Protein Kinase C (PkcA) of *Aspergillus nidulans* Is Involved in Penicillin Production

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The biosynthesis of the β -lactam antibiotic penicillin in the filamentous fungus *Aspergillus nidulans* is catalyzed by three enzymes that are encoded by the *acvA*, *ipnA*, and *aataA* genes. A variety of *cis*-acting DNA elements and regulatory factors form a complex regulatory network controlling these β -lactam biosynthesis genes. Regulators involved include the CCAAT-binding complex AnCF and AnBH1. AnBH1 acts as a repressor of the penicillin biosynthesis gene *aataA*. Until now, however, little information has been available on the signal transduction cascades leading to the transcription factors. Here we show that inhibition of protein kinase C (Pkc) activity in *A. nidulans* led to cytoplasmic localization of an AnBH1-enhanced green fluorescent protein (EGFP) fusion protein. Computer analysis of the genome and screening of an *A. nidulans* gene library revealed that the fungus possesses two putative Pkc-encoding genes, which we designated *pkcA* and *pkcB*. Only PkcA showed all the characteristic features of fungal Pkc's. Production of *pkcA* antisense RNA in *A. nidulans* led to reduced growth and conidiation in *Aspergillus* minimal medium, while in fermentation medium it led to enhanced expression of an *aataA-lacZ* gene fusion, reduced penicillin production, and predominantly cytoplasmic localization of AnBH1. These data agree with the finding that inhibition of Pkc activity prevented nuclear localization of AnBH1-EGFP. As a result, repression of *aataA* expression was relieved. The involvement of Pkc in penicillin biosynthesis is also interesting in light of the fact that in the yeast *Saccharomyces cerevisiae*, Pkc plays a major role in maintaining cell integrity.

Penicillins and cephalosporins belong chemically to the group of β -lactam antibiotics. The biosyntheses of both penicillins and cephalosporins have the first two steps in common. They are synthesized from the same three amino acids, L- α -amino adipic acid (L- α -AAA), L-cysteine, and L-valine (reviewed in reference 9). In the first reaction of the cephalosporin and penicillin biosynthesis pathway, the amino acid precursors are condensed to the tripeptide δ -(L- α -amino adipyl)-L-cysteiny-L-D-valine (ACV). This reaction is catalyzed by a single enzyme, ACV synthetase. ACV synthetase is encoded by a single structural gene designated *acvA* (*pcbAB*). In the second step, oxidative ring closure of the linear tripeptide leads to formation of a bicyclic ring, i.e., the four-membered β -lactam ring fused to the five-membered thiazolidine ring which is characteristic of all penicillins. This reaction is catalyzed by isopenicillin N synthase (IPN synthase), encoded by the *ipnA* (*pcbC*) gene. IPN is the branch point of penicillin and cephalosporin biosyntheses. In the third and final step of penicillin biosynthesis, the hydrophilic L- α -AAA side chain of IPN is exchanged for a hydrophobic acyl group catalyzed by acyl coenzyme A (acyl-CoA):isopenicillin N acyltransferase (IAT). The corresponding gene was designated *aataA* (*penDE*) (reviewed in references 10 and 13). It is generally believed that the activated forms of the side chains consist of their CoA-thioesters, but the mechanism behind this activation is still not fully elucidated (19). The formation of hydrophobic penicillins has been reported in

fungi only, notably *Penicillium chrysogenum* and *Aspergillus nidulans*, whereas the hydrophilic cephalosporins are produced by both fungi and bacteria, e.g., *Acremonium chrysogenum* and *Streptomyces clavuligerus*, respectively (reviewed in reference 13).

Within the last few years, several studies have indicated that the β -lactam biosynthesis genes of fungi are controlled by a complex regulatory network, and a comparison with known regulators and DNA elements involved in the regulation of genes of primary metabolism is of interest. A variety of *cis*-acting DNA elements and regulatory factors are involved. In *A. nidulans*, among others, these regulators include AnCF and AnBH1. AnCF was shown to bind to a CCAAT box (I) in the intergenic region between the *acvA* and *ipnA* genes and to a CCAAT box (II) present in the promoter of the *A. nidulans* *aataA* gene. AnCF consists of the subunits HapB, HapC, and HapE (27, 45). AnBH1 belongs to the family of basic-region helix-loop-helix (bHLH) transcription factors. AnBH1 binds in vitro as a homodimer to an asymmetric E-box (not previously described) within the *aataA* promoter which overlaps with the AnCF binding site (CCAAT box II). Since deletion of *anbH1* appeared to be lethal, the *anbH1* gene was replaced by a regulatable *alcAp-anbH1* gene fusion. Analysis of *aataA-lacZ* expression in such a strain indicated that AnBH1 acts as a repressor of *aataA* gene expression and therefore counteracts the positive action of AnCF (15). Until now, however, little information has been available on the signal transduction cascades leading to the transcription factors. Based on the amino acid sequence of AnBH1 and because all bHLH transcription factors were shown to be phosphorylated (25), it was likely that AnBH1 was phosphorylated. Therefore, analysis of phosphorylation and putative kinases involved might give hints on the

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TABLE 1. Fungal strains, bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype and/or phenotype	Source or reference
Strains		
<i>Aspergillus nidulans</i>		
R21	<i>pabaA1 yA2</i>	20
LOGOAnBH1	<i>pyrG89 biA1 fwA1 bga0 argB2::pOLAB1 (aatAp-lacZ) pRG1 pLOGO-AnBH1 (anbH1-egfp) ArgB⁺ PyrG⁺</i>	29
OLAB1URA	<i>pyrG89 biA1 fwA1 bga0 argB2::pOLAB1 (aatAp-lacZ) ArgB⁺</i>	15
AXB4A	<i>bga0 biA1 argB2::pAXB4A (acvAp-uidA ipnAp-lacZ) ArgB⁺</i>	11
HapB-eGFP	<i>pyrG89 pabaA1 fwA1 bga0 argB2::pAXB4A (acvAp-uidA ipnAp-lacZ) pHapB-GFP (hapB-egfp) ArgB⁺ PyrG⁺</i>	44
Palp32.3	<i>pyrG89 pabaA1 fwA1 bga0 argB2::pAXB4A (acvAp-uidA, ipnAp-lacZ) ΔsuAprgA1 paba1p32 ArgB⁺ PyrG⁺ PabaA⁺</i>	A. Gehrke, unpublished data
R21OLAB7	<i>pabaA1 yA2 argB2::pOLAB1 (aatAp-lacZ) ArgB⁺</i> ; progeny from a cross of strains R21 and OLAB1URA	This study
C2.3	<i>pyrG89 biA1 fwA1 bga0 argB2::pOLAB1 (aatAp-lacZ) pLOGO-AnBH1 (anbH1-egfp) ArgB⁺</i>	This study
PkcAinv1.1, -1.2, -1.3, -1.4, -1.5	<i>pyrG89 fwA1 bga0 biA1 argB2::pOLAB1 (aatAp-lacZ); pLOGO-AnBH1 (anbH1-egfp) (pAL4-pkcAinv)_n ArgB⁺ PyrG⁺</i>	This study
<i>Escherichia coli</i> TOP10F'	F' [<i>lacI^q Tn10 (Tet^r) mcrA Δ(mrr-hsdRMS-mcrBC), φ80d/lacZΔM15 ΔlacX74 recA1 deoR araD139 Δ(ara-leu)7697 galU galK rpsL (Str^r) endA1 nupG</i>]	Invitrogen, The Netherlands
<i>Bacillus calidolactis</i> C953		11
Plasmids		
pCR2.1TOPO	Amp ^r	Invitrogen, The Netherlands
pAL4	Amp ^r <i>pyr-4 alcAp</i>	52
pAL4-pkcAinv	Amp ^r <i>pyr-4 alcAp-pkcA^{antisense}</i>	This study

signal transduction cascades controlling the biosynthesis of the secondary metabolite penicillin. Here we provide evidence that the protein kinase C (Pkc) PkcA is involved directly or indirectly in the regulation of AnBH1 and penicillin biosynthesis.

