pct1⁺, which encodes a new DNA-binding partner of p85^{cdc10}, is required for meiosis in the fission yeast *Schizosaccharomyces pombe*

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The transcriptional activation of genes at late G₁ is an important regulatory step in the commitment to a new cell division cycle. In *Schizosaccharomyces pombe*, this regulation is mediated by MCB elements that serve as binding sites for the MBF/DSC-1 complex. The *cdc10*⁺-encoded protein is a component of this complex. We report the cloning of a new gene, *pct1*⁺, encoding a 73-kD protein that interacts with p85^{cdc10} to form an MCB-binding heteromer. *Pct1*⁺ is related to, but distinct from, the *res1*⁺/*sct1*⁺ gene that also encodes a p85^{cdc10} partner. p73^{pct1} has centrally located ankyrin repeats and a putative amino-terminal DNA-binding domain that has extensive sequence similarity to the DNA-binding domains of the *Saccharomyces cerevisiae* SWI4 and MBP1 proteins. The p73^{pct1}/p85^{cdc10} complex binds both in vitro and in vivo to MCB but not SCB or E2F sites. Overexpression of *pct1*⁺ is sufficient to rescue the growth of the *cdc10-129* temperature-sensitive mutant at the restrictive temperature, although it is unable to rescue a *cdc10* null mutation. A deletion of *pct1*⁺ is not lethal but does result in a severe meiotic defect. Our results indicate that there are two *cdc10*-containing heteromeric complexes that bind to MCB elements and play differential roles in mitotic division and meiosis.

[Key Words: *pct1*⁺ gene; fission yeast; transcriptional activation; DNA binding; meiosis]

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In all eukaryotic cells, a key regulatory step of the cell cycle occurs at the G₁/S transition. In yeast, G₁ cells can continue to divide, enter a quiescent state (stationary phase), or undergo sexual differentiation depending on the environmental and nutritional conditions. Once cells have reached a point in G₁ called Start, they become committed to a new round of DNA synthesis and division irrespective of the prevailing conditions. A similar decision point called the "restriction point" has been described for the mammalian cell cycle (for review, see Cross et al. 1989; Forsburg and Nurse 1991).

In the yeast *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, classical genetics has identified genes crucially involved in Start. *S. pombe* cells need the *cdc2*⁺ gene encoding a serine/threonine kinase, which is also necessary for the initiation of mitosis (Nurse and Bisset 1981). In addition, they require the *cdc10*⁺ gene encoding a protein that is a component of a transcription complex that regulates late G₁-specific expression (Lowndes et al. 1992b). As well as their critical role in mitotic growth, both *cdc2*⁺ and *cdc10*⁺ are also re-

quired for cells to undergo meiosis successfully (Grallert and Sipiczki 1991).

In yeast, it is now well established that gene activation is an important feature of Start. In *S. cerevisiae*, Start-specific transcription involves specific *cis*-acting regulatory sequences known as the SCB and MCB elements that serve as binding sites for the SBF and MBF complexes, respectively. SBF consists of a heteromer of the SWI4 and SWI6 proteins (Andrews and Herskowitz 1989a,b; Taba et al. 1991), whereas the MBF complex is a heteromer between the SWI6 and MBP1 proteins (Lowndes et al. 1991, 1992a; Dirick et al. 1992; Koch et al. 1993). SWI4 and MBP1 proteins are structurally related, containing a conserved central domain consisting of two or more ankyrin repeats (Koch et al. 1993) and highly similar amino-terminal DNA-binding domains (Primig et al. 1992; Koch et al. 1993). Several target genes for these complexes have been identified. MCB elements in the promoters of many genes involved in DNA synthesis ensure their transcription prior to the initiation of the S phase (Epstein and Cross 1992; Merrill et al. 1992; Schwob and Nasmyth 1993). SCB elements control the expression of *CLN1* and *CLN2*, which encode G₁-specific cyclins (Cross and Tinkelenberg 1991; Dirick and

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Nasmyth 1991; Nasmyth and Dirick 1991; Ogas et al. 1991). The activation of *CLN1* and *CLN2* transcription in late G₁ is critical for entry into S phase. The mechanism that underlies the cell cycle-regulated activity of SBF and MBF is not understood. Both are dependent on the CDC28 kinase (Cross and Tinkelenberg 1991; Dirick and Nasmyth 1991), suggesting that they may be direct targets of this kinase and, as a consequence, be regulated by phosphorylation.

In fission yeast, transcriptional regulation at Start is also mediated by MCB elements. The product of the *cdc10⁺* gene, p85^{cdc10}, is a component of the complex that binds to this element (Lowndes et al. 1992b). It shows significant similarity to the *S. cerevisiae* SWI4/SWI6 family of proteins, including centrally located ankyrin repeats (Breedon and Nasmyth 1987). Like the *S. cerevisiae* SWI6 protein, it does not appear to bind MCB elements directly. A new gene required for Start was recently identified (*res1⁺/sct1⁺*) that encodes a 72-kD protein (Tanaka et al. 1992; Caligiuri and Beach 1993) that binds to MCB elements in partnership with p85^{cdc10}. The amino-terminal domain of *res1⁺/sct1⁺* shows a high degree of conservation with the DNA-binding domains of MBP1 and SWI4. Three different *S. pombe* genes regulated by MBF (also known as DSC1) have been identified. One of these, *cdc22⁺*, encodes a subunit of ribonucleotide reductase (Gordon and Fantes 1986; Fernandez-Sarabia et al. 1993), whereas the exact function of the other two genes, *cdc18⁺* (Kelly et al. 1993) and *cdt1⁺* (Hofmann and Beach 1994), remains obscure.

In this paper we describe the cloning and characterization of an *S. pombe* gene that encodes a new partner of p85^{cdc10}, which we call *pct1* (partner of *cdc ten*). Like *res1⁺/sct1⁺*, *pct1⁺* contains an amino-terminal domain highly similar to the SWI4/MBP1 DNA-binding domain and centrally located ankyrin repeats. We show that the *pct1⁺* protein forms a complex with p85^{cdc10} that specifically binds to MCB elements. *pct1⁺* plays a minor role in mitotic growth but is essential for meiosis. Our results indicate, therefore, that different *cdc10⁺*-containing heteromeric complexes are of crucial importance in mitotic and meiotic division.

