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Transcription during meiosis in the fission yeast *Schizosaccharomyces pombe*

A thesis submitted for the degree of
Doctor of Philosophy at the University of Glasgow

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The research reported in this work is my own work except where otherwise stated, and has not been submitted for any other degree.

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Abstract

The meiotic cell cycle is the process by which diploid organisms divide to produce haploid gametes and consists of one round of DNA replication followed by two successive nuclear divisions. In the fission yeast, *Schizosaccharomyces pombe*, meiosis is initiated from G1 phase and involves a complex series of cellular events that lead to the production of four haploid ascospores.

The periodic regulation of gene expression is an important mechanism of control of both mitotic- and meiotic-cell-cycle progression. During the mitotic cell cycle of fission yeast a number of genes, including *cdc22*$^{+}$, *cdc18*$^{+}$ and *cdt1*$^{+}$, are expressed specifically at the G1–S phase boundary. These genes are known to be under the control of MCB (*Mlu*I cell-cycle box) upstream-activating-sequence motifs and the MBF (MCB binding factor; also known as DSC1) complex.

Here we show that control of gene expression during pre-meiotic G1–S-phase is mediated by an MBF-related transcription-factor complex acting upon similar MCB promoter motifs. Several genes, including *rec8*$^{+}$, *rec11*$^{+}$, *cdc18*$^{+}$, and *cdc22*$^{+}$, which contain MCB motifs in their promoter regions, are shown to be coordinately regulated during pre-meiotic S-phase. These genes can be divided into 'mitotic and meiotic' and 'meiotic specific' groups, which contain related but distinct arrangements of MCB motifs. MCB motifs are shown to be physiologically relevant during the meiotic cell cycle and to confer meiotic-specific transcription to a heterologous reporter gene.

An MBF-like transcription factor complex that binds to MCB motifs is identified in meiotic cells. The effect of mutating and over-expressing individual components of MBF (*cdc10*$^{+}$, *res1*$^{-}$, *res2*$^{+}$, *rep1*$^{-}$ and *rep2*$^{+}$) on *cdc22*$^{+}$, *rec8*$^{+}$ and *rec11*$^{+}$ transcription during meiosis was examined. We found that *cdc10*$^{+}$, *res2*$^{+}$ and *rep1*$^{+}$ are required for meiotic transcription, while *res1*$^{+}$ has no role in this process. Surprisingly, manipulation of the mitotic-specific *rep2*$^{+}$ gene had an effect on 'meiotic specifc' but not 'mitotic and meiotic' MCB-regulated transcription during the meiotic cell cycle. This indicates that Rep2p might have a role in allowing the MBF complex to distinguish between 'mitotic and meiotic' and 'meiotic specific' MCB motifs. This work is the first demonstration in yeast of a role for MCB motifs in control of transcription during meiosis, and identifies a meiotic MBF-like transcription-factor complex.

Abbreviations

A	adenine
Ac	acetate
ACS	ARS consensus sequence
ARS	autonomously replicating sequence
ATP	adenosine triphosphate
bp	basepairs
BSA	bovine serum albumin
C	cytosine
Cdc	cell division cycle mutant
CDK	cyclin dependant kinase
cm	centimetres
dATP	deoxy-adenosine triphosphate
dCTP	deoxy-cytosine triphosphate
dH_2O	distilled water
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DSC1	DNA synthesis control 1
EDTA	ethylenediaminetetraacetic acid
EMM	Edinburgh minimal media
g	gram
G	guanine
G1/2	Gap 1/2
h	hour
kb	kilobasepairs
kV	kilovolts
l	litre
M	molar
mM	millimolar
Mb	megabasepairs
MBF	MCB binding factor
MCB	*Mlu*I cell cycle box
min	minute
ml	millilitre
mRNA	messenger RNA

MW	molecular weight
N	Nitrogen
nm	nanometers
nmt	no message in thiamine
ORC	origin recognition complex
orf	open reading frame
PCR	polymerase chain reaction
PEG	polyethyleneglycol
PMSF	phenylmethanesulfonylfluoride
Pre-RC	pre-replicative complex
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RT	room temperature
S (phase)	synthesis (phase)
SBF	SCB binding factor
SDS	sodium dodecyl sulphate
sec	second
T	Thymine
TE	Tris/EDTA buffer
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
Tyr	Tyrosine
UAS	upstream activating sequences
UV	ultra violet
µg	microgram
µl	microlitre
µM	micromolar
w/v	weight/volume
YE	Yeast extract
°C	degrees Celsius
Δ	deletion

Acknowledgements

I would like to thank my supervisor Dr. Chris McInerny for all his help and generosity and also all members of the Goldberg lab past and present for their help and support. I would particularly like to thank Tracy Riddell, whose friendship and encouragement made the whole experience infinitely more enjoyable, and Alan Dunlop whose eternal optimism and eye for a good menu helped get me through the days. Most especially, however, I would like to thank Martin Kerr without whose unfailing emotional, financial and practical support the completion of this thesis would not have been possible.

Contents

Appendices

Index of Figures

Index of Tables

Chapter 1

Introduction

The cell cycle is a phrase used to describe the series of events through which a cell progresses that lead to the production of two daughter cells. This involves the duplication and segregation of all the constituents of the cell, events that must be spatially and temporally controlled to the finest degree, before cell division can produce viable progeny: two daughters in the case of mitotic growth or four progeny during meiosis. The cell cycle has been widely studied in several different experimental systems, from genetic models such as yeast and biochemical systems such as the eggs of marine invertebrates, to human cells themselves. All have contributed significantly to the current understanding of the subject, producing a unified view of the cell cycle, which is believed to apply to most eukaryotic cells (Lew *et al.*, 1997; MacNeill & Nurse, 1997; Nurse *et al.*, 1998). Understanding the cell cycle, as it applies to individual cell types and as a model to provide insight into the behaviour of eukaryotic cells in general, are much longed for goals in basic scientific research. Its relevance to modern medicine cannot be overstated as the control of the cell cycle is at the basis of the growth and development of all organisms, defects in which are responsible for a multitude of diseases; most notably the uncontrolled cell division associated with cancer.

In this study we are particularly interested in the meiotic cell cycle. This counterpart to the mitotic cell cycle provides the mechanism by which diploid cells are able to differentiate into haploid gametes, therefore allowing sexual reproduction to occur; a process vital for the maintenance of genetic diversity. The transition between mitosis and meiosis is a basic developmental step and the fact that meiosis differs so dramatically from mitosis should allow us an insight into the regulatory mechanisms that govern both processes (Kupiec *et al.*, 1997). Furthermore, the correct regulation of meiosis is essential for the production of healthy gametes; chromosomal mis-segregation is responsible for several devastating medical conditions, placing even greater importance on the elucidation of this complex process (Sluder & McCollum, 2000).

Yeast cell systems have been widely used as models for the study of the eukaryotic cell cycle for various reasons. As single-celled organisms they are extremely amenable to genetic analysis and are relatively easily manipulated in the laboratory, whilst retaining many important features of higher eukaryotic systems. The budding yeast *Saccharomyces cerevisiae* has a long experimental history and as a result its biology is well understood, which makes it an extremely attractive model system. The fission yeast *Schizosaccharomyces pombe* has a shorter history as a model eukaryotic system but various features, such as the fact that it grows by apical extension, have made it a popular choice in cell cycle studies (cell cycle mutants can be easily detected by cell length). Indeed the fact that the genomes of both budding yeast (Goffeau *et al.*, 1996) and fission yeast (Wood *et al.*, 2002) are now sequenced has afforded us an excellent

basis for comparison of two highly divergent eukaryotic systems, both of which are equally far removed from the human genome (Wainright *et al.*, 1993; Paquin *et al.*, 1997).

The regulation of gene expression plays a fundamental role in many aspects of the life of a cell, from cell growth, morphology and development, to the cellular response to environmental conditions and disease. Regulation at the level of transcription remains a major point of control, complementing various other mechanisms that can be used along the pathway that leads from DNA to functional gene product (Struhl, 1989). Specifically, it has been recognized that certain genes are expressed periodically over the course of the cell cycle. And it seems logical to assume that this sequential activation and repression of genes involved in subsequent stages of the cell cycle would itself play a role in the progression of that cycle (Lew *et al.*, 1997; MacNeill & Nurse, 1997). This has lead to studies of global cell-cycle-regulated transcription in various organisms, particularly the easily manipulated yeasts (Mata *et al.*, 2002; Vishwanath *et al.*, 2001).

In this thesis we have undertaken the study of transcription during meiosis in the fission yeast *S. pombe*. We are particularly focusing on the initial stages of the cell cycle during the transition point from G1 to pre-meiotic S phase, as it is after this point that cells become committed to the meiotic cell cycle. Features of this part of the cell cycle, therefore, may be important in the developmental switch between mitosis and meiosis.

In this introduction I will discuss the life cycle and genetics of the model organism fission yeast. I will also briefly describe the biology of budding yeast, as many studies that provided an essential background for this project were carried out in *S. cerevisiae*. The eukaryotic cell cycle and its regulation will be covered, in an attempt at brevity, mainly with respect to the detail of the fission yeast cycle, although I will relate this information to other eukaryotic systems. I will also discuss the current understanding of the mechanisms known to operate during meiosis in fission yeast, again relating this to both budding yeast and higher eukaryotes. Finally, I will review the current knowledge of the transcriptional controls operating around the G1–S phase transition of the cell cycle. Particular attention will be paid to the transcription factor DSC1/MBF of fission yeast, as it is this system, and how it may operate during meiosis, which forms the basis for this study.

1.2.1 Experimental yeast species

The use of model organisms in biological research has proved to be an important way of elucidating gene function. As single-celled micro-organisms, yeast species represent simple eukaryotic systems that can be easily manipulated in the laboratory, both practically and genetically. Two particular species, the budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe*, have been extensively studied, providing a wealth of knowledge about basic eukaryotic cell function and proving themselves to be excellent model systems (Bartlett & Nurse, 1990).

The phylogenetic relationships between budding yeast, fission yeast and higher eukaryotic organisms is controversial, as various methods of analysis produce differing views on the position of these organisms on the tree of life. This has a bearing on their relevance as model eukaryotes, although, it is now accepted that the two yeasts are highly diverged from one another, with neither fungus being closer to humans than the other (Paquin *et al.*, 1997; Sipiczki, 2000). This observation provides an excellent opportunity for comparison between the two yeasts, which is likely to enhance our insight into the mechanics of eukaryotic cells in general (Forsburg, 1999).

1.2.2 Saccharomyces cerevisiae

The budding yeast *S. cerevisiae* is a single-celled, eukaryotic, ascomycete fungus. Its haploid genome, of 14 Mb, is distributed among sixteen chromosomes, which range from 230 kb to over 1 Mb in size. The budding yeast genome has been sequenced, providing an indispensable resource for the genetic analysis of this organism (Goffeau *et al.*, 1996).

Budding yeast grows as a stable haploid or diploid cell and employs an unusual budding mode of division. A small bud is produced which grows continuously throughout the cell cycle. The nucleus migrates into the neck of the bud where it undergoes mitosis. The bud then pinches off and a new, smaller, daughter cell is formed. This bud formation and nuclear migration, requires spindle pole body duplication and mitotic spindle formation relatively early in the cell cycle, precluding a clear comparison of the budding yeast S, G2 and M phases with those of other eukaryotes.

During G1 phase budding yeast can embark upon one of three alternative developmental fates (Figure 1.1). If nutrients are plentiful, cells can proceed through the cell cycle and divide. However, if starved, they can enter stationary phase, becoming resistant to heat and chemical treatment. Alternatively, if the cells are haploid they can mate. Wild type haploid cells exist as one of two opposite mating types: **a** and α. However, they are capable of switching their mating

type to increase their chances of diploid formation. During conjugation a haploid cell will produce peptide mating pheromones, which induces a transient G1 arrest in cells of the opposite mating type. This serves to synchronize the cell cycles of the two opposite haploid cells, before fusion occurs to produce a single diploid cell. This diploid will then continue to grow mitotically, unless starved, at which point it can undergo meiosis to produce four haploid spores (Forsburg & Nurse, 1991).

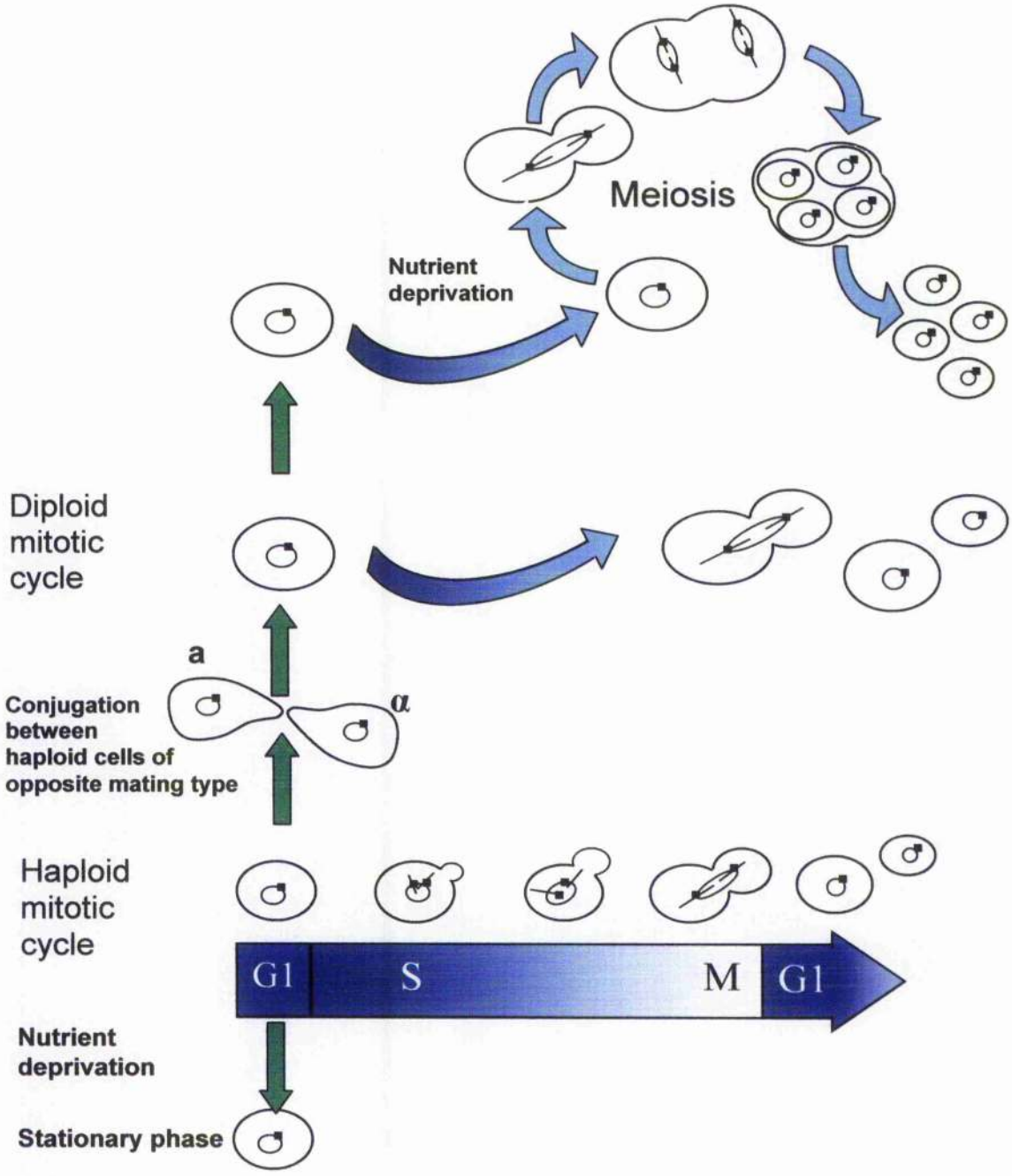


Figure 1.1: The life cycle of budding yeast

Budding yeast grows by a process of bud formation in either the haploid or the diploid state. When haploid cells are starved of nutrients they will enter stationary phase, unless they are in the presence of cells of the opposite mating type. If the opposite mating types **a** and α meet and are starved, they will conjugate to form diploid cells. If nutrients are returned, the diploid cells will enter into diploid mitotic growth. If the diploid cells are also starved, the programme of meiosis and sporulation is entered into, resulting in the formation of four haploid spores, which can then enter the mitotic cell cycle when conditions become favourable again.

1.2.3 Schizosaccharomyces pombe

The fission yeast *S. pombe* is also a eukaryotic unicellular ascomycete fungus. It has rod-shaped cells of 7–14μm in length and 3–4μm in diameter, that grow by apical extension, and divide by septation followed by medial fission (Forsburg & Nurse, 1991; MacNeill & Nurse, 1997). Fission yeast normally exists in a haploid state and has a genome of similar size to budding yeast, at around 14 Mb, which has also been sequenced (Wood *et al.*, 2002). Unlike budding yeast however, its genome is divided between three chromosomes, of 5.7, 4.7 and 3.5 Mb, which become microscopically visible when condensed during mitosis or meiosis. Fission yeast was first developed as a genetically useful organism in the 1950s by Urs Leupold who isolated a homothallic strain called h^{90} from which he derived two heterothallic strains of opposite mating type h^+ and h^- (Leupold, 1993).

In the presence of nutrients, haploid fission yeast cells enter the mitotic cycle. When cells are starved of nutrients several developmental fates are open to them (Figure 1.2). If cells of only a single mating type are present, fission yeast will exit mitosis and accumulate in stationary phase. If both mating types are present, conjugation occurs, resulting in the formation of a transient diploid zygote. Unlike budding yeast the diploid state is not normally stable and the diploid zygote will usually undergo meiosis and sporulation to produce four haploid spores. However, if diploid cells are transferred into fresh nutrient-rich media before meiosis has been initiated, a diploid mitotic cycle will be entered into. Subsequent removal of nutrients, then initiates entry into meiosis and sporulation, without the need for intervening conjugation (Forsburg & Nurse, 1991; Hayles, 1992).

The cell cycle of fission yeast is very similar to that of higher eukaryotes as it has distinct G1, S, G2 and M phases. In rapidly growing cells G1 phase is very short, approximately 0.1 of a cell cycle. S phase is also rapid, again lasting for about 0.1 of a cell cycle. This results in G1–S cells appearing as a doublet, because by the time cell separation occurs, the cells have already entered G2 phase. G2 lasts for 0.7 of the cell cycle before the cells enter M phase which accounts for the remaining 0.1 of the cycle. The cell cycle will be discussed in more detail in section 1.3 (Forsburg & Nurse, 1991; MacNeill & Nurse, 1997).

Figure 1.2 The life cycle of fission yeast

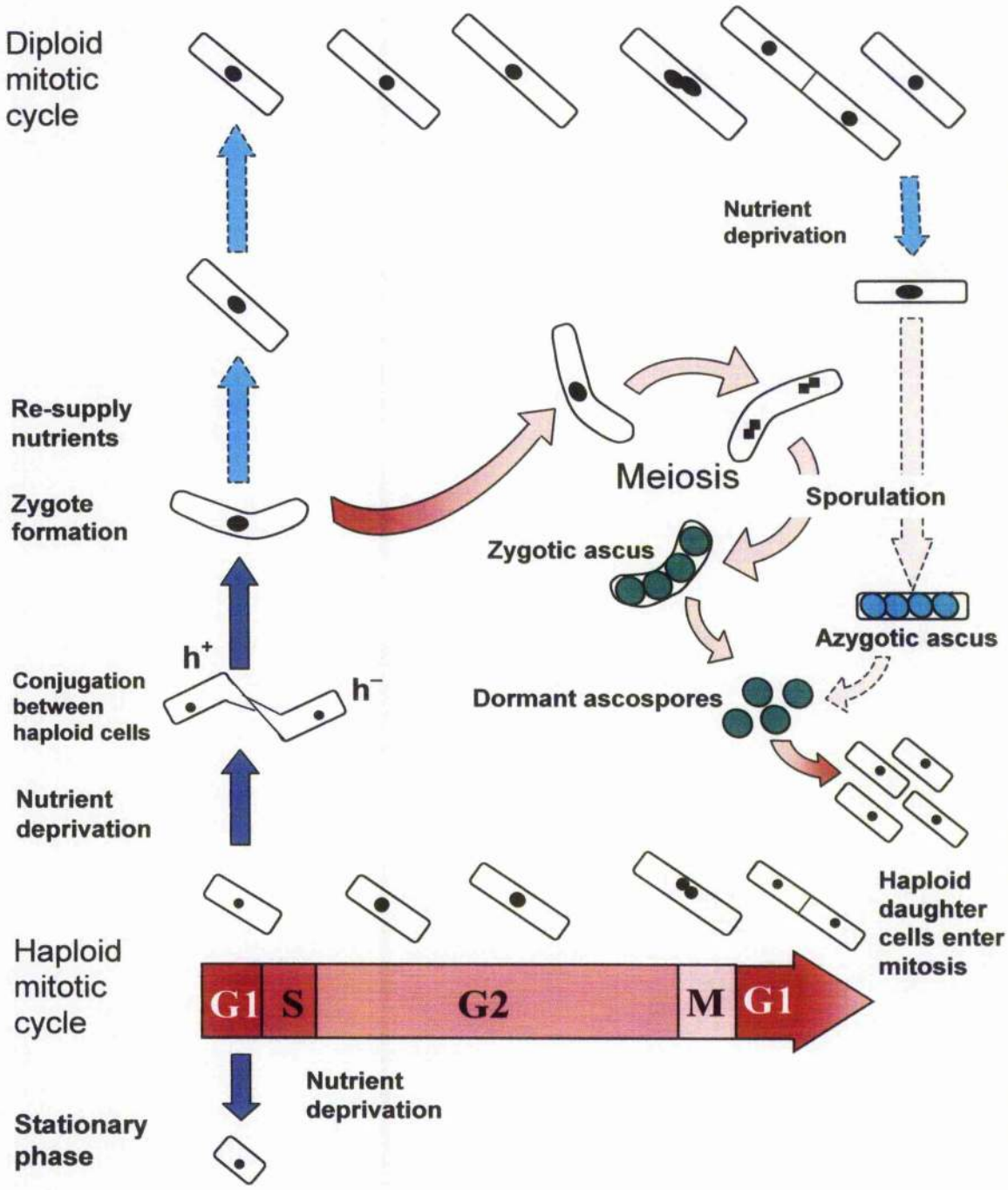


Figure 1.2 The life cycle of fission yeast

Fission yeast cells normally grow in the haploid state. When starved they will enter stationary phase unless cells of the opposite mating type are present. If h^+ and h^- cells meet and are starved, conjugation occurs and a zygote is formed. The diploid zygote is unstable and will normally proceed directly into the meiotic cell cycle. A zygotic ascus is formed that contains four haploid ascospores. The spores can exist in a dormant state until conditions are favourable again.

However, if nutrients are reintroduced to the diploid zygote directly after conjugation, fission yeast can be induced to undergo diploid mitotic growth. Starved diploid cells will then enter the meiotic cell cycle, in which an azygotic ascus is formed, and which results in the formation of four haploid spores as before.

1.2.4 Overview of advantageous experimental methods

Several features of the life cycles of these two yeast species have proven extremely useful in genetic analysis. As the generation times of both species are only a few hours long, large numbers of cells can be easily screened using standard microbial techniques, providing, for example, the ability to isolate conditional mutations, such as temperature sensitivity in essential genes. The fact that both budding yeast and fission yeast can be grown in either the haploid or diploid states enables the isolation of recessive mutants whose phenotype can be easily explored. Examination of meiotic products using tetrad dissection or random spore analysis, as well as complementation analysis using diploid cells have also been proven to be extremely powerful techniques. The development of plasmid vector systems and the fact that homologous recombination occurs readily in these cells provides us with molecular genetic tools for the manipulation of these organisms (Forsburg & Nurse, 1991).

1.2.4.1 Mitotic cell cycle studies

Both yeast species have been widely used in the study of the mitotic cell cycle, as it has been possible to identify several *cdc* (cell division cycle) mutants (e.g. Nurse *et al.*, 1976). These mutations block cell cycle progression without perturbing cellular growth i.e. the increase in the mass of a cell. This allowed their identification by visual screens, as they continue to grow in size when arrested at certain cell cycle points (Lew *et al.*, 1997; MacNeill & Nurse, 1997). In budding yeast, *CDC*$^-$ cells arrest with a uniform bud phenotype and, even more distinctly, in fission yeast, *cdc*$^-$ cells continue to elongate, becoming easily distinguishable from normal rod-shaped cells by their longer length (Nurse *et al.*, 1976).

The ability to synchronize a population of cells is also vital in cell cycle studies and is relatively easily achieved in either yeast. Temperature sensitive *cdc*$^-$ mutants may be used to arrest cells by shifting them to the restrictive temperature for long enough to block the entire population at the arrest point. Releasing them back to the permissive temperature then allows all the cells to recover and return to the cell cycle in synchrony. Similarly, chemical treatments such as hydroxyurea, which blocks DNA replication, or treatments with mating pheromones can also be used to induce synchrony. However it should be noted that the synchrony only lasts for a few generations and the possible physiological effects of blocking the system in such a way should be considered (Forsburg & Nurse, 1991). Perhaps a more practical way of synchronizing a population of cells, therefore, is to employ centrifugal elutriation. Centrifugal elutriation uses both elutriation and centrifugal force to separate cells on the basis of cell size. As the size of the cell is directly related to its position in the cell cycle, this method allows the extraction of a synchronous sub-population of cells from an asynchronous culture, avoiding the need for mutants or chemical treatments (Walker, 1999).

1.2.4.2 Meiotic cell cycle studies

With specific reference to this thesis, it should be noted that a particularly elegant method exists in fission yeast, which has greatly facilitated the study of the cell cycle during meiosis. A diploid fission yeast strain, heterologous for mating type, can be induced to enter meiosis by shifting the strain from growth medium to sporulation medium (Egel & Egel-Mitani, 1974); but such meioses are only poorly synchronous. However, the temperature sensitive *pat1-114* mutation can be used to induce a highly synchronous meiosis (Iino & Yamamoto, 1985; Nurse, 1985; Bähler *et al.*, 1991). The *pat1*$^{+}$ gene encodes a protein kinase, which plays an important role in the regulatory cascade that leads to the initiation of meiosis. Artificial inactivation of *pat1-114* by shifting the mutant to the restrictive temperature (32–36°C) leads to the activation of the *mei2*$^{+}$ gene, which then induces meiosis irrespective of mating type or nutritional starvation (Watanabe *et al.*, 1997). Starving the cells for nitrogen prior to inducing the *pat1-114* meiosis is also employed however, partly in order to increase synchronicity (nitrogen starved cells arrest at G1), and partly to replicate the natural conditions under which fission yeast cells would enter meiosis.

1.3.1 Overview of the mitotic cell cycle

The eukaryotic mitotic cell cycle is a complex and highly regulated series of cellular events, which ensures that the genome is duplicated faithfully and the chromosomes distributed evenly between the two daughter nuclei, before cytokinesis separates the two new daughter cells (Lewin, 1997b). The mitotic cell cycle of eukaryotes has been extensively studied in several different model systems, leading to the view that the basic principles of the cell cycle apply to all eukaryotic cells. These cellular events can be divided into four discrete phases:

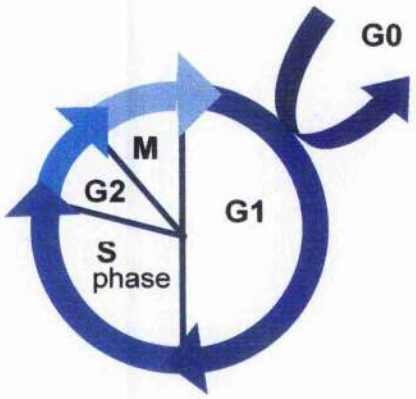


Figure 1.3: The phases of the eukaryotic mitotic cell cycle

The eukaryotic cell cycle can be described as passing through four discrete phases: an initial gap phase, G1; the DNA synthesis phase, S phase; a second gap phase, G2; and the mitosis 'M' phase. G0 represents stationary phase, when cells become starved of nutrients or growth factors and enter a state of quiescence.

The initial gap phase (G1 phase) is characterized by RNA and protein synthesis. During G1, the DNA is primed for replication, a process known as DNA licensing, in preparation for the DNA synthesis phase of the cycle (S phase). The first so-called control point of the mitotic cell cycle occurs towards the end of G1 phase and is known as START in both budding and fission yeast and as the restriction point (R) in higher eukaryotes. At this point, dependent on external conditions, the cell can either proceed through the mitotic cell cycle or enter one of various alternative phases of development.

S phase begins when DNA replication is initiated and is defined as lasting until DNA replication is completed. The second gap phase (G2 phase) then occurs, during which the cells continue to grow in size, and which is characterized by the cell having double its usual DNA content. At this stage of the mitotic cell cycle the second major control point is active, ensuring that the cell does not enter mitosis unless DNA replication is complete and the cell has reached a minimum size. These initial three phases, which precede the cell division phase (M phase), are collectively known as interphase.

Mitosis then follows during M phase and is the process used to distribute the chromosomes correctly between the two daughter cells:

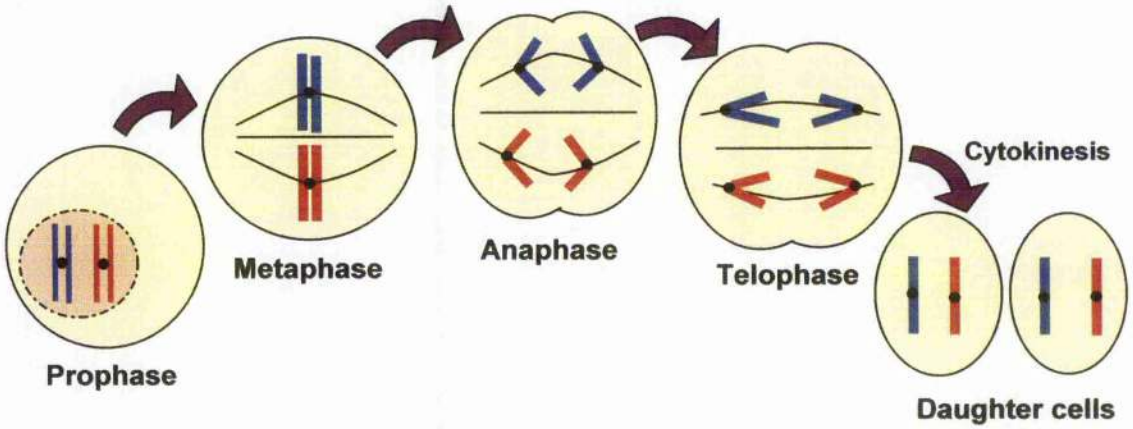


Figure 1.4: Mitosis

Chromosomes become visible as pairs of sister chromatids during prophase and align on the mitotic spindle during metaphase. During anaphase the sister chromatids begin to separate and they reach opposite ends of the cell during telophase. Cytokinesis then occurs producing two new identical daughter cells.

The mitotic M phase itself can be further divided into five stages. During the initial prophase the chromosomes begin to condense and become visible as long threadlike pairs of replicated sister chromatids which are joined by a centromere. During prometaphase the chromosomes become thicker and shorter and begin to migrate towards the equator of the cell as the mitotic spindle begins to form. Metaphase then follows and is characterized by the alignment of the chromosomes in the equatorial plane. During anaphase the centromeres divide and the sister chromosomes are separated and migrate towards opposite poles of the cell. Telophase is characterized by the completion of this chromosomal migration resulting in the equal distribution of the chromosomes between the daughter nuclei. Once mitosis is complete, cytokinesis then divides the cell to produce two new daughter cells (Lewin, 1997a).

