Regulation of gene expression during M-G1-phase in fission yeast through Plo1p and forkhead transcription factors

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Accepted 16 October 2007
Journal of Cell Science 121, 38-47 Published by The Company of Biologists 2008
doi:10.1242/jcs.019489

Summary

In fission yeast the expression of several genes during M-G1 phase is controlled by binding of the PCB binding factor (PBF) transcription factor complex to Pombe cell cycle box (PCB) promoter motifs. Three components of PBF have been identified, including two forkhead-like proteins Sep1p and Fkh2p, and a MADS-box-like protein, Mbx1p. Here, we examine how PBF is controlled and reveal a role for the Polo kinase Plo1p. *plo1*$^+$ shows genetic interactions with *sep1*$^+$, *fkh2*$^+$ and *mbx1*$^+$, and overexpression of a kinase-domain mutant of *plo1* abolishes M-G1-phase transcription. Plo1p binds to and directly phosphorylates Mbx1p, the first time a Polo kinase has been shown to phosphorylate a MADS box protein in any organism. Fkh2p and Sep1p interact in vivo and in vitro, and Fkh2p, Sep1p and Plo1p contact PCB promoters in vivo. However, strikingly, both Fkh2p and Plo1p bind to PCB promoters only when PCB-controlled genes are not expressed during S- and G2-phase, whereas by contrast Sep1p contacts PCBs coincident with M-G1-phase transcription. Thus, Plo1p, Fkh2p and Sep1p control M-G1-phase gene transcription through a combination of phosphorylation and cell-cycle-specific DNA binding to PCBs.

Key words: Cell cycle, Fission yeast, Mbx1p, Plo1p, Forkhead, Transcription

Introduction

Periodic gene expression is an important mechanism that contributes to the orderly execution of cell cycle events (Breeden, 2003; McInerny, 2004; Bähler, 2005; Ng et al., 2006). Transcriptome analyses of the budding yeast *Saccharomyces cerevisiae* genome indicate that over 800 genes are periodically expressed during the mitotic cell cycle in this organism (Cho et al., 1998; Spellman et al., 1998). These genes fall into at least ten groups, where the expression of each group peaks at a distinct stage of the cell cycle and is regulated by a different transcription factor complex. Some periodically expressed genes encode cell regulators that activate the next wave of gene expression and/or repress the previous one. However, in many cases it has not been established how periodic gene expression is coordinated with cell cycle progression.

In budding yeast, one of the most studied waves of transcription is known as the CLB2 cluster. Approximately 30 genes are co-regulated, and these include *CLB2*, *CDC5*, *CDC20*, *SPO12, SWI5* and *DBF2,* which control mitotic entry, sister chromatid separation, mitotic exit and cytokinesis (Spellman et al., 1998; Jorgensen and Tyers, 2000). The promoters of these genes contain a Mcm1p-binding motif, flanked by a forkhead transcription factor site, which together are responsible for G2-M-specific expression through the mitotic cell cycle (Althoefer et al., 1995; Maher et al., 1995; Koranda et al., 2000; Kumar et al., 2000; Pic et al., 2000). Mcm1p recruits the forkhead transcription factor Fkh2p and this complex remains bound to promoters throughout the cell cycle (Althoefer et al., 1995; Hollenhorst et al., 2000; Koranda et al., 2000; Kumar et al., 2000; Pic et al., 2000; Zhu et al., 2000). A second forkhead transcription factor, Fkh1p, supports periodic transcription of the CLB2 cluster in the absence of Fkh2p but, unlike its relative, does not bind cooperatively with Mcm1p (Kumar et al., 2000; Hollenhorst et al., 2002).

In addition to Fkh1p, Fkh2p and Mcm1p, activation of the CLB2 cluster requires Ndd1p. *NDD1* is an essential gene; however, deletion of Fkh2p or truncation at its C-terminus relieves the requirement for *NDD1*, implying that Ndd1p functions with Fkh2p (Koranda et al., 2000). Conversely, overexpression of *NDD1* enhances levels of CLB2-cluster transcription (Loy et al., 1999). Unlike Mcm1p and Fkh2p, which bind throughout the cell cycle, Ndd1p is recruited to *CLB2* and *SWI5* promoters only during G2-M phase and this recruitment is dependent on either Fkh1p or Fkh2p (Koranda et al., 2000). Recently, it has been shown that phosphorylation of Ndd1p on threonine 319 by Clb2p and Cdc28p cyclin-dependent kinase promotes association of Ndd1p with the FHA domain of Fkh2p and transcriptional activation (Reynolds et al., 2003), and that Cdc5p (a Polo kinase) phosphorylates serine 85 of Ndd1p to ensure normal temporal expression of the CLB2 cluster (Darieva et al., 2006).

In the fission yeast *Schizosaccharomyces pombe* approximately 400 genes can be grouped into at least four clusters of periodic expression during the mitotic cell cycle (Rustici et al., 2004; Oliva et al., 2005; Peng et al., 2005). One cluster of coordinate expression occurs during late mitosis and early G1-phase and includes the *cdc15*$^+$, *spo12*$^+$, *fin1*$^+$, *slp1*$^+$, *ace2*$^+$, *fkh2*$^+$ and *plo1*$^+$ genes (Anderson et al., 2002). The products of these genes regulate spindle formation, the onset of anaphase, the formation and placement of the actomyosin ring and cytokinesis. As in budding

yeast, this cluster is controlled by two forkhead transcription factors, Sep1p and Fkh2p, and a MADS box protein, Mbx1p (Zilahi et al., 2000; Buck et al., 2004; Bulmer et al., 2004). Two of these, Fkh2p and Mbx1p, are phosphoproteins, whose phosphorylation status varies through the cell cycle peaking in late M-phase, with genetic experiments implying a role for the Polo protein kinase Plo1p (Anderson et al., 2002; Buck et al., 2004; Bulmer et al., 2004). In this paper we examine how this transcription factor complex is regulated, and reveal a role for phosphorylation of Mbx1p by Plo1p, together with cell-cycle-specific binding by Fkh2p, Sep1p and Plo1p to PCB promoters. These observations suggest a stepwise mechanism by which Plo1p, Mbx1p, Fkh2p and Sep1p control gene expression at this cell cycle time.

Results

Genetic interactions between Plo1p and components of PBF

Earlier observations have implicated Plo1p in the regulation of PBF. This was explored here by searching for genetic interactions between mutants of *plo1* and components of PBF. *plo1-ts35* is a temperature-sensitive mutant containing a mutation in the kinase domain, and is known to affect PBF binding to PCB motifs in vitro and M-G1-phase-specific gene expression in vivo (Anderson et al., 2002; Buck et al., 2004). Strong synthetic genetic interactions were seen in *plo1-ts35 sep1Δ*, *plo1-ts35 fkh2Δ* and *plo1-ts35 mbx1Δ* double mutants, which all showed striking septation and cell separation defects at the permissive temperature of 25°C (Fig. 1A). In all cases these phenotypes were more severe than those of the single mutants, with cells both more elongated and showing asymmetrically placed septa. Microscopic analysis of the number and position of nuclei revealed that these cells contained only one nucleus per compartment, as did the single mutants (data not shown).

Overexpression of *plo1* mutants affects M-G1 transcription

Previously, we showed that overexpressing wild-type *plo1*$^+$ caused induction of M-G1 transcribed genes (Anderson et al., 2002) (Fig. 1B). Using a similar approach we discovered that overexpressing *plo1K69R*, with a mutation in the kinase domain (Reynolds and Ohkura, 2003), abolished M-G1 transcription (Fig. 1B). As this repression occurred in cells containing endogenous wild-type *plo1*$^+$, this suggests that *plo1K69R* behaves as a dominant-negative mutant when overexpressed. Overexpressing *plo1DHK625AAA*, with a mutation in the Polo-box domain, did not have the same effect but, instead, caused induction of M-G1 gene transcription as seen upon overexpression of wild-type *plo1*$^+$ (Fig. 1B). This latter observation suggests that the Polo-box domain is not required for the transcription function of Plo1p.