Results

Cloning of *pct1⁺*

It had been shown previously that the product of the *S. pombe cdc10⁺* gene (p85^{cdc10}) is a component of the DSC1 or MBF complex (Lowndes et al. 1992b) that specifically binds to MCB elements in the promoters of *S. pombe* genes activated at the G₁/S transition of the cell cycle. We used a genetic screen in *S. cerevisiae* to determine whether the p85^{cdc10} protein could bind directly to DNA. The assay was carried out in a strain (YZ100) that is disrupted for the *SWI6* gene and contains an integrated copy of a hybrid gene consisting of the *lacZ*-coding region fused downstream of a 69-bp fragment of the *S. cerevisiae* thymidine synthase gene (*TMP1*) promoter. This

region of the *TMP1* promoter contains two direct copies of the MCB element. In a wild-type strain this fusion gene is activated by the *S. cerevisiae* MBF complex (a complex between the SWI6 and MBP1 proteins). However, because of the SWI6 disruption in YZ100, this complex cannot form and the level of *lacZ* expression is consequently reduced. At present, it is not known whether p85^{cdc10} contains a transcriptional activation domain and, if so, whether it could function in the heterologous *S. cerevisiae* system. Therefore, to ensure that direct or indirect binding of p85^{cdc10} to the 69-bp *TMP1* promoter fragment would result in strong *trans*-activation of the *lacZ* gene, we constructed and expressed a fusion gene encoding a chimeric protein consisting of p85^{cdc10} fused at its amino terminus to the activation domain of the herpes simplex virus VP16 protein. This activation domain of VP16 has been shown to function in a variety of eukaryotic backgrounds, including yeast. Expression of the fused gene was controlled by a promoter containing the galactose-inducible GAL1-10 upstream activating sequence (UAS). Expression of this chimeric protein did not result in any increase in *lacZ* expression (Table 1). This indicated that p85^{cdc10} could neither bind to the MCB elements on the *TMP1* gene promoter directly nor bind indirectly in conjunction with endogenous *S. cerevisiae* factors. This suggests that p85^{cdc10} interacts with MCB elements as a result of heterodimerization with additional *S. pombe* proteins. To identify partners of p85^{cdc10} that enable such interaction, we transformed YZ100 expressing the *vp16-cdc10⁺* fusion with an *S. pombe* cDNA library, expressed in a high-copy *S. cerevisiae* vector (Fikes et al. 1990), and driven by the *S. cerevisiae* alcohol dehydrogenate (ADH) promoter (Fig. 1). Approximately 1 million transformants were screened for increased *lacZ* expression following growth on galactose-containing medium, and a number of transformants (150) showing increased β -galactosidase activity were selected. However, only one transformant showed increased *lacZ* activity in a manner that was strictly dependent on galactose induction (Table 1), indicating a reliance on the expression of the *vp16-cdc10⁺*

Table 1. Cooperative interaction between *pct1* and *cdc10* in strain YZ100

Constructs	β -Galactosidase level	
	glucose	galactose
None	1.0	1.4
pGAL1-10-VP16-CDC10	1.5	1.0
pADH-PCT1, pGAL1-10-VP16-CDC10	2.5	40
pADH-PCT1	1.5	<1

The level of β -galactosidase expression in the strain YZ100 harboring the indicated plasmids was measured following growth on glucose- and galactose-containing medium (Materials and methods). The results are expressed as the fold increase in comparison to the level of β -galactosidase expression in the YZ100 carrying no plasmid and growing on glucose containing medium.

Figure 1. Schematic representation of the screening strategy. A genetic screen was carried out in a *SWI6*-disrupted strain of *S. cerevisiae* containing a *TMP1* promoter-*lacZ* hybrid gene integrated at the *URA3* locus. The promoter contains two copies of the MCB element. These cells remain white in a *lacZ* assay. A plasmid was introduced into this strain containing a *vp16-cdc10*⁺ hybrid gene under the transcriptional control of a galactose-inducible promoter. These transformed cells also remained white in a *lacZ* assay. This derivative was used as the recipient for transformation with a cDNA library, and the resulting transformants were assayed for *lacZ* activity. Positive colonies that turned blue upon assay were chosen for further study.

fusion gene. Curing this transformant of either the *vp16-cdc10*⁺-containing plasmid or the cDNA-containing plasmid resulted in loss of high *lacZ* expression levels (Table 1). The cDNA plasmid present in this transformant was therefore chosen for further study and named *pct1*⁺.

pct1⁺ is homologous to the *S. cerevisiae* *SWI4* and *MBP1* genes and the *S. pombe* *res1*⁺/*sct1*⁺ gene

The nucleotide sequence of the *pct1*⁺ cDNA revealed an open reading frame of 1971 bp predicted to encode a polypeptide of 657 amino acids with a molecular mass of 73 kD (Fig. 2A). It shares two principal regions of homology to the *S. cerevisiae* *SWI4* and *MBP1* proteins and the *S. pombe* *res1*⁺/*sct1*⁺ protein (Fig. 2B; Andrews and Herskowitz 1989a; Tanaka et al. 1992; Caligiuri and Beach 1993; Koch et al. 1993). The first corresponds to the amino terminus, which in the case of *SWI4*, has been shown to constitute the DNA-binding domain (Primig et al. 1992). The homology among the four proteins in this region ranges from 45% to 50% identity, with *pct1*⁺ being most homologous to *res1*⁺/*sct1*⁺.

The second region of homology is centrally located and is conserved in all known components of SBF and MBF complexes from *S. cerevisiae* and *S. pombe*. The conservation in this region consists of two ankyrin repeats that closely resemble the ankyrin consensus sequence. The residues that lie between the two repeats

are ankyrin repeat-like and may therefore represent highly degenerate examples. The function of the repeats in these proteins is presently unclear, although the high level of conservation that exists suggests that they must play a critical role.

In addition to these two principal regions of homology, some sequence similarity is also evident in the carboxyl terminus (Fig. 2B). The carboxy-terminal region of *SWI4* has been shown to be the region that interacts with *SWI6* (Sidorova and Breeden 1993).

p73^{pct1} and *p85^{cdc10}* form an MCB-binding complex in vitro

Results of the genetic screen suggested that *p73^{pct1}*, together with *p85^{cdc10}*, can bind to the 69-bp promoter fragment of the *TMP1* gene. To test this directly, we performed electrophoretic mobility-shift assays (EMSAs) with *pct1*⁺- and *cdc10*⁺-encoded proteins translated in rabbit reticulocyte lysates.

Three different probes were used for this assay: (1) the 69-bp *TMP1* fragment used in the genetic screen, which contains two perfect MCB sites (Primig et al. 1992); (2) a 131-bp promoter fragment from the *S. pombe* *cdc22*⁺ gene, containing one perfect MCB and three near-match MCB sites; and (3) a 142-bp fragment from the 5' upstream region of the *S. pombe* *cdc18*⁺ gene (Kelly et al. 1993), which contains six near-match MCB sites (all of the near-match MCBs have at least the core CGCG se-

quence conserved). All three of these genes are transcriptionally activated at the G₁/S transition and are dependent on a functional SWI6 protein in the case of *TMP1* in *S. cerevisiae* (Dirick et al. 1992; Lowndes et al. 1992a) and a functional *cdc10* protein in the case of *cdc22*⁺ (Lowndes et al. 1992b) and *cdc18*⁺ (Kelly et al. 1993) in *S. pombe*.

