Cdk9-like kinases in complex with T-type cyclins are essential components of the eukaryotic transcription elongation machinery. The full spectrum of Cdk9/cyclin T targets, as well as the specific consequences of phosphorylations, is still largely undefined. We identify and characterize here a Cdk9 kinase (PtkA) in the filamentous ascomycete *Aspergillus nidulans*. Deletion of *ptkA* had a lethal effect in later stages of vegetative growth and completely impeded asexual development. Overexpression of *ptkA* affected directionality of polarized growth and the initiation of new branching sites. A green fluorescent protein-tagged PtkA version localized inside the nucleus during interphase, supporting a role of PtkA in transcription elongation, as observed in other organisms. We also identified a putative cyclin T homolog, PchA, in the *A. nidulans* genome and confirmed its interaction with PtkA in *vivo*. Surprisingly, the Pcl-like cyclin Pca1, previously described to be involved in asexual development, was also found to interact with PtkA, indicating a possible role of PtkA in linking transcriptional activity with development and/or morphogenesis in *A. nidulans*. This is the first report of a Cdk9 kinase interacting with a Pcl-like cyclin, revealing interesting new aspects about the involvement of this Cdk-subfamily in differential gene expression.
organisms that lack a canonical CTD sequence, remains largely undefined.

In mammals, several cyclin subunits have been found to activate Cdk9-like kinases (T1, T2a, T2b, and K), with cyclin T1 being the predominant binding partner (11, 36). Unlike for cell cycle-related cyclins, the protein levels of the transcriptional cyclin T subfamily do not oscillate during different cell cycle phases. However, there is an upregulation of cyclin T expression during different developmental programs (13, 22, 41). Accordingly, the Cdk9 homologs in budding and fission yeast interact with regulatory cyclin partners that show significant sequence similarities to cyclin T proteins from higher organisms (34, 59). However, an upregulation of expression levels during certain cellular situations has not been reported.

Thus far, four Cdns have been characterized in the filamentous ascomycete A. nidulans. The Cdk1 homolog NimX in complex with the essential regulatory subunit NimEF52ch represents the key cell cycle regulator (28). Together with another protein kinase, NimA, NimX is required for mitosis entry and septation (31, 33). It is noteworthy that nimX expression is upregulated during asexual development (60), which implies an interaction between developmental regulators and cell cycle regulators in A. nidulans. This was further emphasized by the discovery of a second cyclin interaction partner of NimX, the Pcl-like cyclin PclA (44, 45). As revealed by the expression pattern and the developmental defect of the deletion mutant, PclA function is limited to sporulation, where its exact role remains to be discovered. It was hypothesized that PclA in complex with NimX is required to accelerate cell cycle progression during the generation of conidiospores by rapid, repeated budding of the phialides (45). Apart from the cell cycle regulator NimX

TABLE 1. A. nidulans strains used in this study

| Strain | Genotype or construction | Source or reference |
|--------|-------------------------|-------------------|
| FGSCA4 | Glasgow wild type       | FGSC®             |
| TN02A3 | pyrG89; argB2 ∆nkaA::argB; pyroA4 | 27                |
| GR5    | pyrG89; wA3; pyroA4     | 53                |
| SO451  | pyrG89; wA3; argB2; pyroA4; ∆nkaA::argB; sE15 | 32                |
| SFB1   | TN02A3 transformed with pFB2; (alcA(p)::GFP-PtkA) | This study |
| SFB2   | TN02A3 transformed with pFB2; (alcA(p)::GFP-PtkA) | This study |
| SFB19  | GR5 transformed with pFB13 and pFB14; (alcA(p)::YFP-PchA and YFP C-PtkA) | This study |
| SFB49  | GR5 transformed with pFB25 (alcA(p)::GFP-PtkA/K540) | This study |
| SFB51  | SO451 transformed with ptkA deletion cassette (ΔptkA, heterokaryotic strain) | This study |
| SFB54  | SO451 transformed with ptkA deletion cassette (ΔptkA, heterokaryotic strain) | This study |
| SFB55  | SO451 transformed with ptkA deletion cassette (ΔptkA, heterokaryotic strain) | This study |
| SFB69  | GR5 transformed with pFB13 and pFB22 (alcA(p)::YFP-PchA and YFP C-PtkA) | This study |
| SFB82  | GR5 transformed with pFB15 (alcA(p)::GFP-PtkA; three ectopic integrations) | This study |
| SFB83  | GR5 transformed with pFB15 (alcA(p)::GFP-PtkA; one ectopic integration) | This study |
| SCK9   | TN02A3 transformed with pFB31 (pchA deletion plasmid, ΔpchA) | This study |
| SCK10  | SCK9 transformed with pFB26 (pchA) | This study |

<sup>a</sup> All strains are veA1.
<sup>b</sup> FGSC, Fungal Genetics Stock Center.

