6. GENERAL DISCUSSION

In this thesis the gene expression program during the fission yeast cell cycle has been investigated. It is the first time that gene expression has been comprehensively studied in vegetatively growing cells of *S. pombe*. These results have provided a useful framework for fission yeast research as well as an interesting point of comparison with other eukaryotic organisms such as budding yeast and humans. Results and their implications are discussed below.

6.1 Cell-cycle periodic genes and their regulation

Conventional methods of RNA measurements have been applied over the last decades to identify cell cycle regulated genes. The availability of the complete genome sequence of an increasing number of organisms and the development of the microarray technology, which enables transcript levels to be measured across the entire genome, has dramatically accelerated the identification of periodic transcripts. It is now possible to obtain a comprehensive picture of how the transcription profiles of all genes vary across the cell cycle in any population of cells that can be synchronised. In this study, fission yeast cells have been synchronised in two independent ways (whole-culture method/temperature sensitive mutants and selective method/elutriation), and microarray analysis of the fission yeast cell cycle was performed. Microarray data were derived from eight timecourse experiments, using microarrays containing > 99.5% of all known and predicted fission yeast genes. 407 genes (out of the 5119 investigated, corresponding to ~8% of the genome) whose expression is periodic were identified using an autocorrelation algorithm combined with visual inspection of each expression profile. The results were validated independently using a Fourier transform approach. Among these genes, 136 showed changes in the levels of expression bigger than 2-fold and they are referred to as ‘high amplitude’ genes (Appendix VI). The remaining 271 showed changes between 1.5 and 2 fold and they are referred to as ‘low amplitude’ genes.

Periodic genes can be grouped into four clusters resulting in four successive waves of transcription (Fig. 3.2), corresponding to different cell cycle phases: cluster 1 to mitosis (including genes involved in chromosome condensation and segregation, cytokinesis and cell separation), cluster 2 to M/G1 (including genes involved in DNA

replication initiation and cell separation), cluster 3 to S (including histones and other DNA replication genes) and cluster 4 to G2 (including several stress related genes). Clusters 1 to 3 occupy only 30% of the cell cycle, as a result of the very short G1 phase in *S. pombe* (MacNeill S.A. and Nurse P., 1997), and they include the majority of fission yeast genes previously characterised as periodic (Table 3.1). The majority (111/136) of the 'high amplitude' genes peaks around mitosis and G1, which consists of an even shorter cell cycle window (~20% of the entire cycle). Cluster 4 is more weakly regulated and covers the majority of the cycle (~70%), corresponding to a long G2 phase. This differs from *S. cerevisiae* where at least twice as many genes are regulated during the cell cycle and periodic transcription is evenly spread throughout the cycle (Cho R.J. *et al.*, 1998; Spellman P.T. *et al.*, 1998). In budding yeast G1 is longer and G2 shorter compared to fission yeast, in which most of the changes are concentrated around a short G1 and little seems to happen during a lengthy G2 phase.

Periodic transcription is usually regulated by transcription factors that can exert either positive or negative roles on their gene targets. Three major transcription factors or complexes (MBF, forkhead and ace2p) are conserved across the two yeasts (Koch C. and Nasmyth K., 1994; Kumar R. *et al.*, 2000; Ribar B. *et al.*, 1997; Martin-Cuadrado A.B. *et al.*, 2003), but there are evident differences between the transcriptional cascades they are part of, reflecting the different strategies adopted by the two species during evolution.

Two transcription factors, the forkhead-type Sep1p (Ribar B. *et al.*, 1997); (Ribar B. *et al.*, 1999); (Zilahi E. *et al.*, 2000) and Ace2p (Martin-Cuadrado A.B. *et al.*, 2003), are part of a cascade that regulates some of the cluster 1 and 2 members, mainly involved in mitosis and cell division. Sep1p controls expression of cluster 1 members, including *ace2*, which in turn regulates cluster 2 members. A third transcription complex (MBF) is the regulator of a different subset of genes also belonging to cluster 2, mainly involved in DNA replication, and it acts in parallel to and independently of Ace2p. This again is in contrast with what has been observed in budding yeast, where periodic gene expression is driven by eight main transcriptional regulators which are all connected through a cascade cycle of serial regulation (Simon I. *et al.*, 2001) (Fig. 6.1). In budding yeast, pairs of transcription factors such as MBF/SBF and Ace2p/Swi5p have similar and overlapping roles, as a consequence of a duplication event of the entire budding yeast genome that resulted in different degrees of divergence of duplicated gene pairs (Wolfe K.H. and Shields D.C., 1997; Kellis M. *et al.*, 2004). In fission yeast only one transcriptional

