4. TRANSCRIPTIONAL REGULATION OF PERIODICALLY EXPRESSED GENES IN FISSION YEAST

This chapter will provide an insight into the transcriptional regulatory network that governs periodic gene transcription in fission yeast. Several deletion mutants of well known, as well as less characterised or putative, transcriptional regulators have been used with the purpose of clarifying the mechanisms that regulate gene expression during the cell cycle.

4.1 Experimental overview

Gene deletion mutants represent a powerful tool to investigate gene function. The first indication of a gene's role often comes from the phenotype of the mutant strain. Ultimately, expression profiling of a gene deletion using microarrays can reveal the relationship between a transcription factor and potential targets. If a gene is responsible for regulating a specific wave of transcription, its deletion will have an effect on the expression profile of its targets. In unsynchronised cells this should lead to higher (in the case the gene acts as repressor) or lower expression (in the case of an activator) compared to wild type cells. Overexpression of the same gene similarly should lead to higher (activator) or lower (repressor) levels of expression of its targets. In reality transcriptional regulatory networks are much more complicated and transcription factors are often interdependent making the understanding of regulation a less straightforward process.

Details concerning the experimental conditions can be found in Appendix IVb. At least two independent biological experiments were carried out for each mutant and a technical replicate (microarray hybridisation with a dye swap) was performed in most cases. Asynchronous mutants were compared to asynchronous wild type cells and differentially expressed genes identified by combining two methods as described in Materials and Methods, section 2.6.3. Overexpression strains transformed with the pREP-3X vector carrying the gene of interest, were compared to a control strain transformed with the pREP-3X vector only.

4.2 Sep1p-dependent regulation

Sep1p, a member of the forkhead family of transcription factors (Ribar B. *et al.*, 1997; Ribar B. *et al.*, 1999), regulates periodic expression of *cdc15*, which encodes a protein involved in cytokinesis and septation (Zilahi E. *et al.*, 2000). *Sep1* mRNA levels are constant throughout the cycle whereas *cdc15* levels peak before septation. In a *sep1* deletion (*sep1Δ*), *cdc15* periodicity is lost (Zilahi E. *et al.*, 2000). *Sep1Δ* displays a distinctive phenotype (Fig. 4.1B) with very elongated, multiseptated and branched cells due to the septation defect. The *S. cerevisiae* homologues, *FKH1* and *FKH2*, are also involved in regulation of cell cycle periodic transcription (see Introduction).

Two *sep1Δ* mutants (in different backgrounds) have been used to investigate the role of sep1p in regulating gene transcription: *sep1*Δ and *sep1*Δ *cdc25*. In parallel, the *pREP3X-sep1* strain, overexpressing sep1p, has also been used (sep1p OE). The overexpression strain did not show any particular phenotype (Fig. 4.2C).

Transcription levels were analysed in asynchronous *sep1*Δ cells versus wild type cells. 67 genes were found to be expressed at lower level and around 70% (45 genes) of these were cell cycle regulated (Table 4.1). The same subset of 45 genes was also found to be induced in cells overexpressing *sep1* (compared to cells transformed with the expression vector only), confirming their dependency on sep1p (Fig. 4.3A). The majority of the 45 sep1p-dependent periodic genes were members of either cluster 1 (15 genes) or 2 (26 genes). Therefore sep1p must be involved in the regulation of both waves of transcription.

Cdc15 is found in this group, as expected, together with several other genes encoding proteins involved in cytokinesis (SPAC14C4.09, *eng1*, *etd1*, *exg1*, *mid2* and *plo1*) and cell wall maintenance (*chs2*) or synthesis (SPAC23H4.19 and SPBC3E7.12c); this correlates well with the *sep1Δ* phenotype. The transcription factor gene *ace2* was also found in this group.

As described in section 1.3.2 of the introduction, in budding yeast the forkhead-type transcription factor complex Mcm1p/Fkh2p/Ndd1p is responsible for activating the G2/M wave of transcription which includes another transcription factor, Ace2p (Simon I. *et al.*, 2001). Since in fission yeast, *ace2* (homologous to *ACE2* in bidding yeast) also appears to be a forkhead/sep1p-dependent gene, an investigation into the role of ace2p in

transcriptional regulation was performed, particularly focusing on a possible interaction between the two transcription factors sep1p and ace2p.

Table 4.1 Sep1p-dependent periodic genes

Biological/systematic name	Gene description	Cluster
ace2	Zinc finger transcription factor	1
bet1 [a, b]	Member of SNARE domain containing family	1
SPAC19B12.02c [a, b]	Protein with high similarity to 1,3-beta-glucanosyltransferase, member of glycolipid anchored surface protein (GAS1) family	1
SPAC23H4.19 [a, b]	Putative cell wall biogenesis protein	1
SPBC27.05 [a, b]	Unknown function	1
SPAC3F10.15c [a, b]	Protein likely to play role in regulating cell cycle progression, possibly at G2 to M phase transition	1
SPBC4F6.12 [a, b]	LIM domain protein, low similarity to paxillin focal adhesion protein that regulates integrin or growth factor-mediated responses	1
SPCC757.12 [a, b]	Protein containing an alpha amylase N-terminal catalytic domain	1
cdc15 [a, b]	Protein involved in cytokinesis	1
chs2 [a]	Member of chitin synthase family, involved in cell wall maintenance	1
etd1 [a]	Protein required for cytokinesis	1
klp5 [a, b]	Kinesin motor protein; KIP3 subfamily	1
myo3: myp2 [a, b]	Myosin-3 isoform, heavy chain (Type II myosin)	1
plo1 [a, b]	Polo kinase involved in regulation of mitosis and cytokinesis	1
slp1 [a]	WD-domain protein of the spindle defect checkpoint and APC activator	1
SPBC1709.12	Unknown function	2
SPCC18.01c	Member of SUN family, contains predicted N-terminal signal sequence	2
SPAC22G7.02 [a]	Unknown function	2
SPBC27.04 [a, b]	Unknown function	2
SPBC2A9.07c	Unknown function	2
SPAC2E1P5.03	Protein containing a DnaJ domain, which mediates interaction with heat shock proteins	2
SPBC31F10.17c [a]	Unknown function	2
SPBC32F12.10 [a, b]	Protein with phosphoglucomutase or phosphomannomutase C-terminal domain	2
SPAC343.20	Unknown function	2
SPBC3E7.12c	Unknown function, possible role in regulation of chitin synthase	2
SPAC644.05c [a]	Protein similar to dUTP pyrophosphatase, which maintains dUTP at low levels to prevent misincorporation into DNA	2
SPBC651.04	Unknown function	2
cdm1	DNA polymerase delta subunit	2
eng1	Endo-beta-1,3-glucanase required for cell separation	2
exg1 [a]	Putative exo-beta-1,3-glucanase	2
klp8 [a]	Protein containing a kinesin motor domain	2
meu19 [a, b]	Non-coding RNA	2
mid2	Protein required for septin function and stability during cytokinesis	2
SPBPB2B2.13 [b]	Protein similar to galactokinase, which catalyzes first step in	2

	galactose metabolism	
SPAPJ760.03c	Unknown function	2
rad21 [a]	Cohesin complex subunit, double-strand-break repair protein	2
rgf3	Protein containing a pleckstrin homology (PH) and a RhoGEF (GTPase exchange factor) domain	2
rpc17	Unknown function	2
SPCC1322.10	Unknown function, similar to cell-surface proteins and proteoglycans	2
SPAC14C4.09	Unknown function, putative glucanase	2
SPAC19G12.17c	Unknown function, similarity to podocalyxin like, a transmembrane sialomucin important for lymphocyte adhesion and homing	2
SPBPJ4664.02	Unknown function, possible cell surface glycoprotein	3
SPAP7G5.06 [a]	Protein similar to amino acid permease, a proton symport transporter for all naturally-occurring L-amino acids	4
SPBC1271.09 [a, b]	Member of sugar (and other) transporter family, possible role in inositol metabolism	N(3,4)
SPCC965.06	Protein similar to potassium voltage-gated channel	N(1,2,3)

