKs may play a role in activation of the kinases by interacting with SH3 domain-containing proteins has been pointed out (Maeda *et al*., 1995) and, by extension of this argument, the N-terminal extension of $PHOA^{M1}$ might be important for interacting with other proteins. Interestingly, a similar sequence is also found in the transcriptional activator NUC-1 that controls the transcription of genes encoding the phosphorus acquisition enzymes in *N.crassa* (Kang and Metzenberg, 1990), in a region that is not required for activation of phosphatase gene expression (Kang, 1993). Both the $PHOA^{M1}$ and NUC-1 sequences contain an SP dipeptide, a potential phosphorylation site for Pho85-related kinases (O'Neill *et al*., 1996), at similar positions. Thus, another possibility is that the extension could be involved in autoregulation of $PHOA^{M1}$ activity. However, we were unable to determine any specific roles for the two different forms of PHOA kinase during these studies.

Our finding that the CDK family member PHOA is required to modulate the differentiation program of *A.nidulans* in response to environmental conditions will enable us to study the role of this important class of

Table II. The *A.nidulans* strains used in this study

Strain	Genotype	Source or cross
GR5	*pyrG89; wA3; pyroA4*	N.R.Morris
R153	*wA3; pyroA4*	FGSC[a]
PHOΔ17	*pyrG89; wA3 phoA1*[b]*; pyroA4*	this study
A122	*riboA1 yA2; nicB8*	FGSC[a]
SO69	*riboA1 yA2; nimX1; nicB8*	this study
SO72	*pyrG89; wA3 nimX3*	this study
A248	*biA1; palcA1*	FGSC[c]
DJX[c]	*pabaA1; palcA40*	H.N.Arst
TTA127.4	*pabaA1 yA2; fluG::trpC;trpC801*	Lee and Adams (1994)
HB9	*pyrG89; phoA1; pyroA4*	PHOΔ17×HB31
HB10	*pyrG89 yA2; phoA1; pyroA4*	PHOΔ17×A122
HB23	*pyrG89 riboA1 yA2*	PHOΔ17×SO69
HB25	*pyrG89; wA3 phoA1 nimX3; pyroA4*	PHOΔ17×SO72
HB26	*yA2; pyroA4*	R153×HB23
HB27	*wA3 nimX3; pyroA4*	R153×SO72
HB28	*pyrG89 riboA1 yA2; phoA1*	PHOΔ17×HB23
HB30[d]	*pyrG89; wA3 palcA1; pyroA4*	PHOΔ17×HB31
HB31	*pyrG89 yA2; palcA1*	A248×HB23
HB32[d]	*pyrG89; wA3 phoA1 palcA1; pyroA4*	PHOΔ17×HB31
HB34	*pyrG89; phoA1 palcA1; pyroA4*	PHOΔ17×HB31
HB35	*pyrG89 yA2; palcA1; pyroA4*	PHOΔ17×HB31
HB37	*pyrG89; palcA1; pyroA4*	PHOΔ17×HB31
HB38	*pyrG89; pyroA4*	PHOΔ17×HB31

[a]Fungal Genetics Stock Center.
[b]The *phoA1* allele consists of a replacement of the major part of the gene with the functional *pyrG* gene.
[c]May contain *palD8*.
[d]May contain *yA2*.

kinase in one of the most fundamental and interesting aspects of biology, controlled differentiation of specific cell types. Limited studies in higher eukaryotes have implicated other CDK family members in differentiation (Nikolic *et al*., 1996; Ohshima *et al*., 1996; Philpott *et al*., 1997). Clearly the potential cyclin partners and inhibitors of the PHOA CDK family member will be of interest and should be readily identifiable. Further study of PHOA, and its interacting partners, should enhance our understanding of the cyclin-dependent class of protein kinase and their role in differentiation within multicellular eukaryotes.

