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A Family of Cyclin-Like Proteins That Interact with the Pho85 Cyclin-Dependent Kinase

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In budding yeast, entry into the mitotic cell cycle, or Start, requires the Cdc28 cyclin-dependent kinase (Cdk) and one of its three associated G_1 cyclins, Cln1, Cln2, or Cln3. In addition, two other G_1 cyclins, Pcl1 and Pcl2, associate with a second Cdk, Pho85, to contribute to Start. Although Pho85 is not essential for viability, Pcl1,2-Pho85 kinase complexes become essential for Start in the absence of Cln1,2-Cdc28 kinases. In addition, Pho85 interacts with a third cyclin, Pho80, to regulate acid phosphatase gene expression. Other cellular roles for Pho85 cyclin-Cdk complexes are suggested by the multiple phenotypes associated with deletion of *PHO85*, in addition to Start defects and deregulated acid phosphatase gene expression. Strains with *pho80*, *pcl1*, and *pcl2* deletions show only a subset of the *pho85* mutant phenotypes, suggesting the existence of additional Pho85 cyclins (Pcls). We used two-hybrid screening and database searching to identify seven additional cyclin-related genes that may interact with Pho85. We found that all of the new genes encode proteins that interacted with Pho85 in an affinity chromatography assay. One of these genes, *CLG1*, was previously suggested to encode a cyclin, based on the protein's sequence homology to Pcl1 and Pcl2. We have named the other genes *PCL5*, *PCL6*, *PCL7*, *PCL8*, *PCL9*, and *PCL10*. On the basis of sequence similarities, the *PCLs* can be divided into two subfamilies: the Pcl1,2-like subfamily and the Pho80-like subfamily. We found that deletion of members of the Pcl1,2 class of genes resulted in pronounced morphological abnormalities. In addition, we found that expression of one member of the Pcl1,2 subfamily, *PCL9*, is cell cycle regulated and is decreased in cells arrested in G_1 by pheromone treatment. Our studies suggest that Pho85 associates with multiple cyclins and that subsets of cyclins may direct Pho85 to perform distinct roles in cell growth and division.

In most eukaryotic cells, mitogenic signals and cell size are primarily sensed in late G_1 phase prior to the initiation of DNA synthesis (S phase). The budding yeast *Saccharomyces cerevisiae* becomes committed to cell division at a regulatory step in late G_1 known as Start, which is analogous to the restriction point in mammalian cells (9, 51). Passage through Start allows the onset of several critical cell cycle events, including DNA synthesis, spindle pole body duplication, and preparation for bud emergence (10, 41).

Start, and other major cell cycle transitions, require Cdc28, a member of the highly conserved cyclin-dependent kinase (Cdk) family. Cdk activity requires binding of an activating subunit called a cyclin (13, 41, 45). As the name implies, cyclins were originally described as proteins whose levels oscillate during the cell cycle (17). Subsequent work has revealed some cyclins whose levels vary little throughout the cell cycle, suggesting a broader definition of cyclins as a family of structurally related proteins which bind and activate Cdk catalytic subunits (39). In yeast, specific cyclin-Cdc28 activities are required at different points in the cell cycle. At Start, Cdc28 is activated by association with the G_1 cyclins, Cln1, Cln2, and Cln3 (reviewed in reference 10), and cells with all three *CLN* genes deleted arrest at Start (6, 54). After Start, B-type cyclin (Clb)-Cdc28 activity is required for cell cycle progression; association of Cdc28 with the S-phase cyclins Clb5 and Clb6 regulates entry into S phase (15, 56), while cyclins that accumulate during G2, Clb1 to Clb4, are required for mitosis (20, 53, 58). The current model of the mammalian cell cycle similarly invokes waves of Cdk activity regulated sequentially by various cyclins. In higher eukaryotic cells, eight Cdks (Cdk1 to Cdk8) that associate with one or more cyclins (cyclins A, B1, B2, B3, C, E, D1, D2, D3, and H) have been identified (44, 45, 60). Whereas Clns and Clbs are thought to associate only with Cdc28 in yeast, vertebrate cyclins can interact with more than one Cdk to regulate cell cycle progression. For example, complexes of D-type cyclins with Cdk4 and Cdk6 are considered key regulators of G_1 progression, while Cdk2-cyclin A and Cdc2-cyclin A complexes regulate S phase and entry into mitosis (reviewed in reference 45).

Although many Cdks function directly in cell cycle progression, roles for Cdks in other cellular processes, most notably transcription, have become apparent (reviewed in references 8, 44, and 52). In budding yeast, three cyclin-Cdk complexes that can phosphorylate the carboxy-terminal repeat domain of the large subunit of RNA polymerase II in vitro have been identified: (i) Ccl1-Kin28, which is homologous to mammalian cyclin H-Cdk7 and is a component of the basal transcription factor TFIIH (5, 18, 65, 66); (ii) Srb11-Srb10, which is a component of the RNA polymerase II holoenzyme and is required for repression of gene expression in response to glucose and mating type (34, 35, 59, 67); and (iii) the Cdk-related kinase, Ctk1, and its cyclin Ctk2 (57). Although genetic and biochemical data support roles for these kinase complexes in the regulation of RNA polymerase II transcription in vivo, the precise function of each individual complex remains to be determined (for reviews see references 44, 50, and 52).

Cyclin-Cdk complexes have also been found to control gene expression through a transcriptional activator protein in re-

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sponse to nutrient availability. *PHO85* encodes a Cdk that interacts with the cyclin Pho80 to regulate the activity of Pho4, a basic helix-loop-helix transcription factor (30, 48, 49). In vivo, Pho4 is phosphorylated by Pho80-Pho85 when cells are exposed to a phosphate-rich environment (30, 49). When phosphorylated by Pho80-Pho85, Pho4 is predominantly localized to the cytoplasm and expression of the acid phosphatase gene *PHO5* is repressed (49). When yeast cells are starved for phosphate, the activity of Pho80-Pho85 is repressed by the Cdk inhibitor Pho81 (26, 55), allowing activation of Pho4.

In addition to Pho80, Pho85 interacts with two other cyclin subunits, Pcl1 and Pcl2 (Pho85 cyclins [16, 38]). Pcl1-Pho85 and Pcl2-Pho85 appear to play a role in regulating cell cycle progression at Start. Although Pho85 is not essential for viability, it is required for G_1 progression when the G_1 cyclin genes *CLN1* and *CLN2* are deleted (16, 38). Likewise, deletion of *PCL1* and *PCL2* causes G_1 arrest when *CLN1* and *CLN2* are absent (38). These genetic observations suggest a role for Pcl1,2-Pho85 kinase complexes in G_1 in the absence of Cln1,2-Cdc28. Two other observations support a role for Pcl1,2-Pho85 kinase complexes at Start. First, the expression of *PCL1* and *PCL2*, like that of *CLN1* and *CLN2*, is controlled by the transcription factor *SWI4* and peaks in G_1 phase (38, 42, 46, 64, 68). Second, the kinase activity of Pcl2-Pho85, as assayed by phosphorylation of Pho4 in vitro, also peaks in G_1 phase (38). The exact roles of Pcl1-Pho85 and Pcl2-Pho85 in G_1 control are unclear, but biological substrates are likely to be different than those of Cln-Cdc28 kinase complexes, as suggested by the distinct substrate preferences of the Pho85 and Cdc28 kinases in vitro (16, 38, 64).

Other cellular roles for Pho85 cyclin-Cdk complexes are suggested by the multiple phenotypes, in addition to Start defects and deregulated acid phosphatase gene expression, caused by deletion of *PHO85*. First, in cells deleted for *PHO85*, glycogen synthase kinase activity is impaired and hyperaccumulation of glycogen is observed (27, 61). Biochemical and genetic data suggest that Pho85 phosphorylates and inhibits glycogen synthase (27, 61). Since *pho80*Δ strains do not hyperaccumulate glycogen, it remains to be determined what activating cyclin functions with Pho85 in its role as a glycogen synthase kinase (61). Second, both *pho80*Δ and *pho85*Δ mutants show a reduced ability to grow on glycerol, ethanol, and acetate, although the effect is more pronounced in a *pho85*Δ mutant (21, 61). Finally, *pho85*Δ strains grow slowly on glucose-containing medium, are enlarged, and show morphological abnormalities not seen in a *pcl1*Δ *pcl2*Δ *pho80*Δ strain.

As summarized above, strains with the known Pho85 cyclins deleted show only a subset of the *pho85* mutant phenotypes, suggesting the existence of other cyclins (Pcls) specific for different Pho85 functions. We report the identification of a family of cyclin-related proteins that interact with and may regulate Pho85. We have used two-hybrid screening and database searching to identify seven additional members of the *PCL*/*PHO80* gene family. On the basis of sequence similarity, the *PCL*s can be divided into two subfamilies: the Pcl1,2 subfamily and the Pho80 subfamily. A functional significance to this subclassification was suggested by our phenotypic analysis of strains with multiple *PCL*s deleted. We found that a diploid with the Pcl1,2 cyclin subfamily (*pcl1*Δ/*pcl1*Δ *pcl2*Δ/*pcl2*Δ *clg1*Δ/*clg1*Δ *pcl5*Δ/*pcl5*Δ *pcl9*Δ/*pcl9*Δ) deleted had an abnormal cell morphology more pronounced than that seen in a strain with *PHO85* deleted. In addition, we report that expression of *PCL9*, a close relative of *PCL1* and *PCL2*, is also cell cycle regulated, with peak expression in G_1 phase.