[a] sep1p-dependent genes only

[b] sep1p-dependent genes upregulated in fkh2Δ

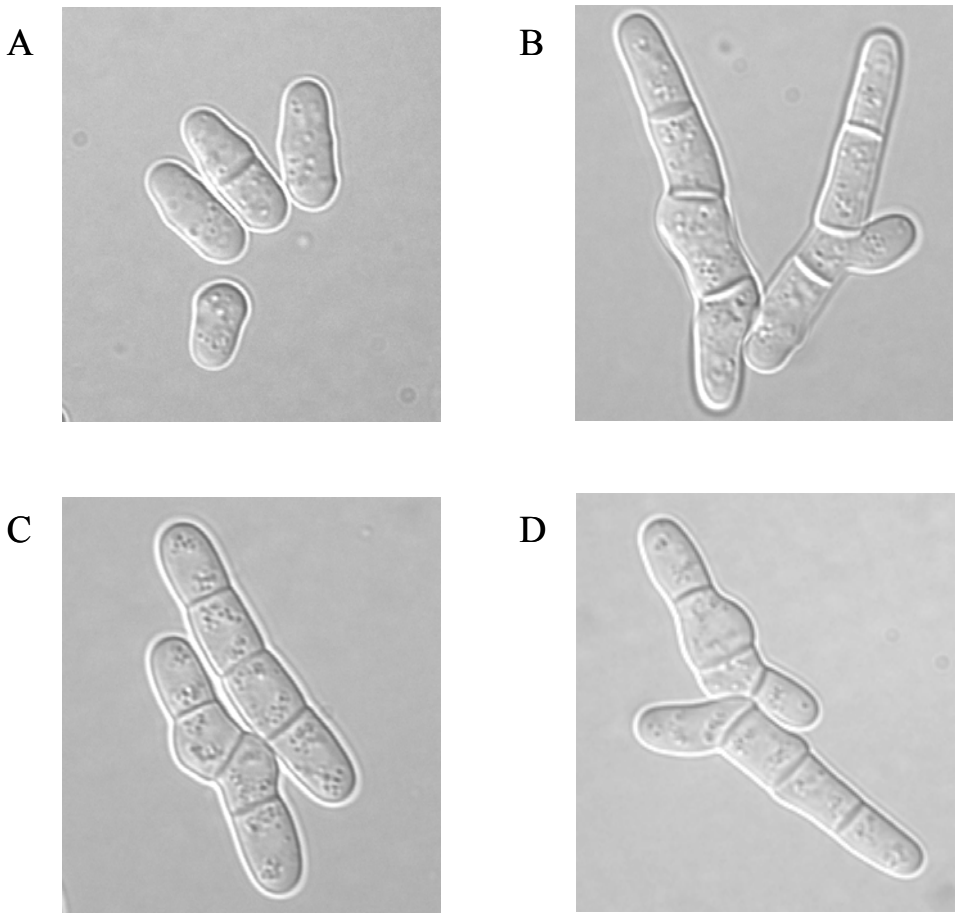

Fig. 4.1 Microscopic appearance of wild type (A), *sep1*Δ (B), *ace2*Δ (C) and *ace2*Δ *sep1*Δ (D) mutant cells. Photographs of differential interference contrast microscopy (DIC).

4.3 Ace2p-dependent transcription

Ace2p, a transcriptional regulator containing a zinc finger domain, has been identified based on homology with *S. cerevisiae* Ace2p, which regulates a group of genes involved in cell separation, including *ENG1*, encoding for a glucanase required for septum digestion at cytokinesis. Similarly in fission yeast, ace2p regulates periodic expression of *eng1* (cluster 2 in this study) and in *ace2Δ* periodic accumulation of *eng1* is lost (Martin-Cuadrado A.B. *et al.*, 2003). Based on the literature and on what was found in this study so far, in fission yeast sep1p regulates expression of *ace2*, a member of cluster 1, which in turn regulates *eng1*, a member of cluster 2. It is reasonable to assume that other genes in cluster 2 are also regulated by ace2p.

An *ace2Δ* and an ace2p OE strain were prepared and their expression profile investigated using microarrays. The phenotype of the *ace2Δ* strain is very similar to the *sep1Δ* strain with cells showing septation defects but cells are less elongated than in *sep1Δ* (Fig. 4.1C). The ace2p OE instead is characterised by round cells (Fig. 4.2B).

23 periodic genes were found to be expressed at lower levels in asynchronous *ace2Δ* versus wild type (Table 4.2), and the same subset of genes were highly induced in ace2p OE. Five additional periodic genes (Table 4.2 – genes marked with [a]) were found to be highly induced in ace2p OE but only slightly downregulated in *ace2Δ*, below the criteria applied to define downregulated genes according to the method used. 23 of these genes belong to cluster 2, as does *eng1*, the only ace2p target known before.

74% of the ace2p-dependent genes are also sep1-dependent genes and most of the overlapping targets are members of cluster 2. The level of induction of those genes in the ace2p OE appears to be higher compared to sep1p OE (Fig. 4.3A), suggesting that ace2 is the main regulator of their expression. Their promoter sequences also contain a binding site (5'-CCAGCC-3' that was here named 'Ace2' –Appendix VI) very similar to the one identified in budding yeast for Ace2p (5'-RRCCAGCR-3'), illustrating once more how conserved the ace2 regulation mechanism is between the two yeasts. Ace2p is therefore responsible for regulating periodic expression of a subset of genes, all members of cluster

2. These genes are only indirect targets of sep1p since sep1p is the regulator of *ace2* periodic expression.

Table 4.2 Ace2p-dependent periodic genes

Biological/systematic name	Gene description	Cluster
SPAC1071.09c[a]	Protein containing a DnaJ domain, which mediates interaction with heat shock proteins	2
SPBC1289.01c[a]	Unknown function, putative involvement in chitin biosynthesis	2
SPCC1322.10	Unknown function, similar to cell-surface proteins and proteoglycans	2
SPAC14C4.09	Unknown function, putative glucanase	2
SPBC1709.12	Unknown function	2
SPCC18.01c	Member of SUN family, contains predicted N-terminal signal sequence	2
SPAC19G12.17c	Unknown function, similarity to podocalyxin like, a transmembrane sialomucin important for lymphocyte adhesion and homing	2
SPBC2A9.07c	Unknown function	2
SPBC2A9.13	Unknown function	2
SPAC2E1P5.03	Protein containing a DnaJ domain, which mediates interaction with heat shock proteins	2
SPBC31F10.17c[a]	Unknown function	2
SPAC343.20	Unknown function	2
SPBC3E7.12c	Unknown function, possible role in regulation of chitin synthase	2
SPBC651.04	Unknown function	2
cdm1	DNA polymerase delta subunit	2
cut2[a]	Securin; required for sister chromatid separation	2
eng1	Endo-beta-1,3-glucanase required for cell separation	2
klp8[a]	Protein containing a kinesin motor domain	2
mid2	Protein required for septin function and stability during cytokinesis	2
par2	Protein phosphatase PP2A, B' regulatory subunit, required for cytokinesis, morphogenesis, and stress tolerance	2
SPAPJ760.03c	Unknown function	2
rgf3	Protein containing a pleckstrin homology (PH) and a RhoGEF (GTPase exchange factor) domain	2
rpc17	Unknown function	2
SPBC28F2.11	Protein with a high mobility HMG-box domain	3
SPBPJ4664.02	Unknown function, possible cell surface glycoprotein	3
SPCC965.06	Protein similar to potassium voltage-gated channel	N(1,2,3)
SPBC800.11	Protein with inosine-uridine preferring nucleoside hydrolase domain	N(2,3)

[a] ace2p-dependent genes highly induced in *Ace2p OE*

Looking back to the list of sep1p-dependent genes that do not appear to be ace2p-dependent, the majority of them are members of cluster 1 (Table 4.2 - genes marked with [a]). Several cluster 1 genes are enriched for a promoter motif (5'-TGTTTAC-3', called 'FLEX' –Appendix VI) similar to the conserved binding sites reported for forkhead

proteins (Alvarez B. *et al.*, 2001; Zhu G. *et al.*, 2000; Horie S. *et al.*, 1998). The overlap between the sep1p-dependent genes and cluster 1 genes containing a forkhead binding site in their promoter sequence is statistically significant ($P \sim 10^{-16}$).