MATERIALS AND METHODS

Strains and plasmids. Fungal and bacterial strains and plasmids used in this study are listed in Table 1. Vectors and plasmids were propagated in *Escherichia coli* TOP10F'. For generation of *A. nidulans* strain C2.3, strain R21 was crossed with strain LOGOAnBH1, and one of the resulting progeny was crossed with strain OLAB1URA (Table 1). Ascospores were plated on *Aspergillus* minimal medium (AMM) (see below) agar plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; 50 μg ml⁻¹) and glucose as the carbon source. Uracil auxotrophic colonies staining the agar blue, which is indicative of the presence of the *aatAp-lacZ* gene fusion, were used. One of the resulting progeny with the desired genotype was designated C2.3 (Table 1).

Media and cultivation of strains. *E. coli* was grown in LB medium supplemented with ampicillin (50 μg ml⁻¹). AMM was prepared as previously described (12). For growth under repressing conditions, glucose (0.8%, wt vol⁻¹) was used as the carbon source. For growth under inducing conditions, lactose (1.6%, wt vol⁻¹) was used as the carbon source, and 10 mM cyclopentanone was added to the medium. Another *alcAp*-inducing compound is threonine. However, threonine was not used, because it is consumed by the fungus, and therefore a constant concentration cannot be maintained. In addition, consumption of threonine changes the pH of the culture and consequently influences penicillin production (48). Fermentation experiments with *A. nidulans* were carried out in fermentation medium (FM) essentially as described previously (49). Experimental cultures were inoculated with 1 ml of freshly harvested and filtered spore suspensions (2 × 10⁸ conidia ml⁻¹) instead of using seed culture suspensions. Experimental cultures (20 ml of FM in 250-ml flasks) contained 4% (wt vol⁻¹) lactose as the carbon source and 10 mM cyclopentanone under inducing conditions and 4% (wt vol⁻¹) glucose under repressing conditions. Cultures were incubated for 72 h. If required, biotin (0.3 μg ml⁻¹) or *p*-aminobenzoic acid (15 μg ml⁻¹) was added to the medium.

Quantification of sporulation. A 50-μl volume of spore suspension containing 1 × 10⁵ conidia of freshly harvested and filtered spore suspensions was plated

onto AMM agar plates containing either glucose (0.8%, wt vol⁻¹) or lactose (1.6%, wt vol⁻¹) plus 10 mM cyclopentanone. Because of the high number of conidia, the plates were covered by mycelium. Four plates for each strain and each condition were incubated for 3 days, and the conidia produced from each plate were harvested with 10 ml saline solution containing 2% (vol/vol) Tween 80 (Merck, Germany). Spore suspensions were filtered, and the number of conidia was counted using a Thoma chamber.

Penicillin bioassay. Penicillin bioassays using "*Bacillus calidolactis*" C953 as an indicator organism were carried out as described previously (11).

Determination of dry weight. Dry weights of cultures grown in AMM or FM were determined as previously described (11). Cultures were grown under both repressing (glucose) and inducing (lactose plus 10 mM cyclopentanone) conditions. Fermentation cultures were harvested after 24 h, 48 h, and 72 h. AMM cultures were harvested after 12 h, 24 h, 36 h, 48 h, and 60 h.

Genetic techniques. Sexual crosses and characterization of the resulting progeny were performed according to Pontecorvo et al. (37).

Standard DNA techniques. For small-scale preparation of *A. nidulans* chromosomal DNA, the technique of Raeder and Broda (39) was used. Standard techniques in the manipulation of DNA were used as detailed in Sambrook et al. (40). Southern blot analysis was performed essentially as previously described (12). Chromosomal DNA of *A. nidulans* was digested with KpnI and XbaI. DNA fragments were separated on an agarose gel and blotted onto Hybond N⁺ nylon membranes (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, United Kingdom). Labeling of DNA probes with fluorescein-11-dUTP and detection of hybridized fragments by Southern blot analysis were carried out using the Gene Images random prime labeling module and CDP-Star detection reagent (both from Amersham Pharmacia Biotech, Germany), respectively, according to the manufacturer's instructions.

Generation of recombinant plasmids and probes for Southern blot analyses. PCR products were cloned using the TOPO cloning kit (Invitrogen, Karlsruhe, Germany). For construction of the *pkcA* antisense RNA plasmid, a 3.0-kb DNA fragment covering about 80% of the *pkcA* coding sequence was amplified from genomic DNA using primers PkcAinv_5 and PkcAinv_3, the latter primer encoding a KpnI site (Table 2; Fig. 3A). The PCR fragment was cloned into the pCR2.1TOPO vector (Invitrogen, Karlsruhe, Germany). After restriction digestion with KpnI and PstI, the *pkcA* fragment was cloned into the KpnI-PstI-digested pAL4 vector (52) (Table 1), which contains the *alcA* promoter of *A. nidulans* and the *pyr-4* gene of *Neurospora*

TABLE 2. Oligonucleotide list

Oligonucleotide	Sequence (5' to 3')
PkcAinv_5	CGTCGAATATGCGACAGTCAACCG
PkcAinv_3	GGGGTACCACAGAAGGTGCTTGTA
PkcAnar_5	GGCGCCTCAGCAGGCTATAGCAGC
PkcAnar_3	GGCGCCGAGGTTGAGAAGGAACGG
PkcAanti5	ATCCATACCACATGTTCTCC
PkcAanti3	ATTTTCCAGGTTCTATGCGG
PkcAanti33	ATGGAGTATATCAGTGGTGG
PkcAintr7	ATTTGGCGATACAACCTGACG
Tef1forw	TTCCCTCCAGGATGTCTACA
Tef1rev	TGCTGGTGGTGCATCTCAAC

crassa as a selectable marker. The resulting plasmid, pAL4-pkcAinv, thus contained a 2.9-kb fragment of the *pkcA* sequence in the antisense orientation under the control of the *alcA* promoter (Fig. 3A). Probes for Southern blot analysis were generated by PCR amplification of a 1.5-kb *pkcA* fragment using oligonucleotides PkcAnar_5 and PkcAnar_3 (Fig. 3A).