**MATERIALS AND METHODS**

**Strains, plasmids, and culture conditions.** Supplemented minimal medium (MM) and complete medium for A. nidulans were prepared as described earlier, and standard strain construction procedures were as described by Hill and Kafer (18). A list of A. nidulans strains used in the present study is given in Table 1. Standard laboratory Escherichia coli strains (XL1-Blue, Top10F+) were used. The plasmids are listed in Table 2.

**Molecular techniques.** Standard DNA transformation procedures were used for A. nidulans (61) and E. coli (43). For PCR experiments, standard protocols were applied by using a Biometra TRIO Thermoblock for the reaction cycles. DNA sequencing was done commercially (MWG Biotech, Ebersberg, Germany). Genomic DNA was extracted from A. nidulans by using a DNeasy plant minikit (Qiagen, Hilden, Germany). DNA analyses (Southern hybridizations) were performed as described previously (43).

**Deletion of ptkA and pchA.** The coding regions of ptkA were replaced by a deletion cassette containing the pyrG gene of A. fumigatus, which encodes the orotidine-5'-phosphate decarboxylase and complements the uridine/uracil auxotrophy that is imposed by the pyrG89 mutations. The deletion cassette was generated by fusion PCR as described previously (50) and consists of the pyrG gene flanked by the upstream and downstream sequences (each ~1,500 bp) of the target genes. The following primers were used to generate the linear DNA
fragments for fusion PCR (the underlined segments are identical to the 5’ end or the 3’ end of the pyrG marker cassette): for the upstream region, ptkA-P3-for (GGCTACGCGACAGTATTAGG) and ptkA-P1-rev (GAAGAGCATTTTGGAGCGTTTACCATTGATCTGCCG); and for the downstream region, ptkA-P5-for (ATCACTGCTCTCCATCAAGGATGTGG) and ptkA-P2-rev (CTTCCAC). The linear deletion fragment was then generated using PCR by using the nested primers ptkA-P2-for (GGCCCTTGTATATTCTCTCG) and ptkA-P7-rev (TGCTTGCTAAGGAGCATCTCGAATGCACTTGCT) (32). The deletion cassette was transformed into the ΔnkuA strain S0451 to increase the frequency of homologous integration (27).

In case of the pchA deletion the flanking regions were amplified by PCR using genomic DNA and the primers pchA-P1-for (CTGCTCGCTCTCAGTGAAGCTACCGTCG) and pchA-P10-S8 (GGCCGATCTGGCGGATAGGACCGACCGACCGAATGGCGGACTACCGTACATCCGAGCAGGCGATTGACGAGGTGG). The resulting PCR product was then cloned into the ΔnkuA strain S0451 to increase the frequency of homologous integration (27).

**Heterokaryon rescue analysis.** The essential ptkA gene function was ascertainment and analyzed by using the heterokaryon rescue technique as described elsewhere (31, 32). In brief, conidia of the primary transformations were carefully removed from the surface and replica streaked on YAG and YAGUU plates. After 1 to 3 days of incubation at room temperature, the plates were observed and scored for growth either per eye or at low power (×40 magnification) in an Axiohot microscope (Carl Zeiss, Jena, Germany). On selective YAG plates almost no growth occurred, because the mononuclear conidia either lacked the nutritional marker gene or the essential ptkA gene. On YAGUU plates, however, pyrG+ ptkA+ conidia still did not form colonies, but the ΔnkuA ptkA+ conidia were able to grow normally, thus indicating the heterokaryotic state of the primary transformants. This was confirmed by diagnostic PCR (see also Fig. 2B). Three individual ΔnkuA heterokaryotic strains were selected for further studies and propagated and stored as described previously (32).

