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Periodic gene expression program of the fission yeast cell cycle

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Cell-cycle control of transcription seems to be universal, but little is known about its global conservation and biological significance. We report on the genome-wide transcriptional program of the *Schizosaccharomyces pombe* cell cycle, identifying 407 periodically expressed genes of which 136 show high-amplitude changes. These genes cluster in four major waves of expression. The forkhead protein Sep1p regulates mitotic genes in the first cluster, including Ace2p, which activates transcription in the second cluster during the M-G1 transition and cytokinesis. Other genes in the second cluster, which are required for G1-S progression, are regulated by the MBF complex independently of Sep1p and Ace2p. The third cluster coincides with S phase and a fourth cluster contains genes weakly regulated during G2 phase. Despite conserved cell-cycle transcription factors, differences in regulatory circuits between fission and budding yeasts are evident, revealing evolutionary plasticity of transcriptional control. Periodic transcription of most genes is not conserved between the two yeasts, except for a core set of ~40 genes that seem to be universally regulated during the eukaryotic cell cycle and may have key roles in cell-cycle progression.

The cell-division cycle is fundamental to the proliferation of all organisms, and knowledge of its regulation helps in understanding various diseases, notably cancer. Periodic gene expression seems to be a universal feature of cell-cycle regulation[1]. Genome-wide studies of cells undergoing growth and division have identified hundreds of periodically expressed genes[2–6], although alternative explanations for these findings have been suggested[7]. Elegant microarray approaches pioneered with the budding yeast *Saccharomyces cerevisiae* have provided global insights into target genes of transcription factors and cell-cycle regulatory networks[8–12]. This work has led to the proposal that serial regulation of transcription factors, whereby transcriptional activators functioning during one cell-cycle stage regulate activators functioning during the next stage, results in a continuous cycle of interdependent waves of transcription[1,9,13,14]. Yet it is not known how well cell cycle–regulated transcription is conserved with regard to both the types of genes periodically expressed and the regulatory networks involved.

In the present study, we examined the global cell-cycle control of gene expression in the fission yeast *Schizosaccharomyces pombe* using DNA microarrays. We have generated a comprehensive data set that provides a framework for understanding periodic transcription and its regulation during the cell cycle. Fission yeast is a useful model organism for research in cell-cycle regulation[15], and it is only distantly related to budding yeast, as these two eukaryotes diverged >1 billion years ago, according to a recent estimate[16]. Therefore, we have compared our data with that from budding yeast to elucidate the basic features and critical targets of transcriptional networks during eukaryotic cell cycles, to improve understanding of the evolutionary plasticity of transcriptional regulation and to give further insight into the biological significance of cell cycle–regulated transcription.

RESULTS

Identification of periodically expressed genes

To identify mRNAs whose levels oscillate during the cell cycle, we obtained microarray data for >99.5% of all known and predicted fission yeast genes[17,18]. We measured gene expression as a function of time in cells synchronized through different approaches: centrifugal elutriation, which generates a homogeneous population of small cells early in their cell cycle, and the use of temperature-sensitive cell-cycle mutants, which arrest at specific stages from which cells undergo synchronous divisions after release (see **Supplementary Fig. 1** online). Use of the cell-cycle mutants gave slightly better synchrony but might introduce artifacts caused by the temperature shift and lengthy cell-cycle arrest[7]. Combining data from 8 different synchronous cultures, we identified 407 genes whose expression is periodically regulated during the cell cycle (**Fig. 1**). But only 136 of these genes showed a >2-fold difference between peak and trough expression in the elutriation experiments ('high-amplitude' genes); most showed smaller changes of between 1.5- and 2-fold ('low-amplitude' genes). More than 30 cell cycle–regulated genes have been reported in fission yeast, and their periodic transcription is reflected by periodicity in the corresponding

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protein abundance in most cases; we validated our results by comparison with these published data (see **Supplementary Table 1** online).

We grouped the periodic genes into four main clusters defining successive waves of transcription (**Fig. 2**). The expression peaks of known periodic genes correlated well with our grouping into the first three clusters (**Fig. 2**; **Supplementary Table 1** online), whereas the fourth cluster contained no previously identified periodic genes. Below, we summarize major characteristics of each cluster together with a few sample genes. Full information on cell cycle–regulated genes is available online, including an overview of 140 periodic genes with known functions for each cluster (see **Supplementary Table 2** online) and a list of all periodic genes ordered by cluster and amplitude (see **Supplementary Table 3** online).

Genes peaking in transcript levels during M and G1

Genes of clusters 1 and 2 show increased transcript levels during mitosis and G1 phase, which occur within a short time in rapidly growing *S. pombe* cells (**Fig. 2**)[15]. Cluster 2 closely follows cluster 1, and some genes are difficult to assign unambiguously to one or the other cluster owing to the close temporal separation. Other differences in regulation further support separation into two clusters, however (see later discussion).

Cluster 1 contains 87 genes whose expression peaks during mitosis and whose Gene Ontology (GO) associations are enriched for the terms 'M phase', 'Cytokinesis' and 'Chromosome condensation' (see **Supplementary Table 4** online). This group includes genes encoding mitotic regulators (such as Aurora and Polo[19] kinases), mitotic spindle or contractile cytokinesis ring proteins (such as Klp5p and Klp6p kinesins and Myo3p myosin) and cell-cycle regulators (including five regulators of the Cdc2p cyclin-dependent kinase); a noncoding RNA gene (*meu16*)[20]; and genes encoding proteins involved in DNA metabolism (such as Mus81p nuclease and Msh6p mismatch repair) and transcriptional regulators (see later discussion).

Cluster 2 contains 78 genes whose expression peaks a little later, around anaphase, cytokinesis and entry into G1 phase. This cluster contains many highly regulated genes (**Fig. 2**) and includes the highest number of known periodic genes, most of which function in cell-cycle control or regulation of DNA replication as reflected in the associated GO terms (**Supplementary Table 4** online). Cluster 2 also contains genes encoding chromosome cohesion and segregation proteins (including Cut2p securin, Psm3p cohesin and the Ams2p GATA factor for centromere-specific binding of CENP-A histone variant[21,22], encoded by a gene that is also regulated in this cluster), genes for DNA metabolism (including Pol1p and Cdm1p polymerases), a noncoding RNA gene (*meu19*)[20] and genes required for cell division (for example, encoding the Par2p PP2A phosphatase regulatory subunit and Mid2p anillin[23]).