MATERIALS AND METHODS

Yeast strains, media, and methods. Standard methods for yeast culture and transformation were followed (23). Standard rich medium (YPD) and supplemented minimal medium (SD) were used (31). Yeast strains are described in Table 1. Strain Y153 was used for the two-hybrid assays and has been previously described (14). All other strains were isogenic to strain BY263 (**a** *trp1*Δ*63 GAL2*$^+$ *ura3-52 lys2-801*^{am} *ade2-107*^{o} *his3*Δ*200 leu2-*Δ*1*; S288C background) with the exceptions noted. Construction of the *pcl2*Δ strain BY271 (*pcl2*Δ*HIS3*) has been described elsewhere (38). A strain with the *PCL1* gene disrupted (BY628) was made by transformation of BY263 with plasmid pBA892 (*pcl1*Δ*LEU2*) that had been digested with *Sal*I and *Sph*I. Disruption of the *CLG1* gene (strain BY393, *clg1*Δ*URA3*) was achieved by transformation of BY263 with a *clg1*Δ*URA3* disruption plasmid (37) that had been digested with *Eco*RI and *Hin*dIII. A strain carrying a *pcl5*Δ*LEU2* disruption allele (*pcl5*Δ*LEU2*; BY527) was made by transformation of BY263 with plasmid pBA895 that had been digested with *Hin*dIII and *Sac*I. A strain in which *PCL5* was disrupted with *TRP1* (BY631) was constructed by transformation with plasmid p*pcl5*Δ*TRP1* digested with *Sph*I and *Kpn*I. In strain BY629, the *PCL8* gene was disrupted by transformation with p*pcl8*::*LEU2* digested with *Eco*RI and *Hin*dIII. Disruption of the *PCL9* gene (*pcl9*Δ*HIS3*; BY694) was achieved by transformation of BY263 with pBA929 that had been digested with *Eco*RI and *Sph*I to release a *pcl9*Δ*HIS3* disruption cassette. A strain disrupted for *PHO80* (BY490) was made by transformation of BY263 with plasmid pOS2 (62) digested with *Eco*RI and *Sal*I. All gene disruptions were verified by Southern blot analysis. Construction of plasmids used for the gene disruptions is described below.

To construct yeast strains deleted for multiple *PCL*s (Table 1), strains BY628 (**a** *pcl1*Δ*LEU2*) and BY647 (α *pcl2*Δ*LYS2 pcl5*Δ*TRP1 clg1*Δ*URA3*) were mated and BY637 (**a** *pcl1*Δ*LEU2 pcl2*Δ*LYS2 clg1*Δ*URA3 pcl5*Δ*TRP1*) was recovered by dissecting tetrads. Other multiple *PCL* deletion strains were recovered as meiotic segregants from a diploid formed by mating BY675 (α *pcl1*Δ*LEU2 pcl5*Δ*TRP1 clg1*Δ*URA3*) and BY702 (**a** *pcl1*Δ*LEU2 pcl2*Δ*LYS2 pcl9*Δ*HIS3*).

Plasmids. The *PCL2* DNA template, pBA686, used for in vitro transcription-translation reactions has been described elsewhere (38). Plasmid pET15b-*CLN1*-HA (a gift of Mike Tyers) allows expression of a hemagglutinin (HA) epitope-tagged Cln1 protein from the T7 promoter. Plasmid pBEF198 (a gift of B. Funnell) expresses the ParA protein from the T7 promoter (11).

Vectors for in vitro transcription and translation of various cyclins were constructed by cloning the cyclin genes into pET19b-HMK, a derivative of pET19b (Novagen), or pRSET vectors (Invitrogen). Cyclin protein produced from both the pET and pRSET vectors carries a polyhistidine tag at the NH_2 terminus. The pET19b-HMK derivatives also carry an NH_2-terminal heart muscle kinase recognition sequence. All cloning steps described below involving PCR amplification used the proofreading enzyme Vent DNA polymerase (New England Biolabs).

Plasmid pET19b-HMK-*CCL1* (pBA825) was constructed by subcloning a *Bam*HI-*Bgl*II fragment containing the *CCL1* gene (66) from a λACT (14) library isolate obtained in the *PHO85* two-hybrid screen (see below) into the *Bam*HI site of pET19b-HMK. Plasmid pBA914b, expressing the *PCL1* gene, was constructed by subcloning a *Bam*HI fragment containing the *PCL1* gene from pBA674 (pBluescript-SK-*PCL1*) into the *Bam*HI site of pRSETB. Plasmid pBA885, expressing the *CLG1* gene, was constructed by PCR amplification of *CLG1* from plasmid p533-7AB (37) with primers CLG1-START(BamHI) (5′-CCGGGATCCAATGGCTAATACTTTCAAGTA) and CLG1-END(BamHI) (5′-CCGGGATCCTTCACTAATACACTGCCGAAT), digestion of the product with *Bam*HI, and cloning into the *Bam*HI site of pET-19b-HMK (underlining indicates restriction sites). Plasmid pBA849 was constructed by PCR amplification of the entire *PCL5* coding sequence from yeast genomic DNA by using the oligonucleotides PCL5-START (5′-CCGGGATCCTATGGATGGAAATCATAGGTT) and PCL5-END (5′CCGGGATCCTAACATGACAGGGCACATA). The PCR product was cleaved with *Bam*HI and cloned into the *Bam*HI site of pET19b-HMK. Plasmid pBA948 was constructed by PCR amplification of the *PCL6* coding sequence from yeast genomic DNA by using primers PCL6-START (5′-CCGCTCGAGATGTCTATCAAAGGTGATTCC) and PCL6-END (5′-CCGCTCGAGTACCTTTGAGAGAAACTGTGT), cleavage of the products with *Xho*I, and cloning into the *Xho*I site of pRSETA. Plasmid pBA949, expressing the *PCL7* coding sequence, was constructed by subcloning an *Xho*I fragment containing the *PCL7* gene from a λACT plasmid obtained in the *PHO85* two-hybrid screen into the *Xho*I site of pRSETA. Plasmid pBA946a, expressing the *PCL8* gene, was constructed by PCR amplification of the *PCL8* coding sequence from yeast genomic DNA by using primers PCL8-START (5′-CCGAGATCTATGGCTAATGATCAAGATCCC3′) and PCL8-END (5′-CCGAGATCTTAAGGGAAGGAACAAATGAAC3′), cleavage of the product with *Bgl*II, and cloning into the *Bgl*II site of pRSETC. The *PCL9* coding sequence was PCR amplified from yeast genomic DNA by using primers PCL9-START (5′-CCGGGATCCAGATGATTTCTGACTACGATGC3′) and PCL9-END (5′-CCGGGATCCCTCGAGAAACAAGACGAATGCC3′), cleaved with *Bam*HI, and cloned into the *Bam*HI site of pRSETB to form plasmid pBA950. Plasmid pBA945a, expressing the *PCL10* gene, was constructed by subcloning an *Xho*I fragment containing the *PCL10* gene from a λACT library isolate obtained in the *PHO85* two-hybrid screen into the *Xho*I site of pRSETB.