Materials and methods

Aspergillus nidulans strains and culture conditions

The *A.nidulans* strains used in this study are listed in Table II and all contain the *veA1* marker. Sexual crosses were performed and minimal medium was as described (Pontecorvo, 1953) but was buffered at pH 8.0 with 20 mM Tris–HCl or at pH 5.0 or 6.0 with 20 mM trisodium citrate, when appropriate, and for media with reduced phosphate, KH_2PO_4 was substituted with KCl. In sulfur-limited media, $MgSO_4$ was replaced by an equimolar amount of $MgCl_2$, and for media with reduced nitrate, $NaNO_3$ was substituted with NaCl. Trace elements based on Vishniac and Santer (1957) as a 5000-fold concentrate consisted of 10 g/l EDTA, 4.4 g/l $ZnSO_4{\cdot}7H_2O$, 1.01 g/l $MnCl_2{\cdot}4H_2O$, 0.32 g/l $CoCl_2{\cdot}6H_2O$, 0.315 g/l $CuSO_4{\cdot}5H_2O$, 0.22 g/l $(NH_4)_6Mo_7O_{24}{\cdot}H_2O$, 1.47 g/l $CaCl_2{\cdot}2H_2O$ and 1.0 g/l $FeSO_4{\cdot}7H_2O$ as modified. MAG medium consisted of 2% malt extract, 0.2% peptone, 1% glucose and trace elements, pH 6.5. P_i-depleted MAG medium was prepared by dissolving malt extract and peptone in half the final volume, followed by alkaline Mg^{2+} precipitation of phosphates (Rubin, 1973) using 10 ml of a 29.5% ammonium hydroxide solution and 10 mmol $MgSO_4$/l medium (final volume), and filtering through Whatman no.1 paper. The filtrate was neutralized, other components (20 mM trisodium citrate, 1% glucose, trace elements, KH_2PO_4 as appropriate) added and the pH adjusted to pH 6.0. The functional *pyrG* gene in *phoA1* strains does not fully restore uridine-independent growth and therefore 10 mM each of uridine and uracil were included in most media, and other supplements as required. Phenotypic characterization was at 37°C using Petri dishes (9 cm) containing 45 ml of growth medium solidified with 1.5% agar (Sigma). Sexual differentiation in transformants obtained with pPAP plamids was scored on small Petri dishes (5.3 cm) containing 20 ml of MAG medium. Confluent plate cultures were made by plating conidia that had been washed three times in 0.85% NaCl/0.02% Tween-20 solution, using 4.0 ml of medium containing 0.6% agar. The number of conidia formed in these cultures were counted by excising the top layer marked with the end of a disposable 1 ml pipet tip (diameter 1.0 cm), placing it in 0.5 ml of saline/Tween solution followed by high-speed vortexing three times for 0.5 min, and counting of appropriate dilutions in a hemacytometer. Counts are based on at least three different plates.

Table III. Oligonucleotides used in this study[a]

Name	Sequence[b,c]	Position[d]
ERK1(F)	CGGGATCCTNGGNSARGGNRCNTAYG	1089–1106(F)
ERK3(R)	CGGAATTCTCNGGNSNNCKRTACCA	1641–1657(R)
ERK4(R)	CGGAATTCCKNGCNARNCCRAARTC	1575–1591(R)
IP1	AGACCCGTGCTCTACTT	770–786(F)
PAU2	ACCGGTACCTTAACGCTCATCACACCAACC	925–945(F)
PAU3	ACAGGTACCGAGGAATTGACCACC	816–831(F)
PF	CTAGGATCCGATAAGTCGCAACAGCCCA	984–1002(F)
PHO2	GTGAATCACATCGTAGAGTG	1333–1352(R)
PHO11[e]	TGAGCTGCCGTAACCAGT	
PHO13	CACCTCTCTAGCCACATACTC	382–402(F)
PR	TATGAATTCCCATACGCTTGTTGCGGTG	2068–2084(R)
RACE1	GGCTTCAGATCTCGGTGTAGGAC	1509–1531(R)

[a]Sequencing primers are not included.
[b]5′ extensions not complementary to *phoA* sequence are underlined.
[c]R = A,G; Y = C,T; K = G,T; S = C,G; N = A,C,G,T.
[d]Positions refer to Figure 1.
[e]Starts 36 bases upstream of the sequence in Figure 1.