1.3.2 The cell cycle in eukaryotes

Although the basic cell cycle can be applied to all eukaryotic systems, the details of regulation and relative amounts of time spent in the gap phases differ (Lewin, 1997b). As this thesis will be predominantly discussing budding yeast, fission yeast and higher eukaryotic systems, a brief comparison of the basic outlines of these three mitotic cell cycles will now be made.

Under optimal conditions, budding yeast will complete the mitotic cell cycle in approximately 90 minutes (Forsburg & Nurse, 1991). As in higher eukaryotic systems, budding yeast remains in G1 for a large proportion of the mitotic cell cycle. However, in budding yeast, certain cytological markers of mitosis either do not exist (e.g. nuclear envelope breakdown) or

are difficult to detect (e.g. chromosome condensation). So, the assembly of a mitotic spindle has been proposed to mark entry into M phase, but this occurs comparatively early in budding yeast. Thus, although it is possible to assign cell cycle phases to the budding yeast mitotic cell cycle, a clear comparison with the discrete phases of the higher eukaryotic cycle is precluded (Forsburg & Nurse, 1991).

The mitotic cell cycle of fission yeast however, closely follows the basic form of the mitotic cell cycle of higher eukaryotes in that it has similarly distinct G1, S, G2 and M phases (Forsburg & Nurse, 1991; MacNeill & Nurse, 1997). Although again, the relative amount of time spent in different phases of the mitotic cell cycle differs between the two systems. Fission yeast cells take approximately three hours to divide when grown under optimal conditions. As discussed in Section *1.2.3*. G1, S, G2 and M phases occupy 0.1, 0.1, 0.7 and 0.1 of the cell cycle, respectively (Forsburg & Nurse, 1991; McNeil & Nurse, 1997).

In higher eukaryotes, actively cycling somatic cells will complete a cell cycle every 18–24 hours (Lewin, 1997b). G1 phase usually occupies the bulk of interphase and also varies depending on cell type, ranging from 6–12 hours. As in budding yeast, once G1 has been completed, cultured cells of higher eukaryotes will go through S phase, which takes about 6–8 hours, and do not usually rest in G2, which in most cases represents the shortest part of interphase. (Exceptions to this include certain cell types that may have to divide again e.g. cells at certain stages of insect embryogenesis rest in a tetraploid G2 phase). Mitosis then occurs, lasting less than 1 hour (Lewin, 1997b).

1.3.3 Events of the eukaryotic mitotic cell cycle

1.3.3.1 Cell cycle control points: START/ The restriction point (R)

In fission yeast, if nutrients are plentiful, the cell will proceed through the first control point known as START. If nutrients are limited however, the cell then faces two alternative fates: it may either enter stationary phase, becoming metabolically dormant awaiting an improvement in nutrient supply; or if cells of the opposite mating type are present, it may conjugate and go through meiosis to produce haploid spores. Essentially similar developmental fates are available to budding yeast (Bartlett & Nurse, 1991; Forsburg & Nurse, 1991; Nurse, 1998).

Before passing the restriction point (R), higher eukaryotic cells may enter a state known as G0, which is often thought of as being homologous to budding yeast/fission yeast stationary phase. However, this occurs when the cell is starved of growth factors rather than nutrients and the cell remains metabolically active, though at a reduced rate. Many cells in a multicellular organism, such as striated muscle cells and nerve cells become terminally differentiated and remain in G0. Others, such as fibroblasts, are able to exit G0 and re-enter the cell cycle, but usually remain quiescent (Lewin, 1997b).

In both budding yeast and higher eukaryotes START/R is the major cell cycle control point, with the duration of G1 phase being altered depending on the prevailing conditions. In fission yeast, although a pre-START G1 size-control point does exist, it is normally cryptic; as rapidly growing fission yeast cells have attained the minimum size required for passage through START even before mitosis has been completed. The G1 size control point of fission yeast only becomes apparent when G2 phase is shortened (MacNeill & Nurse, 1997). This results in a lengthened G1 phase in which fission yeast cells grow to a minimum size before traversing START. This fission yeast control point was identified in so called 'wee' cells as a result of inactivation of Wee1p, a regulatory kinase (MacNeill & Nurse, 1997). This shortens G2 phase, allowing mitosis and cytokinesis to occur in cells of an inappropriately small size. The subsequent lengthening of G1 phase causes the overall length of the mitotic cell cycle to remain the same, although *wee* cells are unusually small as they enter M phase at half the usual length (7μm).

1.3.3.2 Cell cycle control points: G2-M control

Controlling the transition from the second growth phase to M phase is vital for correct progression of the mitotic cell cycle. Dividing at a reduced cell size could be deleterious for the survival of daughter cells. Similarly, if DNA replication were not completed before mitosis was initiated, there would be disastrous consequences for the cell, as correct segregation of the nuclear material between daughter cells could not occur. Regulatory checkpoints concerning DNA integrity and cell size are therefore active in late G2 phase to prevent the cell from initiating mitosis before it is properly prepared (MacNeill & Nurse, 1997; Humphrey, 2000). This control point plays a more prominent role in the fission yeast cell cycle however, than it does in either budding yeast or higher eukaryotes. Indeed it is the length of fission yeast G2 phase that is typically altered in response to nutritional conditions, as opposed to G1 phase in budding yeast and higher eukaryotes. This situation is advantageous to a haploid organism as it means that the majority of the cell cycle is spent in possession of two full copies of the genome, which may facilitate protection against DNA damage (Forsburg & Nurse, 1991; Humphrey, 2000).

1.3.4 The molecular mechanisms of mitotic cell cycle progression: Interphase

Many experimental systems have been important in dissecting the molecular basis of the control of the eukaryotic cell cycle and a great deal is now known about the mechanisms that drive cells through their phases of growth. However, for the sake of brevity it would be inappropriate to discuss the mitotic cell cycles of alternative systems in great detail in this thesis. In this section therefore, the mitotic cell cycle, as it is currently understood to function in fission yeast, will be discussed with brief reference to the mechanisms operating in other experimental systems.

It is known that coordination of events during the mitotic cell cycle, such that these events occur in the proper order with respect to each other, is vital in ensuring successful completion of that cycle. Studies have revealed two different controlling mechanisms by which this complex regulation is achieved (Moser & Russell, 2000). First, a set of serine/threonine protein kinases or CDKs (cyclin dependant kinases) varies cyclically in its activity, depending on various covalent modifications and associations with different regulatory proteins. These CDKs phosphorylate various substrate proteins in a sequential manner, as they become active during the cell cycle. This sets up the progression of a series of cell cycle events (Moser & Russell, 2000; Morgan, 1995).

Secondly, several regulatory checkpoint controls are imposed on this cycle (Moser & Russell, 2000). Using their ability to delay the cell cycle at particular points, these checkpoints function to control and link the various events of the cell cycle such that they occur in the proper order. Ensuring, for example, that DNA is fully replicated and the cells are of a satisfactory size before entry into mitosis, or that assembly of the mitotic spindle is complete before mitosis is initiated (MacNeill & Nurse, 1997; Humphrey, 2000). In the following sections, the current knowledge of these molecular events and controls will be discussed in the order that they occur during the mitotic cell cycle, after a brief introduction to CDKs.

1.3.4.1 Cyclin dependant kinases (CDKs)

Cyclin dependant kinases (CDKs) have been identified as the most significant molecules involved in driving the progression of the cell cycle (Morgan, 1995). The level of CDKs within the cell remains constant throughout the cell cycle but the kinase activity of CDKs is tightly regulated and oscillates over the course of the cell cycle.

CDKs range from 30–40 kDa in size and are all closely related in sequence (>40% identity). Cdc2p (Nurse & Bissett, 1981; Nurse *et al.*, 1976) is the single major CDK involved in cell cycle regulation in fission yeast, the budding yeast counterpart being ScCdc28p (Nasmyth, 1993; Reed, 1992). In human cells, the first CDK HsCdc2p/HsCdk1p was identified on the basis of its functional homology to the fission yeast Cdc2p (Lee & Nurse, 1987) although it is clear that several CDKs are present, and involved in cell cycle regulation at various stages, in this organism (Morgan, 1995).

CDKs are catalytically inactive when they are present as a monomer and must associate with a cyclin to become active (Morgan, 1995). All cyclins share a region of homology known as the 'cyclin box' which is responsible for both for CDK association and activation (Kobayashi *et al.*, 1992; Lees & Harlow, 1993). Fission yeast Cdc2p associates with at least four different cyclins: Cdc13p, Cig1p, Cig2p and Puc1p (Booher *et al.*, 1989; Fisher & Nurse, 1995; Forsburg & Nurse, 1991; Martin-Castellanos *et al.*, 1996; Mondesert *et al.*, 1996; Moreno *et al.*, 1989; Obara-Ishihara & Okayama, 1994; Stern & Nurse, 1996). Similarly budding yeast ScCdc28p

associates with several ScClnp and ScClbp cyclins (Low *et al.*, 1997). However, in higher eukaryotes, several CDKs are required throughout the cell cycle and they each associate with various cyclins. For example, the D cyclins (D1, D2 and D3) are synthesized when cells re-enter the cell cycle from G0 and their interaction with HsCdc2, HsCdc4 and HsCdc6 is required during G1. HsCdc2 must then interact with Cyclin E to cause entry into S phase. HsCdc2 then associates with Cyclin A to cause progression through S phase and this complex is required again to promote entry into mitosis. The HsCdk2–Cyclin-B complex is required in mitosis as well but is also important for the G2–M transition (Lewin, 1997b; Morgan, 1995).

1.3.4.2 The molecular events of the G1–S transition

Passage through START in late G1 is a vital control point representing a point of commitment to the mitotic cell cycle (Section *1.3.3.1*). Two different mechanisms are important for controlling the passage of START. First, cell-cycle-regulated periodic transcription has been shown to play a vital role at this point in both fission and budding yeast cell cycles. In fission yeast, a transcription factor complex, MBF (MCB binding factor, also known as DSC1) has been shown to control the periodic transcription of several genes vital for cell cycle progression (Lowndes *et al.*, 1992). These genes contain common UASs (upstream activating sequences) called MCBs (*Mlu1* cell cycle boxes) in their promoters and their transcription is coordinately initiated in late G1 (McIntosh, 1993). However as this particular topic is central to this thesis, the mechanisms and control of cell-cycle-regulated transcription will be given detailed consideration later in the text (Section *1.5.4*).

Second, activity of the CDK Cdc2p is required for passage through START (Nurse & Bissett, 1981). In early G1 phase Cdc2p is predominantly associated with Cdc13p; the cyclin required for the previous M phase (Booher *et al.*, 1989; Moreno *et al.*, 1989). The Cdc2p inhibitor Rum1p accumulates in mitotic anaphase and is present throughout G1 phase, keeping Cdc2p activity low throughout G1 phase (Benito *et al.*, 1998). Rum1p acts to inhibit the Cdc2p–Cdc13p kinase activity as well as targeting Cdc13p for ubiquitin-mediated proteolysis (Correa-Bordes *et al.*, 1997).

The anaphase-promoting complex (APC) is the cell-cycle-regulated ubiquitin ligase which is responsible for mediating the proteolysis of mitotic cyclins (Amon *et al.*, 1994). During early G1, a molecule called Ste9p associates with the APC (Moser & Russell, 2000). Fission yeast Ste9p/Srw1p is a WD-repeat protein and a homologue of budding yeast ScHct1p/ScCdhp and the Fizzy-related protein of higher eukaryotes (Kitamura *et al.*, 1998; Blanco *et al.*, 2000). During the period of low Cdc2 kinase activity in G1, Ste9p is dephosphorylated and acts as an activator of the APC, specifically promoting the ubiquitination and proteolysis of Cdc13p and Cig1p (Kitamura *et al.*, 1998; Yamaguchi *et al.*, 2000). Ste9p therefore helps to further regulate the activity of the CDK during G1. Ste9p is not required for

the degradation of Cdc13p and Cig1p under normal circumstances; however, it is absolutely required for the ability of cells to undergo a G1 arrest, for example in nitrogen starved conditions (Blanco *et al.*, 2000).

During G1, the regulator of periodic transcription, MBF, induces *cig2*$^{+}$ transcription, allowing accumulation of Cig2p, the S-phase cyclin (Conolly & Beach, 1994; Obara-Ishihara & Okayama, 1994). Cig2p associates with Cdc2p but again the presence of Rum1p inhibits the Cdc2p–Cig2p kinase activity (Benito *et al.*, 1998). At this point however, Cdc2p is also associated with two other cyclins, Puc1p and Cig1p (Martin-Castellanos *et al.*, 1996; Moser & Russell, 2000). Both Cdc2p–Puc1p and Cdc2p–Cig1p are insensitive to Rum1p inhibition and begin to actively phosphorylate Rum1p. Phosphorylated Rum1p is targeted for degradation allowing the Cdc2p–Cig2p kinase activity to rise and eventually induce entry into S phase (Benito *et al.*, 1998).

1.3.4.3 Assembly of the pre-replication complex (pre-RC)

1.3.4.3.1 Origins of replication

Although there is a certain amount of divergence in the DNA sequences which mark sites of replication initiation in eukaryotes, the molecular mechanisms and protein factors involved appear to be highly conserved across all systems that have been studied (Lei & Tye, 2001). Budding yeast origins of replication are relatively simple and are known as autonomously replicating sequences (ARSs), due to their ability to promote autonomous replication of plasmids. ARSs consist of 100–200 bp stretches of DNA containing an 11 bp consensus sequence, the ACS (ARS consensus sequence), which is essential for their function (Bell & Stillman, 1992; Diffley & Cocker, 1992; Marahrens & Stillman, 1992). Fission yeast origins also promote autonomous replication of plasmids but consist of much longer chromosomal regions of over 1 kb in length, with multiple, asymmetrically spaced, stretches of adenine/thymine residues being vital for function, rather than specific consensus sequences (Okuno *et al.*, 1999; Takahashi *et al.*, 2003). Similarly, studies in higher eukaryotes (Aladjem *et al.*, 1998; Delidakis & Kafatos, 1989) have implied that, as is the case in fission yeast, long regions of DNA containing discrete stretches of essential sequences are important in these organisms.

1.3.4.3.2 The origin recognition complex

In fission yeast, a hexameric structure known as ORC (origin recognition complex) is constitutively bound to the ARSs throughout the cell cycle. ORC consists of Orc1p to Orc6p, encoded by *orp1*$^{+}$ to *orp6*$^{+}$ respectively (Lygerou & Nurse, 1999; Moon *et al.*, 1999). Analogous hexameric complexes exist in both budding yeast and higher eukaryotes (Austin *et al.*, 1999; Bell & Stillman, 1992). Studies in budding yeast have shown that ORC exists in two

conformations: an extended conformation bound to pre-replicative dsDNA and a bent conformation bound to post-replicative ssDNA (Lee *et al.*, 2000).

1.3.4.3.3 Cdc23p/Mcm10p

The fission yeast Cdc23 protein is a homologue of Mcm10p found in budding yeast and humans (Aves *et al.*, 1998; Izumi *et al.*, 2000; Merchant *et al.*, 1997). Several studies have implied that Cdc23p/Mcm10p is also bound to DNA at the origin of replication and acts as an anchor for the inactive form of the MCM (mini chromosome maintnance) complex, via interaction with MCM subunits (Merchant *et al.*, 1997; Kawasaki *et al.*, 2000; Homesley *et al.*, 2000; Forsburg *et al.*, 2001; Lei & Tye, 2001). So, formation of the pre-RC is thought to begin with ORC and Cdc23p/Mcm10p complexes bound to ARS sequences at the origins of DNA replication (see Figure 1.5).

1.3.4.3.4 Timing of DNA replication

Pre-RC formation begins with recruitment of the initiation factors Cdc18p, which is homologous to budding yeast and human Cdc6p (Cocker *et al.*, 1996; Kearsey *et al.*, 2000; Kelly *et al.*, 1993; Nishitani & Nurse, 1995; Saha *et al.*, 1998; Williams *et al.*, 1997; Zhou *et al.*, 1989) and Cdt1p, which has homologues in *Drosophila* (DUP), *Xenopus* and humans (Hofman & Beach, 1994; Maiorano *et al.*, 2000; Nishitani *et al.*, 2000; Whittaker *et al.*, 2000; Wohlschlagel *et al.*, 2000).

Cdc18p is transcribed in an MBF-dependent manner and appears during mitosis in metaphase. However, Cdc18p levels are kept low due to Cdc2p–Cdc13p dependent phosphorylation of Cdc18p and its subsequent degradation (Baum *et al.*, 1998; Jallepalli *et al.*, 1997; Lopez-Giorna *et al.*, 1998). The mitotic cyclin Cdc13p begins to be degraded during mid-anaphase (Booher *et al.*, 1989; Moreno *et al.*, 1989) which is thought to allow accumulation of Cdc18p during late anaphase. A study by Kearsey *et al* (2000) suggests that this accumulation of Cdc18p may allow the MCM complex to associate with the replication origins during late mitosis, even before entry into G1. This situation may facilitate the rapid passage of fission yeast cells through G1 and S phases (Kearsey *et al.*, 2000). In any case, the MBF-dependent transcription of both *cdc18*$^{+}$ and *cdt1*$^{+}$ allows both proteins to accumulate over the course of late M-phase/G1; and results in Cdc18p and Cdt1p associating with ORC and acting synergistically to recruit the MCM complex (Kearsey *et al.*, 2000; Nishitani *et al.*, 2000). In both budding yeast and higher eukaryotes pre-RC formation is thought to occur over the course of G1, and in higher eukaryotes, also requires APC-mediated removal of negative regulators such as geminin, which interacts tightly with Cdt1p (Lei & Tye, 2001; Wohlschlagel *et al.*, 2000). Several mechanisms are therefore in place to help prevent the formation of pre-RC complexes and origin firing at

inappropriate cell cycle times, from transcriptional control of vital proteins, to their regulation by CDK phosphorylation and interaction with negative regulators.

1.3.4.4 The MCM complex

The MCM complex is a hexamer, formed from six different MCM proteins Mcm2p to Mcm7p (Tye & Sawyer, 2000), which are recruited to the origin in a Cdc18p/Cdt1p dependent manner (Kearsey *et al.*, 2000; Nishitani *et al.*, 2000). All six subunits are highly conserved in eukaryotic systems and are essential for DNA replication (Kearsey & Labib, 1998; Tye & Sawyer, 2000). Various studies have suggested that this complex acts as a DNA helicase, unwinding DNA at the replication fork to allow access of DNA polymerases to the unwound strands (Labib & Diffley, 2001). It is thought that whilst ORC/Cdc18p/Cdt1p are required for the recruitment of the MCM complex, it is the Cdc23p complex that facilitates subsequent anchoring of the MCM complex to the origin (Hart *et al.*, 2002; Homesley *et al.*, 2000). However, once MCM has been recruited to the origin the DNA is licensed and ready to initiate replication (see Figure 1.5).

1.3.4.5 S phase: Initiation of DNA replication

S phase begins with the initiation of DNA replication at multiple origins throughout the genome. Activation of the pre-RC, which causes the initiation of DNA synthesis, requires the action of two separate kinases: an S phase CDK and the Hsk1p kinase that relies upon binding to Dfp1p for its activity (Lei & Tye, 2001; Moser & Russell, 2000). First, at the end of G1 phase the S phase CDK Cdc2p–Cig2p becomes active (due to degradation of the Rum1p inhibitor) and phosphorylates Cdc18p (Jallepalli *et al.*, 1997; Lopez-Girona *et al.*, 1998), which results in Cdc18p dissociating from the pre-RC and being degraded. This degradation of Cdc18p, coupled with the decline in *cdc18*$^{+}$ transcription during S phase, results in there being only a small window of opportunity in which Cdc18p is able to initiate pre-RC formation. This helps to prevent re-initiation of DNA synthesis until the following M phase has passed. Once cells have passed START Cdc2p becomes associated with the Cdc13p cyclin, which eventually is responsible for promoting entry into the mitotic division phase. However, activity of Cdc2p–Cdc13p is kept low during S phase by Wee1p/Mik1p phosphorylation of Cdc2p–Cdc13p on Tyr-15 (Moser & Russell, 2000).

The second kinase, Hsk1p, is essential for DNA replication and is present at constant levels throughout the mitotic cell cycle (Brown & Kelly, 1998; Masai *et al.*, 1995). The regulatory subunit of Hsk1p is Dfp1p/Him1p, the protein levels of which peak, periodically, at the G1–S transition (Brown & Kelly, 1999; Takeda *et al.*, 1999). Hsk1p binding to Dfp1p results in the formation of an active kinase complex that is recruited to the pre-RC at the end of G1 (Brown & Kelly, 1998; Brown & Kelly, 1999; Takeda *et al.*, 1999). Functionally

homologous complexes exist in budding yeast (ScCdc7p–ScDbf4p; Sclafani, 2000) and humans (HsCdc7p–HsAskp; Jiang *et al.*, 1999; Masai *et al.*, 2000)

Hsk1p–Dfp1p then phosphorylates the MCM complex on the MCM2p subunit at the G1–S transition resulting in a conformational change in the MCM complex (Diffley & Labib, 2002; Nishitani & Lygerou, 2002). The MCM complex then becomes an active helicase capable of melting the dsDNA (Labib & Diffley; 2001). The Sld3p-dependant association of Sna41p then acts to disengage the MCM complex from the Cdc23p anchor, initiating the melting of the DNA (Miyake, 1998; Nakajima & Masukata, 2002). Sna41p is homologous to Hs/ScCdc45p (Uchiyama *et al.*, 2001).

Figure 1.5: Control of DNA licensing in the fission yeast cell cycle

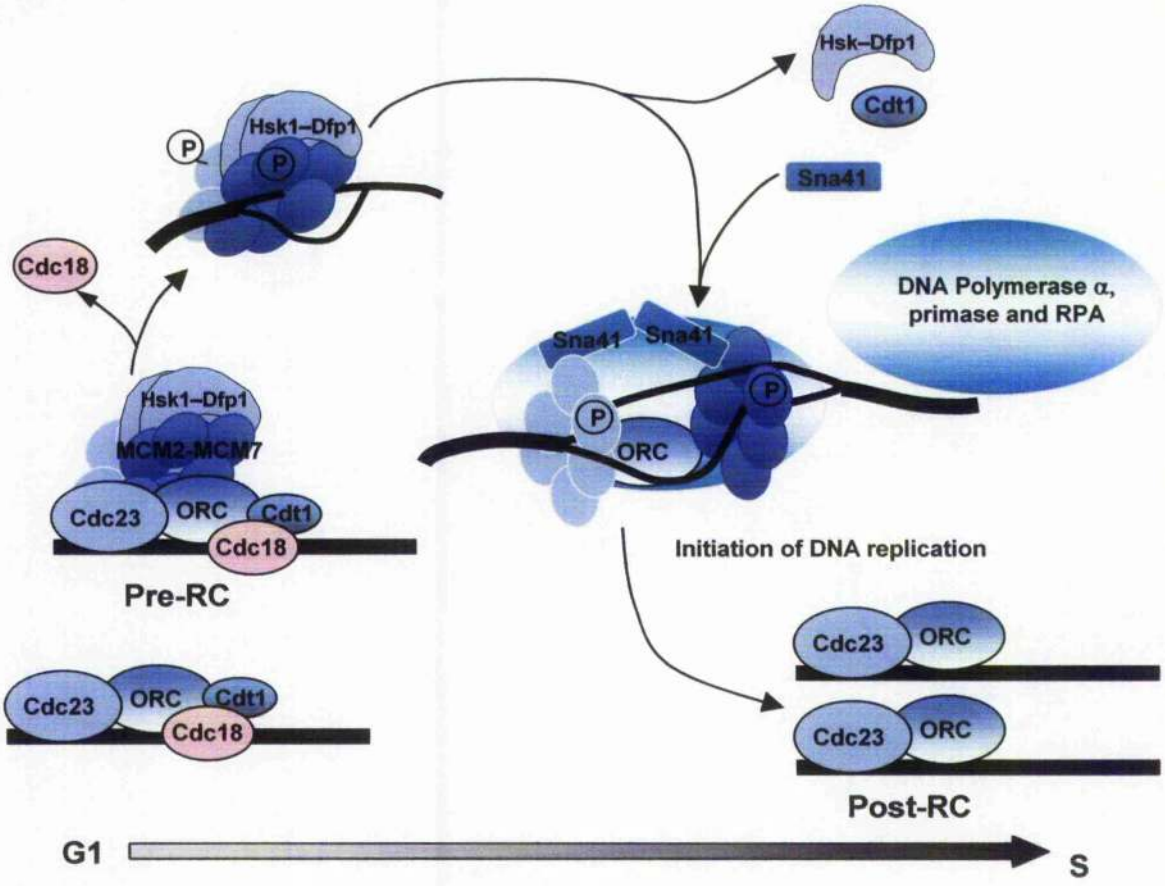


Figure 1.5: Control of DNA licensing in the fission yeast cell cycle
The ORC complex, consisting of Orc1p to Orc6p is associated with the multi-subunit Cdc23p/Mcm10p at replication origins on DNA. Cdc18p and Cdt1p also associate with chromatin at the origins as cells complete mitosis, where they function to load the MCM complex onto the DNA. Once the MCM complex, consisting of Mcm2p to Mcm7p, is loaded onto the origins the pre-replicative complex (pre-RC) is formed. Once the pre-RC is formed Cdc18p is removed from the complex and Hsk1p–Dfp1p is recruited. Hsk1p–Dfp1p phosphorylates the MCM complex, which results in the activation of its helicase activity. Hsk1p–Dfp1p and Cdt1p dissociate from the complex and Sna41p is then recruited. Sna41p recruitment results in dissociation of the MCM complex from the origins and recruitment of DNA polymerase α, primase and RPA. This figure was adapted from figure 1 of Lei & Tye (2001).

1.3.4.6 S phase: DNA synthesis

Sna41p association with the pre-RC at the point of DNA melting also recruits the DNA synthesis machinery to the initiation site (Uchiyama *et al.*, 2001) and at this point the RPA complex is also thought to have a role (MacNeill & Nurse, 1997). DNA polymerase α (DNApolα) then acts in concert with a DNA primase complex to produce short tracts of ribonucleotides which act as primers for DNA synthesis (MacNeill & Nurse, 1997). DNA synthesis from replication origins typically occurs bidirectionally, but DNA polymerases can only synthesize DNA in the 5'→3' direction — so while there is one 'leading strand' that is synthesized in the 5'→3' direction, the other 'lagging strand' is synthesized in short fragments (Okazaki fragments) as the DNA becomes progressively unwound. In fission yeast, these discrete fragments are 'filled in' by the action of Dna2p and, by analogy to other eukaryotes, Rad2p (Kang *et al.*, 2000). The coordination of lagging-strand synthesis and DNA unwinding is crucial and is thought to involve DNApolα interactions with both Sna41p and Mcm2p to Mcm7p. DNApolα, however, does not have a 3'→5' exonuclease (proofreading) activity so once replication is initiated other DNA polymerases take over to elongate the newly synthesized strands. DNA polymerase δ (DNApolδ) and DNA polymerase ε are thought to be necessary for this (Diffley & Labib, 2002; Dahlen *et al.*, 2003).

In order for DNApolδ to progress along the DNA the processivity factor Pcn1p (also known as PCNA or the 'sliding clamp'; Tallada *et al.*, 2002) is required — the loading of the Pcn1p–DNAPolδ complex on to the DNA requires the RFC (also known as the 'clamp loader'). RFC is a complex of five related proteins, Rtf1p to Rtf5p, which uses ATP to load Pcn1p on to the DNA (Shimada *et al.*, 1999; MacNeill & Nurse, 1997). As the DNA is unwound its topology changes and positive supercoils are formed in front of the replication fork, these are relaxed by the action of topoisomerases Top1p and Top2p. Termination of replication then occurs when two replication forks meet (Diffley & Labib, 2002).

Before progression into G2 phase the replicated DNA is assembled into chromatin. This requires the S-phase-specific expression of the four core histones H2A, H2B, H3 and H4 and the action of various chromatin assembly factors (Diffley & Labib, 2002). Cohesin complexes, which mediate the association of the replicated sister chromatids, are also loaded on to the DNA during S phase. The budding yeast cohesins ScSmc1p and ScSmc3p are thought to form a dimer which is joined by a hinge region at one end and then interacts with the cohesin ScScc1p to encircle the two sister chromatid arms (Haering *et al.*, 2002). The fission yeast cohesins Psm1p, Psm3p and Rad21p are, respectively, homologues of the budding yeast ScSmc1p, ScSmc3p and ScScc1p cohesins (Hagstrom & Meyer, 2003).

1.3.4.7 G2–M transition

The onset of the mitotic division phase from G2 is controlled by the activity of Cdc2p–Cdc13p, which is held in balance by the phosphorylation state of the regulatory Tyr-5 residue (MacNeill & Nurse, 1997). As the cells progress through G2 phase the level of Mik1p decreases and Wee1p becomes mainly responsible for preventing entry into mitosis through Cdc2p–Cdc13p phosphorylation (Moser & Russell, 2000). Wee1p is inhibited at the transition into mitosis by the kinases Cdr1p, Cdr2p and the Slm9p protein, and, Cdc2p–Cdc13p is gradually activated by the dephosphorylation of Tyr-15, which is brought about by the protein tyrosine phosphatase Cdc25p (MacNeill & Nurse, 1997). The abundance of Cdc25p is under the control of the translation factor Tif1p, which seems to be required for the increase in abundance of Cdc25p over the course of the cell cycle (Daga & Jiminez, 1999). A peak of Cdc25p abundance corresponds to an increase in Cdc2p–Cdc13p activity and the onset of M phase (Moser & Russell, 2000).

Two major checkpoint pathways also govern entry into M phase: the DNA damage checkpoint, the main effector of which is Chk1p; and the replication checkpoint, the main effector of which is Cds1p. Both checkpoints seem to act by upregulating Mik1p, as well as downregulating Cdc25p (Rhind & Russell, 2000). Chk1p becomes phosphorylated after DNA damage in a Rad3p-dependent manner. This causes Chk1p to bind and phosphorylate Cdc25p therefore reducing Cdc25p activity as well as reducing its nuclear localization (Russell, 1998; Rhind & Russell, 2000). Chk1p also seems to cause both upregulation of the activity of Mik1p and, in a prolonged cell cycle arrest, an increase in the abundance of Mik1p, which seems to be required to maintain cell cycle arrest (Rhind & Russell, 2001). Similarly, Mik1p accumulates in a Cds1p-dependent manner in response to activation of the DNA replication checkpoint, and Cdc25p activity is downregulated (Rhind & Russell, 2000; McGowan, 2002)

1.3.5 Mitosis

In fission yeast, mitosis is initiated by the Cdc25p-mediated activation of Cdc2p–Cdc13p, which phosphorylates numerous substrates within the nucleus that are involved in the progression of mitosis. This triggers the two important events of prophase: nuclear envelope breakdown and chromosome condensation (Figure 1.4; Lewin, 1997a).

The condensation of chromosomes requires the five-subunit complex, condensin, which, in fission yeast, consists of Cut14p, Cut3p, Cnd1p, Cnd2p and Cnd3p (Hagstrom & Meyer, 2003). The action of condensin allows chromosomes to undergo the morphological change from an amorphous mass at the beginning of prophase to a tightly packed two-armed, rod-shaped structure by entry into metaphase (Hagstrom & Meyer, 2003).

The kinetochores of each sister chromatid must then attach to microtubules of opposite orientations (known as amphitelic attachment) to allow their eventual separation to opposite

poles of the cell (Petronczki *et al.*, 2003). Aurora kinase, which in fission yeast is encoded by *Ark1*$^+$ and in budding yeast is encoded by *IPL1*$^+$, is essential for this interaction between the cytoskeleton and the chromosomes (Leverson *et al.*, 2002). Aurora kinases, of which mammalian systems have three, seem to mediate the correct attachment of kinetochores to the mitotic spindle by the dissolution of incorrect attachments. The attachment of sister chromatids to the microtubules produces tension in the mitotic spindle as the chromatids are pulled in opposite directions by their kinetochores, while the cohesion between the sister chromatids resists this pull. This results in the alignment of the pairs of chromosomes at the equator of the cell on the 'metaphase plate' (Petronczki *et al.*, 2003).