Plo1p binds to Mbx1p in vivo

The possibility that Plo1p influences M-G1-specific expression by interacting with components of PBF was tested using the budding yeast two-hybrid system. Placing the *plo1*$^+$ gene in a bait vector in combination with a prey vector containing either *mbx1*$^+$, *fkh2*$^+$ or *sep1*$^+$ revealed a strong positive response with *mbx1*$^+$, but only a very weak or no response with *fkh2*$^+$ and *sep1*$^+$ (Fig. 2A). These observations suggest an in vivo interaction between Plo1p and Mbx1p. The interaction between Plo1p and Mbx1p requires both the kinase domain and Polo-box domain of Plo1p, because bait vectors containing *plo1* with mutations in these domains resulted in no two-hybrid colour response (Fig. 2B).

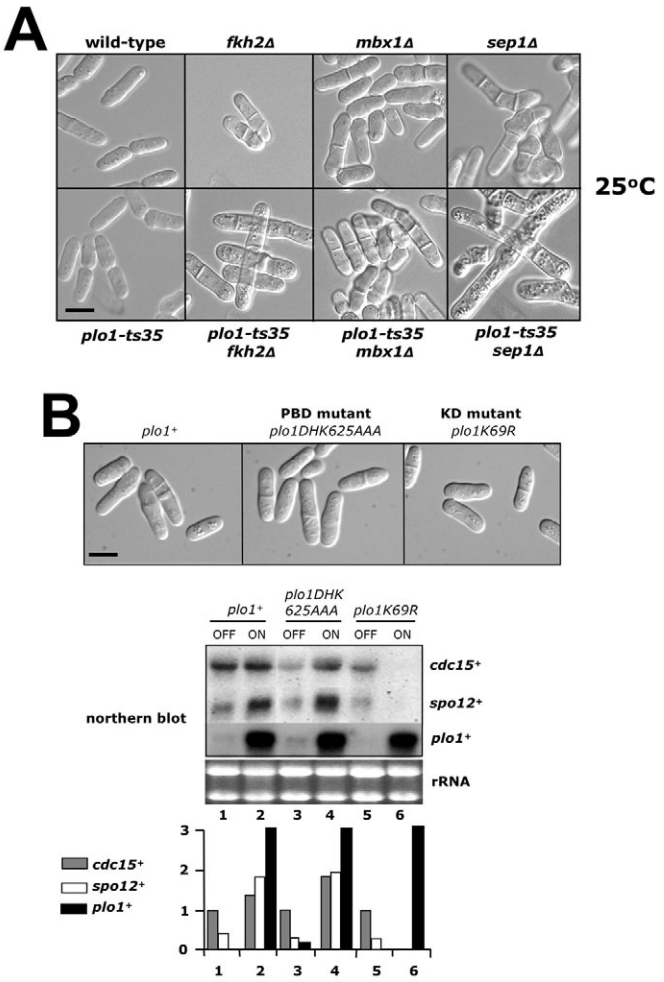
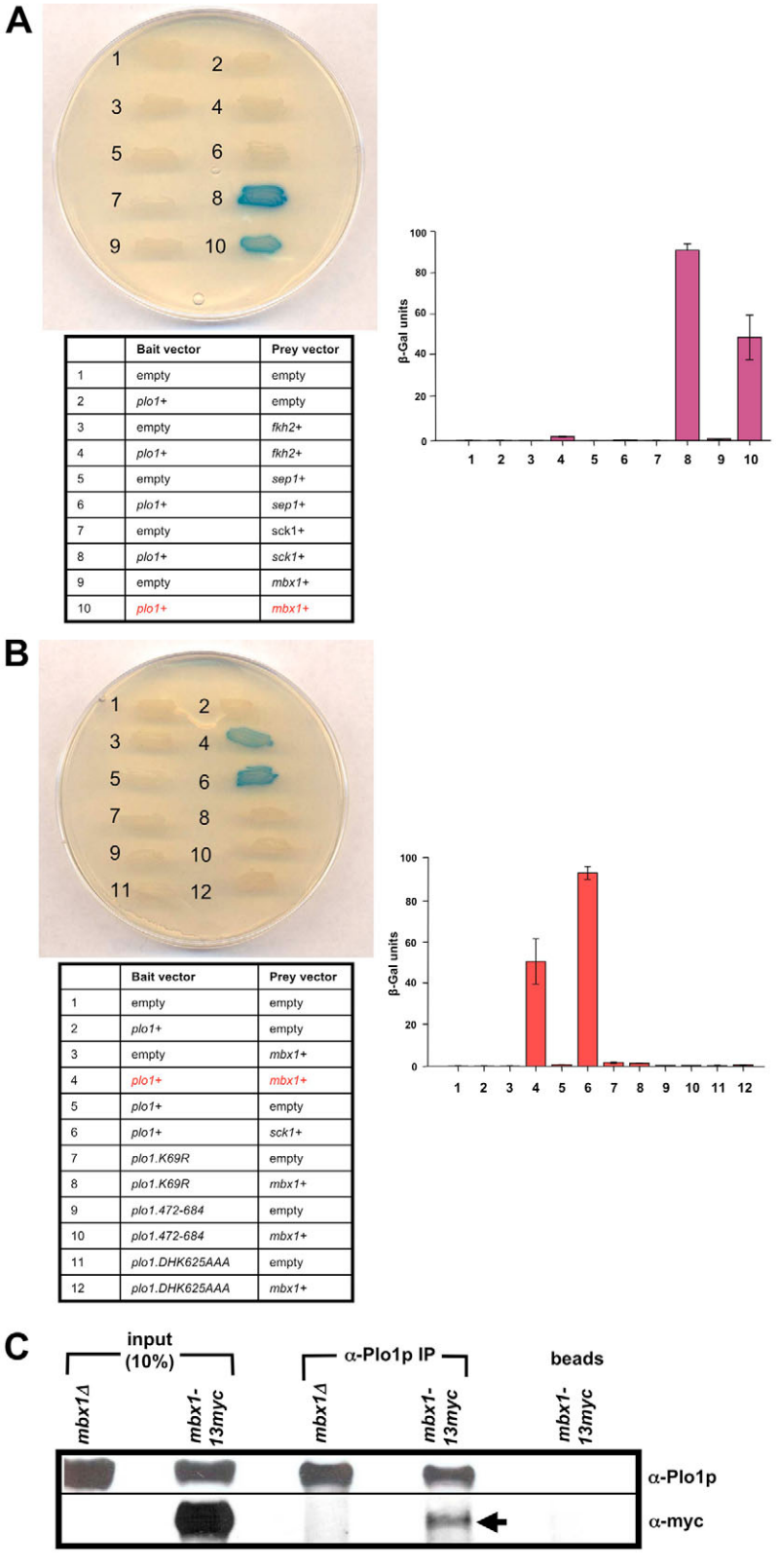


Fig. 1. Genetic interactions between *plo1*$^+$, components of PBF and M-G1 transcription. (A) Synthetic defective cell separation phenotypes revealed by double mutants in *plo1*, *fkh2*, *sep1* and *mbx1*. Cells containing combinatorial mutants of *fkh2Δ*, *sep1Δ*, *mbx1Δ* and *plo1-ts35* were grown on solid YE medium at 25°C, and viewed with DIC optics. Scale bar, 5 μm. (B) Effect on transcription of genes expressed at M-G1 phase by overexpressing mutant versions of Plo1p. Cells containing either pREP1: *plo1*$^+$ (wild-type), pREP1: *plo1DHK625AAA* [Polo-box domain (PBD) mutant], or pREP1: *plo1K69R* [kinase domain (KD) mutant] were grown to mid-exponential stage before the removal of thiamine to induce overexpression. After 16 hours cells were removed for microscopic examination and northern blot analysis of *cdc15*$^+$, *spo12*$^+$ and *plo1*$^+$ mRNA levels. Quantification of each transcript against rRNA is shown. Scale bar, 5 μm.