PCR amplification, DNA sequence analysis, and computer programs. For PCR amplification, sequence-specific oligonucleotides were synthesized (Invitrogen, Karlsruhe, Germany). PCRs were carried out using BIOTAQ Red DNA polymerase (Bioline, Luckenwalde, Germany). The DNA fragments produced were purified using the NucleoSpin purification kit (Macherey-Nagel, Dueren, Germany). The sequences of DNA fragments were determined on both strands by the dideoxy chain termination method (41) using fluorescent dyes analyzed by an Applied Biosystems ABI 310 sequencer. DNA sequence data were edited by using the "Sequence Navigator" and "Auto Assembler" programs (Applied Biosystems, Darmstadt, Germany). Similarities between different amino acid sequences were analyzed using the ClustalW program (16) (<http://www.ebi.ac.uk/clustalw>).

Preparation of RNA. Cultures of *A. nidulans* were incubated in AMM containing either glucose (repressing conditions) or lactose plus 10 mM cyclopentanone (inducing conditions) at 180 rpm and 37°C for 36 h. Mycelia were harvested and broken using liquid nitrogen as previously described (26). Total RNA was prepared using the RNeasy Plant minikit (QIAGEN, Hilden, Germany). The amount of RNA was determined both spectrophotometrically and by agarose gel electrophoresis. DNA remaining in the RNA solutions was digested with RNase-free DNase I (Roche, Mannheim, Germany). DNase I (2 µl; 10 U ml⁻¹) was added to a 20-µl reaction volume containing 10 to 20 µg RNA, 4.4 µl 25 mM MgCl₂, and 2 µl 10× TaqMan RT buffer (Applied Biosystems, Darmstadt, Germany). The reaction mixture was incubated at 37°C for 2 h, followed by heat denaturation at 95°C for 5 min. Control PCRs using primers Tef1forw and Tef1rev (Table 2) were carried out to ensure that the RNA extracts did not contain any DNA contamination.

Reverse transcription of RNA. cDNA was created using the TaqMan reverse transcription reagents (Applied Biosystems, Darmstadt, Germany). Twenty-five-microliter reaction volumes contained 2.0 to 5.0 µg RNA, 2.5 µl 10× TaqMan RT buffer, 5.5 µl 25 mM MgCl₂, 5 µl deoxynucleoside triphosphate mix, 3.0 µl of each reverse primer (1.2 pmol µl⁻¹), 0.5 µl RNase inhibitor, and 31.25 U MultiScribe reverse transcriptase. A mixture of RNA and oligonucleotides was first incubated at 70°C for 10 min and then cooled to 4°C. After addition of the other reagents listed above, the reaction mixture was incubated at 48°C for 60 min. Finally, the enzyme was heat inactivated at 95°C for 5 min. Reverse transcription of *pkcA* antisense RNA was carried out using the specific primer PkcAanti3. cDNA of *pkcA* mRNA was generated using the specific primer PkcAanti5. cDNA of *tef1* as an endogenous control was obtained using the specific primer Tef1rev (Table 2; Fig. 5A). Subsequent PCRs with cDNA as the template were carried out using oligonucleotides PkcAanti5 and PkcAanti3, or Tef1forw and Tef1rev. cDNAs obtained from both *pkcA* mRNA and *pkcA* antisense RNA were cloned (TA cloning kit; Invitrogen, Karlsruhe, Germany) and sequenced.

Transformation of *A. nidulans*. *A. nidulans* strain C2.3 was transformed to uracil prototrophy according to a method previously described (6). For transformation, plasmid DNA was purified using the E.Z.N.A. Plasmid Miniprep kit (peqlab Biotechnologie GmbH, Erlangen, Germany) according to the manufacturer's instructions.

β-Gal activity assays. β-Galactosidase (β-Gal) activities were determined in crude extracts from mycelia grown in FM as previously described (49). β-Gal activities at each time point were measured in cell extracts from three simultaneously incubated cultures. Protein concentrations were determined according to Bradford (8) using Roti-Nanoquant (Roth, Karlsruhe, Germany). Specific activities of β-Gal were calculated as previously described (11).

Nucleotide sequence accession numbers. The nucleotide sequence of *A. nidulans pkcA* and the deduced amino acid sequence were previously deposited at the NCBI GenBank (<http://www.ncbi.nlm.nih.gov>) under accession no. AB103459.1. The *pkcB* sequence was obtained by screening of an *A. nidulans* gene bank using a probe targeting the catalytic domain of protein kinase C and subsequent cloning and sequencing steps (data not shown). The sequence of the *pkcB* gene is part of the contigs ANI61C10640, ANI61C2064, ANI60C210894_1, ANI61C7279, and ANI61C9348. The nucleotide sequence of the *pkcB* mRNA and the deduced amino acid sequence were previously deposited as "Aspergillus nidulans FGSC A4 hypothetical protein" (AN5973.2; REFSEQ accession no. XM 658485). The nucleotide sequence of the *A. nidulans tef1* cDNA and the deduced amino acid sequence are accessible at the NCBI GenBank (accession no. XM 408355).

RESULTS

Addition of Pkc inhibitor to culture medium led to localization of an AnBH1-EGFP fusion in the cytoplasm. AnBH1 contains several putative phosphorylation motifs, making it likely that Pkc could be involved in the regulation of this transcription factor and consequently in penicillin biosyn-

FIG. 1. Cellular localization of AnBH1-EGFP and HapB-EGFP fusion proteins. Strains LOGOAnBH1 (containing AnBH1-EGFP) and HapB-eGFP were incubated in AMM either with or without the Pkc inhibitor calphostin C. Nuclei were stained with 4',6'-diamidino-2-phenylindole (DAPI) (panels 2). Samples were analyzed by fluorescence microscopy (panels 1, showing localization of the fusion proteins, and panels 2, showing nuclei) or light microscopy (panels 3). (A and B) AnBH1-EGFP-derived fluorescence of *A. nidulans* strain LOGOAnBH1 without calphostin C (A) and after addition of calphostin C (B). (C and D) HapB-EGFP-derived fluorescence of *A. nidulans* strain HapB-eGFP without calphostin C (C) and after addition of calphostin C (D).

FIG. 2. (A) Structures of *A. nidulans* PkcA and PkcB. Domains are based on the overall structure of fungal Pkc as given by Ambra and Macino (2). (B) Comparison of the deduced amino acid sequences of Pkc in *A. nidulans* (PkcAAn and PkcBAAn) with the Pkc sequences of *A. niger* (PkcAAng; Swiss-Prot accession no. Q00078), *Aspergillus fumigatus* (PkcAf; REFSEQ accession no. XM 748361.1), *T. reesei* (Pkc1Tr; Swiss-Prot accession no. Q99014), *N. crassa* (Pkc1Nc; SWISS-PROT accession no. P87253), and *S. cerevisiae* (Pkc1Sc; REFSEQ accession no. NC 001134.7). Amino acids that are identical in at least five of the seven different amino acid sequences at the same position are boxed. Numbers on the right indicate the position of the last amino acid shown in each protein. Also indicated are residues identical in all sequences of the alignment (*), conserved substitutions (:), and semiconserved substitutions (.). Conserved features and domains are indicated (2).

FIG. 2—Continued.

thesis. Therefore, *A. nidulans* strain LOGOAnBH1 was analyzed. This strain contains an *anbH1-egfp* gene fusion integrated into the genome (29). The strain was cultivated in AMM either with or without the Pkc inhibitor calphostin C.