**Tagging of proteins with GFP and YFP/YFP’.** To create a N-terminal green fluorescent protein (GFP) fusion construct of PtkA, a 0.7-kb N-terminal fragment of ptkA (starting from ATG) was amplified from genomic DNA, using the primers ptkA-Efi-for (GGGGCGCGCCCAATGGGCATAGCGTCACTCGAA CGG) and ptkA-Efi-rev (GGTTAAATTAAACCGCGTTTACTATGAGA TTGGCCAGCGCCGGCGGTCAGGCAGCGGCTAGG) (32). The deletion cassette was transformed into the ΔnkuA strain S0451 to increase the frequency of homologous integration (27).

| Plasmid          | Construction                                  | Source or reference |
|------------------|-----------------------------------------------|---------------------|
| pCMB17apx        | aLe(A):GFP, for N-terminal fusion of GFP to proteins of interest, contains N. crassa pyr-4 | 9                   |
| pCR21-TOPO       | Cloning vector                                | Invitrogen          |
| pDV7             | GFP replaced by N-terminal half of YFP in pCMB17apx | 51                  |
| pDV8             | GFP replaced by C-terminal half of YFP in pCMB17apx | 51                  |
| pSM14            | GFP replaced by 3×HA in pCMB17apx             | 40                  |
| pNZ2             | 1.7-kb pyrA fragment in pC51                  | 62                  |
| pFB2             | N-terminal 0.7-kb ptkA fragment in pCMB17apx  | This study          |
| pFB10            | N-terminal ptkA CDNA fragment                  | This study          |
| pFB13            | Full-length ptkA in pDV8                      | This study          |
| pFB14            | Full-length pchA in pDV7                      | This study          |
| pFB15            | Full-length pchA in pCMB17apx                 | This study          |
| pFB22            | Full-length pchA in pDV7                      | This study          |
| pFB25            | Full-length ptkA with point mutation K54Q in pCMB17apx | This study          |
| pFB26            | Full-length pchA in pSM14                     | This study          |
| pFB27            | 1.5-kb 5-flanking region of pchA with Sfi site in pCR2.1-TOPO | This study          |
| pFB28            | 1.5-kb 3-flanking region of pchA with Sfi site in pCR2.1-TOPO | This study          |
| pFB31            | pchA deletion plasmid: flanking regions from pFB27 and pFB28 ligated with pyrA from pNZ12 | This study          |
| pCK1             | N-terminal ptkA CDNA fragment (aa 24 to 330) in pGBK7 | This study          |
| pCK2             | Full-length pchA CDNA in pGADT7               | This study          |
| pCK3             | Full-length pchA CDNA in pGADT7               | This study          |

**TABLE 2. Plasmids used in this study**
pcha-pGADT7-1rev (GCCCGCGGCTACATCTCCCCTTCTTCCCTTC) and cloned into pGADT7 using NdeI/XmaI yielding pC3K. pGBK7-derived plasmids were transformed into the yeast strain AH109 ( mating type Matα), and pGAD7-derived plasmids were transformed into the yeast strain Y187 ( mating type Matα). The system uses three reporter genes (His3, Ade2, and LacZ) under the control of the GAL4-responsive UAS. Protein interactions were analyzed on selective plates lacking tryptophan, leucine, and histidine (triple-dropout medium [TDO]) or lacking tryptophan, leucine, adenine, and histidine (quadro-dropout medium [QDO]).

**Protein extracts and Western blotting.** To prepare protein extracts, *A. nidulans* strains were incubated in liquid MM for 24 h at 37°C. To induce the alcA promoter, this medium was supplemented with 0.2% glucose and 2% threonine (derepression of the alcA promoter, and thus moderate gene expression), or MM plus 2% glycerol (promotion and thus high gene expression), or MM plus 2% glucose (repression of the alcA promoter). For pictures of young hyphae, cells were incubated at 25°C for 1 day. For nuclear staining, coverslips were mounted on microscope slides with mounting medium containing DAPI (4',6'-diamidino-2-phenylindole) and VectorShield (Vector Laboratories, Burlingame, CA). Images were captured at room temperature by using an Axiosimager microscope (Zeiss, Jena, Germany). Images were collected and analyzed by using the AxiosVision system (Zeiss).

**Gene expression analyses.** Transferring development-competent vegetative hyphae from liquid medium to agar plates, thus inducing development by air exposition synchronized the asexual development of wild-type *A. nidulans* cells. At different time points, the RNA was isolated. For that, the mycelium was harvested, dried, frozen in liquid N₂, and ground to a powder. The total RNA was isolated by using Trizol (Invitrogen, NV Leek, Netherlands) according to the manufacturer’s instructions. After removal of DNA contaminations with RNase-free DNase I (Invitrogen), cDNA was synthesized from 1 µg of RNA from each sample by using a SuperScript first-strand synthesis kit (Invitrogen). The resulting cDNA was then used as a template for PCRs with the primers ptkA-F1 (AAGGGAAAGCGCTATCGCTCTCC) and ptkA-R1 (CGGGCGGCCTAGA AATGC) for ptkA expression analysis, pC1-A3 (AACACGACCCAAGCT TGC) and pC1-R3 (AAGGTGCGACTATCCACTGC) for pclA expression analysis, and pcha-A2 (CCACGCCGAATAAACCGCC) and pcha-R2 (CTT CTTAAGTTCACCACCCCTCC) for pclA expression analysis. To check for genomic DNA contaminations, all reverse transcription-PCRs (RT-PCRs) were performed in the absence of reverse transcriptase; however, no bands were produced.

