

# *plo1*<sup>+</sup> regulates gene transcription at the M–G<sub>1</sub> interval during the fission yeast mitotic cell cycle

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**The regulation of gene expression plays an important part in cell cycle controls. We describe the molecular machinery that co-ordinates gene transcription at the M–G<sub>1</sub> interval during the fission yeast mitotic cell cycle. A sequence is identified in the *cdc15*<sup>+</sup> promoter that we call a PCB (pombe cell cycle box), which confers M–G<sub>1</sub>-specific transcription. Sequences similar to the PCB are present in the promoters of seven other genes, *spo12*<sup>+</sup>, *cdc19*<sup>+</sup>, *fin1*<sup>+</sup>, *sid2*<sup>+</sup>, *ppb1*<sup>+</sup>, *mid1*/*dmf1*<sup>+</sup> and *plo1*<sup>+</sup>, which we find to be transcribed at M–G<sub>1</sub>. A transcription factor complex is identified that binds to the PCB sequence, which we name PBF, for PCB-binding factor. Finally, we show that PBF binding activity and consequent gene transcription are regulated by the Plo1p protein kinase, thus invoking a potential auto-feedback loop mechanism that regulates mitotic gene transcription and passage through septation and cytokinesis.**

**Keywords:** cell cycle/fission yeast/*plo1*<sup>+</sup>/transcription

## Introduction

Many forms of control have been shown to regulate the mitotic cell division cycle, such as protein kinase activity, specific proteolytic degradation and changes in intracellular location. Transcription has also been found to play an important role in controlling cell cycle progress, and the cell cycle-specific regulation of gene expression is widespread. Microarray analysis has revealed in the budding yeast, *Saccharomyces cerevisiae*, that the transcript profile of ~700 genes, out of a total of ~6000, varies throughout the mitotic cell cycle (Cho *et al.*, 1998; Spellman *et al.*, 1998). These genes fall into a number of groups whose transcript abundance peaks at different cell cycle times. Each group of genes is regulated co-ordinately by a common DNA sequence present in their promoters, which are bound by a transcription factor complex. Examples of such groupings are the MCB–MBF group of genes at

G<sub>1</sub>–S, and Mcm1p/Fkhp genes during mitosis (Futcher, 2000).

Cell cycle-regulated transcription has also been studied in the fission yeast, *Schizosaccharomyces pombe*. A group of genes, including *cdc22*<sup>+</sup>, *cdc18*<sup>+</sup>, *cig2*<sup>+</sup>, *cdt1*<sup>+</sup> and *mik1*<sup>+</sup>, are transiently expressed at the beginning of S phase, and their products are required, either directly or indirectly, for DNA synthesis (Fernandez-Sarabia *et al.*, 1993; Kelly *et al.*, 1993; Connolly and Beach, 1994; Hofmann and Beach, 1994; Ng *et al.*, 2001). The molecular components that control G<sub>1</sub>–S transcription in fission yeast comprise a transcription factor complex named DSC1 (DNA synthesis control; also called MBF), that contains the products of the *cdc10*<sup>+</sup>, *res1*<sup>+</sup>, *res2*<sup>+</sup>, *rep1*<sup>+</sup> and *rep2*<sup>+</sup> genes (Lowndes *et al.*, 1992; Tanaka *et al.*, 1992; Caligiuri and Beach, 1993; Miyamoto *et al.*, 1994; Sugiyama *et al.*, 1994; Zhu *et al.*, 1994; Nakashima *et al.*, 1995). DSC1 binds to MCB sequences (*Mlu*I cell cycle box; ACGCGT) that are present in the promoters of *cdc22*<sup>+</sup>, *cdc18*<sup>+</sup>, *cdt1*<sup>+</sup> and *cig2*<sup>+</sup>, all of which are expressed maximally at the G<sub>1</sub>–S boundary during the mitotic cell cycle.

We are interested in identifying other groups of genes that are regulated co-ordinately in fission yeast at different cell cycle times, with the aim of characterizing the molecular components that control their transcription. To this end, we have studied *cdc15*<sup>+</sup>, a gene that is transcribed specifically during the M–G<sub>1</sub> phase of mitotic division (Fankhauser *et al.*, 1995; Utzig *et al.*, 2000). We report the identification of seven other genes that are expressed coincidentally with *cdc15*<sup>+</sup>, and describe a promoter sequence and a transcription factor complex that regulates their cell cycle transcription. Finally, we show that Plo1p regulates the expression of these genes. We propose that Plo1p controls M–G<sub>1</sub> cell cycle transcription during mitosis in fission yeast, as part of an auto-activatory loop that results in cytokinesis and septation.

## Results

### **Mitotic cell cycle transcription of *cdc15*<sup>+</sup>**

*cdc15*<sup>+</sup> was first identified by mutation in the original fission yeast cell cycle screens (Nurse *et al.*, 1976), and ascribed a function late in the cell cycle between nuclear division and early cell plate formation. Subsequent characterization of the gene product (Fankhauser *et al.*, 1995) showed that *cdc15*<sup>+</sup> is a key element in the control of the re-organization of the F-actin ring at cytokinesis (Marks *et al.*, 1986). This ring later constricts to effect cytokinesis, once genomic segregation has occurred (Demeter and Sazer, 1998). Fankhauser *et al.* (1995) also showed that *cdc15*<sup>+</sup> transcript abundance varied during the cell cycle, with a peak at metaphase during vegetative growth, which suggested that control of *cdc15*<sup>+</sup> transcription may play a role in promoting cytokinesis.

We confirmed and extended the observation concerning *cdc15<sup>+</sup>* mRNA abundance during the mitotic cell cycle, by two different synchronization methods. In synchronously dividing cells, which were either wild type (972h<sup>-</sup>) size selected by centrifugal elutriation (Figure 1A), or synchronized by transient arrest at the G<sub>2</sub>-M boundary by reversible temperature shifts of a *cdc25-22* mutant (Figure 1B), *cdc15<sup>+</sup>* mRNA varied in abundance in a cell cycle manner. By directly comparing the peak level of *cdc15<sup>+</sup>* transcript with that of *cdc22<sup>+</sup>*, we confirmed that *cdc15<sup>+</sup>* peaks slightly earlier in the cell cycle. This was especially apparent in the *cdc25-22* mutant experiment (Figure 1B), where we took samples every 10 min after temperature release for northern analysis. As *cdc22<sup>+</sup>* mRNA peaks at the G<sub>1</sub>-S boundary (White *et al.*, 2001), these two experiments suggested that *cdc15<sup>+</sup>* mRNA was present at M-G<sub>1</sub>.

*cdc22<sup>+</sup>* is a member of a group of genes whose transcription is regulated co-ordinately at the G<sub>1</sub>-S boundary by the combination of a common promoter sequence present in all the genes' promoters which is bound by a transcription factor complex. The promoter sequence is called an MCB, and the transcription factor complex, DSC1, contains the *cdc10<sup>+</sup>* gene product (Lowndes *et al.*, 1992).

*cdc15<sup>+</sup>* is unlikely to be under DSC1-MCB control, because it is not transcribed at the G<sub>1</sub>-S interval. Furthermore, *cdc15<sup>+</sup>* does not contain MCB sequences in its promoter region. Finally, a mutant of a component of DSC1, *cdc10-C4*, which results in overexpression of all known MCB-regulated genes compared with wild type (McInerny *et al.*, 1995; Ng *et al.*, 2001), did not affect *cdc15<sup>+</sup>* transcription (Figure 1C).

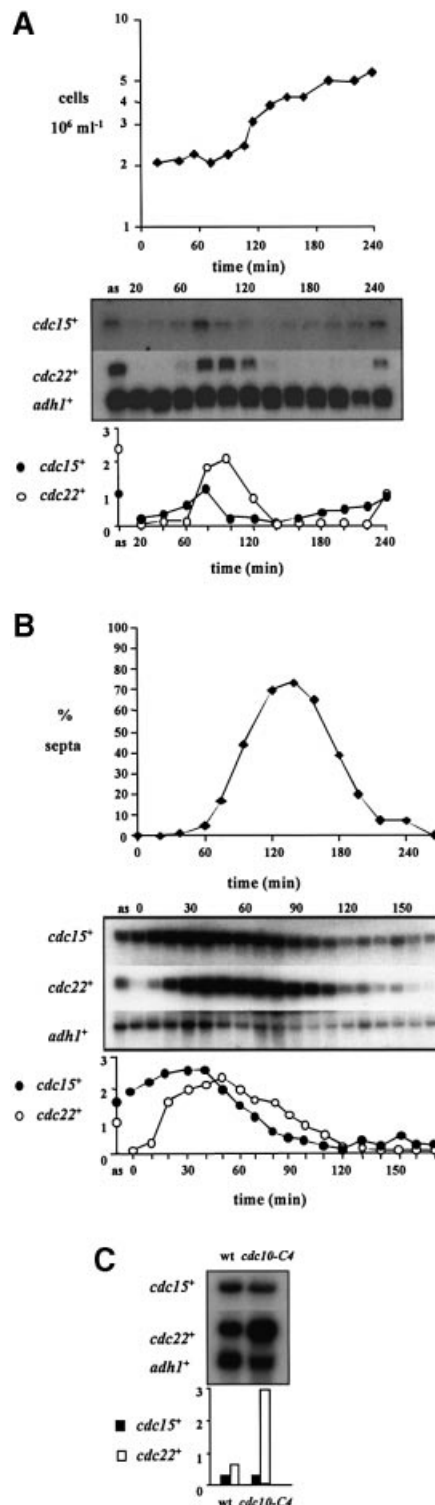
These experiments showed that *cdc15<sup>+</sup>* is representative of a new type of mitotic cell cycle-expressed gene in fission yeast, which is transcribed at the M-G<sub>1</sub> boundary.