Genes peaking in transcript levels during S and G2

Cluster 3 contains only 46 genes whose expression peaks during DNA replication (S phase; **Fig. 2**). The ten most highly regulated genes in this cluster all encode histones, which form a tight subcluster. Accordingly, the GO term 'Chromatin' was over-represented among GO associations of cluster 3 genes (**Supplementary Table 3** online). No genes encoding proteins of other known biological functions were prevalent in cluster 3.

Cluster 4 contains 147 genes, but most are only weakly regulated (low-amplitude genes). This is the most heterogeneous cluster, with genes peaking at different times during G2 phase, although a majority peak in early G2. Only ~20% of these genes have characterized functions and no term was significantly enriched among their GO associations. One of the more highly regulated cluster 4 genes is *spd1*, a negative regulator of DNA replication[24] that shows a broad peak in early G2 phase. Beside other cell-cycle control genes, this cluster also includes numerous genes involved in cell growth and metabolism and all nine genes for Tf2-type transposons (although the latter are too similar to each other to permit distinguishing whether one or all of them are periodically expressed). During early G2, fission yeast cells switch from monopolar to bipolar growth, which increases overall growth rate and may account for the activation of the cell growth and metabolism genes at this stage. There may be an advantage to cells if transposition occurs during G2 phase, because the presence of two gene copies would prevent transposon insertions within essential genes from being lethal; this could explain the presence of transposon genes in cluster 4. Notably, a significant portion of cluster 4 genes overlap with genes modulated during environmental stress[25] ($P \sim 10^{-22}$). The weak periodicity of these genes could be triggered by stress caused during the induction of synchrony[7], implying that cells are more responsive or more sensitive to stress during G2 and repeat the response in the subsequent cycle. Alternatively, expression of these genes might reflect a somewhat higher activity during G2 of the stress-activated MAP kinase Sty1p, which is required for the core environmental stress response as well as for G2-M progression[25,26].

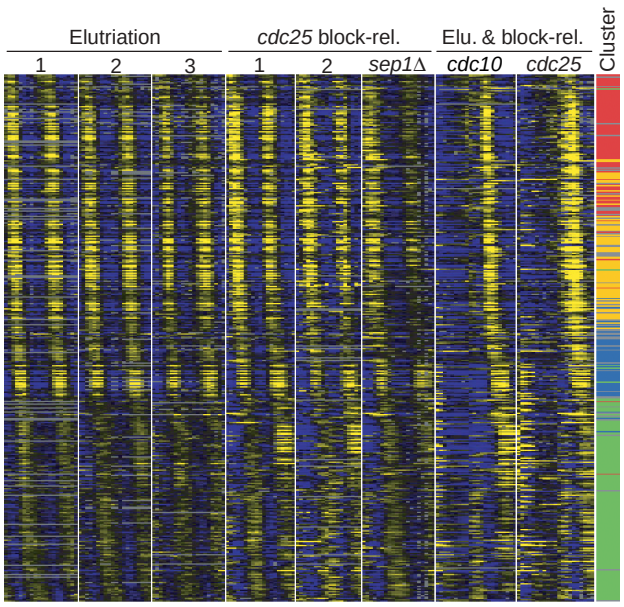

Figure 1 Phaseogram of all periodically expressed genes identified in this study. Horizontal rows represent the profiles of 407 cell cycle-regulated genes ordered along the *y* axis by the time of their peak expression (Methods). Columns represent synchronized experimental samples (8 time-course experiments of 18–22 time points each collected at 15-min intervals: 160 time points total). The mRNA levels at the time points relative to levels in unsynchronized cells are color coded (yellow, induced expression; blue, repressed expression; gray, no data). Shown left to right are cell-cycle synchronization by elutriation (three independent biological repeats), by *cdc25* 'block-release' (two independent biological repeats, and one experiment in a *sep1*Δ background) or by a combination of both methods: elutriation of *cdc10* and *cdc25* cells followed by a shift to restrictive temperature to synchronously block in G1 and late G2 phases, respectively, before release at permissive temperature. All experiments, except the last two, followed two full cell cycles. The bar at right reflects our grouping of genes into four clusters (using the same color code as in **Fig. 2**; gray indicates genes not assigned to any cluster).

Transcriptional regulation of periodically expressed genes

Given the transcriptional network that controls the cell cycle in budding yeast[1,13,14], how similar is the regulation of periodic transcription in fission yeast? A gene in cluster 1, *cdc15*, requires the forkhead transcription factor Sep1p for periodic expression[27]; forkhead proteins have conserved roles in regulating the progression of mitosis in budding yeast[8,28–30] and human cells[31]. We therefore used microarrays to analyze transcription levels in unsynchronized *sep1* deletion mutants (*sep1*Δ) relative to wild-type cells. More than 70% of the genes expressed less in *sep1*Δ cells showed periodic transcription. Of 46 *sep1*-dependent periodic genes, most were in either cluster 1 or 2 (16 and 26 genes, respectively). The same genes were induced in cells overexpressing Sep1p relative to the wild type (**Fig. 3a**), confirming their dependency on Sep1p.

Although *sep1* itself was not periodically expressed, another gene encoding a forkhead protein (SPBC16G5.15c; *fkh2*) was co-regulated with cluster 1 genes. We also deleted and overexpressed *fkh2*, resulting in elongated, branched and slowly growing cells with a delay in cell separation. Both strains were rather sick, making it difficult to extract meaningful cell-cycle transcriptome information; among hundreds of modulated genes, most were involved in the stress response[25], probably because of the distressed state of the mutants. We found, however, that Sep1p-dependent genes were more highly expressed in the absence of *fkh2*, raising the possibility that Fkh2p might have negative regulatory functions similar to its ortholog (Fkh2p) in budding yeast[28] (data not shown). The periodic expression of *fkh2* and its effects on cell separation suggest roles in cell-cycle control, but further work is required to establish whether and how Sep1p and Fkh2p function together.