activator seems to operate at each stage of the cycle. A recent study has proved that gene duplication events in the two yeasts have affected 56 independent gene families, resulting in the adaptation of the two species to their characteristic life cycles (Hughes A.L. and Friedman R., 2003). Some additional transcription factors may still be missing in the transcriptional regulatory network that governs the fission yeast cell cycle. In budding yeast, the forkhead protein Fkh2p is known to have overlapping roles with another forkhead-type protein, Fkh1p, and interacts with a MADS-box transcription factor, Mcm1p. The fission yeast genome encodes for another forkhead gene, *fkh2* that from the results of this study appears to have a possible negative role in regulating some sep1p-dependent genes. The gene SPBC19G7.06, coding for a MADS-box protein, also appears to have a regulatory role on a subset of genes that contain a forkhead binding motif. An involvement of these genes in regulating periodic gene expression in fission yeast is possible.

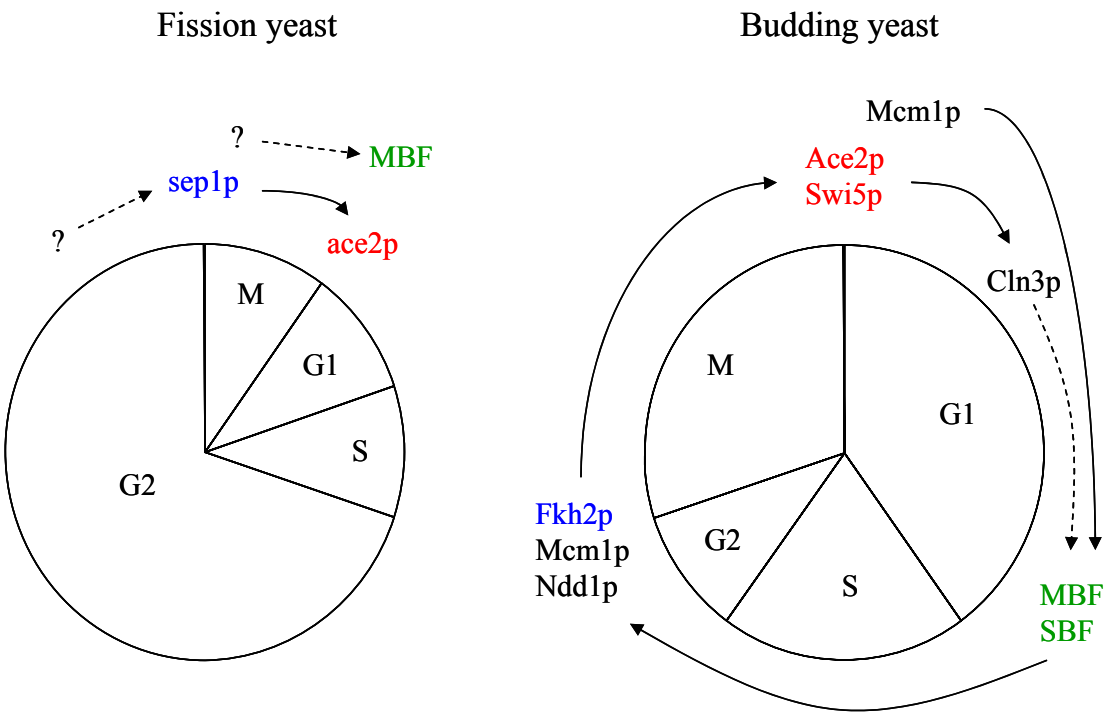

Fig. 6.1 Transcriptional regulation cascade in fission and budding yeast.
Orthologous transcription factor complexes are shown in corresponding colours and approximate cell cycle phases are represented within the cycles. Solid arrows indicate transcriptional regulation, dashed arrows posttranscriptional regulation. Question marks refer to still unidentified posttranscriptional mechanisms which might regulate transcription factors in *S. pombe*.

The part of the cascade from forkhead to ace2p, responsible of regulating M and G1 transcripts, is conserved in the two yeasts. The MBF complex, which operates at G1/S, is also conserved in the two organisms. However, it acts downstream of Ace2p/Swi5p and upstream of forkhead (Fkh2p) in budding yeast (Simon I. *et al.*, 2001; Futcher B., 2002), whereas in fission yeast it acts in parallel with ace2p and independently of forkhead (sep1p). The reason why ace2p and MBF are functioning in fission yeast at the same time is a consequence of the short G1 phase. In fission yeast cell division and initiation of DNA replication are coincident and this is reflected in the overlap of ace2p- and MBF-dependent transcriptional waves.

In *S. pombe*, the transcriptional regulators identified so far are not fully cyclically connected as in *S. cerevisiae*. Around 70% of the entire cycle corresponds to G2 phase were no transcription factors seem to be acting and transcriptional control alone is unlike to be able to bridge such a lengthy phase.