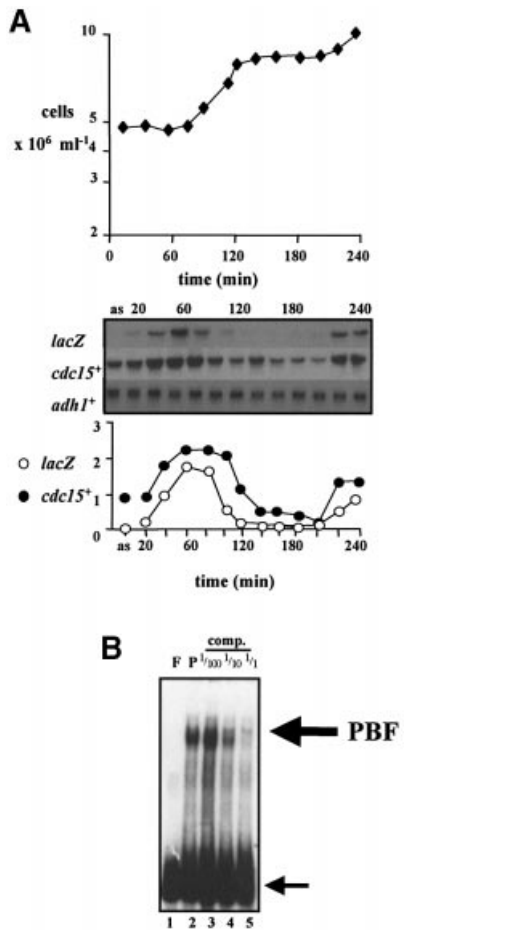
### Regulation of *cdc15<sup>+</sup>* transcription

To see whether variation in *cdc15<sup>+</sup>* mRNA levels during the cell cycle was due to transcriptional regulation, we tested the ability of DNA fragments from the promoter region of *cdc15<sup>+</sup>* to confer M-G<sub>1</sub> transcription to a heterologous gene. A number of *cdc15<sup>+</sup>* promoter fragments were cloned into the reporter plasmid pSPΔ178

**Fig. 1.** *cdc15<sup>+</sup>* is expressed before MCB-regulated genes in the fission yeast cell cycle, and is not controlled by DSC1. (A) A population of wild-type cells (972h<sup>-</sup>), synchronous for division, were size selected by centrifugal elutriation at 32°C, and cell samples taken every 20 min for northern blot analysis of RNA. 'as' indicates RNA prepared from asynchronously dividing cells prior to elutriation. The blot was hybridized consecutively with *cdc15<sup>+</sup>*, *cdc22<sup>+</sup>* and *adh1<sup>+</sup>* probes, the latter as a loading control. Quantification of each transcript against *adh1<sup>+</sup>* is shown. (B) *cdc25-22* cells were synchronized for cell division by transient temperature shifts. Northern blot analysis was performed on RNA samples prepared from cell samples taken at 10 min intervals following release from restrictive temperature. The blot was hybridized consecutively with *cdc15<sup>+</sup>*, *cdc22<sup>+</sup>* and *adh1<sup>+</sup>* probes, the latter as a loading control. Quantification of each transcript against *adh1<sup>+</sup>* is shown. The degree of synchrony is indicated by the septation index. (C) RNA was prepared from cultures of wild-type (972h<sup>-</sup>) and *cdc10-C4* cells grown at 25°C, and subjected to northern blot analysis. The membrane was hybridized consecutively with *cdc15<sup>+</sup>*, *cdc22<sup>+</sup>* and *adh1<sup>+</sup>* probes, the latter as a loading control. Quantification of each transcript against *adh1<sup>+</sup>* is shown.

(Lowndes *et al.*, 1992), and the cell cycle-dependent transcription of *lacZ* analysed by northern blot analysis. One fragment, corresponding to bases -157 to -53 relative to the *cdc15<sup>+</sup>* ATG, conferred M-G<sub>1</sub> transcription to *lacZ*, coincident with endogenous *cdc15<sup>+</sup>* cell cycle expression in synchronized wild-type cells size selected by elutriation (Figure 2A). Similar results were obtained in *cdc25-22* cells synchronized by transient temperature arrest (data not shown). These experiments showed that the cyclic



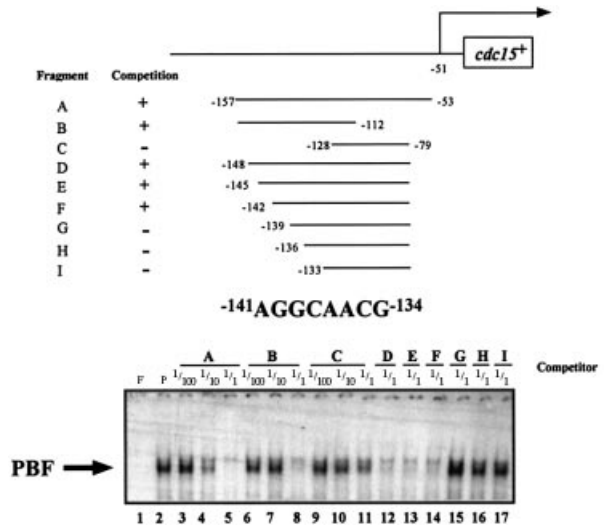


**Fig. 2.** Characterization of a region of the *cdc15*<sup>+</sup> promoter that confers M-G<sub>1</sub> transcription, and identification of a transcription factor complex, PBF, that binds to it. (A) A fragment from the *cdc15*<sup>+</sup> promoter was inserted into pSPΔ178 (Lowndes *et al.*, 1992) to create pSPΔ178. 15UAS, transformed into wild-type cells, and cells synchronous for division were size selected by centrifugal elutriation at 32°C, with samples taken every 20 min for northern blot analysis of RNA. ‘as’ indicates RNA prepared from asynchronously dividing cells prior to elutriation. The blot was hybridized consecutively with *lacZ*, *cdc15*<sup>+</sup> and *adh1*<sup>+</sup> probes, the latter as a loading control. Quantification of each transcript against *adh1*<sup>+</sup> is shown. (B) The same *cdc15*<sup>+</sup> promoter fragment as in (A) was used as labelled probe in gel retardation analysis with total fission yeast protein extracts. Lane F, free probe; lane P, 20 μg of protein with probe. Competition reactions were performed with the same unlabelled *cdc15*<sup>+</sup> promoter DNA with 1/100, 1/10 and 1 M excess. The large arrow indicates PBF, and the small arrow indicates free probe.

behaviour of *cdc15*<sup>+</sup> mRNA abundance was due to transcriptional regulation and not mRNA stability, and that this fragment of DNA contained the *cis*-acting DNA element that controlled this expression.

This same *cdc15*<sup>+</sup> promoter fragment was then labelled and used as the probe in gel retardation experiments with protein extracts from wild-type fission yeast cells to identify the protein complex that bound to this sequence (Figure 2B). Because this complex potentially regulates *cdc15*<sup>+</sup> cell cycle transcription, we named it PBF for pombe cell cycle box (PCB)-binding factor (see later).

We mapped the region of contact between PBF and the *cdc15*<sup>+</sup> promoter by competition experiments using fragments of DNA from the *cdc15*<sup>+</sup> promoter region (Figure 3).



**Fig. 3.** Mapping of the interaction site between PBF and the *cdc15*<sup>+</sup> promoter. Gel retardation analysis was performed using the *cdc15*<sup>+</sup> promoter fragment as labelled probe. Lane F, free probe; lane P, 20 μg of protein with probe. Competition reactions were performed with unlabelled DNAs corresponding to different fragments of the *cdc15*<sup>+</sup> promoter, designated with the letters A–I, with 1/100, 1/10 and 1 M excess, as indicated.