The separation of sister chromatids then occurs during anaphase (Figure 1.4). Anaphase onset is induced by the phosphorylation, and activation, of the APC/C by Cdc2p–Cdc13p and perhaps other mitotic kinases (Nigg, 2001). The activated APC/C, in combination with the Slp1p regulatory subunit, then ubiquitylates the anaphase inhibitor Cut2p (securin), this liberates Cut1p (separin) which cleaves the Rad21p cohesin subunit, therefore releasing sister-chromatid cohesion (Yanagida, 2000). The APC/C is responsible for the degradation of numerous mitotic cyclins and kinases, which occurs in an exquisitely regulated progression over the course of the mitotic division phase (Nigg, 2000). Towards the end of mitosis in fission yeast the APC/C becomes associated with a second activator subunit Ste9p, which maintains the activity of the APC through G1 phase where it is responsible for the degradation of mitotic cyclins Cdc13p and Cig1p (Blanco *et al.*, 2000; Tournier & Millar, 2000).

In budding yeast and fission yeast progression through the final stages of mitosis is regulated by a conserved signalling cascade, known as the mitotic exit network (MEN) in budding yeast and the septation initiation network (SIN) in fission yeast (McCollum & Gould, 2001; Bardin & Amon, 2001). In budding yeast, the MEN controls mitotic CDK inactivation through a signalling cascade that includes the phosphatase ScCdc14p. In fission yeast, the SIN regulates the formation of the septum and cell division in a remarkably similar signalling cascade after CDK inactivation (Bardin & Amon, 2001).

1.4.1 Overview of the meiotic cell cycle

The specialized meiotic cell cycle is the means by which diploid eukaryotic organisms ensure the maintenance of genetic diversity by exchanging genetic material between parental chromosomes (Shaw & Moore, 1998). For this to occur, haploid gametes must be formed and so meiosis consists of a single round of DNA replication followed by two consecutive nuclear divisions, which halves the chromosome complement of the resulting gametes (Petronczki *et al.*, 2003; Villeneuve & Hillers, 2001). The DNA is replicated during pre-meiotic S phase to produce two identical sister chromatids for each chromosome. At prophase of Meiosis I, homologous pairs of chromatids align on the equator of the cell and undergo the process of recombination, in which connections (chiasmata) form between the homologous chromatid arms. Chiasmata formation results in the exchange of portions of the genome between the two parental chromosomes to produce new chromosomes containing an entirely novel combination of genetic information. At the transition from metaphase I to anaphase I homologous pairs of chromatids are separated during a process of reductional division which halves the genetic content of the resulting daughter cells. A second round of meiotic division then occurs immediately (Meiosis II, which resembles a normal mitotic division) in which sister chromatids are separated and four new, genetically non-identical, cells are formed (Roeder, 1997; Lee & Amon, 2001b).

The study of meiosis is important because it is a basic developmental pathway and the switch between the mitotic and meiotic cell cycles represents one of the most fundamental developmental progressions seen in even the most basic eukaryotes. Furthermore, disruptions to the meiotic process are the cause of several clinically significant disorders in humans (Hassold & Hunt, 2001; Petronis, 1999; Sluder & McCollum, 2000). Approximately 10–30% of all conceptions have chromosomal abnormalities caused by mistakes made during meiosis (Hassold & Hunt, 2001; Sluder & McCollum, 2000). The most severe defects, such as autosomal monosomy, result in spontaneous abortion but several devastating medical conditions are the result of the mis-regulation of meiosis. These range from fertility problems to conditions such as Down syndrome (trisomy of chromosome 21), Patau syndrome (trisomy of chromosome 13) and Edwards syndrome (trisomy of chromosome 18), which result in severe physical and mental abnormalities (Hassold & Hunt, 2001; Petronis, 1999; Sluder & McCollum, 2000).

The basic meiotic pathway is highly conserved among eukaryotes and has been extensively studied in several organisms (Hassold & Hunt, 2001; Lee & Amon, 2001). The budding and fission yeast systems, however, provide the most clearly understood models for the progression of this specialized cell cycle (Lee & Amon, 2001) and so will be predominantly discussed here.

Figure 1.6 Meiosis

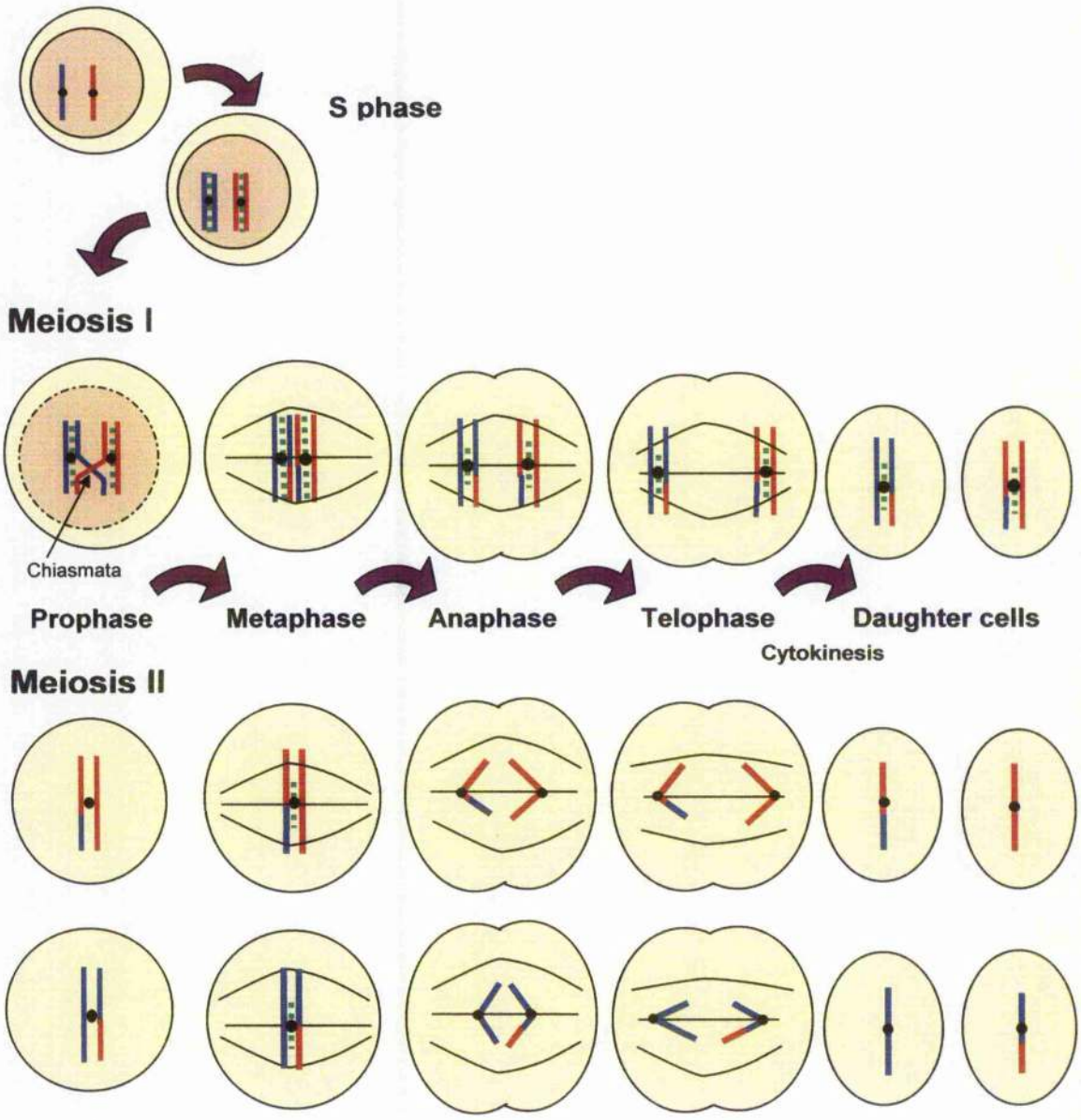


Figure 1.6 Meiosis

During pre-meiotic S phase chromosomes (blue, paternal; red, maternal) are replicated and sister chromatids are fixed together by cohesin complexes (green dots), which are laid down along the whole length of the chromosomes. Homologous parental chromosomes pair up and recombination occurs between chromosome arms of the two homologues, producing chiasmata, which also helps hold the sister chromatids together. Release of cohesin from the distal arms of the chromosomes triggers Meiosis I. Sister chromatids are held together by the maintenance of cohesin complexes at the centromeres and, unlike in mitosis, begin to migrate to the same poles of the cell during Anaphase I. Completion of Meiosis I results in two products containing recombined sister chromatids. Meiosis II is similar to mitotic division, cohesion of the sister chromatids is lost completely by the onset of anaphase II allowing the two chromatids to separate and results in four haploid meiotic products which differ form the parental genotype.

1.4.2 Mechanisms of the meiotic cell cycle

1.4.2.1 Initiation of meiosis in budding yeast

Entry into meiosis in budding yeast is controlled by the expression and activation of the master regulator ScIme1p; the regulation of which is extremely complex and requires various inputs at both transcriptional and post-transcriptional levels (Mitchell, 1994; Vershon & Pierce, 2000). The promoter region of *IME1* is over 2 kb in length and contains multiple regulatory transcriptional control regions that integrate various nutritional and cell-state-specific signals into the control of *IME1* expression (Sagee *et al.*, 1998; Vershon & Pierce, 2000).

In haploid cells *IME1* expression is repressed by the haploid-specific ScRme1p, which is thought to repress transcription, via the Mediator transcription factor, by altering the chromatin structure of the *IME1* promoter (Mitchell, 1994; Sagee *et al.*, 1998). This is accompanied by inhibition of the ScIme1p pathway via the budding yeast mitotic G1 Clnp–CDK cyclins (Lee & Amon, 2001). To promote entry into meiosis a diploid-cell-specific signal is required. Haploid budding yeast cells exist as one of two different mating types **a** or α, depending on whether they possess *MAT***a** or *MAT*α at the mating type locus. Diploid **a**/α cells have both *MAT***a** and *MAT*α alleles, which encode the transcriptional repressor subunits a1 and α2, respectively (Mitchell, 1994; Kupiec *et al.*, 1997). In diploid cells the **a**1–α2 transcription factor specifically represses *RME1* transcription and promotes *IME4* transcription, which is itself a transcriptional activator of *IME1* (Mitchell, 1994; Sagee *et al.*, 1998; Vershon & Pierce, 2000).

Initiation of the meiotic cell cycle in budding yeast occurs only under conditions of nutritional starvation and several regulatory elements in the *IME1* promoter repress *IME1* transcription under nutrient rich conditions. Similarly, the lack of various essential nutrients positively regulates *IME1* transcription under starvation conditions (Sagee *et al.*, 1998; Vershon & Pierce, 2000). These signals are briefly summarized in Figure 1.6.

Once ScIme1p is expressed it is phosphorylated by the protein kinase ScRim11p, which renders it capable of interacting with ScUme6p (Lee & Amon, 2001). ScUme6p binds to *URS1* sites in early-meiotic-gene promoters and is itself phosphorylated by ScRim11p (Malathi *et al.*, 1997). Once these components are phosphorylated the ScRim11p–ScIme1p complex interacts with ScUme6p which effectively tethers the activation domain of ScIme1p to the promoters of the early meiotic genes (Lee & Amon, 2001). Of particular importance is the induction of *IME2* expression by ScIme1p. *IME2* encodes an essential meiotic-specific protein kinase that is required for the induction of the next wave of meiotic transcription; the induction of the middle genes (Murakami & Nurse 2000; Foiani *et al.*, 1996). *URS1* sites are only weakly activating however, and additional transcriptional activator elements are often found in early gene promoters (Lee & Amon, 2001). These activating elements are prevented from functioning inappropriately in mitosis due to the mitotic interaction of ScUme6p with the ScSin3p–ScRpd3p

complex in which the histone deacetylase ScRpd3p functions to inhibit transcription (Kadosh, 1998; Lee & Amon, 2001).

Figure 1.7: Initiation of meiosis in budding yeast

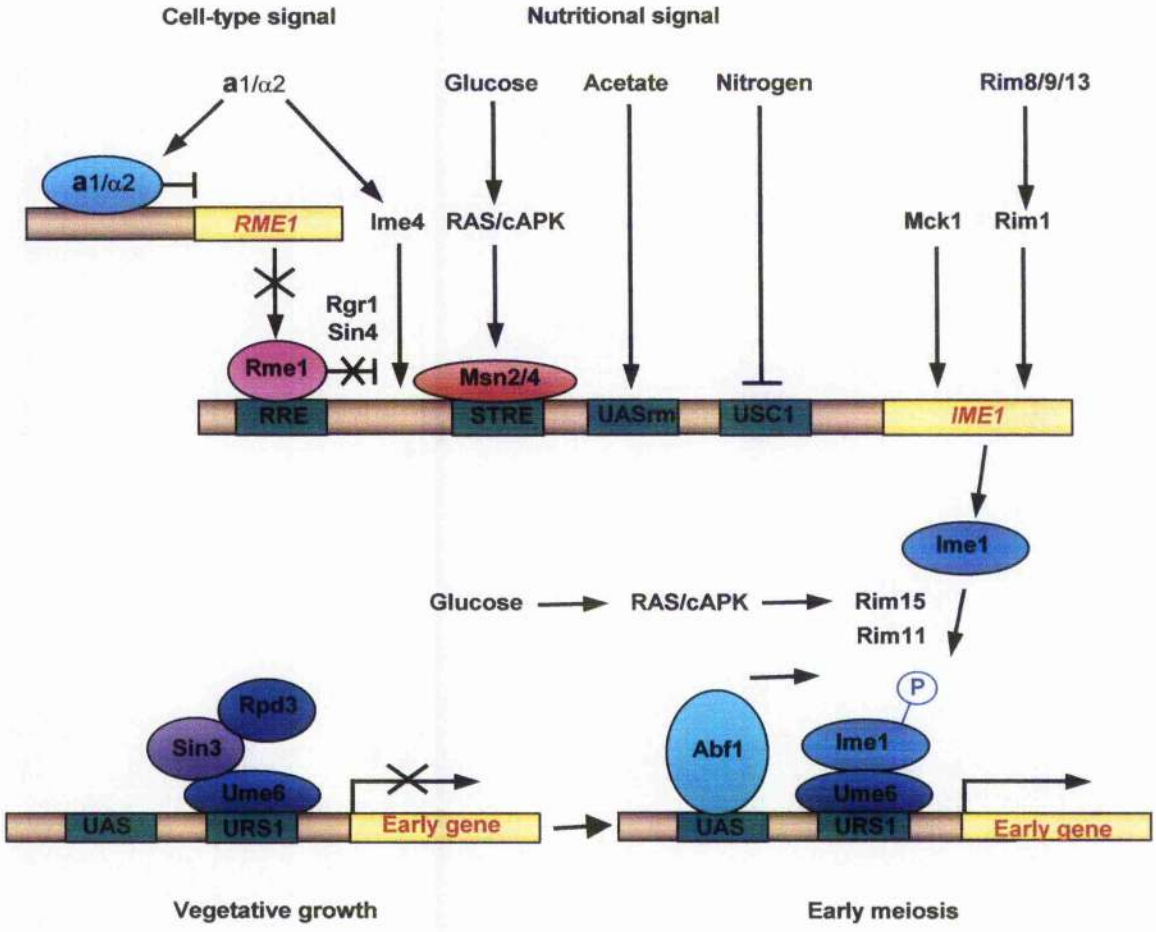


Figure 1.7: Initiation of meiosis in budding yeast
In haploid cells ScRme1p binds to repressor elements (RREs) in the *IME1* promoter and in combination with ScRgr1p and ScSin4p represses its transcription. Low glucose levels activate *IME1* transcription through ScMsn2p and ScMsn4p binding to stress response elements (STRE's) in the *IME1* promoter. Actetate and nitrogen signals also affect *IME1* transcription through UASrm and USC1, respectively. The ScRim8p–ScRim9p–ScRim13p complex, ScMck1p and ScRim1p are also required for full *IME1* expression. ScIme1p is then phosphorylated by ScRim11p, which allows it to interact with ScUme6p that is bound to URS1 elements in early-gene promoters. Additional regulatory factors such as ScAbf1p are also often required to increase expression of these genes. In vegetative cells early gene promoters are repressed by the recruitment of the ScRpd3p–ScSin3p histone-deacetylase complex by ScUme6p.
This figure was adapted from Vershon & Pierce (2000).

1.4.2.2 Initiation of meiosis in fission yeast

In the laboratory, fission yeast cells normally exist in the haploid state as either h^+ (*mat1-P*) or h^- (*mat1-M*) cells and initiate meiosis following nutritional starvation and conjugation. However, they can be induced to undergo vegetative growth as diploids if transferred to rich medium immediately after conjugation (see Figure 1.2). Nevertheless, fission yeast cells must be in the diploid state (h^+/h^-) and starved of essential nutrients before meiosis is initiated (Davis & Smith, 2001; Yamamoto, 1996). First, starvation induces the phosphorylation of the transcription factor Atf1p–Pcr1p via the stress-induced Wis1p–Spc1p protein kinase cascade (Davis & Smith, 2001). Second, starvation causes the intracellular levels of cAMP to be reduced — the G-protein Gpa2p is thought to have a role in detecting nutrient limitation and is known to regulate the activity of the adenylate cyclase Cyr1p (Yamamoto, 1996). This results in the cAMP-dependent protein kinase Pka1p becoming inactivated (Davis & Smith, 2001). These two pathways result in the expression and activation of the *ste11*$^+$ transcription factor (Davis & Smith, 2001). Ste11p induces transcription by binding the TR-box in the promoter regions of various genes including *mat1-P*, *mat1-M* and *mei2*$^+$ (Kjaerulff *et al.*, 1997; Sugimoto *et al.*, 1991). Mat1-Pp and Mat1-Mp directly induce transcription of the critical meiotic regulator *mei3*$^+$ (vanHeeckeren *et al.*, 1998). Mei3p is a pseudosubstrate of the Pat1p protein kinase and when Mei3p is expressed Pat1p is inactivated (Watanabe *et al.*, 1997). This leads to dephosphorylation of both Ste11p and Mei2p, resulting in the production of active Mei2p, which binds meiRNA. It is then is transported into the nucleus where it is required for the induction of pre-meiotic DNA synthesis and meiosis itself (Ohno & Mattaj, 1999; Peng *et al.*, 2003; Watanabe *et al.*, 1997). See Figure 1.8.

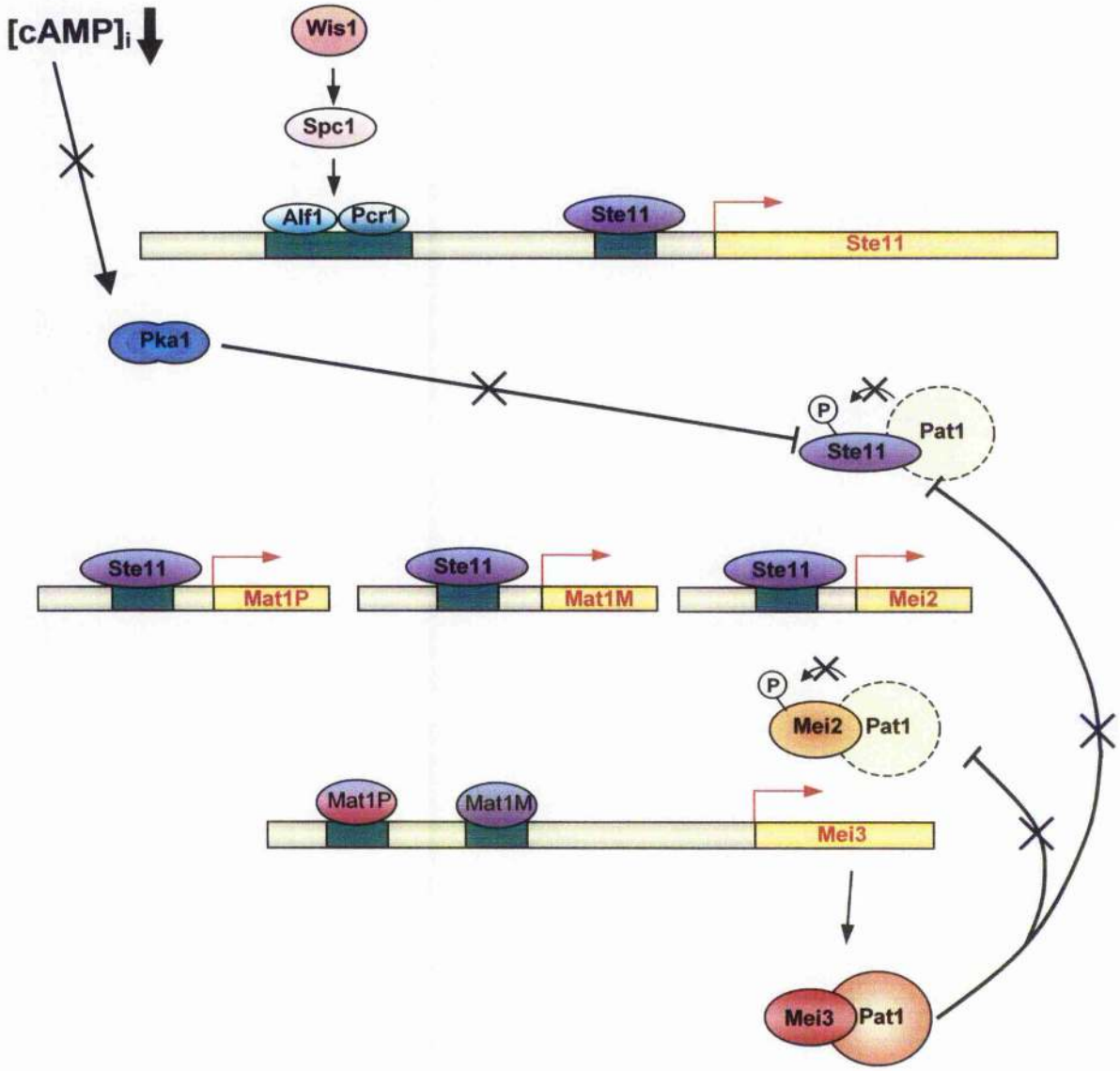


Figure 1.8: Initiation of meiosis in fission yeast
Fission yeast cells must be diploid (h^+/h^-) and starved to initiate meiosis. First, starvation induces the phosphorylation of the transcription factor Atf1p–Pcr1p via the stress-induced Wis1p–Spc1p protein-kinase cascade. Second, starvation causes the intracellular levels of cAMP to be reduced via the action of Gpa2p and Cyr1p. This results in the cAMP-dependent protein kinase Pka1p becoming inactivated. This results in the expression and activation of the *ste11*$^+$ transcription factor. Ste11p induces transcription by binding the TR-box in the promoter regions of genes important for meiosis initiation including *mat1-P*, *mat1-M* and *mei2*$^+$. Mat1-Pp and Mat1-Mp directly induce transcription of the critical meiotic regulator *mei3*$^+$. Mei3p binds and inactivates Pat1p. This leads to dephosphorylation of both Ste11p and Mei2p resulting in the production of active Mei2p, which binds meiRNA and is transported into the nucleus where it is required for the induction of pre-meiotic DNA synthesis and meiosis itself.

1.4.2.3 Pre-meiotic S phase

Once meiosis has been initiated from G1 the cells must then enter pre-meiotic S phase and replicate their DNA before progression into meiotic division (Petronczki *et al.*, 2003). Just as in mitosis, this requires the initiation of cell-cycle-regulated transcription of DNA-replication genes (Murakami & Nurse, 2000). In both fission yeast and budding yeast this requires the same basic transcription machinery that functions to induce mitotic G1–S transcription, although, in fission yeast, the Cdc10p–Res1p–Res2p complex is replaced by a Cdc10p–Res2p complex, possibly in combination with Rep1p (Section *1.5*; Lee & Amon, 2001; Ding & Smith, 1998). Similarly, in fission yeast, the action of the CDK Cdc2p is also required for progression through the meiotic cell cycle, with Cdc2p phosphorylation appearing at the beginning of pre-meiotic S phase and decreasing during the meiotic division phase (Murakami & Nurse, 2000).

In budding yeast meiosis, the Cdc2p homologue ScCdc28p does not seem to be absolutely required for pre-meiotic S phase, although it is required for the meiotic division phase. The B-type cyclins ScCln5p and ScCln6p are, however, required for pre-meiotic DNA synthesis (Murakami & Nurse, 2000). The meiosis-specific protein kinase Sclme2p — itself an early gene induced by Sclme1p — is required for the initiation of both early- and middle-gene transcription (Lee & Amon, 2001; Foiani *et al.*, 1996). Similarly, the transcription factor ScNdt80p — which is also induced by Sclme1p — is then required for the transcription of both middle and late genes that are involved in the spore-formation pathway (Chu *et al.*, 1998; Murakami & Nurse, 2000).

As in mitosis, DNA-replication checkpoints operate at this point in the meiotic cell cycle to prevent cells entering the meiotic-division phase without having replicated their DNA. In fission yeast, the *rad*$^{+}$ genes and *cds1*$^{+}$ are required for both mitotic and meiotic DNA-replication checkpoints but Cds1p has a more prominent role during meiosis (Murakami & Nurse, 1999). If DNA replication is blocked, Cdc2p phosphorylation on Tyr15 is maintained, therefore keeping Cdc2 protein-kinase-activity low and preventing progression into the meiotic division phase (Murakami & Nurse, 1999).

Pre-meiotic S phase is known to be much longer than pre-mitotic S phase and it is thought that preparation for meiotic recombination is responsible for this (Lee & Amon, 2001). Many genes are required for recombination in fission yeast and budding yeast (Davis & Smith, 2001), but the formation of cohesin complexes along the length of the sister chromatids is particularly essential for recombination and the successful completion of meiotic division (Petronczki *et al.*, 2003). Meiotic cohesins, Rec8p and Rec11p in fission yeast and ScRec8p and Scirr1p/ScScc3p in budding yeast hold the sister chromatids together (Murakami & Nurse, 1999; Davis & Smith 2001). After pre-meiotic DNA replication, homologous chromosomes are also held together by chiasmata, which are formed at sites of reciprocal recombination between the two homologues.

1.4.2.4 Meiotic division

Once pre-meiotic DNA replication has occurred, cells progress into the meiotic division phase. The prophase of Meiosis I can itself be divided into discrete phases based on cytological events. In leptotene, meiotic chromosomes become visible and homologous chromosomes recognize each other. In zygotene, the homologues become closely associated and a synaptonemal complex (SC) is formed in which both chromatids of each homologue are held together by cohesin complexes and close association of the two parental chromosomes is achieved by the SC (Shaw & Moore, 1998; Petronczki *et al.*, 2003). In fission yeast, linear elements (LEs) — which are similar to the axial elements of other eukaryotes — take the place of the SC and form along the length of the chromosome (Davis & Smith, 2001). Entry into the following phase, pachytene, occurs when the SC is fully formed. Recombination then occurs at points, called chiasmata, at which the arms of the two homologous chromosomes have crossed over (see Figure 1.6). Chiasmata formation begins in leptotene and is resolved through double-strand breakage of the DNA followed by the formation of double Holliday junctions (Petronczki *et al.*, 2003). By the following phase, diplotene, the chromosomes have been recombined, the SC begins to disassemble and the chromosomes become more condensed. Further condensation then occurs during diakinesis (Petronczki *et al.*, 2003).

During metaphase I the chromosomes become attached to the meiotic spindle (Shaw & Moore, 1998). Entry into anaphase I is then caused by the dissolution of the cohesin complexes along the arms of the chromosomes distal to the centromeres, while the centromeric cohesin complexes seem to be protected. In budding yeast, this is achieved by the cleavage of ScRec8p by separase (ScEsp1p), which is thought to be regulated in a manner similar to that which occurs in mitosis — that is, by the APC/C-regulated interaction with securin (ScPds1p; Lee & Amon, 2001). In vertebrates, however, transition into anaphase I of meiosis appears to be regulated by an APC/C-separase-independent mechanism although anaphase II does depend on APC/C-separase (Lee & Amon, 2001). Once the cohesin complex is removed from the chromosome arms, Meiosis I is completed resulting in reductional division, in which the two copies of each homologous chromosome migrate along the spindle to opposite poles of the cell (see Figure 1.6; Petronczki *et al.*, 2003).

Meiosis II then takes place, which is very similar to mitotic division in that the paired homologous chromosomes are separated and migrate to opposite poles of the cell, before cell division occurs, resulting in four haploid meiotic products. This involves the dissolution of the centromeric cohesin complexes at anaphase II (see Figure 1.6; Lee & Amon, 2001). The mechanism by which centromeric Rec8p (the meiotic-specific HsScc1p homologue) is protected from cleavage during anaphase I is unknown, but this appears to be specific to Rec8p as, in budding yeast, expression of *SCC1* instead of *REC8* results in complete loss of cohesin during Meiosis I (Toth *et al.*, 2000). The budding yeast protein ScSlk19p localizes to meiotic

kinetochores prior to Meiosis I and cells lacking *SLK19* show reduced ScRec8p staining after anaphase I (Kamieniecki *et al.*, 2000). Also, in a *SPO13* mutant, ScRec8p is lost from the whole length of the chromosome during anaphase I (Klein *et al.*, 1999). This strongly implicates ScSlk19p and ScSpo13p in the mechanism of ScRec8p protection, although how this works remains to be elucidated (Lee & Amon 2001).

In eukaryotic cells, gene transcription is carried out by one of three different RNA polymerases (RNAPols) depending on the type of gene being transcribed. RNAPol I transcribes ribosomal (r)RNA, RNAPol II transcribes messenger (m)RNA and RNAPol III transcribes transfer (t)RNA and other small RNAs (Lewin, 1997c).

1.5.1 RNA polymerase I transcription

RNAPol I is found in the nucleolus where it transcribes the large rRNA molecules which are an integral part of the ribosomal protein-synthesis machinery. Initiation of rRNA-gene transcription requires many individual protein factors. In budding yeast, the upstream activating factor (UAF), which is a complex of several polypeptides and two histones, interacts with an upstream element in the rRNA gene promoter (Lewin, 1997c; Grummt, 2003). The TATA-binding protein (TBP) and a second protein complex, known as CF (Core Factor) are recruited to the downstream core promoter and are themselves required for the recruitment of the functional holoenzyme, which includes RNAPol I and RRN3p (Grummt, 2003). It has been suggested that, in budding yeast, UAF remains associated with the upstream element acting as a scaffold for reinitiation, and other RNAPol I components cycle on and off the promoter during each successive round of transcription (Aprikian *et al.*, 2001).

In mammalian systems, the upstream binding factor (UBF) binds the upstream control element and its synergistic action with the TBP-containing 'promoter-selectivity factor' (TIF-IB) recruits various TBP-associated factors (TAFs), which provide the DNA-binding/promoter-recognition function. UBF and TIF-IB interaction with TIF-IA (the homologue of budding yeast RRN3p) then recruits the RNAPol I holoenzyme (Lewin, 1997c; Grummt, 2003). In human systems, however, only the RNAPol-I–TIF-IA components are thought to be recycled (Panov *et al.*, 2001).

1.5.2 RNA polymerase III transcription

Much of the known detail of RNAPol III transcription has come from studies of budding yeast and human systems (Lewin, 1997c; Huang & Maraia, 2001; Paule & White, 2000) although recent studies have revealed both strong similarities and differences between budding yeast, human and fission yeast systems (Huang & Maraia, 2001).