The data just discussed indicate that expression of some genes in clusters 1 and 2 requires the Sep1p forkhead protein. In budding yeast, a forkhead transcription complex indirectly activates a transcriptional wave during M-G1 phase by regulating two homologous transcription factors (Ace2p and Swi5p)[1,8,9]. Is there a similar transcriptional cascade functioning in fission yeast? *S. pombe* Ace2p (the ortholog of *S. cerevisiae* Ace2p) controls *eng1* expression[32], which is induced in cluster 2. We found that *ace2* was itself periodically transcribed in cluster 1 and its expression required Sep1p (**Fig. 3**). This raised the possibility that the Sep1p-dependent genes in cluster 2 are Ace2p targets and only indirectly regulated by Sep1p. To test this, we analyzed transcriptomes in cells that had a deletion of *ace2* (*ace2*Δ) or overexpressed Ace2p relative to wild-type cells. Whereas the *ace2*Δ mutant had a phenotype similar to that of the *sep1*Δ mutant (chains of cells defective in cell separation[27,32]), Ace2p overexpression resulted in small, round cells (data not shown). Genes expressed less in *ace2*Δ cells were highly induced in Ace2p overexpressors (**Fig. 3a**). More than 90% (22) of the genes expressed at lower levels in *ace2*Δ cells were periodic, and 17 of these were in cluster 2. The strong induction of potential target genes in Ace2p overexpressors (unlike in Sep1p overexpressors, **Fig. 3a**) suggests that Ace2p either acts alone or is the limiting factor for regulating these genes. Many of the Sep1- and Ace2p-dependent genes overlapped (**Fig. 3a**, green branches), and these were all in cluster 2. Some of the Ace2p-dependent genes, such as *mid2* (ref. 23), *eng1* (ref. 32) and SPAC14C4.09 (a putative glucanase), have known or predicted roles in cell division that may explain the cell separation defects in *ace2*Δ and *sep1*Δ cells. Cluster 2 and Ace2p-dependent genes were enriched for a promoter motif similar to the one recognized by the Ace2p orthologs in budding yeast[2] (**Fig. 4**; **Supplementary Table 5** online), indicating that Ace2p recognizes the same regulatory motif in both yeasts.

About 70% of the Sep1- but not Ace2p-dependent genes were in cluster 1. They encode several mitosis and cytokinesis proteins, such as Plo1p (Polo kinase), which may provide positive feedback for the transcription of mitotic genes[19]. Forty cluster 1 genes contained promoter motifs similar to the conserved binding sites of forkhead proteins (**Fig. 4**; **Supplementary Table 5** online)[8,30,31,33,34]. The Sep1p-dependent genes significantly overlapped with genes containing forkhead motifs ($P \sim 10^{-16}$). (Several cluster 1 genes with forkhead motifs are also induced during meiosis in response to the Mei4p forkhead protein[34,35].) Most potential Sep1p target genes were marginally induced in *ace2*Δ cells and strongly repressed in Ace2p overexpressors (**Fig. 3a**, red branches), raising the possibility that Ace2p inhibits expression of these genes. Together, our data suggest a transcriptional cascade whereby the Sep1p forkhead protein activates cluster 1 genes including the Ace2p transcription factor, which in turn activates cluster 2 genes and possibly helps to switch off the Sep1p targets of cluster 1 (**Fig. 3b**).

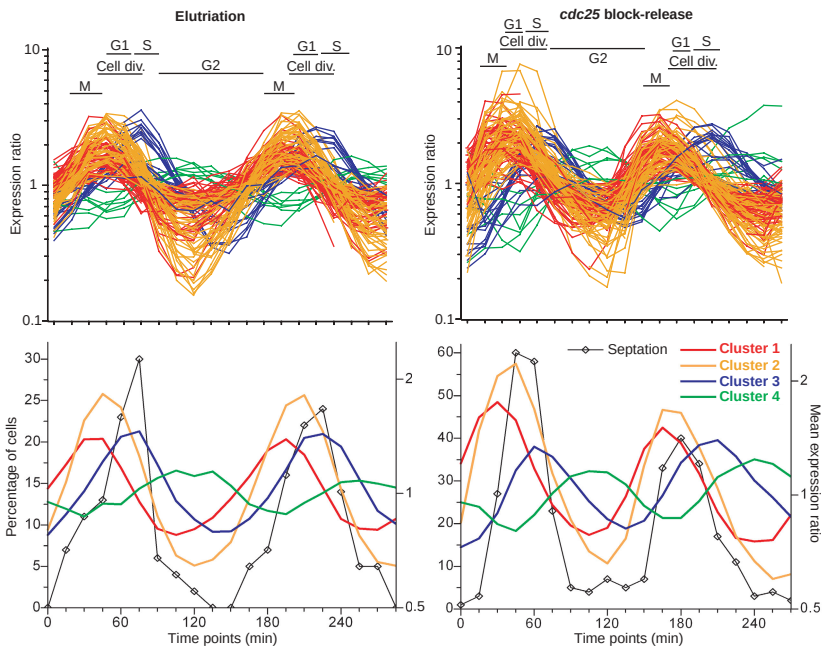

Figure 2 Grouping of periodically expressed genes into four main clusters. Top, relative mRNA levels at different time points as compared to unsynchronized cells are shown for 123 high-amplitude genes that were assigned to cluster 1 (red), 2 (orange), 3 (blue) or 4 (green) on the basis of their profiles in all time-course experiments (Methods). The expression profiles in an elutriation and a *cdc25* block-release experiment are shown as examples (corresponding to elutriation 2 and *cdc25* block-release 1 in **Fig. 1**). Approximate timings of key cell-cycle events are indicated in the graphs (for corresponding data, see **Supplementary Fig. 1** online). Bottom, average gene expression profiles of 360 genes, assigned to clusters 1–4, for the same experiments as above, together with the percentages of cells undergoing septation (cell division) as a function of time.