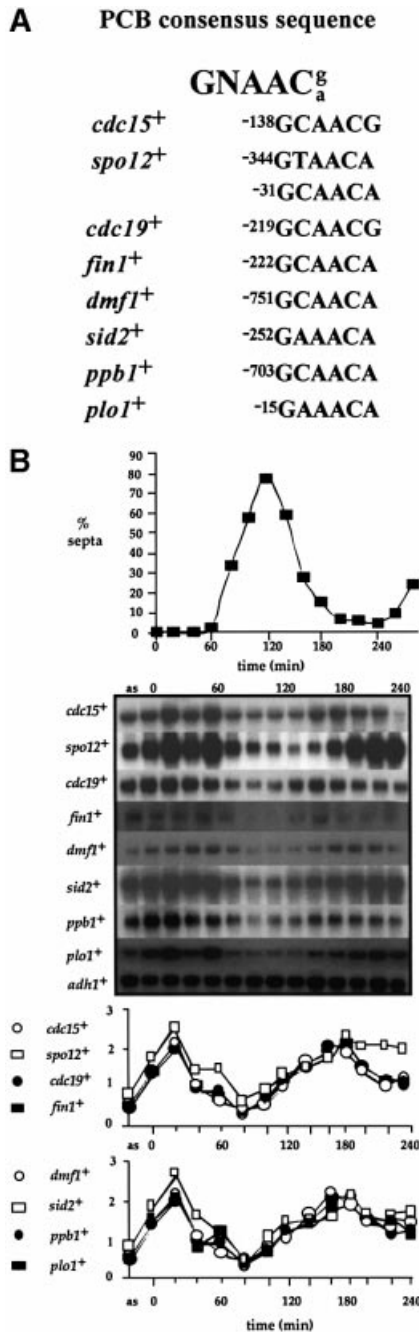
Significantly, whereas fragment F was able to compete, fragment G could not (compare lanes 14 and 15), with the difference between these two DNAs being only 3 bp. This observation implicated this region of DNA as being important for interaction with PBF. The sequence around this region, which corresponded to –141 to –134 relative to the ATG of *cdc15*<sup>+</sup>, is AGGCAACG.

### A family of M-G<sub>1</sub>-regulated genes

Using the sequence defined by gel retardation analysis in the *cdc15*<sup>+</sup> promoter, we searched the fission yeast genome database (Wood *et al.*, 2002) for other genes that contained similar sequences in their promoter regions. We took two approaches to this, both examining gene promoters randomly and looking specifically in the promoter regions of genes that are known to be cell cycle regulated in budding yeast at M-G<sub>1</sub>. Having identified potential genes (Figure 4A), we analysed their transcription profile during a mitotic cell cycle. These data are shown in Figure 4B. Interestingly, *spo12*<sup>+</sup>, *cdc19*<sup>+</sup>, *fn1*<sup>+</sup>, *mid1*<sup>+</sup>/*dmf1*<sup>+</sup>, *sid2*<sup>+</sup>, *ppb1*<sup>+</sup> and *plp1*<sup>+</sup> all showed a similar transcription profile to that of *cdc15*<sup>+</sup>, suggesting that the same molecular processes may regulate transcription of all of these genes. *spo12*<sup>+</sup> previously has been reported to be cell cycle expressed, coincident with *cdc15*<sup>+</sup> (Samuel *et al.*, 2000). It may be significant that most of these genes form a group that is implicated in the execution or regulation of cytokinesis and septation. Outside this group is *cdc19*<sup>+</sup>, a member of the MCM class of protein required for DNA replication that have been shown to be loaded onto chromosomes during metaphase (Kearsey *et al.*, 2000).

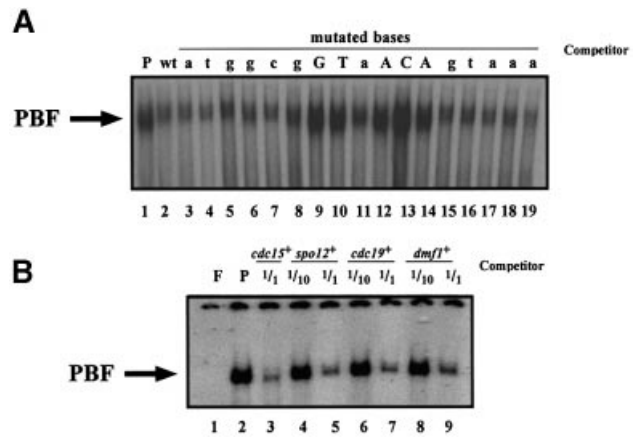
### Defining the PCB sequence

To define more precisely the consensus sequence required for M-G<sub>1</sub> transcription in fission yeast, we adopted two approaches. First, we carried out a series of competitive



**Fig. 4.** A group of seven genes are expressed during mitosis in fission yeast coincident with *cdc15*<sup>+</sup>. (A) Predicted consensus sequence for the PCB element in fission yeast. Sequences related to the PCB consensus present in M-G<sub>1</sub>-expressed gene promoters are listed, with numbers referring to their position relative to each gene's ATG. (B) *cdc25-22* cells were synchronized for cell division by transient temperature shifts. Northern blot analysis was performed on RNA from cell samples taken at 20 min intervals following release from the restrictive temperature. 'as' indicates RNA prepared from asynchronously dividing cells prior to temperature shifts. The blot was hybridized consecutively with *cdc15*<sup>+</sup>, *spo12*<sup>+</sup>, *cdc19*<sup>+</sup>, *mid1*/*dmf1*<sup>+</sup>, *fin1*<sup>+</sup>, *sid2*<sup>+</sup>, *ppb1*<sup>+</sup>, *plo1*<sup>+</sup> and *adh1*<sup>+</sup> probes, the latter as a loading control. Quantification of each transcript against *adh1*<sup>+</sup> is shown. The degree of synchrony achieved is indicated by the septation index.

gel retardation experiments using a fragment of DNA from the *spo12*<sup>+</sup> promoter that contained a sequence related to the sequence defined in the *cdc15*<sup>+</sup> promoter. A 20 bp



**Fig. 5.** Defining the PCB sequence. (A) Base pairs required in *spo12*<sup>+</sup> PCB to bind PBF. Gel retardation analysis was performed using the *cdc15*<sup>+</sup> promoter fragment as labelled probe. Lane P, 20 µg of protein with probe. Competition reactions were performed using cold DNA fragments corresponding to the *spo12*<sup>+</sup> PCB, where single consecutive bases were mutated in separate DNAs, with A/T to G, or C/G to T, with 1 M excess. The central GT and ACA are required for successful competition with PBF. (B) PBF binds promoter fragments containing PCB sequences from other genes transcribed at M-G<sub>1</sub> during mitosis. Gel retardation analysis was performed using the *cdc15*<sup>+</sup> promoter fragment as labelled probe. Lane F, free probe; lane P, 20 µg of protein with probe. In alternate lanes, 1 and 1/10 M excess unlabelled competitor promoter DNA fragments from various fission yeast mitotic M-G<sub>1</sub>-expressed genes was added to the reaction mixture prior to electrophoresis.

fragment from the *spo12*<sup>+</sup> promoter containing the putative upstream sequence (position -344; Figure 4A) successfully competed with PBF (Figure 5A, lane 2), demonstrating that both promoters recognize the same transcription factor complex. Individual base pairs required for PBF to bind *spo12*<sup>+</sup> DNA were determined by assaying the effect of mutating single base pairs on competitive binding activity. A series of DNAs were synthesized containing consecutive single base pair mutations (A/T to G, or C/G to T), and their ability to bind PBF assayed. As shown in Figure 5A, the central GT and ACA (lanes 9, 10 and 12-14) were all critical for binding, as their individual mutation resulted in loss of PBF binding activity. The fact that mutating the T in the central GT had such an effect was surprising, as this base pair is not conserved amongst other genes (Figure 4A); possibly this base pair is important in the *spo12*<sup>+</sup> promoter in combination with some other base(s).

In the second approach, we tested whether PBF bound DNA from the promoters of other M-G<sub>1</sub>-transcribed genes containing sequences related to the PCB sequence (Figure 5B). We performed competitive gel retardation experiments using fragments from the *cdc19*<sup>+</sup> and *mid1*/*dmf1*<sup>+</sup> promoters, and demonstrated that these DNAs also bound PBF.

These experiments allowed us to predict GNAACg/a as a putative consensus sequence for the fission yeast M-G<sub>1</sub> *cis*-acting promoter element (Figure 4A). We suggest naming this sequence PCB for pombe cell cycle box.