RNAPol III is responsible for the transcription of a large variety of small RNAs including tRNAs, 5S rRNA and a variety of snRNAs (small nuclear RNAs) and these can be divided into three different basic types of genes (Lewin, 1997c). Type I promoters are found in the 5S rRNA genes and, unlike promoters for RNAPol-I- and RNAPol-II-transcribed genes the regulatory elements reside within the transcribed region and consist of an A-block, an initiator element (IE) and a C block (Huang & Maraia, 2001; Paule & White, 2000). The most common RNAPol III promoter element is the Type II element, which is seen in the tRNA genes e.g.

budding yeast SUP4. Type II promoters are also internal and consist of two highly conserved sequence elements, an A-block close to the site of transcription initiation and a B-block which is usually ~30–60 bp downstream of the A-block (Paule & White, 2000). Both Type I and Type II RNAPol III transcriptional units contain a conserved terminator sequence (Tn) that is important for the reinitiation of successive rounds of transcription (Huang & Maraia, 2001; Paule & White, 2000). Type III RNAPol III promoters have a TATA element upstream of the initiation site and also possess conserved distal and proximal sequence elements (DSEs and PSEs, respectively), which function in transcription factor recognition.

In Type I RNAPol III promoters TFIIIA binds to DNA sequences that encompass the C-block and is required for the binding of TFIIIC (Huang & Maraia, 2001; Paule & White, 2000). In Type II RNAPol III promoters TFIIIC recognizes the B-block and binds DNA sequences that encompass both the A-block and B-block sequence elements (Huang & Maraia, 2001; Paule & White, 2000). In both Type I and II RNAPol III promoters, TFIIIC binding directs the subsequent binding of the TBP-containing TFIIIB complex. The TFIIIC complex of budding yeast resembles a dumbell shape and is able to contact both the A-block and B-block in various Type II promoters despite the widely differing distances between the two binding sites in different promoters. Human TFIIIC, however, consists of two subunits TFIIIC1 and TFIIIC2, of which TFIIIC2 recognizes the B-block and then recruits TFIIIC1 and TFIIIB (Paule & White, 2000). Once TFIIIB is bound by Type I or II promoters it then directs the binding of RNAPol III to the initiation site and can remain stably bound to the DNA during several successive rounds of transcription (Huang & Maraia, 2001).

Type III RNAPol III promoters, however, require a different set of accessory factors to Type I and II promoters. The most well studied Type III promoters are the vertebrate 7SK and U6 snRNA genes (Paule & White, 2000). An accessory factor, Oct-1, binds to the DSE and promotes the binding of the PSE by a five-subunit complex known as SNAPc or PTF. The distance between the PSE and the TATA box is precisely conserved across Type III promoters and SNAPc/PTF binding to PSE is thought, in turn, to aid recruitment of TFIIIB to the TATA box, which then goes on to recruit RNAPol III (Paule & White, 2000).

Unlike RNAPol II, RNAPol III transcription initiation does not require ATP hydrolysis; the initiating DNA sequences melt spontaneously after RNAPol III binding, but this process requires the participation of TFIIIB. While TFIIIB remains bound at the site of initiation, the RNAPol III complex progresses through the gene along with the bubble of melted DNA and, surprisingly, its progress is not hindered by passage through the transcription complexes bound at internal regulatory sites. The continued binding of TFIIIB is thought to be important to prevent the displacement of accessory factors TFIIIA and TFIIIC from their internal sequences (Paule & White, 2000).

Following the sequencing of the fission yeast genome, database searches have revealed the presence of fission yeast homologues of the vast majority of RNApol III proteins and their

associated factors (Huang & Maraia, 2001). However, significant differences do exist between these systems, for example, differences that were noted in the downstream fission yeast TFIIIC subunits are thought to account for mechanistic variances that were observed in RNAPol III transcription termination in this yeast. Similarly, in human RNAPol III Type-III-promoter systems a distinct class of TFIIIB-related accessory factors are required which have no homologues in either yeast (Huang & Maraia, 2001). Furthermore, unlike budding yeast and human RNAPol III systems, which employ TATA-less promoters, fission yeast RNAPol III systems have been shown to absolutely require a TATA box indicating that there are also mechanistic differences in the recruitment of RNAPol III machinery in fission yeast (Hamada *et al.*, 2001; Huang & Maraia, 2001).

1.5.3 RNA polymerase II transcription

RNAPol II is responsible for the transcription of mRNA, which provides the template for protein translation. But, while RNAPol II itself causes the basal transcription of these protein-encoding genes, alone, this is extremely inefficient and interactions with a variety of other transcription factors are required to produce activated transcription — the often intricately regulated physiological expression pattern of an individual gene. In fact, initiation of transcription by RNAPol II can range from using the relatively simple transcription units seen in yeast species — that normally depend on DNA sequences immediately 5' to the transcriptional start site for the regulation of their expression — to extremely complex metazoan transcription-control modules. A complex arrangement of multiple upstream, or downstream, enhancer, silencer and insulator elements, which can be located 10–50 kb away from the core promoter, often occurs in metazoan systems and can generate complex gene expression patterns, which depend on multiple regulatory factors (Figure 1.9).

1.5.3.1 RNAPol II promoter sequences

Basal gene transcription is initiated through the interaction of RNAPol II with the core promoter. The structure of RNAPol II promoters can vary widely from gene to gene but there are certain common elements that are found in a large number of cases and are known to be both necessary and sufficient to cause transcription *in vivo*. Genes that lack good matches to these sequences are often inefficiently transcribed and may have multiple start sites, which is attributed to weak and inaccurate recruitment of RNAPol II (White, 2001). In a simple yeast transcription unit, the core promoter consists of a TATA box, which is the binding site for TBP and a pyramidine-rich initiator element (Inr) that marks the site of transcription initiation (Levine & Tjian, 2003; Struhl, 1989). In budding yeast, transcription initiation occurs ~40–100 bp from the edge of the TATA element whereas in fission yeast this occurs ~25–40 bp downstream of the TATA box (Choi *et al.*, 2002). The binding of TBP to the core promoter is

the initial event that selects a gene for transcription by initiating RNAPol II recruitment, but, to attain activated physiological levels of transcription, interactions with other regulatory transcription factors are required. TBP binding is regulated by upstream activating sequences, which consist of between one and several binding sites for specific transcription factors. Different UAS sequences, which recruit more than one sequence-specific transcription factor, might be involved in the regulation of any one gene (Levine & Tjian, 2003; Struhl, 1989).

Figure 1.9: RNA Polymerase II promoter sequences

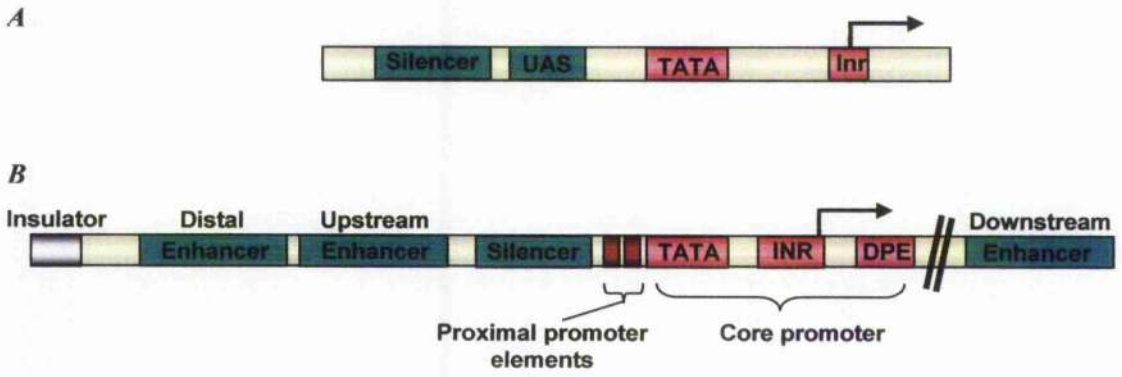


Figure 1.9: RNA Polymerase II promoter sequences

A: A typical yeast RNAPol II promoter contains a TATA box and an initiator element (Inr) as well as upstream activating sequences (UASs) and silencer elements which control RNAPol II association with the TATA box.

B: A Typical mammalian RNAPol II promoter sequence contains a complex arrangement of multiple, clustered, enhancer elements as well as silencer and initiator elements, which can be located 10–50 kb upstream or downstream of the core promoter. The core promoter contains a TATA box, initiator elements (INRs) and downstream promoter elements (DPEs). This figure was adapted from Levine & Tijan (2003).

In addition to the core promoter and other sequence elements that are closely associated with the promoter, typical metazoan transcription modules contain several enhancer sequences that are necessary for activated transcription and that can be located at great distances from the gene itself and even in introns. Each enhancer is responsible for a subset of the total expression pattern of the gene and they typically mediate tissue-, cell-type- or cell-cycle-specific gene expression (Kadonaga, 2002; Levine & Tjian, 2003). Enhancer sequences are usually on the order of 500 bp in length and contain multiple recognition sequences for the binding of sequence-specific transcription factors. Commonly, one enhancer will be able to recruit two different activators and one repressor (Levine & Tjian, 2003). The metazoan core promoter is ~60–80 bp in length and straddles the transcription initiation site (Kadonaga, 2002; Levine &

Tjian, 2003). It contains DNA sequences including TATA (the AT-rich TBP binding sequence) and/or DPE (the downstream promoter element also known to interact with TBP) sequences as well as an initiator element (Kadonaga, 2002; Levine & Tjian, 2003). These sequence elements cooperatively recruit the TBP-containing TFIID initiation complex (Kadonaga, 2002; Levine & Tjian, 2003). In a similar manner to fission yeast, in mammalian systems, initiation of transcription occurs close to the TATA box, typically within 25 bp (Choi *et al.*, 2002). Proximal promoter elements are also often present in the promoter and can act like enhancer elements themselves, or alternatively, act as 'tethering elements' to recruit distal enhancers (Kadonaga, 2002; Levine & Tjian, 2003). Other DNA sequences that flank these multiple regulatory sites act as insulator sequences to prevent the inappropriate regulation of adjacent genes (Levine & Tjian, 2003). This highly complex organisation of the transcriptional module allows the expression of a single metazoan gene to integrate inputs from multiple signalling pathways and transcription factors, to accurately control tissue-, cell-type- and cell-cycle-specific gene expression.

1.5.3.2 RNAPol II transcription machinery

The initial step in transcription initiation is the binding of TFIID to the core promoter. TFIID contains TBP and 10–12 TBP-associated factors (TAFIIs); it is recruited to the core promoter where it acts to nucleate the assembly of the preinitiation complex (PIC) (Wasserman & Saur, 2001). To initiate nucleation TFIID is thought to carry out several functions. First, TFIID provides activator-dependent recognition of specific DNA-sequence elements within the core promoter (Wasserman & Saur, 2001). Second, it alters the chromatin structure of the core promoter to generate a conformation that is favourable for the further assembly of the PIC and subsequent transcription initiation (Wasserman & Saur, 2001). Third, TFIID interacts with, and modifies, some of the general transcription factors TFIIA, TFIIB, TFIIE, TFIIF, and TFIIH that associate with RNAPol II, which facilitates PIC assembly and contributes to transcription initiation and elongation (Wasserman & Saur, 2001).

RNAPol II itself consists of twelve protein subunits Rpb1–Rpb12 the sequence and functions of which are conserved from yeast to humans (Myer & Young, 1998; Shpakovski *et al.*, 2000) and current evidence suggests that the general RNAPol II machinery is highly conserved across eukaryotes (Myer & Young, 1998). However, it is becoming more apparent that metazoan systems have evolved many more diverse layers of transcriptional control (Levine & Tjian, 2003). At the level of PIC assembly, tissue-specific TAFs and TBP-related factors (TRFs), which are not found in yeast, provide a further level of control over gene expression in metazoans (Levine & Tjian, 2003).

1.5.3.3 mRNA production

Once the PIC has been assembled, the DNA strands at the promoter must be melted to allow RNAPol II access to the template strand. This is achieved by the helicase activity of TFIIH, but, at first, this helicase activity is inhibited by TFIIE. Instead, TFIIE stimulates the kinase activity of TFIIH, which phosphorylates the C-terminal domain of RNAPol II. Hyperphosphorylated RNAPol II no longer binds TBP and RNAPol II begins to be released from the promoter. TFIIE then dissociates from the PIC allowing the helicase activity of TFIIH to cause DNA melting, this stimulates the initiation of RNA synthesis and RNAPol II releases TFIIB, TFIIH and TFIIF, and begins to progress along the DNA strand (White, 2001).

Elongation of mRNA then occurs and requires the assistance of elongation factors to protect against stalling or arrest of RNAPol II. The TFIIF complex, which was also required in the PIC, reassociates with RNAPol II and, along with another complex called elongin and a third factor called ELL, facilitates elongation of the mRNA — possibly by keeping the growing mRNA strand aligned with respect to the catalytic site. RNAPol II arrest sometimes occurs however, and is dealt with by a monomeric elongation factor known as TFIIS. TFIIS binds RNAPolII and stimulates an intrinsic endoribonulease activity, which cleaves away any displaced nucleotides and allows RNAPol II to proceed along the template strand (White, 2001).

Termination of transcription is achieved by the cooperative action of several more factors. Cleavage and polyadenylation specificity factor (CPSF), cleavage stimulation factor (CstF), cleavage factors I and II (CFI and CFII), and poly A polymerase (PAP) work together to cause cleavage of the RNA strand between nucleotide sequences that are common to the 3′ ends of most mRNA molecules. PAP, in cooperation with poly-A binding protein (PAB), then adds multiple adenylates to the end of the RNA molecule; ~250 in mammals and ~80 in yeast. (White, 2001).

1.5.3.4 Control of RNAPol II transcription

Eukaryotic enhancer sequences can ultimately act to control the transcription of a promoter in two distinct ways. First, enhancer sequences can bind transcription factors that directly contact the basal transcription machinery. Second, enhancer sequences can interact with transcription factors that then contact intermediary cofactors, which interact with the basal machinery. Thus transcription cofactor complexes provide a further level of transcriptional regulation that also has a vital role in the control of gene expression (Levine & Tjian, 2003). The multi-protein complex Mediator was first identified in budding yeast but has counterparts in metazoan systems and is thought to act as an interface between gene-specific regulatory proteins and the general eukaryotic transcription machinery (Myers & Kornberg, 2000). Mediator is a 20-protein complex that interacts with RNAPol II to form a holoenzyme. It is thought to modulate the binding and/or function of RNAPol II at the core promoter under the influence of enhancer and

operator sequences and can transfer both positive and negative regulatory effects to the transcription machinery (Levine & Tjian, 2003; Myers & Kornberg, 2000). Several other multi-protein complexes that function in a similar manner are also found in metazoans but are more diversified between species compared with the well-conserved general transcription machinery (Levine & Tjian, 2003; Myers & Kornberg, 2000).

Transcription factors and/or their associated cofactors can also act at several possible stages of transcription initiation to stimulate the process of mRNA production. A significant cause of the repression of transcription is the tightly folded packaging of DNA into chromatin. Activators of transcription can often identify their binding sites even in the context of packaged chromatin, or alternatively, are able to bind their recognition sequences quickly enough after DNA synthesis that they become associated with their binding sites before they are hidden in chromatin. Once bound, such factors can then remodel the chromatin to allow access of the basal machinery. Transcription factors that form direct interactions with the basal transcription machinery can work to affect transcription in various ways. Contacts between their activation domains and components of the basal transcription machinery can work to recruit the basal machinery to the promoter, can cause conformational changes or covalent modifications of the basal machinery that affect transcription rates, or can affect the rate of promoter clearance and elongation of the mRNA, therefore also affecting transcription rates (White, 2001).

Such enhancers and their associated transcription factors are able to work at the, often significant, distances from the site of transcription initiation that are commonly seen in higher eukaryotic systems by using various mechanisms. The 'tracking' of the transcription factor along the DNA from the enhancer site to the promoter has been identified as one mechanism. And the transmitting of the effects of enhancer binding through imposing changes on the DNA superstructure is thought to be another. Most commonly, however, such long-distance interactions are thought to work by 'looping-out' the intervening DNA. This can work if the DNA is naturally bent due to intrinsic properties of the DNA sequence, or, if curvature of the DNA is enforced by the binding of specific proteins. Finally, folding of the intervening DNA into chromatin is also known to be a significant method of bringing distant sites into close proximity (White, 2001).

1.5.3.4.1 Chromatin and RNAPol II transcription

DNA is packaged into chromatin via its association with histone proteins, which organize the superhelical DNA into nucleosomes and then chromatin fibres. This has a significant effect on the ability of transcription factors and the basal transcription machinery to recognize their DNA-binding sites and initiate transcription; as such packaging restricts the accessibility of the DNA strands. This does not result in a simply restrictive mechanism, however, as the existence of

various types of factors that are able to influence the chromatin structure allows DNA superstructure and chromatin to have a significant role in gene-specific regulation.

Chromatin remodelling and modifying enzymes, which can either remodel chromatin (e.g. SWI/SNF) (Levine & Tjian, 2003) or modify histones and also transcription factors (the histone acetyl transferases and deacetylases; HATs and HDACs respectively) (Kouzarides, 2000), cause this type of RNAPol II transcriptional regulation. Several chromatin-remodelling complexes exist, but SWI/SNF, which is conserved from yeast to humans, is the prototypic example of this type of factor and was first discovered in budding yeast. SWI/SNF has 11 subunits and is thought to remodel chromatin by catalysing the tracking of nucleosomes along the DNA (White, 2001). HAT enzymes affect chromatin structure by acetylating histones, particularly histone tails, on lysine residues. This promotes decompaction of chromatin, but whether this works by neutralizing the electrostatic interactions of histones with the DNA backbone, by altering the secondary structure of histone tails or by providing acetyl groups that act as signals for other chromatin destabilizing complexes is unclear. The budding yeast protein ScGcn5p is one example of a HAT. It is present in various complexes, the largest of which is SAGA (Spt-Ada-Gcn5-acetyltransferase) and which, in addition to SpGcn5p, contains the products of several *SPT* genes and *ADA2* and *ADA3*. HDAC enzymes, such as budding yeast ScRPD3p, catalyse the deacetylation of histones and are therefore thought to reduce the accessibility of DNA to transcription factors (White, 2001).

The *HO* gene of budding yeast encodes an endonuclease that is required for mating-type switching and is a known example of a gene that relies on the effects of chromatin remodelling complexes for its transcription. Cosma *et al.* (1999) carried out extensive studies on the transcription of this gene using chromatin immunoprecipitation assays to establish what factors are involved in the transcription of *HO* and in what order they are required. Initially, ScSwi5p binds chromatin at two sites that are about 1.2 kb upstream of the *HO* gene — ScSwi5p is a zinc-finger protein that can penetrate chromatin to get access to its binding sites. ScSwi5p binding to chromatin causes the recruitment of the SWI/SNF chromatin remodelling factor to the vicinity of these same upstream binding sites almost immediately, but then ScSwi5p quickly dissociates from the chromatin. This indicates that ScSwi5p might fix the chromatin in a remodelled state but is then no longer needed. Approximately 5 min later, SWI/SNF is also found close to a group of SCB motifs (ScSwi4/6p-dependent cell-cycle box). The ScGcn5p HAT is then recruited to the vicinity of these SCB motifs, which are situated 100–700 bp upstream of the transcription start site. Only then is the transcription factor SBF (SCB-binding factor) able to bind its cognate DNA motifs and help recruit the basal transcription machinery to initiate transcription of *HO* (Cosma *et al.*, 1999; White, 2001).

To pass successfully through the various different phases of growth that lead to mitosis or meiosis and the production of daughter cells, all eukaryotic cells must undergo a coordinated series of cellular events known as the cell cycle. One prominent feature of cell cycle progression and control is the cell-cycle-phase-specific regulation of transcription. Microarray studies in budding yeast have examined the transcription profiles of genes as they pass through the mitotic cell cycle (Cho *et al.*, 1998) and similar studies in fission yeast have examined global transcription during the meiotic cell cycle (Mata *et al.*, 2002). These studies have both revealed that several successive waves of coordinated gene transcription occur during the studied cell cycles.

1.5.4.1 G1–S cell-cycle-regulated transcription

The programme of coordinated cell cycle transcription that occurs at the G1–S cell-cycle-phase transition has been well studied in budding yeast, fission yeast and human systems. Budding and fission yeast both pass through a significant cell cycle control point in late G1 phase, known as START, after which the cells become committed to the division process (Nurse *et al.*, 1998). This control point is also considered to be analogous to the restriction point (R) seen in mammalian cells after which cells must complete the cell cycle regardless of further growth-factor signalling (Nurse *et al.*, 1998; Stevens & LaThangue, 2003). At this point the transcription factors SBF and MBF in budding yeast, MBF (DSC1) in fission yeast and E2F in mammalian cells initiate coordinated programmes of transcription in their respective systems.

1.5.4.2 G1–S cell-cycle-regulated transcription in budding yeast

Initially, in budding yeast, it was shown that several genes were coordinately transcribed shortly after START in late G1 phase and that this pattern of transcription was due to the presence of one of two different UAS sequences in the promoter regions of these genes (McIntosh, 1993). One of the conserved sequence elements in these UAS regions corresponded to the recognition sequence for the restriction endonuclease *Mlu*I (5'-ACGCGT-3') and so this UAS became known as the *Mlu*I cell cycle box or MCB motif (McIntosh, 1993). The other consensus sequence (5'-CACGAAAA-3') was designated the SCB, for ScSwip4,6-dependent cell-cycle box, as it was shown to interact with a complex of the *SWI4* and *SWI6* gene products (Merill *et al.*, 1992).

A protein complex, known as MBF (MCB-binding factor), which contains ScSwi4p and ScMbp1p, binds MCB elements and is closely related to the SBF (SCB-binding factor) protein complex which contains ScSwi4p and ScSwi6p and binds SCB elements (Andrews & Herskowitz, 1989; Dirick *et al.*, 1992; Koch *et al.*, 1993; McIntosh, 1993; Primig *et al.*, 1992).

A combination of chromatin immunoprecipitation and microarray hybridisation revealed that SBF activated genes are predominantly involved in budding and membrane and cell wall biosynthesis, whereas MBF activated genes are mostly involved in DNA synthesis and repair pathways (Vishwanath *et al.*, 2001). Thus, these two closely related regulatory mechanisms provide a method for the independent regulation of two different general molecular processes even though they normally occur in synchrony during the cell cycle (Vishwanath *et al.*, 2001). In agreement with this hypothesis, it has recently been shown that the ScSwi6p binding protein ScStb1p differentially regulates SBF and MBF, as it seems to have an MBF-specific function (Costanzo *et al.*, 2003).

While SBF and MBF bind the promoters of 235 genes, this still does not account for all the budding yeast genes that are expressed with G1–S specificity (Horak *et al.*, 2002; Vishwanath *et al.*, 2001). However, the promoters of several other transcription factors are bound by these complexes which indicates that the SBF/MBF system might be involved in a complex network of transcriptional circuitry that controls gene expression at the G1–S boundary (Horak *et al.*, 2002).

1.5.4.3 G1–S cell-cycle-regulated transcription in fission yeast

In fission yeast, a transcription factor that is highly related to the budding yeast SBF/MBF system was identified as being responsible for the periodic transcription of the *cdc22*$^{+}$ ribonucleotide reductase subunit (Lowndes *et al.*, 1992; Merrill *et al.*, 1992). The *cdc22*$^{+}$ gene has seven copies of the MCB motif in its promoter and the protein complex which interacted with these elements was shown to contain the Cdc10p gene product (Lowndes *et al.*, 1992). Cdc10p shares significant homology with budding yeast ScSwi6p and ScSwi4p and was therefore designated as being the fission yeast DSC1/MBF (DNA synthesis control/MCB binding factor; Lowndes *et al.*, 1992).

Fission yeast MBF is now known to contain at least four gene products; Cdc10p, Res1p, Res2p and Rep2p, and has been widely studied during the mitotic cell cycle (Lowndes *et al.*, 1992; Caligiuri & Beach, 1993; Tanaka *et al.*, 1992; Zhu *et al.*, 1994; Miyamoto *et al.*, 1994; Nakashima *et al.*, 1995). At least 11 genes including *cdc22*$^{+}$, *suc22*$^{+}$, *cdc18*$^{+}$, *cdt1*$^{+}$, *cig2*$^{+}$, *ste6*$^{+}$, *rad21*$^{+}$, *rad11*$^{+}$, *cdt2*$^{+}$, and *mik1*$^{+}$ (Birkenbihl & Subramani, 1995; Conolly & Beach, 1994; Fernandez-Sarabia *et al.*, 1993; Hofman & Beach, 1994; Kearsey *et al.*, 2000; Kelly *et al.*, 1993; Ng *et al.*, 2001; Parker *et al.*, 1997; Tournier & Millar, 2000; Yoshida *et al.*, 2003) have been directly shown to be under the control of MBF during mitosis. However, microarray analysis has identified a set of about 100 genes that are transcribed specifically during late-G1–S phase of the meiotic cell cycle and which also have promoter regions that are enriched for the MCB motif (Mata *et al.*, 2002).

1.5.4.3.1 MBF components: Cdc10p

As a major component of an essential cell cycle transcription factor, Cdc10p is required for both the mitotic and the meiotic cell cycles (Baum *et al.*, 1998; Beach *et al.*, 1985; Lowndes *et al.*, 1992; Nurse & Bissett, 1981). Cdc10p shows significant homology to budding yeast ScSwi4p and ScSwi6p (Merrill *et al.*, 1992) but is not able to bind DNA itself and relies on an interaction with the DNA binding components Res1p and Res2p (McInerny *et al.*, 1995). Deletion of *cdc10*$^{+}$ is lethal but experiments with the viable double mutant *cdc10Δsct1-1* show that in the absence of Cdc10p MCB-regulated transcription is constant and low during the mitotic cell cycle (Baum *et al.*, 1998). However, overexpressing *cdc10*$^{+}$ to a moderate level during the mitotic cell cycle had little effect on MCB-regulated transcript levels (Baum *et al.*, 1998; White *et al.*, 2002). Fission yeast strains containing a particular mutant version of Cdc10p, Cdc10-C4p, in which the carboxy-terminal 61 amino acids of the protein are missing, show interesting effects on MCB-regulated transcription. At permissive temperatures *cdc10-C4* cells show constant overexpression of MCB-regulated genes, indicating that Cdc10p has both positive and negative roles in the control of MCB-regulated gene expression (McInerny *et al.*, 1995).

1.5.4.3.2 MBF components: Res1p

Res1p was identified as both a suppresser of the fission yeast *pat1-114* mutation (Tanaka *et al.*, 1992) and as a Cdc10p interaction partner (Caligiuri & Beach, 1993). Res1p shares significant homology with Res2p (Sturm & Okayama, 1996) and has been shown to specifically interact with MCB motifs and to heterodimerize with Cdc10p (Ayté *et al.*, 1995). Although *res1*$^{+}$ is not essential (Tanaka *et al.*, 1992) it has a significant role in G1–S-phase-specific transcription during the mitotic cell cycle, as in a *res1Δ* fission yeast strain MCB-regulated transcription is low and constant (Baum *et al.*, 1998) and overexpression of *res1*$^{+}$ causes an increase in MCB-regulated gene transcription (Ayté *et al.*, 1995; Baum *et al.*, 1998). However, Res1p is not thought to have a significant role in the meiotic cell cycle, as *res1Δ* fission yeast strains show no impairment in their progression through meiosis (Caligiuri & Beach, 1993; Tanaka *et al.*, 1992) and Res1p levels decrease during meiosis (Ayté *et al.*, 1997). In fact, Res1p is thought to inhibit entry into meiosis (Caligiuri & Beach, 1993; Tanaka *et al.*, 1992).

1.5.4.3.3 MBF components: Res2p

Res2p was isolated as both an extragenic suppresser of a fission yeast *res1Δ* mutation (Miyamoto *et al.*, 1994) and as a protein product from a fission yeast cDNA library that interacted with Cdc10p to induce MCB-regulated gene expression (Zhu *et al.*, 1994). Although it is not essential for mitotic MCB-regulated transcription, during the mitotic cell cycle, deletion of *res2*$^{+}$ caused the periodicity of MCB-regulated transcription to be lost (Baum *et al.*, 1998).

Furthermore, Res2p has been shown to be present in the MBF complex throughout the mitotic cell cycle (Ayté *et al.*, 1997; Whitehall *et al.*, 1999). Moderate overexpression of *res2*$^{+}$ during the mitotic cell cycle had little effect on MCB-regulated gene transcription (Baum *et al.*, 1998) but Res2p is part of the Cdc10-C4p MBF complex which causes constitutively high MCB-regulated gene expression (Whitehall *et al.*, 1999). So, like Cdc10p, Res2p does not act simply as either an activator or repressor of transcription during the mitotic cell cycle. However, the Cdc10p–Res2p complex is thought to require activation by both the mitotic-specific *trans*-activator Rep2p (Nakashima *et al.*, 1995; Tahara *et al.*, 1998) and the Pcl-like cyclin Pas1p (Tanaka & Okayama, 2000) for its mitotic cell cycle function.

Res2p has an essential role during the meiotic cell cycle: *res2*Δ fission yeast cells are unable to complete meiosis (Miyamoto *et al.*, 1994; Zhu *et al.*, 1994) and overexpressing *res2*$^{+}$ enhances entry into meiosis (Ayté *et al.*, 1997). In agreement with this Res2p is induced under nitrogen starvation conditions in diploid cells (Miyamoto *et al.*, 1994). Res2p levels increase and Res1p levels decrease following entry into the meiotic cell cycle although, concomitantly, the MBF complex becomes undetectable *in vitro* (Ayté *et al.*, 1997).

1.5.4.3.4 MBF components: Rep2p

Rep2p was identified as a multicopy suppresser of the *cdc10-129* fission yeast mutation and was shown to specifically interact with the Res2p-Cdc10p protein complex (Nakeshima *et al.*, 1995). Indeed, reconsitution experiments in budding yeast have shown that, in the absence of Rep2p, Res2p–Cdc10p can interact with MCB motifs but is unable to initiate MCB-regulated transcription (Tahara *et al.*, 1998). In *rep2*Δ fission yeast cells, MCB-regulated genes still show a periodic increase in transcript abundance but at greatly reduced overall levels in comparison to wild type fission yeast cells, further indicating that Rep2p has activating role in the MCB–MBF transcription system (Baum *et al.*, 1998). In agreement with this, overexpression of *rep2*$^{+}$ during mitosis causes constitutive, high levels of expression of MCB-regulated genes (White *et al.*, 2001). Furthermore, in wild type cells, the levels of *rep2*$^{+}$ mRNA decrease in response to G1-arrest through nitrogen starvation (Nakeshima *et al.*, 1995). Also, deletion of *rep2*$^{+}$ facilitates, whereas overexpressing *rep2*$^{+}$ inhibits, a nitrogen-starvation-induced G1-arrest, indicating that the mitotic-specific Rep2p might mediate the nitrogen signal in fission yeast (Nakeshima *et al.*, 1995).

1.5.4.3.5 The Rep1 protein

Rep1p was also identified as a multicopy suppressor of the *cdc10-129* fission yeast mutation (Sugiyama *et al.*, 1994). However, its function was found to be specific to the meiotic cell cycle as *rep1*Δ fission yeast cells were unable to initiate pre-meiotic DNA synthesis and no effect was

seen in this fission yeast strain during vegetative growth (Baum *et al.*, 1998; Sugiyama *et al.*, 1994). In agreement with its role in the meiotic cell cycle, the *rep1*$^{+}$ gene is moderately induced after nitrogen starvation and strongly induced during conjugation in a *ste11*$^{+}$ dependent fashion (Sugiyama *et al.*, 1994). Furthermore, *rep1*$^{+}$ is known to control expression of both *res2*$^{+}$ and the *rec*$^{+}$ family of meiotic-specific recombination genes during the meiotic cell cycle (Ding & Smith, 1998; Sugiyama *et al.*, 1994).