Besides the Ace2p targets, cluster 2 contains several genes known to be regulated by *Mlu*I cell-cycle box (MCB) binding factor (MBF), a conserved transcription complex functioning in G1-S progression[1,15,36,37]. The genes *cdc10* and *rep2* (encoding a component and regulator of MBF, respectively)[15,36] showed periodic transcription in cluster 2, although with low amplitude. To further investigate the regulation of MBF-dependent genes, we analyzed the transcriptome of a *cdc10-C4* mutant, which shows increased expression of MBF-regulated genes[38]. Among 62 genes more highly expressed in *cdc10-C4* cells relative to wild-type, 32 were periodic, with a majority of these in cluster 2. These genes included nine of the ten previously known MBF target genes. Promoters of cluster 2 genes and, even more, of the MBF-dependent genes were enriched for sequences similar to the conserved MCB motif recognized by MBF (**Fig. 4**; **Supplementary Table 5** online)[1,36]. In two cases, pairs of divergently expressed genes shared a promoter with clustered MCB motifs (*cdt2* with *psc3*; *ssb1* with *mik1*).

None of the MBF-dependent genes overlapped with the Ace2p-dependent genes; together, Ace2p and MBF seemed to regulate ~80% of cluster 2. Unlike the Ace2p-dependent genes, the MBF-dependent genes did not require Sep1p for high expression (**Fig. 3a**). To further examine the roles of Sep1p in Ace2p- and MBF-dependent transcription, we synchronized *sep1*Δ cells and found that the expression amplitudes of many genes were reduced (**Fig. 1**). Sep1p- and Ace2p-dependent genes were still periodically expressed in *sep1*Δ cells but with lowered amplitudes as compared to control cells (**Fig. 3c**). Periodic expression of the MBF-dependent genes was not affected, however, although the second peak was ~30 min later than in control cells. Although the peaks of Ace2p- and MBF-dependent genes coincided in the elutriated cultures, MBF targets tended to peak before the Ace2p targets in the block-release synchronized cultures, especially during the first cycle (**Fig. 3c**). Consistently, DNA replication occurred earlier in the elongated *cdc25* cells relative to other cell-cycle events (**Fig. 2**). This further suggests that these two gene groups are independently regulated, although they normally peak at a similar time. Sep1p-Ace2p- and MBF-dependent genes also behaved differently in cells arrested in S phase by hydroxyurea: whereas the former were repressed, the MBF targets were strongly induced under these conditions (**Fig. 3a**). We conclude that MBF regulates some of the cluster 2 genes independently of Sep1p and Ace2p and controls different but coincident cell-cycle events (**Fig. 3b**).

We searched for other potential regulatory motifs upstream of periodically expressed genes (**Fig. 4**; **Supplementary Table 5** online). The Mcm1 motif, which is conserved in mammals[39], has several functions in *S. cerevisiae* cell-cycle transcription, either on its own or in combination with the forkhead motif[2,30,40,41]. Cell cycle–regulated genes in fission yeast did not show enrichment for any similar motif. However, cluster 1 was enriched for two rarer motifs (New 1 and 2), which frequently occurred in combination with forkhead motifs but only marginally overlapped with Sep1p-dependent genes. Many promoters contained combinations of different motifs (**Supplementary Table 3**

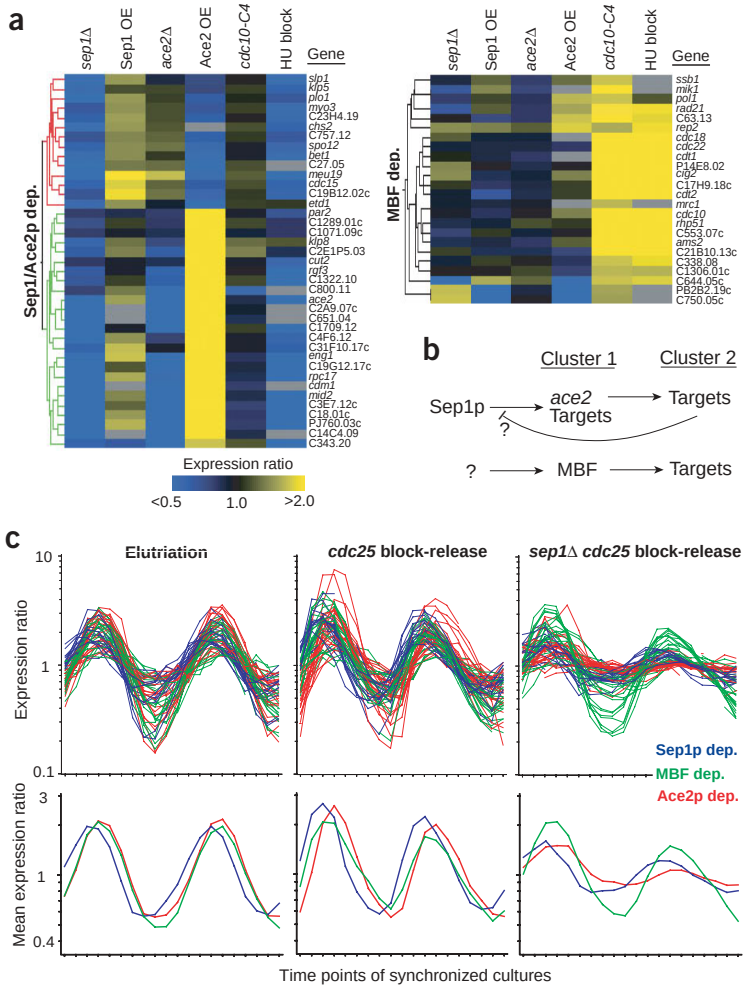

Figure 3 Transcriptional regulation of genes in clusters 1 and 2. (**a**) Hierarchical clustering of genes on the basis of their relative expression levels in *sep1*Δ cells, Sep1p overexpressors (OE), *ace2*Δ cells, Ace2p overexpressors, *cdc10-C4* cells and cells arrested in S phase 3 h after addition of hydroxyurea (HU). Genes are represented by horizontal strips, with changes in expression levels relative to control cells color-coded as indicated in the bar (gray, no data). Average data of biological repeated experiments are shown (Methods). Left, 39 high-amplitude genes of clusters 1 and 2 that are either Sep1p-dependent (red branches; 14 genes) or Sep1- and Ace2p-dependent (green branches; 25 genes, including *ace2*). Right, 24 high-amplitude genes of cluster 2 that are MBF dependent. (**b**) Schematic model for regulation of genes in clusters 1 and 2. The Sep1p-Ace2p–dependent genes function mainly in mitosis and cell division, whereas most MBF-dependent genes have roles in DNA replication. (**c**) Top, expression profiles of the genes clustered in **a** are shown in an elutriation experiment (left, as in **Fig. 2**) and two *cdc25* block-release experiments, without (middle, as in **Fig. 2**) or with (right) *sep1*Δ mutation. Bottom, average gene expression profiles of the three gene groups are shown for the same experiments as above. Blue, Sep1p- but not Ace2p-dependent genes (cluster 1); red, Ace2p-dependent genes (cluster 2); green, MBF-dependent genes (cluster 2).