The *pcl1*Δ*LEU2* plasmid (pBA892) was made by digesting pJO125 (a

TABLE 1. Yeast strains used in this study

Strain	Genotype	Reference or source
BY262	*MAT***a**/α *trp1*Δ*63/trp1*Δ*63 GAL2*$^+$*/gal2 ura3-52/ura3-52 lys2-801*^{am}*/lys2-801*^{am} *ade2-107*^{o}*/ade2-107*^{o} *his3*Δ*200/his3*Δ*200 leu2-*Δ*1/leu2-*Δ*1*	This study
BY263	*MAT***a** *trp1*Δ*63 GAL2*$^+$ *ura3-52 lys2-801*^{am} *ade2-107*^{o} *his3*Δ*200 leu2-*Δ*1*	38
BY177[a]	*MAT***a** *swi5*Δ*LEU2*	C. Peterson
BY271	*MAT***a** *pcl2*Δ*HIS3*	38
BY391	*MAT***a** *pho85*Δ*LEU2*	38
BY393	*MAT***a** *clg1*Δ*URA3*	This study
BY431	*MAT***a** *cln2*Δ*URA3*	This study
BY490	*MAT***a** *pho80*Δ*HIS3*	This study
BY527	*MAT***a** *pcl5*Δ*LEU2*	This study
BY559[b]	*MAT***a**/α *pho85*Δ*LEU2/pho85*Δ*LEU2*	This study
BY628	*MAT***a** *pcl1*Δ*LEU2*	This study
BY629	*MAT***a** *pcl8*::*LEU2*	This study
BY631	*MAT***a** *pcl5*Δ*TRP*	This study
BY634	*MAT***a** *pcl1*Δ*LEU2 pcl2*Δ*LYS2*	This study
BY637	*MAT***a** *pcl1*Δ*LEU2 pcl2*Δ*LYS2 clg1*Δ*URA3 pcl5*Δ*TRP1*	This study
BY647	*MAT*α *pcl2*Δ*LYS2 clg1*Δ*URA3 pcl5*Δ*TRP1*	This study
BY675	*MAT*α *pcl1*Δ*LEU2 clg1*Δ*URA3 pcl5*Δ*TRP1*	This study
BY694	*MAT***a** *pcl9*Δ*HIS3*	This study
BY702	*MAT***a** *pcl1*Δ*LEU2 pcl2*Δ*LYS2 pcl9*Δ*HIS3*	This study
BY708	*MAT***a** *pcl1*Δ*LEU2 pcl2*Δ*LYS2 pcl5*Δ*TRP1 pcl9*Δ*HIS3*	This study
BY710	*MAT***a** *pcl1*Δ*LEU2 pcl2*Δ*LYS2 clg1*Δ*URA3 pcl9*Δ*HIS3*	This study
BY712	*MAT***a** *pcl1*Δ*LEU2 clg1*Δ*URA3 pcl5*Δ*TRP1 pcl9*Δ*HIS3*	This study
BY714	*MAT***a** *pcl1*Δ*LEU2 pcl2*Δ*LYS2 clg1*Δ*URA3 pcl5*Δ*TRP1 pcl9*Δ*HIS3*	This study
BY715	*MAT*α *pcl1*Δ*LEU2 pcl2*Δ*LYS2 clg1*Δ*URA3 pcl5*Δ*TRP1 pcl9*Δ*HIS3*	This study
BY723	*MAT***a**/α *pcl9*Δ*HIS3/pcl9*Δ*HIS3*	This study
BY724	*MAT***a**/α *pcl2*Δ*LYS2/pcl2*Δ*LYS2 pcl9*Δ*HIS3/pcl9*Δ*HIS3*	This study
BY725	*MAT***a**/α *pcl5*Δ*TRP/pcl5*Δ*TRP pcl9*Δ*HIS3pcl9*Δ*HIS3*	This study
BY726	*MAT***a**/α *pcl1*Δ*LEU2/pcl1*Δ*LEU2 pcl2*Δ*LYS2/pcl2*Δ*LYS2 pcl9*Δ*HIS3/pcl9*Δ*HIS3*	This study
BY727	*MAT***a**/α *pcl1*Δ*LEU2/pcl1*Δ*LEU2 pcl2*Δ*LYS2/pcl2*Δ*LYS2 clg1*Δ*URA3/clg1*Δ*URA3 pcl9*Δ*HIS3/pcl9*Δ*HIS3*	This study
BY728	*MAT***a**/α *pcl1*Δ*LEU2/pcl1*Δ*LEU2 pcl2*Δ*LYS2/pcl2*Δ*LYS2 pcl5*Δ*TRP/pcl5*Δ*TRP pcl9*Δ*HIS3/pcl9*Δ*HIS3*	This study
BY729	*MAT***a**/α *pcl1*Δ*LEU2/pcl1*Δ*LEU2 clg1*Δ*URA3/clg1*Δ*URA3 pcl5*Δ*TRP/pcl5*Δ*TRP pcl9*Δ*HIS3/pcl9*Δ*HIS3*	This study
BY730	*MAT***a**/α *pcl1*Δ*LEU2/pcl1*Δ*LEU pcl2*Δ*LYS2/pcl2*Δ*LYS2 clg1*Δ*URA3/clg1*Δ*URA3 pcl5*Δ*TRP1/pcl5*Δ*TRP1 pcl9*Δ*HIS3/pcl9*Δ*HIS3*	This study
BY731	*MAT***a**/α *pcl1*Δ*LEU2/pcl1*Δ*LEU2 pcl2*Δ*LYS2/pcl2*Δ*LYS2 clg1*Δ*URA3/clg1*Δ*URA3 pcl5*Δ*TRP/pcl5*Δ*TRP*	This study

[a] This strain and all following haploid strains are isogenic to BY263 unless noted otherwise.
[b] This strain and all following diploid strains are isogenic to BY262 unless noted otherwise.

*pcl1*Δ*HIS3* plasmid [16]) with *Bgl*II to disrupt the *HIS3* gene and filling in the ends with Klenow enzyme. The blunted fragment was then ligated to a fragment containing the *LEU2* gene from plasmid pJJ250 (29) to create plasmid pBA892. The *pcl5*Δ*LEU2* allele was constructed by amplification of *PCL5* flanking sequences from plasmid pUC19*PCL5* by PCR using primers designed to allow deletion of the *PCL5* coding sequences starting at the initiator codon: 5′ primer (5′CCGGTCGACGGAAACAGATTAAATCTTG3′) and 3′ primer (5′CCGGGATCCGGAGTAAACCTATGATTTCC3′). Plasmid pUC19*PCL5* was made by cloning a 2.6-kb *Pst*I-*Eco*RV genomic fragment carrying *PCL5* into *Pst*I-*Sma*I-digested pUC19. The PCR product was digested with *Bam*HI and *Sal*I and ligated to a *Bam*HI-*Sal*I restriction fragment from pJJ250 carrying the *LEU2* gene to create plasmid pBA895. The *pcl5*Δ*TRP1* plasmid was made by ligating a *Bam*HI-*Sal*I fragment carrying the *TRP1* gene (from pJJ281 [29]) to pBA895 that had been digested with *Bam*HI and *Sal*I to release *LEU2*. Plasmid p*pcl8*::*LEU2* was constructed by cloning an *Eco*RI-*Hin*dIII fragment encoding the carboxy-terminal half of *PCL8* into *Eco*RI-*Hin*dIII-digested pBluescript KS+ to create pKS-*PCL8C*. A *Pst*I fragment from Yep13 carrying the *LEU2* gene was inserted into the *Nsi*I site of pKS-*PCL8C* to generate the disruption plasmid p*pcl8*::*LEU2*. A *pcl9*Δ*HIS3* allele (pBA929) was made by cloning a 4.6-kb *Eco*RI-*Hin*dIII genomic fragment containing *PCL9* into *Eco*RI-*Hin*dIII-digested pUC18 to create pUC18*PCL9*. A PCR fragment carrying *PCL9* flanking sequences but lacking *PCL9* coding sequences was obtained by amplification with a 5′ primer (5′CCGGGATCCCAATAATTCTAAGTTACTCGC3′) and a 3′ primer (5′CCGGGATCCCAGAAATCATCTCTTTGTGCG3′). The PCR product was digested with *Bam*HI and ligated to a *Bam*HI fragment containing the *HIS3* gene from pJJ215 (29) to create pBA929.

Plasmid AD-*PCL1*, expressing a Gal4 activation domain (AD)-*PCL1* fusion, has been described previously (38). Plasmid pBA938, expressing an AD-*PCL9* fusion, was constructed by PCR amplification of *PCL9* from genomic DNA with primers PCL9-START and PCL9-END (described above), cleaved with *Bam*HI, and cloned into the *Bam*HI site of pACTII (gift of S. Elledge). AD-*PCL5* (pBA942) was constructed by digesting pBA849 (described above) with *Bam*HI, blunting the ends with Klenow enzyme, and cloning the fragment into the *Nco*I-digested, Klenow-treated pACTII. AD-*PCL8* was constructed by isolating an *Eco*RI-*Sal*I fragment from pKS-*PCL8*C and cloning it into *Eco*RI-*Sal*I-cut pGAD-GH, creating an in-frame fusion of the Gal4 AD to the carboxy-terminal half of *PCL8*.

In vitro transcription-translation reactions. HIS-*PCL1* (pBA914b), HIS-*PCL2* (pBA686), HIS-*CLG1* (pBA885), HIS-*PCL5* (pBA849), HIS-*PCL6* (pBA948), HIS-*PCL7* (pBA949), HIS-*PCL8* (pBA946a), HIS-*PCL9* (pBA950), HIS-*PCL10*, HIS-*CCL1* (pBA825), HIS-*CLN1*-HA (pET15b-*CLN1*-HA), and HIS-*PARA* were transcribed and translated in vitro in rabbit reticulocyte lysates with [^{35}S]methionine as recommended by the supplier (TNT kit; Promega). Supercoiled templates for the reactions were prepared by CsCl gradient purification. Reaction mixtures were incubated at 30°C for 1 h and used immediately in affinity chromatography assays or stored at −70°C.

Preparation of Gst fusion proteins. The glutathione *S*-transferase (Gst)–Pho85 expression construct, pGEX-*PHO85* (pBA700), has been previously described (38). To prepare Gst or Gst-Pho85 proteins, mid-log-phase cultures (1 liter) of *Escherichia coli* CAG456 [*lac*(Am) *trpC*(Am) *pho*(Am) *supC*(Ts) *rpsL mal*(Am) *htpR165*] harboring either vector pGEX-1 or pGEX-*PHO85* (pBA700) were induced with 0.4 mM isopropylthiogalactopyranoside (IPTG) for 2 h at 30°C. All subsequent steps were performed at 4°C. Cells were pelleted, washed once with water, resuspended in 10 ml of phosphate-buffered saline (PBS), pH 7.3, containing 1% Triton X-100, 20 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride, and lysed by sonication. Cell debris was removed by centrifugation (10,000 rpm for 20 min, Sorvall SS-34 rotor), and the supernatant was added to 200 μl of glutathione-Sepharose 4B (50% slurry in PBS; Pharmacia). The lysate-resin mixture was rocked at 4°C for 2 h. The beads were then pelleted, and the lysate supernatant was subjected to an additional round of batch binding using a fresh 200-μl resin aliquot (50% slurry). After both batch-binding steps, the beads were washed four times with 10 ml of PBS followed by a final wash in PBS–10% glycerol–2 mM dithiothreitol. Batches were then pooled, and the protein was eluted with 50 mM glutathione–50 mM Tris (pH 8.0). The glutathione was

TABLE 2. Interaction of Pho85 with Pcls and other cyclins in the two-hybrid system

Plasmid	β-Galactosidase activity (Miller units)[a]		Fold increase in activation	No. of isolates[b]	Database no.[c]	Chromosome no.
	pAS1	pAS1-*PHO85*				
pAD	0.2	0.0	0	NI[d]		
AD-*PCL1*	0.1	2.1	21	NI		
AD-*PCL2* (PIP9.9)	0.2	8.0	40	1		
AD-*CLG1* (PIP10.36)	0.3	4.2	14	3		
AD-*PCL5*	0.1	0.3	3	NI	YHR071W	VIII
AD-*PCL6* (PIP10.5)	0.1	171	1,709	3	YER059W	V
AD-*PCL7* (PIP8.3)	0.1	70	703	14	YIL050	IX
AD-*PCL8*	0.2	7.0	35	4	YPL219W	XVI
AD-*PCL9*	0.1	7.1	71	NI	Z67750	IV
AD-*PCL10* (PIP7.2)	0.3	98	326	19	YGL134W	VII
AD-*CCL1* (PIP1.1)	0.1	0.2	2	1		
AD-*UME3* (PIP10.106)	0.4	4.2	11	1		
AD-*CLN1* (PIP10.23)	0.3	19	63	1		