Reticulocyte lysates programmed with *pct1*⁺ RNA synthesized a product close to the predicted size of 73 kD. This in vitro-translated product failed, on its own, to generate specific complexes on any of the three probes used (Fig. 3, lanes 1,5,9). This was also the case with in vitro-translated *p85*^{cdc10} (Fig. 3, lanes 2,6; data not shown), which is consistent with our in vivo results (Table 1). In contrast, incubation of the probes with a mixture of *p73*^{pct1} and *p85*^{cdc10} proteins resulted in all cases in a single prominent slow migrating complex with each probe (Fig. 3, lanes 3,4,7,8,10,11). Increasing the amount of each protein in the reaction increased the extent of complex formation (Fig. 3, lanes 4,8,11). The results suggest that *p73*^{pct1} and *p85*^{cdc10} generate a heteromeric complex capable of binding in vitro to probes containing MCB sites.

In *S. cerevisiae*, the SWI6 protein associates with two different DNA-binding subunits, giving rise to complexes that have related but distinct binding specificity.

Figure 3. *p73*^{pct1} and *p85*^{cdc10} can form a heteromeric complex that can bind to MCB sites. Three different radioactive probes containing MCB sites were incubated with rabbit reticulocyte lysates containing *p73*^{pct1} or *p85*^{cdc10}. The different probes used are indicated and derived from either the *S. cerevisiae* *TMP1* promoter, the *S. pombe* *cdc18*⁺ promoter, or the *S. pombe* *cdc22*⁺ promoter. The amount of each lysate used in the binding reaction is indicated at the top. The arrowheads indicate the position of the free probe, and the arrows indicate the positions of the specific complexes.

The SWI4/SWI6 heteromer binds to the SCB element [consensus sequence: CACGAAA (Andrews and Herskowitz 1989a,b)] whereas the MBP1/SWI6 heteromer binds to MCB sites [consensus: ACGCGTNA (Lowndes et al. 1991, 1992a; Dirick et al. 1992; Koch et al. 1993)]. The observation that *p85*^{cdc10} can also associate with two different DNA-binding partners, namely, *p73*^{pct1} and the previously described product of the *res1*⁺/*sct1*⁺ gene (Caligiuri and Beach 1993), prompted us to examine the binding specificity of the *p73*^{pct1}/*p85*^{cdc10} complex. The fact that *p73*^{pct1} can bind to MCB sites does not rule out the possibility that other sites can also be recognized.

The specificity of the *p73*^{pct1}/*p85*^{cdc10} complex was examined by investigating complex formation on two additional probes. One of these probes, *HOSRS2*, derives from the promoter of the *S. cerevisiae* *HO* gene and contains four copies of the SCB element. The other was a 50-bp fragment derived from the adenovirus *E2A* promoter containing two binding sites for the mammalian factor E2F. E2F is involved in late G₁-specific regulation of transcription and binds to a sequence (consensus: CGCGAAA) that shows similarity to the SCB and MCB elements. Incubation of either the *HOSRS2* or *E2A* promoter fragments with *p73*^{pct1}, *p85*^{cdc10}, or both did not result in any specific complexes similar to those seen with the *cdc18*⁺ and *cdc22*⁺ probes, indicating that the *p73*^{pct1}/*p85*^{cdc10} heteromer binds to MCB but not SCB or E2F sites (Fig. 4A). The faster migrating complex observed in all reactions utilizing the *E2A* probe was the result of E2F-binding activity in the rabbit reticulocyte lysate; its presence was not dependent on the synthesis in the lysate of any exogenous protein (Fig. 4A, lanes 11,12).

The binding specificity was confirmed by competition analysis. The binding of the *p73*^{pct1}/*p85*^{cdc10} complex to the *cdc18*⁺-derived probe was carried out in the presence of excess of unlabeled oligonucleotides or DNA fragment containing MCB-, SCB-, or E2F-binding sites. Competition was observed with *cdc18*⁺, *cdc22*⁺, and *TMP1* promoter fragments, although the efficiency did vary, with the *cdc18*⁺ fragment being the most efficient (Fig. 4B, lanes 2–7). In contrast, however, no competition was observed with *HOSRS2* or *E2A* promoter fragments or with a nonspecific fragment (Fig. 4B, lanes 8–13).

pct1⁺ can rescue a *cdc10*^{ts} phenotype

The biochemical evidence described above clearly indicates that the proteins encoded by the *pct1*⁺ and *cdc10*⁺ genes can form a heteromer that specifically binds to MCB elements. However, expression of a *vp16*-*pct1* fusion in YZ100 indicated that in vivo, *p73*^{pct1} could bind to MCB sites alone (data not shown). Accordingly, we investigated whether overexpression of the *pct1*⁺ gene could suppress mutations in the *cdc10*⁺ gene. We first examined whether *pct1*⁺ could rescue a *cdc10*^{ts} phenotype. Two different *pct1*⁺-containing expression plasmids were constructed: In one (*pADH*-*pct1*⁺) the *pct1*⁺

Figure 4. Specificity of $p73^{pct1}/p85^{cdc10}$ binding. (A) Mobility retardation assays using rabbit reticulocyte lysates containing $p73^{pct1}$ and $p85^{cdc10}$ and radioactive probes that contain MCB, SCB, or E2F sites. The different probes are indicated. The $cdc18^+$ and $cdc22^+$ probes both contain MCB elements. The HOSRS2 probe derives from the *S. cerevisiae* *HO* gene promoter and contains four copies of the SCB element. The E2A probe derives from the adenovirus E2A gene promoter and contains two copies of the E2F site. (B) The $cdc18^+$ probe was incubated with $p73^{pct1}$ - and $p85^{cdc10}$ -containing lysates in the presence of the indicated unlabeled competitor probes at a molar excess ratio of 50:1 or 100:1.

cDNA was driven by the strong constitutive *adh⁺* promoter, whereas in the other ($pRep-pct1^+$) it was driven by the thiamine-regulated *nmt1⁺* promoter. *cdc10-129* cells were transformed with these plasmids, and growth at 37°C was assessed. As shown in Figure 5A, growth was observed in both cases, whereas no growth was obtained with cells transformed with the same vectors but containing no *pct1⁺* insert. The results indicate that ectopic expression of *pct1⁺* can rescue the defective growth of the *cdc10^{ts}* mutant at the restrictive temperature. The suppression was not absolute, however. When exponentially growing cultures of the $pADH-pct1^+$ transformants at 25°C were shifted to 37°C, ~30% of the cells displayed an elongated cell shape characteristic of *cdc10* mutants (data not shown). This population was slightly larger, with the $pRep-pct1^+$ transformant possibly reflecting different levels of *pct1⁺* expression.