PCR-generated probes for protein kinase genes

A first round of PCR was performed using *Taq* DNA polymerase and the buffer recommended by the supplier (Perkin Elmer), genomic *A.nidulans* R153 DNA as template and 200 pmol each of the degenerate primers ERK1(F) and ERK3(R) (Table III). The hot-start technique was applied (AmpliWax, Perkin Elmer) and cycling was preceded by incubating at 94°C for 5 min. PCR amplification was with three cycles of 1 min at 94°C, 1 min at 46°C and 1 min at 72°C, followed by 30 cycles at higher stringency (1 min at 94°C, 1 min at 55°C, 1 min at 72°C) with a final extension at 72°C for 10 min. The reaction mixture was diluted 1000-fold in sterile water and 5 μl was used for amplification employing primers ERK1(F) and ERK4(R) under the same conditions as before. The products were separated on a 1.5% agarose gel, individual bands were cut out and eluted in water, re-amplified in 20 cycles of PCR, isolated from a 0.8% low-melting agarose gel and then cloned in pT7Blue T-Vector (Novagen). Clones that carried sequence of the *crkA* gene (M.Mischke and N.R.Morris, personal communication) were identified by hybridization to a probe that had been amplified from a *crkA* cDNA using primers ERK1(F) and ERK4(R). Additional PCR reactions were performed to ensure that the clones obtained represented all the PCR products initially isolated, and a number of clones were then sequenced. To ascertain that the probes obtained originated from *A.nidulans* DNA, they were used, successfully, to identify strongly hybridizing phages in an *A.nidulans* genomic library (Holt and May, 1993).

Plasmids

Two plasmids, pPHO3 and pPHO5, that together comprise the complete *phoA* gene and its flanking regions were obtained by screening an *A.nidulans* genomic library in the vector λpAn (Holt and May, 1993) using the *phoA*-specific PCR fragment as a probe and by subsequent excision of these plasmids from their parent phages in *E.coli* JM107(λKC) (Elledge *et al*., 1991). pPHO3 contains sequence upstream of position 1833 (Figure 1) and pPHO5 contains sequence downstream of position 1029. The inserts of these plasmids were shown to be co-linear with genomic *A.nidulans* DNA by Southern blotting experiments. Fragments of pPHO3 and pPHO5 were inserted in M13 vectors and sequenced using gene-specific primers and Sequenase (USB).

Plasmid pPHO/G was constructed to obtain a fragment for use in the

deletion of the chromosomal copy of the *phoA* gene. The 2.7 kb *Hin*dIII–*Dra*I fragment derived from pPHO3 was fused to the 1.46 kb *Sca*I–*Bam*HI fragment of pPYRG (Oakley *et al*., 1987) by ligating these fragments into *Hin*dIII- and *Bam*HI-digested pBluescript II SK+ (Stratagene), and the fusion product was recovered as a 4.2 kb *Cla*I–*Spe*I fragment (fragment A). An ~4.5 kb *Eco*RV–*Hin*dIII fragment of pPHO5 was ligated into *Hin*cII- and *Hin*dIII-digested pUC18 and most of it was recovered as a 3.5 kb *Xba*I–*Bgl*II fragment (fragment B). Fragments A and B were ligated into *Cla*I- and *Bam*HI-digested pBluescript II SK+, resulting in plasmid pPHO/G. The 6.8 kb *Eco*RV–*Nsi*I fragment isolated from this plasmid essentially consists of, in this order: (i) 2.5 kb of upstream sequence of the *phoA* gene, from an upstream *Eco*RV site to the *Dra*I site at position 617; (ii) the functional *A.nidulans pyrG* gene; and (iii) 2.8 kb of *phoA* sequence downstream of the *Eco*RV site at position 1697.

The origin of the components of the pUC19-based pPAP series of plasmids is described, rather than giving the precise details of their construction. The *A.nidulans pyroA* gene on a 2.7 kb *Bam*HI–*Pst*I fragment isolated from plasmid p14 (a gift from Dr G.May) was incorporated for use as a selectable marker in *A.nidulans* transformations. The promoter of the alcohol dehydrogenase-encoding *alcA* gene on a 0.4 kb *Eco*RI–*Kpn*I fragment originated from plasmid pPAL3 (Waring *et al*., 1989). The larger part of *phoA* sequence in these constructs is from a 1.8 kb *Nde*I–*Bam*HI restriction fragment of pPHO5. Sequences from the *Kpn*I site flanking the *alcA* promoter to the *Nde*I site in the *phoA* structural gene were PCR-amplified in 13 cycles from a subclone of pPHO3. Two versions of the plasmid that direct the synthesis of PHOA from different translation initiation codons were obtained by using two different forward primers that both had an 5′ extension with a *Kpn*I site that was used for fusion of the fragments to the *alcA* promoter; oligonucleotides PAU2 and PAU3 (Table III) were used for the construction of pPAP(M47) and pPAP(M1), respectively. A plasmid having a frameshift mutation in the *phoA* structural gene, pPAPnc, was made by digesting pPAP(M47) with *Csp*45I, filling in the sticky ends with Klenow fragment and recircularizing with T4 DNA ligase.