**RESULTS**

**PtkA is a novel member of the Cd9 family.** The developmental Pel-like cyclin Pela forms a complex with the main cell cycle regulator NimX; however, in contrast to yeast, it does not interact with the Pho85 homologues kinases PhoA and PhoB. To further investigate the functional role of Pela in *A. nidulans*, we aimed to identify other possible kinase interaction partners by performing a BLASTP search of the *A. nidulans* genome at the Broad Institute (Cambridge, MA) using the NimX sequence. We revealed a novel gene that encodes a predicted protein with 544 aa (gi/14530079/emb/CAC42219.1). The N-terminal part of the coding sequence (aa 24 to 326) comprises a kinase catalytic domain (17), and sequence comparison with several Cdk8s revealed extensive similarities, clearly grouping the new protein into this superfamily (Fig. 1).

Within the cyclin-binding region the new protein comprises a characteristic PITALRE motif instead of the PSTAIRE motif present in cdc2-like kinases. Thus, it was designated PtkA, for PITALRE kinase in *A. nidulans*.

The catalytic domain of PtkA shares 44% identical amino acids with the human Cdk9 protein. Highest similarity was found with Cdk9 from *S. pombe* (56% identical and 70% similar residues), followed by the Crk1 kinase in *Candida albicans* with 48% identities and 67% similarities. Both are functional orthologs of the Burat/Gyvl kinase from *S. cerevisiae* that shares 44% identical and 59% similar residues with PtkA. Based on these sequence similarities, we deduced PtkA as a new member of the Cdk9 family in *A. nidulans*. Outside the catalytic domain, there is only sequence conservation with homologue kinases in closely related filamentous fungi (e.g., *Aspergillus niger* and *fumigatus*, *Neurospora crassa*, and *Penicillium marneffei*), but almost no sequence similarities were found with non-catalytic domains of Cdk9s kinases from other organisms.

**Generation of a ptkA deletion strain.** To analyze the function of the deduced Cdk9 homologue PtkA in *A. nidulans*, we constructed a ptkA deletion strain by replacing the coding sequence with a gene deletion construct obtained by fusion PCR (SFB54 [see Materials and Methods]). Transformation of the deletion construct in a KU70-deficient recipient strain resulted in the generation of heterokaryons, implying an essential cellular function of ptkA in *A. nidulans*. The deletion could only be maintained in a heterokaryotic state, where two genetically nonidentical nuclei are present in a common cytoplasm, with the essential ptkA gene provided by the undeleted nucleus, and the nutritional marker provided by the nucleus containing the deletion construct (32). The heterokaryon formation was verified by diagnostic PCR, using primer sets that confirm the presence of both, the correctly integrated deletion cassette and the parental ptkA gene (Fig. 2B). In addition, we tested the deleted heterokaryotic state by plating conidia obtained from the heterokaryon on selective plates. Since conidia are mononuclear, only those with the ΔptkA::pyrG-containing nucleus would be able to grow. However, due to the lack of the ptkA gene, positive transformants only produced very small colonies that are barely visible per eye (Fig. 2A).

**PtkA is essential for effective vegetative growth and asexual development in *A. nidulans.* To examine the growth characteristics of *ptkA* cells, conidia of the heterokaryotic primary transformants were streaked onto nutritionally selective (YAG) or nonselective (YAGU) plates, incubated for several days at room temperature, and observed at low magnification (Fig. 3A). On nonselective plates, the undeleted, ΔpyrG conidia exhibited normal growth, with the projection of long germ tubes after 1 day and the appearance of conidia after 3 to 4 days. On selective plates, ΔpyrG conidia are unable to germinate, whereas, in contrast, the deleted ΔpyrG+ conidia grow to the extent allowed by the lack of the essential ptkA gene. After 1 day, the projection of short germ tubes was observed that continued hyphal growth up to the formation of very small colonies, barely visible per eye. After 4 days, hyphal growth stopped. Asexual conidia could not be observed, indicating that ptkA is required for effective vegetative growth and asexual development in *A. nidulans*. Next, we examined a possible effect of the ptkA deletion on the nuclear state. For that, the conidia of deletion transformants were incubated in liquid medium lacking uracil/uridine on coverslips and incubated
overnight. DAPI staining of \( \Delta ptkA \) cells revealed no significant differences in morphology, number, or distribution of nuclei compared to the wild type (Fig. 3B). This indicates that PtkA, similar to other members of the Cdk9 family, is not involved in regulation of the nuclear state or mitosis but possibly in transcriptional regulation.