In contrast to the results with the *cdc10^{ts}* mutant, we were unable to rescue the growth of a *cdc10* null mutant by *pct1⁺* overexpression (data not shown). A heterozygous diploid strain (NT7) containing a deletion of one of the *cdc10* alleles was transformed with the $pADH-$

pct1⁺ plasmid and induced to sporulate. No *ura⁺ leu⁺* colonies that would derive from haploid cells containing both the *cdc10* null mutation and $pADH-pct1^+$ plasmid could be recovered. We conclude, therefore, that *pct1⁺* cannot rescue a *cdc10* null phenotype.

To identify functional domains of *pct1⁺*, a number of deletion mutants were generated and tested for their ability to complement the *cdc10-129* temperature-sensitive phenotype. The results are summarized in Figure 5B. Removal of carboxy-terminal sequences, including one of the two ankyrin repeats, resulted in mutants ($pRep-pct1-\Delta PstI$ and $pRep-pct1-\Delta ClaI$) that could still rescue, although less efficiently than full-length *pct1⁺*. However, extension of the deletion to include both ankyrin repeats led to complete loss of complementing activity ($pRep-pct1-\Delta EcoRI$). Although not tested directly, we would predict that the amino-terminal sequences would be essential because the homology with SWI4 and Res1⁺/Sct1⁺ strongly implies that this region is directly involved in DNA binding. Expression of the amino-terminal domain alone, however, is not sufficient for complementation ($pRep-pct1-\Delta SalI$).

Figure 5. Complementation of *cdc10-129* by *pct1*⁺. (A) *cdc10-129* (NT8) cells transformed at 25°C with the indicated plasmids were streaked onto EMM media and incubated at 37°C for 4 days. The plasmid pADH-*pct1*⁺ is a *pct1*⁺-containing derivative of the vector pEVP11, and the plasmid pRep-*pct1*⁺ is a *pct1*⁺-containing derivative of the vector pRep3X. (B) A schematic of the full-length *pct1*⁺ cDNA isolated in this study is shown at the top. (Solid areas) Conserved ankyrin repeat; (hatched areas) conserved amino-terminal putative DNA-binding region. Restriction sites used for the construction of a number of *pct1*⁺ deletion mutants are indicated as follows: (S) *SalI*; (E) *EcoRI*; (C) *ClaI*; (P) *PstI*; (H) *HindIII*. The structure of the different mutants is shown below. *cdc10-129* cells were transformed with each construct and growth at 37°C analyzed as described previously. (+++, ++, and +) The relative colony size after growth for 4 days.

pct1⁺ is not essential for mitotic division

To investigate further the physiological role of *pct1*⁺, a null allele was constructed by replacing an internal 1-kb *EcoRI*–*PstI* fragment of the *pct1*⁺ cDNA with a 1.8-kb *ura4*⁺-containing fragment (Fig. 6A). This disrupted gene was unable to rescue the *cdc10-129* temperature-sensitive mutation, confirming its inactivation by the *ura4*⁺ insert (data not shown). A *HindIII* fragment containing the disrupted gene was introduced into the spor-

genic *S. pombe* diploid (NT1) from which stable *ura*⁺ colonies were selected. A number of these were subjected to Southern hybridization analysis, and one was chosen for further study (NT28). As shown in Figure 6B (lanes 1,6), this transformant contained two *pct1* alleles, one disrupted and one wild-type copy. The heterozygous diploid was induced to sporulate, and tetrads dissected. In all cases, all four spores gave rise to viable colonies. Southern hybridization analysis confirmed the normal segregation of two normal and two disrupted alleles [Fig. 6B, lanes 2–5, 7–10]. These results indicate clearly that *pct1*⁺ is not essential for mitotic growth. The growth characteristics of a *pct1*[–] strain were compared with that of a *pct1*⁺ strain by following growth in minimal SD medium at various temperatures. As shown in Table 2, at 30°C, a small difference in growth rate was seen,

Figure 6. Disruption of the *pct1*⁺ gene. (A) The construct used for the disruption of the *pct1*⁺ gene is shown schematically. The 1.0-kb *EcoRI*–*PstI* fragment in the 2-kb *pct1*⁺ cDNA was replaced by the *S. pombe ura4*⁺ 1.8-kb fragment, and the resulting disrupted *pct1* cDNA was used to transform a sporogenic *S. pombe* diploid (NT1) from which a stable *ura*⁺ clone (NT28) was selected. Restriction sites in the disrupted *pct1* and genomic *pct1*⁺ DNA are indicated as follows: (B) *BamHI*; (E) *EcoRI*; (EV) *EcoRV*; (H) *HindIII*; (P) *PstI*. (B) Substitution of the chromosomal *pct1*⁺ gene with the disrupted allele was demonstrated by Southern hybridization analysis. *HindIII*-digested DNA isolated from NT28 (lanes 1,6) and a set of tetrads (lanes 2–5, 7–10) was separated by agarose gel electrophoresis and blotted onto a nylon filter. The filters were hybridized to two probes: a *PstI*–*HindIII* 0.3-kb fragment containing *pct1*⁺ sequences (lanes 1–5) and the 1.8-kb *ura4*⁺-containing fragment (lanes 6–10).

Table 2. Generation time of *pct1*⁻ cells

Strain	Temperature (°C)		
	30	25 (in hours)	18
NT16 (<i>pct1</i> ⁺)	3.0	3.4	9.5
NT38 (<i>pct1</i> ⁻)	3.4	3.4	13.5

NT16 (*pct1*⁺) and NT38 (*pct1*⁻) cells were grown in SD minimal medium at 30°C. Each culture was diluted to a concentration of 2×10^6 cells/ml and shifted to the indicated temperature. Growth of the cells was followed by measuring the OD₅₉₅, and the generation time was calculated.

with the mean doubling time of the *pct1*⁻ cells being slightly longer. However, the difference in growth rate between *pct1*⁺ and *pct1*⁻ cells was accentuated at lower temperatures. At 18°C, *pct1*⁻ cells took at least 40% longer to divide (Table 2), and this was accompanied by a significant increase in cell length (data not shown). The results are qualitatively similar to those described by Tanaka et al. (1992) with cells deleted of the *res1*⁺ gene, which were found to be viable at 30°C but growth inhibited at lower temperatures. In the case of *pct1*⁻ cells, however, the phenotype was not as severe.

Flow cytometry was used to analyze the DNA content of *pct1*⁺ and *pct1*⁻ cells following their shift down from 30°C to 18°C. At 20 hr after the shift down, there was a significant increase in the number of *pct1*⁻ cells that had a 1N DNA content (Fig. 7C,D). No such increase occurred with *pct1*⁺ cells (Fig. 7A,B). The results suggest that at 18°C, *pct1*⁻ cells were partially arrested in G₁.