online), and some of them may reflect combinatorial control by different transcription factors. The nine histone genes strongly induced in cluster 3 all contained versions of the known histone motif, with three pairs of divergently expressed genes sharing their promoters[42]. Five nonhistone genes in clusters 2 and 3 also contained this motif. The gene *pht1*, encoding a histone variant[43], showed no histone motif within 600 bp of its start codon, but a related pattern (AACACTCAC) was located further upstream and could be responsible for the weaker regulation of this gene. The histone motif is not conserved between budding and fission yeasts, and how histone genes are regulated in either yeast is not well understood. Finally, a potential new motif (New 3) was apparent within cluster 4 and enriched among some genes induced in early G2 phase, although the great majority of cluster 4 genes were not linked to any motif. The low periodicity of these genes could reflect poor motifs with weak regulator binding, or the genes could be regulated at the level of mRNA stability.

Conservation of cell cycle–regulated gene expression

How well is periodic transcription conserved through evolution? The most complete published data sets concerning cell cycle–regulated gene expression are from budding yeast[2,3], where two studies have identified ~400–800 periodically transcribed genes comparable to our high-amplitude genes. The overlap in these two budding-yeast data sets is rather limited, with only 301 genes reported as being periodic in both studies. We compared these genes with those identified in our study. Among 87 high-amplitude fission yeast genes that have budding yeast orthologs, ~40 genes were also found to be periodic in the budding yeast studies (**Table 1**). Although this overlap of periodically expressed genes is highly significant ($P \sim 2 \times 10^{-35}$), it is surprisingly small. Comparisons of larger, less conservative lists increased the overlap to >50 genes but reduced the significance of the overlap; see **Supplementary Table 6** online). This suggests that cell-cycle regulation of the majority of genes is not conserved. The genes listed in **Table 1** define a conservative core set of cell cycle–regulated genes, most of which have well-characterized and conserved regulatory functions in DNA replication, mitosis and/or cell division. This is also reflected by the highly enriched GO terms associated with these core cell cycle–regulated genes (**Supplementary Table 4** online). We propose that periodic expression of these genes is more crucial for cell-cycle control. Despite the small overlap in periodic genes, significant numbers of these orthologs are regulated at corresponding cell-cycle stages in the two yeasts: clusters 1–4 overlapped most significantly with the budding yeast G2-M-, G1-, S- and S-G2-phase genes, respectively (**Supplementary Table 6** online)[2]. Cluster 4 and budding yeast M-G1 genes showed the least overlap with any lists in the other yeast. We conclude that cell cycle–regulated transcription of most genes has been rather poorly conserved through evolution, although there is a small but highly significant set of genes whose periodic transcription is conserved and that function mainly in basic cell-cycle processes.

DISCUSSION

We have generated a comprehensive dataset of gene expression during the fission yeast cell cycle and have identified 407 genes (~8% of the genome) whose expression is periodic, 136 of which show a >2-fold difference between peak and trough levels. These genes can be grouped into four clusters defining successive transcriptional waves during the cell cycle. Three of these clusters occupy a window of ~30% of a cell cycle spanning mitosis, cytokinesis, G1-, and S-phases. A fourth cluster is more weakly regulated, extends through G2-phase and occupies the rest of the cell cycle. Two transcription factors, Seplp and Ace2p, act sequentially in a cascade regulating some of the genes in clusters 1 and 2, most of which are required for mitosis and cell division. A third transcription factor (MBF) acts in parallel with Ace2p to regulate further genes in cluster 2 required mainly for DNA replication (**Fig. 3b**).

Among the genes in our data set regulated with high amplitude, most (111/136) are concentrated in the short window spanning mitosis and G1 (~20% of cell cycle[15]). In this fission yeast differs from budding yeast, in which periodic transcription is spread more evenly throughout the cycle and the main transcriptional regulators are connected through a cascade cycle of serial activation[8,9,28–30,41,44,45] (**Fig. 5**). The part of the transcriptional cascade from forkhead to Ace2p regulators acting during M and G1 is conserved between the two yeasts (**Fig. 5**). The MBF complex, which regulates G1-S progression, is also conserved, but it acts downstream of Ace2p and Swi5p and upstream of forkhead proteins in budding yeast, whereas it functions parallel to and independently of Ace2p and Seplp forkhead in fission yeast (**Fig. 5**). The coincident functions of Ace2p and MBF in fission yeast, as compared to their temporal separation in budding yeast, may reflect the short G1 phase in fission yeast. In contrast to budding yeast, in fission yeast transcriptional activators do not seem to form a fully connected