1.5.4.3.6 A model for MCB–MBF transcription during the mitotic cell cycle

It has been demonstrated that the MBF complex contains the Cdc10p Res1p and Res2p components throughout the course of a synchronous mitosis (Ayté *et al.*, 1997; Whitehall *et al.*, 1999). Also, the interaction of this complex with Rep2p has been shown to be important in the activation of MCB-regulated transcription (Nakeshima *et al.*, 1995; Tahara *et al.*, 1998). Finally, genetic analysis has shown that, when in combination with its kinase partner Pef1p, a Pcl-like cyclin, Pas1p, is also required for activation of the Cdc10p–Res2p complex (Tanaka & Okayama, 2000). How Pas1 is regulated remains to be discovered as the expression of the *pas1*$^{+}$ gene is not cell cycle regulated (Tanaka & Okayama, 2000). However, as Pas1p is difficult to detect in protein extracts and contains two PEST-rich domains (which target proteins for degradation) it is likely that Pas1p activity is regulated by programmed proteolysis (Tanaka & Okayama, 2000).

On the basis of current knowledge, therefore, we propose the following model for the regulation of MCB-regulated transcription during the mitotic cell cycle (Figure 1.10). The MBF complex is likely to work by targeting RNAPol II to MCB-containing promoter sequences. Whether this occurs via direct interaction with RNAPol II or via intermediate accessory transcription factors remains to be discovered. However, current evidence indicates that the Cdc10p–Res1p–Res2p complex is activated by the action of mitotic-specific Rep2p. Furthermore, the cyclin–CDK Pas1p–Pef1p complex is a possible candidate for instigating the periodic activity of the MBF complex via the phosphorylation and consequent induction of a conformational change in Res2p.

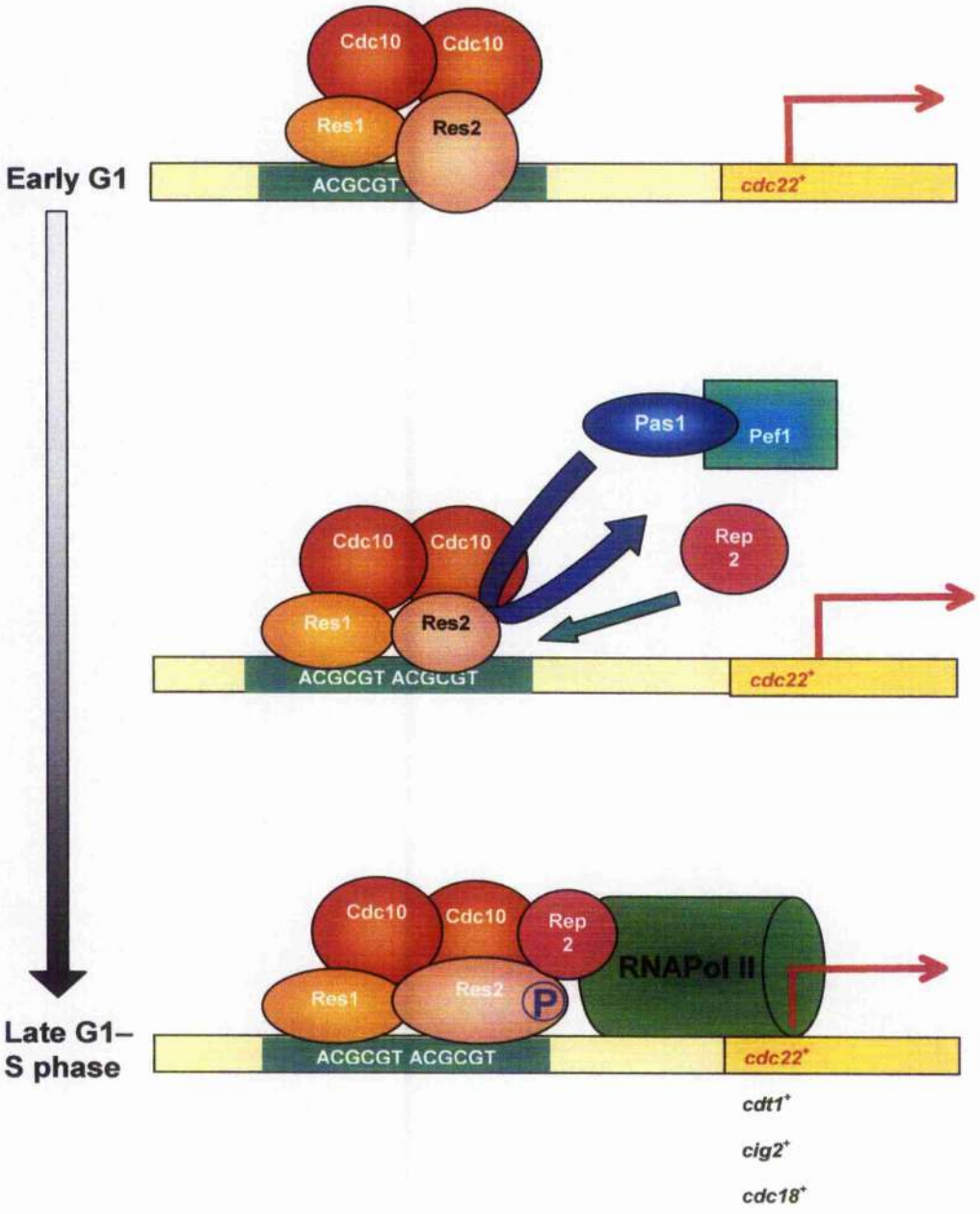


Figure 1.10: A model for MCB-MBF transcription during the mitotic cell cycle
In early G1 the Cdc10p–Res1p–Res2p complex is bound to MCB motifs but is unable to activate transcription. Interaction with both Rep2p and the Pas1p–Pef1p cyclin–CDK complex is required to induce MCB-regulated transcription. Rep2p interacts with Res2p to make MBF 'transcription competent' whereas phosphorylation of Res2p by Pas1p–Pef1p induces a conformational change in Res2p that is required for transcription activation.

1.5.4.4 G1–S cell-cycle-regulated transcription in humans

While the human E2F protein recognizes very similar DNA sequences to the budding yeast and fission yeast G1–S transcription factors and also controls the transcription of similar target genes (cyclins and proteins required for DNA synthesis), there is very little homology between these proteins at the level of protein sequence. By contrast, the ScSwi6p, ScSwi4p and ScMbp1p proteins of budding yeast and the Cdc10p, Res1p and Res2p proteins of fission yeast all contain four copies of the highly conserved ankyrin repeat domain as well as sharing highly homologous DNA binding domains between the ScSwi4p, ScMbp1p, Res1p and Res2p proteins (Whitehall, 1999; White, 2001).

There are six members of the E2F family in mammals, which all share homologous DNA-binding and dimerization domains with two related DP proteins — heterodimerization of an E2F protein with a DP protein is required for its function. E2F1 to E2F3 also share related CDK-binding domains and, similarly, E2F1 to E2F4 share related Retinoblastoma (RB)-binding domains. The mammalian RB family (RB, p107 and p130) of nuclear phosphoproteins also share several regions of homology, the most extensive of which is the 'pocket' domain that provides a binding site for several other proteins including E2F proteins.

The E2F transcription factor controls G1–S cell-cycle transcription by being, itself, under the control of the RB pocket proteins. E2F proteins are bound to the promoters of their target genes during G0 and early G1 phases but they are rendered transcriptionally inactive by being bound to an RB protein. In fact, the RB-bound E2F protein recruits the histone deacetylase HDAC1 to the associated promoter regions, which keeps the chromatin of the promoter region in a repressed state. To induce transcription, the synthesis of D-type cyclins must first be stimulated by external growth factors, which bind and activate HsCdk4 and HsCdk6. The active cyclin-D–HsCdk2/4 complexes are then able to phosphorylate the RB-family proteins, which dissociate from the E2F proteins allowing transcription of the genes under their control. This occurs as the cells are passing the restriction point towards the end of G1. The cyclin E protein is one of the proteins produced at this time and it replaces the D-type cyclins to bind HsCdk2, which further phosphorylates the RB proteins preventing them from binding the E2F proteins and providing a positive feedback loop that prolongs expression of E2F-responsive genes. This periodic transcription is turned off at the end of S phase by the action of another cyclin. Cyclin A eventually replaces cyclin E as the partner of HsCdk2 as S phase progresses and eventually cyclin-A–HsCdk2 binds to E2F1 to E2F3 and phosphorylates the E2F–DP complexes, which can then no longer bind to the promoter regions and dissociate from the DNA switching off periodic gene expression (White, 2001).

Chapter 2

Materials and Methods

2.1 Propagation and storage of fission yeast strains

All yeast strains used in this thesis are listed in Appendix I. Fission yeast strains were maintained in either liquid or in solid YE as appropriate (Appendix II; Moreno *et al.*, 1991). Solid media was made using 2% Difco Bacto agar. Strains containing plasmids or being used in experiments were propagated in EMM with the appropriate supplementary amino acids at a concentration of 100 μg ml^{-1} (Appendix II; Moreno *et al.*, 1991). Nitrogen free EMM was used to synchronise cultures in G1 prior to time course experiments (Appendix II; Moreno *et al.*, 1991). Freezer stocks were maintained in 25% glycerol 75% YE at –70°C.

Cell number per ml of liquid culture was determined from a sample fixed in a 0.1% formaldehyde/0.1% sodium chloride solution. Following brief sonication, cells were counted electronically with a Z2 Coulter Counter.

2.2 Propagation and storage of bacterial strains

All plasmids used in this thesis are listed in Appendix III. Basic bacterial culture was carried out as described in Sambrooke *et al.* (1989). Plasmids were manipulated and stored in the *Escherichia coli* strain DH5α. Bacterial strains were grown in Luria-Bertini media plus antibiotic (100 μg ml^{-1} ampicillin or 70 μg ml^{-1} kanamycin as appropriate). Freezer stocks were maintained in 70% LB 30% glycerol at –70°C.

2.3 Electro-transformation of Escherichia coli *DH5α*

In the electro-transformation of bacterial strains we used a BIORAD *E. coli* pulser and a method adapted from the BIORAD manual.

2.3.1 Preparation of competent cells

500 ml of LB were inoculated with 1/100 volume of fresh overnight culture. This culture was then left to shake vigorously at 37°C until an A_{600} of approximately 0.5–0.7 was obtained. The cells were harvested in 50 ml centrifuge tubes by centrifugation at 3000 rpm for 15 min in a chilled desktop centrifuge. As much supernatant as possible was removed and the cells were gently resuspended in a total of 100 ml of ice-cold 10% glycerol. The cells were centrifuged as before and resuspended in 50 ml ice-cold 10% glycerol. This was repeated and the cells resuspended in 20 ml ice-cold 10% glycerol. Finally the cells were centrifuged and resuspended in 1–2 ml ice cold 10% glycerol before being frozen at –70°C in 50 μl aliquots.

2.3.2 Electro-transformation

Aliquots of competent cells were thawed at room temperature and immediately stored on ice. 1–2 μl of the appropriate plasmid DNA was added to the cell suspension, mixed gently and left on

ice for 1–2 min. The cell–DNA suspension was transferred to a pre-chilled 0.2 cm electroporation cuvette and pulsed at 2.5 kV. 1 ml of SOC (2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) was added as quickly as possible. The cells were then transferred to microfuge tubes and incubated at 37°C for 30 min–1 h before plating out on selective medium.

2.4 Nucleic Acid isolation and quantification

2.4.1 Plasmid DNA isolation from bacteria

Small scale plasmid DNA preparation was performed from 10 ml overnight cultures. 3–10 ml of this culture were spun down into a 1.5 ml microfuge tube (2 x 1 min at 12000 rpm) using a microcentrifuge. The supernatant was removed and the pellet resuspended in 125 µl of P1 (50 mM Tris/HCl pH 8.0, 10 mM EDTA, RnaseA 100 µg ml^{-1}). 125 µl P2 (0.2 M NaOH, 1% SDS) was added and the tube gently inverted several times to mix. 125 µl P3 (2.55 M KOAc pH 4.8) was added and the tube immediately inverted several times before spinning at RT at 12000 rpm for 10 mins. The supernatant was carefully removed to a clean microfuge tube and 1 ml of 100% ethanol was added. This tube was then spun for a further 10 min at 12000 rpm, the supernatant discarded and the pellet washed in 100 µl 70% ethanol. The pellet was then allowed to dry at RT before it was resuspended in 35–50 µl dH_2O

Alternatively when clean DNA was required (e.g. for sequencing) a Qiaprep Spin Miniprep kit (Qiagen) was used according to the manufacturer's instructions.

2.4.2 Genomic DNA isolation from fission yeast

10 ml fission yeast cultures were grown to saturation (2–3 days) in 50 ml centrifuge tubes. Cells were collected by spinning for 5 min, at 3000 rpm, in a chilled desktop centrifuge. The supernatant was removed and the cells resuspended in 0.5 ml dH_2O. The resuspended cells were then transferred to screw top tubes and spun for 30 sec at 12000 rpm in a microcentrifuge to pellet the cells. The supernatant was decanted and the tubes vortexed to resuspend the cells in the residual liquid. 0.2 ml solution A (2% Triton X-100, 1% SDS, 100 mM NaCl, 100 mM Tris pH8, 1 mM EDTA), 0.2 ml 1:1 phenol/chloroform and 0.3 g acid washed glass beads were added to each tube. The tubes were then agitated in a ribolyser (Hybaid) for 5 x 40 sec at setting 4.0.

0.4 ml TE was added and the tubes spun for 5 min at 12000 rpm in a microcentrifuge. The aqueous layer was removed to a fresh microfuge tube leaving behind the liquid directly at the protein interface. 1 ml 100% ethanol was added and the tubes spun again for 5 min in a microcentrifuge at 12000 rpm to precipitate the nucleic acid. All the ethanol was carefully removed and the pellet resuspended in 0.4 ml TE and 0.3 µg RnaseA. This mixture was left at

37°C for 5 min to remove the RNA from the pellet. 8 µl 5M ammonium acetate and 1 ml 100% ethanol were then added to each tube and the contents thoroughly mixed before freezing at –70°C for 30 min. The tubes were spun for 5 min at 12000 rpm in a microcentrifuge and the supernatant was removed. The DNA pellets were air dried and left to resuspend in 100 µl dH_2O with gentle mixing to avoid shearing of the DNA.

2.4.3 Total RNA isolation from fission yeast

Total RNA was extracted from fission yeast using a method adapted from Kaufner *et al.* (1988). Cells were harvested by centrifugation at 2000 rpm for 5 min from a 30 ml experimental sample. The cells were washed in 1 ml of sterile dH_2O, transferred to a 1.5 ml microfuge tube, pelleted again by spinning for 30 sec at 12000 rpm and the supernatant discarded. The cells were usually stored at –70°C at this stage.

To extract the RNA the cells were resuspended in 200 µl STE and pelleted by spinning for 30 sec at 2000 rpm before resuspending in 100 µl STE. 600 µl NTES was added to each microfuge tube. 500 µl aliquots of phenol were heated to 65°C in flat-bottomed, screw top tubes containing approximately 7 mm depth of glass beads. The treated cells were then transferred to appropriately labelled phenol-containing tubes and the tubes pulsed for 3 x 40 sec in a Ribolyser (Hybaid) at setting 4.0. The mixture was then spun for 5 min in a microcentrifuge at 12000 rpm and the aqueous phase and protein interface removed to a second 500 µl aliquot of hot phenol. This mixture was then pulsed for 40 sec in a Ribolyser at setting 4.0 before spinning at 12000 rpm for 5 min in a microcentrifuge. The aqueous phase only was then removed to a 400 µl aliquot of hot phenol and pulsed for 40 sec in a Ribolyser before being spun down again for 5 min at 12000 rpm.

The aqueous phase only was then removed to a 400 µl aliquot of phenol chloroform at RT, which was pulsed in a Ribolyser and spun down as before. This step was then repeated twice. The aqueous phase was then removed to a 300 µl aliquot of chloroform at RT pulsed in a Ribolyser and spun down as before. The aqueous phase was then removed to a clean microfuge tube, 40 µl 3M NaOAc and 1 ml 100% ethanol added and the RNA left to precipitate at –70°C for at least 2 h. The next day the RNA was spun down for 10 min at 12000 rpm and the supernatent discarded. The pellet was then washed in 100 µl 70% ethanol (made with RNase-free water — Gibco). All the ethanol was carefully removed but the pellet was not allowed to dry completely as this makes it difficult to resuspend. The pellet was dissolved in 55 µl of RNase-free water, 5 µl of which was removed for quantification by spectrophotometry before the RNA was stored at –70°C.

2.4.4 Nucleic acid quantification

Nucleic acid concentrations were estimated by spectrophotometry at 260 nm and 280 nm, where an OD of 1 corresponds to 50 μg ml^{-1} of double-stranded DNA and 40 μg ml^{-1} of single-stranded DNA or RNA. Readings were zeroed with the solution in which the samples had been diluted. The ratio of $A_{260/280}$ provided an estimation of nucleic acid purity. Values between 1.8 and 2.0 indicated pure preparations (Sambrooke *et al.*, 1989).

2.4.5 Flow cytometry

Flow cytometry was performed to measure the DNA content of cells during cell cycle experiments. This provided a method for monitoring progression of the cells through S phase. 0.5–1 ml cell samples were taken at hourly time points during a cell cycle experiment. The cells were spun down (30 sec, 12000 rpm) and washed in 1 ml ice-cold dH_2O. The cells were then spun down again before being gradually resuspended in 1 ml of ice-cold 70% ethanol whilst vortexing. These fixed cells were then stored at 4°C until required.

For analysis, 300 μl of the fixed cell sample was spun down (30 sec, 12000 rpm) and rehydrated by washing in 1 ml 50 mM sodium citrate. The cells were then resuspended in 1 ml of 50 mM sodium citrate containing 0.5 μg RNase and 0.4 μg ml^{-1} propidium iodide and left to stain at 37°C for 2–4 h. A Becton Dickinson FACScan machine and CellQuest software were used.

2.5 DNA manipulation

2.5.1 Polymerase chain reaction (PCR)

All oligonucleotides used in this thesis are listed in Appendices III and IV. Standard PCR reactions were carried out in a Primus 25 Machine (MWG) with Taq DNA polymerase (Promega) using the following basic protocol:

95°C for 2 min

30 cycles {
- 95°C for 15 sec
- 50°C for 30 sec (temperature adapted as appropriate for primers)
- 68°C for 1 min (time adapted as appropriate — 1 min per kb of product)
- 68°C for 4 min

}

PCR reactions for products over 2 kb in length were carried out using the adapted protocol as follows:

95°C for 2 min

10 cycles {
- 95°C for 15 sec
- 50°C for 30 sec (temperature adapted as appropriate for primers)
- 68°C for 1 min (time adapted as appropriate — 1 min per kb of product)

}

95°C for 15 sec

25 cycles
- 50°C for 30 sec (temperature adapted as appropriate for primers)
- 68°C for 1 min (+ 5 sec every cycle)
- 68°C for 7 min

2.5.2 DNA fragment purification

DNA fragments to be used in ligations, or for use as DNA probes, required purification after identification on a 1% agarose gel. The appropriate fragments were excised with a scalpel and a QIAquick gel extraction kit was used as according to the manufacturer's instructions (Qiagen).

Alternatively, fragments less than 500 bp in length were purified using a method adapted from Sambrook *et al.* (1989). DNA was separated on a 6% acrylamide gel and the desired fragment excised with a scalpel. The end of a 500 µl pipette tip was sealed with a Bunsen flame and a siliconized glass wool plug inserted. The acrylamide pieces were transferred to the tip and mashed with a toothpick before resuspending in 200 µl elution buffer (0.2 M NaOAc, 1% SDS, 10 mM $MgCl_2$). The suspension was left at 37°C overnight. The following day, the end was cut from the blue tip with a scalpel and the liquid allowed to drain into a microfuge tube. A further 200 µl of elution buffer was passed through the tube and drained into the same microfuge tube. The tube was then spun for two mins at 12000 rpm in a microcentrifuge. The liquid was carefully removed to a clean eppendorf tube, being sure to leave any pelleted acrylamide behind. 1 µl of glycogen was added to the solution and the contents mixed thoroughly before 40 µl of 3 M sodium acetate and 800 µl 100% ethanol were added and the tube placed at –70°C for at least 2 h. The tube was then spun at 12000 rpm in a microcentrifuge for 20 min, the supernatant removed and the pellet washed in 90% ethanol before resuspending in 20 µl dH_2O.

2.5.3 Restriction digests

Restriction digests to check miniprep DNA were carried out in a 37°C incubator as follows:

Miniprep DNA	3 µl
Appropriate buffer	2 µl
Enzyme	0.5 µl
dH_2O	14.5 µl

Exceptions were *Apa*II digests, which required 25°C incubation, and *Nco*I digests, in which a second 0.5 µl aliquot of enzyme was added after 15 min due to the short half-life of the enzyme.

2.5.4 Ligation of DNA

Ligation of DNA was typically performed using T4 DNA ligase (Promega) according to the manufacturers instructions. A ratio of 3:1 insert to vector was normally used and the reactions left for several hours at 4°C.

2.6 Northern analysis

2.6.1 Preparation of DNA probes

DNA probes were made by PCR corresponding in each case to ~1 kb of the orf of each gene. DNA probes were labelled with [α-^{32}P] dCTP using the random hexa-nucleotide labelling procedure of Feinberg & Volgelstein (1983).

2.6.1.2 Purification of radiolabelled DNA probes

Sephadex G-50 (Amersham Pharmacia-Biotech) was prepared by adding two volumes of TE and autoclaving. The plunger was removed from a 1 ml syringe (Plastipak) and a small wad of siliconized glass wool was used to plug the end before the end was placed inside a microfuge tube. Sephadex G-50 was added to the syringe, the syringe and the microfuge tube were placed inside a 50 ml centrifuge tube and then centrifuged at 2000 rpm for 2 min to remove the TE. This was repeated until ~7 ml Sephadex G-50 remained in the syringe. A fresh screw-top microfuge tube was then placed at the bottom of the syringe and the radiolabelled probe added to the Sephadex G-50 column. This was spun again at 2000 rpm for 2 min and the syringe was monitored with a Geiger counter to confirm that unincorporated nucleotides had been removed from the probe. The screw-top microfuge tube containing the purified probe was then heated for 5 min at 95°C and chilled on ice for 5 min to denature the probe before use.

2.6.2 Northern analysis

Running gel. Samples containing 10 µg of RNA were prepared in 20 µl of RNA buffer (600 µl formaldehyde, 200 µl formamide, 240 µl 5x MNE buffer, 160 µl H_2O. 1 µl of a 1 in 50 dilution of ethidium bromide was added to each sample and they were incubated at 65°C for 5 minutes before loading. A 1% agarose gel containing 0.65 % formaldeyde and 20% 5 x MNE buffer was used and run in 1 x MNE buffer at either 70 volts for 3–4h or overnight at 15 volts. (5 x MNE buffer: 0.12 M MOPS, 25 mM NaOAc, 5 mM EDTA (pH 7)).

Transfer of RNA to nitrocellulose membrane. Before blotting, the RNA gel was soaked in 50 mM NaOH for 15 min to facilitate transfer of the larger transcripts and then in 10 mM Na_2HPO_4/NaH_2PO_4 (pH 6.5) for 15 min to remove the formaldehyde from the gel. The nylon membrane (Genescreen) was also briefly soaked, firstly in water, then in 10 mM

Na_2HPO_4/NaH_2PO_4 (pH 6.5) before blotting. The capillary method was then used to blot the gel overnight. Transfered RNA was fixed to the membrane by crosslinking in a UV oven for 1 min and baking at 80°C for 3 h.

Probing membrane. The membrane was prepared for hybridization of the probe by incubation at 42°C for 1–2 h in pre-hybridization buffer (10 ml formamide, 4 ml P buffer, 4 ml 33% (w/v) Dextrane sulphate, 1.16 g NaCl, 200 µl denatured salmon sperm DNA. (P buffer: 1% BSA, 1% pyrolidine, 1% FICOLL, 250 mM TrisCl, 0.5% Na Pyrophosphate, 5% SDS)). The appropriate radiolabelled DNA probes were denatured and added directly to the pre-hybridization buffer and left to probe overnight.

Washing membrane. The following day the membrane was washed twice for five minutes in 2 x SSPE at 42°C and then in 0.2 x SSPE, 0.5% SDS at 65°C for periods of 15 min until an acceptable signal was obtained. (20 x SSPE; 3 M NaCl, 0.2 M NaH_2PO_4, 0.02 M EDTA (pH 7.4)).

Stripping membrane. When required for re-hybridization, membranes were stripped by washing for 30 min–2 h in strip solution at 70°C until no radioactivity could be detected. (Strip solution; 5 mM TrisCl (pH 8.0), 0.2 mM EDTA, 0.05% Na pyrophosphate, 0.002% polyvinyl pyrolidine, 0.002 % BSA, 0.002% FICOLL).

2.6.3 Quantification of northern analysis

Intensity of radioactive signals was quantified using the Image J programme and normalized to the corresponding rRNA gels. Triplicate measurements were made for each time point and the results from at least two separate experiments were averaged to give the results shown.

2.7 Electophoretic mobility shift assay (EMSA)

2.7.1 Probe preparation

DNA fragments were 5′ end-labelled using T4 polynucleotide kinase (PNK). To 5–10 µg of DNA in 5 µl of dH_2O on ice was added 10 units of T4 PNK in 1 x T4 PNK buffer (Promega), followed by the addition of 10 µCi of [γ-^{32}P] dATP (Amersham, 10 µCi µl^{-1}) to give a total volume of 8 µl. The reaction was incubated at 37°C for 1 h, and the DNA was purified using the Sephadex G-50 column as in Section *2.6.1.2* except that the purified probe was not denatured.

2.7.2 Protein extraction from fission yeast

200 ml cultures of fission yeast cells, in mid-exponential stage of growth, were prepared and harvested by centrifugation at 5000 rpm for 10 minutes in screw cap centrifuge tubes. The cell pellet was re-suspended in 200 µl of ice cold lysis buffer [50 mM KCl, 50 mM tris.Cl pH 8,

25% glycerol, 2 mM DTT (dithiothreitol, Sigma), 0.1% Triton X-100, 5 μg of protease inhibitors: chymostatin, pepstatin, antipain, leupeptin, aprotonin (Sigma), 0.2 mM PMSF (phenylmethanesulfonylfluoride)] in 2 ml screw capped micro-centrifuge tubes. The cells were pelleted at 13,000 rpm for 1 min in a high-speed micro-centrifuge and again re-suspended in 200 μl of lysis buffer. Acid-washed glass beads (425–600 micron, Sigma) were added to just beneath the meniscus and the tubes were chilled on ice for 2–3 min before being disrupted using a Ribolyser (Hybaid Ltd, UK) with 1 burst at 40 sec, setting 4. The cell debris was pelleted by centrifugation at 13,000 rpm for 5 min at 4°C and the protein supernatants were transferred to a fresh chilled micro-centrifuge tube, and clarified by centrifugation at 13,000 rpm for 30 min at 4°C.

Supernatants were transferred to a fresh chilled micro-centrifuge tube and 5 μl was removed to determine protein concentration, which was estimated using Bradford's reagent (Biorad) according to the manufacturer's instructions, and the remainder of the protein sample was snap frozen on solid CO_2, and stored at –70°C.

2.7.3 EMSA

Each EMSA was performed by adding 10 μl of sample buffer [1 M Tris.Cl pH 7.5, 1 M KCl, 50% Glycerol, 100 mM DTT, 100 mM protease inhibitors: chymostatin, pepstatin, antipain, leupeptin, aprotonin (Sigma), 100 mM PMSF, 1 M $MgCl_2$] to 20 μg of protein, 1 μg of dIdC (1 mg ml^{-1}; Pharmacia) and 1–2 μl of labelled probe. If required, non-specific or specific competitor DNA was also added. The samples were left for 5 min on ice before the addition of each reagent. Analysis of the formation of protein–DNA complexes was achieved by electrophoresis of samples on a 10% acrylamide gel in 1 x TAE buffer (40 mM Tris-acetate, 1 mM EDTA pH 8) for 1.5–2 hours at 180 V. The gel was dried for 1.5 h at 80°C and exposed to autoradiography film at –70°C.

2.8 Fission-yeast-specific methods

2.8.1 Random spore mating

Strains to be mated were spread out on fresh medium in a small patch and left to grow overnight. Half the patch of each strain was transferred to the centre of an ME plate and the two strains were mixed together. A separate, unmixed patch of each strain was transferred to the side of the plate as control patches and the plates were left for three days at 25°C.

The mated cells were lifted into 100 μl dH_2O and 20 μl glucilase (Sigma) and incubated overnight at 36°C. A series of dilutions of the glucilase-treated mixture were then plated onto YE and left at 25°C until colonies formed (5–7 days). 100–300 single colonies were picked and streaked in small patches onto YE, the plates were left at 25°C overnight and then replica plated

onto selective EMM to determine their phenotype. Patches of cells determined to be of the correct genotype were then streaked out on YE to obtain single colonies and the single colonies replica plated again to re-check the genotype.

2.8.2 Mating type determination

Strains to be tested were patched out on fresh medium and grown overnight along with a known h^+ strain and a known h^- strain. Samples of each strain to be tested were then mixed with each strain of known mating type and incubated for three days at 25°C. A few iodine crystals were then poured into the lid of the ME plate and left for 10–20 min. Where mating was successful the patched strain mixtures stained dark brown/black as the iodine vapour stains the starch reserves found in spores.

2.8.3 Electro-transformation of fission yeast

In the electro-transformation of fission yeast we used a BIORAD *E. coli* pulser and a method adapted from the BIORAD manual.

Preparation of competent cells. 500 ml of EMM plus appropriate supplements were inoculated with a 10 ml overnight culture. This culture was grown at 25°C until a density of approximately 1 x 10^7 cells per ml was obtained. The cells were harvested in 50 ml centrifuge tubes by spinning at 3000 rpm for 5 min in a chilled desktop centrifuge. As much supernatant as possible was removed and the cells were gently resuspended in a total of 100 ml of ice-cold 1 M sorbitol. The cells were centrifuged as before and resuspended in 50 ml ice-cold 1 M sorbitol. This was repeated and the cells resuspended in 20 ml ice-cold 1 M sorbitol. Finally the cells were centrifuged and resuspended in 1–2 ml ice cold 1 M sorbitol before being frozen at –70°C in 50 µl aliquots.

Electro-transformation. Aliquots of cells were thawed at room temperature and immediately stored on ice. 1–2 µl of the appropriate miniprep DNA was added to the cell suspension, mixed gently, and left on ice for 1–2 min. The cell–DNA suspension was transferred to a pre-chilled 0.2 cm electroporation cuvette and pulsed at 1.5 kV. 1 ml of 1 M sorbitol was added immediately and the cells were plated out on selective medium.

2.8.4 Lithium acetate transformation of fission yeast

The appropriate strain was grown to a density of 1 x 10^7 cells per ml in 500 ml of YE. The cells were harvested by spinning for 5 min in a chilled desktop centrifuge and resuspended in a total of 200 ml ice-cold dH_2O. The cells were spun down again, transferred to a microfuge tube and washed once with 1 ml LiAc/TE (prepared from 10 x filter-sterilized stocks of 1 M LiAc pH 7.5 and TE (0.1 M Tris.Cl, 0.01 M EDTA pH 7.5). The cells were spun down for 30 sec at 12000 rpm in a microcentrifuge and resuspended at an approximate density of 2 x 10^9 cells per ml.