Further support for some involvement of the p73^{pct1}/p85^{cdc10} complex in cell growth came from the characterization of a diploid strain produced by crossing a *cdc10-129* mutant (NT9) and a *pct1*⁻ mutant (NT36). This strain (NT58) was induced to undergo meiosis, and the resulting spores were analyzed for viability. The results demonstrated that spores containing both mutations were unable to grow even at the permissive temperature of 25°C. Germination took place, but growth ceased within the first few divisions. Under these conditions, therefore, *res1*⁺/*sct1*⁺ was unable to compensate for loss of *pct1*⁺ function, presumably as a result of lower *cdc10* activity in these cells at the permissive temperature as compared with wild-type cells. The results imply that p73^{pct1}/p85^{cdc10} activity is present during mitotic growth and contributes to the overall MBF activity.

pct1⁺ is required for meiotic division

We then considered whether *pct1*⁺ has a role during meiosis. A *pct1*⁻ null derivative (NT43) of the homo-thallic *h*⁹⁰ strain was isolated from a genetic cross between *h*⁹⁰ cells (NT17) and *pct1*⁻ cells (NT38). When these cells were induced to undergo conjugation and sporulation by nitrogen starvation, we observed normal levels of conjugation and zygote formation (91.3% for NT43 as compared with 89.2% for NT17, an *h*⁹⁰*pct1*⁺

strain). However, the zygotes did not produce normal asci, indicating a defect at some stage during meiosis (data not shown).

To examine the meiotic defect in more detail, a homozygous *pct1*⁻ diploid strain (NT40) was constructed and induced to undergo meiosis. As shown in Figure 8, these *pct1*⁻ cells produced asci that were clearly abnormal. Unlike *pct1*⁺ cells that produced normal four-spore asci (Fig. 8A; Table 3) with evenly distributed nuclei (Fig. 8E; Table 3), the majority of the asci from *pct1*⁻ cells contained two or even fewer spores (Fig. 8B; Table 3). The distribution of nuclei in the asci was also highly irregular (Fig. 8F; Table 3), with some spores appearing to contain more than one nucleus. Clearly, therefore, *pct1*⁺ is required for the normal meiotic process. Ectopic expression of *pct1*⁺ by transformation of *pct1*⁻ cells with pRep-*pct1*⁺ efficiently complemented the meiotic defect (Fig. 8D,H; Table 3), whereas no complementation was detected with the pRep3X vector alone (Fig. 8C,G; Table 3). The series of *pct1* deletion mutants described previously were also tested for their ability to complement the meiotic defect of the *pct1*⁻ cells. As shown in Table 3, none of the mutants efficiently rescued the defect indicating the importance of the carboxy-terminal region in the function of *pct1*⁺. It is possible that pRep-*pct1*-ΔPstI and pRep-*pct1*-ΔClnI had some residual activity because the percentage of asci with no spores was decreased. However, there was no increase in the percentage of four-spore asci. These results are similar to

Figure 7. Flow cytometry analysis of *pct1*⁺ and *pct1*⁻ cells growing at 30°C and at 18°C. NT16 (*pct1*⁺) and NT38 (*pct1*⁻) cells were grown to mid-exponential phase (1×10^7 cells/ml) at 30°C in YES medium, and aliquots were analyzed by flow cytometry as described in Materials and Methods. A portion of the remaining cells was diluted (to 2×10^6 cells/ml), shifted to 18°C, and grown for 20 hr before flow cytometry analysis. (A) NT16 at 30°C; (B) NT16 at 18°C; (C) NT38 at 30°C; (D) NT38 at 18°C. In all cases, the major peak represents a 2N DNA content.

Figure 8. Terminal morphology of a *pct1*[−] diploid strain following nitrogen starvation. The following diploid strains were analyzed for meiotic defects: NT15 (*pct1*⁺; A,E); NT40 (*pct1*[−]; B,F); NT40+pRep3X (C,G), and NT40+pRep-*pct1*⁺ (D,H). Strains were grown to a cell concentration of 0.5×10^6 /ml in PM medium and then transferred to PM-N medium to induce sporulation at 30°C. In the case of NT15 and NT40, the PM medium was supplemented with leucine. After incubation for 24 hr, cells were collected, fixed, and stained with DAPI (4,6-diamidino-2-phenylindole) as described in Materials and methods. (A–D) Phase-contrast micrography; (E–H) fluorescent micrography. Bar, 10 μ m.

those obtained when the same mutants were tested for their ability to rescue the *cdc10-129* mutation. In both cases the extreme carboxy-terminal sequences were found to be important.

We explored the possibility that *pct1*⁺ was important for premeiotic DNA synthesis because this event had been shown previously to require *cdc10*⁺ function (Beach et al. 1985). *pct1*⁺ and *pct1*[−] cells were grown to stationary phase and transferred to nitrogen-free medium to induce meiosis. Samples were analyzed by flow cytometry at various times thereafter. In both cell populations, at the time nitrogen starvation was initiated, the majority of the cells had a 2N DNA content (Fig. 9A,E). In the case of the *pct1*⁺ cells, over the following 7.5 hr there was a gradual decrease in the proportion of 2N cells and a corresponding increase in the number of

cells with a 4N DNA content as a result of premeiotic DNA synthesis (Fig. 9B–D). In contrast, in the *pct1*[−] population there was a significant delay in the accumulation of 4N cells such that at 7.5 hr at least 50% of the cells still had a 2N DNA content (Fig. 9F–H). These results indicated that deletion of the *pct1*⁺ gene affected premeiotic DNA synthesis.

Discussion

In this paper we describe the cloning and sequencing of a new gene, *pct1*⁺, that encodes a protein partner of p85^{cdc10}. It has been shown previously that p85^{cdc10} is a component of the MBF complex that specifically binds to the MCB DNA element and directs the transcription of a number of genes at late G₁ (Lowndes et al. 1992b). The p85^{cdc10} protein, however, does not bind to this sequence directly but, rather, as a heteromer with specific partners that provide a DNA-binding function. The product of *pct1*⁺, p73^{pct1}, is one such partner. The evidence for this is twofold. First, a chimeric protein encoded by a *vp16-cdc10*⁺ fusion gene was found to specifically associate in vivo with an MCB-containing promoter in a *pct1*⁺-dependent manner. Second, specific DNA-binding complexes could be generated in vitro by combining reticulocyte lysates containing p85^{cdc10} and p73^{pct1}; either lysate alone failed to generate such specific complexes.

A second DNA-binding partner of p85^{cdc10}, encoded by the *res1*⁺/*sct1*⁺ gene, has been described recently (Tanaka et al. 1992; Caligiuri and Beach 1993). Like p73^{pct1}, this product can form a complex with p85^{cdc10} and bind specifically to MCB elements. It is clear, therefore, that p85^{cdc10} has at least two different partners, forming two heteromeric complexes that may have different activities and/or be regulated in different ways.