#### Cell cycle-dependent behaviour of PBF binding to PCBs

A possible mechanism for PBF and PCB regulation of cell cycle transcription of *cdc15*<sup>+</sup> is by periodic binding of the

transcription factor to the promoter sequence during the cell cycle. We tested this hypothesis in three different experiments. In the first experiment, we size selected small wild-type cells (972h<sup>-</sup>) by centrifugal elutriation, and followed a synchronous population during two divisions. Gel retardation analysis of PBF of PBF in such synchronized cells revealed constitutive binding of PBF to PCBs during the cell cycle (Figure 6). Similar results were obtained with synchronized cells made by transient temperature arrest using the *cdc10-M17* mutation (data not shown; Kim and Huberman, 2001).

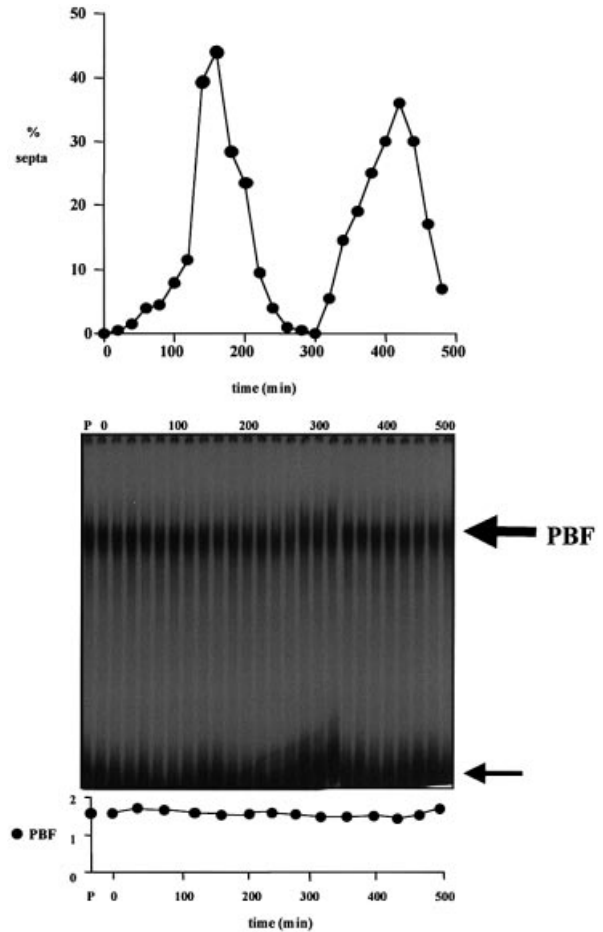
In the third experiment, we examined PBF binding to PCBs in protein extracts from fission yeast cells arrested at discrete stages of the cell cycle (G<sub>1</sub>, S, G<sub>2</sub> and M), using a variety of *cdc*<sup>-</sup> mutants. In agreement with the previous experiments, PBF bound to PCBs in cells arrested at all cell arrest points (data not shown). These three experiments suggested that PBF binds to PCBs throughout the cell cycle.

***plo1-ts35* affects PBF binding activity in vitro and PCB-regulated gene transcription in vivo**

We were interested in identifying genes that regulated cell cycle expression of the *cdc15*<sup>+</sup> group of genes through the PBF transcription factor complex. One potential candidate is the *plo1*<sup>+</sup> polo-like kinase, which is known to have a central role in regulating late mitotic events in fission yeast (Ohkura *et al.*, 1995; Bahler *et al.*, 1998; Tanaka *et al.*, 2001).

If Plo1p controls PCB-regulated genes, then compromising Plo1p function might affect both PBF activity and the periodic transcription of these genes. We therefore initially determined PBF binding activity *in vitro* by gel retardation analysis in five different temperature-sensitive *plo1*<sup>-</sup> alleles. Interestingly, the mutant with the strongest phenotype, *plo1-ts35*, resulted in the loss of PBF binding at both permissive and restrictive temperatures (Figure 7A, lanes 5–7). *plo1-ts35* cells block mitotic progression as cells are unable to form a mitotic spindle. We therefore asked whether the lack of PBF binding in *plo1-ts35* at 36°C was an indirect consequence of the early mitotic arrest, by assessing PBF activity in *cut7-24*. *cut7-24* mutant cells arrest at the same stage of mitosis as *plo1-ts35*, because of defects in a mitotic motor protein that is required to form the spindle (Hagan and Yanagida, 1990). *cut7-24* cells contained PBF at 36°C, indicating that loss of PBF in *plo1-ts35* was not a consequence of the cell cycle arrest in this strain (Figure 7A, lanes 11–13). Furthermore, the loss of PBF activity in *plo1-ts35* was not due to dominant inhibition of binding in *plo1-ts35* cells, as mixing extracts from wild-type and mutant cells had no effect on PBF (Figure 7A, lanes 8–10). *plo1-ts35* is a recessive, loss-of-function, allele: haploid *plo1-ts35* cells containing a single copy of *plo1*<sup>+</sup> are phenotypically wild type (data not shown). Therefore, loss of PBF in *plo1-ts35* was likely to be due to loss of a Plo1p function, rather than dominant interference with activation by another effector.

To determine whether the loss of PBF activity affected the periodic transcription of its target genes, we examined the cell cycle profile of *cdc15*<sup>+</sup> and *spo12*<sup>+</sup> mRNA in *plo1-ts35* cells synchronized by centrifugal elutriation at the permissive temperature of 25°C. The profiles of both transcripts were altered, and they were no longer expressed



**Fig. 6.** Cell cycle binding of PBF to PCBs. A population of wild-type cells (972h<sup>-</sup>) synchronous for division were size selected by centrifugal elutriation at 25°C, and cell samples taken every 20 min for gel retardation analysis. PBF was detected using the *cdc15*<sup>+</sup> promoter fragment as labelled probe with 20 µg of protein in each sample. Lane P, 20 µg of protein from asynchronous cells with probe. The large arrow indicates PBF, and the small arrow indicates free probe. The ratio of PBF to free probe is plotted.

at the M-G<sub>1</sub> boundary but instead were delayed and transcribed coincident with *cdc22*<sup>+</sup> at G<sub>1</sub>-S (Figure 7B). Furthermore, *plo1*<sup>+</sup> itself was no longer transcribed in a cell cycle-dependent manner in *plo1-ts35* cells, but mRNA levels were constant at low levels throughout the cell cycle. The level of *plo1*<sup>+</sup> mRNA in *plo1-ts35* cells was similar to the lowest trough level of the profile seen in a wild-type cell cycle (Figure 7B; compare lane 3 with 5–14). This observation implies that *plo1*<sup>+</sup> regulates its own expression, consistent with the presence of a PCB sequence in its promoter (Figure 4A). A reduction in *plo1*<sup>+</sup> transcript levels at 25°C is consistent with the minor (5%) misshapen septum phenotype seen at this temperature in these cells (data not shown). The alteration in transcript periodicity in *plo1-ts35* was specific to *cdc15*<sup>+</sup>, *spo12*<sup>+</sup> and *plo1*<sup>+</sup>, as the *cdc22*<sup>+</sup> transcript, which is under different cell cycle regulation, peaked in G<sub>1</sub> phase (Figure 7B).

We also tested the effect of a *plo1-ts35* temperature arrest on PCB-regulated gene transcription. A *plo1-ts35 cdc2-33* culture was incubated at 36°C for 150 min after enrichment for early G<sub>2</sub> cells by elutriation. Cells taken for

northern blot analysis contained very low levels of *cdc15<sup>+</sup>*, *spo12<sup>+</sup>* and *plol<sup>+</sup>* transcript (Figure 7C, lane 6), consistent with the suggestion that *plol<sup>+</sup>* is regulating the expression of these genes. These transcripts were present at higher levels in similarly arrested *cdc2-33* cells (data not shown), and in *cdc25-22* cells arrested at the same cell cycle stage (Figure 1B).

To summarize these three experiments, *plol-ts35* affected PBF binding *in vitro*, and *cdc15<sup>+</sup>*, *spo12<sup>+</sup>* and *plol<sup>+</sup>* transcription *in vivo*, suggesting that *plol<sup>+</sup>* controls M-G<sub>1</sub> PCB-regulated gene expression through PBF.