100 µl of concentrated cells were mixed with 2 µl denatured salmon sperm DNA and 10 µl of transforming DNA and left at room temperature for 10 mins. 260 µl of PEG/LiAc/TE was added (8 g PEG 4000 (polyethyleneglycol average MW 4000), 2 ml 10 x LiAc, 2 ml 10x TE, 9.75 ml dH_2O). The mixture was gently agitated and incubated at 25°C for 30–60 min. 43 µl DMSO were added and the cells heat shocked for 5 min at 42°C. The cells were washed once in 1 ml dH_2O and resuspended in 0.5 ml dH_2O before plating out onto selective medium.

2.8.5 Confirmation of plasmid instability in yeast strains

Transformed cells from a selective master plate were streaked onto rich media (YE). Growth was established and colonies replica plated back onto selective media. After 2–3 days at 25°C plates were examined for replica colonies that failed to grow. This indicates plasmid loss has occurred and that growth was not due to plasmid integration into the chromosome or a reversion mutation of the yeast nutritional markers.

2.8.6 Meiosis induction

500 ml cultures of the appropriate *pat1-114* fission yeast strain were grown at 25°C to a cell density of approximately 2–4 x 10^6 cells per ml, in EMM containing the appropriate supplements at a concentration of 100 µg l^{-1}. The cells were harvested (2000 rpm 2 min) and washed twice in nitrogen-free EMM before being re-suspended in nitrogen free EMM plus supplements at a concentration of 50 µg l^{-1}. The culture was incubated at 25°C overnight to arrest the cells in the G1 phase of the cell cycle. The next day, to give a more synchronous meiosis, ammonium chloride was added back to the cultures to a concentration of 0.5 g l^{-1} and a further 50 µg l^{-1} of the appropriate supplements were added. To raise the temperature as quickly and evenly as possible, the flasks were shaken in a 50°C water bath until the contents reached 35°C and immediately transferred to a 35°C shaking water bath. 35 ml samples were then taken at time 0 and every 15 min for 3 h. The cells were harvested (2000 rpm 2 min) and the pellet washed in 500 µl RNase-free water. The cells were then removed to a microfuge and spun (12000 rpm, 30 sec) to remove the supernatant and the pellets stored at –70°C until required.

2.8.7 Induction of gene overexpression

Several fission yeast strains containing the pREP1 vector with the appropriate insert were used to overexpress components of the MBF complex. The pREP1 vector uses the *nmt1*$^+$ (no message in thiamine) promoter to control expression of the inserted genes.

Strains were streaked from glycerol stocks onto EMM plates containing the appropriate amino acids for nutritional selection of the plasmid, plus thiamine at a concentration of 5 µg μl^{-1} (*nmt1*$^+$ promoter 'off'). 15 ml cultures were grown to saturation at 25°C with the appropriate

amino acids and 5 μg μl^{-1} thiamine. These were used to inoculate identical 500 ml cultures, which were harvested at the mid-exponential stage of growth. Cells were washed three times in thiamine-free EMM, and then grown for 16 h in EMM without thiamine (*nmt1*$^{+}$ promoter 'on') and nitrogen at 25°C, before thermal induction of meiosis was undertaken as before.

2.8.8 Protoplast fusion

This method was used to make a stable diploid fission yeast strain containing the *pat1-114* mutation from two haploids of the same mating type. For SP solutions see Appendix 1. The two haploid strains to be fused (strains A and B) were cultured in 10 ml EMM plus supplements for 1–3 days at permissive temperature, then transferred to 100 ml EMM plus supplements, and incubated overnight with shaking until the culture was in exponential phase ($2 \times 10^6 - 1 \times 10^7$ cells per ml). The cells were pelleted by centrifugation at 3000 rpm for 5 min and the supernatant poured off. The cells were resuspended in 10 ml SP1 and 20 μl 2-β mercaptoethanol and incubated at room temperature for 10 minutes. Cells were pelleted by centrifugation at 3000 rpm for 5 min, the supernatant was discarded, and they were resuspended in 10 ml SP2 (see Section *2.6.2.2*). 20 mg of zymolyase 100T (Seikagaku, cat. no: 120493), which hydrolyses the glucose bonds in the yeast cell wall, was then added and incubated at 35°C for 30 min–1 h (25°C if *ts* mutant), checking regularly until 50% of the cells were spheroplasted. This was tested by placing a 5 μl droplet of cells onto a glass slide with 5 μl 0.5 % SDS. This lysed spheroplasted cells giving them a 'ghostly' appearance when compared to cells without SDS. 30 ml of SP3 was then added and the cells pelleted at 3000 rpm for 5 minutes. Pellets were washed twice in 30 ml SP3, discarding the supernatant, and resuspended in 1 ml of SP4 (see Section *2.6.2.4*).

Three microfuge tubes were set up containing: (i) 100 μl strain A, (ii) 100 μl strain B, (iii) 50 μl strain A + 50 μl strain B. 1 ml of SP5 was added to each tube, the cells gently mixed, then incubated at room temperature for 30 min. The cells were gently pelleted at 2000 rpm for 5 min and the supernatant was poured off. Cells were then resuspended in 100 μl SP4. Strains A and B carried complementing *ade*$^{-}$ alleles, so that their successful fusion formed an *ade*$^{+}$ diploid. To isolate *ade*$^{+}$ diploids, cells were spread onto EMM plates plus supplements containing 1 M sorbitol and lacking adenine, and incubated at permissive temperature for 7–14 days, until large white colonies had grown.

Chapter 3

Periodic gene expression during meiosis in fission yeast

During S phase of the mitotic cell cycle a eukaryotic cell must replicate its genome to prepare for cell division. In yeast species, entry into S phase and progression through the mitotic cell cycle is controlled at a point in late G1 phase known as START, which is analogous to the restriction point in metazoans (Nurse, 1998; Stevens & LaThangue, 2003). Passage through this control point is governed by two major mechanisms: specific Cdk activity is required to modulate the function of various proteins essential for the initiation of, and passage through, S phase, and transcriptional activation of a subset of genes — many of which are essential for S phase onset and progression — is also required (Moser & Russell, 2000). The transcriptional control of these genes results in their periodic expression between late G1 and S phase of the mitotic cell cycle, the mechanism of which has been extensively studied in both budding yeast and fission yeast (MacNeill & Nurse, 1997).

3.1.1 Budding yeast G1–S transcription

In budding yeast, two parallel transcription systems operate at the start of S phase, ScSBF and ScMBF. The ScSBF transcription factor is a heterodimer of ScSwi4p and ScSwi6p and regulates genes containing the consensus sequence 5' -CGCGAAAA- 3' in their promoter region. The ScMBF transcription factor is a heterodimer of ScSwi4p and ScMbp1p and recognises a similar consensus sequence of 5' -ACGCGN- 3' in gene promoter sequences (McIntosh, 1993). DNA microarray analysis has shown that the ScSBF activated genes are predominantly involved in budding and membrane and cell wall biosynthesis, whereas ScMBF activated genes are biased towards having DNA replication and repair functions. Thus, these highly related mechanisms allow distinct molecular processes to be regulated independently during the mitotic cell cycle whilst remaining temporally coordinated (Vishwanath *et al.*, 2001).

3.1.2 Fission yeast mitotic G1–S transcription

In fission yeast a transcriptional control mechanism analogous to the budding yeast ScSBF/ScMBF system has been identified, although, a single transcription factor complex controls one group of genes in this organism (MacNeill & Nurse, 1997). The consensus sequence 5' -ACGCGT- 3', or MCB element, is present in the promoter regions of a number of genes that undergo periodic transcription at the G1–S boundary of the mitotic cell cycle (Mata *et al.*, 2002). MCB motifs are recognized by the multi-subunit transcription factor MBF, also known as DSC1. Fission yeast mitotic MBF however, consists of at least four different components: Cdc10p, Res1p, Res2p and Rep2p (MacNeill & Nurse, 1997). The genes *cdc22*$^{+}$, *suc22*$^{+}$, *cdc18*$^{+}$, *cdt1*$^{+}$, *cig2*$^{+}$, *ste6*$^{+}$, *ste9*$^{+}$, *rad21*$^{+}$, *rad11*$^{+}$, *cdt2*$^{+}$ and *mik1*$^{+}$ (Fernandez-Sarabia, *et*

al., 1993; Kelly *et al.*, 1993; Conolly & Beach, 1994; Hofman & Beach, 1994; Kersey, 1995; Birkenbihl & Subramani, 1995; Parker *et al.*, 1997; Tournier & Millar, 2000; Ng *et al.*, 2001; Yoshida *et al.*, 2003) are known to be expressed under the control of this complex during mitosis and this transcriptional regulation is required for passage through START and entry into the pre-mitotic S phase (MacNeill & Nurse, 1997).

Similarly, entry into pre-meiotic S phase is also known to depend upon the activity of the MCB–MBF system (Zhu *et al.*, 1994; Miyamoto *et al.*, 1994; Ayté *et al.*, 1997). Passage through START during meiosis however, is thought to use a different form of the MBF complex. Meiosis is initiated in a complex cascade following the action of Mei2p after conjugation (Yamamoto, 1997), but in order to enter pre-meiotic S phase the action of the meiotic-specific protein Rep1p is required (Sugiyama *et al.*, 1994) and Rep1p is also known to affect the transcription of the *rec*$^{+}$ genes (Ding & Smith, 1998). The Res2p subunit of MBF is particularly significant during meiosis whereas levels of the Res1p subunit are decreased in comparison with the mitotic MBF complex (Ayté *et al.*, 1997). However, it is likely that this altered MBF transcription complex coordinates the transcription of genes required for pre-meiotic S phase in a similar manner as occurs in the mitotic cell cycle. Furthermore, a recent study, which characterized global gene transcription during meiosis, suggested that the meiotic MCB–MBF transcription system might control a large number of genes (Mata *et al.*, 2002).

3.1.3 Global transcription during meiosis

Mata *et al.* (2002) used microarray technology to examine transcription of all fission yeast genes during both a wild type diploid meiosis and a highly synchronous *pat1-114* induced meiosis. Over one thousand genes were discovered to be upregulated more than fourfold during meiosis, when compared to expression during vegetative growth. These genes were categorized into four distinct groups: delayed, early, middle and late, using the timing of their expression profiles as their defining feature (Mata *et al.*, 2002). When gene function was considered, the early group was found to represent genes required for S phase and recombination, and includes the genes previously identified as being under MBF control, i.e. *cdc22*$^{+}$ *et al.* This group has 100 members and computer analysis showed that the promoter regions of these genes were significantly enriched in MCB motifs (Mata *et al.*, 2002). Thus, the coordinated expression profiles and abundance of MCB elements in the promoters of these genes suggests that many, if not all, of these early genes may be regulated by the same MCB–MBF related system.

Interestingly, another group of genes, which belong to the *rec*$^{+}$ gene family, also fell within this early group (Mata *et al.*, 2002). *rec*$^{+}$ genes were isolated in a screen for fission yeast mutants which affected meiotic recombination (DeVeux *et al.*, 1992), and, consistent with their function during the process of meiosis, *rec*$^{+}$ gene expression was thought to be exclusively limited to the meiotic cell cycle (Ding & Smith, 1998).

That a large group of genes appear to be under MBF control, but subsets of genes within this group are expressed specifically during the different developmental cell cycles, indicates that there must be significant differences in the mechanism of G1–S transcription regulation between the mitotic and meiotic cycles in fission yeast. These differences might occur on two levels: first, the components of MBF might differ significantly during the meiotic cell cycle, and second, there might be differences in the MCB motifs, which allow the alternative forms of the MBF complex to recognize different subsets of genes in the two cell cycles.

In this thesis, we wish to address the issue of whether meiotic-specific G1–S transcription is under the control of MCB elements and the MBF complex in fission yeast. As periodic G1–S transcription is a significant controlling factor in the passage into both pre-mitotic and pre-meiotic S phase, the switch between the mitotic and the meiotic MCB–MBF programmes of transcription represents a crucial step in the developmental transition between the two life cycles. We want to identify differences between the mitotic and meiotic MCB–MBF systems of transcription, as these differences might be of developmental significance for this organism.

Genes of the *rec*$^{+}$ family were first identified in a screen for mutations that caused reduced recombination efficiency between the *-216* and *-210* alleles of the *ade6* gene (DeVeaux *et al.*, 1992; Lin *et al.*, 1992). Several of these *rec*$^{+}$ genes were initially found to be specific to meiosis, in particular, the *rec6*$^{+}$, *rec7*$^{+}$, *rec8*$^{+}$, *rec10*$^{+}$, *rec11*$^{+}$, *rec12*$^{+}$ and *rec15*$^{+}$ genes were shown to be transiently expressed during the meiotic cell cycle (Lin & Smith, 1992; Lin & Smith, 1994; Lin & Smith, 1995a; Lin & Smith, 1995b; Li *et al.*, 1997). Their transcription was subsequently found to require the global regulator Rec16p, which is identical to Rep1p (Ding & Smith, 1998).

The *rep1*$^{+}$ gene was independently isolated as a multicopy suppressor of the *cdc10-129* mutation that causes the mitotic cell cycle to arrest at START (Sugiyama *et al.*, 1994). Rep1p expression is induced under conditions of nitrogen starvation and it has been shown to be required for the initiation of pre-meiotic DNA synthesis — possibly owing to its ability to induce expression of, or activate Res2p (Sugiyama *et al.*, 1994). This implies that Rep1p might be part of, or act through, MBF to induce meiotic-specific *rec*$^{+}$ gene expression. However, the mechanism which confines the expression of *rec*$^{+}$ genes to meiosis is unclear, as other genes known to be under MBF control are expressed in a periodic fashion in both mitosis and meiosis (Ayté *et al.*, 1997; Mata *et al.*, 2002).

The fact that several genes have similar transcription profiles during the cell cycle, and also contain specific UAS sequences in their promoters, supports the idea that these genes might be under the control of a single transcription factor. Initially, therefore, we wanted to confirm that the transcription profiles of the *rec*$^{+}$ genes during the meiotic cell cycle closely resemble those of genes known to be under MCB–MBF control, such as *cdc22*$^{+}$. To this end, a 'wild type' diploid fission yeast culture was induced to undergo a synchronous meiosis, and samples were taken every 15 minutes to allow the mRNA profiles of several genes to be closely monitored throughout a meiotic cell cycle.

3.2.1 pat1-114 *synchronous meiosis*

Under normal circumstances, fission yeast cells enter the meiotic cell cycle from G1 phase, after having been starved for nutrients (particularly nitrogen) and having undergone conjugation to become diploid (Yamamoto, 1996). In the context of cell cycle experiments, a high degree of synchrony is required to allow the examination of cell cycle processes. For this reason, a temperature sensitive mutation in the *pat1*^{ts} gene, which plays a pivotal role in the induction of meiosis, has been extensively used to achieve a highly synchronous meiosis in fission yeast (Bähler *et al.*, 1991). Pat1p is a serine/threonine protein kinase, which phosphorylates and inactivates Mei2p, a protein critical for the induction of meiosis. When diploid cells become starved for nitrogen, Pat1p is inactivated by Mei3p, allowing functional Mei2p to accumulate

and induce meiosis. However, *pat1-114* becomes inactivated at higher temperatures causing immediate entry into meiosis due to the accumulation of active Mei2p. Therefore, shifting a *pat1-114* culture to a higher temperature can be used to induce a highly synchronous meiosis in either haploid or diploid cells, as Pat1-114p is simultaneously inactivated throughout the culture (Yamamoto, 1996; Nurse, 1985; Iino & Yamamoto, 1985; Bähler, 1991).

A diploid fission yeast culture homozygous for *pat1-114* (*h⁻ /h⁻, pat1-114/ pat1-114, leu1-32/leu1-32, ura4-294/ura4-294, ade6-M210/ade6-M216*; GG 375) was grown in minimal media at 25°C to a cell density of between 5 and 8 x 10^6 cells per ml before being transferred to nitrogen-free EMM overnight to arrest the cells in G1 phase. The cell density was found to be critical to the success of the experiment, as a less dense culture yielded too few cells for the subsequent RNA extraction. Also, a higher cell density meant that the cells would reach stationary phase and stop growing before they became starved for nitrogen, which resulted in failure to induce meiosis. Leucine and uracil were added to the nitrogen free media but at 50 μg l^{-1}, half the normal concentration, to reduce the possibility that the amino acids may act as a nitrogen source. To encourage a more synchronous meiosis, the following day more leucine and uracil were added to restore the amino acid concentration to 100 μg l^{-1}, and NH_4Cl was added back to the culture at a concentration of 0.5g l^{-1} (Muramaki & Nurse, 1999). The temperature of the culture was then raised to 36°C and 35 ml samples taken every 15 minutes for RNA extraction and 1 ml samples taken hourly for flow cytometry analysis. Flow cytometry analysis permitted the quantification of the DNA content of cells, which allowed the time of meiotic S phase to be calibrated, thus giving an indication of cell synchrony.

Total RNA was extracted from the time course samples and 10 μg aliquots separated on a denaturing formaldehyde gel (Section *2.4.3*). Northern blot analysis of the RNA was then carried out using denatured double stranded DNA probes (Section *2.6*). The probes were amplified by PCR and corresponded to the ORFs of the various genes of interest (Appendix IV) and labelled with (α^{32}P) dCTP using the random hexa-nucleotide primer method (Feinberg & Vogelstein, 1983), before being purified on a Sephadex column to remove unincorporated nucleotides (Section *2.6.1.2*).

3.2.2 Meiotic G1–S transcript analysis

The northern blot of the meiotic time course was hybridised with probes for *cdc22⁺*, *cdc18⁺*, *cdt1⁺*, *cig2⁺*, *mik1⁺* and *suc22⁺* to identify their transcript profiles during meiosis (Figure 3.1). In each case transcript abundance was exceptionally low or absent directly after nitrogen starvation. A gradual increase in mRNA levels occurred around 30 minutes onwards after meiosis induction, reaching a peak of expression at about 90 minutes. Therefore, transcript levels peaked just before the cells entered S phase at two hours after meiotic induction — as

indicated by flow cytometry analysis. This corresponds to the peak of transcription seen just before S phase in the mitotic cell cycle (White *et al.*, 2001).

When the same membrane was probed for meiosis specific *rec*$^+$ genes, multiple transcripts for the *rec7*$^+$, *rec12*$^+$ and *rec15*$^+$ genes were detected on a northern blot. However, in each case an abundant, periodic meiotic-specific transcript was identified: it is this transcript which is depicted in Figure 3.1A. A detailed analysis of the transcription products of the *rec7*$^+$ gene has been undertaken (Molnar *et al.*, 2001) and it identified several mRNA products that originate from the complementary DNA strand, as well as mRNA products larger than expected, which the authors attribute to incomplete transcription termination (Molnar *et al.*, 2001). Thus, multiple transcripts and unusually long mRNAs are a recognized feature of recombination genes — including several instances in budding yeast — although the biological function of this feature is not understood (Menees *et al.*, 1992; Pittman *et al.*, 1993; Molnar *et al.*, 2001). Nevertheless, functional length transcripts for each of the *rec*$^+$ genes were found to conform to a similar pattern of expression as the *cdc22*$^+$ group of genes whilst remaining meiotic specific. Specifically, transcripts are detected at around 30 minutes after meiosis induction and rise to a peak of expression between 105–120 minutes.

The expression profiles characterized in this work by northern blot analysis are consistent with previously published work (Fernandez-Sarabia *et al.*, 1993; Kelly *et al.*, 1993; Connolly & Beach, 1994; Hofmann & Beach, 1994; Ding & Smith, 1998; Yoshida *et al.*, 2003) and with the data recently obtained using microarray analysis (Mata *et al.*, 2002). One notable exception, however, was the *rec6*$^+$ gene, which appears to be significantly induced in meiosis in both the microarray analysis and previous studies (Mata *et al.*, 2002; Lin & Smith, 1994). In the data presented here *rec6*$^+$ was only weakly expressed, although an abundant transcript was detected in the mitotic sample.

Figure 3.1: G1–S-specific gene expression during a synchronous diploid* pat1-114 *meiosis in fission yeast

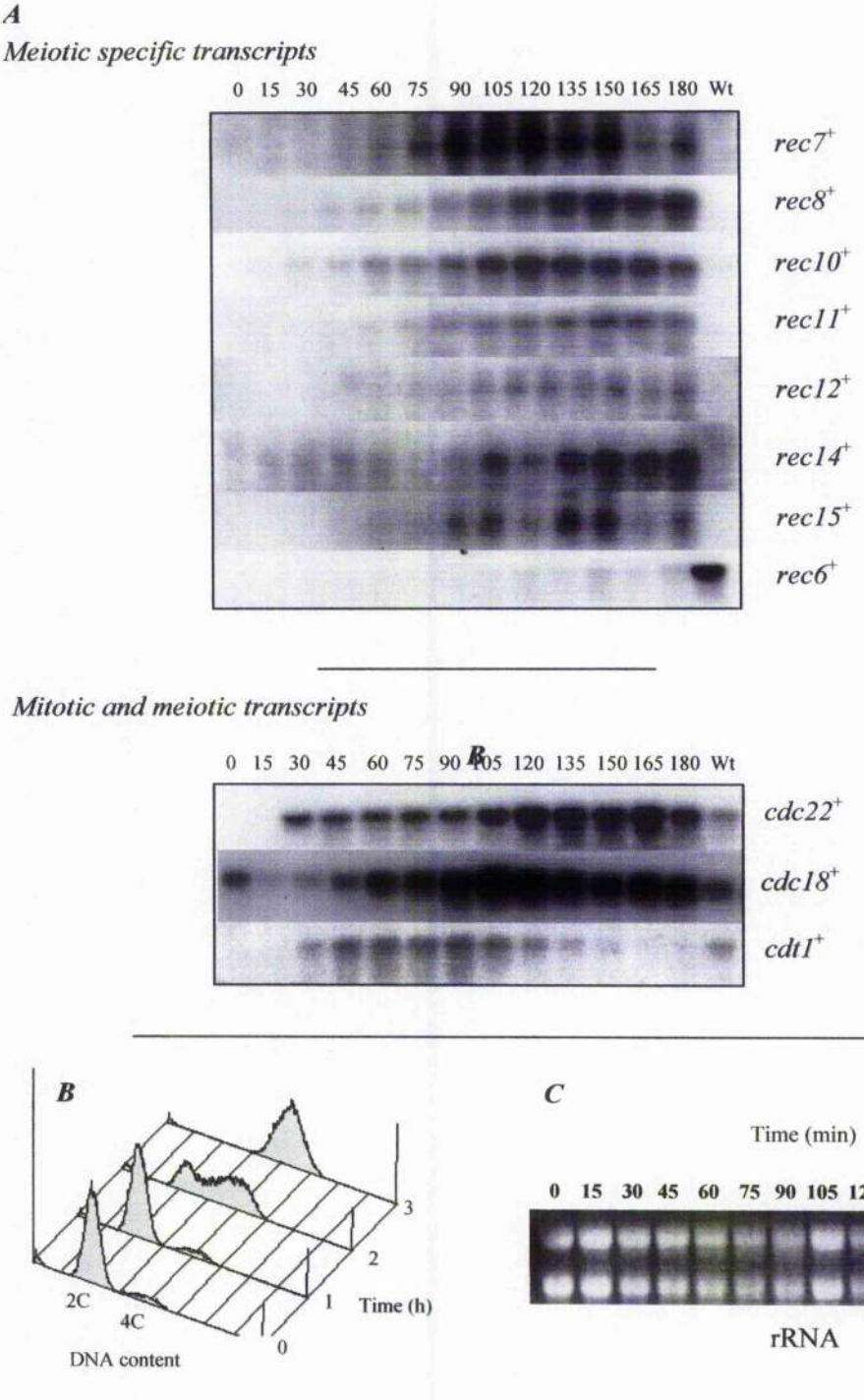
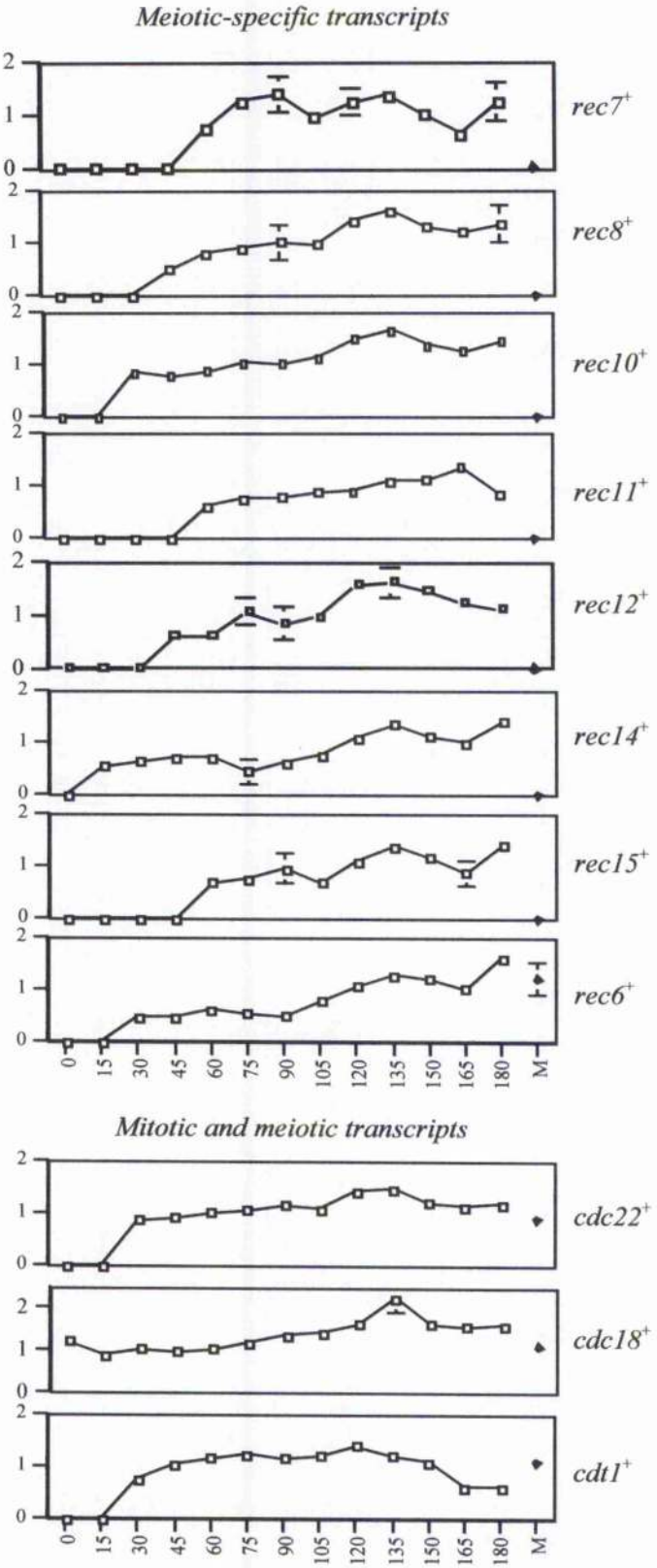


Figure 3.1: G1–S-specific gene expression during a synchronous diploid* pat1-114 *meiosis in fission yeast

A: Diploid *pat1-114 cells* were induced to undergo a synchronous meiosis (see Section *2.7.6*) and samples were taken every 15 minutes. RNA was extracted and northern blots performed to probe for meiotic-specific genes and for genes expressed during both mitosis and meiosis. ***B***: Flow cytometry analysis was used to monitor the DNA content of the cells. ***C***: The ethidium bromide stained rRNA gel is shown as a loading control to which the quantified graphs were normalized. The northern blots were quantified using Image J software and graphs for each transcript are shown over the page in ***D***, the results are measured in arbitrary units.

Figure 3.1: G1–S-specific gene expression during a synchronous diploid* pat1-114 *meiosis in fission yeast

D



One mechanism by which *rec*$^{+}$ gene transcription could be confined to meiosis is if different forms of the MCB motif operate in the two different life cycles. Differences in the number or arrangement of MCB motifs within the promoters of *rec*$^{+}$ genes might be responsible for their specificity to meiosis. The promoters of the genes previously examined for transcript abundance during meiosis were therefore examined for the presence of MCB elements. Further genes, that were classified as early genes using the microarray technique (Mata *et al.*, 2000), were also included in this analysis to assess whether other genes, which are known to be periodically expressed during meiosis, followed similar patterns of MCB element distribution.

MCB motifs from various genes that are known to be expressed in meiosis were considered at two complementary levels. First, individual MCB sequences were considered (Figure 3.2) to determine if there was any correlation in specific nucleotide sequences between mitotic specific, meiotic specific or mitotically and meiotically expressed genes. Second, the gross arrangement of MCB motifs within each promoter (Figure 3.3), was examined to determine whether the arrangements of MCB motifs correlate with specificity of expression. The position of the MCB motifs was considered in relation to the position of the ATG of the genes as there is very little information available on the position of transcription start sites in fission yeast. Entire intergenic regions, upstream from the genes considered, were scanned for MCB motifs. However, MCB sequences did not occur greater than 1kb away from the ATG with any frequency and so were not considered in this analysis. MCB motifs downstream from the ATG did occur quite often, so they have been included on the schematic diagram. But these motifs did not appear to have a particular pattern and as UAS sequences appearing in the ORF are not thought to be functional (Struhl, 1989) they were not taken into account when the promoters were grouped. The analysis revealed that there are several different arrangements of MCB motifs within promoters, which have been grouped into five different categories of increasing complexity.