\textbf{PtkA overexpression affects hyphal morphology.} In order to study the essential function of PtkA in more detail, we constructed an \textit{A. nidulans} strain expressing the only \( ptkA \) copy as a GFP fusion protein under the control of the conditional \( alcA \) promoter (SFB1 and SFB2 [see Materials and Methods]). Under repressing conditions (grown on glucose) the mutant showed a pronounced effect on vegetative growth, leading to very small colonies compared to the wild type and thus reflecting the phenotype of the deletion mutant to a weaker extent (Fig. 4A). Under derepressing conditions (glycerol) the wild-type morphology was restored, providing an internal control for the functionality of the N-terminal GFP fusion construct. Under inducing conditions (threonine), the phenotype of the \( alcA(p)GFPptkA \) colonies was also very similar to that of the wild type. However, individual hyphae of the mutant showed different defects in polarized growth (Fig. 4B and C). Most notably, a high proportion of mutant hyphae displayed a curvy phenotype under inducing conditions (56%). Partially, this effect could be due to the pourer carbon source threonine since the wild-type strain also displayed an increased proportion of curved hyphae in threonine medium (32%). However, the increase in the mutant strain under overexpressing conditions was still significantly higher (Fig. 4C), indicating that an increased abundance of PtkA somehow interferes with the directionality of polarized growth. In addition, under inducing conditions lateral and apical branching was also much more frequently observed in mutant hyphae than in the wild type. To confirm that these effects are really due to the overexpression of \( ptkA \) and not an individual characteristic of SFB1, two \textit{A. nidulans} strains were generated that ectopically express one (SFB83) or two (SFB82) additional \( ptkA \) copies under \( alcA \) control. These strains also displayed high proportions of curved hyphae under inducing conditions, as well as lateral branching and hyphal tip splitting. (Fig. 4B and C). Hyperbranching was even more pronounced in strains with additional ectopic \( ptkA \) copies compared to SFB1. These results point to a role of PtkA...
in maintaining the directionality of hyphal growth and the regulation of the initiation of new branching sites.

Active PtkA is concentrated in the nuclei but changes localization during mitosis. To investigate the subcellular localization of PtkA, the alcA(p)GFP-ptkA strain was grown in glycerol medium and observed in a fluorescence microscope. The protein clearly localized inside the nucleus, which was confirmed by costaining with DAPI (Fig. 5A). However, in some hyphae, a faint cytoplasmic staining was also visible, indicating that the protein may shuttle between the nucleus and the cytoplasm. For human Cdk9 it has been shown that nuclear localization is regulated by autophosphorylation since mutants that either lack kinase activity or certain phospho-acceptor sites at the C termini fail to enter the nucleus (26). To investigate whether PtkA localization is regulated by a similar mechanism, we generated a PtkA point mutant where a strictly conserved lysine residue (K54, see Fig. 1) that is essential for ATP-binding, phosphate transfer, and kinase regulation (17, 42) was exchanged against glutamine. We constructed an A. nidulans strain expressing the GFP-labeled PtkA-K54Q mutant under the control of the alcA promoter and examined localization of the fusion protein under inducing conditions (Fig. 5B). The inactive kinase still enters the nucleus. However, a much bigger proportion of the protein resided in the cytoplasm compared to the active kinase. To exclude the possibility that the cytoplasmic localization of the mutant PtkA is caused by overexpression and leakage from the nucleus, we compared the protein levels of the mutant and the wild-type GFP fusion by Western blot analysis (Fig. 5B). We found a lower protein level for the inactive PtkA than for the wild type, indicating that the catalytic activity of PtkA and possibly autophosphorylation is indeed required for the correct localization of the protein. In addition, the localization of GFP-PtkA was observed by time-lapse confocal microscopy throughout the cell cycle (see Fig. S1 in the supplemental material). When mitosis started the fluorescence signal dispersed into the cytoplasm. After the completion of mitosis, indicated by the doubling of nuclei number, it relocalized back into the nucleus. Since A. nidulans undergoes a partially open mitosis (30), the mitosis specific dispersal of PtkA indicates that it does not remain associated with nuclear components during nuclear division.

