Fkh2p and Sep1p regulate mitotic gene transcription in fission yeast

Vicky Buck[1,*], Szu Shien Ng[2,*], Ana Belen Ruiz-Garcia[1,*,‡], Kyriaki Papadopoulou[2], Saeeda Bhatti[2], Jane M. Samuel[1], Mark Anderson[2], Jonathan B. A. Millar[1,§] and Christopher J. McInerny[2,§]

[1]Division of Yeast Genetics, National Institute for Medical Research, The Ridgeway, Mill Hill, London, NW7 1AA, UK
[2]Division of Biochemistry and Molecular Biology, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow, G12 8QQ, UK
*These authors contributed equally to this work
‡Present address: Departament de Bioquimica i Biologia Molecular, Universitat de València, Dr Moliner 50, 46100 Burjassot, Valéncia, Spain
§Authors for correspondence (e-mail: jmillar@nimr.mrc.ac.uk; c.mcinerny@bio.gla.ac.uk)

Accepted 30 July 2004
Journal of Cell Science 117, 5623-5632 Published by The Company of Biologists 2004
doi:10.1242/jcs.01473

Summary

In the fission yeast *Schizosaccharomyces pombe*, several genes including *cdc15*$^+$, *spo12*$^+$, *fin1*$^+$, *slp1*$^+$, *ace2*$^+$ and *plo1*$^+$ are periodically expressed during M phase. The products of these genes control various aspects of cell cycle progression including sister chromatid separation, septation and cytokinesis. We demonstrate that periodic expression of these genes is regulated by a common promoter sequence element, named a PCB. In a genetic screen for cell cycle regulators we have identified a novel forkhead transcription factor, Fkh2p, which is periodically phosphorylated in M phase. We show that Fhk2p and another forkhead transcription factor, Sep1p, are necessary for PCB-driven M-phase-specific transcription. In a previous report we identified a complex by electrophoretic mobility shift assay, which we termed PBF, that binds to a 150 bp region of the *cdc15*$^+$ promoter that contains the PCB element. We have identified Mbx1p, a novel MADS box protein, as a component of PBF. However, although Mbx1p is periodically phosphorylated in M phase, Mbx1p is not required for periodic gene transcription in M phase. Moreover, although PBF is absent in strains bearing a C-terminal epitope tag on Fkh2p, simultaneous deletion of *fkh2*$^+$ and *sep1*$^+$ does not abolish PBF binding activity. This suggests that Mbx1p binds to gene promoters, but is not required for transcriptional activation. Together these results suggest that the activation of the Fkh2p and Sep1p forkhead transcription factors triggers mitotic gene transcription in fission yeast.

Key words: Fission yeast, Cell cycle, Transcription, Forkhead, MADS box

Introduction

Periodic gene expression is an important mechanism by which the orderly execution of cell cycle events is ensured (Futcher, 2000; Breeden, 2003). A comprehensive survey of the budding yeast *Saccharomyces cerevisiae* genome indicates that over 800 genes are periodically expressed through the mitotic cell cycle in this organism (Cho et al., 1998; Spellman et al., 1998). These genes fall into at least ten clusters, each of which peaks at a distinct stage of the cell cycle and is regulated by a different transcription factor complex. The products of some of these genes are transcriptional activators of the next wave of gene expression and/or repressors of the previous one. This observation has given rise to a model to explain how successive waves of transcription are induced (Lee et al., 2002). Although many periodically expressed genes are cell cycle regulators, it is not clear how periodic gene expression is precisely co-ordinated with cell cycle progression.

One of the best studied waves of transcription in budding yeast is known as the 'CLB2 cluster' (Spellman et al., 1998). This cluster contains approximately 30 genes including *CLB2*, *CDC5*, *CDC20*, *SPO12*, *SWI5* and *DBF2*, which control mitotic entry, sister chromatid separation, mitotic exit and cytokinesis (Jorgensen and Tyers, 2000). The promoters of these genes contain a Mcm1p-binding motif which is flanked by a forkhead transcription factor site. These elements 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). Fkh1p and Fkh2p additionally contain a forkhead-associated (FHA) domain which binds target molecules that have been specifically phosphorylated on threonine residues (Durocher and Jackson, 2002).

Several lines of evidence suggest that in addition to Fkh1p, Fkh2p and Mcm1p, activation of the CLB2 cluster requires the Ndd1p protein. *NDD1* is an essential gene that was originally identified as a multi-copy suppressor of the temperature-sensitive *cdc28-1N* allele (Loy et al., 1999). However, deletion of Fkh2p or truncation at its C-terminus relieves the requirement for *NDD1*, implying that Ndd1p works through

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 and this recruitment is dependent on either Fkh1p or Fkh2p (Koranda et al., 2000). Recently, Reynolds and colleagues (Reynolds et al., 2003) have shown that phosphorylation of Ndd1p on threonine 319 by Clb2p/Cdc28p kinase promotes association of Ndd1p with the FHA domain of Fkh2p and transcriptional activation.

In fission yeast approximately 400 genes are periodically expressed through the mitotic cell cycle and these can be grouped into at least four clusters (Rustici et al., 2004). The expression of one of these gene clusters peaks in G1 and is regulated by the MBF-DSC1 transcription factor complex. This is followed by the core histone wave during S-phase and then a group of genes during G2 including *spd1*$^+$, *psu1*$^+$ and *rds1*$^+$. Recently, a new cluster has been identified which is coordinately expressed during mitosis and early G1-phase and includes the *plo1*$^+$, *cdc15*$^+$, *spo12*$^+$, *ppb1*$^+$, *slp1*$^+$, *fin1*$^+$ and *sid2*$^+$ 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. In this study we have identified transcription factors which are responsible for the periodic expression of these genes.

Materials and Methods

Media and general techniques

General molecular procedures were performed as described by Sambrook et al. (Sambrook et al., 1989), and the standard methodology and media used for the manipulation of *S. pombe* was as described by Moreno et al. (Moreno et al., 1991). To construct the double and triple deletion strains, some crosses required 'covering' the single deletions with the equivalent wild-type genes on plasmids. The strains used in this study are shown in Table 1. For physiological experiments, cells were routinely grown in minimal medium (EMM) with shaking, at 25°C or 28°C. Synchronisation of cultures for cell cycle experiments was achieved using transient temperature shifts in *cdc25-22* strains as follows: mid-log cells growing at 25°C were shifted to 36°C for 4 hours before being shifted back to 25°C. Samples were removed at various times thereafter for microscopic examination and either northern or western blot analysis. Overexpression of genes was from pREP1 (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 thiamine (*nmt1* promoter 'on').

Cloning *fkh2*$^+$

A genomic library (pURB1) (Barbet et al., 1992) was introduced into *mcs3-12 wee1-50 cdc25-22 ura4-D18* strain (JM 1666) by electroporation and plated onto medium lacking uracil. Of 60,000 transformants screened, 55 complementing colonies were identified at 37°C. Sub-cloning, DNA sequencing and BLAST searches revealed that one of these suppressors was a gene with strong similarity to *S. cerevisiae FKH2* (SPBC16G5.15C) and it was therefore designated *fkh2*$^+$. Other genes isolated in this screen have been described elsewhere (Samuel et al., 2000). To test for allelism between *fkh2* and *mcs3*, *fkh2-3HA*:*kanR wee1-50 cdc25-22* (JM 2275) was mated with *mcs3-12 wee1-50 cdc25-22 ura4-D18* (JM 1666) and the asci analysed by tetrad dissection.

Table 1. Strains used in this study

Collection	Genotype	Origin
GG 1	h$^-$ 972 (wild-type)	Lab stock
GG 214	h$^-$ *ura4*$^+$	Lab stock
GG 308	h$^-$ *cdc25-22*$^+$	Lab stock
JM 1666	h$^-$ *mcs3-12 wee1-50 cdc25-22*	Lab stock
GG 515	h$^-$ *sep1*::*ura4*$^+$	This study
JM 2286	h$^-$ *fkh2*::*kanR*	This study
JM 2331	h$^-$ *mbx1*::*kanR ade6- his7-366*	This study
GG 543	h$^+$ *sep1*::*ura4*$^+$ *mbx1*::*kanR ade6- his7-366*	This study
GG 551	h$^+$ *fkh2*::*kanR mbx1*::*kanR ade6- his7-366*	This study
GG 631	h$^+$ *fkh2*::*kanR sep1*::*ura4*$^+$ *ura4*$^+$	This study
GG 746	h$^+$ *fkh2*::*kanR sep1*::*ura4*$^+$ *mbx1*::*kanR ade6-*	This study
JM 2285	h$^-$ *fkh2*::*kanR cdc25-22*	This study
JM 2805	h$^-$ *sep1*::*kanR cdc25-22*	This study
A 62	h$^-$ *sep1*::*ura4*$^+$ *cdc25-22*	M. Sipiczki*
JM 2332	h$^-$ *mbx1*::*kanR cdc25-22 ade6-*	This study
GG 311	h$^-$ *sep1-3HA*:*kanR*	This study
JM 2255	h$^-$ *fkh2-13myc*:*kanR ade6-*	This study
JM 2257	h$^+$ *fkh2-3HA*:*kanR*	This study
JM 2253	h$^+$ *fkh2-3HA*:*kanR cdc25-22*	This study
JM 2275	h$^+$ *fkh2-3HA*:*kanR wee-150 cdc25-22*	This study
JM 2349	h$^-$ *mbx1-13myc*:*kanR ade6- his7-366*	This study
JM 2252	h$^-$ *mbx1-13myc*:*kanR cdc25-22 ade6- his7-366*	This study

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

*University of Debrecen, Debrecen, Hungary.