A major question that arises from these results is the question of the extent, if any, to which the activities of the two different complexes are unique. The phenotypes of *pct1*[−] and *res1*[−]/*sct1*[−] mutants demonstrate that their major roles are different. The *pct1*⁺ gene is not essential for mitotic division, its deletion having little adverse effect on cell viability or growth rate at 30°C. Deletion of the *res1*⁺/*sct1*⁺ gene, however, results in poor growth at 30°C and no growth at higher or lower temperatures (Tanaka et al. 1992; Caligiuri and Beach 1993). The p85^{cdc10}/p72^{res1/sct1} complex must therefore play a more important role in mitotic growth than the p85^{cdc10}/p73^{pct1} complex. In contrast to its minor role in mitosis, *pct1*⁺ performs a major role during meiosis that is severely affected in a *pct1*[−] mutant. However, at least under some conditions, meiosis can take place normally in *res1*[−]/*sct1*[−] cells (Caligiuri and Beach 1993). These results suggest that *pct1*⁺ has a more critical role than *res1*⁺/*sct1*⁺ in the meiotic process.

Although *pct1*⁺ activity is not essential for mitotic growth, it is likely nevertheless to contribute, along with *res1*⁺/*sct1*⁺, to the overall MBF activity during mitosis. An overlap in the function of these two genes can be demonstrated. *pct1*⁺ overexpression can suppress the mitotic defect of *cdc10-129* temperature-sensitive cells,

Table 3. Functional characterization of *pct1*⁺

Population (%)												
Strain	Plasmid introduced	spores						nuclei				
		0	1	2	3	4	>5	1	2	3	4	>5
NT15 (<i>pct1</i> ⁺)	—	1.1	2.1	4.8	2.6	88	1.2	2.4	4.3	2.8	90	1.0
NT40 (<i>pct1</i> [−])	—	29	25	36	4.2	5.1	0.8	28	50	11	10	0.8
NT40	pRep3x	28	24	38	4.0	5.3	0.6	26	46	13	14	0.7
NT40	pRep− <i>pct1</i> ⁺	13	8.2	6.5	7.0	64	1.2	15	10	8.2	66	1.2
NT40	pRep− <i>pct1</i> −Δ <i>PstI</i>	18	29	41	5.2	5.9	0.5	14	51	19	15	0.8
NT40	pRep− <i>pct1</i> −Δ <i>Cl</i> <i>a</i> <i>I</i>	16	31	42	4.8	5.8	0.3	20	51	15	13	0.9
NT40	pRep− <i>pct1</i> −Δ <i>EcoRI</i>	26	26	37	4.6	5.5	0.8	30	45	11	14	0.7
NT40	pRep− <i>pct1</i> −Δ <i>S</i> <i>al</i> <i>I</i>	29	20	41	3.8	5.9	0.4	27	44	14	14	0.8

The indicated diploid strains (see Table 4) were grown and induced to undergo sporulation as described in Fig. 8. After 24 hr, spore formation was analyzed by phase-contrast micrography and the number of nuclei measured following DAPI staining. The percent of the population of cells or asci with the indicated number of spores or nuclei is given.

and the *pct1*⁺ gene has been isolated independently by virtue of its ability to suppress the growth defect of *res1*[−]/*sct1*[−] cells (H. Okayama, pers. comm.). *pct1*⁺, therefore, can perform the mitotic role of *res1*⁺/*sct1*⁺, although less efficiently. The *pct1*⁺ gene is expressed during mitotic growth (T. Takeda and N. Jones, unpubl.), and the p85^{*cdc10*}/p73^{*pct1*} complex probably contributes to the regulation of transcription at Start. This possibility is supported by the finding that *cdc10-129*, *pct1*[−] double mutants are inviable even at the permissive temperature of 25°C. We suggest that this reflects lower *cdc10*⁺ activity in the temperature-sensitive mutant at 25°C than is found in wild-type *cdc10*⁺ cells. MBF-binding activity has been shown to be lower in *cdc10-129* cells (Caligiuri and Beach 1993; Raymond and Simanis 1993), and if this MBF activity is an amalgam of the activities of both *cdc10*-containing heteromeric complexes, then deletion of *pct1*⁺ would lower it still further. At this point, the total MBF activity may be lower than the necessary threshold for growth. A more direct indication that *pct1*⁺ plays a role at Start comes from investigating the growth of *pct1*[−] cells at 18°C. At this temperature, the growth rate of *pct1*[−] cells is consider-

ably slower than wild-type cells, and this is accompanied by an increase in the average length of the cells as well as an increase in the proportion of cells with a 1N DNA content. Both of these observations are consistent with a delay in the execution of Start.

The different roles of the two heteromeric complexes pose the question of how their activities might differ. An obvious possibility would be that they have different DNA-binding specificities and, hence, activate a different spectrum of target genes. The two different SWI6-containing heteromeric complexes in *S. cerevisiae* have clearly distinct binding specificities (Andrews and Herskowitz 1989a,b; Dirick et al. 1992; Lowndes et al. 1992a). However, this is not readily apparent with the *cdc10*⁺-containing complexes. Both in vitro and in vivo studies suggest that the specificities of *pct1*⁺ and *res1*⁺/*sct1*⁺ must be very similar, both being able to bind specifically to MCB elements (this paper; Caligiuri and Beach 1993). An alternative model would infer that the heteromeric complexes have similar binding specificities but that they respond to different regulatory signals. There is precedence for this model because recent evidence suggests that the MBF and SBF complexes in *S.*

Figure 9. Flow cytometry of nitrogen-starved *pct1*⁺ and *pct1*[−] cells. NT1 (*pct1*⁺/*pct1*⁺ diploid) and NT40 (*pct1*[−]/*pct1*[−] diploid) cells were grown in SSL + N medium to stationary phase (3 × 10⁷ cells/ml), washed in SSL-N medium, and resuspended in SSL-N medium at the same density. The cultures were incubated at 30°C, and aliquots were taken for flow cytometry analysis at 0, 2.5, 5, and 7.5 hr following the initiation of nitrogen starvation. (A) NT1, 0 hr; (B) NT1, 2.5 hr; (C) NT1, 5 hr; (D) NT1, 7.5 hr; (E) NT40, 0 hr; (F) NT40, 2.5 hr; (G) NT40, 5 hr; (H) NT40, 7.5 hr.

cerevisiae are differentially regulated (Amon et al. 1993), although the activation of both is dependent on CDC28 kinase activity (Cross and Tinkelenberg 1991; Dirick and Nasmyth 1991). Thus, during mitosis and meiosis in *S. pombe*, the signals involved in regulating *cdc10*⁺-containing complexes may be qualitatively different. For example, the *cdc2*-encoded kinase may be associated with different cyclins during mitosis and meiosis, resulting in altered target specificity of the kinase.