This same set of sep1-dependent genes was slightly induced in *ace2Δ* and downregulated in ace2p OE as if ace2p was acting as an inhibitor of their expression (Fig. 4.3A).

From the results presented so far, it can be concluded that sep1p and ace2p are members of the same transcriptional cascade whereby sep1p directly regulates transcription of cluster 1 members, including *ace2*, which in turn activates transcription of cluster 2 members and possibly inhibits transcription of sep1p/cluster 1 targets (Fig. 6.1).

A double mutant strain *ace2*Δ *sep1*Δ was also constructed in this study. Since the mechanism of regulation proposed here for sep1p and ace2p implies that ace2p is acting downstream of sep1p, this double mutant is expected to show a phenotype and expression profile similar to the one of sep1p. As can be seen in Fig. 4.1D, cells have a very similar morphology to *sep1*Δ, slightly less elongated and they tend to form bigger clumps of cells, due to the separation defect. No major differences were found between the expression profile of the *ace2*Δ *sep1*Δ double mutant and the single *sep1*Δ mutant. The same genes found to be downregulated in *sep1*Δ showed the same pattern in the *ace2*Δ *sep1*Δ double mutant (Fig. 4.3A). This is consistent with our model for the sep1p/ace2p regulatory cascade.

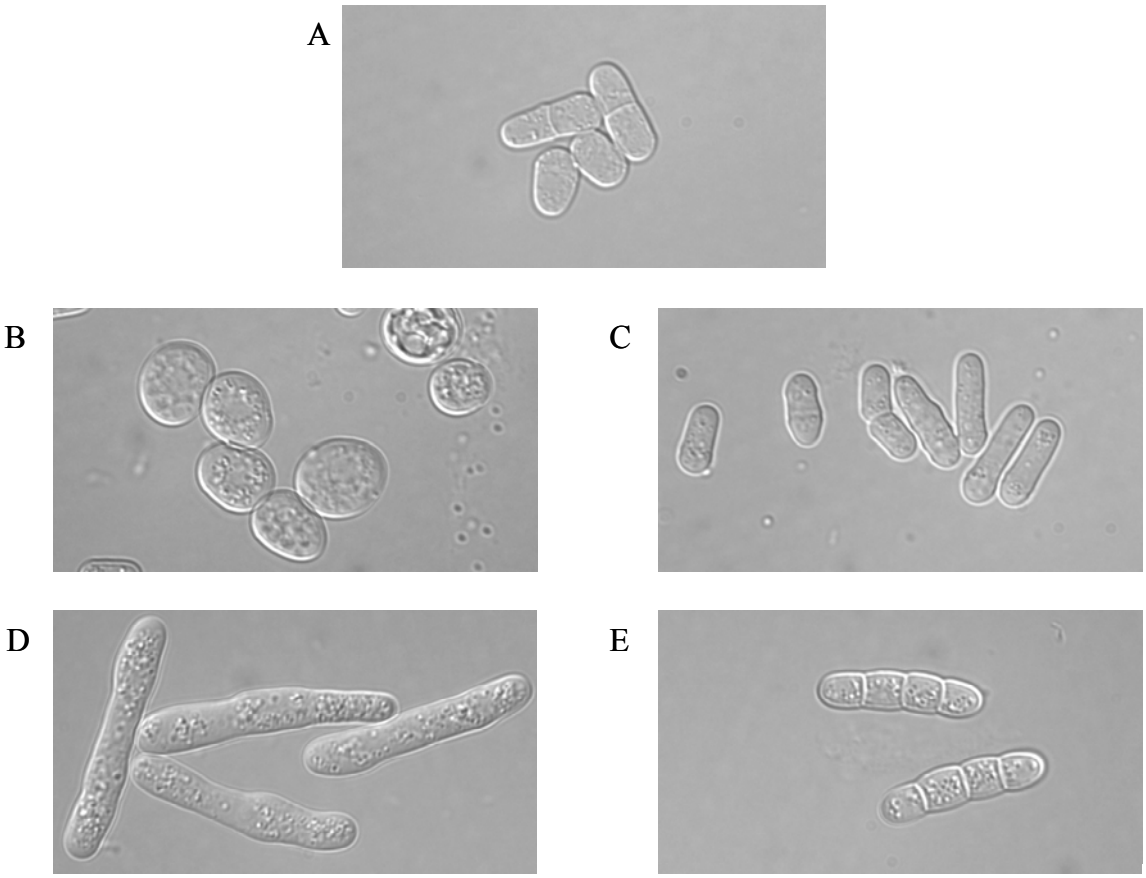

Fig. 4.2 Microscopic appearance of *leu1-32 h*$^{-}$ overexpressing the following vectors: *pREP3X* only (A), *pREP3X-ace2* (B), *pREP3X-sep1* (C), *pREP3X-fkh2* (D) and *pREP3X-fhl1* (E). DIC photographs are shown.

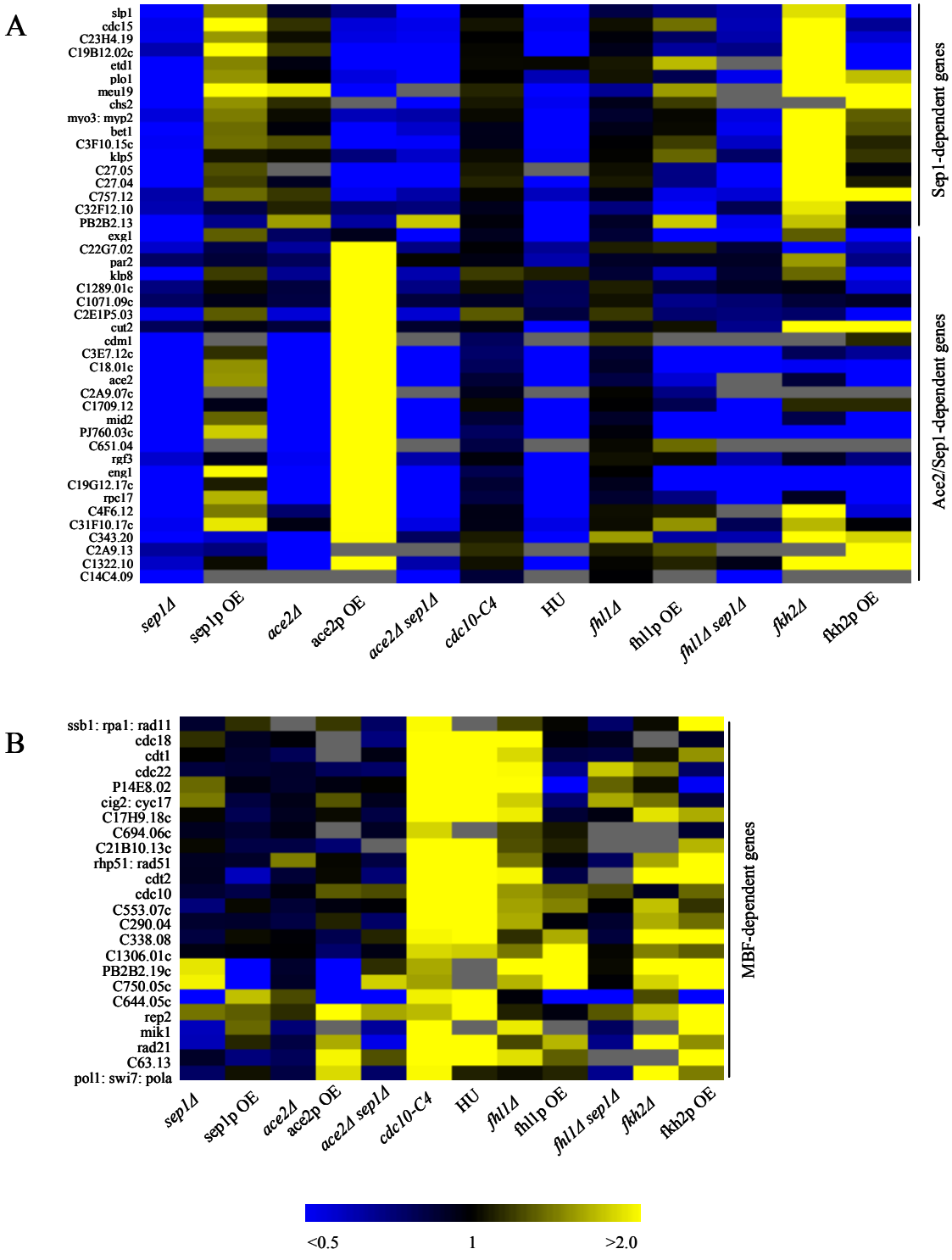

Fig. 4.3 Transcriptional regulation of selected cluster 1 and 2 genes.