### Overexpression of *plol<sup>+</sup>* causes overexpression of PCB-regulated genes

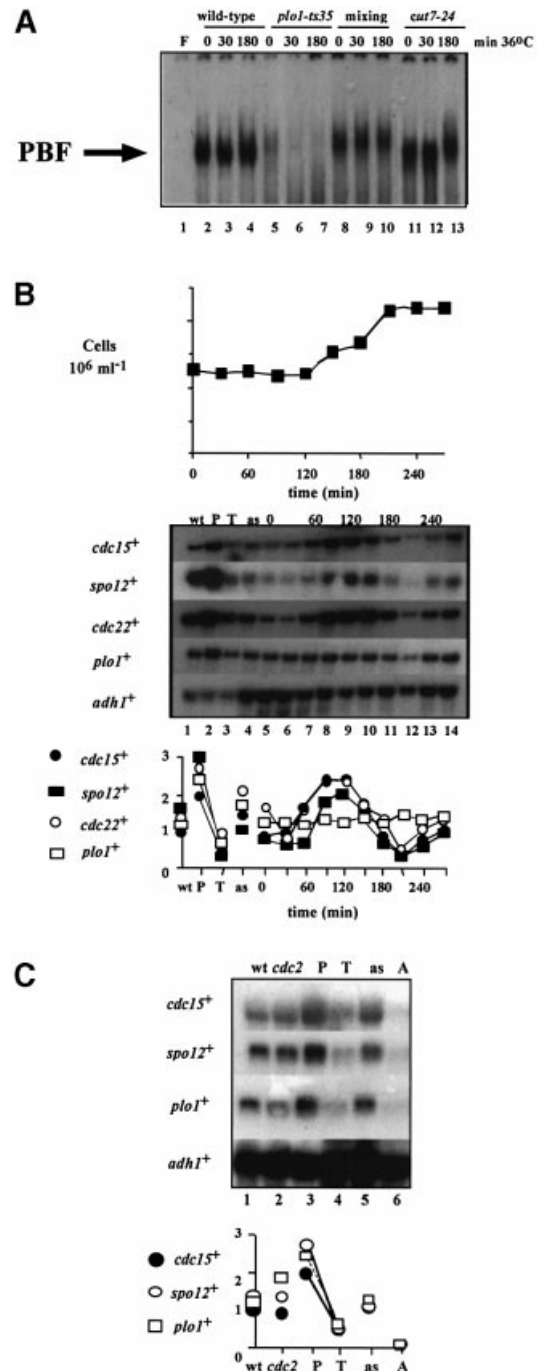
Previous experiments have demonstrated the critical role of *plol<sup>+</sup>* in controlling late cell mitotic events, as its overexpression in interphase cells resulted in premature septation (Ohkura *et al.*, 1995). This control of septation by *plol<sup>+</sup>* is thought to be, in part, through it activating the regulatory septation initiation network (SIN; Tanaka *et al.*, 2001). However, the active SIN needs to have the relevant target molecules in the F-actin ring in order to stimulate contraction of this ring and concomitant septation. Given that Plolp is required for PBF activity and cell cycle transcription of PCB-regulated genes, we next asked whether overexpression of *plol<sup>+</sup>* would also provide the target molecules for the SIN by inducing transcription of PCB-regulated genes in interphase cells.

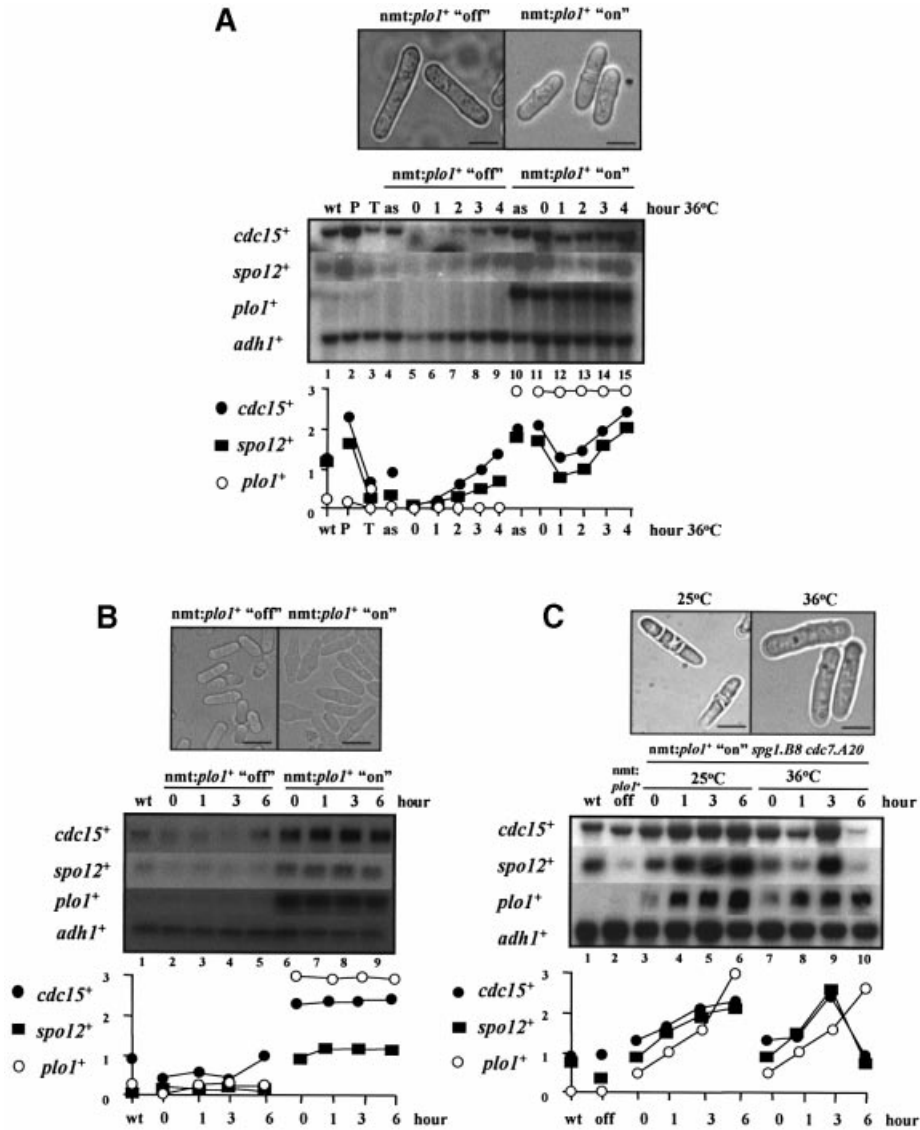
*plol<sup>+</sup>* transcription was induced in elutriated *cdc10-129* cells, immediately transferred to 36°C and so arrested in G<sub>1</sub> (Ohkura *et al.*, 1995), and *cdc15<sup>+</sup>* and *spo12<sup>+</sup>* mRNA levels were monitored. *plol<sup>+</sup>* overexpression caused multiple rounds of septation and resulted in abnormally high levels of both transcripts (Figure 8A; compare lanes 4–9 with 10–15). Significantly, the level of *cdc15<sup>+</sup>* and *spo12<sup>+</sup>* mRNA in these cells was similar to the levels seen at the peak of cell cycle expression of either gene in

wild-type cells at M-G<sub>1</sub> (Figure 8A; compare lane 2 with 10–15). We observed similar levels of overexpression for other PCB-regulated genes in G<sub>1</sub>-arrested cells overexpressing *plol<sup>+</sup>* (data not shown).

To confirm that inappropriate activation of PCB-regulated genes by *plol<sup>+</sup>* overexpression was not due to an artefact of the *cdc10-129* arrest, we performed a similar experiment in wild-type cells. *plol<sup>+</sup>* was overexpressed in a culture of asynchronous wild-type cells, and *cdc15<sup>+</sup>* and *spo12<sup>+</sup>* transcript levels were monitored. Transcripts of both genes were present at higher levels in these cells relative to cells where *plol<sup>+</sup>* was not overexpressed (Figure 8B).

**Fig. 7.** *plol-ts35* affects PBF *in vitro* and PCB-regulated gene transcription *in vivo*. (A) Gel retardation analysis was performed using the *cdc15<sup>+</sup>* promoter fragment as labelled probe with 20 µg of protein extracts from wild-type (972h<sup>-</sup>), *plol-ts35* and *cut7-24* cells grown at permissive (25°C) and restrictive temperatures (36°C), for the indicated times. In the mixing experiment, protein extracts from wild-type and *plol-ts35* cells were mixed before electrophoresis. Lane F indicates free probe. (B) Cell cycle transcription of *cdc15<sup>+</sup>*, *spo12<sup>+</sup>* and *plol<sup>+</sup>* in *plol-ts35* cells. A population of *plol-ts35* cells, synchronous for division, was size selected by centrifugal elutriation at 25°C. Cell samples were taken before elutriation (as) and at 30 min intervals after elutriation for northern blot analysis of RNA. Control RNA samples from asynchronous wild-type (972h<sup>-</sup>) cells (wt), and peak (P) and trough (T) *cdc15<sup>+</sup>* mRNA cell cycle samples from the experiment shown in Figure 1A were included. The blot was hybridized consecutively with *cdc15<sup>+</sup>*, *spo12<sup>+</sup>*, *cdc22<sup>+</sup>* and *adh1<sup>+</sup>* probes, the latter as a loading control. Quantification of each transcript against *adh1<sup>+</sup>* is shown. (C) Transcription of *cdc15<sup>+</sup>*, *spo12<sup>+</sup>* and *plol<sup>+</sup>* in *plol-ts35 cdc2-33* arrested cells. *plol-ts35 cdc2-33* cells were cell cycle arrested by temperature shift after enriching for early G<sub>2</sub> cells by elutriation (Tanaka *et al.*, 2001). Cell samples were taken before elutriation (as), and at the arrest (A) after elutriation for northern blot analysis of RNA. Control RNA samples from asynchronous wild-type (972h<sup>-</sup>) cells (wt), asynchronous *cdc2-33* cells (*cdc2*), and peak (P) and trough (T) *cdc15<sup>+</sup>* mRNA cell cycle samples from the experiment shown in Figure 1B were included. The blot was hybridized consecutively with *cdc15<sup>+</sup>*, *cdc22<sup>+</sup>* and *adh1<sup>+</sup>* probes, the latter as a loading control. Quantification of each transcript against *adh1<sup>+</sup>* is shown.