PHOA-coding sequence was PCR-amplified from a cDNA library (Osmani *et al*., 1988) using oligos PF and PR. The *Bam*HI- and *Eco*RI-digested fragment was ligated into pET21a vector (Novagen) digested with the same enzymes to give rise to pET21a-PHO. The absence of introns was verified by sequencing.

Transformation of A.nidulans

Aspergillus nidulans was transformed essentially as described (Osmani *et al*., 1987) except that selection for pyridoxine prototrophy was on minimal medium containing 0.6 M KCl as osmotic stabilizer. To select *phoA* deletion strains, *A.nidulans* GR5 was transformed to uridine prototrophy using the 6.8 kb *Eco*RV–*Nsi*I fragment of pPHO/G; genomic DNA was isolated from transformants that had been streaked repeatedly to single colony, digested with *Eco*RI and *Eco*RV and analyzed by Southern blotting. Hybridization was first with a radiolabeled fragment internal to the deletion to identify deletion strains on the basis of absence of a hybridization signal, and the blot was then reprobed with radiolabeled plasmid pPHO/G to check expected shifts of bands when compared with the wild-type pattern and the absence of other bands. Three independent strains showing a 'clean' deletion of the *phoA* gene were identified.

Phosphatase assays

Acid phosphatase activity was assayed as described (Caddick and Arst, 1986) using 0.1 M sodium maleate buffer at pH 6.0 containing 2 mM EDTA. Detection of phosphatase activities in colonies followed the methods of Caddick and Arst (1986). To test for extracellular phosphatase activities on solid media, colonies were grown at pH 5 when staining for acid phosphatase and phosphodiesterase and at pH 8 when staining for alkaline phosphatase.

Western blot analysis

His-tagged PHOA expressed from plasmid pET21a-PHO in *E.coli* BL21(DE3)pLysS was induced, purified and used to raise antisera in rabbits essentially as described (Ye *et al*., 1995). One rabbit was selected, administered another booster injection 4 months after the previous injection using ~50 µg of SDS–PAGE gel-purified PHOA, and serum E70(3) was collected 2 weeks later. *Aspergillus* mycelia were harvested and cell-free extracts prepared as described previously (Ye *et al*., 1995). Proteins (100 µg/lane) were separated by SDS–PAGE (10% gels) and electroblotted onto nitrocellulose. Blots were blocked overnight in 5% bovine serum albumin (BSA) in TBST (10 mM Tris–HCl at pH 7.5, 150 mM NaCl, 0.05% Tween-20) containing 0.02% sodium azide and then incubated for 3 h with E70(3) antiserum diluted 1:500 in BSA/TBST. Blots were washed at least three times in TBST and then incubated for 1 h with a 1:30 000 dilution of donkey anti-rabbit secondary antibody conjugated with horseradish peroxidase (Amersham) in TBST with 3% non-fat dry milk. After five washes in TBST, blots were developed using an enhanced chemiluminescence (ECL) detection system (Amersham) according to the manufacturer's recommendations. Pre-stained molecular weight markers (Sigma) and biotinylated markers (Amersham) in combination with ECL detection were used.

cDNA procedures

Total RNA was isolated from *A.nidulans* R153 mycelium grown in liquid minimal medium for 16 h using Ultraspec RNA reagent as recommended by the manufacturer (Biotecx Laboratories), and poly(A)$^+$ mRNA was purified further using the PolyATtract mRNA Isolation System (Promega). One µg of poly(A)$^+$ RNA was used to obtain a library of adaptor-ligated ds cDNA or RACE1-primed first-strand cDNA, using the Marathon cDNA Amplification Kit as specified by the manufacturer (Clontech Labaratories). For 3′-RACE, amplification was with oligos PHO11 and AP1, and reamplification with oligos PHO13 and AP2. RACE1-primed cDNA was amplified using primers IP1 and PHO2. Fragments were cloned in pT7Blue T-vector prior to sequencing.