PtkA interacts with the cyclin T homolog PchA and the Pcl-like cyclin PclA. Since PtkA is a Cdk, an integral part of its regulation is the interaction with a cyclin partner. Different members of the transcriptional cyclin family have been established to activate Cdk9 in metazoans, including cyclin T1, T2a,
T2b, and cyclin K, with cyclin T1 being the predominant binding partner (11, 35). Accordingly, the *S. pombe* Cdk9 interacts with the cyclin T-homolog Pch1. To identify possible cyclin interaction partners for PtkA, we searched the *A. nidulans* genome for cyclin T homologs and found an uncharacterized ORF (AN4981.2), encoding a predicted 513-aa protein (Fig. 6A). Within the N-terminal half, including the predicted cyclin box fold (aa 51 to 145), the protein sequence is closely related to the Pch1 cyclin from *S. pombe* (40% identical and 63% similar residues); therefore, it was named PchA. Lower sequence similarities exist to the N-terminal halves of the human transcriptional cyclins K (32% identical, 49% similar residues) and T1 (29% identities, 32% similarities). Based on the sequence characteristics, we considered the new protein a likely cyclin partner of PtkA. We tested this by performing yeast two-hybrid experiments and indeed found an interaction between the catalytic domain of PtkA and PchA (Fig. 7A). To confirm this result and to analyze the localization of the PtkA-PchA complex in vivo, we used the BiFC method (1). For that, the N-terminal half of YFP (YFP<sup>N</sup>) was fused to PchA and the C-terminal half (YFP<sup>C</sup>) was fused to PtkA. Strains expressing only PchA-YFP<sup>N</sup> or PtkA-YFP<sup>C</sup> did not show any YFP fluorescence (not shown). However, the expression of both fusion proteins together produced a signal inside the nuclei (Fig. 7B), in agreement with the GFP-PtkA localization observed before (Fig. 5A). Almost no fluorescence signal was detected in the cytoplasm, indicating that the interaction of PtkA with PchA is confined to the nucleus.

To further analyze the function of the newly identified cyclin PchA, we generated a deletion strain by replacing the coding sequence with a deletion cassette (see Materials and Methods). Diagnostic PCR and Southern blotting (Fig. 6B and see Fig. S2 in the supplemental material) verified the correct integration and the absence of the *pchA* gene, and one of the strains (SCK9) was selected for further analysis. The deletion mutant displayed a severe growth defect with colony sizes of ca. 10% compared to wild-type colonies and a severe reduction in conidial production. All phenotypes were rescued after transformation with the *pchA* gene (SCK10, Fig. 6C). These results are in agreement with the effects of the deletion of
cyclin T homologue genes in *S. cerevisiae* or *S. pombe*, which also resulted in severe growth defects or lethality, a finding comparable to the phenotype of ptkA repression (12, 59). Since in *A. nidulans* the deletion of the kinase ptkA is lethal, whereas the deletion of *pchA* is not, we concluded that there are one or more other cyclins interacting with PtkA that have not been identified in our genome search and that allow for survival in the absence of *pchA*, albeit with severe defects.

Our original aim was to identify kinase interaction partners for the developmental cyclin PclA in addition to the already-reported interaction with NimX (44). Therefore, we again performed yeast-two hybrid experiments with the full-length PclA protein and indeed found a strong interaction with the catalytic domain of PtkA (Fig. 7A). To confirm this result, we used BiFC analysis, which also supported the formation of PtkA-PclA complexes in vivo (Fig. 7B). The hypothesis that PchA and PclA are the two main interaction partners of PtkA was further supported by the fact that the generation of a *pclA-pchA* double mutant was unsuccessful, indicating a lethal phenotype comparable to the ptkA deletion.

*pchA* and *pclA* are differentially expressed during asexual development. Next, we examined the expression profiles of ptkA and its interacting cyclins during different phases of development. For that, asexual development of the wild-type strain FGSCA4 was synchronized by air exposition of a liquid culture, and the total RNA was isolated at different time points of development (Fig. 8A). RT-PCR experiments with ptkA primers gave similar bands for all time points (Fig. 8B), indicating...
cating that ptkA is constitutively expressed throughout development. In contrast, both cyclins exhibited differential expression profiles. pchA mRNA levels were unregulated between 7 and 11 h of development, which is about when the conidio- phores are formed. Interestingly, after 11 h the expression went down again, indicating that PchA may be not required for later stages of development. pclA mRNA levels also increased after 7 h of development but remained at that level for the whole time course. This is in agreement with Northern blot analysis results published earlier (45) and reflects the role of PclA during the generation of conidiospores.