**Fig. 8.** Effect of overexpressing *plo1*<sup>+</sup> on PCB-regulated gene transcription. (A) Time course of *cdc15*<sup>+</sup> and *spo12*<sup>+</sup> mRNA levels during the overexpression of *plo1*<sup>+</sup> in *cdc10-129* arrested cells, following enrichment of G<sub>2</sub> cells by centrifugal elutriation (Mulvihill *et al.*, 1999). Two cultures of asynchronous *cdc10-129* cells containing pREP1:*plo1*<sup>+</sup> were grown for 15 h in EMM at 25°C, in one case in the presence of thiamine (*nmt:plo1*<sup>+</sup> off), and in the other in the absence of thiamine (*nmt:plo1*<sup>+</sup> on). G<sub>2</sub> cells were size selected by centrifugal elutriation, and immediately transferred to 36°C. Cell samples were taken before elutriation (as) and at hourly time points after elutriation (0–4), for northern blot analysis of RNA. Control RNA samples from asynchronous wild-type (972h<sup>-</sup>) cells (wt), and peak (P) and trough (T) *cdc15*<sup>+</sup> mRNA cell cycle samples from the experiment shown in Figure 1B were included. The blot was hybridized consecutively with *cdc15*<sup>+</sup>, *spo12*<sup>+</sup>, *plo1*<sup>+</sup> and *adh1*<sup>+</sup> probes, the latter as a loading control. Quantification of each transcript against *adh1*<sup>+</sup> is shown. Representative phase contrast micrographs of cells in both cultures are shown. Bar = 10 μm. (B) Time course of *cdc15*<sup>+</sup> and *spo12*<sup>+</sup> mRNA levels during the overexpression of *plo1*<sup>+</sup> in wild-type cells. A culture of asynchronous wild-type cells containing pREP1:*plo1*<sup>+</sup> was grown in the presence of thiamine (*nmt:plo1*<sup>+</sup> off) to early exponential stage of growth at 25°C. The culture was then split in two: one half grown in EMM in the absence of thiamine (*nmt:plo1*<sup>+</sup> on), the other half in EMM in the presence of thiamine (*nmt:plo1*<sup>+</sup> off), both for 15 h. Cell samples were taken for northern blot analysis of RNA at the times indicated. A control RNA sample from asynchronous wild-type (972h<sup>-</sup>) cells (wt) was included. The blot was hybridized consecutively with *cdc15*<sup>+</sup>, *spo12*<sup>+</sup>, *plo1*<sup>+</sup> and *adh1*<sup>+</sup> probes, the latter as a loading control. Quantification of each transcript against *adh1*<sup>+</sup> is shown. Representative phase contrast micrographs of cells in both cultures are shown. Bar = 10 μm. (C) Time course of *cdc15*<sup>+</sup> and *spo12*<sup>+</sup> mRNA levels during the overexpression of *plo1*<sup>+</sup> in *cdc7.A20 spg1.B8* arrested cells (Tanaka *et al.*, 2001). A culture of asynchronous *cdc7.A20 spg1.B8* cells containing pREP1:*plo1*<sup>+</sup> were grown for 15 h in EMM at 25°C, in the absence of thiamine (*nmt:plo1*<sup>+</sup> on). The culture was then split in two: one half grown at 25°C, and the other half transferred to 36°C. Cell samples were taken after temperature shift (0–6) for northern blot analysis of RNA. Control RNA samples from asynchronous wild-type (972h<sup>-</sup>) cells (wt), and *cdc7.A20 spg1.B8* cells containing pREP1:*plo1*<sup>+</sup> grown in the presence of thiamine (*nmt:plo1*<sup>+</sup> off) were included. The blot was hybridized consecutively with *cdc15*<sup>+</sup>, *spo12*<sup>+</sup>, *plo1*<sup>+</sup> and *adh1*<sup>+</sup> probes, the latter as a loading control. Quantification of each transcript against *adh1*<sup>+</sup> is shown. Representative phase contrast micrographs of cells in both cultures are shown. Bar = 10 μm.

***Plo1p* control of PCB-regulated genes does not require the active SIN**

*Plo1p* activates the SIN to control the timing of septation. It was possible that *Plo1p* controls the transcription of

PCB-regulated genes indirectly by activating the SIN. To test this possibility, we asked whether overexpression of *plo1*<sup>+</sup> promoted *cdc15*<sup>+</sup> and *spo12*<sup>+</sup> transcription in cells where SIN function was inactivated by temperature-

sensitive mutation. *plo1<sup>+</sup>* was overexpressed in *cdc7.A20 spg1.B8* cells at 36°C. As both the *cdc7.A20* and *spg1.B8* gene products are inactive at this temperature, *plo1<sup>+</sup>* overproduction cannot activate the SIN and so is unable to promote septation (Schmidt *et al.*, 1997; Tanaka *et al.*, 2001). *cdc15<sup>+</sup>* and *spo12<sup>+</sup>* were transiently overexpressed in these cells (Figure 8C; compare lanes 5 and 9). This result suggests that induction of *cdc15<sup>+</sup>* and *spo12<sup>+</sup>* transcription by *plo1<sup>+</sup>* is not dependent on the SIN, although SIN activity is required to maintain PBF activity.

## Discussion

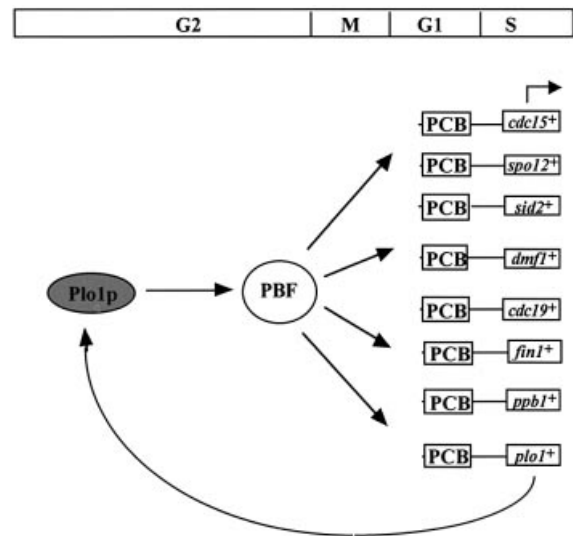
### *plo1<sup>+</sup>* regulates M–G<sub>1</sub> transcription

In fission yeast, the formation of the septum is coordinated with mitotic progression (Le Goff *et al.*, 1999). After commitment to mitosis, an F-actin ring forms in the middle of the cell. This ring contains the products of the *mid1<sup>+</sup>/dmf1<sup>+</sup>* and *cdc15<sup>+</sup>* genes. Later in mitosis, after the nuclei have separated, constriction of the F-actin ring and subsequent formation of the primary septum are induced by the activation of a protein kinase cascade called the septum initiation network (SIN). The activity of the small G protein, Spg1p, controls the activity of this cascade: septation is inhibited if Spg1p is maintained in an inactive state by the bipartite GAP complex Cdc16p–Byr4p.

Plo1p has already been shown to regulate more than one aspect of septation. It physically binds to Mid1p/Dmf1p and is required for the recruitment of this protein to the F-actin ring (Bahler *et al.*, 1998). Loss of either Plo1p or Mid1p/Dmf1p function results in malformed or inappropriately positioned septa (Chang *et al.*, 1996; Sohrmann *et al.*, 1996; Bahler *et al.*, 1998). Plo1p also acts later in mitosis when it activates the SIN (Tanaka *et al.*, 2001). Furthermore, a screen for mutations that are dependent upon elevated levels of Plo1p for survival has identified links with genes encoding modulators of the actin cytoskeleton, *sce3<sup>+</sup>*, *cdc8<sup>+</sup>* and *rho1<sup>+</sup>* (Cullen *et al.*, 2000). Thus, *plo1<sup>+</sup>* controls septation through regulating actin ring formation, the co-ordinated constriction of this ring and septum deposition.