Nucleotide sequence accession number

The nucleotide sequence of *phoA* has been assigned DDBJ/EMBL/GenBank accession No. U59215.

Acknowledgements

We thank our colleagues for their help, Dr G.May for generously providing us with the *A.nidulans* genomic DNA library and the *pyroA* gene, Drs H.Arst and T.Adams for strains and discussions, and Drs M.Mischke and N.R.Morris for the *crkA* probe. This work was supported by a grant (GM42564) from the NIH.

References

Adams,T.H. (1994) Asexual sporulation in higher fungi. In Gow,N.A. and Gadd,G.M. (eds), *The Growing Fungus*. Chapman & Hall, London, UK, pp. 367–382.

Arst,H.N. (1994) Regulation of gene expression by oxygen, phosphorus and pH. In Martinelli,S.D. and Kinghorn,J.R. (eds), *Aspergillus: 50 Years On.* Elsevier, London, UK, pp. 369–380.

Beach,D., Durkacz,B. and Nurse,P. (1982) Functional homologous cell cycle control genes in budding and fission yeast. *Nature*, **300**, 705–709.

Boulton,T.G., Nye,S.H., Robbins,D.J., Lp,N.Y., Radziejewska,E., Depinho,R.A., Panayotatos,N., Cobb,M.H. and Yancopoulos,G.D. (1991) ERKs: a family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. *Cell*, **65**, 663–675.

Brachmann,C., Sherman,J.M., Devine,S.E., Cameron,E.E., Pillus,L. and Boeke,J.D. (1995) The *SIR2* gene family, conserved from bacteria to human, functions in silencing, cell cycle progression and chromosome stability. *Genes Dev.*, **9**, 2888–2902.

Caddick,M.X. and Arst,H.N. (1986) Structural genes for phosphatases in *Aspergillus nidulans*. *Genet. Res. Camb.*, **47**, 83–91.

Caddick,M.X., Brownlee,A.G. and Arst,H.N. (1986) Phosphatase regulation in *Aspergillus nidulans*: responses to nutritional starvation. *Genet. Res. Camb.*, **47**, 93–102.

Champe,S.P. and Simon,L.D. (1992) Cellular differentiation and tissue formation in fungus *Aspergillus nidulans.* In Rossomando,E.F. and Alexander,S. (eds), *Morphogenesis: An Analysis of the Development of Biological Structure*. Marcel Dekker, Inc., New York, NY, pp. 63–91.

Champe,S.P., Rao,P. and Chang,A. (1987) An endogenous inducer of sexual development in *Aspergillus nidulans*. *J. Gen. Microbiol.*, **133**, 1383–1387.

Champe,S.P., Nagle,D.L. and Yager,L.N. (1994) Sexual sporulation. In Martinelli,S.D. and Kinghorn,J.R. (eds), *Aspergillus: 50 Years On.* Elsevier, London, UK, pp. 429–454.

Cismowski,M.J., Laff,G.M., Solomon,M.J. and Reed,S.I. (1995) *KIN28* encodes a C-terminal domain kinase that controls mRNA transcription in *Saccharomyces cerevisiae* but lacks cyclin-dependent kinase-activating kinase (CAK) activity. *Mol. Cell. Biol.*, **15**, 2983–2992.

Clutterbuck,A.J. (1993) *Aspergillus nidulans*. In O'Brien,S.J. (ed.), *Genetic Maps: Locus Maps of Complex Genes*. Cold Spring Harbor Press, Cold Spring Harbor, NY, pp. 371–384.

Elledge,S.J., Mulligan,J.T., Ramer,S.W., Spottswood,M. and Davis,R.W. (1991) λYES: a multifunctional cDNA expression vector for the isolation of genes by complementation of yeast and *Escherichia coli* mutations. *Proc. Natl Acad. Sci. USA*, **88**, 1731–1735.

Espinoza,F.H., Ogas,J., Herskowitz,I. and Morgan,D.O. (1994) Cell cycle control by a complex of the cyclin HCS26 (PCL1) and kinase PHO85. *Science*, **266**, 1388–1391.