[a] See Materials and Methods and reference 1; average of four independent transformants.
[b] Number of independent cDNAs isolated in the two-hybrid screen.
[c] *Saccharomyces* genome database locus or GenBank accession numbers are listed for novel genes only.
[d] NI, not isolated in the two-hybrid screen.

removed from the eluted protein through iterative dialysis using 50 mM Tris (pH 8.0) and Microcon-10 microconcentrators (Amicon). The concentrated protein was quantified by the Bradford assay (Bio-Rad) before being rebound at desired concentrations to 200 μl of glutathione-Sepharose 4B (50% slurry in PBS) by rocking for 2 h at 4°C. Aliquots of affinity beads were then added to 2× sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis sample buffer (100 mM Tris [pH 6.8], 20% glycerol, 4% SDS, 0.1% bromophenol blue, 2% β-mercaptoethanol), boiled, run on a 10% SDS-polyacrylamide gel, and stained with Coomassie blue. The concentrations of Gst or Gst fusion proteins on beads were estimated through comparison with unstained molecular weight markers (Bio-Rad). The amounts of Gst and Gst-Pho85 used in the affinity chromatography assays are the approximate amounts of full-length protein (micrograms) which occupy a 25-μl bead bed volume.

Batch affinity chromatography. For in vitro binding assays, 5 μl of each translation mixture was incubated with beads containing 25 μg of Gst or 12.5 μg of full-length Gst fusion protein in 50 μl of buffer D (2) for 2 h at 4°C with rocking. Following a 2-min centrifugation, the supernatant was saved as the unbound fraction and boiled in SDS sample buffer. The beads were washed three times with 1 ml of buffer D and boiled in SDS sample buffer. The bound and unbound fractions were loaded on a 10% SDS-polyacrylamide gel. The gels were fixed, treated with Amplify (Amersham), dried, and exposed to X-ray film.

Two-hybrid assay. The *PHO85* coding sequence was isolated in a previous two-hybrid screen (38) and subcloned into pAS1 (14) to create a fusion of Pho85 to the Gal4 DNA binding domain. The pAS1-*PHO85* plasmid was used to transform yeast strain Y153 (14), and this transformant was cotransformed with a yeast cDNA library in vector λACT (gift of Steve Elledge). Interacting clones were selected as described previously (14) from an estimated pool of 880,000 yeast colonies on plates containing 50 mM 3-aminotriazole (Sigma). Clones which supported reporter gene expression with pAS1-*PHO85* but not pAS1 alone were sequenced with a Gal4 AD primer. Gal4 AD fusions isolated in this screen were called Pho85-interacting proteins (PIPs). The number of independent isolates of each coding sequence isolated in the screen together with the PIP designation for a representative isolate is given in Table 2. Other Gal4-AD fusions to *PCL* sequences not identified in the screen were constructed for use in the two-hybrid assay as described above. Liquid β-galactosidase assays were performed as described previously (1).

Northern blot analysis. To examine *PCL* gene expression in response to pheromone (α-factor) treatment, strain BY263 (Table 1) was grown to an optical density at 600 nm of 0.3 and arrested with 5 μM α-factor (Vetrogen). Samples of cells were taken prior to addition of pheromone and at the indicated times after α-factor addition and analyzed for DNA content by fluorescence-activated cell sorting (FACS) as described previously (38) and for RNA. To examine *PCL* expression throughout the cell cycle, RNA was isolated and Northern blotting was done as described previously (38). RNA was also prepared from log-phase samples of various *pcl*Δ strains. The strains used were BY177, BY271, BY393, BY431, BY490, BY527, BY628, BY629, and BY694 (Table 1).

The probes used for Northern blot analysis were a 600-bp *Eco*RI-*Hin*dIII fragment of the *ACT1* gene (38); a 864-bp PCR product containing the *PCL1* coding sequence; a 1.3-kb PCR product containing the *PCL2* gene; a 1-kb *Bam*HI fragment from plasmid HA-*PHO80* (30) containing the *PHO80* coding sequence; a 1.7-kb *Xho*I fragment from pAD-*CLG1* (PIP10.36); a 793-bp PCR product from a genomic *PCL5* clone; a 1.3-kb *Xho*I fragment from plasmid pBA948 (pRSETA-*PCL6*) containing the *PCL6* coding sequence; a 1-kb *Xho*I fragment from plasmid pBA949 (pRSETA-*PCL7*) containing the *PCL7* coding sequence; a 980-bp *Eco*RI-to-*Hin*dIII fragment of *PCL8* from pKS-*PCL8*C; a 1.1-kb *Bam*HI fragment from pBA950 (pRSETB-*PCL9*) containing the *PCL9* coding sequence; a 1.1-kb *Xho*I fragment from plasmid pBA945a (pRSETB-*PCL10*) containing the *PCL10* coding sequence; a 1.3-kb *Xho*I-*Nco*I fragment of *CLN2* (24); and a 2.5-kb *Bam*HI fragment from pCITE2a-*SWI5* (3).

Protein alignments and phylogenetic analysis. Multiple alignments and phylogenetic trees were generated using the Megalign program (DNASTAR). The alignment was generated by the Clustal method, using a PAM250 weighting table.

Microscopy. Cells were grown in YPD medium to log phase, spun down, and concentrated approximately 10-fold. Cells were observed at a magnification of ×400, using Nomarski optics and a charge-coupled device camera mounted on a Leica DM-LB microscope. Images from the camera were analyzed by using a Northern Exposure imaging system (Empix Imaging, Inc., Mississauga, Ontario, Canada).

PhosphorImager analysis. Gels were exposed on a Molecular Dynamics screen and scanned with a Molecular Dynamics PhosphorImager. To quantitate protein binding in the Gst-Pho85 binding assay, Imagequant software (version 3.3) was used to draw a rectangle around the signal corresponding to the protein in a lane. The same size rectangle was used for bound and unbound fractions for every lane. Background was established by placing the same size rectangle in each lane either above or below the proteins. For a particular gel, the background rectangles were placed at the same latitude in every lane. For each lane, both rectangles were integrated by volume and the background value was subtracted. The percentage bound for either Gst or Gst-Pho85 was the integrated value for the bound lane divided by the sum of the integrated value for the unbound lane plus the bound lane. Therefore, each Pcl protein had a percentage bound to Gst value and a percentage bound to Gst-Pho85 value. The bar graph on Fig. 2D represents the percentage bound to Gst-Pho85 after subtraction of the percentage bound to Gst.

RESULTS

Identification of proteins that interact with Pho85 in the two-hybrid system. We undertook a yeast two-hybrid screen to search for proteins that associate with Pho85. A library of yeast cDNAs fused to the Gal4 AD was used to transform a yeast strain expressing a fusion of the Pho85 coding sequence to the Gal4 DNA binding domain (pAS1-*PHO85*). Colonies were screened for proteins that interact with Pho85 by testing for transcriptional activation of β-galactosidase and *HIS3* reporter genes (see Materials and Methods and references 14, 19, and 25). Clones encoding proteins which appeared to interact specifically with Pho85 in the two-hybrid assay were called PIPs. The number of independent isolates of each gene obtained in the two-hybrid screen is given in Table 2 together with *Saccharomyces* genome database locus numbers and chromosomal locations for the previously unidentified genes. From this

screen, we isolated one cDNA (PIP9.9) encoding Pcl2, which is known to associate with Pho85 in late G_1 phase to form an active cyclin-Cdk complex (38). We also recovered one cDNA encoding the Pho81 protein, which is also known to interact with and inhibit the Pho80-Pho85 kinase (26, 55). We identified three cDNAs encoding a putative cyclin called Clg1. Clg1 was previously noted to be similar to Pcl1 and Pcl2 within a region known as the cyclin box that is conserved among known cyclins (28, 37). In addition, we isolated four cDNAs which encoded novel proteins with sequence similarity to the known Pho85 cyclin, Pho80 (Fig. 1; see below). On the basis of the sequence similarity of these genes to Pho80 and data presented below, we have renamed these genes *PCLs*, for Pho85 cyclin genes (*PCL6*, chromosome V; *PCL7*, chromosome IX; *PCL8*, chromosome XVI; and *PCL10*, chromosome VII [Table 2]). In addition to isolating genes in the two-hybrid screen that are similar to known Pho85 cyclin genes, we also isolated the known cyclins Ccl1, Srb11/Ume3, and Cln1 as PIPs. As described earlier, Ccl1 is an essential protein that associates with the cyclin-dependent kinase Kin28, Srb11/Ume3 is a cyclin subunit for the Srb10/Ume5 Cdk, and Cln1 is a G_1 cyclin for Cdc28.

Pho85 cyclins classified into subfamilies. In addition to the genes identified in the two-hybrid screen, database searches revealed only two other genes with sequence similarity to the Pho85 cyclins. These genes are located on chromosome VIII and chromosome IV and have been renamed *PCL5* and *PCL9*, respectively (Table 2). Based on a phylogenetic analysis of the cyclin box regions of various yeast cyclins (Fig. 1A), the Pho85 cyclins appear to define a distinct cyclin family. This Pcl family can be further divided into two subfamilies. Pcl6, Pcl7, Pcl8, and Pcl10 are most similar to Pho80, and we refer to these proteins (and the encoding genes) as the Pho80 subfamily, whereas Pcl1, Pcl2, Clg1, Pcl5, and Pcl9 comprise the Pcl1,2 subfamily. An alignment of the cyclin box regions of the entire Pho85 cyclin family is shown in Fig. 1B. This corresponds to, approximately, the carboxy-terminal 50% of the cyclin box as defined by Hunt (28).