Hierarchical clustering of genes based on their expression level in the following experiments: *sep1* deletion (*sep1Δ*), sep1p overexpression (sep1p OE), *ace2* deletion (a*ce2Δ*), ace2p overexpression (ace2p OE), *ace2Δ sep1Δ* double deletion (*ace2Δ sep1Δ*), *cdc10-C4* cells, S-phase 3 hours hydroxyurea (HU) arrest, *fhl1* deletion (*fhl1Δ*), fhl1p overexpression (fhl1p OE), *fhl1Δ sep1Δ* double deletion (*fhl1Δ sep1Δ*), *fkh2* deletion (*fkh2Δ*) and fkh2p overexpression

(fkh2p OE). Panel A shows the behaviour of sep1p- and sep1p/ace2p-dependent genes, panel B of MBF-dependent genes. Clusters were obtained in GeneSpring using Pearson correlation as the distance measurement.

4.4 Other fission yeast forkhead genes

In budding yeast, at least two forkhead genes are involved in regulation of G2/M periodic transcription, *FKH1* and *FKH2*, with overlapping and distinct functions (Hollenhorst P.C. *et al.*, 2000; Kumar R. *et al.*, 2000). The role of other fission yeast forkhead genes was therefore investigated in order to unmask possible roles of them in regulating gene transcription.

Excluding *sep1*, three other fission yeast genes encode proteins containing a forkhead motif: *mei4*, encoding a meiosis-specific transcription factor; SPAC1141.08 (hereafter referred as *fhl1*), whose protein shows similarity to budding yeast transcriptional activator Fhl1p, and SPBC16G5.15c (hereafter referred as *fkh2*), whose protein shows similarity to budding yeast Fkh2p. In order to investigate the function of the last two genes, the expression profiles of two gene deletions (*fhl1Δ* and *fkh2Δ*) and two overexpression strains (fhl1p OE and fkh2p OE) were analysed. *Mei4* was not investigated any further because of its presumed specific role in meiosis. Only *fkh2* was cell cycle regulated in this study, belonging to cluster 1.

Fhl1Δ did not show any particular phenotype (Fig. 4.4C); on the contrary *fkh2Δ* (strain kindly provided by Dr. Brian Morgan) showed a severe phenotype with elongated, branched cells (Fig. 4.4B) and slow growth rate (4.5-5 hours at 30°C in YE). The fhl1p OE phenotype was characterised by multiseptated cells (Fig. 4.2E) and fkh2p OE by very elongated and sometimes branched cells (Fig. 4.2D).

In *fhl1Δ* cells very few genes were found to be expressed at lower levels and no correlation was found between this short list and the genes upregulated in fhl1p OE. Although, several sep1p-dependent genes appear expressed at higher levels in fhl1p OE (Fig. 4.3A), suggesting a regulatory role of fhl1p on a subset of sep1p targets. This could account for the septation defect of the cells overexpressing fhl1p. In *fkh2Δ* cells at least 100 genes appeared to be upregulated and a large portion of them was represented by stress genes (Chen D. *et al.*, 2003), probably reflecting the state of sickness of the strain itself.

16 sep1p-dependent genes (Table 4.1 – genes marked with [b]) were found among the upregulated genes in *fkh2Δ* (Fig. 4.3A). This, together with the septation defect of the deletion, could suggest a possible role of fkh2p in regulating gene expression with a possible negative function on the expression of sep1p-dependent genes. This would once again draw a correlation with the budding yeast regulatory mechanism since Fkh2p has also negative transcriptional roles in *S. cerevisiae* (Koranda M. *et al.*, 2000). This hypothesis requires further work to be proved. It has to be mentioned here that most of these 16 genes were not downregulated in the fkh2p OE.

A double mutant strain *fhl1Δ sep1Δ* was also made and analysed in order to find out if any cumulative effect could result from the deletion of both genes. The phenotype of the strains once again resembles the one of the *sep1Δ* cells (Fig. 4.4D) with multiseptated cells. Similarly, the expression profile did not present any difference when compared with the one obtained for the single mutant *sep1Δ* (Fig. 4.3A).

Based on these data, no obvious role in regulating periodic gene expression during the fission yeast cell cycle could be assigned to fhl1p. Further work is needed to clarify its potential role in regulating some of the sep1p targets. Fkh2p instead could play an inhibitory role on the expression of some sep1p-dependent genes.

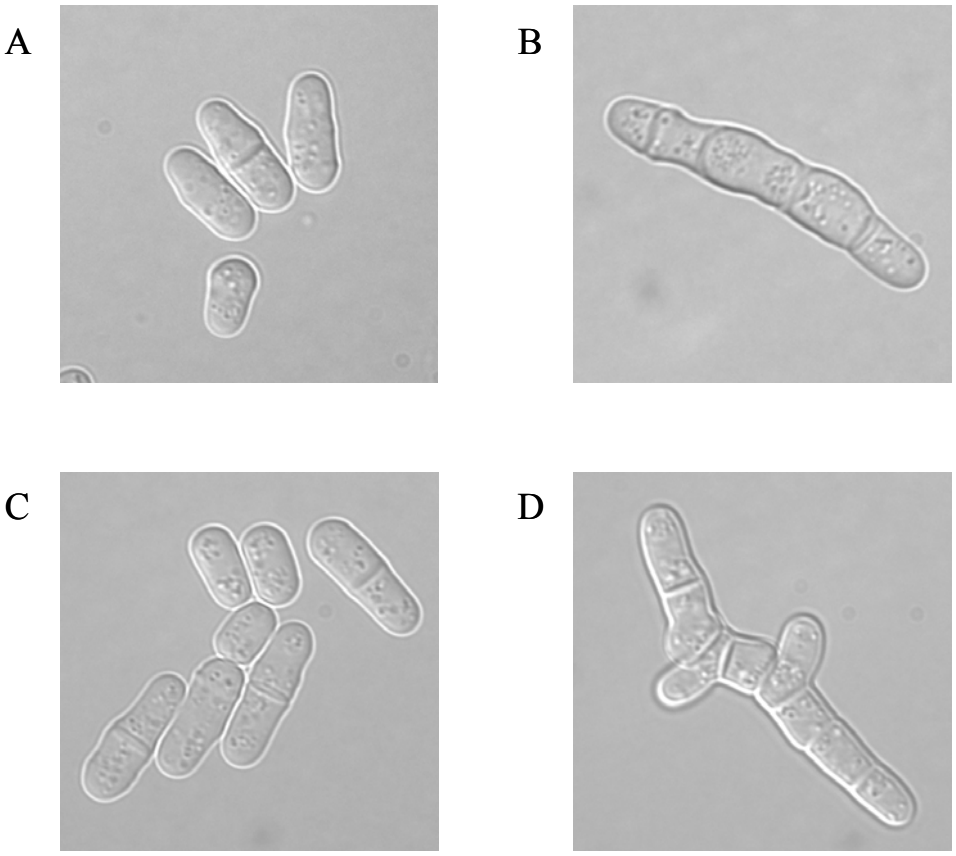

Fig. 4.4 Microscopic appearance of wild type (A), *fkh2*Δ (B), *fhl1*Δ (C) and *fhl1*Δ *sep1*Δ (D) mutant cells. DIC photographs are shown.

4.5 Cdc10p-dependent transcription

As presented in section 3.5, cluster 2 includes several genes known to be regulated by the MBF transcription factor complex, whose components are cdc10p, res1p, res2p and the regulator rep2p (see Introduction). *Cdc10* and *rep2* were identified as periodic in this study and assigned to cluster 2, although the change in *cdc10* expression was of low amplitude.