To confirm the interaction between Plo1p and Mbx1p we undertook immunoprecipitation experiments. Antibody against native Plo1p was used to make Plo1p immunoprecipitates from fission yeast cells that express a Myc-tagged version of Mbx1p controlled by its native promoter, and so present at normal levels. Under such conditions we could detect Mbx1p in co-immunoprecipitates by using antibodies against Myc (Fig. 2C; arrow). This experiment confirms that Plo1p and Mbx1p bind to each other in vivo.

Plo1p binds directly to and phosphorylates Mbx1p in vitro

As Plo1p appears to interact with components of PBF, we next tested whether this interaction leads to phosphorylation. 6His-Plo1p was purified and shown to be active through its ability to



	Bait vector	Prey vector
1	empty	empty
2	*plo1+*	empty
3	empty	*fkh2+*
4	*plo1+*	*fkh2+*
5	empty	*sep1+*
6	*plo1+*	*sep1+*
7	empty	sck1+
8	*plo1+*	*sck1+*
9	empty	*mbx1+*
10	*plo1+*	*mbx1+*

	Bait vector	Prey vector
1	empty	empty
2	*plo1+*	empty
3	empty	*mbx1+*
4	*plo1+*	*mbx1+*
5	*plo1+*	empty
6	*plo1+*	*sck1+*
7	*plo1.K69R*	empty
8	*plo1.K69R*	*mbx1+*
9	*plo1.472-684*	empty
10	*plo1.472-684*	*mbx1+*
11	*plo1.DHK625AAA*	empty
12	*plo1.DHK625AAA*	*mbx1+*

Fig. 2. Plo1p binds to Mbx1p in vivo. (A) Two-hybrid interaction between Plo1p and Mbx1p. Wild-type *plo1*$^+$ in the two-hybrid bait vector was transformed into budding yeast, together with empty prey vector, or prey vector containing either *mbx1*$^+$, *fkh2*$^+$, *sep1*$^+$ or *sck1*$^+$ (the latter encoding a known Plo1p-interacting protein) (Reynolds and Ohkura, 2003). Colour reactions were quantified in cells grown in liquid medium and are plotted; error bars indicate two standard errors. Table indicates combinations of two-hybrid plasmids used. (B) Two-hybrid interaction between Plo1p and Mbx1p requires both the kinase and the Polo-box domains. Wild-type *plo1*$^+$ or either kinase (*plo1K69R*) or Polo-box domain (*plo1.472-684* and *plo1DHK625AAA*) mutants of *plo1* in the two-hybrid bait vector were transformed into budding yeast, together with the empty prey vector, or the prey vector containing either *mbx1*$^+$ or *sck1*$^+$. Colour reactions were quantified in cells grown in liquid medium and are plotted; error bars indicate two standard errors. Table indicates combinations of two-hybrid plasmids used. (C) Mbx1p is co-immunoprecipitated with Plo1p from fission yeast extract. Plo1p was immunoprecipitated with antibody against the native protein from soluble extracts of fission yeast expressing Mbx1p-13myc from its endogenous promoter. The soluble extracts and the immunoprecipitates were analysed by western blotting with antibodies against Plo1p and Myc (the latter to detect Mbx1p-13myc). As controls, extracts from *mbx1Δ* and precipitates without antibodies (beads only) from tagged strains are also included. Approximately ten times more of the precipitates were loaded than of the soluble input fractions. Arrow indicates co-immunoprecipitated Mbx1p-13myc with Plo1p.

autophosphorylate (Fig. 3A) and phosphorylate myelin basic protein (data not shown). We also observed specific phosphorylation of purified GST-Mbx1p, but not GST-Sep1p nor 6His-Fkh2p (Fig. 3A; arrow).

To confirm this result, and use a system that allowed us to separate phosphorylated GST-Mbx1p and 6His-Plo1p – which have similar mobilities under SDS-PAGE conditions – an alternative purification protocol was adopted. GST-Mbx1p was overexpressed and purified using glutathione-Sephadex beads; however, the immobilised protein was subjected to a kinase assay with 6His-Plo1p without eluting with reduced glutathione. This method allowed for the recovery of GST-Mbx1p-beads after the mix had been subjected to extensive washes with wash buffer supplemented with 1% NP-40 to remove 6His-Plo1p. GST-Mbx1p was then removed from the beads by boiling in Laemmli buffer, and separated on an SDS-PAGE gel. An autoradiogram revealed the presence of a 90-kDa band, corresponding to Mbx1p (Fig. 3B; arrow). The fact that faster mobility phosphorylated bands were also observed, presumably indicating breakdown products of Mbx1p, implies that Mbx1p is phosphorylated in its N-terminus, the location of the GST-tag. This observation confirms that Plo1p binds directly to and specifically phosphorylates Mbx1p in vitro.

Plo1p binds to Mbx1p throughout the cell cycle

To probe how Plo1p may regulate M-G1 gene expression, we examined its binding to Mbx1p through the cell cycle. *mbx1-13myc* was expressed from its endogenous promoter in a *cdc25-22* strain, and cell cycle progression was synchronised by a temporary temperature shift to 36°C. On return to the permissive temperature of 25°C samples were removed at 20-minute intervals and processed for immunoprecipitation of Plo1p. Binding of Mbx1p-

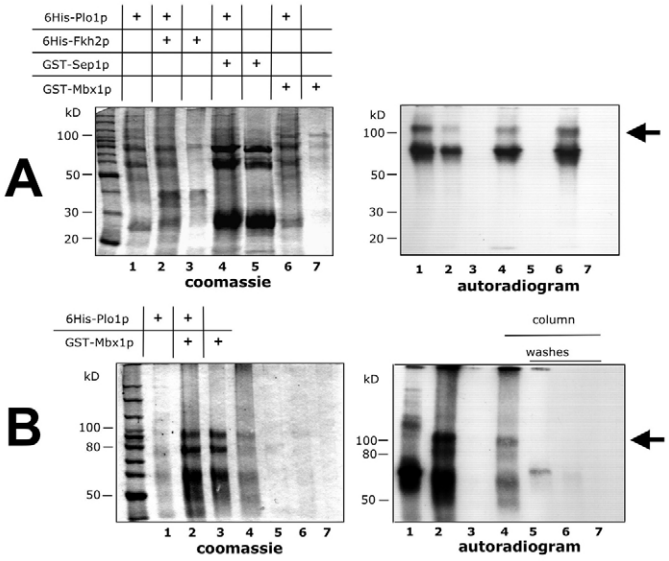


Fig. 3. Plo1p binds directly to and phosphorylates Mbx1p in vitro. (A) In vitro kinase assay with purified 6His-Plo1p and recombinant components of PBF. Separate kinase reactions with 6His-Plo1p and either 6His-Fkh2p, GST-Sep1p or GST-Mbx1p were separated by SDS PAGE, stained with Coomassie Blue and autoradiographed. Arrow indicates phosphorylated GST-Mbx1p, near to autophosphorylated 6His-Plo1p. Table indicates combinations of tagged proteins used. (B) In vitro kinase assay with purified 6His-Plo1p and GST-Mbx1p bound to glutathione-Sephadex beads after washes with 1% NP-40 to remove 6His-Plo1p, separated by SDS PAGE, stained with Coomassie Blue and autoradiographed. Arrow indicates phosphorylated GST-Mbx1p. Table indicates combinations of tagged proteins used.