Results are shown in Fig. 1. Without the addition of the inhibitor, the AnBH1-enhanced green fluorescent protein (EGFP) fusion protein was found to be located in the nucleus (Fig. 1A). By contrast, addition of the Pkc inhibitor led to

PkcBAn	←-----●-----→	SLKLEDF	303
PkcAAn	AMQQVAAKEDIP-QQPK-----	VRIGLDHF	738
PkcAAng	PAMQVVSQQPPEAPQQPK-----	VRIGLDHF	771
PkcAf	PMQQQVAVKEEIPQQPK-----	VRIGLDHF	781
Pkc1Tr	PGQQYQQQPAQAQKPPQPPATAQG-----	AAAGPPGSQRKALPSATDPGTGARIGLDHF	814
Pkc1Nc	QVS PMYQQNPQTPI SKPPQVPAS YDNQVVPASGVVPPTKKPLPSATDPGTGMRIGLDHF		817
Pkc1Sc	QTEQDHASKEVLQETVSLAPTSTHPSRTTDDQSQPKSQTSTSAKHKRAAKRRKVS LDNE		824
		: * : *	
	Kinase domain		
PkcBAn	DLKLVGKGSFGKVMQHRKDTARLHIIYALKTIRKAHTISRSQVTHLAERSVLAQISN		363
PkcAAn	NFLAVLGKGNFGKVMLAETKATK---KLYAIKVLKKEFIIENDEVESTKSEKRVFLIANK		795
PkcAAng	NFLAVLGKGNFGKVMLAETKSTK---RLYAIKVLKKEFIIENDEVESTKSEKRVFLIANK		828
PkcAf	NFLAVLGKGNFGKVMLAETKSTK---KLYAIKVLKKEFIIENDEVESTKSEKRVFLIANK		838
Pkc1Tr	NFLAVLGKGNFGKVMLAETKRSK---RLFAIKVLKKEFIIENDEVESTKSEKRVFLIANR		871
Pkc1Nc	NFLAVLGKGNFGKVMLAETKRSR---KLYAIKVLKKEFIIENDEVESTKSEKRVFLIANR		874
Pkc1Sc	VLLKVLGKGNFGKVLKSKNTD---RLCAIKVLKKNIIQNHDIESARA EKKVFLIATK		881
	: * : * : * : * : * : * : * : * : * : *		
PkcBAn	---PFIVPLKFSFQSPKLYFVLAFVNGGELFHHLOREQRFVNRARFYTAELLCAECL		420
PkcAAn	ERHPFLLNLHACFQTETRVYFVMEYISGGDLMLHIQRGQ-FGL---FYAAEVLLALKYF		850
PkcAAng	ERHPFLLNLHACFQTETRVYFVMEYISGGDLMLHIQRGQ-FGLKRAQFYAAEVLLALKYF		887
PkcAf	ERHPFLLNLHACFQTETRVYFVMEYISGGDLMLHIQRGQ-FGLKRAQFYAAEVLLALKYF		897
Pkc1Tr	ERHPFLLNLHACFQTETRVYFVMEYISGGDLMLHIQRGQ-FGTRKRAQFYAAEVLLALKYF		930
Pkc1Nc	ERHPFLLNLHACFQTETRVYFVMEYISGGDLMLHIQRM-FGTRKRAQFYAAEVLLALKYF		933
Pkc1Sc	TKHPFLLNLHACFQTETRVYFVMEYISGGDLMLHVNQR-LSVRRAQFYAAEVLLALKYF		940
	** : * : * : * : * : * : * : * : * : * : *		
PkcBAn	←-----●-----→	HGFKVIYRDLKPENILLDYTGIALCDFGLCKLDMKDEDRNTFCGTPFYLAPPELLGQG	480
PkcAAn	HENGVIYRDLKLDNILLTLDGHIKIDYGLCKENMWYGSTTSTFCGTPFEMAPEILLDKK		910
PkcAAng	HENGVIYRDLKLDNIMLTLDGHIKVADYGLCKENMWYGATTSTFCGTPFEMAPEILLDKR		947
PkcAf	HENGVIYRDLKLDNILLTLDGHIKIADYGLCKENMWYGSTTSTFCGTPFEMAPEILLDKK		957
Pkc1Tr	HENGVIYRDLKLDNIMLTLDGHIKIADYGLCKEDMWYGSTTSTFCGTPFEMAPEILLDKK		990
Pkc1Nc	HENGVIYRDLKLDNILLTLDGHIKIADYGLCKEDMWYGSTTSTFCGTPFEMAPEILLDKK		993
Pkc1Sc	HDNGVIYRDLKLENILLTPEGHIKIADYGLCKEDMWYGNRTSTFCGTPFEMAPEILKEQE		1000
	* : * : * : * : * : * : * : * : * : * : *		
PkcBAn	←-----●-----→	YTKTVDWWTGLVLLIYEMLTGLPPFYDENTNDMYRKILQEPLTFPSSDIVPPAARDLITRL	540
PkcAAn	YGRAVDWWAFGVLIYQMLLQSPFRGEDEDEIYDAILADEPLYPIH--MPRDSVSLQKL		968
PkcAAng	YGRAVDWWAFGVLIYQMLLQSPFRGEDEDEIYDAILADEPLYPIH--MPRDSVSLQKL		1005
PkcAf	YGRAVDWWAFGVLIYQMLLQSPFRGEDEDEIYDAILADEPLYPIH--MPRDSVSLQKL		1015
Pkc1Tr	YGRAVDWWAFGVLIYQMLLQSPFRGEDEDEIYDAILADEPLYPIH--MPRDSVSLQKL		1048
Pkc1Nc	YGRAVDWWAFGVLIYQMLLQSPFRGEDEDEIYDAILADEPLYPIH--MPRDSVSLQKL		1051
Pkc1Sc	YTKAVDWWAFGVLLIYQMLLQSPFSGDDEDEVFNAILTDEPLYPID--MAGEIVQIFQGL		1058
	* : * : * : * : * : * : * : * : * : * : *		
PkcBAn	←-----●-----→	LDRDPNRRLGAN--GAEEKSHHFFANIDWRKLLQRKYEPSEFRPNVADARDTKNFDAEFT	598
PkcAAn	LTREPELRLGSGPTDAQEVMSHAFFRNINWDDIYHKRVPPPFMTISSATDTSNFDQEF		1028
PkcAAng	LTREPELRLGSGPTDAQEVMSHAFFRNINWDDIYHKRVPPPFPLQISSPTDTSNFDQEF		1065
PkcAf	LTREPELRLGSGPTDAQEVMSHAFFRNINWDDIYHKRVPPPFPLTISPTDTSNFDQEF		1075
Pkc1Tr	LTREPDQRLGSGPTDAQEVMSQPFRRNINWDDIYHKRVQPPFLQIKSATDTSNFDSEFT		1108
Pkc1Nc	LTREPDQRLGSGPTDAQEVMSQPFRRNINWDDIYHKRVQPPFLQIKSATDTSNFDSEFT		1111
Pkc1Sc	LTKDPEKRLGAGPRDADEVMEEPFRRNINFDLILNLRVKPPYIPEIKSPEDTSFEQEF		1118
	* : * : * : * : * : * : * : * : * : * : *		
PkcBAn	←-----●-----→	SEAPKDSYVDGPMLSSTQQQFEGWSYNRPVAGLDAGGSVKDPSFASIPEDSRF	653
PkcAAn	SVTPVLTPVQS-VLSQAMQEEFRGFSYTADFA-----		1059
PkcAAng	SVTPVLTPVQS-VLSQAMQEEFRGFSYTADFA-----		1096
PkcAf	SVTPVLTPVQS-VLSQAMQEEFRGFSYTADFA-----		1106
Pkc1Tr	SVTPVLTPVQS-VLSQAMQEEFRGFSYTADLD-----		1139
Pkc1Nc	SVTPVLTPVQS-VLSQAMQEEFRGFSYTADFE-----		1142
Pkc1Sc	SAPPTLTPLPS-VLTTSQEEFRGFSFMPDDL-----		1151
	* : * : * : * : * : * : * : * : * : * : *		