To further investigate the role of cdc10p in regulating gene transcription, the transcription profile of a well characterised *cdc10* mutant, *cdc10-C4*, was analysed. In this mutant cdc10p is truncated by 61 aminoacids before the C-terminus resulting in a protein that retains some activity when grown at low temperature and looses it at high temperature. As a consequence of the loss of function cdc10p targets are expressed at very high levels throughout the cell cycle (McInerny C.J. *et al.*, 1995).

62 genes were expressed at high levels in *cdc10-C4* cells compared to wild type and 32 of them were periodic genes, most of them members of cluster 2 (Table 4.3 and Fig. 4.3B). Among those were included nine of the ten previously characterised MBF targets (Table 4.3 – genes marked with [a]). The promoter regions of these genes were also enriched for regulatory sequences (MCB1, 5'-AACGCG-3' and MCB2, 5'-CGCGNCGCG-3') similar to the consensus MCB sequence (5'-ACGCGT-3') recognised by MBF in budding yeast and to the E2F transcription factor site in mammalian genes (5'-TTTTGCGCG-3' or 5'-CGCGCAAAA-3') (McIntosh E.M., 1993). Cdc10p is therefore responsible for the regulation of a subset of the cluster 2 genes.

The changes in gene expression in a *cdc10* deletion were also studied. Since *cdc10* is an essential gene, its deletion results in an unviable strain. In the mutant *cdc10Δ* used in this thesis, the *cdc10* mutation is rescued by the overexpression of the res1p N-terminus (1 to 192 aa) (Ayte J. *et al.*, 1995). When cells are grown in normal conditions, res1p directly binds to the MBF targets, substituting cdc10p and allowing normal expression of MBF targets. When thiamine is added to the media, overexpression of rep1p is switched off resulting in the development of the *cdc* phenotype. Levels of *cdc18* decrease by 50% within 2 hours after thiamine addition. Microarray analysis of this strain was not conclusive. Despite a general tendency to a reduction in the expression levels of the MBF-dependent genes so far identified, the changes were not as dramatic as expected and some of the targets were not affected at all by thiamine addition. Some of the known targets such as *cdc18* and *cdc22* decreased within 4 hours, after thiamine was added but their concentration started to increase again after 6 hours.

Similarly the *nda3-KM311* strain was used to better understand the MBF mechanism of regulation. It has been reported that in this strain MBF targets are already transcribed in mitotic cells during a metaphase block (Baum B. *et al.*, 1998). When cells are shifted to the restrictive temperature, the MBF targets accumulate as a result of a metaphase block caused by the mutation in β-tubulin which results in spindle anomalies and therefore in cell cycle arrest at the metaphase-anaphase transition (Hiraoka Y. *et al.*, 1984), (Baum B. *et al.*, 1998). Again the microarray analysis of this strain was not conclusive. Despite the level of some MBF targets increasing, the changes in gene expression were relatively low. *Cig2* increased in this experiment more than *cdc18* and *cdc22*, contrary to what was reported (Baum B. *et al.*, 1998). Therefore, we could not confirm previously published data and both *cdc10Δ* and *nda3* experiments could not be

used to validate the results obtained with the other mutants. It cannot be excluded that this was due to experimental errors.

Based on what has been discussed so far, both cdc10p and ace2p seem to regulate genes in cluster 2 but no overlap was seen between the MBF-dependent and the ace2p-dependent genes. Additional experiments have been performed with the intent of better understanding the relationship between MBF, sep1p and ace2p and the results will be presented in the next section.

Table 4.3 MBF-dependent periodic genes

Biological/systematic name	Gene description	Cluster	Motifs
psc3	Cohesin complex component, required for sister chromatid cohesion and normal mitosis	1	MCB1
rhp51: rad51 [a]	Required for DNA repair and meiotic recombination	1	MCB1
ams2	Protein that binds binds chromatin at centromere and is involved in chromosome segregation	2	MCB 1
SPAC17H9.18c	Unknown function	2	FLEX
SPBC21B10.13c	Homeobox domain (homeodomain) protein, putative transcription factor	2	
SPCC63.13	Protein containing a DnaJ domain, which mediates interaction with heat shock proteins	2	
SPAC644.05c	Protein similar to dUTP pyrophosphatase, which maintains dUTP at low levels to prevent misincorporation into DNA	2	MCB 1, MCB 2
cdc10	Component of MBF transcriptional activation complex involved in control of START	2	
cdc18 [a]	Protein that couples cell cycle signals to DNA replication machinery and induces replication	2	MCB 1, MCB 2
cdc22 [a]	Ribonucleoside-diphosphate reductase large chain, likely required for initiation of DNA replication	2	MCB 1, MCB 2
cdt1 [a]	Protein that coordinates completion of S phase with onset of mitosis	2	MCB 1, MCB 2
cdt2 [a]	Protein required for DNA replication	2	MCB 1, MCB 2
cig2: cyc17 [a]	Major G1/S-phase cyclin, promotes onset of S phase	2	FLEX
cnp1	CENP-A-like protein, histone H3 variant specific to inner centromeres and required for chromosome segregation	2	FLEX, MCB2
eso1	DNA polymerase eta, involved in sister chromatid cohesion	2	FLEX, MCB1
mrc1	Protein required for DNA replication checkpoint	2	MCB 1, MCB 2
mik1 [a]	Protein kinase that inhibits Cdc2p kinase	2	FLEX, MCB1, MCB2
pol1: swi7: pola	DNA polymerase alpha catalytic subunit	2	FLEX, histone
rad21 [a]	Cohesin complex subunit, double-strand-break repair protein	2	
rep2	Zinc finger transcriptional activator, MBF transcriptional complex	2	

ssb1: rpa1: rad11 [a]	Single-stranded DNA-binding protein subunit, required for DNA replication	2	MCB1, MCB2, Novel 1
SPAP14E8.02	Unknown function	2	MCB 1, MCB 2
SPBC16D10.06	Member of ZIP zinc transporter family	4	
SPBC25B2.08	Unknown function	4	FLEX
SPAC869.02c	Member of globin family of oxygen transporters, similar to flavohemoglobin that protects from stress	4	
SPBC1683.07	Protein similar to alpha-glucosidase	N(1,2)	Ace2
SPCC338.08	Unknown function	N(1,2)	
SPBC428.17c	Unknown function	N(1,4)	
SPBC1306.01c	Protein with elongation factor Tu GTP binding domain, similar to mitochondrial translation elongation factor G	N(2,3)	
SPCC553.07c	Member of impB, mucB or samB family, possible role as translesion DNA repair polymerase	N(2,3)	
CSPA750.05c	Telomeric protein of unknown function, highly similar to S. pombe SPAC977.01, SPAC1348.02 and SPBPB2B2.19c	N(2,3)	
SPBPB2B2.19c	Telomeric protein of unknown function, highly similar to S. pombe SPAC977.01, SPAC1348.02 and SPAC750.05C	N(2,3)	

[a] previously known cdc10p targets

4.6 Additional experiments addressing regulation by sep1p, ace2p and cdc10p

The roles of MBF, sep1p and ace2p transcription factors were also investigated in a *sep1Δ cdc25* strain in cells synchronised in a 'block and release' experiment. Sep1p-dependent and ace2p-dependent genes still maintained their periodic behaviour but the amplitude of the expression was reduced when compared to the profiles of the same genes in wild type elutriated cells or in a *cdc25* 'block and release' experiment (Fig. 4.5). The expression amplitude of the MBF-dependent targets was not affected but a delay of 30 min in the second peak of expression of those genes could be observed when compared with a typical *cdc25* 'block and release' experiment.

The relative timing of the peaks of expression for MBF-dependent and ace2p-dependent genes seems to be different (especially in the first cycle) when the two synchronisation methods were compared. They are coincident when elutriation is used whereas the MBF-regulated wave seems to precede the ace2p-regulated one when temperature sensitive mutants are used. This is consistent with the timing of the cell cycle

events in *cdc25* cells where DNA replication occurs earlier than in wild type elutriated cells as shown in Fig. 3.2.