We describe an additional mechanism by which *plo1<sup>+</sup>* regulates late mitotic events. We show that Plo1p controls expression of a group of genes late in the cell cycle (Figure 9). These genes include *cdc15<sup>+</sup>*, *ppb1<sup>+</sup>* and *sid2<sup>+</sup>*, which have important roles in different aspects of septation. *cdc15<sup>+</sup>* controls deposition of the actin ring (Fankhauser *et al.*, 1995; Utzig *et al.*, 2000), *ppb1<sup>+</sup>* regulates the degradation of the primary septum (Yoshida *et al.*, 1994), and *sid2<sup>+</sup>* is a component of the SIN that determines the timing of actin ring constriction (Balasubramanian *et al.*, 1998; Sparks *et al.*, 1999).

Our evidence for the role of *plo1<sup>+</sup>* in controlling the PCB-regulated genes comes partly from using a temperature-sensitive mutant of *plo1<sup>+</sup>*, *plo1-ts35*. *plo1-ts35* is a recessive, loss-of-function, mutant allele, which arrests mitotic progression due to an inability to form the mitotic spindle at the restrictive temperature. The septa in *plo1-ts35* cells at 36°C resemble those in *mid1-/dmf1-* cells in being misshapen and positioned inappropriately. The *plo1-ts35* mutant not only abolished PBF binding to the *cdc15<sup>+</sup>* promoter DNA *in vitro* (Figure 7A), but delayed cell cycle transcription of *cdc15<sup>+</sup>* and *spo12<sup>+</sup>*



**Fig. 9.** Model outlining the regulation of M–G<sub>1</sub>-specific transcription in fission yeast by Plo1p. A group of eight genes which are expressed periodically during the fission yeast cell cycle, with a peak of expression at M–G<sub>1</sub>, are required for cytokinesis and septation. This coordinate expression is controlled by a combination of a promoter sequence present in the gene promoters called a PCB (pombe cell cycle box), bound by a transcription factor complex PBF (PCB-binding factor). PBF activity is regulated by Plo1p; thus, potentially, *plo1<sup>+</sup>* regulates its own expression in a positive feedback loop.

*in vivo* (Figures 7B). This latter phenotype was specific to the PCB-regulated genes, as *cdc22<sup>+</sup>*, a G<sub>1</sub>–S cell cycle-regulated gene under DSC1–MCB control, was not affected. Importantly, these effects were seen at the permissive temperature for *plo1-ts35*, when mitotic progression was essentially normal. Therefore, they were not the secondary consequence of a mitotic defect in *plo1-ts35*, but were a direct consequence of compromised Plo1p function. A minority (5%) of *plo1-ts35* septa are misplaced or misshapen, which suggests that the periodicity of transcription that is missing in *plo1-ts35* may be important to ensure fidelity of septation.

The consequence of ectopic overexpression of *plo1<sup>+</sup>* confirmed the role of Plo1p in regulating PBF function, as expression in both G<sub>1</sub>-arrested and wild-type cells elevated transcription of *cdc15<sup>+</sup>* and *spo12<sup>+</sup>* (Figure 8A and B). Overexpression of *plo1<sup>+</sup>* in G<sub>1</sub> and G<sub>2</sub> has been shown previously to cause premature SIN activation and septation (Ohkura *et al.*, 1995; Mulvihill *et al.*, 1999; Tanaka *et al.*, 2001). We have established that overexpression of *plo1<sup>+</sup>* promoted transcription of the PCB-regulated genes in cells in which the SIN had been inactivated (Figure 8C). This indicates that *plo1<sup>+</sup>* induces PCB-mediated transcription independently of its function in regulating the SIN. It is interesting, however, to note that PBF activation by *plo1<sup>+</sup>* overproduction was only transient when SIN function was absent. This suggests that the SIN is required to maintain PBF-mediated transcription, once it has been activated by Plo1p. The transient nature of PBF activation is reminiscent of the transient induction of the EMTOC upon *plo1<sup>+</sup>* induction in SIN-defective cells (Heitz *et al.*, 2001). We suggest that overexpression of *plo1<sup>+</sup>* results in septation in G<sub>1</sub> and G<sub>2</sub> cells in part because it activates the SIN, but also because it results in expression of PCB-regulated genes, some of which are either components or substrates of the SIN.



**Table I.** Strain list

Collection No.	Genotype	Origin
GG1	972h <sup>-</sup> (wild type)	Laboratory stock
GG 194	h <sup>-</sup> <i>cdc2-33</i>	Laboratory stock
GG 202	h <sup>-</sup> <i>cdc10-M17</i>	Laboratory stock
GG 249	h <sup>+</sup> <i>cdc10-C4</i>	Laboratory stock
GG 394	h <sup>-</sup> <i>cdc25-22 ura4-D18 pSPΔ178.15UAS</i>	This study
GG 479	h <sup>-</sup> <i>cdc10 129 leu1-32 pREP1:plp1<sup>+</sup></i>	Laboratory stock
GG 481	h <sup>-</sup> <i>plp1-ts35 cdc2-33</i>	This study
GG 603	h <sup>-</sup> <i>ura4-D18 pSPΔ178.15UAS</i>	This study
GG 615	h <sup>-</sup> <i>leu1-32 pREP:plp1<sup>+</sup></i>	This study
IH 136	h <sup>-</sup> <i>cut7-24 leu1-32</i>	Laboratory stock
IH 1751	h <sup>-</sup> <i>cdc7.A20 spg1.B8 leu1-32 ura4-D18 pREP1:plp1<sup>+</sup></i>	Laboratory stock
IH 1758	h <sup>-</sup> <i>plp1-ts35 ura-D18 leu1-32</i>	This study

To summarize these experiments, we have shown that manipulating *plp1*<sup>+</sup> resulted in changes in the expression of PCB-regulated genes. Overexpressing *plp1*<sup>+</sup> elevated PCB-regulated gene transcription, whereas compromising *plp1*<sup>+</sup> function by mutation (implying reduced *plp1*<sup>+</sup> activity) delayed or abolished *cdc15*<sup>+</sup> expression. Combining these observations with the fact that *plp1-ts35* also affected PBF binding *in vitro*, we suggest that *plp1*<sup>+</sup> regulates septation by both SIN activation and through controlling expression of the *cdc15*<sup>+</sup> group of genes through the PCB and PBF transcription machinery.

**Feedback model**

*plp1*<sup>+</sup> itself was also cell cycle regulated at M–G<sub>1</sub>, raising the possibility that there is a positive feedback loop regulating late mitotic events (Figure 9). Potentially, *plp1*<sup>+</sup> could regulate its own expression through controlling the activity of PBF. *plp1-ts35* affected *plp1*<sup>+</sup> transcription (Figure 7B and C), and *plp1*<sup>+</sup> contains a putative PCB sequence in its promoter (Figure 4A). *plp1*<sup>+</sup> transcription was affected differently from other PCB-regulated genes in *plp1-ts35*, with mRNA being present at low levels throughout the cell cycle (Figure 7B). A possible explanation for this difference is that *plp1-ts35* initially results in reduced levels of *plp1*<sup>+</sup> transcription and Plo1p protein, which subsequently results in delayed *cdc15*<sup>+</sup> and *spo12*<sup>+</sup> transcription. At the very least, this result suggests that other levels of control of the *plp1*<sup>+</sup> gene are likely, which is consistent with the observation that Plo1p protein levels are not cell cycle regulated (Mulvihill *et al.*, 1999).

The fact that Plo1p is a protein kinase suggests that Plo1p may regulate PBF through phosphorylation. Modulation of transcription factor activity by phosphorylation is widespread and well studied (Whitmarsh and Davis, 2000).

**Components of PBF**

What are the molecular components of PBF? In budding yeast, the transcription factor complex that operates late in the cell cycle, and controls the homologue to *plp1*<sup>+</sup>, *CDC5*, is composed of Mcm1p, Fkh1p and Fkh2p (Koranda *et al.*, 2000; Kumar *et al.*, 2000; Pic *et al.*, 2000; Zhu *et al.*, 2000). Fkh1p and Fkh2p are both forkhead-type transcription factors. A gene containing a forkhead motif, named *sep1*<sup>+</sup>, has been identified in fission yeast in a mutant screen for genes required for septation (Ribar *et al.*, 1997)

and, provocatively, a *sep1Δ* mutant has been shown to affect *cdc15*<sup>+</sup> transcription during the cell cycle (Zilahi *et al.*, 2000). Unfortunately, we have been unable to show that Sep1p is part of PBF in gel retardation studies, with either epitope-tagged or deletion versions of the gene (S.S.Ng, M.Anderson, C.J.McInerny and V.Simanis, data not shown).