In addition all genes coding for transcriptional regulators in budding yeast are periodically transcribed themselves, peaking ahead of their targets, whereas in fission yeast this appears to be true for *ace2* only. *Cdc10* (component of the MBF complex) also shows a weak periodicity but its peak of expression is coincident with its targets. It is therefore likely that fission yeast has evolved different mechanisms such as post-transcriptional regulation to orchestrate periodic gene expression. A clear example of an *S. pombe* gene whose expression is regulated at the post-transcriptional level is *rum1* (Daga R.R. *et al.*, 2003). *Rum1* mRNA is periodically transcribed and regulated by mRNA stability. Rum1 protein level is also controlled, post-translationally via phosphorylation and ubiquitin-dependent degradation. Certain types of regulation can have advantages compared to others; changes in mRNA half-life are quicker than *de novo* protein synthesis and might be preferred in certain conditions.

The eukaryotic cell cycle is driven by CDK complexes formed by a kinase and its phase-specific cyclin partners (Morgan D.O., 1997). The budding yeast genome encodes at least eight different cyclins that contribute to cell cycle progression and that are regulated at many different levels, including the transcriptional one. The cyclin genes are periodically expressed and the function of the various cyclins coincides with their appearance. In fission yeast, only expression of one cyclin (*cig2*) is strongly cell cycle regulated whereas expression of *cig1* and *cdc13* is weak. This could be the consequence of evolutionary divergence or reflect the fact that *cig1* and *cdc13* regulation might

become more crucial in a particular situation such as changes in the environmental conditions that would cause the cell cycle to slow down until normal conditions are restored. This again underlies the fact that fission yeast might have evolved other mechanisms than transcriptional regulation for controlling cell cycle progression. Posttranslational modifications are good candidates in playing such a crucial role and are well characterised in many cases. Many fission yeast proteins are known to be regulated by phosphorylation and subsequent degradation via ubiquitination or by anaphase promoting complex mediated proteolysis.

Differences in the regulatory mechanisms between the two yeasts are probably linked to the fact that what needs to be preserved in terms of cell cycle regulation is the activity of the gene in itself and this can be achieved regulating it at many different levels. Integration of all different levels of regulation is what ultimately governs the cell cycle machinery and orchestrates cell cycle events, making sure they take place at the right time and in the right order.

6.2 Conservation of periodic transcription across eukaryotes

Comparisons between budding yeast, fission yeast and humans have revealed that conservation of periodic transcription is limited to a restricted core set of genes, around 40 when both yeasts are considered and lower when the comparison is extended to humans. Such a limited level of conservation suggests that transcriptional regulation is not necessarily a universal feature. From the point of view of efficiency, a protein that is needed throughout the entire cycle can be continuously expressed whereas a protein that is needed only once can be synthesised just before being used. Therefore certain genes are expressed only at a particular stage of the cycle when their product is needed. Taking into account the differences in the life cycle of different eukaryotic cells can explain why some genes have lost their periodic behaviour. A more detailed analysis of the individual functions reveals that most of these genes are involved in basic cell cycle processes such as DNA replication, mitosis and cytokinesis (Table 5.2).

Why has this small set of genes maintained a periodic behaviour across eukaryotes? It is possible that periodic transcription of those genes is a limiting step for cell cycle progression. Their regulation might be responsible for ensuring that cell cycle events are taking place in the right order or that the their products are available in large

quantities at a specific stage of the cycle, e.g. the histones during DNA replication. In some cases, it might also be the strategy adopted to ensure that a fresh pool of a given protein is available in order to override previous posttranslational modifications. In conclusion, only a small core-set of genes are universally transcriptionally regulated in eukaryotes and these genes are probably key players in controlling cell cycle progression.

6.3 Cell cycle periodic genes and their behaviour in meiosis

The transcriptional program of meiosis has also been studied in fission yeast using microarrays (Mata J. *et al.*, 2002). Almost half of the genes encoded by the *S. pombe* genome were upregulated at least 2-fold when compared to vegetatively growing cells and 700 genes were induced more than 5-fold.

Are the cell cycle periodic genes also regulated in meiosis? To answer this question the behaviour of the 136 'high amplitude' genes was checked during meiosis. 18 MBF-dependent genes (Table 4.3) were upregulated during premeiotic S-phase, including *cdc18*, *cdc22*, *cdt2*, *cig2*, *rhp51* and *ssb1*, all previously known cdc10p targets. Some genes such as *ams2* and *mik1* show two peaks of expression, one during premeiotic S-phase and a second one during meiotic division. Cdc10p is responsible for the regulation of its targets both during mitotic and meiotic cell cycle (Cunliffe L. *et al.*, 2004). Interestingly, both cdc10p targets and meiotic recombination genes are enriched for MCB elements, but the recombination genes are not regulated during the mitotic cell cycle. This raises the interesting question of how genes carrying the same promoter sequence are distinguished by a transcription factor. Histone genes peak just after the MBF-dependent genes in meiosis as in mitosis.