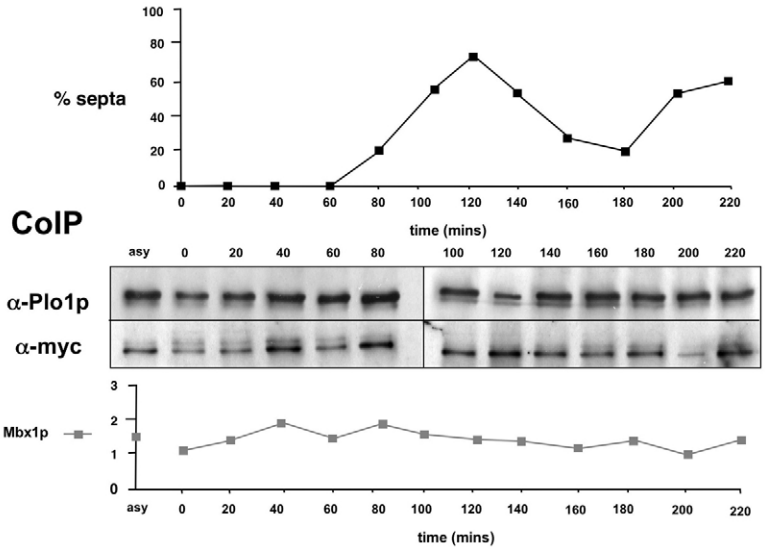


Fig. 4. Mbx1p binds to Plo1p throughout the cell cycle. *cdc25-22 mbx1-13myc* cells were synchronised by transient temperature arrest and samples taken every 20 minutes after returning the cells to the permissive temperature. Septation indices were counted microscopically and are plotted to indicate the synchrony of the culture. Soluble extracts from each time point were prepared, Plo1p was immunoprecipitated with antibody against the native protein, and the immunoprecipitates analysed by western blotting with antibodies against Plo1p and Myc (the latter to detect Mbx1p-13myc). asy, control sample from asynchronous cells prior to synchronisation. Quantification of co-immunoprecipitated Mbx1p against immunoprecipitated Plo1p is shown.

13myc to Plo1p was detected using antibodies against Myc, and this revealed that Mbx1p interacts with Plo1p throughout the mitotic cell cycle (Fig. 4).

Fkh2p and Sep1p bind to each other in vivo and in vitro

To analyse further the in vivo interactions between components of PBF, we completed co-immunoprecipitation experiments with tagged versions of Sep1p, Fkh2p and Mbx1p expressed from their endogenous promoters. This analysis revealed binding between Sep1p-3HA and Fkh2p-13myc (Fig. 5A), but not Mbx1p-13myc with either Sep1p-3HA or Fkh2p-3HA (data not shown). To determine whether the binding between Fkh2p and Sep1p is direct we completed in vitro 'pull-down' experiments with bacterially expressed and purified tagged versions of these two proteins. Such experiments revealed an interaction (Fig. 5B), and so confirmed that Sep1p and Fkh2p bind to each other directly.

Fkh2p, Sep1p and Plo1p bind in vivo to the promoters of genes transcribed during M-phase

As another way to examine the role of Plo1p in controlling gene expression in M-G1 phase we examined the binding of Plo1p and components of PBF to promoter regions in vivo by chromatin immunoprecipitation assays (ChIPs). Using antibodies against

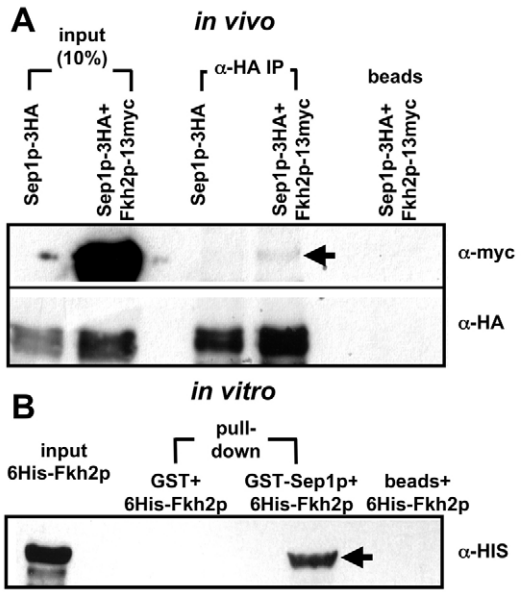


Fig. 5. Sep1p binds to Fkh2p both in vivo and in vitro. (A) Fkh2p is co-immunoprecipitated with Sep1p from fission yeast extract. Sep1p-3HA was immunoprecipitated with antibody against HA from soluble extracts of fission yeast expressing Fkh2p-13myc from its endogenous promoter. The soluble extracts and the immunoprecipitates were analysed by western blotting with antibodies against HA and Myc (the latter to detect Fkh2p-13myc). As controls, extracts from Sep1p-3HA-expressing cells alone and precipitates without antibodies (beads only) from the double-tagged strain are also included. Approximately ten times more of the precipitates were loaded than of the soluble input fractions. Arrow indicates Fkh2p-13myc co-immunoprecipitated with Sep1p-3HA. (B) 6His-Fkh2p is pulled down by GST-Sep1p in vitro. Bacterially expressed 6His-Fkh2p and GST-Sep1p were purified and mixed together. When GST-Sep1p was bound to GST beads, 6His-Fkh2p was present in the pull-down fraction, whereas GST alone was unable to pull down 6His-Fkh2p. Arrow indicates pulled-down 6His-Fkh2p with GST-Sep1p.

tagged versions of the proteins under the control of their native promoter (Fkh2p and Sep1p), or following mild overexpression with *nmt41* (Plo1p), we were able to detect Fkh2p-13myc, Fkh2p-3HA, Sep1p-13myc and Plo1p-3HA bound to the promoters of *cdc15*$^+$, *fkh2*$^+$ and *plo1*$^+$ (Fig. 6B-E). The only exception was that we were unable to detect specific binding of Sep1p to the *fkh2*$^+$ promoter (Fig. 6D). These three genes contain PCB sequences in their promoters and it was these specific regions that were amplified during the ChIP procedure, consistent with these DNA motifs having a role in M-G1-phase transcription. To explore how Plo1p and Mbx1p might control M-G1 transcription we analysed the binding of Fkh2p in the *plo1-ts35* mutant, and found that Fkh2p still bound to PCB DNA at the restrictive temperature of 36°C (Fig. 7A). Similarly, we found that the absence of Mbx1p or Sep1p had no effect on the ability of Fkh2p to bind to promoter DNA (Fig. 7B,C).