Figure 3.2: Position of MCB motifs in the promoters of fission yeast genes expressed at G1–S

Type I

rec7$^+$	−32	ACGCGT	−27															
rec10$^+$	−109	ACGCGT	−104															
SPCC4G3.07C	−251	ACGCGCGT	−244															
res2$^+$	−34	TCCGCGCT	−27															

Type II

rec6$^+$	−732	TCGCGA	−727								−562	ACGCGT	−557					
rec8$^+$	−49	ACGCGT	−44								−20	ACGCGT	−15					
rec12$^+$	−263	TCGCGT	−258								−64	ACGCGCT	−59	−22	ACGCGT	−17		
meu13$^+$	−94	ACGCGT	−89								−77	ACGCGT	−72	−42	TCGCGCT	−36		
SPCC290.04	−285	ACGCGATAATTCACGCGT	−268								−176	GCGCGA	−171	−9	TCGCGA	−4		
SPBC1718.02	−81	ACGCGTTCGACGCGT	−67															

Type III

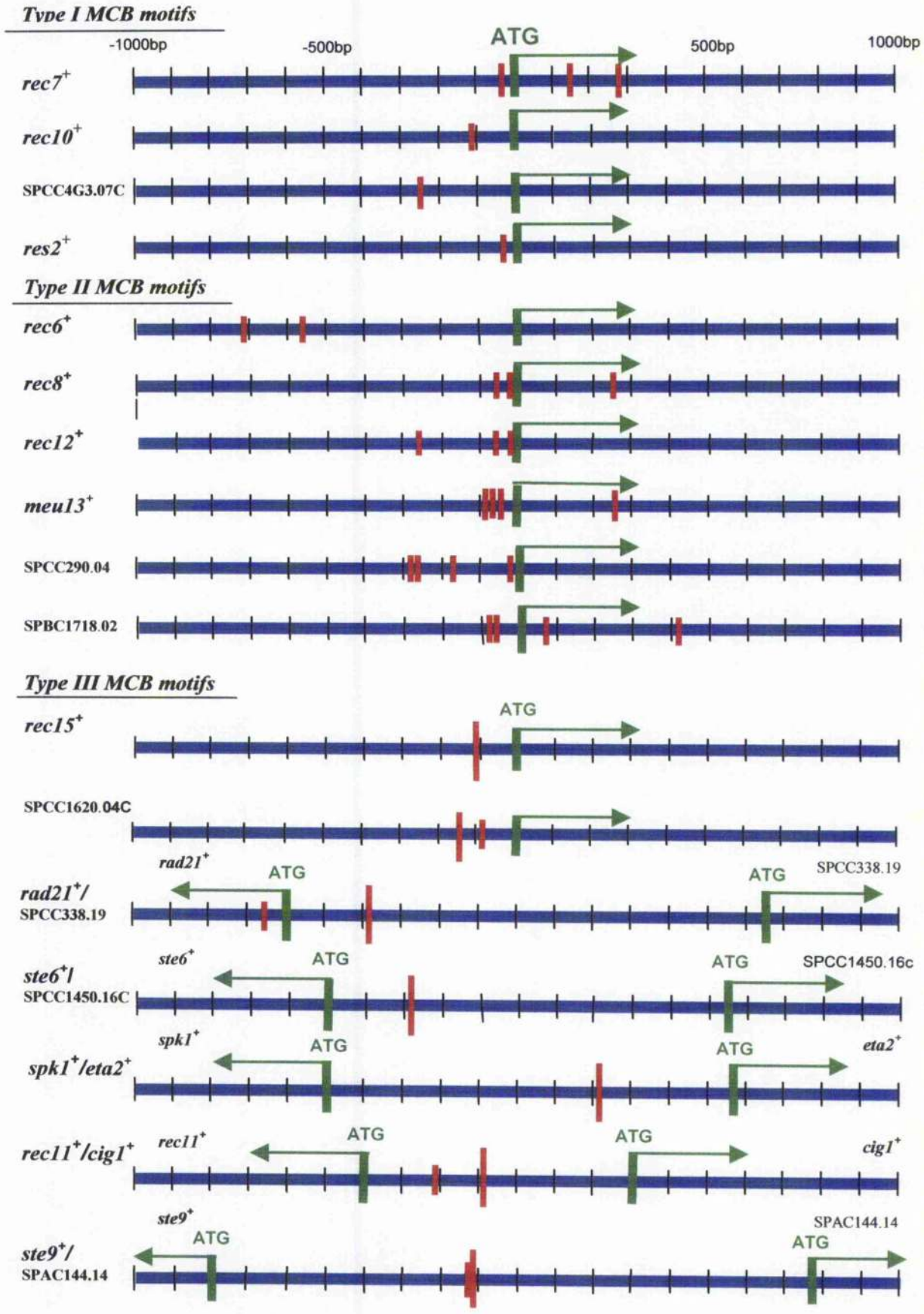
rec15$^+$	−104	TCGCGTCGCGT	−94															
mei3$^+$	−417	TCGCGCCGCGT	−407															
rad21$^+$	−223	ACGCGCAGCGCGT	−212															
ste6$^+$	−234	ACGCGACGCGCCA	−222															
spk1$^+$	−725	GCGCGACGCGTCGCGC	−710															
rec11$^+$	−212	ACGCGT	−207								−95	ACGCGACTCGT	−85					
ste9$^+$	−701	ACACGAACGCGA	−690								−676	TCGCGT	−671					
SPCC1620.04C	−146	ACGGGCGCGT	−137								−89	ACGCGT	−84					

Type IV

suc22$^+$	−917	TCGCGCT	−911								−874	TCGCGTCGCAT	−864					
	−707	ACGCGT	−702								−86	TCGCGGT	−80					
SPCC21B10.13c	−102	GCGCGATATAGTGCAACGCGC	−82								−66	AGCGCGACGCGTCG	−52					
cdc18$^+$	−246	TCGCGCGTAGAACGCGACACGACACGTAGAGTTGAACGCGA	−206															
	−173	TCGCGTTTACGTGTCGCGTCGCGT	−150															
cdt1$^+$	−72	AGCGCGACACGATAGCTACGCGACGCGTCAACAAACGCGA	−33															
cdt2	−444	CCGCGA	−439															
	−382	ACGCGT	−377															
	−175	ACGCGT	−170															
	−116	ACGCGTCGCGTTCACGCGT	−98															
mik1$^+$	−383	CCGCGTCGCGTTATCCCAAATGAAAAACGCGAACGCGTCATGACGCGT	−336															
cdc22$^+$	−105	ACGCGACGCGGCATCACGTTATATTAGCGTGACGCGTCTGAACGCGT	−59															
	−311	TCGCGT	−306															
	−493	ACCGCGTGTTTAATTTATGTAAACAGTCGCGTCGCGTTGCAATTGAG																
		ACGCGT	−441															
spo6$^+$	−337	ACGCGACGTGTCGCGCGTTTAATTTACTGGCTTAATACGCGAGGCGA	−291															

Figure 3.2: Position of MCB motifs in the promoters of fission yeast genes expressed at G1–S
The specific DNA sequence of the individual MCB elements considered in this chapter is shown. Close sequence matches to the single or double MCB consensus sequences are shown in red and the position in the genome relative to the ATG of the gene is indicated.

Figure 3.3: Distribution of MCB motifs around the ATG of fission yeast G1–S expressed genes



Type IV MCB elements

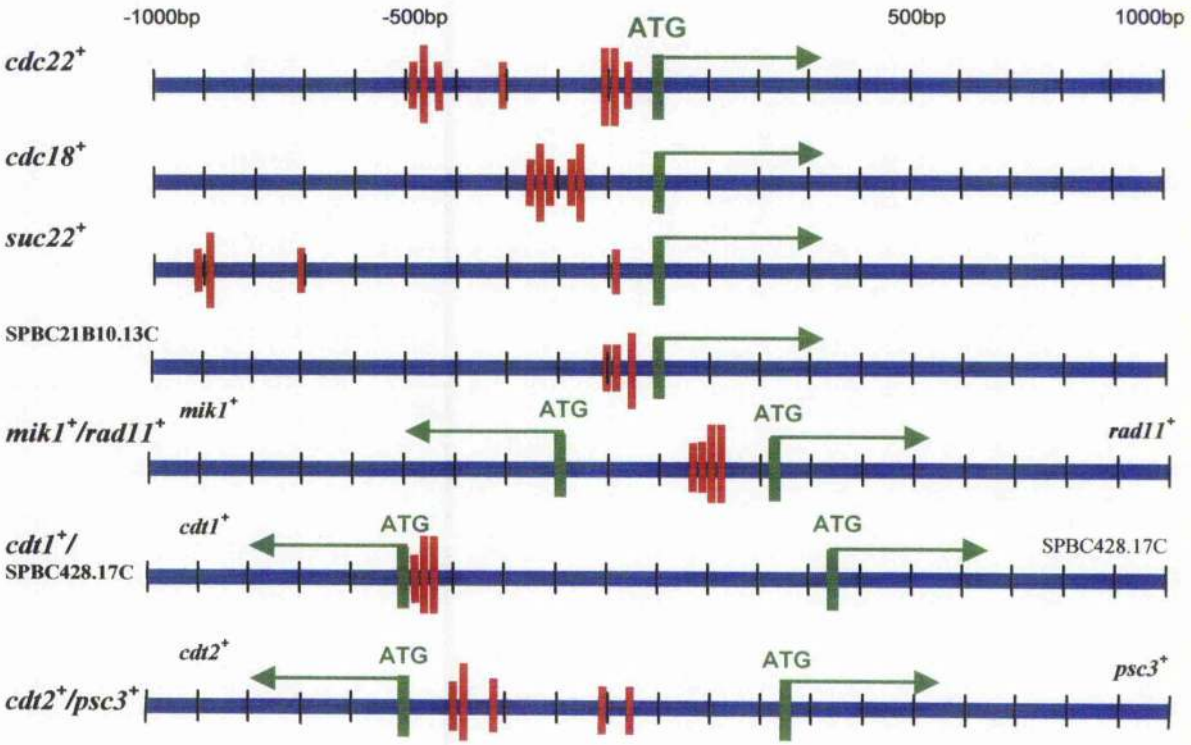


Figure 3.3: Distribution of MCB motifs around the ATG of fission yeast G1–S expressed genes
A schematic representation of the position of MCB elements relative to the ATG of periodically expressed genes. Single MCB elements are represented by a short red line and double MCB elements are represented by a long red line.

3.3.1 Type I MCB promoters

Type I MCB promoters contain a single 5'-ACGCGT-3' motif within 1 kb of the ATG of the gene and include *rec7*$^+$, *rec10*$^+$ and *res2*$^+$. *rec7*$^+$ and *rec10*$^+$ are both involved in meiotic recombination and in our analysis produce multiple transcripts which are recognized by a double stranded DNA probe in northern blot analysis. It is known that several early recombination genes in both fission yeast and budding yeast often produce both shorter, and unusually longer, transcripts. In the case of *rec7*$^+$ the short transcripts have been shown to derive from the non-coding strand and an *in vivo* function for these transcripts has not been excluded. The longer transcripts are thought to be caused by incomplete transcription termination, as they do not extend beyond the usual 5' region of the transcript (Molnar *et al.*, 2000). However, in each case functional length meiotic-specific transcripts are identified, although in the case of *rec7*$^+$, two of the larger transcripts appear in the meiotic control lane. Both the *rec7*$^+$ and *rec10*$^+$ data are consistent with the profiles obtained by microarray analysis. The MBF component *res2*$^+$ is also a member of this group and shows a distinct G1–S peak of transcript abundance in both the microarray analysis (Mata *et al.*, 2002) and this study. As a member of the early group of genes SPCC4G3.07C was also found to contain a single MCB motif and is a transcriptional regulator that has similarity to human retinoblastoma binding protein 2 although the specificity of its expression is unknown (Wood *et al.*, 2002). The MBF component *res2*$^+$ is known to be expressed with weak G1–S periodicity during mitosis (Obara-Ishihara & Okayama, 1994). It is also a member of this Type I group and shows a distinct G1–S peak of transcript abundance during meiosis (Miyamoto *et al.*, 1994).

3.3.2 Type II MCB promoters

Type II MCB promoters contain several single 5'-ACGCGT-3' motifs in their promoters and include *rec6*$^+$, *rec8*$^+$ and *rec12*$^+$. The *rec6*$^+$, *rec8*$^+$ and *rec12*$^+$ genes all produce G1–S peaks of mRNA in our meiotic experiment, which agree with the transcription profiles found in the microarray analysis (Mata *et al.*, 2002). However, the *rec6*$^+$ probe detects a single transcript in our experiment which is only weakly expressed during the meiotic time course, but which is detected strongly in the mitotic control lane. Again, *rec12*$^+$ also has a larger transcript that appears in the mitotic control lane. *meu13*$^+$ was identified as being a meiotic-specific gene (Nabeshima *et al.*, 2001). It gives a distinct G1–S peak in the microarray analysis and has an excellent fit to the Type II MCB motif pattern. SPCC290.04 and SPBC1718.02 are both putative transcriptional regulators identified by the microarray analysis as early genes. They have close matches to the Type II MCB motif in their promoters and show distinct G1–S transcript profiles which mirror closely those of other genes in this class of MCB type (Mata *et al.*, 2002).

3.3.3 Type III MCB promoters

Type III MCB promoters contain one double MCB motif which has at least 9 out of 11 matches to the consensus of GXCGCGTCGCGT (Kersey, 1995), and in some instances also contain a single MCB motif at a distance from the double motif. This group includes *rec15*$^{+}$ and SPCC1620.04C, and the gene pairs *rad21*$^{+}$/SPCC338.19, *rec11*$^{+}$/*cig1*$^{+}$, *ste6*$^{+}$/SPCC1450.16C, *spk1*$^{+}$/*eta2*$^{+}$ and *ste9*$^{+}$/SPAC144.14. The *rec15*$^{+}$ and SPCC1620.04C single genes both give a G1–S peak in transcript abundance in microarray analysis and our results for *rec15*$^{+}$ agree with this. SPCC1620.04C is a WD repeat protein of the Cdc20p/Fizzy family, which was identified as an early gene by the microarray analysis and found to conform to the Type III MCB pattern.

The *rad21*$^{+}$/SPCC338.19, *rec11*$^{+}$/*cig1*$^{+}$, *ste6*$^{+}$/SPCC1450, *spk1*$^{+}$/*eta2*$^{+}$ and *ste9*$^{+}$/SPAC144.14 gene pairs represent interesting cases in the family of MCB containing promoters. In each case, the pairs of genes are situated next to each other in the genome but their ORFs read in opposite directions, therefore the two genes in each pair share the same upstream intergenic region. Thus, the identified MCB motifs may potentially apply to either or both genes.

Interestingly, *rad21*$^{+}$ is known to be under MCB control in the mitotic cell cycle (Maqbool *et al.*, 2003), but neither *rad21*$^{+}$, nor its partner SPCC338.19, which is a pseudogene, gives a periodically transcribed product during the meiotic cell cycle. Thus, the MCB motif in this intergenic region is likely to apply to *rad21*$^{+}$ but seems to confer mitotic-specific periodic expression.

In the case of *ste9*$^{+}$ and SPAC144.14 neither gene produces a G1–S transcript peak in the microarray analysis even though *ste9*$^{+}$ is MCB regulated in mitosis (Tournier & Millar, 2000) suggesting that these might also be mitotic-specific MCB motifs. *ste9*$^{+}$ is a regulator of the APC complex and its partner SPAC144.14 is a kinesin-like protein.

The *ste6*$^{+}$ transcript peaks in abundance immediately after nitrogen starvation in the microarray analysis, which classifies it in the delayed group of genes. *ste6*$^{+}$ is a Ras nucleotide-exchange factor (Hughes *et al.*, 1994) and was classified as being under MCB control in the mitotic cell cycle (Kersey, 1995) but has been shown to be under the control of the Ste11p transcriptional activator during the meiotic cell cycle (Hughes *et al.*, 1994). Thus *ste6*$^{+}$ may be under the control of different transcriptional regulators in the different cell cycles. The *ste6*$^{+}$ partner SPCC1450.16C encodes a predicted phospholipase, but does not show a periodically transcribed profile in the microarray analysis (Mata *et al.*, 2002) so it is unlikely that the double MCB motif applies to that gene.

spk1^{+} is also classified in the delayed group of genes by microarray analysis (Mata *et al.*, 2002). However, it too appeared to be under MCB control in the mitotic cycle (Kersey, 1995). Recent data suggests that *spk1*$^{+}$ is not cell cycle regulated in mitosis (Maqbool, 2003). The *spk1*$^{+}$ promoter also contains several Ste11p recognition sequences, suggesting it is, in fact,

under the control of Ste11p. The partner of *spk1*$^+$, *eta2*$^+$, does give a strong G1–S transcript peak during the meiotic cell cycle so it is possible that this MCB cluster may apply to *eta2*$^+$ rather than *spk1*$^+$. *eta2*$^+$ is a member of the myb family and is predicted to be a transcription factor for RNA polymerase II (Wood *et al.*, 2002) and as such it is likely that it would be needed during S phase when the transcription of several genes is required. It is not known whether this gene is expressed during mitosis, but if, as predicted, it is a general transcription factor it is probable that it would be transcribed in both mitosis and meiosis.

rec11$^+$ was identified as a meiotic-specific recombination gene (Li *et al.*, 1997) and in this analysis appears to be periodically transcribed at the G1–S phase boundary specifically during the meiotic cell cycle, which agrees with the transcript profile identified in the microarray study (Mata *et al.*, 2002). Its partner *cig1*$^+$ does not appear to be periodically transcribed in the mitotic (Conolly & Beach, 1994) or meiotic cell cycles (Mata *et al.*, 2002) suggesting the MCB motifs apply solely to the *rec11*$^+$ gene and confer meiotic-specific expression.

3.3.4 Type IV MCB promoters

Type IV MCB promoters contain complicated arrangements of several MCB motifs within a few hundred bases of translational start (the ATG 'start' codon). At least one double and one single motif are found within a few bases of each other in these promoters. However, often several double and single motifs are found in discrete clusters. *cdc22*$^+$ and *cdc18*$^+$ are known to be periodically transcribed under the control of the MBF complex during the mitotic cell cycle. *cdc22*$^+$ and *cdc18*$^+$ also give a clear peak of mRNA abundance at the G1–S boundary in meiotic microarray analysis (Mata *et al.*, 2002) — in agreement with results presented here (Figure 3.1). The gene SPBC21B10.13C, which is a putative transcriptional regulator, produces a strong G1–S peak in transcript abundance in microarray analysis (Mata *et al.*, 2002) and contains Type IV MCB motifs.

The genes *cdt1*$^+$, *cdt2*$^+$ and *mik1*$^+$ all share upstream intergenic regions with other genes; SPBC428.17C, *psc3*$^+$ and *rad11*$^+$, respectively. And, *cdt1*$^+$, *cdt2*$^+$ and *mik1*$^+$ are already known to be under MBF control in the mitotic cell cycle (Hofman & Beach, 1994; Tournier & Millar, 2000; Ng *et al.*, 2001; Yoshida *et al.*, 2003).

mik1$^+$ and *rad11*$^+$ are both known to be MCB–MBF controlled during mitosis (Parker *et al.*, 1997; Ng *et al.*, 2000). Both genes show a distinct increase in transcription during the G1–S phase of the meiotic microarray analysis, with *rad11*$^+$ particularly showing a distinct peak in transcript abundance (Mata *et al.*, 2002). Both genes, however, also display separate, later peaks in meiotic transcript abundance. It is likely therefore that this cluster of MCB motifs acts on both genes at the G1–S period of the meiotic cell cycle but that alternative transcription factors are acting on these genes to cause a second peak of expression later in the meiotic cell cycle.

A meiotic G1–S peak in *cdt1*$^{+}$ transcript abundance is shown in this thesis and also in microarray analysis (Mata *et al.*, 2002). Interestingly, the opposing gene SPBC428.17C also shows a peak in transcript abundance at G1–S in the microarray analysis, which indicates that this MCB cluster may be controlling expression of both genes. Similarly, the *cdt2*$^{+}$ and *psc3*$^{+}$ genes both show G1–S peaks in transcript abundance in meiosis (Mata *et al.*, 2002). The *psc3*$^{+}$ gene however, does not seem to be under MBF control in mitosis (Maqbool *et al.*, 2003). The MCB motifs in this intergenic region are arranged in two smaller clusters, which are separated by over one hundred base pairs, so it is possible that these clusters act independently of each other on *cdt2*$^{+}$ and *psc3*$^{+}$. The cluster closest to *cdt2*$^{+}$ may confer periodic expression in both mitosis and meiosis, and the cluster closest to *psc3*$^{+}$ may confer periodic expression in meiosis alone.

3.4.1 G1–S transcript analysis

An investigation was undertaken into the transcript profiles of the *rec*$^+$ genes during meiosis to analyse whether they were under the control of the MCB–MBF system. The *rec*$^+$ genes have previously been noted to contain MCB elements in their promoter regions and to require Rep1p for expression (Ding & Smith, 1998) — Rep1p is known to act on Res2p, which is part of the MBF complex (Sugiyama *et al.*, 1994; Ding & Smith, 1998). Previously, these *rec*$^+$ genes were classified as being specific to meiosis (Ding & Smith, 1998). However, in our investigation we found that, in the case of those genes which produced multiple transcripts (*rec15*$^+$, *rec12*$^+$ and *rec7*$^+$), the larger transcripts were also detected in mitotic cells. Similarly, a mitotic transcript was detected for the *rec6*$^+$ gene. In each *rec*$^+$ gene considered, except *rec6*$^+$, a functional length transcript was identified which conformed to the same pattern of G1–S induced transcription as is seen in the *cdc22*$^+$ group of genes. This confirms that the *rec7*$^+$, *rec8*$^+$, *rec10*$^+$, *rec11*$^+$, *rec12*$^+$ and *rec15*$^+$ mRNA profiles are consistent with their control by the MCB–MBF system in the meiotic cell cycle.

3.4.2 MCB-motif distribution analysis

An analysis of the promoter regions of several meiotic G1–S transcribed genes was also undertaken in order to determine if there was any correlation in the incidence of MCB motifs between mitotic specific, meiotic specific or mitotically and meiotically expressed genes. The results will be discussed in relation to their putative MCB-motif specificity.

3.4.2.1 Mitotic-specific MCB motifs

Gene name (MCB motif type)	*Specificity of expression*	*Gene function*	*Reference*
rad21$^+$ (III)	Mitotic-specific G1–S transcript	Mitotic cohesin	(Birkenbihl & Subramani, 1995; Mata *et al.*, 2002)
ste9$^+$ (III)	Mitotic-specific G1–S transcript plus meiotic-specific G2–M transcript	WD repeat protein, regulator of APC complex	(Yamaguchi *et al.*, 1997; Mata *et al.*, 2002)
ste6$^+$ (III)	Mitotic-specific G1–S transcript plus meiotic-specific G2–M transcript	Guanine nucleotide-releasing factor involved in conjugation	(Sipizki, 1988; Mata *et al.*, 2002)

Table 3.1: Mitotic-specific MCB motifs

Summary of the genes, considered in this chapter, which contain putative mitotic-specific MCB elements.

The genes *rad21*$^{+}$, *ste6*$^{+}$ and *ste9*$^{+}$ appear to be expressed at the G1–S boundary exclusively in mitosis. Unlike *rad21*$^{-}$ however, which is entirely specific to mitosis, *ste6*$^{+}$ and *ste9*$^{+}$ are also highly induced during the meiotic cell cycle but with a different periodicity to G1–S transcription (Mata *et al.*, 2002). Interestingly, all three genes possess similar MCB motifs, which indicates that these MCB motifs may confer mitotic specific, G1–S induced expression. *ste6*$^{+}$ and *ste9*$^{+}$ are likely to be under the control of alternative transcription factors in the meiotic cell cycle.

3.4.2.2 Meiotic-specific MCB motifs

Gene name (MCB motif type)	***Specificity of expression***	***Gene function***	***Reference***
Group A			
rec8^{+} (II)	Meiotic-specific G1–S transcript	Meiotic cohesin	DeVeaux *et al.*, 1992; Lin & Smith, 1992; Mata *et al.*, 2002
rec10^{+} (I)	Meiotic-specific G1–S transcript	Required for proper segregation of sister chromatids during MII	DeVeaux *et al.*, 1992; Lin & Smith, 1995a; Mata *et al.*, 2002
rec11^{+} (II)	Meiotic-specific G1–S transcript	Required for proper segregation of sister chromatids during MII	DeVeaux *et al.*, 1992; Li *et al.*, 1997; Mata *et al.*, 2002
meu13^{+} (II)	Meiotic-specific G1–S transcript	Meiotic specific. Promotes homologous chromosome pairing.	Watanabe *et al.*, 2001; Mata *et al.*, 2002
Group B			
rec6^{+} (II)	Meiotic G1–S transcript which also appears in mitotic control lane	Involved in meiotic recombination	DeVeaux *et al.*, 1992; Lin & Smith, 1994; Mata *et al.*, 2002
rec7^{+} (I)	Functional length meiotic-specific G1–S transcripts plus larger non-periodic mitotic and meiotic transcripts	Involved in intra- and intergenic meiotic recombination	DeVeaux *et al.*, 1992; Lin & Smith, 1992; Mata *et al.*, 2002
rec12^{+} (II)	Meiotic-specific G1–S transcript plus larger non-periodic mitotic and meiotic transcripts	Involved in meiotic recombination	DeVeaux *et al.*, 1992; Lin & Smith, 1994; Mata *et al.*, 2002
rec15^{+} (III)	Meiotic-specific G1–S transcript plus larger non-periodic mitotic and meiotic transcripts	Involved in meiotic recombination	DeVeaux *et al.*, 1992; Lin & Smith, 1995b; Mata *et al.*, 2002
psc3^{+} (IV)	Meiotic G1–S transcript mitotic transcription non-periodic	Cohesin subunit involved in normal mitotic progression	Tomonaga *et al.*, 2000; Mata *et al.*, 2002

Gene name (MCB motif type)	*Specificity of expression*	*Gene function*	*Reference*
Group C			
SPCC4G3.07C (I)	Meiotic G1–S transcript mitotic profile unknown	Transcriptional regulator, similarity to human retinoblastoma binding protein 2	Wood *et al.*, 2002; Mata *et al.*, 2002
SPCC290.04 (II)	Meiotic G1–S transcript, mitotic profile unknown	Putative transcriptional regulator (GATA zinc finger)	Wood *et al.*, 2002; Mata *et al.*, 2002
SPBC1718.02 (II)	Meiotic G1–S transcript, mitotic profile unknown	Putative transcriptional regulator (PHD zinc finger — possible chromatin regulation)	Wood *et al.*, 2002; Mata *et al.*, 2002

Table 3.2: Meiotic-specific MCB motifs

Summary of the information known about the genes considered in this chapter, which contain putative meiotic-specific MCB elements.

Group A. Genes whose expression is known to be exclusive to meiosis are considered in Group A in Table 3.2. Of the *rec*$^+$ genes only *rec8*$^+$, *10*$^+$ and *11*$^+$ are exclusive to meiosis. The only other gene considered which is known to be exclusive to the meiotic cell cycle is *meu13*$^+$. *meu13*$^+$ was identified in a study which catalogues a number of meiotic-specific genes using a subtracted cDNA library (Watanabe *et al.*, 2001). The *meu13*$^+$ gene encodes a meiotic cohesin, so it is interesting that it falls into the same MCB category as meiotic-specific *rec*$^+$ genes, particularly *rec8*$^+$, another known meiotic-specific cohesin (Watanabe & Nurse, 1999).

Group B. Group B in Table 3.2 includes those genes which produce detectable transcripts during mitosis, but which only appear to be regulated at the G1–S phase transition during meiosis. *rec7*$^+$, *rec12*$^+$ and *rec15*$^+$ appear in this category as, possibly, does *psc3*$^+$. The *psc3*$^+$ gene is a cohesin, which is known to play a role in mitosis where it is constitutively expressed (Maqbool, 2003). *psc3*$^+$ shares its upstream intergenic region with *cdt2*$^+$ which is periodically expressed in both mitosis and meiosis (Yoshida *et al.*, 2003; Mata *et al.*, 2002). The arrangement of MCB motifs in this region is such, however, that *psc3*$^+$ is likely to be under the control of a Type II pair of MCB motifs which are distinct from those which apply to *cdt2*$^+$ (see Figure 3.3). This again raises the possibility that these genes might be under the control of different transcriptional control mechanisms in the two different life cycles.

Group C. Group C in Table 3.2 includes genes whose promoter sequences were investigated after their identification as early genes by microarray analysis (Mata *et al.*, 2002). SPCC4G3.07C, SPCC290.04 and SPBC1718.02 are putative transcription factors, which all

contain Type I or Type II MCB motifs within their promoters. The nature of the mRNA profiles of these genes in mitosis is unknown but they show a G1–S peak in transcription during meiosis, as would be predicted from the presence of Type I or Type II MCB motifs in their promoter regions. Therefore, they have been included in the meiotic-specific table as the majority of other genes containing Type I or Type II MCB motifs are confirmed as being periodically expressed only in meiosis. Thus, it would be interesting to investigate the expression profiles of these genes further.

3.4.2.2.1 Comparison with a global meiotic-specific gene analysis

A study designed to identify meiotic-specific genes (Watanabe *et al.*, 2001) did not identify any of the *rec*$^{+}$ genes. This is unsurprising in the case of *rec6*$^{+}$, *rec7*$^{+}$, *rec12*$^{+}$ and *rec15*$^{+}$, which all produce large mitotic transcripts, and so would have been eliminated in the subtracted cDNA library used. However, it is surprising that *rec8*$^{+}$, *rec10*$^{+}$ and *rec11*$^{+}$ were not detected, particularly as the study did identify *meu13*$^{+}$, which is not only expressed at the same cell cycle time but has a similar gene function. This study also identified three other meiotic-specific genes (Watanabe *et al.*, 2001) that are classed as early genes by microarray analysis (Mata *et al.*, 2002). *meu10*$^{+}$, which is required for spore-cell-wall formation, gives a later peak in transcript abundance (Mata *et al.*, 2002) but does have an MCB motif 355 bp away from its ATG. *meu3*$^{+}$ and *meu20*$^{+}$ transcripts also appear at meiotic G1–S phase but are both non-coding RNAs and neither contain MCB motifs in their promoters. Furthermore, several *meu*$^{+}$ genes that do not appear as early genes in microarray analysis (Mata *et al.*, 2002) do contain MCB motifs in their promoters. *meu9*$^{+}$, *meu11*$^{+}$, *meu15*$^{+}$, *meu23*$^{+}$, *meu25*$^{+}$, *meu26*$^{+}$ and *meu28*$^{+}$ all contain matches to Type I or II MCB motifs in their intergenic regions but do not produce a G1–S-specific peak in transcript abundance (Mata *et al.*, 2002). Further investigation of these specific gene-transcription profiles would be useful to help understand what exactly constitutes a meiotic-specific MCB motif.

3.4.2.3 MCB motifs of mitotically and meiotically expressed genes.

Gene name (MCB motif type)	*Specificity of expression*	*Gene function*	*Reference*
cdc22$^+$ (IV)	Mitotic and meiotic G1 S MCB-regulated transcription	Large subunit of ribonucleotide reductase	Nurse *et al.*, 1976; Mata *et al.*, 2002
cdc18$^+$ (IV)	Mitotic and meiotic G1–S MCB-regulated transcription	Required for assembly of pre-RC at ORI	Nurse *et al.*, 1976; Mata *et al.*, 2002
res2$^+$ (I)	Mitotic and meiotic G1 S transcript	Transcriptional regulator, part of DSC1 complex	Miyamoto *et al.*, 1994; Zhu *et al.*, 1994; Mata *et al.*, 2002
cdt2$^+$ (IV)	Mitotic and meiotic G1–S MCB-regulated transcription	WD repeat protein couples START with cytokinesis.	Hofman & Beach, 1994; Mata *et al.*, 2002
rad11$^+$ (IV)	Meiotic G1–S transcript mitotic profile unknown	Large subunit of Replication Protein A	Parker *et al.*, 1997; Mata *et al.*, 2002
cdt1$^+$ (IV)	Mitotic and meiotic G1–S MCB-regulated transcription	Essential, involved in DNA replication	Hofman & Beach, 1994; Mata *et al.*, 2002
SPBC428.17C (IV)	Meiotic G1–S transcript mitotic profile unknown	Hypothetical serine-rich protein	Wood *et al.*, 2002; Mata *et al.*, 2002
SPBC21B10.13C (IV)	Meiotic G1–S transcript mitotic profile unknown	Hypothetical DNA-binding protein	Wood *et al.*, 2002; Mata *et al.*, 2002
SPCC1620.04C (III)	Meiotic G1 S transcript mitotic profile unknown	CDC20/Fizzy family, APC regulator	Wood *et al.*, 2002; Mata *et al.*, 2002
cta2$^+$ (III)	Meiotic G1–S transcript mitotic profile unknown	Myb DNA-binding-domain protein	Wood *et al.*, 2002; Mata *et al.*, 2002

Table 3.3: Mitotic and Meiotic MCB motifs

Summary of the information known about the genes considered in this chapter, which contain putative mitotic and meiotic MCB elements.

cdc22$^+$, *cdc18*$^+$ and *cdt2*$^+$ are all known to be periodically expressed at the G1–S boundary in mitosis and appear to have a similar transcription profile in meiosis (see Table 3.3 and Mata *et al.*, 2002). They all contain complex Type IV MCB motifs within their promoters. *res2*$^-$ is also expressed during both mitosis and meiosis but contains only a close match to a Type I motif within its promoter. *res2*$^+$ however, is an unusual case as it is part of the MBF transcription complex known to control MCB regulated genes.

The *mik1*$^+$/*rad11*$^+$ and *cdt1*$^+$/SPBC428.17C gene pairs also contain Type IV MCB motifs within their shared intergeneic regions which are likely to be acting on both genes in the two pairs. It would, therefore, be interesting to identify the mitotic transcription profiles of *rad11*$^+$ and SPBC428.17C.

SPBC21B10.13C is a putative transcriptional regulator identified by microarray analysis (Mata *et al.*, 2002) as an early gene, which has a Type IV MCB motif within its promoter region. It is therefore predicted that, as is the case with other Type IV motif containing genes,

this gene would be under MCB–MBF control in both mitosis and meiosis, an extensive examination of its transcript profiles should therefore be undertaken.