Deletion and tagging

Deletions and tagged versions of *fkh2*$^+$, *sep1*$^+$ and *mbx1*$^+$ were made using the PCR-based gene targeting method of Bähler et al. (Bähler et al., 1998) or a refinement of this (Krawchuk and Wahls, 1999). For deletions, replacements were performed in wild-type diploids, potential heterozygotes identified by PCR and confirmed by southern blot before being sporulated and the asci dissected. Tagging was performed directly into wild-type haploids, potential positives identified by PCR and back-crossed to wild-type. Tagged genes were confirmed by western blot of protein extracts using anti-HA or anti-myc antibodies.

DNA constructs

The mutated *cdc15*$^+$ PCB reporter construct was made by changing the PCB sequence (GCAACG to AAGGTT) in pSPΔ178.15UAS (Anderson et al., 2002), to create pSPΔ178.15UAS.MUT (GB 344). pREP3X:*fkh2*$^+$ (GB 263) and pREP3X:*sep1*$^+$ (GB 290) were made by amplifying the open reading frames of each gene by PCR using oligonucleotides containing restriction sites for cloning into pREP3X.

RNA manipulations

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

Electromobility Bandshift Assay (EMSA)

Whole cell extracts were generated from cells and gel retardation analysis was performed with a *cdc15*$^+$ promoter fragment containing the PCB, as previously described (Anderson et al., 2002).

Immunoprecipitations and western blot analysis

Preparation of whole cell extracts, immunoprecipitations and western blot analysis were performed exactly as previously described (Buck et al., 1996).

Results

Identification of *fkh2*$^+$ as a regulator of mitosis in fission yeast

To identify novel regulators of mitosis we screened for multi-copy suppressors of the G2/M cell cycle arrest phenotype of *mcs3-12 cdc25-22 wee1-50* cells at restrictive temperature (Molz et al., 1989; Samuel et al., 2000). The *cdc25-22 wee1-50* double mutant strain is able to proliferate at 37°C, whereas the triple mutant strain carrying the *mcs3-12* mutation undergoes G2 arrest, implying a role for Mcs3p in controlling entry into mitosis. In addition to *cdc25*$^+$, *spo12*$^+$ and *swo1*$^+$, two identical genomic clones were identified that efficiently suppressed the triple mutant phenotype (Samuel et al., 2000). These unidentified clones contained a truncated version of a gene with strong sequence similarity to that encoding the *S. cerevisiae* Fkh2p forkhead-type transcription factor (Koranda et al., 2000; Kumar et al., 2000; Pic et al., 2000; Zhu et al., 2000). This novel *S. pombe* forkhead gene (SPBC16G5.15c) (Wood et al., 2002) was therefore designated *fkh2*$^+$. A schematic representation of the predicted full-length, 642 amino acid, 71 kDa Fkh2p polypeptide is shown in Fig. 1A. Fkh2p contains both a forkhead-binding domain (FHD) and a forkhead-associated domain (FHA) in the N-terminal half of the protein (Kaufmann and Knochel, 1996). The shortened protein responsible for rescue of *mcs3-12 cdc25-22 wee1-50* cells, Fkh2p (1-392), is truncated at its C-terminus by 250 amino acids. Surprisingly, full-length *fkh2*$^+$ was unable to rescue *mcs3-12 cdc25-22 wee1-50* cells at restrictive temperature, suggesting that the C-terminus of Fkh2p contains a regulatory domain (data not shown). Using standard genetic techniques, *fkh2*$^+$ and *mcs3*$^+$ were shown to be non-allelic (Materials and Methods). Database analysis reveals that the fission yeast genome contains three additional forkhead-type transcription factors, Sep1p, Mei4p and Fhl1p. Phylogenetic analysis of the forkhead-type transcription factors from budding and fission yeasts suggests that *S. pombe* Fkh2p is most closely related to the *S. cerevisiae* Fkh1p and Fkh2p proteins (Fig. 1A), which are involved in cell cycle-regulated transcription (Koranda et al., 2000; Kumar et al., 2000; Pic et al., 2000; Zhu et al., 2000).

fkh2$^+$ regulates the timing and position of the division septum

To investigate the function of *fkh2*$^+$, we first deleted the gene in a diploid (Bähler et al., 1998; Krawchuk and Wahls, 1999). After sporulating the heterozygous *kan*^{r}-tagged chromosomal deletion (*fkh2Δ/fkh2*$^+$), we found that all four progeny spores gave rise to viable haploids that segregated 2:2 with respect to G418 resistance (Fig. 1B), indicating that *fkh2*$^+$ is a non-essential gene. We noted that colonies of *fkh2Δ* cells grew more slowly than the wild-type and this was confirmed in liquid culture (Fig. 1B; data not shown). In addition, exponentially growing *fkh2Δ* cells were heterogeneous in size at division, with a greater mean length (17±5.3 μm) than wild-type cells (14.7±1.1 μm) (Fig. 1C). To investigate these phenotypes more closely, exponential *fkh2Δ* cells were stained for chromatin and septal material. Although 81% of the population appeared normal, 11% of the cells had multiple nuclei with unconstricted septa, 4% contained two nuclei but multiple septa and 4% showed incorrectly positioned or misoriented septa (Fig. 1D). We conclude that Fkh2p is required for the correct timing, positioning and contraction of the division septum in fission yeast.

fkh2$^+$ regulates periodic gene expression in M phase

In *S. cerevisiae* the *FKH1* and *FKH2* transcripts are

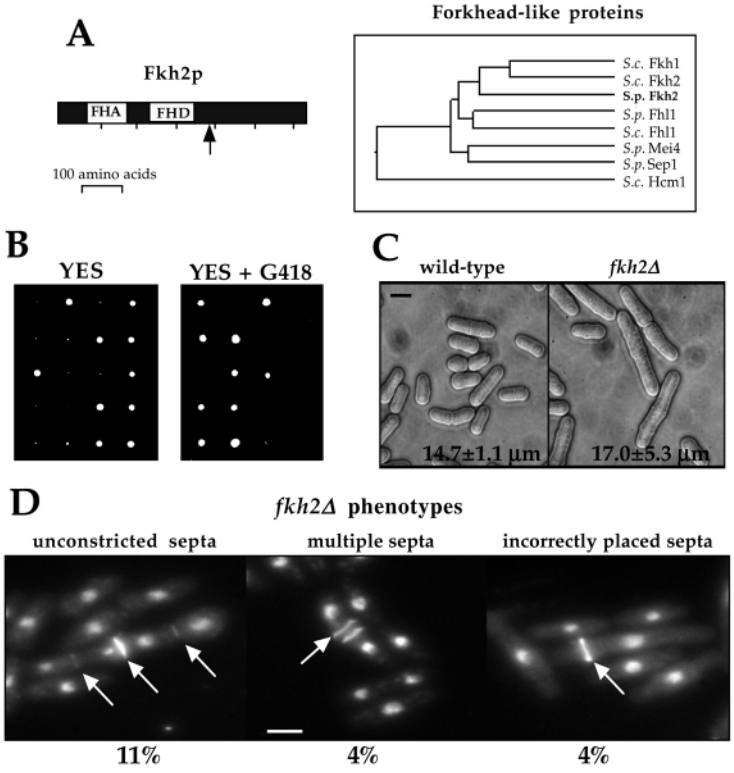
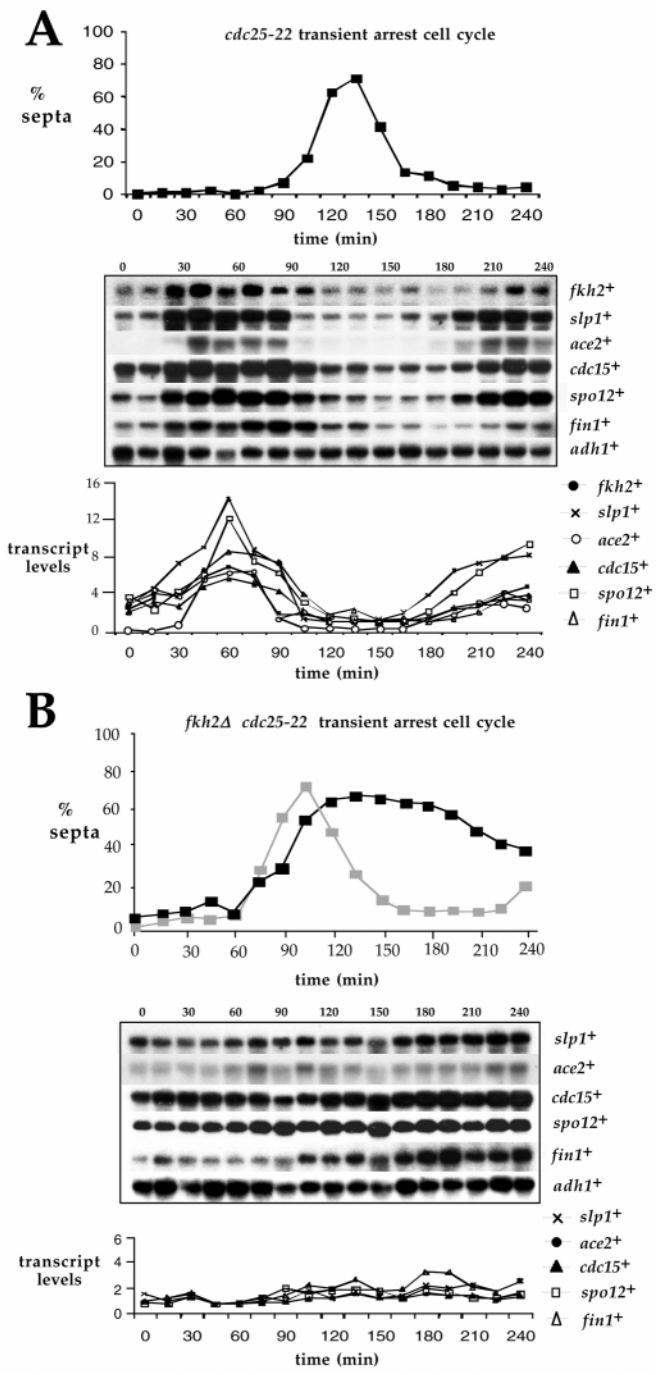