Gilliquet,V. and Berben,G. (1993) Positive and negative regulators of the *Saccharomyces cerevisiae* 'PHO system' participate in several cell functions. *FEMS Microbiol. Lett.*, **108**, 333–340.

Gurr,S.J., Unkles,S.E. and Kinghorn,J.R. (1987) The structure and organization of nuclear genes of filamentous fungi. In Kinghorn,J.R. (ed.), *Gene Structure in Eukaryotic Microbes*. IRL Press, Oxford, UK, pp. 93–139.

Hanks,S.K., Quinn,A.M. and Hunter,T. (1988) The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. *Science*, **241**, 42–52.

Hermann,T.E., Kurtz,M.B. and Champe,S.P. (1983) Laccase localized in hulle cells and cleistothecial primordia of *Aspergillus nidulans*. *J. Bacteriol.*, **154**, 955–964.

Hirst,K., Fisher,F., McAndrew,P.C. and Goding,C.R. (1994) The transcription factor, the Cdk, its cyclin and their regulator: directing the transcriptional response to a nutritional signal. *EMBO J.*, **13**, 5410–5420.

Holt,C.L. and May,G.S. (1993) A novel phage lambda replacement Cre-*lox* vector that has automatic subcloning capabilities. *Gene*, **133**, 95–97.

Huang,D., Farkas,I. and Roach,P.J. (1996) Pho85p, a cyclin-dependent protein kinase and the Snf1p protein kinase act antagonistically to control glycogen accumulation in *Saccharomyces cerevisiae*. *Mol. Cell Biol.*, **16**, 4357–4365.

Irie,K., Takase,M., Lee,K.S., Levin,D.E., Araki,H., Matsumoto,K. and Oshima,Y. (1993) Mkk1 and Mkk2, which encode *Saccharomyces cerevisiae* mitogen-activated protein kinase-kinase homologs, function in the pathway mediated by protein kinase C. *Mol. Cell. Biol.*, **13**, 3076–3083.

Jeffrey,P.D., Russo,A.A., Polyak,K., Gibbs,E., Hurwitz,J., Massage,J. and Pavletich,N.P. (1995) Mechanism of CDK activation revealed by the structure of a cyclinA–CDK2 complex. *Nature*, **376**, 313–320.

Kaffman,A., Hershowitz,I., Tjian,R. and O'Shea,E.K. (1994) Phosphorylation of the transcription factor PHO4 by a cyclin–CDK complex, PHO80–PHO85. *Science*, **263**, 1153–1156.

Kang,S. (1993) Functional domains of the transcriptional activator NUC-1 in *Neurospora crassa*. *Gene*, **130**, 259–264.

Kang,S. and Metzenberg,R.L. (1990) Molecular analysis of *nuc-1*$^{+}$, a gene controlling phosphorus acquisition in *Neurospora crassa*. *Mol. Cell. Biol.*, **10**, 5839–5848.

Lee,B. and Adams,T.H. (1994) The *Aspergillus nidulans fluG* gene is required for production of an extracellular developmental signal and is related to prokaryotic glutamine synthetase I. *Genes Dev.*, **8**, 641–651.

Lenburg,M.E. and O'Shea,E.K. (1996) Signaling phosphate starvation. *Trends Biochem. Sci.*, **21**, 383–387.

Maeda,T., Takekawa,M. and Saito,H. (1995) Activation of yeast PBS2 MAPKK by MAPKKKs or by binding of an SH3-containing osmosensor. *Science*, **269**, 554–558.

Measday,V., Moore,L., Ogas,J., Tyers,M. and Andrews,B. (1994) The PCL2 (ORFD)–PHO85 cyclin-dependent kinase complex: a cell cycle regulator in yeast. *Science*, **266**, 1391–1395.

Measday,V.K., Moore,L., Retnakaran,R., Lee,J., Donoviel,M., Neiman,A.M. and Andrews,B. (1997) A family of cyclin-like proteins that interact with the Pho85 cyclin-dependent kinase. *Mol. Cell Biol.*, **17**, 1212–1223.

Meyerson,M., Enders,G.H., Wu,C., Su,L., Gorka,C., Nelson,C., Harlow,E. and Tsai,L. (1992) A family of human cdc2-related protein kinases. *EMBO J.*, **11**, 2909–2917.