Fkh2p, Sep1p and Plo1p bind to PCB promoter DNA in a cell-cycle-specific manner

To examine how Fkh2p, Sep1p and Plo1p might contribute to the control of M-G1-phase-specific gene expression, we used ChIP to reveal their binding to PCB promoter DNA through the cell cycle. *fkh2-3HA*, *sep1-13myc* and *plo1-3HA* were separately introduced into a *cdc25-22* strain to allow the production of synchronously dividing cells. After release from the temperature cell cycle arrest, samples were taken every 20 minutes and

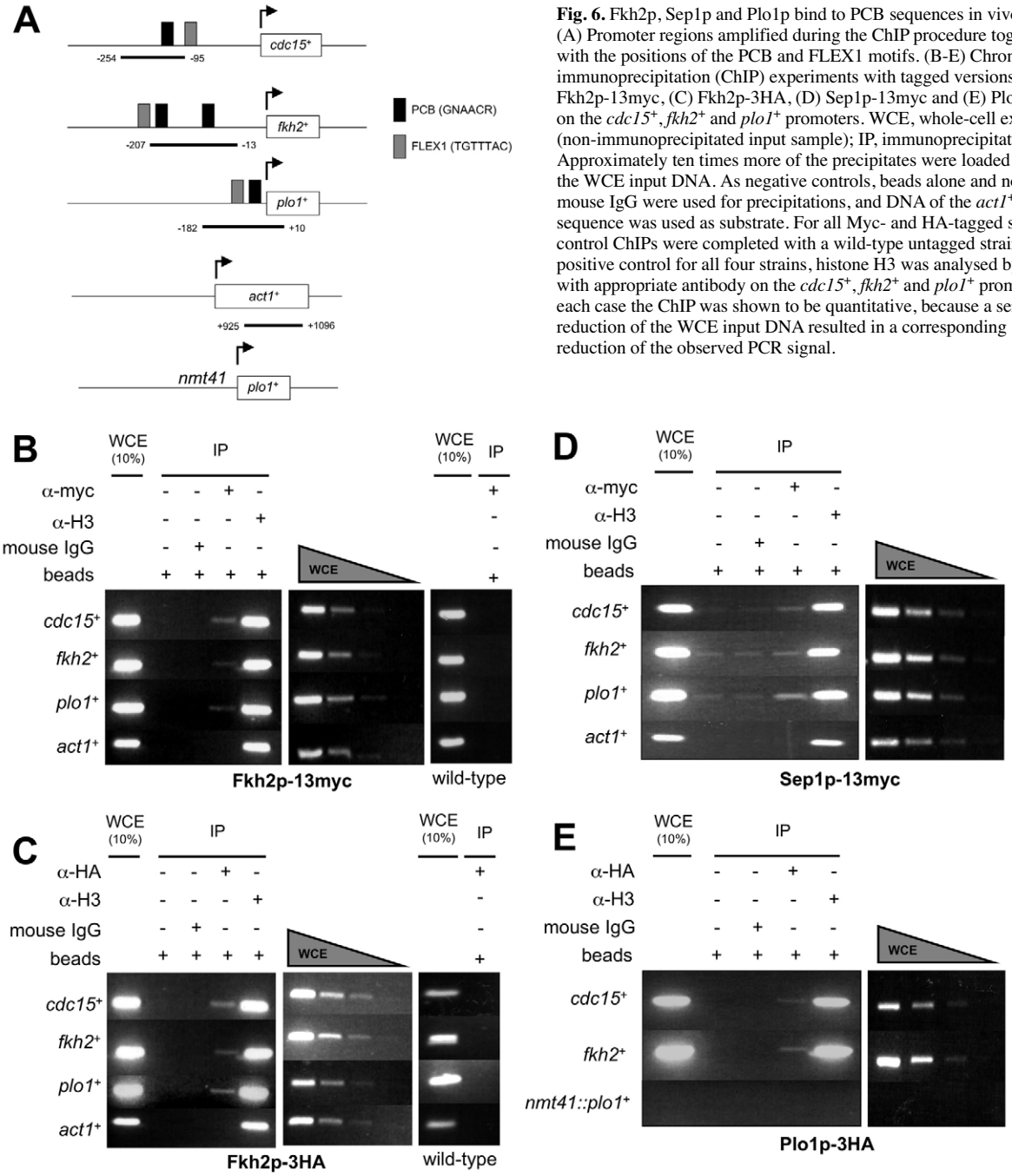


Fig. 6. Fkh2p, Sep1p and Plo1p bind to PCB sequences in vivo. (A) Promoter regions amplified during the ChIP procedure together with the positions of the PCB and FLEX1 motifs. (B-E) Chromatin immunoprecipitation (ChIP) experiments with tagged versions of (B) Fkh2p-13myc, (C) Fkh2p-3HA, (D) Sep1p-13myc and (E) Plo1p-3HA, on the *cdc15*$^+$, *fkh2*$^+$ and *plo1*$^+$ promoters. WCE, whole-cell extracts (non-immunoprecipitated input sample); IP, immunoprecipitates. Approximately ten times more of the precipitates were loaded than of the WCE input DNA. As negative controls, beads alone and normal mouse IgG were used for precipitations, and DNA of the *act1*$^+$-coding sequence was used as substrate. For all Myc- and HA-tagged strains, control ChIPs were completed with a wild-type untagged strain. As a positive control for all four strains, histone H3 was analysed by ChIP with appropriate antibody on the *cdc15*$^+$, *fkh2*$^+$ and *plo1*$^+$ promoters. In each case the ChIP was shown to be quantitative, because a serial reduction of the WCE input DNA resulted in a corresponding reduction of the observed PCR signal.

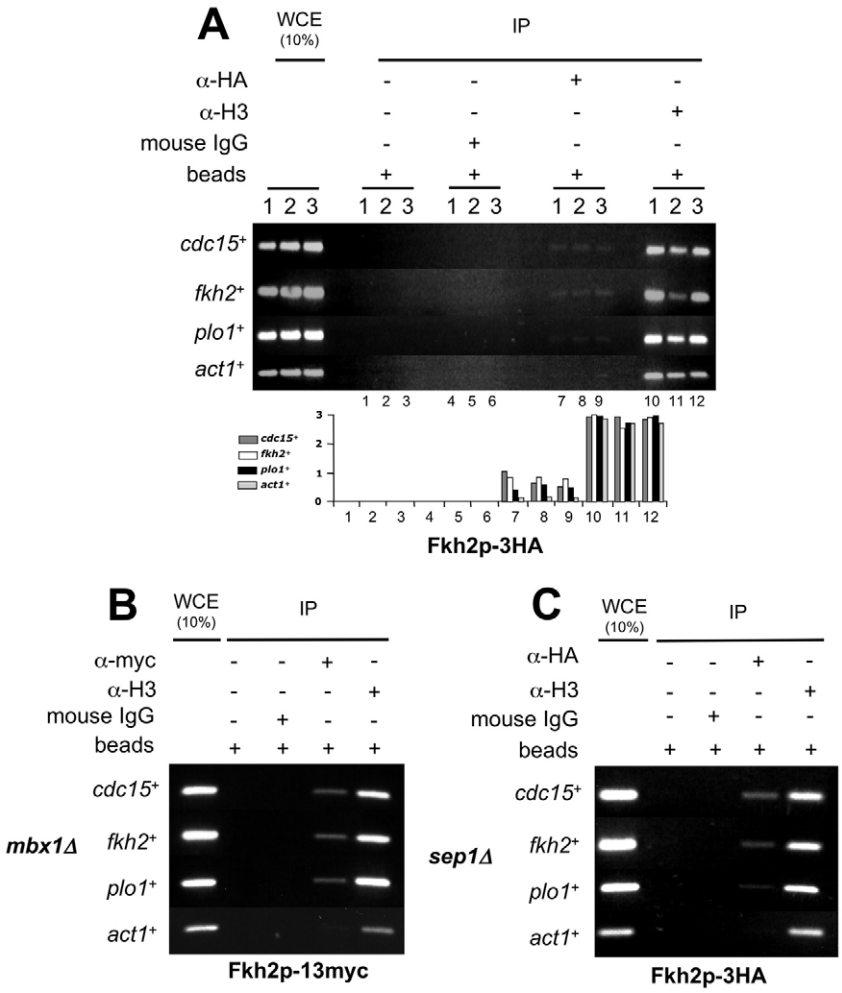


Fig. 7. Requirement of Plo1p, Mbx1p and Sep1p for Fkh2p promoter binding in vivo. ChIP experiments with Fkh2p-13myc and Fkh2p-3HA from extracts of cells containing mutants of *plo1*, *mbx1* and *sep1*. (A-C) Crosslinked DNA was prepared from (1) wild-type and (A) *plo1-ts35* cells at (2) permissive and (3) restrictive temperatures, or from cells with chromosome deletions of (B) *mbx1Δ* or (C) *sep1Δ*. In each case, Fkh2p-13myc and Fkh2p-3HA were analysed by ChIP using anti-Myc and anti-HA antibodies respectively, and binding to PCB promoter fragments from *cdc15*$^+$, *fkh2*$^+$ and *plo1*$^+$ detected by PCR. WCE, whole-cell extracts (non-immunoprecipitated input sample); IP, immunoprecipitates. Approximately ten times more of the precipitates were loaded than of the WCE input DNA. As negative controls, beads alone and normal mouse IgG were used for precipitations, and DNA of the *act1*$^+$-coding sequence was used as substrate. As a positive control, histone H3 was analysed by ChIP with appropriate antibody. Graph in A shows the quantification of Fkh2p binding against WCE input DNA. In each case the ChIP was shown to be quantitative, because a serial reduction of the WCE input DNA resulted in a corresponding reduction of the observed PCR signal (data not shown).

processed to detect binding to PCB promoter DNA of *cdc15*$^+$, *fkh2*$^+$ and *plo1*$^+$.