Fig. 1. *S. pombe fkh2*$^+$. (A) Schematic representation of the predicted 642 amino acid *S. pombe* Fkh2p, forkhead-like protein (SPBC16G5.15c) (Wood et al., 2002). Positions of the forkhead domain (FHD; 232-342) and the forkhead associated domain (FHA; 78-158) are shown. Arrow indicates position of the C-terminal truncation clone (1-392) that suppresses *mcs3-12 wee1-50 cdc25-22*. Phylogenetic tree showing the relationship between *S. cerevisiae* and *S. pombe* forkhead polypeptides, based on multiple sequence alignment, generated using the DNA cluster method in the Megalign programme based on a Jotun Hein algorithm (DNASTAR). (B) *fkh2*$^+$ is non-essential. Heterozygous diploids containing a *fkh2::kan*^{R} (*fkh2Δ*) and a wild-type allele were sporulated on EMM. Asci were separated by tetrad dissection and spores germinated on YE for 4 days, before replica plating on YE plus G418. (C) *fkh2Δ* cells have a variable length at division. Micrographs of wild-type (GG 1) and *fkh2Δ* (JM 2286) cells, growing in liquid EMM at 28°C. The length of 100 septating cells was measured and the mean length and standard deviation measured. Bar, 10 μm. (D) *fkh2Δ* (JM 2286) cells show abnormal septation. Fluorescent micrographs of *fkh2Δ* cells growing in liquid EMM at 28°C. Cells were fixed, the DNA stained with DAPI and septa marked with calcofluour. Representative images illustrating the range and incidence of septation defects (estimated from 100 cells) are shown. Arrows indicate positions of septa. Bar, 5 μm.

periodically transcribed, with mRNA levels peaking in S-phase (Cho et al., 1998; Spellman et al., 1998). This prompted us to examine whether *fkh2*$^+$ is periodically expressed during the fission yeast cell cycle. *cdc25-22* cells were transiently arrested in late G2 and released to the permissive temperature to enter a synchronous cell cycle. Northern blot analysis of mRNA isolated from successive time points revealed that *fkh2*$^+$ is periodically expressed during the fission yeast cell cycle. Similar results were obtained with synchronous wild-type cells, size selected by elutriation (data not shown). The peak of *fkh2*$^+$ mRNA expression is coincident with that of *cdc15*$^+$, *fin1*$^+$ and *spo12*$^+$ (Fig. 2A). We recently identified a sequence motif, known as a PCB element, in the *cdc15*$^+$, *fin1*$^+$ and *spo12*$^+$ promoters (Anderson et al., 2002). We note that *fkh2*$^+$ contains two putative PCB motifs (consensus GNAACG/A) in its promoter region (GCAACG at –208bp and GCAACG at –85bp, relative to the initiating ATG). We also found that *slp1*$^+$, the fission yeast homologue of the *S. cerevisiae CDC20* gene (Matsumoto, 1997) and *ace2*$^+$, the fission yeast homologue of the *S. cerevisiae ACE2* gene (Martin-Cuadrado et al., 2003), are periodically expressed at the same time (Fig. 2A). Both genes contain putative PCB sequences in their promoters: *slp1*$^+$ (GCAACA at –289 bp) and *ace2*$^+$ (GCAACG at –241 bp and GCAACA at –389 bp).



The similarity between Fkh2p and its homologues in budding yeast prompted us to examine the effect of deleting *fkh2*$^+$ on the periodic transcription of PCB-regulated genes. Separate cultures of *cdc25-22* and *fkh2Δ cdc25-22* were synchronised by transient temperature arrest and after release to the permissive temperature, samples were taken at intervals for both northern blot analysis and microscopic examination. In *fkh2Δ* cells the onset of septation was delayed by ~15 minutes and remained high at later time points relative to the control culture (Fig. 2B). The rate of binucleate formation in *fkh2Δ* cells was also delayed by ~15 minutes (data not shown), showing that either mitotic entry or the onset of anaphase was also delayed. Importantly, periodic transcription of *spo12*$^+$, *fin1*$^+$, *slp1*$^+$, *ace2*$^+$ and *cdc15*$^+$ was not observed in the absence of *fkh2*$^+$ (compare Fig. 2A and 2B). We conclude that *fkh2*$^+$ regulates periodic gene transcription in M phase. This may account for the role of *fkh2*$^+$ in controlling mitotic progression and the timing, positioning and contraction of the division septum.

sep1$^+$ and *fkh2*$^+$ have both common and distinct functions in cytokinesis

Previous studies have shown that another forkhead transcription factor, Sep1p, regulates septation and periodic transcription of the *cdc15*$^+$ gene (Ribar et al., 1997; Zilahi et al., 2000). To examine the relationship between *sep1*$^+$ and *fkh2*$^+$, each gene was expressed ectopically in *fkh2Δ* and *sep1Δ* cells, respectively. We found that mild overexpression of full-length *fkh2*$^+$ did not relieve the septation defect of *sep1Δ* cells, nor did mild overexpression of *sep1*$^+$ from its own promoter rescue loss of *fkh2*$^+$ (data not shown). However, we noted that strong overexpression of full-length *fkh2*$^+$ from the *nmt1*

Fig. 2. Role of *fkh2*$^+$ in M/G1-specific transcription. (A) *fkh2*$^+$, *ace2*$^+$ and *slp1*$^+$ are periodically expressed during M-phase. *cdc25-22* (GG 308) cells were synchronised by transient temperature arrest and samples taken every 15 minutes upon release to the permissive temperature. RNA was analysed by northern blot using *fkh2*$^+$, *slp1*$^+$, *ace2*$^+$, *cdc15*$^+$, *spo12*$^+$, *fin1*$^+$ and *adh1*$^+$ as probes, the latter as a loading control. Quantification of each transcript against adh1$^+$ is shown. Septation indices were counted microscopically and are plotted to indicate the synchrony of the culture. (B) Loss of M/G1 periodic transcription in *fkh2Δ* cells. *cdc25-22* (GG 308) and *fkh2Δ cdc25-22* (JM 2285) cells were synchronised by transient temperature arrest and samples taken every 15 minutes upon release to the permissive temperature. RNA was analysed by northern blot using *slp1*$^+$, *ace2*$^+$, *cdc15*$^+$, *spo12*$^+$, *fin1*$^+$ and *adh1*$^+$ as probes, the latter as a loading control. Quantification of each transcript against *adh1*$^+$ is shown. Septation indices were counted microscopically and are shown in black (*fkh2Δ cdc25-22*) or grey (*cdc25-22*).

promoter is lethal in wild-type cells but not in *sep1Δ* cells, suggesting that Sep1p is required for Fkh2p function (Fig. 3A). Conversely, strong overexpression of *sep1*$^+$ is lethal in both wild-type and cells lacking *fkh2*$^+$, indicating that Sep1p can function in the absence of Fkh2p (data not shown). It is interesting to note that the terminal phenotype of wild-type cells overexpressing full-length, but not truncated, *fkh2*$^+$ is elongated, suggesting that the C-terminus of Fkh2p inhibits mitotic entry in a dominant-negative manner (Fig. 3A, data not shown).