Despite their peaking at nearly the same time, those two sets of genes clearly show differences in the mechanism of their regulation. This was confirmed also by the different response in their behaviour when wild type cells were synchronised using hydroxyurea (HU). This drug inhibits DNA replication causing cell cycle arrest in S-phase. As a consequence of the HU treatment, sep1p- and ace2p-dependent genes were repressed whereas MBF-dependent genes were strongly induced (Fig. 4.3A and B).

A further attempt was made to temporally separate the MBF and ace2p-waves using *cig1*Δ *cig2*Δ *puc1*Δ strain synchronised by elutriation. Fission yeast cells lacking the three G1 cyclins cig1p, cig2p and puc1p have an extended G1 phase compared to wild type cells in which G1 is relatively short and difficult to study (Martin-Castellanos C. *et al.*, 2000). MBF and ace2p waves still seemed coincident in this experiment (Fig. 4.6). It is worth mentioning that elutriating this strain was more difficult than any of the other mutants. Despite it being repeated several times (3 independent attempts), interpretation of the FACS profile was difficult, making it hard to establish how good cell synchrony was and therefore judge the validity of the experiment.

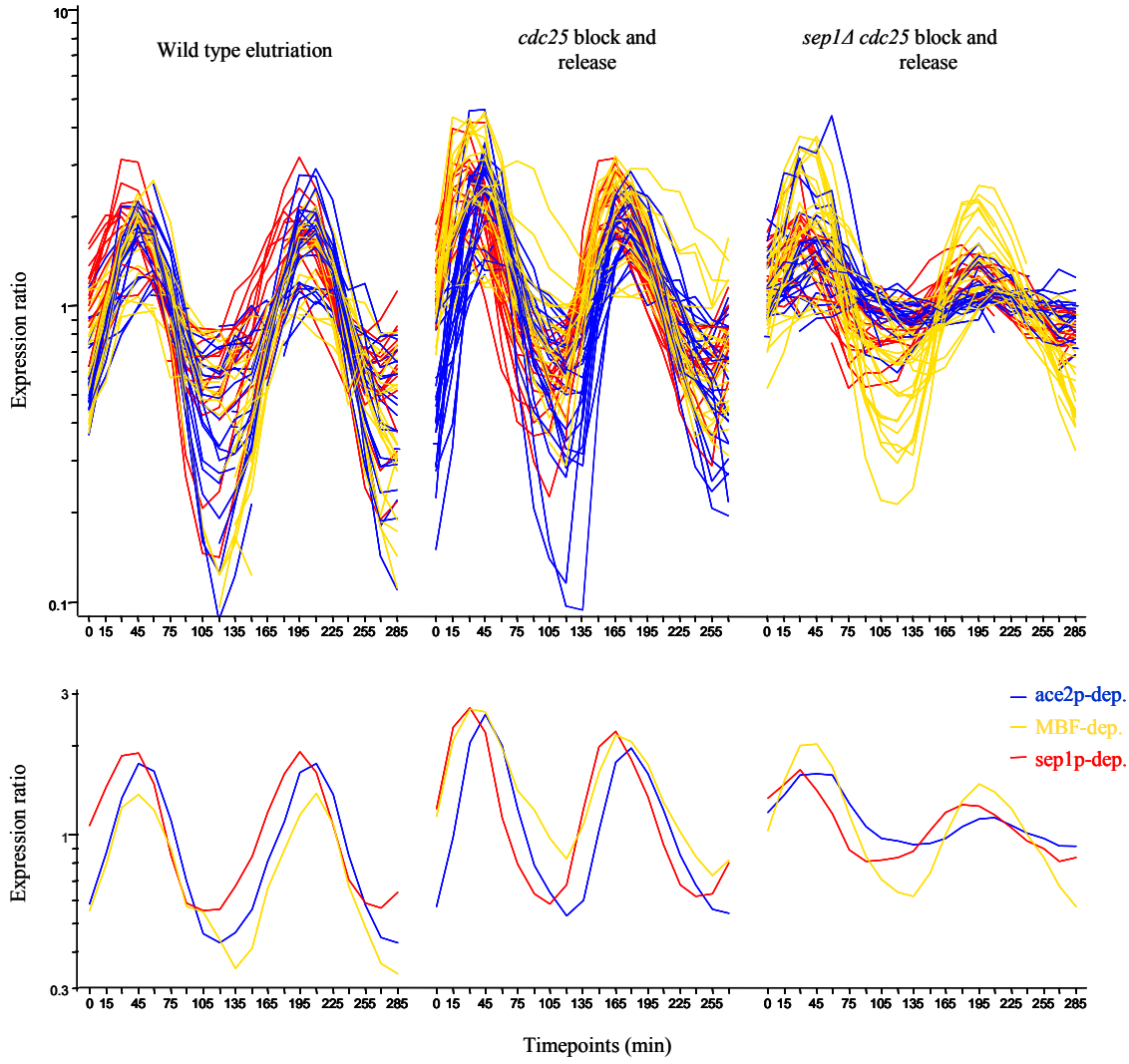

Fig. 4.5 MBF, ace2p and sep1p transcriptional regulation of cluster 1 and 2 genes in a *sep1Δ cdc25* 'block and release' experiment.

Expression profiles of MBF-, ace2- and sep1-dependent genes are shown (from right to left) in a wild type elutriation, in a *cdc25* 'block and release' and in a *sep1Δ cdc25* 'block and release' experiment (top graphs). The bottom graphs show the average profiles for the same subset of genes. Red: sep1p-dependent; yellow: MBF-dependent; blue: ace2p-dependent.