**Meiotic transcription**

The PCB-regulated genes are also transcribed specifically during meiosis, suggesting that these genes have a role in regulating the sexual life cycle in fission yeast (Mata *et al.*, 2002; our unpublished data). The induction of *cdc15*<sup>+</sup> occurred after pre-meiotic S phase, at a time corresponding to the first and second meiotic divisions, implying that these genes may be important for these events. While little is known about the function of the PCB-regulated genes during meiosis, it is clear that Plo1p displays discrete localization to the SPB and spindle during this alternative life cycle (Mulvihill, 1999).

The meiotic regulation of *cdc15*<sup>+</sup> may occur through PBF and the PCB elements. There is precedence in fission yeast for groups of genes being regulated by a similar transcription factor complex in both mitosis and meiosis: DSC1 and MCB sequences control *cdc22*<sup>+</sup> and the *rec*<sup>+</sup> genes during the two life cycles (L.Cunliffe, S.White and C.J.McInerny, unpublished data).

**Conservation of controls**

Homologues to a number of the genes that we have identified as being regulated at the M–G<sub>1</sub> interval in fission yeast are also cell cycle regulated in budding yeast. These include *CDC5* (*plp1*<sup>+</sup>), *DBF2* (*sid2*<sup>+</sup>), *SPO12* (*spo12*<sup>+</sup>) and *MCM2* (*cdc19*<sup>+</sup>), and are transcribed slightly earlier in the cell cycle during M phase (Cho *et al.*, 1998; Spellman *et al.*, 1998). This difference may arise from the alternative approaches to cytokinesis that have been adopted by the two organisms. Budding yeast generate the F-actin ring used for cytokinesis when they commit to the cell cycle at START. This ring persists until after anaphase when the SIN equivalent, the MEN, is activated and promotes both mitotic exit and cytokinesis (Bardin and Amon, 2001). Given that MEN activation follows so rapidly after commitment to anaphase, transcription of the *CDC5* group of genes at the metaphase–anaphase boundary

would prepare the cell for rapid and efficient utilization of the ring during cytokinesis.

In the case of MCM genes, these have also been shown to be expressed during late mitosis in budding yeast (Cho *et al.*, 1998; Spellman *et al.*, 1998). This control is probably relevant to the fact that these proteins are loaded onto chromosomes during late mitosis at anaphase, as an important part of regulating initiation of the subsequent S phase (Kearsey *et al.*, 2000).

The fact that genes with similar functions are transcribed specifically late in the cell cycle in both organisms suggests that such regulation of expression is significant. It will be important to establish if related mechanisms are conserved in other eukaryotes.

## Materials and methods

### Media and general techniques

General molecular procedures were performed as described by Sambrook *et al.* (1989), while the media used for the propagation of *S.pombe* were as described by Moreno *et al.* (1991). The standard genetic procedures of Gutz *et al.* (1974) and Kohli *et al.* (1977) were followed.

The strains used in this study are shown in Table I. *plol-ts35* was identified in a screen for diploidizing mutants (Broek *et al.*, 1991), which had a spindle formation defect, and is mutated in the kinase domain (F.H.MacIver, F.E.Stevens, D.M.Glover and I.M.Hagan, unpublished data).

For physiological experiments, cells were grown routinely in minimal medium (EMM) with shaking, at 25 or 32°C. Temperature-sensitive mutants were incubated at the restrictive temperature of 36°C in order to display their mutant phenotype.

Populations of synchronously dividing fission yeast cells were prepared by use of a Beckman elutriator rotor (Creanor and Mitchison, 1982). Synchronization of cells by transient temperature shifts in the *cdc25-22* mutant was achieved by growing cells to mid-exponential growth at 25°C, before shifting to 36°C; cells were then shifted back to 25°C after 3.5 h to enter the mitotic cell cycle in synchrony. *plol-ts35 cdc2-33* and *cdc2-33* cells, grown in YE, were elutriated to produce early G<sub>2</sub> cells, before transfer to 36°C for 2.5 h. Samples subsequently were removed for RNA extraction, and to measure septation indices by microscopic examination.

To overexpress *plol*<sup>+</sup> using the pREP1 vector (Maundrell, 1993), cells were grown in EMM with 5 µg/µl thiamine (*nmt1*<sup>+</sup> promoter 'off') to the early exponential stage of growth. Cells were washed three times in thiamine-free EMM, and then grown for 15 h in thiamine-free EMM (*nmt1*<sup>+</sup> promoter 'on'), at the same temperature. For *cdc* arrest experiments, the next day cells were synchronized by elutriation, and immediately transferred to 36°C (Ohkura *et al.*, 1995).

Cell number per ml of liquid culture was determined from a sample fixed in a 0.1% formaldehyde/0.1% sodium chloride solution. Following sonication, cells were counted electronically with a Z2 Coulter Counter. Flow cytometry analysis (fluorescence-activated cell sorting; FACS) was performed as previously described (McInerney *et al.*, 1995), using the FACScan system and the Cell Quest analysis program (Becton Dickinson, USA); 10 000 cells were analysed per time point.

### DNA constructs

The PCB-containing fragment DNA from the *cdc15*<sup>+</sup> promoter was amplified by PCR with *XhoI* restriction sites with oligos GCGCTCGAG-TATTGTGCACTCAGATAGGCA and GCGCTCGAGAAATTCCTG-GTAGTCGATTTC, and cloned into pSPΔ178 (Lowndes *et al.*, 1992) to create pSPD178.15UAS (GB 194). pREP1:*plol*<sup>+</sup> (pHN204; Ohkura *et al.*, 1995) was transformed into *leu1-32*, *cdc10-129* and *cdc7.A20 spg1.B8* cells.

### RNA manipulations

*Schizosaccharomyces pombe* total RNA was prepared (McInerney *et al.*, 1995) using a Ribolyser (Hybaid Ltd, UK), and northern blot analysis carried out using GeneScreen membrane (NEN, Life Science Products Inc., USA), following the manufacturer's suggested protocol. Northern blots were hybridized with the DNA probes made by PCR corresponding in each case to ~1 kb of each gene's open reading frame. DNA probes

were labelled with [ $\alpha$ -<sup>32</sup>P]dCTP using the random hexanucleotide labelling procedure of Feinberg and Volgelstein (1983). Equal loading of RNA in each lane was confirmed by hybridization with an *adh1*<sup>+</sup> probe. Transcripts were quantified using NIH software, and the ratios, relative to the invariant *adh1*<sup>+</sup> mRNA, estimated and plotted.

### Gel retardation analysis (band shift)

Whole-cell protein extracts were generated from wild-type (972h<sup>-</sup>) cells, and gel retardation analysis was performed as previously described (Ng *et al.*, 2001), using a labelled *cdc15*<sup>+</sup> promoter fragment made with oligos GCGGAATTCTATTGTGCACTCAGATAGGCA and GCGCTCGAG-AAATTCCTGGTAGTCGATTTC. This fragment corresponds to 'A' in Figure 3. To generate other *cdc15*<sup>+</sup> promoter fragments for competition experiments shown in Figure 3, the following oligos were used: 'B', GCGGTGCGAGGTGACAACCGTCCCTAGCAAG and GCGGAATTG-TATTGTGCACTCAGATAGGCA; 'C', TAGGGACGGTTGTACCCG; 'D', CTCAGATAGGCAACGGTTG; 'E', AGATAGGCAACGGTTGCTA; 'F', TAGGCAACGGTTGATAGG; 'G', GCAACGGTTGCTAGG-GAC; 'H', ACGGTGCTAGGGACGGTTG; and 'I', GTTGCTAGG-GACGGTTGTC were each amplified with the oligo GCGGTGAGAG-GATAAACATGTTTGTAG.

For the *spo12*<sup>+</sup> promoter analysis, two 20mer oligos containing the *spo12*<sup>+</sup> PCB, ATGGCGTAACAGTAAAAGT and ACTTTTACTGT-TACCGAACT, were annealed and used in competition reactions. Individual base pair requirements for PBF binding were established by creating 20mers as before, but with consecutive base pair substitutions A/T to G, or C/G to T.

Competition reactions were completed using promoter fragments amplified with oligos from the gene promoters *mid1*<sup>+</sup>/*dmf1*<sup>+</sup>, CGTTGCTATCAACAACTC and TTGTATTTTACTGACTGATAGC; and *cdc19*<sup>+</sup>, GGAAAATGTAGTGATACCTG and CAACTAAAC-GTTGGCAAATA.

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