Our results suggest that *pct1*⁺ plays a significant role in premeiotic DNA synthesis that is known to require *cdc10*⁺ activity (Beach et al. 1985; Grallert and Sipiczki 1991). Flow cytometry analysis of *pct1*[−] cells induced to undergo meiosis showed a considerable delay in the completion of premeiotic S phase. Consistent with this was the observation that a number of *pct1*[−] cells arrested with single nuclei and a significant percentage produced no spores. The role of *pct1*⁺ in the premeiotic S phase presumably involves the activation of essential target genes in conjunction with *cdc10*⁺. However, the identity of such targets remains unknown. Of the previously described targets of *cdc10*⁺-containing complexes, *cdc22*⁺ is the only one that has been shown to be essential for premeiotic DNA synthesis and would therefore be a good candidate for a *pct1*⁺-dependent target gene. However, our preliminary analysis suggests that *cdc22*⁺ expression following meiotic induction is unaffected by deletion of the *pct1*⁺ gene (Y. Zhu, T. Takada, and N. Jones, unpubl.). The premeiotic function of *cdc10*⁺ need not derive only from the p73^{pct1}/p85^{cdc10} complex but, rather, from a combination of the two *cdc10*⁺-containing heteromeric complexes, and we suggest that the expression of *cdc22*⁺ is being maintained in a *pct1*[−] background by the *res1*⁺/*sct1*⁺-dependent activity. Other targets must exist that are likely to be more dependent on the p73^{pct1}/p85^{cdc10} complex.

The terminal phenotype of the *pct1*[−] mutants is variable. Cells containing two, three, or four nuclei are observed, perhaps reflecting arrest at different points during meiosis. The reason for this leakiness is unknown but could be attributable to the ability of *res1*⁺/*sct1*⁺ to partially compensate for the loss of *pct1*⁺ function or variable residual expression of critical target genes that are activated by the p73^{pct1}/p85^{cdc10} complex. It is also possible that *pct1*⁺ has multiple roles in meiosis. The coordination between nuclear division and spore wall formation is also disrupted in *pct1*[−] mutants. For example, some spores contain two nuclei, a result that is explained most easily by the initiation of spore wall formation prior to the completion of meiotic division. Interestingly, a similar observation has been made with *cdc13-117*, which at the restrictive temperature predominantly gives rise to two-spore asci with each spore containing a single nucleus but in addition to spores with two nuclei (Grallert and Sipiczki 1991).

The sequence of *pct1*⁺ is related to all known genes in *S. pombe* and *S. cerevisiae* that are involved in late G₁-specific transcription, namely the *SWI4*, *MBP1*, and *SWI6* genes of *S. cerevisiae* and the *res1*⁺/*sct1*⁺ and *cdc10*⁺ genes of *S. pombe*. A very high degree of simi-

larity is found between the amino-terminal regions of *pct1*⁺, *res1*⁺/*sct1*⁺, *SWI4*, and *MBP1*-encoded proteins. These proteins all bind specifically to DNA either alone or in conjunction with partner proteins. This domain in *SWI4* and *MBP1* constitutes the DNA-binding domain (Primig et al. 1992; Koch et al. 1993), and considering the similarity in binding specificity, it is very likely to be the case with *pct1*⁺ and *res1*⁺/*sct1*⁺ also. The *pct1*⁺ gene can rescue a *cdc10*^{ts} phenotype that presumably necessitates binding of p73^{pct1} to DNA. The carboxy-terminal region of *pct1*⁺ is not essential for this rescue, although it does contribute. This is consistent with the DNA-binding domain being located in the amino terminus.

The high degree of conservation between *pct1*⁺ and *res1*⁺/*sct1*⁺ and the corresponding transcriptional regulators in *S. cerevisiae* suggests the possibility that similar proteins may exist in higher eukaryotes. The only known mammalian factor that regulates transcription at late G₁ is the E2F factor. A number of functional similarities exist between E2F and the *pct1*⁺/*SWI4* family. It has also been suggested that some structural similarity exists between the DNA-binding domain of *SWI4* and that of E2F1 and DP1, two members of the E2F family (LaThangue and Taylor 1993). However, the overall similarity is very weak with only two residues being conserved. It is unlikely, therefore, that E2F represents the mammalian equivalent of the *pct1*⁺/*SWI4* family, and the possibility must remain that true equivalents exist.

Materials and methods

Strains and media and genetic methods

S. pombe strains used in this study are summarized in Table 4. Complete medium YES and minimal medium SD (Sherman et al. 1986) or EMM (Mitchison 1970) were used for routine culture of *S. pombe* strains. SPA medium (Gutz et al. 1974) was used to induce mating and sporulation. Minimal medium PM and its nitrogen-free derivative PM-N (Beach et al. 1985) and SSL medium and its nitrogen-free derivative SSL-N (Egel and Egel-Mitani 1974) were used for nitrogen-starvation experiments. General genetic methods for *S. pombe* were according to Gutz et al. (1974). Transformation and gene replacement were done as described (Beach et al. 1982).

The *S. cerevisiae* strain YZ100 was derived from strain K2866 (*mata*, *ade2-1*, *trp1-1*, *can1-100*, *leu2-3,112*, *his3-11*, *URA3*, *swi6::TRP1*). The strain K2866 has an integrated copy of a *lacZ* gene driven by the 69-bp promoter region of the *S. cerevisiae* thymidine synthase gene containing two MCB elements. In YZ100, the *URA3* gene has been replaced by a functional *HIS3* gene. The strain By 600 has been described previously (Breedon and Nasmyth 1987); it contains a *swi6::TRP1* deletion and an integrated *lacZ* gene driven by the SRS2 region of the HO gene promoter (Tabata et al. 1991).

All routine growth and maintenance of *S. cerevisiae* strains were as described previously (Ausubel et al. 1987).

Cloning and sequencing of *pct1*⁺

A *S. pombe* cDNA library in a *S. cerevisiae* high-copy expression vector (Fikes et al. 1990) was transformed into the *S. cerevisiae* strain YZ100 using the DMSO-enhanced lithium ace-