12 sep1-dependent genes (Table 4.1) were upregulated during the meiotic divisions including *etd1*, *klp5*, *myo3* and *plo1*, all involved in progression through mitosis and cytokinesis. The ace2-dependent genes (Table 4.2) showed a broader spectrum of behaviours: some genes peak at premeiotic S-phase, some others at meiotic division (*cut2, par2, rpc17* and *eng1*) and then immediately decrease whereas some others remain highly expressed.

The 10 genes encoding for the APC components, which are required for progression through mitotic anaphase, were strongly induced during the meiotic divisions

but only *apc1* appeared periodic in mitosis. These genes might have additional functions during meiosis.

6.4 Future work

This work has provided comprehensive gene expression profiles of the fission yeast cell cycle, allowing global identification of genes showing a periodic behaviour.
In order to better understand the mechanisms that govern periodic transcription, future work will include:

- Analysis of the forkhead gene *fkh2*. The main objective of this would be the identification of fkh2p targets and unmasking possible interactions between fkh2p and other transcription factors, most notably sep1p. In budding yeast, Fkh2p interacts with a MADS-box protein and a transcriptional activator. The results presented in Chapter 4 showed (despite the sickness of the strain) that some periodic genes in *fkh2Δ* are highly expressed, suggesting a negative role for this factor in regulating gene transcription.
- Analysis of the MADS-box gene *mbx1*. This is obviously linked to the further characterisation of *fkh2*, as just described. Again the results presented in Chapter 4 suggested a possible role for mbx1p in interacting with forkhead genes, as reported in budding yeast. Further analysis would help clarifying if this interaction between forkhead/MADS-box protein is a conserved mechanism.
- Analysis of cluster 4 regulation. The attempts made so far for the identification of a transcription factor responsible for the regulation of the forth wave of transcription have weakened the possibility of a direct involvement of transcriptional regulators such as atf1p, pcr1p and prr1p (which are part of the stress response cascade) despite the presence in this wave of many stress related genes. Performing a timecourse experiment for each deletion (*atf1Δ*, *pcr1Δ* and *prr1Δ*) would be the only way of excluding their involvement in the regulation of cluster 4 members.
- Chromatin immunoprecipitation (ChIP) combined with DNA microarrays (ChIP-chip). *In vivo* genomic binding sites for a specific transcription factor can be defined by combining chromatin immunoprecipitation and DNA microarrays.

Proteins are crosslinked with formaldehyde to their target sites *in vivo* and the crosslinked DNA subsequently purified by immunoprecipitation using an antibody against the transcription factor. Once the crosslink is reversed, DNA can be amplified, fluorescently labelled and consequently hybridised onto microarrays. For this purpose chromosome tiling arrays that include all intergenic regions as well as all predicted coding sequences in the fission yeast genome would be used. Traditional expression profiling and ChIP-chip experiments are complementary. From expression data it cannot be distinguished if a transcription factor directly regulates its targets or acts indirectly regulating another transcription factor, such in the case of sep1p and ace2p in fission yeast. ChIP-chip instead identifies targets for a specific transcription factor but does not provide any information concerning the type of regulation, if negative, positive or neutral. In addition, when a transcription factor binds to a promoter that is found in between two divergent genes, ChIP-chip would not help in clarifying which of the two genes is under the control of the transcription factor binding to that specific promoter sequence. Combining those two approaches will contribute to improve our understanding of the action of *S. pombe* transcription factors.

- Systematic gene deletion of all 136 open reading frames coding for the 'high amplitude' genes. Comparison with *S. cerevisiae* allowed identification of a core set of about 40 genes which have a conserved periodic behaviour in both yeasts. Many of those genes have a well characterised functions and the effect of their deletion on the cell cycle is already well known. If the systematic deletion was extended to the 136 'high amplitude' genes, this would reveal the degree of importance of each 'high amplitude' gene in regulating cell cycle progression and show the effect of each deletion on the cell cycle.
- The importance of periodic gene expression can also be investigated placing a periodic gene under the control of a constitutive promoter or swapping promoters between periodic genes. Suppressing periodicity or changing the time of expression for a specific gene should reveal the importance of its periodic behaviour.
- Systematic comparison with microarray datasets from other eukaryotic model organisms. This will include comparison of orthologous expression profiles across different genomes and analysing gene regulatory mechanisms in these

organisms. This will allow investigation of the basal gene network responsible for driving the cell cycle in eukaryotes.