We next examined the effect of a deletion of *sep1*$^+$ on the periodicity of transcripts of the M/G1 gene cluster. *sep1Δ cdc25-22* cells were synchronised by transient temperature arrest and release before samples were subjected to northern blot analysis. We found that periodic expression of *cdc15*$^+$, *fkh2*$^+$, *spo12*$^+$, *slp1*$^+$, *ace2*$^+$ and *fin1*$^+$ (Fig. 3B) is abolished in the absence of Sep1p, thus confirming and extending previous observations (Zilahi et al., 2000). By contrast, deleting *fhl1*$^+$ (SPAC1142.08), which encodes a distinct forkhead-domain containing transcription factor in fission yeast, had no effect on periodic transcription of M/G1 genes (data not shown). These data suggest that a common function shared by *sep1*$^+$ and *fkh2*$^+$, but not *fhl1*$^+$, is the regulation of periodic expression of genes in M phase.

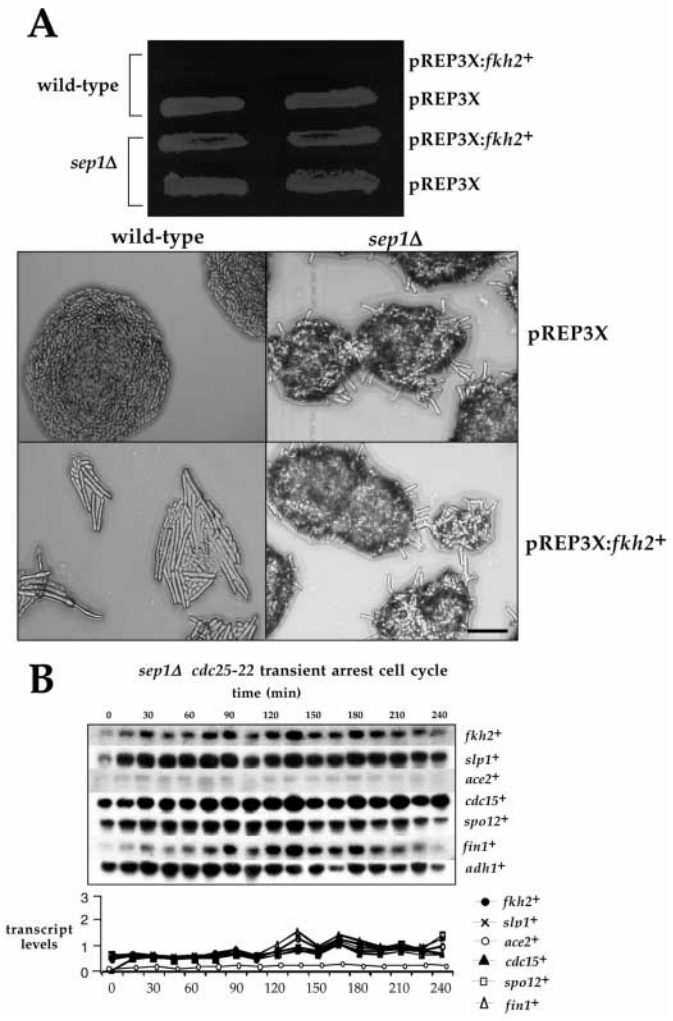


Fig. 3. *fkh2*$^+$ and *sep1*$^+$ share an overlapping function. (A) *sep1Δ* rescues the lethal phenotype caused by overexpression of *fkh2*$^+$. pREP3X:*fkh2*$^+$ and pREP3X were transformed into wild-type and sep1Δ cells, and colonies streaked on EMM agar to induce overexpression of *fkh2*$^+$. Micrographs of wild-type and *sep1Δ* cells overexpressing *fkh2*$^+$, grown on EMM agar. Bar, 30 μm. (B) Loss of periodic transcription in *sep1Δ* cells. *sep1Δ cdc25-22* (A 62) cells were synchronised by transient temperature arrest and samples taken every 15 minutes for northern blot analysis after release to the permissive temperature. The blot was probed consecutively with *fkh2*$^+$, *slp1*$^+$, *ace2*$^+$, *cdc15*$^+$, *spo12*$^+$, *fin1*$^+$ and *adh1*$^+$ probes, the latter as a loading control. Quantification of each transcript against *adh1*$^+$ is shown.

fkh2$^+$ and *sep1*$^+$ regulate M/G1-specific transcription through the PCB sequence

We previously showed that a *cdc15*$^+$ promoter fragment containing the PCB sequence in a reporter plasmid (pSPΔ178.15UAS) (Anderson et al., 2002) confers periodic M/G1 expression to *lacZ* in fission yeast (Fig. 4A) (Anderson et al., 2002). To examine the role of the core PCB sequence we changed it from GCAACG to AAGGTT in the reporter plasmid and found that this mutation resulted in a complete loss of cell cycle-regulated transcription of *lacZ*, but not of the endogenous wild-type *cdc15*$^+$ gene (Fig. 4D). We further found that periodic expression of *lacZ* was similarly abolished in both *fkh2Δ* (Fig. 4B) and *sep1Δ* cells (Fig. 4C). The *cdc15*$^+$ mRNA and septation profiles were different in these experiments compared with others (for example, Fig. 2A); this is likely to be because of the presence of the nutritionally selected plasmids in these strains, which affected growth rates.

Combined, these observations imply that *fkh2*$^+$ *and sep1*$^+$ control M/G1-specific transcription in fission yeast through the PCB sequences present in the promoters of M/G1 transcribed genes, although other sequences may also have a role.

Fission yeast contains three MADS box proteins

In *S. cerevisiae*, Fkh1p and Fkh2p combine with Mcm1p, a MADS box-type protein to control G2/M-specific transcription (Koranda et al., 2000; Kumar et al., 2000; Pic et al., 2000; Zhu et al., 2000). We therefore searched for MADS box-containing gene products (Triesman and Ammerer, 1992) in the fission yeast database (Wood et al., 2002) (Fig. 5A) and identified three. The first, Map1p, is not required for mitotic growth and displays no obvious cell cycle phenotype (Yabana and Yamamoto, 1996). A second (SPBC19G7.06), which we named Mbx1p (MADS box protein 1) (Fig. 5A), is most similar to *S. pombe* Map1p and *S. cerevisiae* Mcm1p and Arg80p. To investigate the function of Mbx1p, we generated a chromosomal deletion (Bähler et al., 1998; Krawchuk and Wahls, 1999). We found that *mbx1Δ* haploid cells are viable and grow at a similar rate to wild-type cells (data not shown). However, *mbx1Δ* cells showed a significant defect in cytokinesis (Fig. 5B), which was exacerbated in double mutants with either *sep1Δ* or *fkh2Δ* (Fig. 5C,D). Intriguingly, we find that the slow growth phenotype of *fkh2Δ* cells is not observed in *mbx1Δ fkh2Δ* cells, suggesting that Fkh2p, Sep1p and Mbx1p share a common, but not fully overlapping, function (Fig. 5C). The third MADS-box protein identified (SPBC317.01), which we named Mbx2p, is most similar to