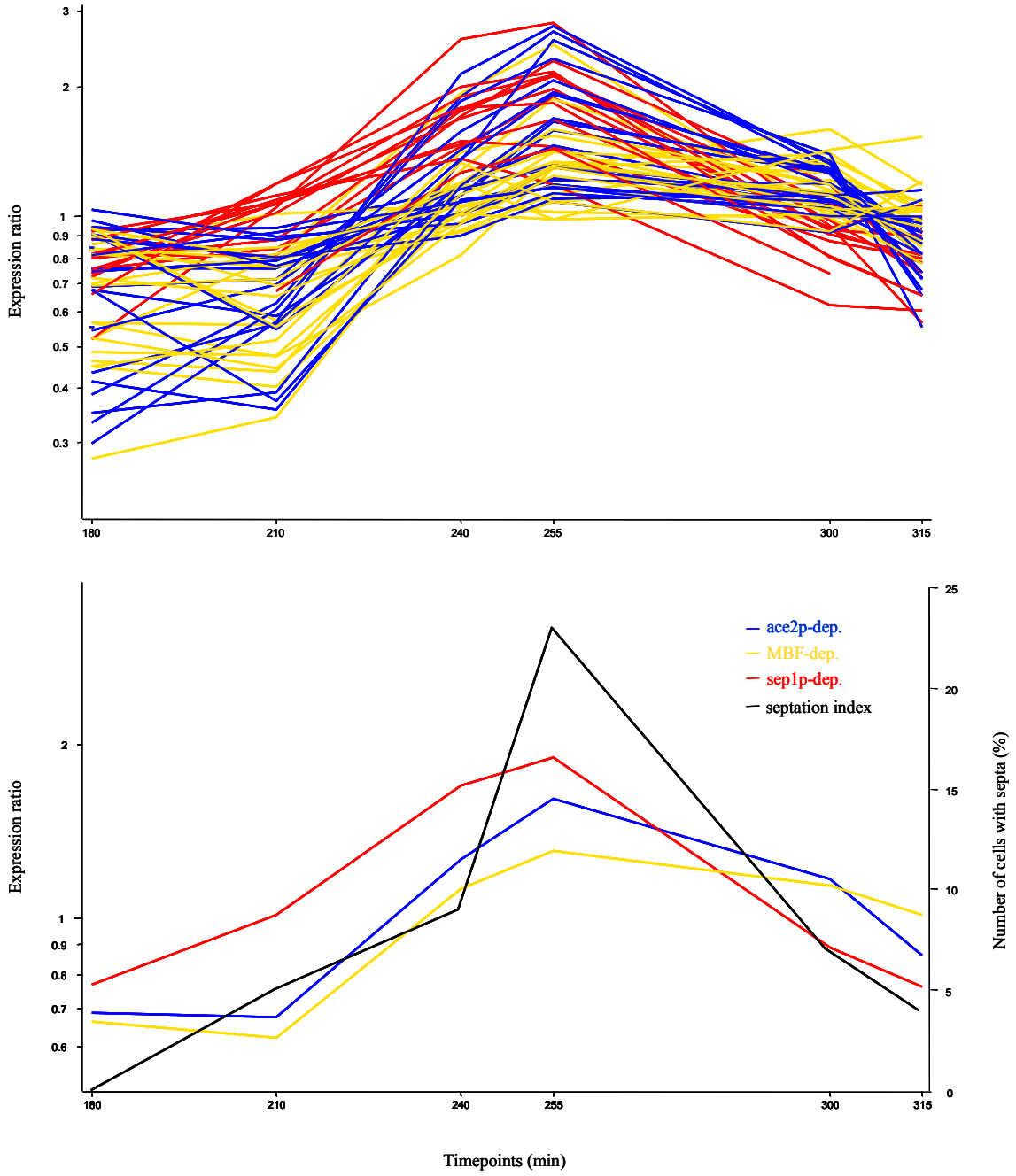

Fig. 4.6 MBF, ace2p and sep1p transcriptional regulation of cluster 1 and 2 genes in a *cig1*Δ *cig2*Δ *puc1*Δ elutriation experiment.

Red: sep1p-dependent; yellow: MBF-dependent; blue: ace2p-dependent; black: septation index. Only selected timepoints around the septation peak have been hybridised.

4.7 Studies with additional potential regulatory genes

Meu3 and *meu19*

Five non-coding RNAs are transcribed by *meu3*, *meu11*, *meu16*, *meu19* and *meu20* genes. It has been speculated that these RNAs might have a regulatory role in meiosis, similarly to meiRNA which specifically binds to mei2p allowing premeiotic DNA synthesis and meiosis I onset (Watanabe T. *et al.*, 2001).

Out of those three genes, only *meu19* was found to be periodically expressed during the cell cycle (member of cluster 2). *Meu3* was periodic in the preliminary data and subsequently removed from the periodic genes. *Meu3* and *meu19* are twin genes; they share an identical 5' half sequence, differ in the 3' half sequence and are located in distinct genomic areas. A *meu3Δ* and a *meu19Δ* were constructed in this study with the intention of investigating a potential regulatory role of these genes in mitosis. *Meu3Δ* did not show any particular phenotype and isolation of *meu19Δ* haploid cells was particularly difficult (Fig. 4.7C and D). None of the periodic genes appeared to be affected by the absence of *meu3* or *meu19*. Based on the results of those experiments, a function of those genes in regulating periodic gene expression is unlikely.

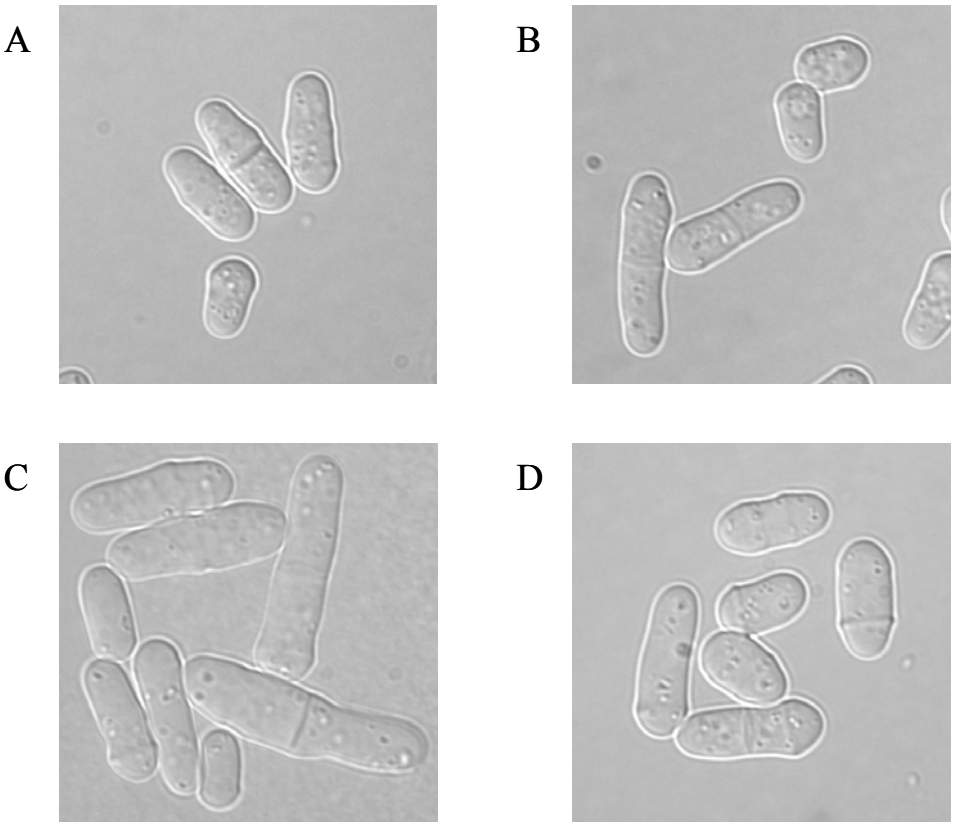

Fig. 4.7 Microscopic appearance of wild type *972 h*$^-$ (A), SPBC19G7.06Δ (B), *meu19*Δ (C) and *meu3*Δ (D). DIC photographs are shown. Information about SPBC19G7.06Δ can be found in the next section.

MADS-box gene

In budding yeast, the transcription factor complex that regulates expression of G2/M is composed of a forkhead-type protein, Fkh2p, a MADS-box protein, Mcm1p and an activator, Ndd1p. It is a clear example of a regulatory complex containing two different kinds of transcription factors, forkheads and MADS-box proteins interacting with each other.

MADS-box proteins are a highly conserved family of transcription factors involved in many biological functions (Messenguy F. and Dubois E., 2003). Two proteins containing a MADS-box motif have been identified in fission yeast: map1p that is involved in transcriptional activation of mating type-specific genes and SPBC19G7.06 encoded protein whose function is still unknown. A strain with SPBC19G7.06 deleted (kindly provided by Dr. J. Millar) was analysed to investigate the potential role of this protein in regulating gene expression. This deletion did not show any particular phenotype (Fig. 4.7B). SPBC19G7.06 expression is not cell cycle regulated.

Few periodic genes (12) were expressed at lower levels in this mutant strain compared to wild type (Table 4.4). Interestingly, a few of them are members of cluster 2 and contain a FLEX binding site, a typical forkhead-binding motif. Interaction between forkhead-type transcription factors and MADS-box has never been reported in *S. pombe.* Although, considering the significant degree of conservation of the forkhead regulation mechanism between the two yeasts and the function of some of the genes downregulated in the SPBC19G7.06 deletion (glucanases and chitin synthase involved in cell separation), a possible interaction between the two factors is possible. Further analysis will be needed to validate this theory.