**DISCUSSION**

In this study we identified and characterized the Cdk PtkA in the filamentous ascomycete *A. nidulans*. Isolated by screening of the *A. nidulans* genome for sequences related to the Cdc2 homolog NimX, the protein sequence reveals extensive similarities to Cdk9-like kinases, including the characteristic PITALRE motif in the cyclin-binding box, and thus clearly grouped into this subfamily. In complex with T-type cyclins, Cdk9-like kinases are involved in the stimulation of transcriptional elongation, chromatin remodeling, and mRNA processing in various organisms (2, 37, 55). We show here that deletion of ptkA in *A. nidulans* causes lethality during vegetative growth and, since no asexual spores are produced, can only be propagated in a heterokaryon. The germination of *H9004* ptkA spores is delayed, but germ tubes otherwise showed no morphological defects, which would account for the detrimental effect of ptkA deletion in later growth stages. This either means that PtkA does not become essential until a certain cell size has been reached, possibly reflecting an increased need for transcriptional activity to continue cellular growth, or that, alter-
natively, PtkA might be sufficiently stable so that a small amount of protein from the heterokaryotic phase supports growth for a limited time. The growth characteristics of a strain carrying a conditional ptkA allele confirm the essential role of PtkA in *A. nidulans*, since under repressing conditions only very small colonies are produced. The absence of any recognizable defect in nuclear morphology and/or nuclei number in the ptkA deletion mutant, which would be expected in case of a functional role in cell cycle regulation, supports the view that PtkA is a transcription-related rather than cell cycle-related Cdk.

A GFP-labeled PtkA version accumulated inside the nuclei. Since no nuclear localization signal was identified in the protein sequence, we suggest that nuclear entry depends on the interaction with either a cyclin binding partner or a target protein. In addition, we discovered that the nuclear localization is partly dependent on the kinase activity, since an inactive GFP-labeled point mutant produced a fluorescent signal in both nuclear and cytoplasmic compartments. A similar effect was described for human Cdk9, where nuclear accumulation is dependent on catalytic activity, as well as phosphoacceptor sites in the C terminus (26). This indicates that autophosphorylation is required for the correct localization of hCdk9, and we suggest a similar mechanism for PtkA. During mitosis, the kinase gets dispersed in the cytoplasm. Since *A. nidulans* undergoes a partially open mitosis (6, 7), this indicates that PtkA does not remain associated with nuclear structures during nuclear division. A similar localization pattern was also observed for RNAPII (47), possibly reflecting the general downregulation of transcription during mitosis (57, 58).

Based on the phenotypes associated with the ptkA and pchA deletions, the protein sequence characteristics, and the localization studies, we hypothesize a role of PtkA in controlling transcriptional activity in *A. nidulans*, although direct proof for this is still missing and subject of our current investigations. Different transcriptional T-type cyclins (cyclins T1, T2a, T2b, and K) have been established to activate Cdk9 kinase activity in various organisms. Accordingly, we identified a novel cyclin protein in *A. nidulans*, PchA, that comprises significant sequence similarities to the cyclin T-homolog Pch1 from *S. pombe* and the human cyclins T1 and K. Using yeast-two-hybrid and BiFC analysis, we show that PchA interacts with PtkA *in vivo*, suggesting PchA to be an activating cyclin for PtkA in *A. nidulans*. Accordingly, the deletion of pchA resulted in a severe growth defect and the complete absence of asexual development, which is comparable to the effects of *cyclin T* deletions in yeast cells (12, 59).

Surprisingly, we identified the Pcl-like cyclin PclA as a second cyclin interaction partner for PtkA *in vivo*. In yeast, members of the Pcl cyclin family interact with the Pho85-kinase and are involved in a variety of cellular processes, including environmental signaling, cell cycle regulation, and polarized growth (20). Although the targets of the different Pho85-Pcl complexes include a number of transcription factors, the Pcl cyclins are not transcription related in the sense of a direct involvement in the transcriptional machinery. We show here in *A. nidulans* that PtkA is an interacting kinase of PclA, which is to our knowledge the first report of a Cdk9-like kinase to form a complex with a canonical cyclin. Since PclA is essential for spore generation, this may indicate a functional role of PtkA/PclA complexes in asexual development. Furthermore, both cyclins PclA and PchA are upregulated during spore generation, and the complete lack of asexual development in the ptkA and pchA deletion mutants supports an involvement of PtkA in this developmental program.