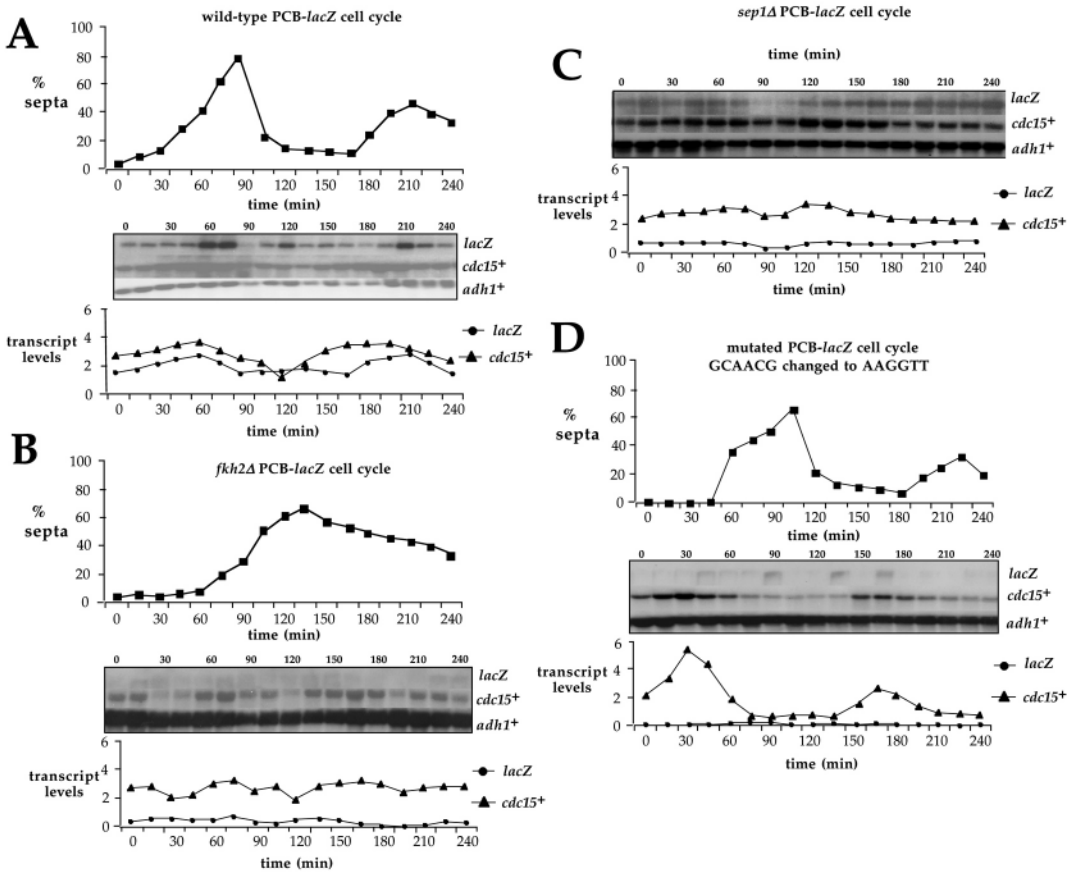


Fig. 4. Requirement of *fkh2*$^+$, *sep1*$^+$ and the PCB sequence for M/G1-specific transcription. (A) A *cdc15*$^+$ promoter fragment containing the PCB sequence confers M/G1-specific transcription. *cdc25-22* (GG 308) cells containing pSPΔ178.15UAS (Anderson et al., 2002) were synchronised by transient temperature arrest and samples taken every 15 minutes upon release to the permissive temperature. RNA was analysed by northern blot using *lacZ*, *cdc15*$^+$ and *adh1*$^+$ as probes, the latter as a loading control. Quantification of each transcript against adh1$^+$ is shown. Septation indices were counted microscopically and are plotted to indicate the synchrony of the culture. (B) Loss of PCB-regulated periodic transcription in *fkh2Δ* cells. *fkh2Δ cdc25-22* (JM 2285) cells containing pSPΔ178.15UAS (Anderson et al., 2002) were synchronised by transient temperature arrest and samples taken every 15 minutes upon release to the permissive temperature. RNA was analysed by northern blot using *lacZ*, *cdc15*$^+$ and *adh1*$^+$ as probes, the latter as a loading control. Quantification of each transcript against *adh1*$^+$ is shown. Septation indices were counted microscopically and are plotted to indicate the synchrony of the culture. (C) Loss of PCB-regulated periodic transcription in *sep1Δ* cells. *sep1Δ cdc25-22* (JM 2805) cells containing pSPΔ178.15UAS (Anderson et al., 2002) were synchronised by transient temperature arrest and samples taken every 15 minutes upon release to the permissive temperature. RNA was analysed by northern blot using *lacZ*, *cdc15*$^+$ and *adh1*$^+$ as probes, the latter as a loading control. Quantification of each transcript against *adh1*$^+$ is shown. Septation indices could not be counted for this culture, because of *sep1Δ* septation defect. (D) PCB sequence is required for M/G1-specific transcription in fission yeast. *cdc25-22* (GG 308) cells containing pSPΔ178.15UAS.MUT (GB 344) were synchronised by transient temperature arrest and samples taken every 15 minutes upon release to the permissive temperature. RNA was analysed by northern blot using *lacZ*, *cdc15*$^+$ and *adh1*$^+$ as probes, the latter as a loading control. Quantification of each transcript against *adh1*$^+$ is shown. Septation indices were counted microscopically and are plotted to indicate the synchrony of the culture.

the *S. cerevisiae* Rlm1p and Smp1p proteins (Dodou and Treisman, 1997). This gene has recently been identified as having a role in the biosynthesis of the pyruvylated Galβ 1,3-epitope in N-linked glycans, and named Pvg4p (Andreishcheva et al., 2004); we now use this annotation. To investigate the function of *pvg4*$^+$, we deleted it in diploid cells and subjected the heterozygotes (*pvg4Δ/pvg4*$^+$) to tetrad dissection. We found that all four progeny were viable and segregated 2:2 with respect to G418 resistance. *pvg4Δ* cells were slow growing, fragile and rounded in shape, suggesting that Pvg4p is required for cell wall integrity (data not shown). Because *pvg4*Δ cells flocculated and frequently lysed in liquid culture, this precluded further analysis.

Mbx1p is a component of PBF

PBF was identified as a factor that binds to a region of the *cdc15*$^+$ promoter that is responsible for periodic gene transcription during M phase (Anderson et al., 2002). This prompted us to analyse PBF binding activity in cells that were either deleted for the *mbx1*$^+$, *fkh2*$^+$ or *sep1*$^+$ genes, and in cells in which these genes were tagged at their respective C-termini (Bähler et al., 1998; Krawchuk and Wahls, 1999). We found that PBF binding activity was present in *map1Δ* cells (data not shown) but absent in both *mbx1Δ* and *mbx1-13myc* cells, suggesting that Mbx1p is a component of PBF (Fig. 6A). Notably, PBF binding activity was still detected in *fkh2Δ*, *sep1Δ* and double mutant *fkh2Δ sep1Δ* cells, suggesting that PBF is

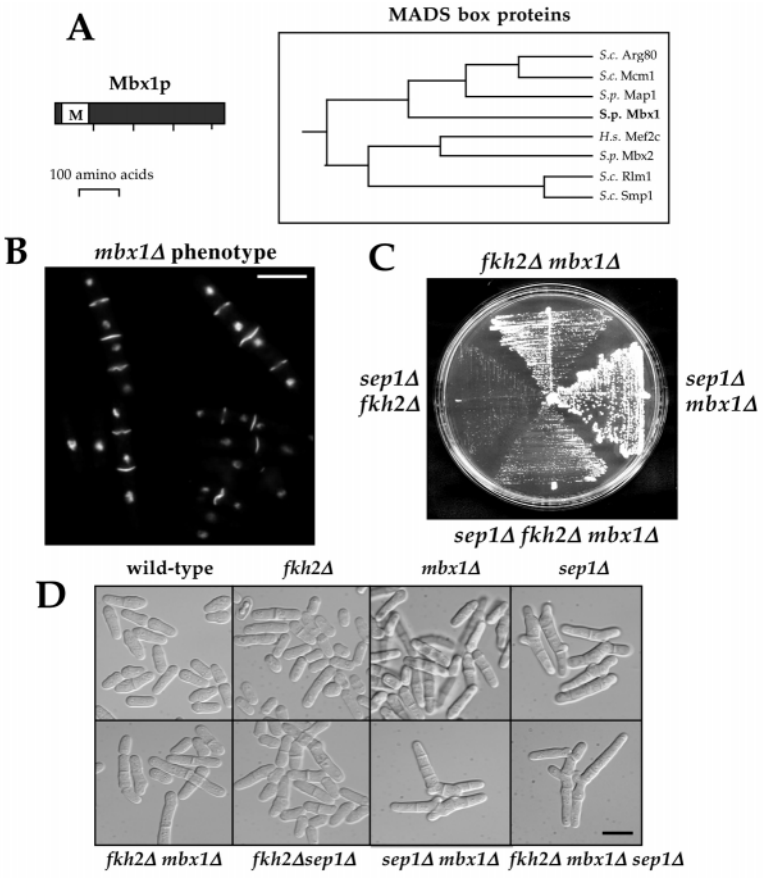


Fig. 5. *S. pombe mbx1*$^{+}$. (A) Schematic representation of the predicted 436 amino acid *S. pombe* Mbx1p, MADS box-like protein (SPBC19G7.06). Position of the MADS box motif (M; 20-80) is shown. Phylogenetic tree showing the relationship between *S. cerevisiae* and *S. pombe* MADS box-like polypeptides, based on multiple sequence alignment, generated using the DNA cluster method in the Megalign programme based on a Jotun Hein algorithm (DNASTAR). (B) Fluorescent micrographs of *mbx1Δ* (JM 2331) cells, growing exponentially in EMM at 28°C. DNA stained with DAPI and septa marked with calcofluor. Representative images illustrating cytokinetic defects are shown. Bar, 10 μm. (C) Genetic interaction between *mbx1*$^{+}$ and *fkh2*$^{+}$. *fkh2Δ sep1Δ* (GG 631), *fkh2Δ mbx1Δ* (GG 551), *sep1Δ mbx1Δ* (GG 543) and *sep1Δ fkh2Δ mbx1Δ* (GG 754) cells were streaked on YE agar and grown at 28°C for 3 days. (D) *sep1Δ*, *fkh2Δ* and *mbx1Δ* show synthetic cytokinetic defects. Micrographs of cells growing in liquid EMM at 28°C: wild-type (GG 1), *fkh2Δ* (JM 2286), *mbx1Δ* (JM 2331), *sep1Δ* (GG 515), *fkh2Δ mbx1Δ* (GG 551), *fkh2Δ sep1Δ* (GG 631), *sep1Δ mbx1Δ* (GG 543) and *fkh2Δ mbx1Δ sep1Δ* (GG 754) were shown. Bar, 10 μm.

not responsible for periodic expression of the M phase gene cluster. However, no complex was observed in *fkh2-3HA* or *fkh2-13myc* cells, indicating that tagging the C-terminus of Fkh2p interferes with binding of Mbx1p to the promoter fragment (Fig. 6A,B). In these cells, binding of the PBF complex was restored by ectopic expression of a plasmid carrying a wild-type copy of *fkh2*$^{+}$ (Fig. 6B). Together these data suggest that Mbx1p is a component of PBF and binds in close proximity to the Fkh2p and Sep1p proteins on the *cdc15+* promoter.