These studies revealed that Fkh2p bound to the *cdc15*$^+$, *fkh2*$^+$ and *plo1*$^+$ promoters in a cell-cycle-specific manner, with Fkh2p in contact when low levels of mRNA were present during S- and G2-phase (Fig. 8A). Maximum Fkh2p binding was observed when mRNA levels of *cdc15*$^+$, *fkh2*$^+$ and *plo1*$^+$ were decreasing, with no detectable binding of Fkh2p when mRNAs for these genes reappeared. Low levels of Fkh2p binding was detectable in the G2-arrested cells at time zero when some gene expression occurred, but this is possibly an artefact of the experimental procedure. Such a pattern of binding is consistent with Fkh2p being a repressor of M-G1-specific transcription.

Similarly, Plo1p binding to the promoters also occurred at a time when mRNAs levels of PCB-regulated genes were low (Fig. 8C), although the time at which highest levels of bound Plo1p were detected was later than that for Fkh2p. This implies that Plo1p binding causes a function later that Fkh2p, when gene repression has already occurred. As other experiments suggest that Plo1p has a positive role in gene expression in M-G1 phase (Anderson et al., 2002) (Fig. 1B), possibly this binding is the first step in re-activating gene expression.

Finally, and in contrast to Fkh2p and Plo1p, highest amounts of Sep1p were found to bind to the *cdc15*$^+$ and *plo1*$^+$ promoters coincident with peak levels of mRNAs of PCB-regulated genes (Fig. 8B), consistent with Sep1p being an activator of M-G1-specific transcription. In this experiment, *cdc15*$^+$ and *plo1*$^+$ transcription, as well as septation, were all delayed by 40 minutes compared with the cell cycle experiments examining Fkh2p and Plo1p binding (Fig. 8A,C). This observation implies that the Myc-tag on Sep1p is affecting its function, resulting in both delayed transcription and septation.

Discussion

The cell division cycle is regulated at multiple levels, with the control of protein abundance playing a major role. This regulation itself occurs in many ways, with the control of both transcription and protein stability being important. In this paper we have examined how in fission yeast a group of genes that encode proteins required for late cell cycle events is periodically transcribed at the M-G1 interval. We have discovered that, as part of this process, the Plo1p protein kinase directly binds to and phosphorylates the MADS-box protein Mbx1p. This is the first time a Polo kinase has been shown to bind and phosphorylate a MADS-box protein in any organism. However, the mammalian MADS box protein serum response factor (SRF) is highly phosphorylated at numerous sites, with a number of signal transduction pathways, including the MAP kinase signalling pathway, shown to have a role in this regulation (Rivera et al., 1993; Miranti et al., 1995; Dodou and Treisman, 1997; Heidenreich