FIG. 2—Continued.

a decrease in the fluorescence in the nucleus, and additionally, fluorescence was visible in the cytoplasm, suggesting that AnBH1-EGFP either could not enter the nucleus any more or was actively exported from the nucleus (Fig. 1B). As a control, the nuclear localization of a HapB-EGFP fusion was studied. HapB does not contain an obvious phosphorylation site. As shown in Fig. 1C and D, the HapB-EGFP fusion protein of the *A. nidulans* strain

HapB-eGFP was located in the nucleus irrespective of whether calphostin C was added to the culture medium. It was thus likely that Pkc influenced the nuclear localization of AnBH1.

***A. nidulans* encodes two putative *pkc* genes.** Analysis of the *A. nidulans* database (<http://www.broad.mit.edu/annotation/fungi/aspergillus/index.html>) indicated that there are two putative Pkc-encoding genes, which we designated *pkcA* and *pkcB*. The

FIG. 3. (A) Schematic map of the *pkcA* gene of *A. nidulans* and of the *alcAp-pkcA*-encoding antisense plasmid pAL4-*pkcA*inv. For the endogenous gene, exon (white arrows) and intron (black line) regions are indicated. *ampR*, ampicillin resistance gene. The *pyr-4* gene of *N. crassa* was used as the selection marker gene in *A. nidulans*. (B) Southern blot analysis of five selected *pkcA* antisense transformants (PkcAinv1.1, PkcAinv1.2, PkcAinv1.3, PkcAinv1.4, and PkcAinv1.5, abbreviated as 1.1, 1.2, 1.3, 1.4, and 1.5, respectively) and two wild-type (wt) strains, R21OLAB7 (left) and AXB4A (right). Genomic DNA was digested with *Xba*I and *Kpn*I. The probe was targeted against both the genomic site of the *pkcA* gene and the integration sites of the *pkcA* antisense RNA-encoding vector.

pkcB gene was originally obtained by screening an *A. nidulans* gene library using a probe targeting the catalytic domain of protein kinase C (see Materials and Methods). Based on computer analysis, the putative *pkcB* gene consists of 2,224 nucleotides coding for 653 amino acids. The putative *pkcA* gene comprises 3,680 nucleotides encoding 1,059 amino acids. Most probably, the coding sequences of *pkcB* and *pkcA* are interrupted by 4 and 9 introns, respectively.

Comparative sequence analysis of the PkcB amino acid sequence revealed that the similarity to other known Pkc's is confined to the amino-terminal region of the protein, which harbors the catalytic region of all Pkc proteins (Fig. 2B). Moreover, PkcB contains several ATP binding sites and putative Pkc phosphorylation regions. However, cysteine-rich regions, usually involved in the binding of diacylglycerol or phorbol esters, as well as a pseudosubstrate sequence involved in the regulation of Pkc activity (5), could not be found (Fig. 2A).

The *A. nidulans* PkcA amino acid sequence greatly resembles those of other fungal Pkc's (Fig. 2B). It contains an amino-terminal-extended regulatory domain, which, so far, has been found in fungal Pkc's only. This region contains the three conserved subdomains CN1, CN2, and CN3. In *Trichoderma reesei* Pkc1 and *Aspergillus niger* PkcA, two consecutive hydrophobic helices within CN1, which are also conserved in *A. nidulans* PkcA, were suggested to interact with cellular membranes (32). The CN3 subdomain shows a high similarity to the Ca^{2+} binding region of classical Pkc's (38). However, the sequence of this binding region lacks aspartate residues that are important for the binding of Ca^{2+} . This has been observed for all fungal Pkc enzymes characterized so far. Thus, fungal Pkc's

have been assigned to the subgroup of novel Pkc's, whose regulation is independent of Ca^{2+} (2, 38). Moreover, the deduced amino acid sequence of PkcA contains a Q/A/P-rich region, which, so far, has been reported exclusively for fungal Pkc's (17). Taken together, the deduced amino acid sequence of *A. nidulans* PkcA shows a higher number of features characteristic of typical Pkc's than the PkcB sequence. These findings suggest that PkcA most probably represents a member of the group of novel Pkc's. Consequently, PkcA was more likely to be involved in phosphorylation of AnBH1 than PkcB, because the Pkc inhibitor calphostin C interacts with the regulatory domain, which is conserved in PkcA but not in PkcB (14, 18) (Fig. 2). Therefore, it is very likely that PkcA is involved in the phosphorylation-dependent nuclear localization of AnBH1. Direct phosphorylation of a transcription factor by Pkc has been described for the *N. crassa* protein WC-1 (4, 22, 28).

Generation of *pkcA* antisense RNA-producing plasmids. To study a possible influence of PkcA on AnBH1 in vivo, deletion of the structural gene was attempted. Efforts to replace the *pkcA* gene by the *pabaA1* gene of *A. nidulans* using a *p*-aminobenzoic acid-auxotrophic *A. nidulans* strain for transformation failed (data not shown). Also, the use of the *N. crassa pyr-4* gene instead of *pabaA1* and the transformation of a uracil-auxotrophic strain as well as of a strain lacking the gene homologous to the human *KU70* gene (*nkuA*), essential for non-homologous end joining of DNA in double-strand break repair (34), did not result in generation of a knockout mutant (data not shown).

Therefore, an antisense strategy using a regulatable pro-

Repressing conditions Inducing conditions

A

B

Repressing conditions

C

Inducing conditions

D

E

moter was followed, i.e., the production of a *pkcA* antisense transcript was controlled by the regulatable *alcA* promoter. For this purpose, the uracil-auxotrophic *A. nidulans* strain C2.3 was transformed with plasmid pAL4-*pkcA*inv, carrying the *alcAp-pkcA*^{antisense} gene and the *pyr-4* gene as the selectable marker gene (Fig. 3A). After transformation, 30 PyrG⁺ transformants were isolated, and most of them were checked by Southern blot analyses for the presence of the plasmid integrated into the genome (data not shown).

For Southern blot analysis, genomic DNA was digested with KpnI and XbaI. The probe used was targeted against the genomic site of the *pkcA* gene and the integration sites of the *pkcA* antisense RNA-encoding vector (Fig. 3A). For further investigations, five transformants, named PkcAinv1.1, PkcAinv1.2, PkcAinv1.3, PkcAinv1.4, and PkcAinv1.5, were selected; these had been shown by Southern blot analysis to carry few (1.2), several (1.1 and 1.4), and many (1.3 and 1.5) copies of the plasmid integrated into the genome at multiple sites (Fig. 3B; Table 1).