Table 4. *S. pombe* strains used in this study

Strain	Genotype
NT1	<i>h⁺/h⁻ ade6-M210/ade6-M216 leu1-32/leu1-32 ura4-D18/ura4-D18</i>
NT7	<i>h⁹⁰/h⁻ ade6-M210/ade6-M216 leu1-32/leu1-32 ura4-D18/ura4-D18 cdc10::ura4⁺/+</i>
NT8	<i>h⁺ cdc10-129 leu1-32</i>
NT9	<i>h⁻ ade6-M216 cdc10-129 leu1-32 ura4-D18</i>
NT15	<i>h⁺/h⁻ ade6-M210/ade6-M216 leu1-32/leu1-32</i>
NT16	<i>h⁻ ade6-M216 leu1-32</i>
NT17	<i>h⁹⁰ ade6-M216 leu1-32</i>
NT28	<i>h⁺/h⁻ ade6-M210/ade6-M216 leu1-32/leu1-32 ura4-D18/ura4-D18 pct1::ura4⁺/+</i>
NT36	<i>h⁺ ade6-M210 leu1-32 ura4-D18 pct1::ura4⁺</i>
NT38	<i>h⁻ ade6-M216 leu1-32 ura4-D18 pct1::ura4⁺</i>
NT40	<i>h⁺/h⁻ ade6-M210/ade6-M216 leu1-32/leu1-32 ura4-D18/ura4-D18 pct1::ura4⁺/pct1::ura4⁺</i>
NT43	<i>h⁹⁰ ade6-M216 leu1-32 ura4-D18 pct1::ura4⁺</i>
NT58	<i>h⁺/h⁻ ade6-M210/ade6-M216 leu1-32/leu1-32 ura4-D18/ura4-D18 cdc10-129/+ pct1::ura4⁺/+</i>

tate transformation procedure (Hill et al. 1991). The transformants were plated directly onto nylon filters layered on glucose containing selective agar medium. One day later the filters were transferred to plates containing galactose and raffinose as the carbon source, and incubation continued for another day. The resulting colonies growing on the filter were assayed for β -galactosidase activity as described previously (Breedon and Nasmyth 1987; Dalton and Treisman 1992). Briefly, the filters were immersed in liquid nitrogen for ~1 min to permeabilize the cells, thawed, and layered on top of 3M Whatman paper absorbed with the *lacZ* substrate X-gal and Z buffer (60 mmoles of $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 40 mmoles of $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 10 mmoles of KCl, 1 mmole of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$). The color of the colonies indicated the level of β -galactosidase activity. Approximately 10^6 transformants were screened, and 150 blue colonies were selected for additional assays. The increased *lacZ* expression in one clone was found to be dependent on galactose induction and was characterized further. The cDNA plasmid in this clone was isolated following electroporation into bacteria. The 2.16-kb insert was subcloned into the *NotI* site of the pBluescript KS (-) vector in both orientations giving pYZA0 and pYZB0, and a series of unidirectional deletions were constructed for sequencing. Both strands were sequenced.

Plasmids

The plasmid pGall-10-vp16cdc10 was constructed by cloning the entire *cdc10⁺* coding region into the vector pSD06a (Dalton and Treisman 1992) such that the VP16 activation domain was fused to the amino terminus of *cdc10*. Two vectors containing either the *S. pombe* ADH promoter (pEVP11; Russell and Nurse 1986) or the *nmt1⁺* promoter (pRep3x, a derivative of pRep1; Maundrell 1989; a gift from S. Forsburg and P. Nurse, ICRF, London, UK) were used for the overexpression of the *pct1⁺* cDNA. The pYZB0 plasmid was digested with *Bam*HI and *Sst*I, which are in the flanking polylinker sequences, and the resulting fragment was ligated with pEVP11 restricted with *Bam*HI and *Sst*I. For the construction of the *pct1⁺*-inducible plasmid, a cDNA fragment derived from pYZA0 digestion with *Bam*HI and *Sst*I was ligated with pRep3x restricted with *Bam*HI and *Sma*I. For the construction of the *pct1⁺* deletion plasmids, *pct1⁺* cDNA was excised with appropriate restriction enzymes from pYZA0 and inserted into pRep3x as described above.

Gene disruption

The 1.0-kb *Eco*RI-*Pst*I fragment from the *pct1⁺* cDNA was replaced with the 1.8-kb *ura4⁺* gene. The disrupted cDNA frag-

ment was used to transform the diploid strain NT1, and stable *ura⁺* transformants were isolated. Transformants were checked by Southern blot hybridization to confirm the presence of a disrupted *pct1⁺* gene. All spores from the disruptants germinated.

In vitro protein expression

The cDNAs of *pct1⁺* and *cdc10⁺* were cloned into the T7plink vector (Dalton and Treisman 1992). The proteins were expressed using the TNT-coupled reticulocyte lysate system (Promega).

EMSAs

The DNA nucleotide fragments used as probes for EMSAs were as follows: The *TMP1* probe consisted of sequences -163 to -110 of the *TMP1* promoter (+1 being the position of the translation initiation codon) with *Eco*RI and *Bam*HI linkers at the 5' and 3' termini. The *Eco*RI-*Bam*HI fragment was dephosphorylated and end-labeled with [32 P]dATP and T4 DNA kinase. The 131-bp *cdc22⁺* probe contained promoter sequences from -409 to -539, and the 142-bp *cdc18⁺* probe contained promoter sequences from -119 to -281. Both probes were labeled upon PCR amplification using *Taq* polymerase and 10 μ Ci of [32 P]dCTP. Probe HOSRS2 has been described previously (Taba et al. 1991); it was end-labeled with [32 P]dATP and T4 DNA kinase. The E2A probe contained promoter sequences extending from -75 to -32 relative to the transcription initiation site and was labeled using reverse transcriptase. The specific activity of these probes was within the range of 5×10^7 to 5×10^8 cpm/ μ g.

The binding reaction was performed at room temperature for 10 min in 20 μ l of binding buffer [50 mM Tris-Cl (pH 7.5), 50 mM KCl, 25 mM MgCl_2 , 25% glycerol, 50 mg/ml of BSA, 5 mM spermidine, 1 mM ATP, 1 mg of poly [d(I-C)], and 1 $\times$ protein inhibitor cocktail (1 mM DTT, 50 μ g/ml of PMSF, 1 mM benzamide, 5 μ g/ml of aprotin, 5 μ g/ml of leuprotin, 5 μ g/ml of pepstatin)]. Programmed or unprogrammed reticulocyte lysate (2-4 μ l) was incubated with the above binding buffer at room temperature for 10 min. About 0.5 ng of the [32 P]dNTP-labeled probe was added into the reaction mixture, and incubation continued for an extra 20 min. The competition assay was carried out by the addition of an excess amount of nonradioactive labeled DNA fragments as competitors at the same time as the labeled probe. The reaction mixtures were loaded onto a 4% acrylamide gel (55:1 cross-linking) and were run at 200 V for 90-120 min.

DAPI staining and flow cytometry

DAPI staining of *S. pombe* DNA was as described previously (Toda et al. 1981). In the nitrogen-starvation experiments, portions of the cultures were fixed with ice-cold ethanol on ice for 5 min. The cells were stained with DAPI (1 μ g/ml; Sigma Chemical Co.) and washed with PBS three times. Slides for fluorescence microscopy were as described by Johnson et al. (1981). For flow cytometry, ethanol-fixed cells were stained with propidium iodide and analyzed using a Becton-Dickinson FACScan and the software LYSIS II (Sazer and Sherwood 1990).

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Note added in proof

The sequence data described in this paper have been submitted to the GenBank data library.

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