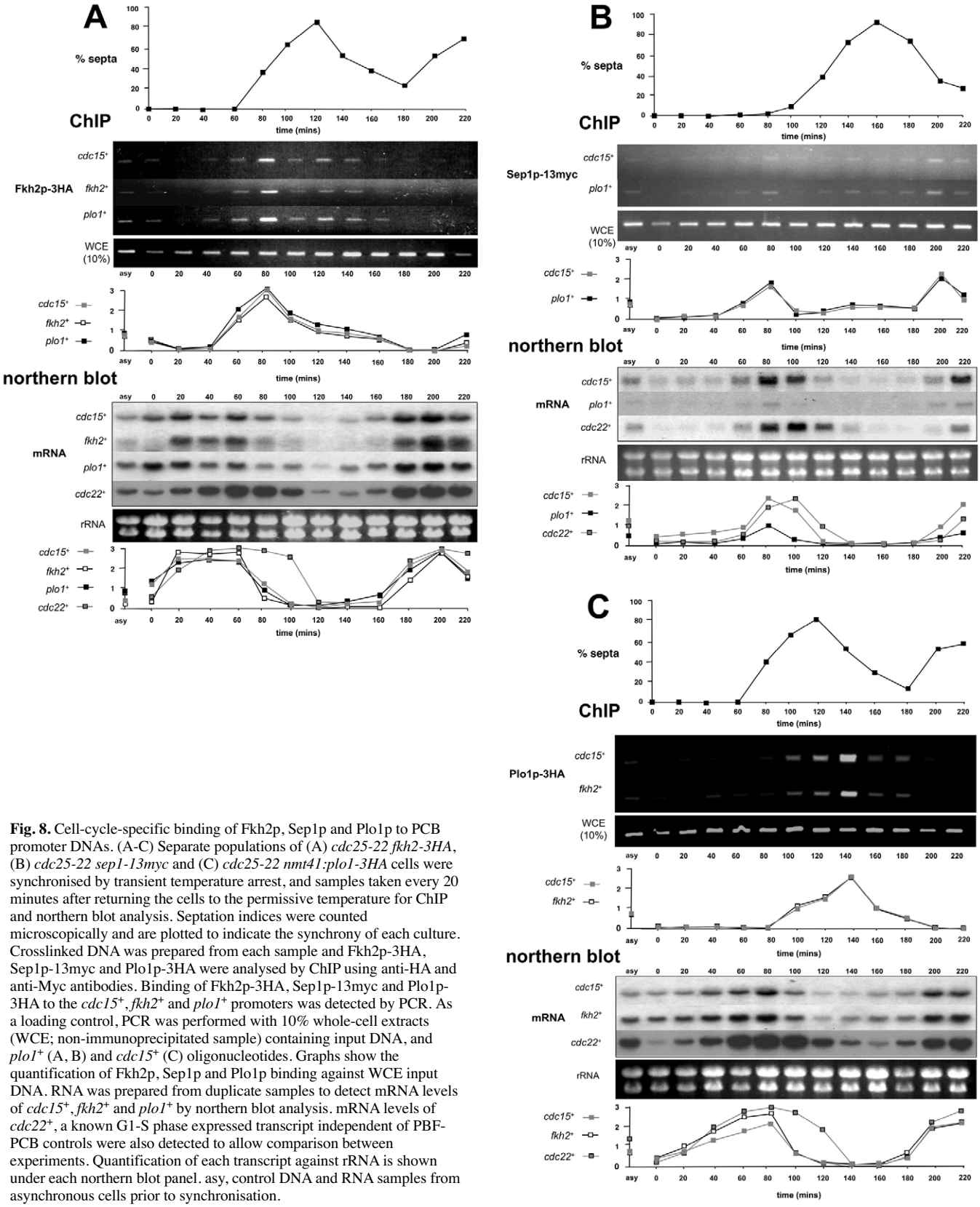


Fig. 8. Cell-cycle-specific binding of Fkh2p, Sep1p and Plo1p to PCB promoter DNAs. (A-C) Separate populations of (A) *cdc25-22 fkh2-3HA*, (B) *cdc25-22 sep1-13myc* and (C) *cdc25-22 nmt41:plo1-3HA* cells were synchronised by transient temperature arrest, and samples taken every 20 minutes after returning the cells to the permissive temperature for ChIP and northern blot analysis. Septation indices were counted microscopically and are plotted to indicate the synchrony of each culture. Crosslinked DNA was prepared from each sample and Fkh2p-3HA, Sep1p-13myc and Plo1p-3HA were analysed by ChIP using anti-HA and anti-Myc antibodies. Binding of Fkh2p-3HA, Sep1p-13myc and Plo1p-3HA to the *cdc15*$^+$, *fkh2*$^+$ and *plo1*$^+$ promoters was detected by PCR. As a loading control, PCR was performed with 10% whole-cell extracts (WCE; non-immunoprecipitated sample) containing input DNA, and *plo1*$^+$ (A, B) and *cdc15*$^+$ (C) oligonucleotides. Graphs show the quantification of Fkh2p, Sep1p and Plo1p binding against WCE input DNA. RNA was prepared from duplicate samples to detect mRNA levels of *cdc15*$^+$, *fkh2*$^+$ and *plo1*$^+$ by northern blot analysis. mRNA levels of *cdc22*$^+$, a known G1-S phase expressed transcript independent of PBF-PCB controls were also detected to allow comparison between experiments. Quantification of each transcript against rRNA is shown under each northern blot panel. asy, control DNA and RNA samples from asynchronous cells prior to synchronisation.

et al., 1999). As SRF, in combination with other transcription factors, controls the expression of a number of groups of genes, it has been proposed that the specific phosphorylation by various kinases may confer and regulate gene expression (Whitmarsh and Davis, 2000). In support of this concept, recent work with replicating mammalian myoblasts has lead to the proposal that

phosphorylation of SRF serine residue 162 permits the specific regulation of two groups of target genes, those required for differentiation and others involved in proliferation (Iyer et al., 2006).

In the context of cell-cycle-regulated transcription, the budding yeast Polo kinase Cdc5p has been shown to contact and directly phosphorylate Ndd1p to contribute to the regulation of the CLB2 cluster of genes at the G2-M interval (Darieva et al., 2006). Thus, it appears that the regulation of cell cycle transcription factors by direct Plo1p phosphorylation may be widespread amongst eukaryotes. As Plo1p in fission yeast is required for normal expression of genes at the M-G1 interval, and *plo1*$^+$ is itself expressed at this time, this implies that *plo1*$^+$ controls its own expression. The fact that *plo1*$^+$ is transcribed at M-G1 phase, contains PCB sequences in its promoter and binds to PCB promoters in a cell cycle specific manner, all support this hypothesis.

Previous experiments showed that, at least in vitro, Mbx1p binds to PCB DNA throughout the cell cycle (Anderson et al., 2002; Buck et al., 2004). Unfortunately, for technical reasons, we have been unable to confirm this occurs in vivo by ChIP. However, the observation that Mbx1p binds to Plo1p throughout the cell cycle and that Plo1p interacts with PCB DNA in a cell-cycle-specific manner, suggests that a sub-population of Mbx1p is not in contact with gene promoters. As deleting *mbx1*$^+$ has little effect on M-G1 transcription this implies that much of the regulation occurs through the forkhead transcription factors Sep1p and Fkh2p, whose deletion results in loss of periodicity of transcription (Buck et al., 2004). Nevertheless, the observation that *mbx1*$^+$, *fkh2*$^+$ and *sep1*$^+$ genetically interact (Buck et al., 2004) supports the idea that this MADS box protein, together with the two forkhead transcription factors, mediate gene expression in M-G1 phase.

Another level of control operates through two forkhead transcription factors. We have shown that Fkh2p contacts promoter DNA in a cell-cycle-specific manner. Strikingly, Fkh2p is only bound to the promoters of *cdc15*$^+$, *fkh2*$^+$ and *plo1*$^+$ when these genes are not being expressed, which implies that Fkh2p is required for the negative regulation of their transcription. This observation is consistent with other studies, which have revealed that deletion of *fkh2*$^+$ causes overexpression of target genes (Rustici et al., 2004) and that its overexpression is lethal, causing the inhibition of transcription of PCB-regulated genes and cell cycle arrest (Buck et al., 2004). By contrast, maximum levels of Sep1p bind to PCB promoters coincident with transcription during M-G1 phase, implying it is required for positive regulation, in agreement with previous data that have suggested an activating role (Rustici et al., 2004). Regulation could occur through one forkhead transcription factor replacing the other in a stepwise manner, to either stimulate or repress gene expression. The fact that Fkh2p and Sep1p interact, and that Sep1p is required for Fkh2p function (Buck et al., 2004), suggests that this process occurs through the two transiently binding to each other.

Quite how this circuitry is first activated and then inactivated is unclear, but it possibly involves the stepwise activation of Plo1p, Sep1p and Fkh2p (Fig. 9) (Ng et al., 2006). In fission yeast Plo1p kinase activity fluctuates during the cell cycle, peaking during M-phase, with Plo1p activated by MPF at G2-M phase (Tanaka et al., 2001; MacIver et al., 2003). These observations imply that the initial activation of Plo1p through MPF triggers gene expression. The data presented here suggest a model whereby Fkh2p binds to promoters to repress gene expression, possibly through the recruitment of other factors that silence transcription, such as histone modifiers. Once this function is completed Fkh2p binding is no longer needed to maintain repression but, instead, the subsequent binding of Plo1p and Sep1p is required to re-activate gene transcription, possibly again through chromatin modification.

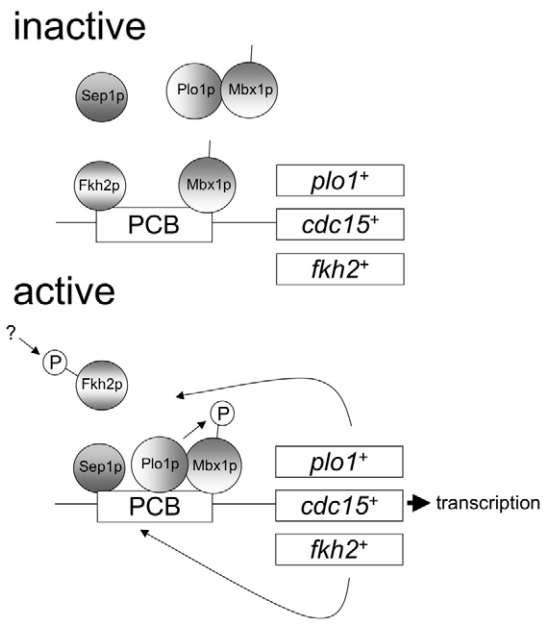


Fig. 9. Plo1p, Fkh2p and Sep1p regulation of genes expressed during the M-G1 phase in fission yeast. Expression of genes during late M phase in fission yeast is controlled by a transcription factor complex containing at least three proteins, the MADS box protein Mbx1p, and the forkhead transcription factors Fkh2p and Sep1p. Fkh2p and Sep1p both control cell cycle expression through cell-cycle-specific binding to PCB sequences, with Fkh2p repressing and Sep1p activating gene expression. Additionally, Mbx1p changes in a cell-cycle-specific manner. It is directly bound to and phosphorylated by Plo1p, whereby Plo1p stimulates transcription by binding to PCB DNA after Fkh2p. As *plo1*$^+$ and *fkh2*$^+$ are also transcribed at M-G1 phase, and both contain PCB sequences in their promoters to which Plo1p and Fkh2p bind, these genes regulate their own expression.