The phenotypes of the different transformants were analyzed. The growth of the transformant strains and that of the untransformed wild-type strain were indistinguishable under *alcAp*-repressing conditions, i.e., on AMM agar plates with glucose as the sole carbon source (Fig. 4A). Growth of the strains under *alcAp*-inducing conditions on AMM agar plates containing 1.6% (wt vol⁻¹) lactose plus 10 mM cyclopentanone led to a different picture (Fig. 4B). In contrast to the wild-type *A. nidulans* strain Palp32.3, which carried the *alcAp* plasmid pabalp32 integrated into the genome (A. Gehrke, unpublished data), the strains exhibited reduced conidiation and increased formation of aerial hyphae. This finding was further supported when the dry weights of mycelia of the different strains were measured from liquid cultures. Under inducing conditions, the dry weights of transformant strains were lower than that of the wild-type strain R21OLAB7 (Fig. 4D), whereas under repressing conditions, there was no difference between wild-type and transformant strains (Fig. 4C). Measurement of the production of conidia under inducing and repressing conditions revealed that the transformant strains produced about 10-fold fewer conidia under inducing conditions than under repressing conditions, whereas there was no difference in the wild-type strain (Fig.

FIG. 4. Phenotypic characterization of *pkcA* antisense-encoding strains PkcAinv1.2, PkcAinv1.3, and PkcAinv1.5 and of control strains Palp32.3 and R21OLAB7 (wild type). Repressing conditions, glucose as the carbon source. Inducing conditions, lactose as the carbon source plus 10 mM cyclopentanone. (A and B) Growth and sporulation on AMM agar plates under repressing conditions (A) and inducing conditions (B). Plates were inoculated with 4 μ l of conidial suspension and incubated at 37°C for 72 h. Strain Palp32.3, which was derived from transformation of strain DE58 (51) with the *alcAp*-containing vector pabalp32 (A. Gehrke, unpublished data), was used as the wild-type control (wt). (C and D) Dry weight. Growth of *A. nidulans* strain R21OLAB7 (wild type) and *pkcA* antisense transformants PkcAinv1.2, PkcAinv1.3, and PkcAinv1.5 grown in AMM under either repressing (C) or inducing (D) conditions at 37°C and 180 rpm for 60 h was determined. (E) Conidiation after 72 h of incubation at 37°C. Cultures were grown under either repressing or inducing conditions.

FIG. 5. Detection of *pkcA* antisense RNA in *A. nidulans* transformant strains. (A) Scheme showing the strategy to detect antisense RNA by RT-PCR. The predicted antisense RNA was transcribed to cDNA using the specific primer PkcAanti3. The cDNA is indicated by a black arrow. Primers PkcAanti5 and PkcAanti3 were used in subsequent PCRs, yielding a 165-bp PCR product. The binding sites of the reverse primers PkcAintr7 and PkcAanti33, which were used in additional RT-PCR assays (see the text), are also indicated. (B) Detection of *pkcA* antisense RNA. RT-PCR was performed using primers specific for antisense RNA (primer PkcAanti3). Cultures for RNA extraction were grown either with glucose (repressing conditions) or with lactose plus 10 mM cyclopentanone (inducing conditions). wt, wild-type strain R21OLAB7; 1.2, 1.3, or 1.5, *pkcA* antisense-encoding transformant strain PkcAinv1.2, PkcAinv1.3, or PkcAinv1.5, respectively; NC, negative control (no template); PC, positive control (chromosomal DNA as the template). (C) Detection of *tef1* RNA. RT-PCR was performed using primers specific for the *tef1* gene (primer Tef1rev) as a control. Cultures for RNA extraction were grown with either glucose (repressing conditions) or lactose plus 10 mM cyclopentanone (inducing conditions).

4E). These data indicated that repression of *pkcA* affected both growth and conidiation of *A. nidulans*.

***pkcA* antisense RNA was detectable only in *alcAp-pkcA*^{antisense}-encoding *A. nidulans* transformant strains.** To study whether *pkcA* antisense RNA was present in the transformant strains, the production of *pkcA* antisense RNA was analyzed by reverse transcription-PCR (RT-PCR). As shown in Fig. 5, antisense RNA was detected only in RNA of transformant strains. *pkcA* antisense RNA was detectable under both *alcAp*-repressing and -inducing conditions. However, the amount of antisense RNA produced under *alcAp*-inducing conditions greatly exceeded that produced under repressing conditions (Fig. 5B). *pkcA* antisense RNA was not detected in the wild-type strain R21OLAB7 (Fig. 5B). Also, the amount of *pkcA* antisense RNA correlated with the number of *pkcA* antisense RNA-encoding plasmids integrated into the genome in the different transformant strains, i.e., the intensity of the cDNA band derived from antisense RNA was strongest for strain PkcAinv1.3, which carried the greatest number of copies of plasmid pAL4-*pkcA*inv integrated into the genome (Fig. 5B). As a control, *tef1* RNA was analyzed. The *tef1* gene encodes translational factor TEF1, which is one of the most abundant proteins in eukaryotic cells (43). The *tef1* gene has been shown to be highly expressed in filamentous fungi (33, 46). RT-PCR using primers specific for the *tef1* gene indicated that *tef1* mRNA was present in all samples, i.e., the wild-type strain and the transformant strains, irrespective of whether they were grown under repressing or inducing conditions (Fig. 5C). These data suggest that *tef1* was constitutively expressed and therefore could be used as an endogenous control.

To prove the authenticity of the PCR fragments obtained, cDNAs obtained from both *pkcA* mRNA and *pkcA* antisense RNA were cloned and sequenced. The sequences agreed with the sequences of *pkcA* without intron and the deduced anti-

sense RNA (data not shown). To analyze the length of the antisense RNA, reverse primers PkcAanti3, PkcAintr7, and PkcAanti33 (Fig. 5A; Table 2) were used in different RT-PCR experiments. Combining the forward primer PkcAanti5 and either of the reverse primers PkcAanti3 and PkcAintr7 in subsequent PCRs with cDNA as the template yielded a PCR product of the expected size of 165 bp or 186 bp, respectively. However, by using the reverse primer PkcAanti33, a PCR product of the expected size of 280 bp could not be obtained. These results suggest that the antisense RNA had at least 218 but fewer than 312 bases, as measured from the assumed starting point of transcription.