Michaelis,C. and Weeks,G. (1993) The isolation from a unicellular organism, *Dictyostelium discoideum*, of a highly-related *cdc2* gene with characteristics of the PCTAIRE subfamily. *Biochim. Biophys. Acta*, **1179**, 117–124.

Nasmyth,K. (1993) Control of the yeast cell cycle by the Cdc28 protein kinase. *Curr. Opin. Cell Biol.*, **5**, 166–179.

Nasmyth,K.A. and Reed,S.I. (1980) Isolation of genes by complementation in yeast: molecular cloning of a cell-cycle gene. *Proc. Natl Acad. Sci. USA*, **77**, 2119–2123.

Nicolson,T.A., Weisman,L.S., Payne,G.S. and Wickner,W.T. (1995) A truncated form of the Pho80 cyclin redirects the Pho85 kinase to disrupt vacuole inheritance in *S.cerevisiae*. *J. Cell Biol.*, **130**, 835–845.

Nikolic,M., Dudek,H., Kwon,Y.T., Ramos,Y. and Tsai,L. (1996) The cdk5/p35 kinase is essential for neurite outgrowth during neuronal differentiation. *Genes Dev.*, **10**, 816–825.

Nurse,P. (1990) Universal control mechanism regulating onset of M-phase. *Nature*, **344**, 503–508.

O'Neill,E.M., Kaffman,A., Jolly,E.R. and O'Shea,E.K. (1996) Regulation of PHO4 nuclear localization by the PHO80–PHO85 cyclin–CDK complex. *Science*, **271**, 209–212.

Oakley,B.R., Rinehart,J.E., Mitchell,B.L., Oakley,C.E., Carmona,C., Gray,G.L. and May,G.S. (1987) Cloning, mapping and molecular analysis of the pyrG (orotidine-5′-phosphate decarboxylase) gene of *Aspergillus nidulans*. *Gene*, **61**, 385–399.

Ohshima,T., Ward,J.M., Huh,C.G., Longenecker,G., Veeranna, Pant,H.C., Brady,R.O., Martin,L.J. and Kulkarni,A.B. (1996) Targeted disruption of the cyclin-dependent kinase 5 gene results in abnormal corticogenesis, neuronal pathology and perinatal death. *Proc. Natl Acad. Sci. USA*, **93**, 11173–11178.

Osmani,A.H., van Peij,N., Mischke,M., O'Connell,M.J. and Osmani,S.A. (1994) A single p34^{cdc2} protein kinase (nimX^{cdc2}) is required at G1 and G2 in *Aspergillus nidulans*. *J. Cell Sci.*, **107**, 1519–1528.

Osmani,S.A., May,G.S. and Morris,N.R. (1987) Regulation of the mRNA levels of *nimA*, a gene required for the G2–M transition in *Aspergillus nidulans*. *J. Cell Biol.*, **104**, 1495–1504.

Osmani,S.A., Engle,D.B., Doonan,J.H. and Morris,N.R. (1988) Spindle formation and chromatin condensation in cells blocked at interphase by mutation of a negative cell cycle control gene. *Cell*, **52**, 241–251.

Paris,J., Guellec,R.L., Couturier,A., Guellec,K.L., Omilli,F., Camonis,J., MacNeill,S. and Philippe,M. (1991) Cloning by differential screening of a *Xenopus* cDNA coding for a protein highly homologous to cdc2. *Proc. Natl Acad. Sci. USA*, **88**, 1039–1043.

Peeper,D.S., Parker,L.L., Ewen,M.E., Toebes,M., Hall,F.L., Xu,M., Zantema,A., van der Eb,A.J. and Piwnica-Worms,H. (1993) A- and B-type cyclins differentially modulate substrate specificity of cyclin–cdk complexes. *EMBO J.*, **12**, 1947–1954.

Peleg,Y., Addison,R., Aramayo,R. and Metzenberg,R.L. (1996a) Translocation of *Neurospora crassa* transcription factor NUC-1 into the nucleus is induced by phosphorus limitation. *Fungal Genet. Biol.*, **20**, 186–191.

Peleg,Y., Aramayo,R., Kang,S., Hall,J.G. and Metzenberg,R.L. (1996b) NUC-2, a component of the phosphate-regulated signal transduction pathway in *Neurospora crassa*, is an ankyrin repeat protein. *Mol. Gen. Genet.*, **252**, 709–716.