Though the sequences common to all of the Pcl family members are limited to this cyclin box region, some of the Pcls display more extensive homology. For example, Pcl6 and Pcl7 are highly similar over a large region containing the cyclin box (64% identical over 186 amino acids [Fig. 1C]), as are Pcl2 and Pcl9 (64% identical over 229 amino acids [Fig. 1C]). Our phenotypic analysis of strains with the Pcl1,2 subfamily deleted suggested a functional significance to this subclassification (see below).

Specific interaction of cyclin proteins with Pho85 in the two-hybrid system. Pcl proteins that were not isolated in our two-hybrid screen (Pcl5 and Pcl9) were fused to the Gal4 AD and tested directly for interaction with pAS1-*PHO85* in the two-hybrid assay. Interaction of the two proteins was determined from their ability to activate transcription of a *GAL1*::*lacZ* reporter gene by assaying β-galactosidase activity (Table 2). The results of β-galactosidase assays for all cyclins isolated in our screen and Pcl5 and Pcl9 are summarized in Table 2. In general, members of the Pho80 subfamily of cyclins showed a higher level of activation over background than the Pcl1,2 subfamily of cyclins. For example, coexpression of AD-*PCL6* and pAS1-*PHO85* led to a 1,700-fold stimulation of reporter gene expression above background. As well, we recovered many independent isolates of *PCL6*, *PCL7*, *PCL8*, and *PCL10* (members of the Pho80 subfamily) in the two-hybrid screen, whereas *PCL1*, *PCL5*, and *PCL9* (the Pcl1,2 subfamily) were not recovered. Members of the Pcl1,2 subfamily may have been recovered less frequently in our screen due to the lower levels of reporter gene expression or a low frequency of cDNAs in the library.

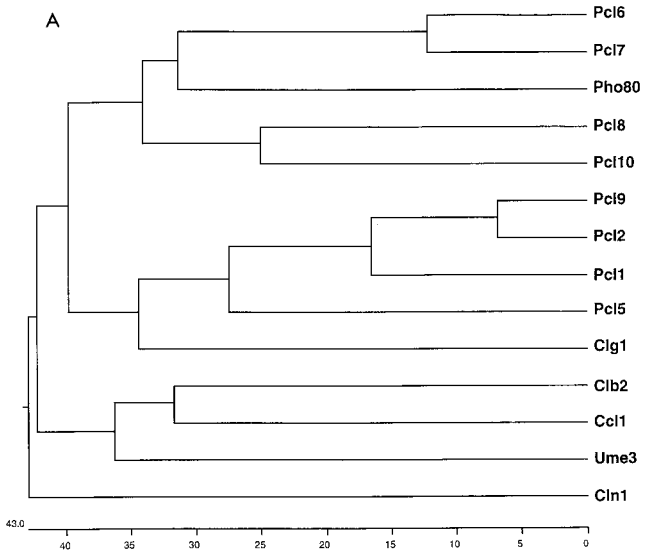

FIG. 1. Alignment of putative Pho85 cyclins. (A) Phylogenetic tree of budding yeast cyclins. The scale bar indicates the relative distance on the tree in arbitrary units. (B) Alignment of Pcls within the cyclin box region. Pcls are grouped into the Pcl1,2 subfamily (Pcl1, Pcl2, Clg1, Pcl5, and Pcl9) and the Pho80 subfamily (Pho80, Pcl6, Pcl7, Pcl8, and Pcl10). -, gap in the sequence; a, acidic residue (D or E); ○, aromatic residue (W, F, or Y); ◐, aliphatic residue (V, I, L, M, or A). Stippled residues are conserved in 4 of 5 members within a subfamily, and the consensus sequence is a match in 7 of 10 family members. Asterisks indicate positions also conserved in a general cyclin box consensus (as described by Hunt [28]). (C) Alignment of Pcl6 with Pcl7 and Pcl2 with Pcl9. The cyclin box region (aligned with other cyclins in panel B) is highlighted. Identical residues are boxed.

Interaction of Pho85 with cyclins in vitro. To test the association of Pho85 with the various Pcls in vitro, we developed a batch affinity chromatography assay using a Gst-Pho85 ligand and in vitro-translated cyclins. We were able to detect specific binding of all of the Pho85 cyclins to the Gst-Pho85 resin compared with binding to a control Gst resin (Fig. 2A and B). Pcl1, Pcl2, and Pho80 have been previously shown to associate with Pho85; therefore, we used Pcl1 and Pcl2 as controls in our assay (16, 30, 38). PhosphorImager analysis was used to quantitate the amount of in vitro-translated protein that was retained on the Pho85 resin compared to that bound to Gst (Fig. 2D). The amount of Pcl-like cyclin retained on the Pho85 resin varied 10-fold, from about 2% of the input protein for Pcl8 (Fig. 2B, lanes 13 to 16; Fig. 2D) to over 20% of the input translation for Pcl5 and Pcl10 (Fig. 2A, lanes 13 to 16; Fig. 2B, lanes 1 to 4; Fig. 2D). Overall, the Pcl-type cyclins bound the Gst-Pho85 resin more efficiently than cyclins which are known to bind other Cdks. Specifically, Ccl1 and Cln1 bound at near background levels in this assay (Fig. 2C, lanes 1 to 8; Fig. 2D). By contrast, only two of the Pcl-type cyclins, Pcl7 and Pcl8, showed less than 5% binding to the Pho85 resin relative to Gst (Fig. 2B, lanes 9 to 16; Fig. 2D). An unrelated bacterial protein, ParA, showed no binding to the Pho85 resin (Fig. 2C, lanes 9 to 12; Fig. 2D).

Cell cycle regulation of *PCL9* gene expression. Expression of *PCL1* and *PCL2* is cell cycle regulated, with peak transcription in G_1 phase of the cell cycle (38, 64). We determined whether the new members of the Pcl1,2 subfamily showed a similar pattern of expression. Yeast cells were arrested at Start with mating pheromone (α-factor) and released into fresh medium, and RNA was prepared from samples of cells as the culture

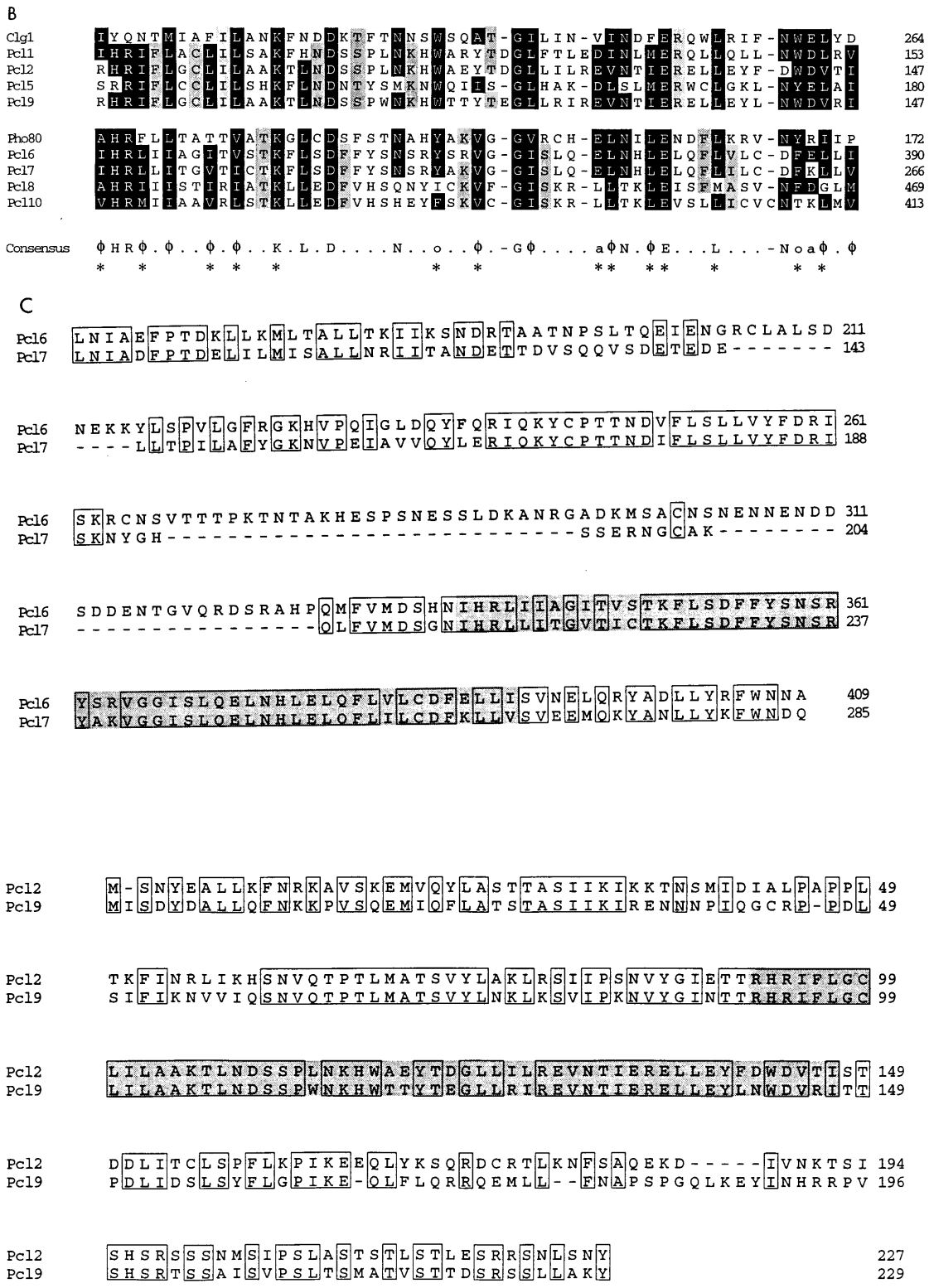

FIG. 1—*Continued.*

progressed synchronously through the cell cycle. Northern blot analysis of the RNA samples showed that *CLG1* transcript levels were relatively constant throughout the cell cycle (Fig. 3A, *CLG1* panel). *PCL5* expression appeared slightly periodic because, as quantified with a PhosphorImager, levels were twofold higher than during log phase at the 20- to 60-min time points compared to *ACT1* transcript levels (Fig. 3A, *PCL5* panel, lanes 4 to 6). By contrast, the amount of *PCL9* mRNA