Induction of *pkcA* antisense RNA led to increased expression of an *aatAp-lacZ* gene fusion and reduced penicillin titers. Since we have shown that *pkcA* antisense RNA was produced, the influence of PkcA on *aatA* gene expression was studied. Previously, it was shown that AnBH1 acted as a repressor of *aatAp-lacZ* expression (15). If PkcA negatively affected AnBH1, this should result in increased *aatAp-lacZ* expression. To test this hypothesis, the expression of an *aatAp-lacZ* gene fusion was analyzed in *A. nidulans* strain R21OLAB7 (wild type) and three *pkcA* antisense-encoding transformants (PkcAinv1.2, PkcAinv1.3, PkcAinv1.5). The strains carry an *aatAp-lacZ* gene fusion integrated in a single copy at the chromosomal *argB* gene locus. In addition, the transformant strains contain inducible *alcAp-pkcA*^{antisense} fusions (Table 1). The strains were grown in FM containing 4% (wt/vol) glucose (repressing conditions) or 4% (wt/vol) lactose plus 10 mM cyclopentanone (inducing conditions) at 27°C and 250 rpm. Mycelia were harvested after 24 h, 48 h, and 72 h for analysis of *aatAp-lacZ* expression by β -galactosidase assays. Results are shown in Fig. 6. As the determination of the dry weight indicated, in contrast to the results in AMM, in FM there was no difference in growth rate

FIG. 6. Fermentation run of *A. nidulans* R21OLAB7 (wild type) and three *pkcA* antisense RNA-encoding transformants (PkcAinv1.2, PkcAinv1.3, PkcAinv1.5) with lactose plus cyclopentanone or glucose as the carbon source. Data for each time point are means and standard deviations for three simultaneously harvested flasks (for dry weight, data represent only one flask). (A, B, and C) Glucose was used as the carbon source (repressing conditions). (D, E, and F) Lactose was used as the carbon source, and 10 mM cyclopentanone was added to the medium (inducing conditions). Results were expressed as dry weight (A and D), expression of the *aatAp-lacZ* fusion gene determined as β -Gal specific activity (B and E), or penicillin titer (C and F).

FIG. 7. Cellular localization of the AnBH1-EGFP fusion protein. Strains PkcAinv1.3 and C2.3, respectively, were incubated in AMM under either *alcAp*-repressing (0.8% [wt vol⁻¹] glucose) or *alcAp*-inducing (1.6% [wt vol⁻¹] lactose plus 10 mM cyclopentanone) conditions. Nuclei were stained with DAPI (panels 2). Samples were analyzed by fluorescence microscopy (panels 1, showing localization of the AnBH1-GFP fusion protein, and panels 2, showing nuclei) or light microscopy (panels 3). (A and B) AnBH1-EGFP-derived fluorescence of *A. nidulans* strain PkcAinv1.3 under *alcAp*-repressing (A) and *alcAp*-inducing (B) conditions. Arrows in panel B1 indicate nuclei. (C and D) AnBH1-EGFP-derived fluorescence of *A. nidulans* strain C2.3 under *alcAp*-repressing (C) and *alcAp*-inducing (D) conditions.

between strains irrespective of the carbon source used, i.e., glucose or lactose plus cyclopentanone (Fig. 6A and D).

The results of the analysis of *aatAp-lacZ* expression are shown in Fig. 6B and E. Under repressing conditions (glucose), expression of the *aatAp-lacZ* gene fusion was similar in all strains, while under inducing conditions (lactose plus cyclopentanone), the expression patterns were different. After 48 h, *aatAp-lacZ* expression in the antisense RNA-producing transformants PkcAinv1.3 and PkcAinv1.5 was threefold higher than in the wild-type strain. Interestingly, the increase in *aatAp-lacZ* expression was less prominent in transformant strain PkcAinv1.2. This finding correlated well with the reduced amounts of antisense RNA-encoding DNA and antisense RNA in this transformant. Also, under *alcAp-pkcA*^{antisense}-inducing conditions, the amount of penicillin produced correlated negatively with the amount of *pkcA* antisense RNA produced in the different strains (Fig. 6F). The amount of penicillin produced was highest in the wild-type strain and lowest in the transformant strains PkcAinv1.3 and PkcA

inv1.5, which produced the greatest amount of *pkcA* antisense RNA. By contrast, the wild-type and transformant strains showed similar penicillin production under repressing conditions (glucose) (Fig. 6C), further indicating the dependence of penicillin production on induction of the *alcAp-pkcA*^{antisense} fusion. Taken together, these results indicated that reduced expression of *pkcA* resulting from the production of *pkcA* antisense RNA led to increased *aatA* expression and reduced penicillin titers. This apparent contradiction is discussed below.

Induction of *pkcA* antisense RNA production led to localization of an AnBH1-EGFP fusion in the cytoplasm. As mentioned above, the increased *aatAp-lacZ* expression under *alcAp-pkcA*^{antisense}-inducing conditions suggests a direct relationship of PkcA and AnBH1. Based on the inhibitory studies with calphostin C (Fig. 1), it is conceivable that nuclear localization of AnBH1 is regulated by PkcA. Consequently, if PkcA positively affected nuclear localization of AnBH1, under *alcAp-pkcA*^{antisense}-inducing conditions an AnBH1-EGFP fusion protein should be located, at least in part, in the cytoplasm. To prove this hypothesis, strain PkcAinv1.3, containing an *anbH1-egfp* gene fusion and the *alcAp-pkcA*^{antisense} construct (Table 1), was analyzed. The strain was cultivated in AMM under either *alcAp-pkcA*^{antisense}-repressing or *alcAp-pkcA*^{antisense}-inducing conditions. Results are shown in Fig. 7.

Under *alcAp-pkcA*^{antisense}-repressing conditions, the AnBH1-EGFP fusion protein was found to be located in the nucleus (Fig. 7A). By contrast, when *pkcA*^{antisense} expression was induced by lactose plus cyclopentanone, fluorescence was also visible in the cytoplasm (Fig. 7B). Nevertheless, even under inducing conditions, the nuclei still showed fluorescence (Fig. 7B), indicating that AnBH1-EGFP only partially entered the nucleus when *pkcA* mRNA was silenced. As a control, the AnBH1-EGFP fusion protein was analyzed in strain C2.3, which does not contain the *alcAp-pkcA*^{antisense} construct (Table 1). As shown in Fig. 7C and D, the AnBH1-EGFP fusion protein was located in the nucleus irrespective of whether incubation occurred under repressing or inducing conditions. These results indicated that reduced expression of *pkcA* due to production of *pkcA* antisense RNA led to reduced localization of AnBH1 in the nucleus and, furthermore, to an accumulation of the protein in the cytoplasm.

DISCUSSION

Until now, little information has been available on the signal transduction cascades involved in β -lactam biosynthesis. Previous work revealed that FadA, the α subunit of a heterotrimeric G protein, is involved in the regulation of penicillin biosynthesis. In an *A. nidulans* strain containing constitutively activated FadA [*fadA*(G42R)], an increased steady-state mRNA level of the *ipnA* gene and concomitantly increased penicillin titers were observed. FadA appears to be a member of a signal transduction cascade activating penicillin biosynthesis in *A. nidulans* (47). Here we showed that PkcA is involved in penicillin biosynthesis. Members of the family of Pkc's are generally serine/threonine kinases and are found exclusively in eukaryotic cells. Many, if not all, Pkc enzymes are central components of signal transduction chains (reviewed in reference 42). Computer analysis of the *A. nidulans* genome revealed that the fungus contains two putative Pkc's. Whereas

PkcA looks like a typical fungal Pkc, PkcB apparently lacks some of the characteristic features of Pkc's, making it likely that PkcB represents only a Pkc-related protein kinase.