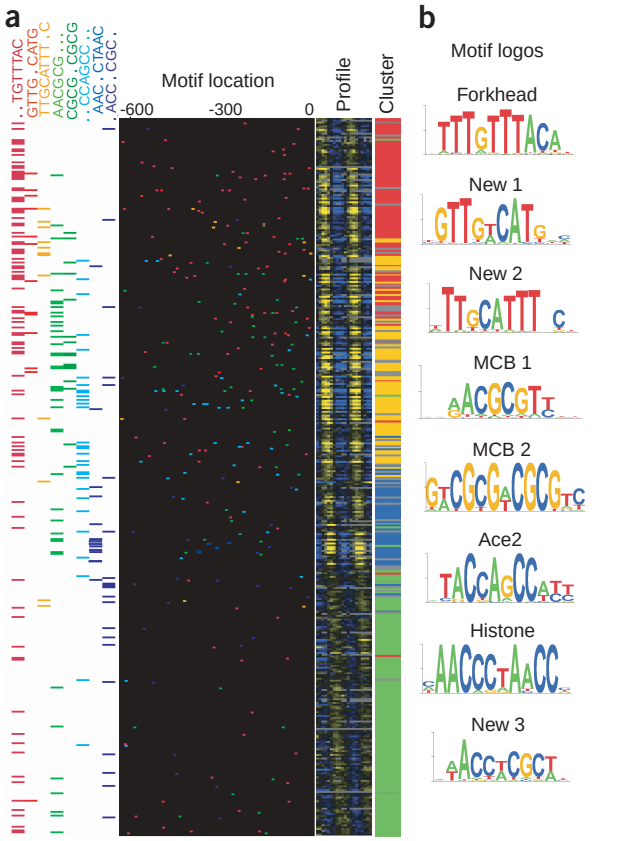

Figure 4 Identification of potential regulatory promoter motifs. (**a**) Genes were ordered by their phase and analyzed for significantly enriched sequence motifs using a running window of 40–60 bases (Methods). The presence of eight motifs is indicated by colored bars, and motif locations within a 600-bp region upstream of the start of the open reading frame are shown in the black box. All motifs found upstream of the periodic genes are indicated in **Supplementary Table 3** online. The corresponding expression profiles of an elutriation experiment and cluster assignment of the genes are also shown (same experiment and color code as in **Fig. 2**; gray, genes not assigned to any cluster). (**b**) Logo versions of eight motifs obtained by compilation of related motifs (Methods).

cyclic cascade. The extended G2 phase (~70% of cell cycle), during which no known or predicted genes encoding transcription factors show increased expression, is unlikely to be bridged by transcriptional control. Furthermore, in fission yeast Ace2p is the only known transcription factor whose transcript is strongly regulated and peaks ahead of its target genes. Post-transcriptional mechanisms may therefore be relatively more important for regulating cell-cycle transcription factors in fission yeast. Thus, despite conservation of three main cell-cycle transcriptional regulators between the two distantly related yeasts, there are differences in circuitry of the regulatory networks, probably reflecting a rewiring during evolution to adjust for differences in cell-cycle phasing between the two yeasts.

At least twice as many genes are strongly regulated during the cell cycle in budding yeast as in fission yeast. In budding yeast, pairs of transcriptional activators have partially redundant functions (for example, Ace2p and Swi5p, MBF and SBF)[9,10,14,45], whereas in fission yeast there seems to be only one activator at each stage, possibly reflecting the absence of a whole-genome duplication during fission yeast evolution[17]. Transcriptional regulation of at least eight different cyclin genes contributes to driving the cell cycle in budding yeast[1,9,13,46,47], whereas in fission yeast only one cyclin gene (*cig2*) is strongly regulated and two others (*cdc13* and *cig1*) show weak regulation. The weak transcriptional regulation of *cdc13* and *cig1* might be an evolutionary remnant, or this regulation could become more important, and more pronounced, when the cell cycle slows down, as during nutritional limitation. These observations indicate that fission yeast relies less on transcriptional control for cell-cycle regulation than does budding yeast, and therefore post-translational mechanisms may be more important in fission yeast.

These differences may reflect the fact that the key issue for cell-cycle control is the final activity of gene products, which can be regulated at multiple, partly redundant levels. It is possible that the regulation of most periodic genes is not absolutely required but enhances efficiency and fidelity in cell-cycle transitions. Periodic transcription that has been conserved through evolution is probably of more general biological significance for proper cell-cycle function. Our comparisons with budding yeast data revealed a surprisingly small core set of genes that are periodically expressed in both yeasts. Although some of the small

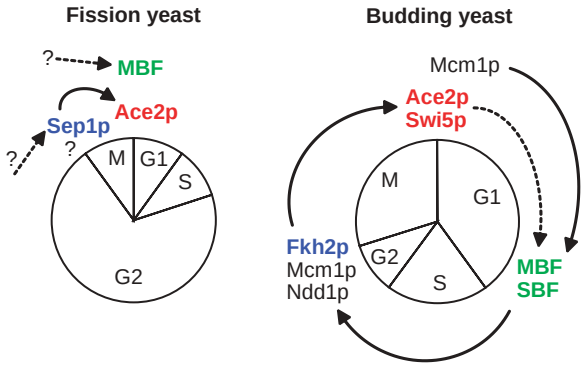

Figure 5 Cell-cycle transcriptional networks in fission and budding yeasts. Orthologous transcription factors are shown in corresponding colors, and approximate cell-cycle phases during which transcription factors function are given within the circles. Arrows with solid lines indicate direct transcriptional regulation by transcription factors, and arrows with dashed lines indicate post-transcriptional regulation. In budding yeast, Ace2p and Swi5p indirectly activate MBF and SBF by transcriptional activation of the Cln3p cyclin[9]. See main text for discussion.

Table 1 Core set of periodically expressed genes in fission and budding yeasts

S. pombe ortholog	*S. cerevisiae* ortholog	Function
DNA replication		
pol1	*POL1*	DNA polymerase α
cdc20	*POL2*	DNA polymerase ε
ssb1	*RFA1*	Single-stranded DNA-binding protein
cdc18	*CDC6*	Regulator of DNA replication initiation
mrc1	*MRC1*	DNA replication checkpoint protein
cdc22	*RNR1*	Ribonucleotide reductase
psm3	SMC3	Cohesin
rad21	MCD1	Cohesin
pht1	*HTZ1*	Histone variant
hta1, *hta2*	*hta1*, *hta2*	Histone H2A
htb1	*htb1*, *htb2*	Histone H2B
hht1, *hht2*, h*ht3*	*hht1*, *hht2*	Histone H3
hhf1, hhf2, hhf3	hhf1, hhf2	Histone H4
Mitosis and cell division		
plo1	*CDC5*	Polo kinase
ark1	*IPL1*	Aurora kinase
fin1	*KIN3*	NimA kinase
cut2	*PDS1*	Securin (sister chromatid separation)[a]
slp1	*CDC20*	Activator of anaphase promoting complex
wis3	*SPO12*	Putative cell-cycle regulator
klp5, *klp6*, *klp8*	*KAR3*, *KIP1*	Kinesin microtubule motor[b]
mob1	*MOB1*	Protein involved in mitotic exit and septation
sid2	*DBF2*	Kinase involved in mitotic exit and septation
myo3	*MYO1*	Myosin II heavy chain
mid2	*BUD4*	Protein involved in cytokinesis
ace2	*ACE2*	Transcription factor
imp2	*HOF1*	Protein involved in cell division
chs2	*CHS2*	Protein involved in septum formation
eng1	*DSE4*	Glucanase for cell separation
mac1	*TOS7*	Putative role in cell separation (*S. pombe*)
Others		
rum1	*SIC1*	Inhibitor of cyclin-dependent kinase[a]
mik1	*SWE1*	Kinase inhibiting cyclin-dependent kinase
cig2	*CLB1–CLB6*	B-type cyclin[b]
msh6	*MSH6*	Mismatch-repair protein
rhp51	*RAD51*	DNA repair protein
SPBC32F12.10	*PGM1*	Phosphoglucomutase, carbohydrate metabolism
SPAP14E8.02	*TOS4*	Transcription factor? (*S. cerevisiae*)