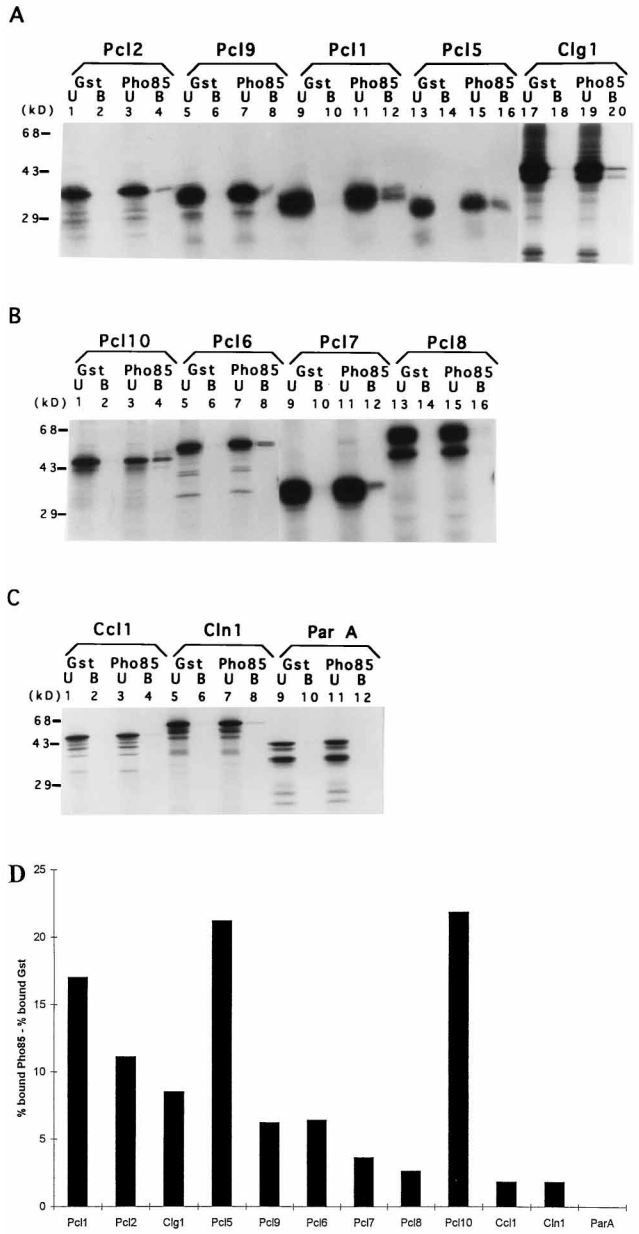

FIG. 2. Binding of cyclins to Gst-Pho85 in vitro. Proteins were translated in vitro and incubated with either Gst or Gst-Pho85 immobilized on glutathione beads (labeled as Gst or Pho85 above the lanes). U indicates the unbound fraction (the supernatant), and B indicates the fraction bound to the beads. The labeled protein included in each set of reactions is indicated above the bracket. The positions of migration of protein molecular weight markers are indicated to the left. (A) Lanes 1 to 16 show Pcl2, Pcl9, Pcl1, and Pcl5 assays as indicated. Shown are the results of a single experiment. The Clg1 assays (lanes 17 to 20) were the result of a separate experiment. Both gels were exposed to X-ray film for 18 h. (B) Lanes 1 to 16 show Pcl10, Pcl6, Pcl7, and Pcl8 assays as indicated. All assays were performed on the same day. The gel was exposed to film for 45 h. (C) Lanes 1 to 12 show Ccl1, Cln1, and ParA reactions as indicated. The assays were done at the same time as those shown in panel B. The gel was exposed for 18 h. (D) Bar graph showing quantitation of protein binding to Gst and Gst-Pho85 resins for assays shown in panels A to C. The data were collected with a PhosphorImager (see Materials and Methods). The values graphed represent the percentages of protein bound to Gst-Pho85 less the percentages of labeled protein bound to Gst.

fluctuated significantly in the cell cycle (Fig. 3A, *PCL9* panel). FACS analysis (data not shown) indicated that the peak in PCL9 transcript levels occurred in early G_1 phase of the second cell cycle following release from the pheromone block. Interestingly, *PCL9* was not expressed for the first cell cycle after the pheromone block; no other cyclins that have been examined show this pattern of regulation. Given that we were looking at expression of *PCL9* in the second cell cycle following release from the pheromone block, we also looked at *SW15* expression, which peaks in late G_2 phase (36, 43). *SWI5* transcript levels peaked before *PCL9*, consistent with an early G_1 expression of *PCL9*. The timing of expression of *PCL9* was similar to that of *PCL2*, although *PCL9* expression peaked at 80 min whereas *PCL2* expression was maximal at both at 80 and 100 min (Fig. 3A, *PCL2* and *PCL9* panels, lanes 7 and 8). *PCL1* transcript levels peaked later coincident with maximal *CLN2* expression (Fig. 3A, *PCL1* and *CLN2* panels). Transcript levels for three members of the Pho80 subfamily (*PHO80*, *PCL6*, and *PCL7*) were constant throughout the cell cycle (data not shown).

Expression of *PCL* genes in response to mating pheromone. *PCL2* transcript has been previously shown to be induced in response to α-factor (38, 47). Also, the expression of other G_1 cyclins, *CLN1* and *CLN2*, is reduced when cells are exposed α-factor, contributing to inhibition of Cln-Cdc28 kinase activity and cell cycle arrest at Start (7, 24, 40, 68). However, response to and recovery from α-factor arrest appeared unaffected by deletion of *PCL2* (37a). Because the function of Pcl2 may be redundant with other Pcls, we tested expression of *PCL* transcripts in cells arrested by α-factor. Cells were exposed to α-factor for 3 h, RNA was isolated at various time points, and *PCL* transcript levels were assessed by Northern blot analysis. *PCL2* was the only *PCL* gene whose expression was induced in response to pheromone treatment (Fig. 3B, *PCL2* panel), consistent with the fact that only *PCL2* contains a putative pheromone response element in its upstream regulatory sequences. However, *PCL1* and *PCL9*, the two *PCL* genes most closely related to *PCL2*, were also significantly affected by pheromone treatment. Levels of *PCL1* transcript were reduced to background levels after a 15-min exposure to α-factor (Fig. 3B, lane 3, *PCL1* panel), while the *PCL9* transcript decreased after 60 min of exposure to α-factor (Fig. 3B, lane 5, *PCL9* panel). *PCL1* transcript reappeared after 3 h (180 min) of pheromone treatment, coincident with recovery of some of the arrested cells (Fig. 3B, lane 7, *PCL1* panel). Levels of other *PCL* transcripts remained relatively constant throughout the arrest, although *PCL6* transcript levels were decreased after 120 min in the presence of pheromone (Fig. 3B, lane 6, *PCL6* panel).

Given the transcriptional response of the *PCL1*, *PCL2*, and *PCL9* genes to α-factor treatment, we tested the sensitivity of *pho85* and *pcl* mutant strains to α-factor in a plating assay. The *pho85* mutant strain failed to form colonies at 0.1 μM α-factor, whereas an isogenic wild type grew in the presence of 1 μM α-factor (data not shown). Thus, the *pho85* mutant showed supersensitivity to the presence of mating pheromone. However, many combinations of *pcl* mutants (in the Pcl1,2 subfamily) were also tested and showed only minor variations in their ability to grow in the presence of pheromone (data not shown). Although not all slow-growing strains are supersensitive to pheromone, we cannot exclude the possibility that the sensitivity of the *pho85* mutant to α-factor is a secondary consequence of its slow-growth phenotype or some other defect.