Mbx1p is not required for periodic gene transcription in M phase

We next examined the requirement for MADS box proteins in controlling cycle-regulated expression of M/G1-specific genes. First, *cdc25-22* and *mbx1Δ cdc25-22* cells were synchronised by temperature arrest and release, when mRNA levels were examined by northern blot. Whereas *cdc25-22* cells underwent normal cytokinesis, the septation profile of *mbx1Δ* cells remained high (Fig. 6C), probably because of a failure in the constriction of the cytokinetic actomyosin ring (Fig. 5B). Surprisingly, the *cdc15*$^{+}$, *spo12*$^{+}$, *slp1*$^{+}$, *fin1*$^{+}$, *ace2*$^{+}$ and *fkh2*$^{+}$ genes were still periodically regulated in *mbx1Δ cdc25-22* cells with maximal expression in M/G1, although the amplitude of expression appeared reduced (Fig. 6C). We reasoned that distinct MADS box protein(s) may substitute for the absence of Mbx1p. However, we found that expression of M/G1 genes in *map1Δ mbx1Δ* double mutants was indistinguishable from that of *mbx1Δ* single mutants (data not shown). This suggests that Fkh2p and Sep1p may not require an associated MADS box protein to drive periodic gene expression in M phase, although we were unable to assess the role of Pvg4p (Mbx2p) being unable to synchronise *Pvg4Δ* cells, or construct compound deletions with either *map1Δ* or *mbx1Δ* mutants.

Fkh2p and Mbx1p are periodically phosphorylated in M phase

A common way to regulate transcription factor activity is via phosphorylation (Whitmarsh and Davis, 2000), which can often be detected by a change in the mobility of proteins on SDS-PAGE. We used this approach to examine Fkh2p, Sep1p and Mbx1p through the cell cycle. To detect the proteins each gene was tagged at its C-terminus and cell extracts analysed by western blot (Bähler et al., 1998; Krawchuk and Wahls, 1999). Although we could readily detect Fkh2p-3HA and Mbx1p-13myc proteins we were unable to detect Sep1p-3HA by western blot despite repeated efforts, the reason for which is unknown. Although the level of the proteins remained largely constant, Fkh2p-3HA and Mbx1p-13myc both exhibited multiple forms, with lower mobility forms appearing in M phase co-incidentally with the disappearance of faster mobility forms (Fig. 7A). These lower mobility forms were because of phosphorylation, because the addition of exogenous phosphatase in vitro resulted in their disappearance (Fig. 7B, data not shown). These data echo observations made in *S. cerevisiae*, in which Fkh2p is transiently hyper-phosphorylated during mitosis (Pic et al., 2000).

Discussion

In a screen for regulators of mitotic progression in fission yeast, we identified a gene encoding a forkhead domain transcription factor which shares highest structural homology to the budding yeast Fkh2p protein. Consequently we named the gene *S. pombe fkh2*$^{+}$. We found that *fkh2*$^{+}$ is periodically regulated in late M- to early G1-phase, co-incidentally with the *plo1*$^{+}$, *spo12*$^{+}$, *ace2*$^{+}$ and *sid2*$^{+}$ gene cluster (Anderson et al., 2002). We noted that homologues of these genes, namely *FKH2*,

CDC5, *SPO12*, *ACE2* and *DBF2* are regulated in *S. cerevisiae* by a transcription factor complex that contains the Fkh1p, Fkh2p, Mcm1p and Ndd1p proteins (Cho et al., 1998; Spellman et al., 1998). We found that deletion of *fkh2*$^+$ abolished periodic expression of this M/G1 gene cluster and led to defects in the position, timing and contraction of the actomyosin ring, as well as a delay in entry into mitosis. The delayed mitotic entry in *fkh2Δ* cells may be because of altered expression of the Plo1p and Fin1p kinases, both of which have been implicated in the activation of cyclin B/Cdc2p kinase (Tanaka et al., 2001; Grallert and Hagan, 2002). This may explain why a C-terminally truncated allele of *fkh2*$^+$ was identified as an activator of the G2/M transition in our initial genetic screen. Previous studies have shown that another forkhead domain transcription factor in fission yeast, Sep1p, is required for periodic expression of *cdc15*$^+$ (Zilahi et al., 2000). We found that Sep1p is also responsible for periodic expression of all other genes in this cluster and that overexpression of full-length *fkh2*$^+$ is lethal in wild-type but not in *sep1Δ* cells. These data suggest that Fkh2p and Sep1p may act together, possibly as a heterodimer, to control periodic gene transcription. This proposal is consistent with recent microarray experiments which have suggested that Fkh2p and Sep1p have respective negative and positive roles in regulating M-phase-specific transcription in fission yeast (Rustici et al., 2004). *fkh2*$^+$ is itself cell cycle regulated, which suggests that its specific expression

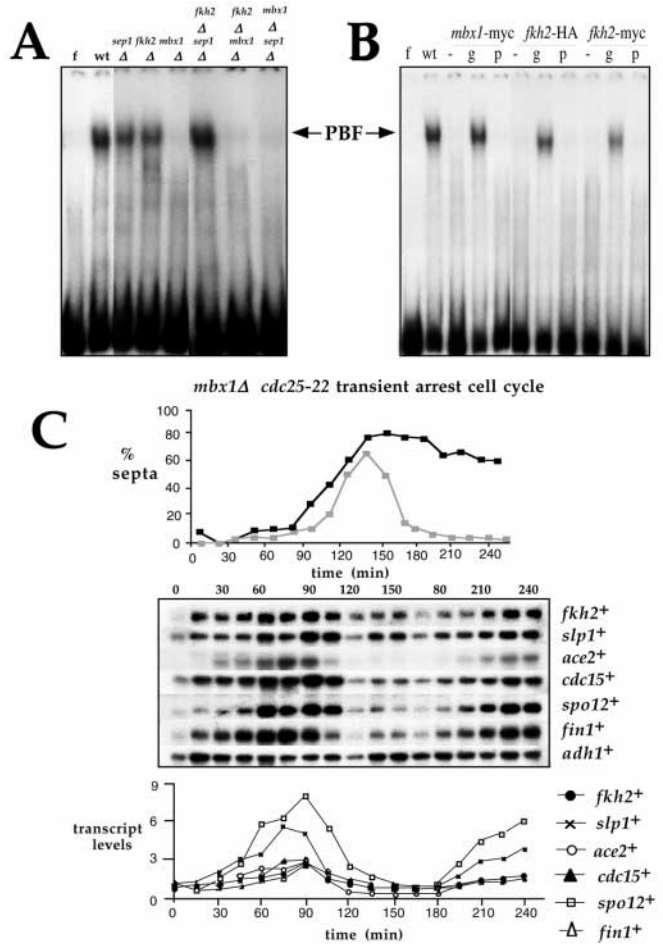


Fig. 6. Mbx1p is a component of PBF and interacts with Fkh2p. (A) Effect of gene deletions on PBF binding activity. Gel retardation analysis was performed using a *cdc15*$^+$ promoter fragment as labelled probe with 20 μg of protein extracts from wild-type (GG 1), *sep1Δ* (GG 515), *fkh2Δ* (JM 2286), *mbx1Δ* (JM 2331), *fkh2Δ sep1Δ* (GG 631), *fkh2Δ mbx1Δ* (GG 551) and *sep1Δ mbx1Δ* (GG 543) cells are shown. Lane 'f' indicates free probe. (B) Effect of gene tags on PBF binding activity. Gel retardation analysis was performed with protein extracts from wild-type (GG 1) cells or protein extracts from *mbx1-13myc* (JM 2349), *fkh2-3HA* (JM 2257) and *fkh2-13myc* (JM 2252) cells, either without plasmid (-) or transformed with a plasmid expressing a genomic copy of the equivalent wild-type gene (g), or transformed with a control plasmid (p). Lane 'f' indicates free probe. (C) *mbx1*$^+$ is not required for periodic transcription in M phase. *cdc25-22* (GG 308) and *mbx1Δ cdc25-22* (JM 2332) cells were synchronised by transient temperature arrest, and upon release to the permissive temperature samples taken every 15 minutes for northern blot analysis. The blot was probed consecutively with *fkh2*$^+$, *slp1*$^+$, *ace2*$^+$, *cdc15*$^+$, *spo12*$^+$, *fin1*$^+$ and *adh1*$^+$ probes, the latter as a loading control. Quantification of each transcript against *adh1*$^+$ is shown. Septation indices were counted microscopically and are shown in black (*mbx1Δ cdc25-22*) or grey (*cdc25-22*).