We failed to generate a knockout strain of the *pkcA* gene by using the usual AMM regeneration agar plates, even when a strain lacking the gene homologous to the human *KU70* gene (*nkuA*), essential for nonhomologous end joining of DNA in double-strand break repair (34), was used (data not shown). Although the reason for this could be that *pkcA* lies in a less recombinogenic region of the genome, we favor the theory that deletion of *pkcA* would be lethal for the cell, even in a heterokaryon. This assumption is supported by the observation that in filamentous fungi the deletion of *pkc*-encoding genes is obviously lethal (35), whereas this is apparently not the case in yeasts. In *Saccharomyces cerevisiae*, a single gene encoding Pkc1p was identified. Mutants carrying a complete deletion were able to proliferate only in the presence of osmotic stabilizing agents and underwent immediate lysis upon transfer to a medium lacking osmotic stabilizers (24). Within the *Schizosaccharomyces pombe* genome, two PKC genes (*pkc1*⁺ and *pkc2*⁺) were identified (50). Different functions for *pkc1*⁺ and *pkc2*⁺ were found. Only the combined deletion of both genes was lethal (3). While the deletion of *pkc1*⁺ only displayed no apparent phenotype, deletion of *pkc2*⁺ showed an aberrant cell morphology (50). Deletion of both endogenous copies of the *CaPKC1* gene, encoding a Pkc in diploid *Candida albicans* cells, resulted in an osmotically remedial cell lysis defect of both the budding and the hyphal growth form and morphologically aberrant cells of the budding form (36). By contrast, in filamentous fungi deletion of *pkc*-encoding genes has not been reported yet, apparently because their deletion appears to be lethal (22). A similar conclusion was drawn by Oeser (35), who failed to delete the Pkc-encoding *pkc1* gene of *Cochliobolus heterostrophus*. Because, as shown here, the deletion of *pkcA* in *A. nidulans* is also potentially lethal, an antisense RNA strategy using an inducible promoter was applied. The production of *pkcA* antisense RNA was an alternative way to study the function of PkcA, which was further investigated in this study because it showed the highest degree of similarity to typical fungal Pkc's. Antisense RNA was successfully applied to filamentous fungi in a number of recent studies (7, 23, 53). As shown here, a good level of *pkcA* antisense RNA was obtained by using the promoter region of the *alcA* gene of *A. nidulans* when transformant strains were grown with lactose as the carbon source plus cyclopentanone. Expression of antisense *pkcA* led to reduced growth of the fungus in AMM but not in FM, indicating that either the effect of the antisense RNA was less prominent when the fungus grew in FM or, alternatively, the phenotype of reduced Pkc activity is less drastic in FM than in AMM.

The observation that under *alcAp-pkcA*^{antisense}-inducing conditions, i.e., when the *pkcA* transcript was silenced, only reduction of growth occurs, and not—as expected for an essential gene—complete growth inhibition, can be explained by the fact that, by using the antisense approach, expression of a given gene usually cannot be completely turned off but can only be reduced (53). The same applies to the observation that under *alcAp-pkcA*^{antisense}-inducing conditions most, but not all, AnBH1-EGFP molecules were located in the cytoplasm. The effect based on the production of antisense RNA was quantified by Moralejo et al. (31), who measured a *pepB* mRNA reduction ranging from 10% to 70% when *pepB*^{antisense} RNA

was produced from the *A. nidulans gpdA* promoter in *Aspergillus awamori*. Therefore, the level of antisense RNA may not be sufficient to titrate out the respective mRNA completely. Nevertheless, when the gene is essential—which is likely for *pkcA*—an antisense approach still allows its functional characterization, especially when formation of the antisense RNA is regulatable (53).

In FM, the penicillin titer was reduced to 10% in multicopy transformant strains expressing antisense RNA and to 40% in low-copy-number transformant strains. The amounts of antisense RNA in the different transformants, the increased expression level of the *aatA* gene, and the copy number of antisense RNA-encoding plasmids integrated into the genome correlated well, i.e., the higher the copy number in the genome, the more antisense RNA was produced, the more *aatAp-lacZ* expression was observed, and the less penicillin was produced. Also, the initial observation that the addition of Pkc inhibitor to the medium led to a decrease in nuclear localization of AnBH1 accords well with the observation that the production of *pkcA* antisense RNA led to higher expression of the *aatAp-lacZ* gene fusion, most likely because the level of AnBH1 present in the nucleus was reduced. These observations suggest that PkcA is involved in the nuclear entry of AnBH1 or in its maintenance in the nucleus, most likely by phosphorylation of AnBH1 either directly or by activating other kinases that phosphorylate AnBH1. This hypothesis is supported by the observation that reduced expression of *pkcA* resulting from production of *pkcA* antisense RNA led to reduced maintenance of AnBH1-EGFP in the nucleus and, furthermore, to an accumulation of the protein in the cytoplasm (Fig. 7). Therefore, taken together, these data suggest that PkcA is a direct target of the Pkc inhibitor calphostin C, because the data obtained from Fig. 7 are in accordance with those of Fig. 1. The latter showed that the addition of calphostin C also led to a decrease in AnBH1-EGFP levels in the nucleus and, furthermore, to an increase in levels of this protein in the cytoplasm.

In yeasts, one of the main functions of Pkc's is to maintain cellular integrity (reviewed in reference 42). In filamentous fungi, it is very likely that Pkc's have pleiotropic effects and affect different, in part essential, cellular functions. Recently, Franchi et al. (22) reported that Pkc modulates light responses in *N. crassa* by regulating the blue light photoreceptor WC-1. To elucidate the function of PkcA, it will also be helpful to identify target proteins by proteome analysis.

An obvious contradiction was the observation that despite higher expression of the *aatAp-lacZ* gene fusion, the penicillin titer was reduced when the *alcAp-pkcA*^{antisense} gene fusion was induced. This indicated that higher expression of the third penicillin biosynthesis gene, *aatA*, leads to reduced penicillin titers. Interestingly, a similar observation supporting this conclusion was made by Fernández-Cañón and Peñalva (21). These authors also reported that overproduction of IAT activity reduced penicillin biosynthesis. They speculated that the negative influence on the synthesis of penicillins exerted by excessive levels of IAT, relative to levels of the other enzymes of the pathway, might be mediated by the penicillin amidase (1) or phenylacetyl-CoA hydrolase (30) activities of IAT. Previously, it was shown that IAT has five enzyme activities (1). The penicillin transacylase activity of IAT exchanges side chains between two hydrophobic penicillin molecules, or between one penicillin molecule and 6-aminopenicillanic acid (6-APA). The penicillin amidase activity of IAT is apparently the reverse of the biosynthetic acyl-CoA:6-APA acyltrans-

ferase activity (1). Therefore, it is conceivable that the increase in these enzyme activities by overproduction of IAT leads to drastically less active penicillins or to an increase in 6-APA which also has no significant antibiotic activity. Our observations fully agree with these assumptions. In addition, it is conceivable that PkcA acts directly on other elements of penicillin biosynthesis.

In summary, PkcA is involved in penicillin biosynthesis via regulation of the nuclear localization of the transcription factor AnBH1. Furthermore, PkcA appears to be involved in other cellular processes. Although the exact function of Pkc's in filamentous fungi has not been resolved yet, it is interesting to speculate that if PkcA were also required for maintaining cellular integrity, this would have a direct effect on penicillin biosynthesis, e.g., hydrolysis of the cell wall of *A. nidulans* by competing bacteria could increase the production of penicillin via activation of PkcA.

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