Cell morphology of *pho85* and *pcl* mutants. In addition to a slow-growth phenotype, we found that *pho85*Δ mutants also showed morphological defects. Haploid *pho85*Δ cells were larger than wild-type cells, with prominent vacuoles visible in Nomarski images (Fig. 4b). Morphological abnormalities were more pronounced in diploid cells lacking *PHO85*. Diploid *pho85*Δ/*pho85*Δ mutants were large, with a wide bud neck, had

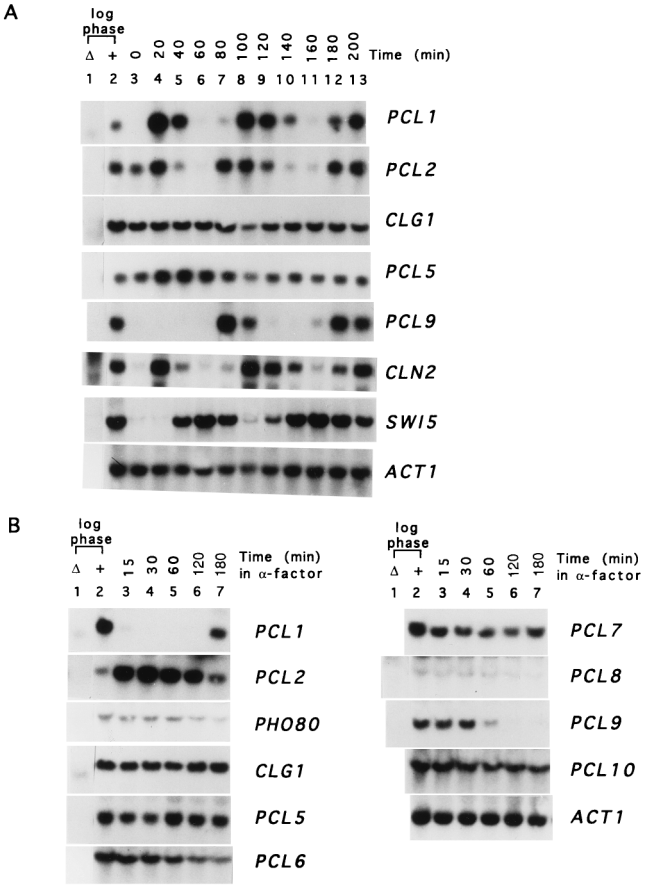

FIG. 3. Analysis of *PCL* gene expression. (A) Cell cycle Northern blot analysis using a pheromone block/release method (see Materials and Methods). Lane 3, RNA isolated from strain BY263 immediately after release from the α-factor block; lanes 4 to 13, RNA isolated every 20 min following inoculation into fresh medium as indicated above the lanes (time). Lane 1 in each panel contains RNA isolated from the following deletion strains: BY628 (*pcl1*Δ), *PCL1* blot; BY271 (*pcl2*Δ), *PCL2* blot; BY393 (*clg1*Δ), *CLG1* blot; BY527 (*pcl5*Δ), *PCL5* blot; BY694 (*pcl9*Δ), *PCL9* blot; BY431 (*cln2*Δ), *CLN2* blot; and BY177 (*swi5*Δ), for *SWI5* blot (Table 1). Lane 2 in each panel shows RNA isolated from wild-type log-phase cells (BY263). Blots were probed with *PCL1*, *PCL2*, *CLG1*, *PCL5*, *PCL9*, *CLN2*, *SWI5*, and *ACT1* as indicated to the right. (B) *PCL* transcript levels in pheromone-treated cells. In all panels, lane 2 shows total RNA isolated from wild-type log-phase cells (BY263) and lanes 3 to 7 show total RNA isolated from cells exposed to α-factor for the times indicated above the lanes. Lane 1 shows RNA isolated from log-phase *pcl* strains if available. The *pcl1*, *pcl2*, *clg1*, *pcl5*, and *pcl9* deletion strains were as described for panel A. In addition, the following deletion strains were used: BY490 (*pho80*Δ), *PHO80* blot; and BY629 (*pcl8*Δ), *PCL8* blot. No knockout strains were available for *PCL6*, *PCL7*, or *PCL10*. Blots were probed with *PCL1*, *PCL2*, *PHO80*, *CLG1*, *PCL5*, *PCL6*, *PCL7*, *PCL8*, *PCL9*, *PCL10*, and *ACT1* as indicated to the right.

a very coarse granular cytoplasm, and often had a somewhat elongated, abnormal cell shape (Fig. 4f).

If the Pcls function redundantly to promote Pho85 function, then deletion of multiple *PCL* genes might lead to morphological abnormalities comparable to those seen in *pho85* mutants. We focused on the Pcl1,2 subfamily because of the known involvement of Pcl1,2 in cell cycle progression. In haploids, *pcl1*Δ *pcl2*Δ *clg1*Δ *pcl5*Δ cells were morphologically indistinguishable from wild-type cells (Fig. 4a compared to Fig. 4c). However, deletion of *PCL9* in this strain, which disrupted all five members of the Pcl1,2 subfamily, resulted in slightly larger cells, some of which were morphologically similar to the *pho85*Δ strain (Fig. 4d).

As for the *pho85*Δ strain, we found that morphological abnormalities of the multiple *pcl* mutants were more apparent in diploid strains. A variety of single and double *pcl* diploid deletion strains were analyzed, and all were similar to the wild type in morphology (Fig. 4g to i), although a *pcl5*Δ *pcl9*Δ strain contained some connected chains of cells (Fig. 4h). Cells lacking *pcl1*, *pcl2*, and *pcl9* exhibited an obvious morphological defect (Fig. 4j). Of 200 *pcl1*Δ *pcl2*Δ *pcl9*Δ diploid cells counted, 5% had highly elongated buds. Some connected chains of cells with multiple nuclei (visible by 4′,6-diamino-2-phenylindole staining [data not shown]) were also evident. These phenotypes are characteristic of diploid pseudohyphal cells (22, 33) and also of cells defective in cytokinesis or cell separation. A higher percentage of cells with pronounced, elongated buds was seen in quadruple *pcl* mutants (Fig. 4k to n). The highest percentage of morphologically abnormal cells (30% elongated buds of 200 cells counted) was seen in the quintuple mutant with all members of the Pcl1,2 subfamily deleted (Fig. 4o). This morphological analysis suggests that the Pcl1,2 subfamily members have a common function, as a more severe phenotype is seen when all members are mutated. However, the quintuple diploid mutant does not look like the *pho85* diploid mutant, most likely because the Pho80 subfamily of cyclins is still present in the cell. Morphological abnormalities in all *pcl* mutant strains were more pronounced in rich medium (YPD) than in synthetic medium (SD).

DISCUSSION

Pho85 is a cyclin-dependent kinase that associates with the cyclin Pho80 to negatively regulate acid phosphatase gene expression and with the cyclins Pcl1 and Pcl2 to play a role in cell cycle progression. Cells with *PHO85* deleted have several phenotypes not seen in *pho80*Δ *pcl1*Δ *pcl2*Δ mutants, suggesting the existence of other Pho85 cyclins. We performed a two-hybrid screen to identify new proteins that interact with Pho85. In this screen, and through database searching, we identified seven other cyclin-like proteins with homology to Pho80, Pcl1, and Pcl2. One of these proteins, Clg1, was previously noted to be similar to Pcl1 and Pcl2 (37). We have named the other cyclins Pcl5, Pcl6, Pcl7, Pcl8, Pcl9, and Pcl10. On the basis of sequence similarity, the Pcls define a cyclin family with two distinct subfamilies (Fig. 1A). Deletion of multiple members of the Pcl1,2 subfamily caused pronounced morphological defects consistent with an overlapping role for this group of cyclin genes in processes related to cell polarity or cell division. Our data suggest that Pho85 interacts with and may be regulated by as many, if not more, cyclin subunits as the closely related kinase, Cdc28.

We tested all of the Pcl-like cyclins except Pho80 for binding to Pho85 both in the two-hybrid assay and in a batch Gst-Pho85 affinity chromatography assay. Others have previously shown that Pho80 binds Pho85 in the two-hybrid assay (26) and in immunoprecipitation experiments (30). The extent of activation of reporter constructs due to interaction of the various Pcls and Pho85 in the two-hybrid assay varied widely, as did the binding of Pcls to Pho85 in vitro (Table 2; Fig. 2). Pcls that bound well in the in vitro assay did not necessarily show high levels of reporter activation in the two-hybrid test. This variability likely reflects differences in expression of fusion constructs in vivo and the quality of translated protein in vitro. Therefore, conclusions about the relative affinities of the various Pcls for Pho85 cannot be drawn. Nonetheless, the two assays clearly show that the Pcl group of cyclins are able to interact with Pho85. A definitive demonstration that the new Pcls function as Pho85 cyclins in vivo awaits the development