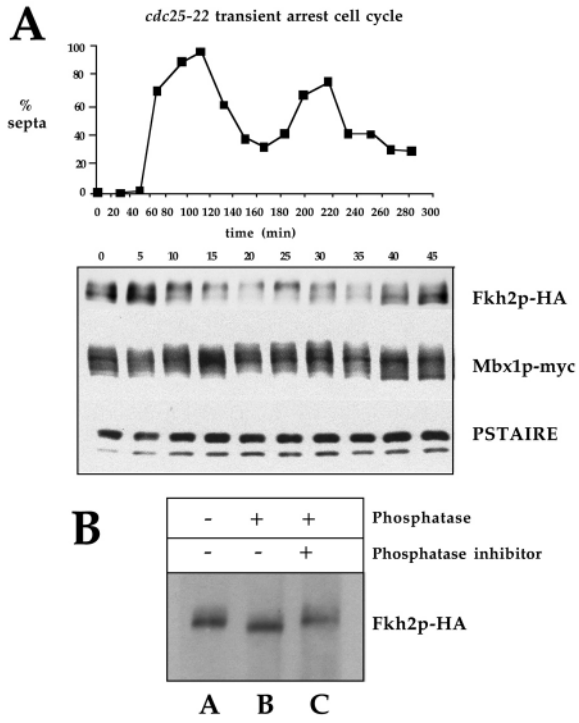


Fig. 7. Fkh2p and Mbx1p are phosphorylated during M-phase. (A) Cell cycle-specific changes in Fkh2p and Mbx1p mobility. Cultures of *fkh2-3HA cdc25-22* (JM 2253) and *mbx1-13myc cdc25-22* (JM 2252) cells were synchronised by transient temperature arrest and upon release to the permissive temperature, samples taken every 5 minutes for western blot analysis. Blots were probed with anti-HA antibodies to detect Fkh2p, anti-myc antibodies to detect Mbx1p, and anti-PSTAIRE antibodies to detect Cdc2p as an invariant loading control. The synchrony of the culture was confirmed by measurement of the septation indices over 300 minutes. (B) Fkh2p is a phosphoprotein. Fkh2-3HAp was immuno-precipitated from lysates prepared from an exponentially growing culture of *fkh2-3HA* cells (JM 2257) using the anti-HA antibody. Sample A was untreated, and samples B and C were incubated with calf intestinal phosphatase, either with or without phosphatase inhibitors. Samples were separated by SDS-PAGE and analysed by western blot with anti-HA antibodies.

may be important for M-phase gene expression, although the fact that its protein levels remain largely constant argues against this proposition. This apparent contradiction may be explained if further post-transcriptional modifications of Fkh2p are important for its cell cycle function.

In budding yeast, recruitment of Fkh2p to promoters requires the MADS box protein, Mcm1p. To identify potential binding partners of Fkh2p and Sep1p, we searched the fission yeast database for MADS box-containing proteins and identified three: Map1p and two novel proteins, Mbx1p and Mbx2p (Pvg4p). Mbx1p has sequence similarity to budding yeast Mcm1p in its DNA binding domain, but contains a C-terminal extension not present in Mcm1p. We found that the fission yeast Mbx1p is non-essential for growth and is required for timely completion of cytokinesis, suggesting that it may act in conjunction with Fkh2p and Sep1p. By contrast, Pvg4p is more closely related to the budding yeast Rlm1p and Smp1p proteins, which are required for cell wall integrity and stress resistance (Dodou and Treisman, 1997). Consistent with this proposal, *pvg4*$^+$ has recently been identified as being required for N-glycan biosynthesis (Andreishcheva et al., 2004), and we found that cells lacking Pvg4p were rounded, fragile and frequently lysed and flocculated in liquid culture. Surprisingly, we found that the M/G1-regulated gene cluster is still periodically expressed in cells lacking both *mbx1*$^+$ and *map1*$^+$ (our unpublished data). This suggests that Fkh2p and Sep1p may control periodic gene transcription in M phase in the absence of an associated MADS box protein, although we were not able to test a role for Pvg4p in this process. This is a surprising result, as in budding yeast Mcm1p appears to have a central role in controlling cell cycle transcription in combination with forkhead proteins. It is possible that in fission yeast, as observed in mammalian systems (Alvarez et al., 2001), MADS box proteins only play a minor role in this process.

We have previously identified a factor by EMSA, called PBF, that binds to a 150 bp region of the *cdc15*$^+$ promoter that is responsible for periodic gene transcription and contains the PCB element (Anderson et al., 2002). Surprisingly, we found that PBF is absent in strains lacking Mbx1p, but persists in cells lacking both Fkh2p and Sep1p. This argues that PBF is not required for periodic expression of the M/G1 gene cluster and may only contain Mbx1p. However, we find that PBF binding activity is absent in cell extracts of strains in which the C-terminus of *fkh2*$^+$ is tagged with an epitope, indicating that tagging the C-terminus of Fkh2p may interfere with Mbx1p function and leads us to suggest that Mbx1p binds in close proximity to the Fkh2p and Sep1p proteins on gene promoters in vivo. Indeed, we propose that Mbx1p may form higher molecular weight complexes with Sep1p and Fkh2p that we were unable to detect by EMSA. This would be consistent with observations in budding yeast in which various complexes of Fkh1p, Fkh2p and Mcm1p can be detected by EMSA, with Fkh1p and Fkh2p only being present in the slower mobility forms (Althoefer et al., 1995; Maher et al., 1995; Koranda et al., 2000; Kumar et al., 2000; Pic et al., 2000). Unfortunately, our attempts to monitor association of Fkh2p, Sep1p and Mbx1p to gene promoters by chromatin immunoprecipitation assay have so far failed (our unpublished data). This may be because epitope tagging of the C-terminus of these genes interferes with their biological function; indeed, we observed that the tags in all three genes caused mild growth phenotypes. Nevertheless, the observation that *mbx1Δ* cells display a defect in cytokinesis that is similar to *fkh2Δ* and *sep1Δ* cells and that *mbx1Δ fkh2Δ* cells do not display the slow growth phenotype of *fkh2Δ* alone suggests that Mbx1p may regulate a subset of genes in the M/G1 cluster. We are currently examining this possibility by microarray analysis. However, Mbx1p is also likely to have functions that are distinct from Sep1p and Fkh2p because the *sep1Δ fkh2Δ mbx1Δ* cells display a more severe cytokinetic defect than *sep1Δ fkh2Δ* cells. We are currently mapping the binding site for Mbx1p on the *cdc15*$^+$ promoter to formally examine the requirement for a MADS box protein in mitotic gene transcription.

In budding yeast, transcriptional activation of the CLB2 cluster is triggered by Clb2p/Cdc28p-mediated phosphorylation of Ndd1p. This promotes association of Ndd1p to the FHA domain of Fkh2p, its recruitment to gene promoters and transcriptional activation (Reynolds et al., 2003). However, the fission yeast genome does not contain any obvious homologues of Ndd1p. Similarly, although forkhead transcription factors regulate G2/M transcription in mammalian cells, these cells also lack a homologue of Ndd1p (Alvarez et al., 2001). Thus, although forkhead transcription factors direct cell cycle-dependent gene transcription in budding yeast, fission yeast and mammalian cells, the mechanisms by which these factors are regulated may be distinct. In this respect it is notable that, in both fission yeast and budding yeast, Fkh2p is transiently hyper-phosphorylated during M-phase, coincident with the peak of M-phase gene transcription (Pic et al., 200). In principle, Fkh2p may be phosphorylated by Cdk1/Cyclin B (Cdc2/Cdc13) or Polo (Plo1p) kinases, both of which are periodically activated in M phase. However, our initial data suggest that mutation of the consensus phosphorylation sites in Fkh2p for Cdc2p/Cdc13p kinase neither abolishes periodic phosphorylation of Fkh2p nor inhibits periodic expression of M-phase gene expression in fission yeast (our unpublished data). This is consistent with our previous observations that overexpression of Plo1p kinase can drive expression of genes in M/G1 cluster in cells lacking Cdc2p/Cdc13p activity (Anderson et al., 2002). We are currently testing whether direct phosphorylation of Fkh2p by Plo1p kinase triggers mitotic gene transcription.

We thank Matthias Sipiczki for strains and plasmids. The work was supported by grants from the Wellcome Trust (to C.J.M.) and the MRC (to C.J.M. and J.B.A.M.). A.B.R-G. was funded by a post-doctoral research fellowship from The Human Frontiers for Science Program (HFSP). K.P. was supported by a Wellcome Trust Studentship. Thanks to other members of the Glasgow and London laboratories for suggestions and encouragement during the course of this work.

References

Althoefer, H., Schleiffer, A., Wassmann, K., Nordheim, A. and Ammerer, G. (1995). Mcm1 is required to coordinate G2-specific transcription in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **15**, 5917-5928.

Alvarez, B., Martinez, A. C., Burgering, B. M. and Carrera, A. C. (2001). Forkhead transcription factors contribute to execution of the mitotic programme in mammals. *Nature* **413**, 744-747.

Anderson, M., Ng, S. S., Marchesi, V., MacIver, F. H., Stevens, F. E., Riddell, T., Glover, D. M., Hagan, I. M. and McInerny, C. J. (2002). *plo1*$^+$ regulates gene transcription at the M-G_1 interval during the fission yeast mitotic cell cycle. *EMBO J.* **21**, 5745-5755.

Andreishcheva, E. N., Kunkel, J. P., Gemmill, T. R. and Trimble, R. B. (2004). Five genes involved in biosynthesis of the pyruvylated Galβ 1,3-epitope in *Schizosaccharomyces pombe* N-linked glycans. *J. Biol. Chem.* **279**, 35644-35655.