Taken together, these observations suggest that another fundamental function for the Plo1p kinase, known to have a central role in controlling cell division in fission yeast and many other organisms (Glover et al., 1998; Barr et al., 2004), is the direct control of phase-specific gene expression. The fact that Polo kinase in budding yeast has been shown to have a related function implies this property may be universally conserved in eukaryotes. Indeed, forkhead-like transcription factors, including human Plo1p homologue Plk1, have been shown to control cell cycle gene expression in humans (Alvarez et al., 2001). Future studies will explore this exciting possibility.

Materials and Methods

Media and general techniques

General molecular procedures were performed as described by Sambrook et al. (Sambrook et al., 1989), whereas the standard methodology and media used for the manipulation of *S. pombe* was as described by Moreno et al. (Moreno et al., 1991). The strains used in this study are shown in Table 1. *mbx1Δ* cells were found to mate with both h$^-$ and h$^+$; and the *fkh2Δ* slow growth phenotype was suppressed on solid minimal medium. For physiological experiments, cells were grown in minimal medium (EMM) while shaking at 25°C. Overexpression of genes was from pREP1 under the control of the *nmt1* promoter (Maundrell, 1993). Cells were grown in EMM with 5 μg ml^{-1} thiamine (*nmt1* promoter 'off') to early exponential stage, washed three times in thiamine-free EMM and then grown for 15 hours in EMM without

Table 1. Strains used in this study

Collection	Genotype	Origin
Fission yeast*		
GG 1	h⁻ 972 (wild-type)	Lab stock
GG 214	h⁻ *ura4⁺*	Lab stock
GG 308	h⁻ *cdc25-22*	Lab stock
GG 311	h⁻ *sep1-3HA:kanR*	Buck et al., 2004
GG 383	h⁻ *plo1-ts35*	Anderson et al., 2002
GG 504	h⁻ *mbx1-13myc:kanR ade6– his7-366*	Buck et al., 2004
GG 507	h⁻ *fkh2-13myc:kanR ade6–*	Buck et al., 2004
GG 508	h⁺ *fkh2-3HA:kanR*	Buck et al., 2004
GG 511	h⁻ *fkh2::kanR*	Buck et al., 2004
GG 515	h⁻ *sep1::ura4⁺*	Buck et al., 2004
GG 555	h⁻ *fkh2-3HA:kanR plo1-ts35*	This study
GG 571	h⁻ *mbx1-13myc:kanR plo1-ts35 ade6–*	This study
GG 596	h⁺ *sep1-3HA:kanR plo1-ts35*	This study
GG 704	h⁺ *leu1:nmt41:plo1-3HA plo1::his3⁺ his3-D1 ade6–*	H. Ohkura (University of Edinburgh, UK)
GG 743	h⁺ *mbx1-13myc:kanR cdc25-22*	This study
GG 745	h⁺ *fkh2-3HA:kanR cdc25-22*	This study
GG 761	h⁻ *sep1::ura4⁺ plo1-ts35*	This study
GG 763	h⁺ *fkh2::kanR plo1-ts35*	This study
GG 760	h⁺/⁻ *mbx1::kanR plo1-ts35 ade6– his7-366*	This study
GG 767	h⁻ *sep1-13myc:kanR*	This study
GG 1030	h⁺ *sep1-3HA:kanR fkh2-13myc:kanR ade6–*	This study
GG 1032	h⁻ *sep1-3HA:kanR mbx1-13myc:kanR his7-366*	This study
GG 1035	h⁻ *mbx1-13myc:kanR fkh2-3HA:kanR his7-366*	This study
GG 1040	h⁻ *sep1::ura4⁺ fkh2-3HA:kanR*	This study
GG 1043	h⁺ *sep1-3HA:kanR cdc25-22*	This study
GG 1044	h⁺ *fkh2::kanR sep1-3HA:kanR*	This study
GG 1047	h⁺/⁻ *mbx1::kanR fkh2-13myc:kanR ade6–*	This study
GG 1049	h⁺/⁻ *mbx1::kanR sep1-3HA:kanR his7-366*	This study
GG 1075	h⁺/⁻ *mbx1::kanR ade6– his7-366*	This study
GG 1097	h⁺ *leu1:nmt41:plo1-3HA cdc25-22 plo1::his3⁺ his3-D1*	This study
GG 1118	h⁺ *sep1-13myc:kanR cdc25-22*	This study
Budding yeast		
GGBY 144	Mat **a** *ura3-1 trp1-28 leu2DO lys2DO his 7 mob6::kanMX4 pep4::LEU2 pMH919: 6HIS:plo1*	This study

*All were *leu1-32 ura4-D18* unless otherwise stated. *ade6–* is either *ade6–M210* or *ade6–M216*.

thiamine (*nmt1* promoter 'on'). Cells were visualised using a Zeiss Axiostar microscope with Nomarski images captured by a Sony DS-75 digital camera.

DNA constructs

cDNAs of *fkh2⁺* and *mbx1⁺*, cloned from fission yeast mRNA using reverse-transcriptase PCR, and a genomic clone of *sep1⁺*, were separately ligated into the vectors pET-28c(+) and pGEX-KG. These allowed bacterial expression and purification using either the N-teminal 6His-tag or the GST-tag of the pET/pGEX vector. The *plo1⁺* coding sequence was cloned by PCR into the budding-yeast expression vector pMH919 (Geymonat et al., 2007) to produce N-terminally tagged 6His-Plo1p.

The *plo1⁺* and *sck1⁺* two-hybrid constructs have been described previously (Reynolds and Ohkura, 2003). To create the other two-hybrid constructs, cDNAs or genomic clones of *fkh2⁺*, *mbx1⁺* and *sep1⁺* were ligated into the pACT2 prey vector (Clontech). All constructs were confirmed by sequencing (MWG Biotech).

RNA manipulations

S. pombe total RNA was prepared according to Schmitt et al. (Schmitt et al., 1990) and analysed by northern blotting, as previously described (Anderson et al., 2002).

Two-hybrid analysis

Two-hybrid analysis using plo1⁺ as the bait was carried out as described (May et al., 2002; Reynolds and Ohkura, 2003). Quantitative β-galactosidase assays were performed in each case for two independent transformants assayed in triplicate.

Protein overexpression and purification

GST-Mbx1p, GST-Sep1p and 6His-Fkh2p were expressed in bacteria and purified using the heterologous tags, by using standard methods. 6His-Plo1p was expressed in budding yeast YAT287, by galactose-induced expression from the *GAL1-10* promoter of the pMH919 expression vector (Geymonat et al., 2007), and purified using Zn-bound sepharose beads; its activity was confirmed by its ability to phosphorylate myelin basic protein (data not shown).

Immunoprecipitations, pull-down assays and western blot analysis

Preparation of whole-cell extracts, immunoprecipitations, pull-down assays and western blot analysis were performed as previously described (Buck et al., 1996; May et al., 2002). The anti-Plo1p antibody has been described (May et al., 2002), and the antibodies against the His (Amersham), GST (Sigma), HA and Myc (Insight Biotechnology) tags are commercially available. Appropriate secondary rabbit and mouse antibodies for western blot analysis are also commercially available (DAKO).

Chromatin immunoprecipitation (ChIP)

Chromatin immunoprecipitations were performed as previously described with modifications (Ekwall and Partridge, 1999; Gomez-Roman et al., 2003). Exponentially growing fission yeast cells were fixed with 1% final concentration of formaldehyde for 30 minutes at 25°C. 4-5 μg of anti-HA or anti-Myc antibodies (Insight Biotechnology) were used against tagged versions of Fkh2p, Sep1p and Plo1p, whereas an antibody against histone H3 (Abcam) was used as a positive control.

The authors thank other members of the lab for help and encouragement. K.P. is supported by a Wellcome Trust Studentship, and SSN by the Wellcome Trust. Work in the C.J.M. laboratory is supported by the MRC and the Wellcome Trust, the H.O. laboratory by the Wellcome Trust, and the S.S. laboratory by the MRC.

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