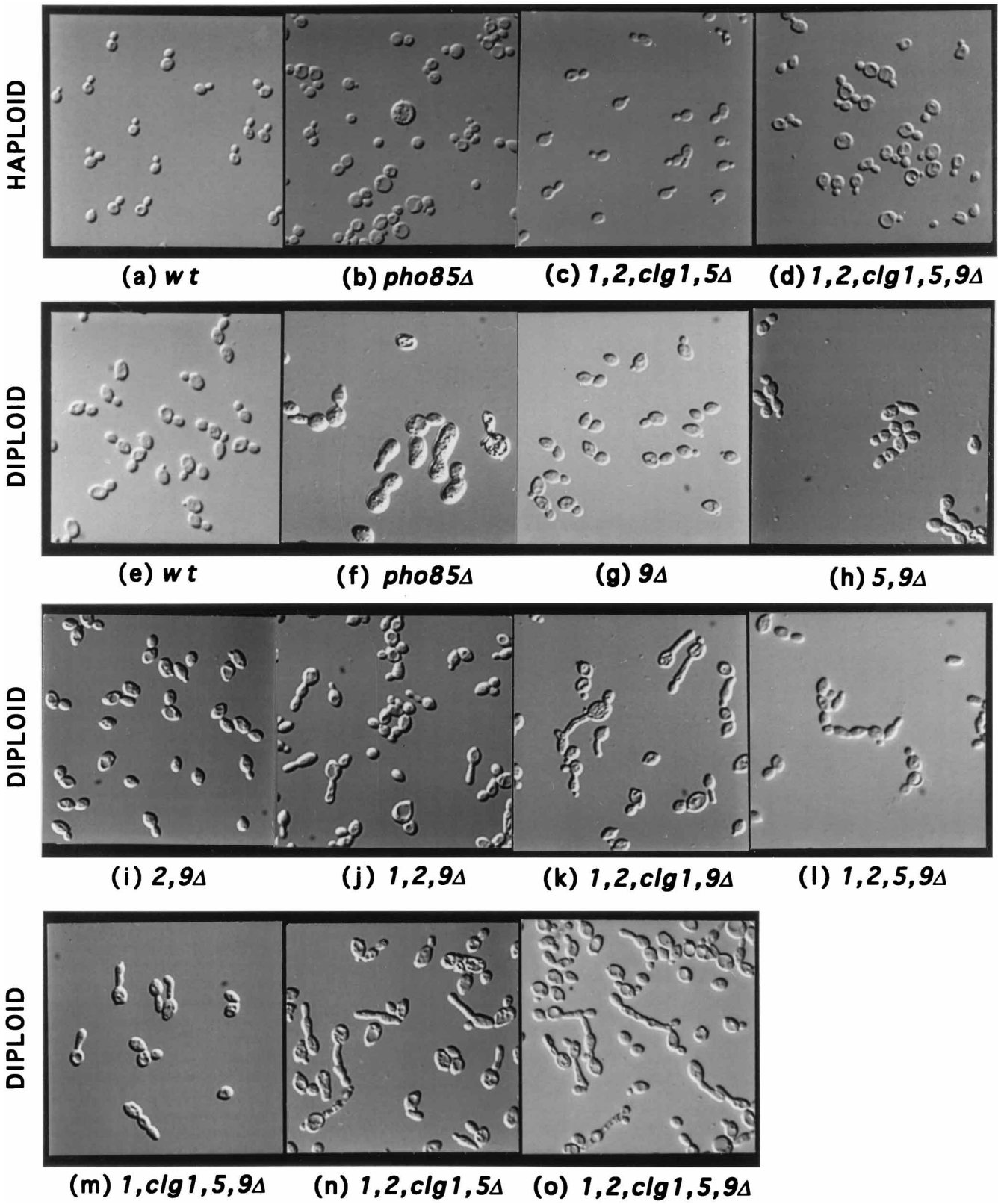

FIG. 4. Morphologies of *pcl* and *pho85* mutant cells. Cells were grown in rich medium to log phase and photographed at magnification of ×400, using Nomarski optics and an imaging system as described in Materials and Methods. Panels a through d show photographs of haploid cells. All other panels show homozygous diploids as indicated to the left. *PCL* gene disruptions are abbreviated to show only the number; for example *pcl2*Δ is indicated as 2. (a) Wild type (wt), BY263; (b) *pho85*Δ, BY391; (c) BY637; (d) BY714; (e) BY262; (f) BY559; (g) BY723; (h) BY725; (i) BY724; (j) BY726; (k) BY727; (l) BY728; (m) BY729; (n) BY731; (o) BY730. Full genotypes are listed in Table 1.

of reagents for immunoprecipitation and kinase assays from yeast extracts.

We also identified in our two-hybrid screen three other cyclins, Ccl1, Srb11, and Cln1, that are known to interact with other Cdks in vivo (reviewed in references 8, 50, and 52). However, Ccl1 and Cln1 do not bind Pho85 well in our in vitro binding assay, and we think that our isolation of Ccl1, Srb11, and Cln1 may be an artifact of the two-hybrid system due to overexpression of the proteins. It is not surprising that a broad range of cyclins, beyond the Pcl family, interact with Pho85 in the two-hybrid assay. Overexpression of cyclins is known to encourage interaction with Cdks that are not natural partners for a given cyclin. For example, overexpression of *CCL1*, which is an activating subunit for the Kin28 Cdk, rescues the inviability of a *cln1*Δ*2*Δ*3*Δ strain, presumably due to association with Cdc28 (66).

Several observations suggest *PCL1*, *PCL2*, and *PCL9* are regulated differently from the rest of the Pho85 cyclins. First, *PCL1*, *PCL2*, and *PCL9* genes were periodically expressed, with *PCL2* and *PCL9* transcripts peaking at a similar time, just slightly earlier than *PCL1* (Fig. 3A). Second, the expression of these three genes was affected by pheromone treatment whereas expression of other *PCL*s remained constant. Finally, Pcl1, Pcl2, and Pcl9 have a high degree of sequence similarity; for example, Pcl2 and Pcl9 are 64% identical over 229 amino acids (Fig. 1C). Since genetic experiments have shown a role for Pcl1,2-Pho85 kinase complexes at Start, it is possible that *PCL9* plays a similar role that will be revealed by appropriate genetic experiments and the identification of relevant substrates for these kinase complexes.

Although the cell cycle-regulated expression of *PCL9* is comparable to that of *PCL2*, there are differences that suggest that expression of *PCL9* may be controlled by different regulatory proteins. The *PCL9* gene was not expressed in the first cell cycle after mating pheromone arrest (Fig. 3), a pattern of expression unique among known cyclins. The expression patterns of the *HO* and *CTS1* genes are similar to that of *PCL9*; neither gene is expressed in the first cell cycle after mating pheromone treatment, and expression of *HO* and *CTS1* is G_1 periodic in subsequent cell cycles (12). Expression of *HO* requires the Swi5 transcription factor, while *CTS1* is regulated by a homologous transcription factor, Ace2 (12). Other genes controlled by both Swi5 and Ace2 include *SIC1*, which encodes a Cdk inhibitor, and *RME1*, which is required for repression of meiosis and appears to play a role in the activation of G_1 cyclin gene expression (32, 63). The *PCL9* upstream regulatory region contains a sequence that fits the consensus for a Swi5 binding site (57a). Thus, *PCL9* may be a novel target of the Swi5 transcription factor.

Transcription of the *PCL5* gene was also detectably cell cycle regulated, with peak transcript levels throughout G_1. G_1-periodic expression of *PCL1*, *PCL2*, *CLN1*, and *CLN2* is controlled largely by the SBF transcription factor that binds to *SWI4*-dependent cell cycle box (SCB) sites upstream of target genes. A related transcription factor, MBF, controls Start-dependent transcription of a number of genes required for S phase and acts through a sequence element called the *Mlu*I cell cycle box (MCB) (reviewed in references 4 and 32). Although *PCL9* transcript appeared in the second cell cycle at about the same time as *PCL2* transcript, the upstream regulatory sequences of *PCL9* do not contain consensus SCB or MCB sites. By contrast, the *PCL5* upstream sequences contain one consensus SCB site (CACGAAA) and two similar sites (CACGA A), suggesting that *PCL5* may be a new target of the SBF transcription factor. Whether expression of *PCL5* and *PCL9* is affected by mutation of *SWI5*, *ACE2*, or the genes encoding components of SBF remains to be determined.

The significance of the transcript regulation of *PCL1*, *PCL2*, and *PCL9* during pheromone-induced G_1 arrest is not clear. Levels of *PCL1* transcript decrease rapidly in response to pheromone, while *PCL9* transcript lingers for almost 1 h. Transcript levels of the G_1 cyclin genes *CLN1* and *CLN2* also decrease rapidly in response to pheromone (68), and this regulation contributes to cell cycle arrest. *PHO85* mutants show marked supersensitivity to pheromone (37a). So far, we have not found a significant defect in cell cycle arrest or recovery in response to pheromone in any combination of *pcl1*, *pcl2*, and *pcl9* mutants or in strains overexpressing *PCL1* and *PCL2*. Perhaps other *PHO85*-encoded cyclins compensate for the loss of the *PCL1,2,9*-encoded cyclins during α-factor arrest.

Deletion of the entire Pcl1,2 subfamily resulted in pronounced morphological defects, suggesting an overlapping role for this group of cyclins in processes determining cell morphology. The morphological phenotype became more pronounced as more *PCL*s were deleted; a larger fraction of quadruple and quintuple mutant cells exhibited elongated cells, connected chains of cells, and a pattern of growth consistent with unipolar budding. These characteristics are reminiscent of diploid cells executing the pseudohyphal growth pathway, which display polarized budding in one direction away from the mother cell among other phenotypes (22). Due to the variable morphology of multiple Pcl knockouts and diffuse Calcofluor staining seen in cells with connected chains of buds, we were unable to determine the number of cells executing a unipolar budding pattern. However, cells in the population that gave localized Calcofluor staining had a random budding pattern, as opposed to the normal diploid pattern of bipolar budding (34a), consistent with a defect in bud site selection and polarized cell growth.

The genetic redundancy suggested for the family of Pho85 cyclins is already well established for the Cdc28 family of cyclins in *S. cerevisiae*. Cdc28 cyclins can be grouped by their essential functions at different stages of the cell cycle. Cln1, Cln2, and Cln3 are essential at Start, Clb1 to Clb4 are essential for mitosis, and Clb5 and Clb6 are important for proper progression through S phase (41). Because Pho85 is not an essential kinase, it is unlikely that any combination of Pho85 cyclin knockouts will reveal an essential function at a particular stage of the cell cycle. However, mutation of the Pcl1,2 subfamily suggests an overlapping role for this subgroup of cyclins. As more Pcls from this subfamily were deleted, a more striking effect on bud morphology was seen. Mutation of the Pho80 group of cyclins may also reveal an overlapping role for this cyclin subfamily. For example, *pho85* mutants are defective in glycogen synthase kinase activity and show a marked hyperaccumulation of glycogen (27, 61), while a strain with multiple members of the Pcl1,2 subfamily of cyclins deleted showed no dramatic glycogen accumulation phenotype (27a). As such, members of the Pho80 subfamily of cyclins may function to activate Pho85 in its role as a glycogen synthase kinase. The Pho85 cyclin-Cdk complexes are likely to provide a useful model for how a single Cdk can act in several distinct biological processes, perhaps allowing coordination of environmental and nutritional signals with cell cycle progression.

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