Bähler, J., Wu, J. Q., Longtine, M. S., Shah, N. G., McKenzie, A., Steever, A. B., Wach, A., Philippsen, P. and Pringle, J. R. (1998). Heterologous modules for efficient and versatile PCR-based gene targeting in *Schizosaccharomyces pombe*. *Yeast* **14**, 943-951.

Barbet, N., Muriel, W. J. and Carr, A. M. (1992). Versatile shuttle vectors and genomic libraries for use in *Schizosaccharomyces pombe*. *Gene* **114**, 59-66.

Breeden, L. L. (2003). Periodic transcription: a cycle within a cycle. *Curr. Biol.* **13**, R31-R38.

Buck, V., Russell, P. and Millar, J. B. A. (1996). Identification of a Cdk-activating kinase in fission yeast. *EMBO J.* **14**, 6173-6183.

Cho, R. J., Campbell, M. J., Winzeler, E. A., Steinmetz, L., Conway, A., Wodicka, L., Wolfsberg, T. G., Gabrielian, A. E., Landsman, D., Lockhart, D. J. et al. (1998). A genome-wide transcriptional analysis of the mitotic cell cycle. *Mol. Cell* **2**, 65-73.

Dodou, E. and Treisman, R. (1997). The *Saccharomyces cerevisiae* MADS-box transcription factor Rlm1 is a target for the Mpk1 mitogen-activated protein kinase pathway. *Mol. Cell. Biol.* **17**, 1848-1859.

Durocher, D. and Jackson, S. P. (2002). The FHA domain. *FEBS Lett.* **513**, 58-66.

Futcher, B. (2000). Microarrays and cell cycle transcription in yeast. *Curr. Opin. Cell Biol.* **12**, 710-715.

Grallert, A. and Hagan, I. (2002). *Schizosaccharomyces pombe* NIMA-related kinase Fin1, regulates spindle formation and an affinity of Polo for the SPB. *EMBO J.* **12**, 3096-3107.

Hollenhorst, P. C., Bose, M. E., Mielke, M. R., Muller, U. and Fox, C. A. (2000). Forkhead genes in transcriptional silencing, cell morphology and the cell cycle. Overlapping and distinct functions for FKH1 and FKH2 in *Saccharomyces cerevisiae*. *Genetics* **154**, 1533-1548.

Hollenhorst, P. C., Pietz, G. and Fox, C. A. (2002). Mechanisms controlling differential promoter-occupancy by the yeast forkhead proteins Fkh1p and Fkh2p: implications for regulating the cell cycle and differentiation. *Genes Dev.* **15**, 2445-2456.

Jorgensen, P. and Tyers, M. (2000). The fork'ed path to mitosis. *Genome Biol.* **1**, 1022.1-1022.4.

Kaufmann, E. and Knochel, W. (1996). Five years on the wing of the fork head. *Mech. Dev.* **57**, 3-20.

Koranda, M., Schleiffer, A., Endler, L. and Ammerer, G. (2000). Forkhead-like transcription factors recruit Ndd1 to the chromatin of G2/M-specific promoters. *Nature* **406**, 94-98.

Krawchuck, M. D. and Wahls, W. P. (1999). High efficiency gene targeting in *Schizosaccharomyces pombe* using a modular, PCR-based approach with long tracts of flanking homology. *Yeast* **15**, 1419-1427.

Kumar, R., Reynolds, D. M., Shevchenko, A., Shevchenko, A., Goldstone, S. D. and Dalton, S. (2000). Forkhead transcription factors, Fkh1p and Fkh2p, collaborate with Mcm1p to control transcription required for M-phase. *Curr. Biol.* **10**, 896-906.

Lee, T. I., Rinaldi, N. J., Robert, F., Odom, D. T., Bar-Joseph, Z., Gerber, G. K., Hannett, N. M., Harbison, C. T., Thompson, C. M., Simon, I. et al. (2002). Transcriptional regulatory networks in *Saccharomyces cerevisiae*. *Science* **298**, 799-804.

Loy, C. J., Lydall, D. and Surana, U. (1999). NDD1, a high-dosage suppressor of *cdc28-1N*, is essential for expression of a subset of late-S-phase-specific genes in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **19**, 3312-3327.

Maher, M., Cong, F., Kindelberger, D., Nasmyth, K. and Dalton, S. (1995). Cell cycle-regulated transcription of the CLB2 gene is dependent on Mcm1 and a ternary complex factor. *Mol. Cell. Biol.* **15**, 3129-3137.

Martin-Cuadrado, A. B., Duenas, E., Sipiczki, M., Vazquez de Aldana, C. R. and del Rey, F. (2003). The endo-beta-1,3-glucanase eng1p is required for dissolution of the primary septum during cell separation in *Schizosaccharomyces pombe*. *J. Cell Sci.* **116**, 1689-1698.

Matsumoto, T. (1997). A fission yeast homolog of CDC20/p55CDC/Fizzy is required for recovery from DNA damage and genetically interacts with $p34^{cdc2}$. *Mol. Cell. Biol.* **17**, 742-750.

Maundrell, K. (1993). Thiamine-repressible expression vectors pREP and pRIP for fission yeast. *Gene* **123**, 127-130.

Molz, L., Booher, R., Young, P. and Beach, D. (1989). *cdc2* and the regulation of mitosis: six interacting *mcs* genes. *Genetics* **122**, 773-782.

Moreno, S., Klar, A. and Nurse, P. (1991). Molecular genetic analysis of the fission yeast *Schizosaccharomyces pombe*. *Methods Enzymol.* **194**, 795-823.

Pic, A., Lim, F. L., Ross, S. J., Veal, E. A., Johnson, A. L., Sultan, M. R., West, A. G., Johnston, L. H., Sharrocks, A. D. and Morgan, B. A. (2000). The forkhead protein Fkh2 is a component of the yeast cell cycle transcription factor SFF. *EMBO J.* **19**, 3750-3761.

Reynolds, D., Shi, B. J., McLean, C., Katsis, F., Kemp, B. and Dalton, S. (2003). Recruitment of Thr 319-phosphorylated Ndd1p to the FHA domain of Fkh2p requires Clb kinase activity: a mechanism for CLB cluster gene activation. *Genes Dev.* **17**, 1789-1802.

Ribar, B., Banrevi, A. and Sipiczki, M. (1997). $sep1^{+}$ encodes a transcription-factor homologue of the HNF-3/forkhead DNA-binding-domain family in *Schizosaccharomyces pombe. Gene* **202**, 1-5.

Rustici, G., Mata, J., Kivinen, K., Lio, P., Penkett, C. J., Burns, G., Hayles, J., Brazma, A., Nurse, P. and Bähler, J. (2004). Periodic gene expression program of the fission yeast cell cycle. *Nat. Genet.* **36**, 809-817.

Sambrook, J., Fritch, E. F. and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Samuel, J. M., Fournier, N., Simanis, V. and Millar, J. B. A. (2000). *spo12* is a multicopy suppressor of *mcs3* that is periodically expressed in fission yeast mitosis. *Mol. Gen. Genet.* **264**, 306-316.

Schmitt, M. E., Brown, T. A. and Trumpower, L. (1990). A rapid and simple method for preparation of RNA from *Saccharomyces cerevisiae*. *Nucleic Acids Res.* **18**, 3091-3092.

Spellman, P. T., Sherlock, G., Zhang, M. Q., Iyer, V. R., Anders, K., Eisen, M. B., Brown, P. O., Botstein, D. and Futcher, B. (1998). Comprehensive identification of cell cycle-regulated genes of the yeast *Saccharomyces cerevisiae* by microarray hybridization. *Mol. Biol. Cell* **9**, 3273-3297.

Tanaka, K., Petersen, J., MacIver, F., Mulvihill, D. P., Glover, D. M. and Hagan, I. M. (2001). The role of Plo1 kinase in mitotic commitment and septation in *Schizosaccharomyces pombe*. *EMBO J.* **20**, 1259-1270.

Triesman, R. and Ammerer, G. (1992). The SRF and MCM1 transcription factors. *Curr. Opin. Genet. Dev.* **2**, 221-226.

Whitmarsh, A. J. and Davis, R. J. (2000). Regulation of transcription factor activity by phosphorylation. *Cell. Mol. Life Sci.* **57**, 1172-1183.

Wood, V., Gwilliam, R., Rajandream, M. A., Lyne, M., Lyne, R., Stewart, A., Sgouros, J., Peat, N., Hayles, J., Baker, S. et al. (2002). The genome sequence of *Schizosaccharomyces pombe*. *Nature* **415**, 871-880.

Yabana, N. and Yamamoto, M. (1996). *Schizosaccharomyces pombe* $map1^{+}$ encodes a MADS-box-family protein required for cell-type-specific gene expression. *Mol. Cell. Biol.* **16**, 3420-3428.

Zhu, G., Spellman, P. T., Volpe, T., Brown, P. O., Botstein, D., Davis, T. N. and Futcher, B. (2000). Two yeast forkhead genes regulate the cell cycle and pseudohyphal growth. *Nature* **406**, 90-94.

Zilahi, E., Salimova, E., Simanis, V. and Sipiczki, M. (2000). The *S. pombe sep1* gene encodes a nuclear protein that is required for periodic expression of the *cdc15* gene. *FEBS Lett.* **481**, 105-108.