We hypothesize that PtkA links the regulation of transcriptional activity with cellular processes such as development or morphogenesis in *A. nidulans*. This idea is supported by the phenotype of ptkA-overexpressing strains that show a significantly increased proportion of curved and hyperbranched hyphae, indicating a functional role of PtkA in the initiation and directionality of polarized growth. The exact mechanism of how PtkA regulates these processes is not elucidated here; however, the nuclear localization of PtkA rather than at hyphal tips or the cytoskeleton indicates that the kinase does not directly influence spatial organization of the cytoskeleton but acts indirectly via gene expression. A similar function has been proposed for the Cdk9 homolog Crk1 in the dimorphic fungus *Candida albicans*. A crk1 deletion mutant is viable but shows defects in hyphal development due to an impaired induction of hyphal specific genes (5).

There are several indications that Cdk9-like kinases are not simply supporting global transcription but are rather involved in differential gene expression in response to certain stimuli. For example, recent microarrays analyses in yeast and human cells after specific inhibition of Cdk9 revealed that certain genes involved in different pathways such as stress response or cell division are especially dependent on Cdk9 activity, whereas others are much less dependent or even independent (15, 52). However, the mechanism of how different gene sets are distinguished with respect to Cdk9 recruitment or activity has not yet been elucidated. One possibility is the local chromatin environment, since in budding yeast Brl1 was found to regulate histone ubiquitination and methylation (23, 55)—a need that could vary at different gene loci (52). Alternatively, several activating factors are known to recruit the P-TEFb complex, such as, for example, the HIV transactivator protein Tat, thereby stimulating transcription of the respective genes (37). Notably, only one of the four known hCdk9-interacting cyclins can bind to Tat (54), suggesting that different Cdk9/cyclin complexes are specifically recruited to certain genes (14). In the present study we show that the Cdk9 homolog PtkA in *A. nidulans* interacts with at least two different cyclins, including PclA. Thus far, Pcl-like cyclins are not known to activate Cdk9-like kinases. However, since they are involved in the response to a large variety of internal and external cues, it is conceivable that this cyclin family plays a role in the differential control of transcriptional activity. Further work is required to confirm this hypothesis, including the elucidation of the mechanism how PtkA/cyclin complexes activate transcription in *A. nidulans*, the identification of genes to which they are recruited, and the discovery of possible other cyclin binding partners.

**ACKNOWLEDGMENTS**

This study was supported by the Deutsche Forschungsgemeinschaft (Research Fellowship for F.B. [BA 3726/1-1]), the Baden-Württemberg Stiftung (Eliteprogramm für Postdoktoranden), and National Institutes of Health grant GM042564 to S.A.O.

We thank Nadine Zekert for helpful discussions and help with the fluorescence microscopy.
57. Xu, Y. X., Y. Hirose, X. Z. Zhou, K. P. Lu, and J. L. Manley. 2003. Pin1 modulates the structure and function of human RNA polymerase II. Genes Dev. 17:2765–2776.
58. Xu, Y. X., and J. L. Manley. 2007. Pin1 modulates RNA polymerase II activity during the transcription cycle. Genes Dev. 21:2950–2962.
59. Yao, S., A. Neiman, and G. Prelich. 2000. BUR1 and BUR2 encode a divergent cyclin-dependent kinase-cyclin complex important for transcription in vivo. Mol. Cell. Biol. 20:7080–7087.
60. Ye, X. S., S.-L. Lee, T. D. Wolkow, S.-L. McGuire, J. E. Hamer, G. C. Wood, and S. A. Osmani. 1999. Interaction between developmental and cell cycle regulators is required for morphogenesis in Aspergillus nidulans. EMBO J. 18:6994–7001.
61. Yelton, M. M., J. E. Hamer, and W. E. Timberlake. 1984. Transformation of Aspergillus nidulans by using a trpC plasmid. Proc. Natl. Acad. Sci. U. S. A. 81:1470–1474.
62. Zekert, N., and R. Fischer. 2009. The Aspergillus nidulans kinesin-3 UncA motor moves vesicles along a subpopulation of microtubules. Mol. Biol. Cell 20:673–684.