Among the *S. pombe* high-amplitude genes with orthologs in *S. cerevisiae*, we identified those genes that were also periodically expressed in both *S. cerevisiae* data sets[2,3] (Methods), providing a conservative core list of cell cycle–regulated genes. For references, see *S. pombe* GeneDB and *Saccharomyces* Genome Database (SGD).

[a]Proteins encoded by these genes show little sequence homology but are functional homologs. [b]Protein families with various functions.

overlap could reflect limitations of the global data sets[7], we suggest that transcriptional regulation is not necessarily a universal feature. Parsimony may explain much of cell cycle–regulated transcription[2,6], whereby genes are expressed when there is a special need for their products at a particular phase in the cell cycle, which can differ between organisms. For example, the periodic induction of genes functioning in metabolism and growth occurs during G2 phase in fission yeast, at a time when cells undergo most of their growth.

That periodic transcription of most genes is not conserved between budding and fission yeasts focuses attention on the ~40 genes whose cell-cycle regulation is conserved. Notably, many of these genes have well-characterized regulatory functions important in basic cell-cycle processes such as DNA replication, mitosis and cytokinesis (**Table 1**). Why has this relatively small core set of cell cycle–regulated genes been conserved through evolution? It is possible that periodic transcription of these genes is crucially important for driving progression through the cell cycle. Perhaps they are genes that must be highly regulated to ensure orderly progression or that are required in relatively large amounts with peak demands, such as histone genes. In some cases it may be to provide a fresh pool of unmodified proteins to override previous post-translational modifications. The majority of genes in **Table 1** are highly conserved throughout the eukaryotes, and many are also regulated during the cell cycle in human cells[5,6]. These include genes encoding protein kinases that regulate mitosis (Polo, Aurora, NimA), genes encoding kinesins, histones and proteins with roles in DNA metabolism (cohesins, RAD51, polymerase α) and genes encoding the dual-specificity kinases that inhibit cyclin-dependent kinases. Other genes are periodically expressed in two of the organisms but not the third. For example, the *CDC2* protein kinase and *CDC25* protein phosphatase genes are periodically expressed in human and fission yeast only and the DNA replication MCM complex genes in human and budding yeast only. This could mean either that some periodic expression was lost during evolution or that periodic expression was acquired later in certain organisms to adjust for their particular needs. We conclude that only a relatively small subset of genes are strongly and universally transcriptionally regulated during the eukaryotic cell cycle and that these are likely to play key roles in controlling cell-cycle progression.

METHODS

Strains and culture conditions. Strains, their constructions and their growth conditions are described in **Supplementary Table 7** online. For centrifugal elutriations, 5 liters of wild-type 972 h^- cells were grown at 30 °C to 5×10^6 cells/ml and loaded into a Beckman J6 centrifuge with JE-5.0 elutriation rotor, and the smallest cells were eluted at 30 °C with 80% fresh Edinburgh minimal medium (EMM), 20% conditioned EMM, 0.005% yeast extract medium. After elutriation, cells were incubated in the same mixture for ~1 h before collection of samples from the synchronized cells every 15 min for ~7 h. As a reference for all time points, we used unsynchronized cells taken from the same culture before elutriation. For the *cdc25* block-release experiments, *cdc25-22* h^- cells were grown at 25 °C to 2×10^6 cells/ml. We then shifted the culture to 36 °C for 3.5 h and collected samples every 15 min for ~5 h after shifting back to 25 °C. As a reference for all time points, we used unsynchronized cells of the same culture before shift to 36 °C. Several stress-related genes were induced in a single expression peak early in the *cdc25* block-release experiments, but these were not periodically expressed in the second cell cycle nor in the elutriation experiments and are not included in the list of periodic genes. In the two experiments with combined elutriation and temperature shift, arrest at the restrictive temperature (after elutriation) was for 2.5 h only. Measurements of cell synchrony are described in **Supplementary Figure 1** online. We grew mutant strains at 25 °C (*sep1*Δ and *cdc10-C4*) or 32 °C (*ace2*Δ) to ~6×10^6 cells/ml. As a reference, we grew wild type cultures of the same genotype (except mutation of interest) in parallel to the same cell density. Overexpression strains were grown at 32°C in EMM plus 15 μM thiamine. Cells were washed twice and grown in EMM without thiamine for 18 h before harvesting (~1–5 × 10^6 cells/ml). Cells carrying the pREP3X control vector were grown in parallel as a reference. For the S-phase block in hydroxyurea (HU), we grew wild-type 972 h^- cells at 32 °C to 2×10^6 cells/ml and added 11 mM HU before harvesting after 3 h. As a reference, we harvested cells before HU addition.

Microarray experiments. Microarray construction, RNA isolation, sample labeling and hybridization, and data acquisition and processing were as described[18]. We chose the first time points of the elutriation experiments such that the time courses start at similar points in the cell cycle as the *cdc25* block-release experiments. We carried out eight independent time-course experiments (**Fig. 1**). Samples for the *cdc25* block-release 2 experiment were hybridized twice (technical repeats) with dye swap. We normalized relative expression data for each gene and time point in the time-course experiments so that the median log(ratio) over the course of the experiment was equal to 0. The mutant and overexpression (OE) experiments were independently repeated (biological repeats) as follows: *sep1*Δ, four repeats; Sep1p OE, two repeats; *ace2*Δ, five repeats; Ace2p OE, two repeats; *cdc10-C4*, four repeats. In total, we used 196 microarrays for the data reported here, and each array contained duplicated array elements in separated regions to give two independent measurements[18].