Table 4.4 Downregulated genes in SPBC19G7.06 deletion

Biological/systematic name	Gene description	Cluster	Motifs
chs2[b]	Member of chitin synthase family, involved in cell wall maintenance	1	MCB 1

C14C4.09[a]	Unknown function, putative glucanase	2	
C18.01c [a]	Member of SUN family, contains predicted N-terminal signal sequence	2	Ace2, FLEX
C965.14c	Member of cytidine and deoxycytidylate deaminase zinc-binding region family	2	FLEX
eng1 [a]	Endo-beta-1,3-glucanase required for cell separation	2	FLEX
PJ760.03c [a]	Unknown function	2	Ace2, FLEX
C19C7.04c	Unknown function	3	
PJ4664.02 [b]	Unknown function, possible cell surface glycoprotein	3	
bgl2	Protein similar to beta-glucosidase, a cell wall endo-beta-1,3-glucanase	4	
C27D7.09c	Unknown function	4	
C27D7.11c	Unknown function	4	Novel 3
nrd1	Protein containing four RNA recognition motifs	4	

[a] ace2p-dependent genes

[b] sep1p-dependent genes

Cluster 4 regulation

It has been mentioned before that several cluster 4 members are genes involved in stress response including the transcription factor pcr1p which plays a role in mating, meiosis and stress response (Watanabe Y. and Yamamoto M., 1996). Pcr1p forms a complex with atf1p, another transcription factor that controls expression of most genes involved in stress defense (Chen D. *et al.*, 2003; Toone W.M. and Jones N., 1998; Takeda T. *et al.*, 1995; Wilkinson M.G. *et al.*, 1996). Another transcription factor, prr1p, is involved in response to oxidative stress and to elevated salt concentrations as well as sexual differentiation, in a pathway that presumably functions in parallel with atf1p (Ohmiya R. *et al.*, 1999; Ohmiya R. *et al.*, 2000; Greenall A. *et al.*, 2002). *Atf1* and *prr1* were not among the 407 cell cycle regulated genes. Since cluster 4 is enriched with stress related genes, it was interesting to investigate a possible involvement of any of these transcriptional regulators (pcr1p, prr1p and atf1p) in controlling the cluster 4/G2 wave of transcription in fission yeast. For this purpose, the expression profiles of *atf1Δ*, *pcr1Δ* and *prr1Δ* have been analysed. *Atf1Δ* and *pcr1Δ* showed a very similar expression profile; most of the genes that require atf1p for their basal level of expression (Chen D. *et al.*, 2003) appeared repressed in both deletions as expected. Similarly, most of the atf1p-repressed genes from the same study were induced in *atf1Δ* and *pcr1Δ*. All the prr1p identified targets (*ste11*, *mam2* and *mei2*) were highly repressed in *prr1Δ*.

In all three deletions some of the cluster 4 members were expressed at low levels but most of the members of this cluster were not affected. Atf1p, pcr1p and prr1p may be involved in the regulation of some cluster 4 members but this does not mean they are involved in controlling their periodic expression. The only way to verify this would be to perform a timecourse experiment for each deletion and observe if any change occurs in the expression of cluster 4 members.

4.8 Potential regulatory promoter motifs

The search for regulatory sequences in the upstream region of the periodically expressed genes identified several motifs, some of them already known as well us some novel ones. All the motifs identified are listed in Table 4.5. The most significant sequence patterns are shown together with the cluster they are most associated with, indicated by the significance (*P* value) of the overlap between genes in the cluster and genes with the given motif among all genes in the genome.

As described before, MBF-dependent genes in cluster 2 were enriched with two motifs, named MCB1 and MCB2, very similar to the MCB motif found in the upstream sequence of well characterised fission yeast MBF targets. Similar sequences are recognised by MBF in *S. cerevisiae* and by E2F in mammals.

A common motif named ACE2 was found in this study in the promoter region of ace2p-dependent genes in cluster 2. This motif shows similarities to the Ace2 consensus sequence recognized by the homologous budding yeast transcription factor.

Several sep1p-dependent genes as well as several other cluster 1 members contained a FLEX promoter sequence, a well known target for forkhead proteins like mei4p. It is the first time a link with the cell cycle is proposed for this motif which until now has been only investigated in relation to meiosis. Other forkhead/MADS box binding motifs have been identified in mammals and *S. cerevisiae* (Maher M. *et al.*, 1995; Messenguy F. and Dubois E., 2003), as an independent transcriptional regulator as well as in combination with Ndd1p/Fkh2p. No motif similar to Mcm1 has been found in fission yeast in this study.

Two novel motifs, named Novel 1 and 2, were often found in combination with forkhead motifs, in particular among cluster 1 members without overlapping with sep1p-dependent genes.

In cluster 3, the nine histone genes, as well as some other non-histone genes (5) in cluster 2 and 3, have in common the Histone motif (Matsumoto S. and Yanagida M., 1985). This motif is not conserved between the two yeasts and not much has been elucidated concerning the regulation of the histone genes. In mammalian cells, histone gene expression is controlled largely at the post-transcriptional level involving mRNA 3'-end formation and RNA stability (Marzluff W.F. and Duronio R.J., 2002).

In cluster 4, only one motif was found, named Novel 3, but the majority of the genes in this cluster do not share any common regulatory sequence. This raises the possibility that these genes might be regulated at a different level, possibly RNA stability.

Table 4.5 Potential regulatory promoter motifs

Motif name	Sequence pattern	Associated cluster (*P* value)/gene list
FLEX	5'-TGTTTAC-3'	1 (<1e-40)
Novel 1	5'-GTTGNCATG-3'	1 (6.1e-07)
Novel 2	5'-TTGCATTTNC-3'	1 (2.0e-05)
MCB 1	5'-AACGCG-3'	2 (1.6e-27)
MCB 2	5'-CGCGNCGCG-3'	2 (2.0e-19)
MCB 2	5'-CGCGNCGCG-3'	MBF-dep.
Ace2	5'-CCAGCC-3'	2 (3.5e-18)
Ace2	5'-ACCAGCCNT-3'	Ace2p-dep.
Histone	5'-AACNCTAAC-3'	3 (4.5e-15)
Novel 3	5'-ACCNCGC-3'	4 (5.2e-11)

N = either A, C, G or T

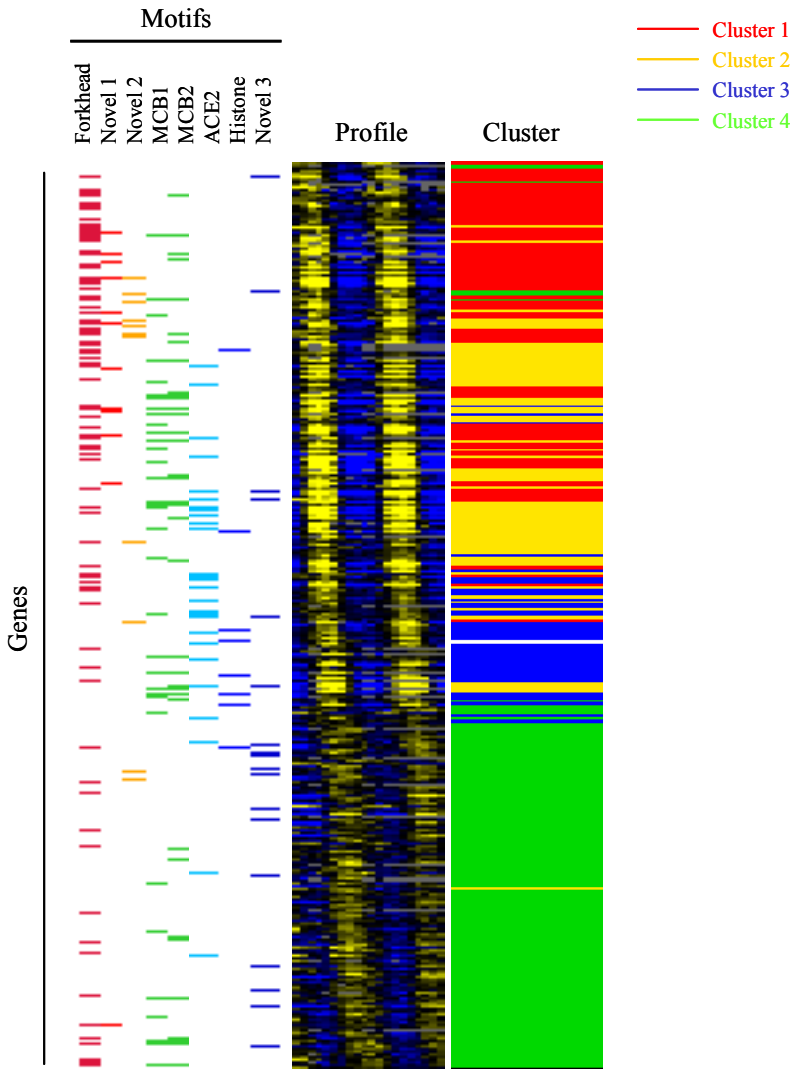

Fig. 4.8 Identification of potential regulatory promoter motifs.

The left hand side graph shows the presence of the eight motifs (indicated by colour bars – the colours chosen have no correlation with the cluster assignment). The middle graph shows the corresponding expression profiles for one elutriation experiment (2201) and the right hand side cluster assignment of the genes.