Philpott,A., Porro,E.B., Kirschner,M.W. and Tsai,L.H. (1997) The role of cyclin-dependent kinase 5 and a novel regulatory subunit in regulating muscle differentiation and patterning. *Genes Dev.*, **11**, 1409–1421.

Pines,J. and Hunter,T. (1991) Cyclin-dependent kinases: a new cell cycle motif? *Trends Cell Biol.*, **1**, 117–121.

Pontecorvo,G. (1953) The genetics of *Aspergillus nidulans*. In Demerec,M. (ed.), *Advances in Genetics*. Academic Press, New York, NY, pp. 141–238.

Qu,Q., Huang,Q., Lee,K., Lew,J. and Wang,J.H. (1995) Reconstitution of neuronal Cdc2-like kinase from bacteria-expressed Cdk5 and an active fragment of the brain-specific activator. *J. Biol. Chem.*, **270**, 10847–10854.

Rubin,G.M. (1973) The nucleotide sequence of *Saccharomyces cerevisiae* 5.8 S ribosomal ribonucleic acid. *J. Biol. Chem.* **248**, 3860–3875.

Santos,R.C., Waters,N.C., Creasy,C.L. and Bergman,L.W. (1995) Structure–function relationships of the yeast cyclin-dependent kinase Pho85. *Mol. Cell. Biol.*, **15**, 5482–5491.

Schneider,K.R., Smith,R.L. and O'Shea,E.K. (1994) Phosphate-regulated inactivation of the kinase PHO80–PHO85 by the CDK inhibitor PHO81. *Science*, **266**, 122–126.

Simon,M., Seraphin,S. and Faye,G. (1986) *KIN28*, a yeast split gene coding for a putative protein kinase homologous to *CDC28*. *EMBO J.*, **5**, 2697–2701.

Som,T. and Kolaparthi,V. (1994) Developmental decisions in *Aspergillus nidulans* are modulated by ras activity. *Mol. Cell. Biol.* **14**, 5333–5348.

Tilburn,J., Sarkar,S., Widdick,D.A., Espeso,E.A., Orejas,M., Mungroo,J., Penalva,M.A. and Arst,H.N. (1995) The *Aspergillus* PacC zinc finger transcription factor mediates regulation of both acid- and alkaline-expressed genes by ambient pH. *EMBO J.*, **14**, 779–790.

Timberlake,W.E. (1990) Molecular genetics of *Aspergillus* development. *Annu. Rev. Genet.*, **24**, 5–36.

Timblin,B.K. and Bergman,L.W. (1997) Elevated expression of stress response genes resulting from deletion of the *PHO85* gene. *Mol. Microbiol.*, **26**, 981–990.

Timblin,B.K., Tatchell,K. and Bergman,L.W. (1996) Deletion of the gene encoding the cyclin-dependent protein kinase Pho85 alters glycogen metabolism in *Saccharomyces cerevisiae*. *Genetics*, **143**, 57–66.

Toh-e,A., Tanaka,K., Uesono,Y. and Wickner,R.B. (1988) PHO85, a negative regulator of the PHO system, is a homolog of the protein kinase gene, CDC28, of *Saccharomyces cerevisiae*. *Mol. Gen. Genet*., **214**, 162–164.

Uesono,Y., Tanaka,K. and Toh-e,A. (1987) Negative regulators of the PHO system in *Saccharomyces cerevisiae*: isolation and structural characterization of PH085. *Nucleic Acids Res*., **15**, 10299–10309.

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

Waring,R.B., May,G.S. and Morris,N.R. (1989) Characterization of an inducible expression system in *Aspergillus nidulans* using *alcA* and tubulin-coding genes. *Gene*, **79**, 119–130.

Ye,X.S., Xu,G., Pu,P.T., Fincher,R.R., McGuire,S.L., Osmani,A.H. and Osmani,S.A. (1995) The NIMA protein kinase is hyperphosphorylated and activated downstream of $p34^{cdc2}$/cyclin B: coordination of two mitosis promoting kinases. *EMBO J*., **14**, 986–994.

Received February 13, 1998; revised and accepted May 29, 1998