Identification, ranking and clustering of periodic genes. Exploratory data analysis using Fourier and wavelet transform, autocorrelation and mutual information suggested that Fourier performs best for detecting periodicity in all data sets. We therefore used fast Fourier transform (FFT) maps to identify genes with maxima of power spectra corresponding to the cell-cycle duration. To determine whether the periodicity in the different experiments could arise from random fluctuations, we computed the *P*-values of each gene by comparing the maximum value of the power spectrum with those obtained from 100,000 permutations of the expression ratios in the six experiments that follow two cell cycles. We also computed *P*-values after combining the data of these six experiments (1 million permutations). About 1,000 genes showed $P < 0.01$ in at least four of the seven time series (including the combined data). Genes with subtle changes in expression are unlikely to be of biological significance; we therefore filtered out genes with ratios at peak divided by ratios at trough <1.5 or with <70% of power in maximum frequency of FFT power spectrum (sum of power for peak frequency plus two adjacent peaks relative to power present at all frequencies of power spectrum) in all three of the most diagnostic experiments for this purpose: elutriations 1 and 2 and *cdc25* block-release 1. The cutoffs were chosen empirically and the profiles of filtered genes were visually double-checked in all time-course experiments for false negatives. We then visually inspected the profiles of the remaining genes in all time-course experiments and discarded ~400 genes that did not seem to show reproducible periodicity in expression consistent with cell-cycle duration. The remaining 407 periodic genes were classified into 136 high-amplitude genes (ratios at peak divided by ratios at trough reproducibly ≥2 in elutriations 1 or 2 and reliable data in the other experiments) and low-amplitude genes (all the remaining genes). Periodic genes were ranked by the time of their peak expression (calculated from the Fourier algorithm) using the mean vectors from elutriations 1 and 2 and *cdc25* block-release 1.

To group periodically expressed genes into expression waves, we carried out clustering based on the gaussian mixture model with Pearson coefficient using ArrayMiner 5 (Optimal Design). This algorithm finds a set of gaussians yielding the highest probability with the ability to detect outliers. This approach produced biologically more coherent clusters from our data than related methods such as *k*-means. The periodically expressed genes were first clustered separately into five groups in all time-course experiments, as this gave the best consistency between experiments. To integrate cluster assignment between different experiments, we calculated the sum of percentage fits (as determined by ArrayMiner) for each gene and cluster in all experiments. Genes were then assigned to the cluster with the highest total fit; if two or more clusters showed similar total fits, we manually assigned the gene to a cluster after visual inspection in diagnostic experiments, or left it unclassified if the expression profiles were not conclusive. The last two clusters were then combined into cluster 4, because separation into two clusters showed poor consistency between experiments.

Identification of Sep1p, Ace2p and MBF-dependent genes. To identify differentially expressed genes from the mutant and overexpression experiments, we used an intensity-dependent calculation of a standard *Z*-score by determining the local mean and standard deviation (s.d.) within a sliding window of 1,000 genes[48]. Genes with expression ratios ≥2 s.d. above the local mean in repeated experiments were regarded as differentially expressed. In experiments with three or more repeats, we also used SAM[49] to confirm differential gene expression. Genes showing no cell-cycle regulation were subtracted from the lists of differentially expressed genes. We hierarchically clustered genes in GeneSpring (Silicon Genetics) using Pearson correlation.

Comparisons with budding yeast data. Lists of cell cycle–regulated genes in *S. cerevisiae* were downloaded from the accompanying websites[2,3]. Genes with a prospective *S. pombe* ortholog were determined and analyzed using a table of curated orthologs as previously described[25]. The total number of orthologs available at the time of analysis was 2,981.

Identification of promoter motifs. We used the pattern discovery tool SPEXS[50] to exhaustively search for all sequence patterns of arbitrary length containing up to two wild-card symbols. Using genomic sequence downloaded from the Sanger Institute FTP server, we extracted for all protein-coding genes the up- and downstream intergenic regions (600 bp and 400 bp, respectively). We discarded 19 genes (transposons, telomeric duplications or very short sequences). We grouped genes in overlapping clusters using a sliding window (40 and 60 genes) along the phase-ranked gene list of periodic genes, shifting the window by 10 genes at a time. Alternatively, we used lists of genes dependent on Ace2p or MBF. To estimate the expected number of pattern occurrences, we used all intergenic regions. We defined cluster-specific significance thresholds from randomized sequence sets of respective sizes. We required that the pattern occurred in at least five sequences in the cluster, that its binomial probability was smaller than the significance threshold and that it occurred in the cluster at least twice more than expected. No significant motifs in downstream regions were found. We grouped all patterns passing the criteria by similarity and reported the best one in each group. Putative regulatory patterns and locations were visualized using PATMATCH and SEQLOGO tools in Expression Profiler. For sequence logos, we introduced pattern variations by allowing one wild card, searched for matches to this approximate pattern and calculated the number of each base occurrence for each position. The letter sizes were rescaled according to the information content in that position. For MCB and Ace2 motif logos, we used the lists of MCB- and Ace2p-dependent genes instead of sliding windows.

URLs. All processed data are available from the authors' website (together with a graphical gene expression viewer to visualize cell-cycle profiles of specific genes) at http://www.sanger.ac.uk/PostGenomics/S_pombe/. The entire raw data set is available from ArrayExpress, accession numbers E-MEXP-54 to E-MEXP-64, at http://www.ebi.ac.uk/arrayexpress/. *S. pombe* GeneDB is available at http://www.genedb.org/genedb/pombe/index.jsp. SGD is available at http://www.yeastgenome.org/. Information on the ArrayMiner algorithm is available at http://www.optimaldesign.com/Download/ArrayMiner/AM2whitepaper.pdf. Expression Profiler and the SPEXS tool are available at http://ep.ebi.ac.uk/EP/.

Note: Supplementary information is available on the Nature Genetics website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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