SPCC1620.04C was identified from the microarray analysis as an early gene with cell cycle regulation properties (Mata *et al.* 2002). *eta2*$^{+}$ is the gene partner of *spk1*$^{+}$, which is not periodically expressed in either mitosis or meiosis (Mata *et al.*, 2002; Maqbool *et al.*, 2003). These genes have Type III MCB motifs but are expressed in meiosis, unlike the Type III genes identified in Table 3.2, which are only periodically transcribed in mitosis. Further investigation of Type III MCB-motif genes would therefore be required to investigate which parts of these intermediately complex motifs are important, and to help identify a mitotic-specific MCB motif.

3.4.2.4 Non-periodic MCB gene partners

Gene name	*Specificity of expression*	*Gene function*	*Reference*
SPCC338.19 (*rad21*$^{+}$ partner)	Non-periodic transcript	Tricothecene 3-O-actyltransferase pseudogene	Wood *et al.*, 2002; Mata *et al.*, 2002
spk1^{+} (*eta2*$^{+}$ partner)	Non-periodic transcript	Serine/threonine protein kinase involved in pheromone signalling pathway.	Gotoh *et al.*, 1993 Neiman *et al.*, 1993; Mata *et al.*, 2002
SPCC1450.16C (*ste6*$^{-}$ partner)	Non-periodic transcript	Predicted triacylglycerol lipase	Wood *et al.*, 2002; Mata *et al.*, 2002
SPAC144.14 (*ste9*$^{+}$ partner)	Non-periodic transcript	Kinesin-like protein	Wood *et al.*, 2002; Mata *et al.*, 2002

Table 3.4: Non-periodic MCB gene partners

Summary of the information known about the genes considered in this chapter, which share the same intergenic regions as MCB regulated genes, but which appear not to be under MCB–MBF control.

Table 3.4 contains genes that share intergenic regions — and therefore, potentially, MCB motifs — with other MCB–MBF regulated genes, but which are not themselves periodically transcribed. This is consistent with data obtained previously (Maqbool *et al.*, 2003), which suggests that MCB motifs are orientation dependent in their ability to stimulate transcription during mitosis.

Table 3.4 however, contains two instances of genes in which a shared MCB cluster has resulted in G1–S periodic transcription for both genes. There may be two explanations for this. Firstly it is possible that MCB motifs are capable of acting in a palindromic fashion but only when they appear in a Type IV motif arrangement. Alternatively, the MCB clusters in the *mik1*$^{+}$/*rad11*$^{+}$ and *cdt1*$^{+}$/SPBC428.17C promoters may actually constitute two separate directional MCB motifs, which are in close proximity to each other due to the tight packing of

the genes in the fission yeast genome. Only further, detailed dissection of these promoter regions will be able to answer these questions.

3.4.3 MCB gene function

Finally, the function of all the genes considered was taken into account after the MCB groupings had been determined, and, genes of related functions did seem to fall into similar categories. All the meiotically expressed recombination and cohesin genes that were considered seem to have simpler Type I and II MCB motifs. Those genes with essential S phase functions, which are required in both developmental cycles, have Type IV MCB motifs, which confer both mitotic and meiotic periodic expression. The functions of Type III MCB-regulated genes were more diverse, although more detailed analysis of these promoter regions may reveal finer motif classifications.

It should be noted, however, that a very limited sample of genes expressed at the G1–S boundary during meiosis was considered. So, while it is interesting to note that the categories of genes considered did tend to share similar MCB motifs, ideally, the MCB motifs of a much wider sample of the early genes (Mata *et al.*, 2002) should be considered before they can be confidently grouped according to function. Nevertheless, these preliminary data suggest that certain predictions can be made with regards to expression specificity for either mitosis or meiosis when the MCB motifs in a promoter region are considered. The MCB Types identified in this chapter may therefore be useful in further investigations into MCB–MBF controlled transcription and have been used as guides for the selection of promoters for use in further experiments in this study.

- The *cdc22*$^{+}$ and *rec*$^{+}$ groups of genes are both coincidentally and periodically transcribed during meiosis at the start of pre-meiotic S phase.

- MCB motifs within the promoters of these and other genes have been examined and appear to fall into three main groups:
  - Type I/ Type II MCB motifs are simple in nature and appear to confer meiotic-specific gene expression.
  - Type III MCB motifs include both mitotic-specific genes and meiotically transcribed genes.
  - Type IV MCB motifs are complex in nature and confer both mitotic and meiotic G1–S expression.

- MCB motifs seem to act in either a directional or a bi-directional manner during the meiotic cell cycle.

- A correlation between general gene function and MCB motif Type has been made in some cases.

Chapter 4

MCB motifs are functional during meiosis in fission yeast

4.1.1 UAS sequences

The functional components of promoter regions can vary widely between individual genes and binding sites for a variety of transcription factors can be found usually within a few hundred bases of the transcription start site of any gene. Some DNA sequence elements are, however, common to a large proportion of the genes that have been studied and these elements form the 'core' promoter. In yeast the core promoter commonly consists of three basic DNA sequence elements: a pyramidine-rich initiator element, which is important for determining the position of transcription initiation; a TATA box, which can vary in distance between 40–100 bps away from the site of transcription initiation and is required for efficient transcription; and upstream activating sequences (UASs), which are also required for transcription and occur between 100–1500bps upstream of the initiation site (Struhl, 1989; White, 2000).

UAS elements of yeast species typically vary between 10–30 bps in length and are usually the major determinant of the specific properties of a promoter as they control the rate and incidence of transcription with regards to physiological or cell cycle conditions. It has been observed that genes subject to a common transcriptional mechanism will contain the same, or highly related, UAS sequences in their promoters. The UAS element is bound by a transcriptional activator, which can function by either overcoming the repressive effects of chromatin structure over the core promoter ('antirepression'), or by interaction with the transcription machinery, to bring about the specific expression of the gene (Struhl, 1989).

These transcriptional activators are not sufficient to initiate transcription alone — they interact with the basal transcription machinery, either directly, or via non-covalent interactions with co-activators that will then contact the basal transcription apparatus (Lewin, 1997c). Repressors of transcription might then act to prevent the upstream transcription factor from contacting or activating the basal transcription machinery (Lewin, 1997c). Therefore UAS sequences play a pivotal role in the control of transcription by recruiting DNA-binding proteins that can activate or repress transcription.

4.1.2 Reporter plasmids.

The study of promoters has been made possible by the use of so called 'reporter plasmids'. Reporter genes, such as *lacZ* (encoding the enzyme β-galactosidase), or the luciferase gene (which encodes a fluorescent protein) are contained within the plasmid downstream of a cloning site and are chosen specifically because their expression is easily and rapidly analysed. Cloning a DNA promoter fragment upstream of the reporter gene allows the transcriptional activity of that DNA sequence to be determined by studying the expression of the reporter gene.

Heterologous UAS elements can be analysed by the use of modified reporter plasmids that still contain a core promoter fused to the reporter gene, but which lack the endogenous UAS motifs that are normally present in a promoter. Theoretically, the reporter gene would then only be expressed if a heterologous UAS element was introduced to the plasmid, upstream of the core promoter, and the cells were in conditions under which that specific UAS element was active. UAS reporter plasmids that are used in yeast species commonly contain the minimal budding yeast promoter *CYC1*, minus the UAS sequences, fused to the bacterial *lacZ* ORF (Guarente, 1983). An example of this is the budding yeast pLGΔ178 series of vectors (Guarente, 1983), from which the fission yeast pSPΔ178 vector, which is used in this thesis, is derived. Δ178 refers to a 178 bp fragment, containing the UAS elements, which is deleted from the *CYC1* promoter in this plasmid series.

4.1.3 MCB motifs

MCB motifs were first identified in the promoters of budding yeast genes as the UAS elements that are essential for cell cycle regulated expression of various genes (Merrill, 1992). MCB motifs consist of the sequence 5′ -ACGCGT- 3′, which, coincidentally, is identical to the recognition sequence of the restriction enzyme *Mlu* I. This led to the UAS being referred to as the *Mlu*I cell cycle box, or MCB element.

These motifs were also found to be present in the promoter regions of a number of fission yeast genes, including *cdc22*$^{+}$, which are periodically transcribed at the G1–S boundary during the mitotic cell cycle. Furthermore, experiments in fission yeast using pSPΔ178 showed that a synthetic DNA oligomer, containing three consecutive MCB motifs, was able to confer mitotic G1–S periodic transcription on *lacZ* (Lowndes *et al.*, 1992). This observation demonstrated that MCB motifs act as UAS elements to regulate mitotic G1–S specific transcription in fission yeast, as they do in budding yeast.

4.1.4 pSPΔ178 experiments

In Chapter 3 of this thesis a survey of the promoter regions of several genes that are known to be expressed during meiosis in this organism was described. A number of genes were identified that contained MCB motifs within their promoters and were subsequently shown to be periodically transcribed at the G1–S boundary of the meiotic cell cycle (Figures 3.1 and 3.2) in agreement with published observations.

However, experiments to demonstrate directly that MCB motifs function during the meiotic cell cycle were required. To this end, haploid *pat1-114* fission yeast strains were created which contained the pSPΔ178 reporter vector with various MCB-containing DNA fragments inserted into its UAS cloning site. After induction of synchronous meioses, northern

blot analysis was used to detect *lacZ* transcript profiles which were determined by the DNA sequences that had been inserted upstream of the minimal promoter in the vector. Using both synthetic MCB DNA sequences and MCB-containing fragments from native promoters, the role of MCB motifs in the fission yeast meiotic cell cycle was investigated.

Unfortunately, the pSPΔ178 vector proved difficult to use, particularly when native genomic MCB sequences were examined. This was due to the large amount of background *lacZ* expression that was seen in the northern blot experiments. The background expression of the reporter gene obscured the specific radioactive signal produced by the UAS-induced transcript. This made the northern blots difficult to interpret, especially when fragments containing fewer MCB consensus sequences (and therefore presumably weaker UAS activities) were used. This is a technical problem that has been noted previously for weak UAS sequences in budding yeast (Griggs & Johnston, 1993; Melcher *et al.*, 2000).

Furthermore, the fact that pSPΔ178 is a multicopy vector may also have contributed to the difficulties experienced, as the presence of several copies of the reporter vector in cells might have the effect of titrating the transcription factor under examination. This could pose two significant problems. First, as cell cycle regulated transcription was being investigated, it is possible that introducing unnaturally high copy numbers of MCB DNA into cells using the plasmid might have an affect on cell division and growth. Consistent with this suggestion, difficulty in obtaining a successful synchronous meiosis was experienced when the strains being used contained pSPΔ178–MCB plasmids. Second, out of the several copies of the vector that were present in each cell, it is possible that not all of the inserted MCB sequences bound a transcription factor. This would increase the proportion of background *lacZ* transcription with regards to specific UAS-induced mRNA and compound the problems experienced with weaker UAS sequences. In an attempt to overcome these problems, therefore, a budding yeast integrating reporter plasmid, which had been specifically designed to remedy the problems associated with using the *CYC1*ΔUAS–*lacZ* reporter cassette, was adapted for use in fission yeast.

4.15 Construction of a new fission yeast integrating vector.

YIPMELβ2. After recognizing that there were significant problems associated with existing budding yeast reporter vectors, Melcher and co-workers set out to design a series of plasmids that would overcome these drawbacks (2000). A detailed analysis of vectors containing the *CYC1*ΔUAS–*lacZ* cassette (identical to that present in pSPΔ178) revealed fortuitous activator binding-sites within pUC19-based plasmid backbone sequences, which contributed to unwanted, background *lacZ* expression.

The plasmid YIPMELβ2 has been constructed to ensure that the bacterial vector sequences are sufficiently far removed from the site of the minimal promoter so as to have no activating effects. YIPMELβ2 still uses the *lacZ* reporter gene, but uses the *MEL1* minimal promoter — which is tightly regulated by a single Gal4p binding site (Melcher & Xu, 2001) — in place of the *CYC1* minimal promoter, which has constitutively accessible TATA sequences. Furthermore the *CEN/ARS* elements — which are responsible for extra-chromosomal plasmid replication — were replaced by integrating vector sequences. This allowed YIPMELβ2 to be integrated into the fission yeast genome in single copy, so avoiding the drawbacks that result from the use of a multi-copy reporter vector.

SpIPMELβ2. To adapt the YIPMELβ2 plasmid for use in fission yeast the *URA3* budding yeast nutritional selection marker had to be replaced with the equivalent fission yeast gene, *ura4*$^{+}$. Although the budding yeast *URA3* gene is functional in fission yeast, native fission yeast sequences are required to permit homologous recombination, which allows linear vector sequences to integrate into the fission yeast genome. The *ura4*$^{+}$ gene was therefore inserted into the *URA3* coding region of YIPMELβ2 to produce *S. pombe* integrating plasmid MELβ2 (SpIPMELβ2, GB 169). This vector, containing various MCB-motif DNA fragments, was then integrated into *pat1-114* fission yeast strains and *lacZ* expression was analysed during synchronous meioses. As an integrating vector this plasmid not only avoids titrating the transcription factor by being present in single copy, but as it is integrated into the genome the DNA sequences to be examined will be correctly packaged into the normal DNA superstructure. They are therefore more likely to interact with transcription factors as they would *in vivo*, which might not be achieved when a non-integrated incompletely packaged plasmid is used.

4.1.6 Summary

In Chapter 3, fission yeast genes were identified that are periodically expressed at the G1–S boundary during meiosis and also contain MCB motifs in their promoters. In this chapter, therefore, an investigation into the function of these MCBs during the meiotic cell cycle was undertaken. This was done by using specifically designed reporter plasmids to monitor the effects of inserting (both synthetic and native) MCB sequences upstream of a UAS-less reporter gene over the course of a *pat1-114*-induced synchronous meiotic cell cycle.

To determine whether MCB motifs function during the meiotic cell cycle, initially, the ability of three synthetic consecutive MCB elements to induce reporter gene expression was examined during a synchronous meiosis. Previously, the UAS-less reporter plasmid pSPΔ178.3M (which contained this synthetic triple-MCB-motif oligomer in its UAS insertion site) had been used to show that these MCB motifs confer periodic mitotic G1–S specific expression in fission yeast (Lowndes *et al.*, 1992). The same construct was therefore used to investigate MCB activity during meiosis in fission yeast.

4.2.1. Synchronous pat1-114 *meiosis*

The vector, pSPΔ178.3M, was transformed by electroporation (Section *2.8.3*) into the *h⁻ pat1-114 leu-1 his1-102 ura-294* (GG190) haploid fission yeast strain to produce GG 206. The pSPΔ178 vector contains the budding yeast *URA3*$^{+}$ gene that complements the fission yeast ura4-294 mutation, permitting plasmid selection in medium lacking uracil. This fission yeast strain (GG 206) was then grown overnight in EMM (plus 100μg l^{-1} leucine and histidine) at 25°C to a cell density of between 5 and 8 x 10^6 cells per ml before being transferred to nitrogen-free EMM overnight to arrest the cells in G1. As stated previously (Section *3.2.1*), the cell density was found to be critical. Leucine and histidine were added to the nitrogen free media at a concentration of 100 μg l^{-1}. Normally, during nitrogen starvation, amino acids were added at half the normal concentration (50 μg l^{-1}), to reduce the possibility that the amino acids were acting as a nitrogen source. However it was found that, in fission yeast strains containing the pSPΔ178 construct, the experiment failed and the culture remained arrested in G1 phase (as judged by flow cytometry analysis), failing to enter meiosis, unless amino acids were added at the full concentration during the nitrogen starvation. The following day NH_4Cl was added back to the culture at a concentration of 0.5g l^{-1}, to encourage a more synchronous meiosis (Muramaki & Nurse, 1999). The temperature of the culture was then raised to 36°C and 35 ml samples were taken every 15 minutes for RNA extraction and 1 ml samples were taken hourly for flow cytometry analysis.

4.2.2 Synthetic MCB motifs are functional during meiosis in fission yeast

Total RNA was extracted from the time course samples and a northern blot was carried out as described previously (Section *2.6.7*). The equal loading of the RNA samples on the northern blot from the haploid meiotic timecourse was confirmed by examination of the 18S and 35S rRNA bands — which were revealed by staining the formaldehyde gel with ethidium bromide prior to the transfer of the RNA to the nitrocellulose membrane (Figure 4.1 *A*). The northern membrane was initially hybridized with probes for two control genes: *cdc22*$^{+}$, a gene that is

periodically expressed at the G1–S boundary during both mitosis and meiosis; and *rec8*$^{+}$, a gene that is periodically expressed at the G1–S boundary specifically during meiosis. The membrane was then hybridized with a *lac*Z DNA probe to assay transcript levels from the reporter gene in pSPΔ178.3M (Figure 4.1 ***B***).

The cells were judged to have entered a synchronous pre-meiotic S phase using flow cytometry analysis, which monitors the doubling of the DNA content of the cells. This occurs approximately two hours after temperature induction of meiosis (Figure 4.1 ***C***). Quantification of the scanned autoradiographs using Image J software (Figure 4.1 ***D***), after normalization to the scanned rRNA levels, showed that RNA profiles, similar to those seen in a wild type diploid meiotic timecourse (Chapter 3, Figure 3.1), were obtained for both *cdc22*$^{+}$ and *rec8*$^{+}$. This confirmed that the cells had successfully entered a synchronous meiosis. The RNA profile obtained using the *lacZ* probe proved difficult to analyse as non-specific, background *lacZ* expression was detected throughout the experiment. However, after optimization of the northern blot technique, a G1–S induced RNA transcript for *lacZ*, the profile of which closely matched those of the *cdc22*$^{+}$ and *rec8*$^{+}$ genes, was consistently distinguished. So, the synthetic triple MCB motif conferred G1–S-specific periodic expression to the *lacZ* reporter gene during meiosis in fission yeast.

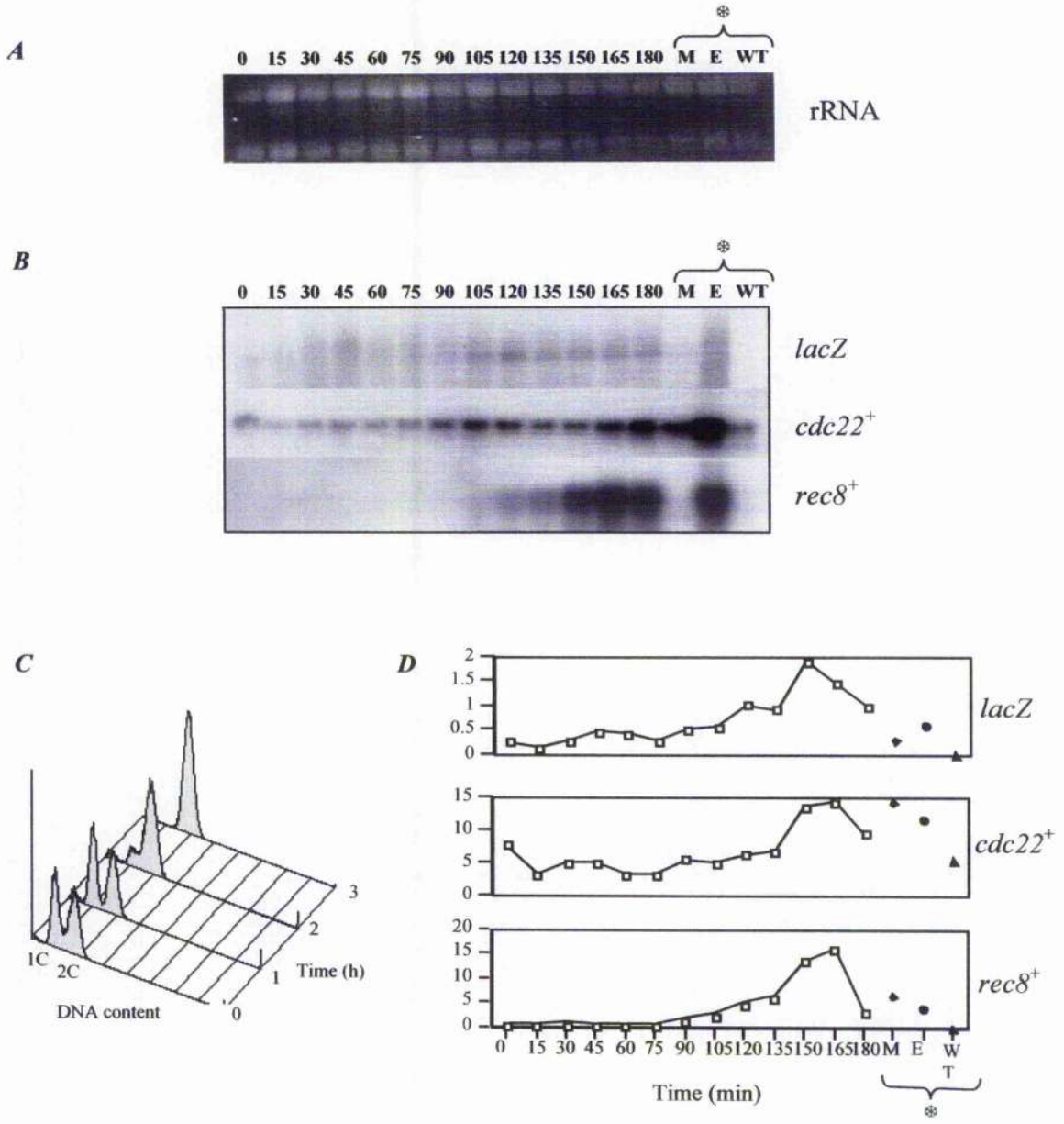


Figure 4.1: Synthetic MCB sequences function during meiosis

A *pat1-114* fission yeast strain containing the pSPΔ178.3M plasmid (GG 206) was induced to undergo a synchronous meiosis. Samples for RNA extraction were taken every 15 minutes. A denaturing formaldehyde gel was run containing 10 μg of RNA per lane and stained with ethidium bromide to confirm equal loading of samples (***A***). The RNA was transferred to a nitrocellulose membrane and northern blot analysis carried out. DNA probes corresponding to the ORFs of *lacZ*, *rec8*$^+$ and *cdc22*$^+$ were used to probe the membrane (***B***). Samples were taken for flow cytometry analysis every hour for three hours to monitor progression of the culture through S phase (***C***). The Image J programme was used to quantify the northern analysis; the results are normalized to the rRNA levels and are measured in arbitrary units (***D***).

❄ = control RNA samples: M, from asynchronous mitotic GG 206 (pSPΔ178.3M) cells; E, from GG 254 cells (pSPΔ178.Empty), 3h meiotic time-course sample; WT, from asynchronous mitotic GG1 cells.

To further investigate the ability of MCB motifs to function during a synchronous meiosis, a study of the ability of native MCB sequences to confer G1–S specific expression was undertaken. Six genes, *rec7*$^{+}$, *rec8*$^{+}$, *rec11*$^{+}$, *rec12*$^{+}$, *rec15*$^{+}$ and *cdc22*$^{+}$ were chosen as candidates for these further experiments. These genes were selected as they represent one gene from each of the MCB 'Type' categories that were identified in Chapter 3. Although both *rec8*$^{+}$ and *rec12*$^{+}$ belong to the Type II category, they represent two slightly different versions of a Type II MCB motif — having two and three single MCB motifs in their promoters respectively, and so both genes were included for this reason.

4.3.1 Cloning MCB-motif DNA sequences into pSPΔ178

In the case of the *rec12*$^{+}$ and *rec15*$^{+}$ genes, DNA oligomers containing engineered *Xho*I sites were designed that would amplify the region of DNA that contained the MCB sequences. Aliquots of the PCR reactions were then used in a TOPO reaction to clone the amplified DNA sequences directly into the plasmid pCRII (Invitrogen). The MCB-motif fragments were excised from pCRII and subcloned into the *Xho*I site of pSPΔ178 to produce pSPΔ178.rec12MCB (GG 675) and pSPΔ178.rec15MCB (GG 672). The pSPΔ178.cdc22MCB2 construct containing the *cdc22*$^{+}$ MCB2 region was obtained from Dr Zakia Maqbool. This construct contains the MCB cluster closest to the ATG of the *cdc22*$^{+}$ gene (Maqbool *et al.*, 2003).

In the case of the *rec7*$^{+}$, *rec8*$^{+}$ and *rec11*$^{+}$ genes the MCB sequences spanned a sufficiently short region of DNA to make a more direct approach to cloning these sequences possible. Two DNA oligomers were designed and synthesized for each gene, one of which represented the upper DNA strand of the MCB-containing sequence and the other of which represented the reverse complement, lower DNA strand. *Xho*I sequences were included in the oligomer design on one end of each strand, to facilitate their subsequent cloning. To clone the MCB DNA fragments, equal amounts of each complementary oligomer were mixed together and incubated for 10 minutes at 95°C. The DNA mixture was then allowed to return slowly to room temperature to give the complementary DNA strands the opportunity to anneal. DNA fragments were therefore produced which contained the synthetic double stranded DNA sequences, plus *Xho*I overhangs, for *rec7*$^{+}$, *rec8*$^{+}$ and *rec11*$^{+}$. These DNA fragments were then directly cloned into the pSPΔ178 to produce pSPΔ178.rec7MCB, pSPΔ178.rec8MCB and pSPΔ178.rec11MCB.

Unfortunately, the *rec11*$^{+}$ sequence proved very difficult to clone. Using both this direct synthetic oligomer method and the normal amplification approach the *rec11*$^{+}$ sequence was consistently cloned into pSPΔ178 in the wrong (reverse) orientation. This occurred several times using both methods of cloning. This can only be put down to either a quirk of secondary

structure within this particular DNA sequence or extreme bad luck. As pSPΔ178 has only a single *Xho*I site in its UAS position, it was not possible to use directional cloning to ensure its correct orientation. So, due to time constraints, attempts to clone the *rec11*$^{+}$ MCB sequences were abandoned.

4.3.2 pSPΔ178 synchronous meiosis experiments

The haploid fission yeast strain GG 190 was separately transformed with each of the different MCB-containing pSPΔ178 plasmids (Appendix IIIb). Synchronous meiosis experiments were completed with each strain as before (Section *4.2*), with samples taken every 15 minutes for RNA extraction and every hour for flow cytometry analysis.

4.3.2.1 pSPΔ178.cdc22MCB2 meiosis

Initially, the pSPΔ178.cdc22MCB2 *pat1-114* strain (GG 677) was analysed. The equal loading of the RNA samples on the northern blot was confirmed by examination of the 18S and 35S rRNA bands as before (Figures 4.2 ***A***). The northern blot was hybridized with probes for *cdc22*$^{+}$, *rec8*$^{+}$ and *lacZ* gene expression (Figure 4.2 ***B***) and the experiment was judged to have entered a synchronous pre-meiotic S phase using flow cytometry analysis (Figure 4.2 ***C***). Quantification of the scanned autoradiographs using Image J software (Figure 4.2 ***D***), showed that RNA profiles, similar to those seen in a wild type diploid meiotic timecourse (Chapter 3, Figure 3.1), were obtained for both *cdc22*$^{+}$ and *rec8*$^{+}$ confirming that GG 677 had entered meiosis. An abundant *lacZ* transcript was detected on this northern blot, the profile of which closely resembled that of the *cdc22*$^{+}$ and *rec8*$^{+}$ genes (Figure 4.2 ***B***). This observation shows that the native *cdc22*$^{+}$ MCB2 cluster (Figure 3.2) confers S phase periodic transcription on to the *lacZ* reporter gene during meiosis.

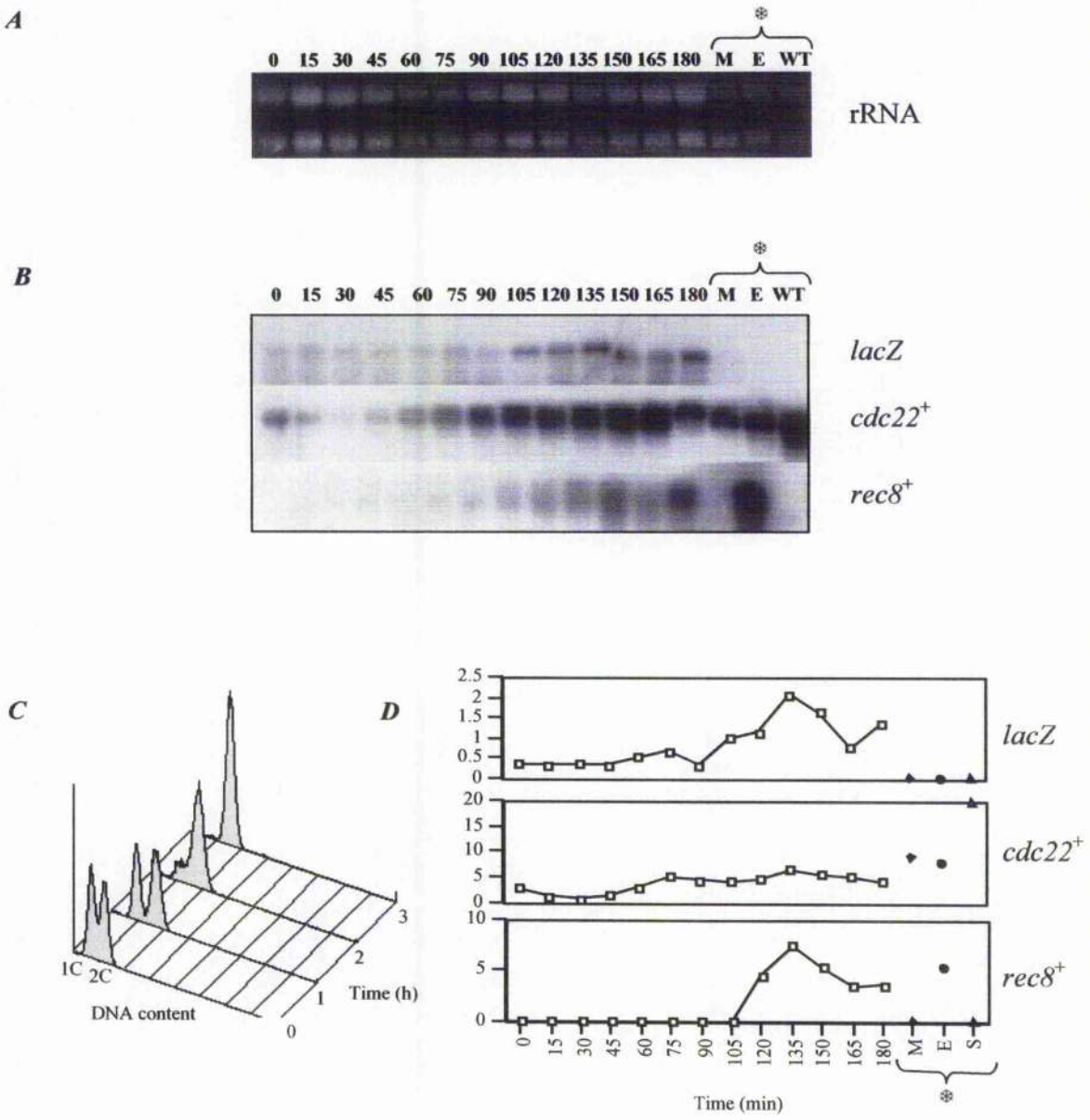


Figure 4.2 pSPΔ178.cdc22MCB2 synchronous meiosis

A *pat1-114* fission yeast strain containing the pSPΔ178.22MCB2 plasmid (GG 677) was induced to undergo a synchronous meiosis. Samples for RNA extraction were taken every 15 minutes. A denaturing formaldehyde gel was run containing 10 μg of RNA per lane and stained with ethidium bromide to confirm equal loading of samples (***A***). The RNA was transferred to a nitrocellulose membrane and northern blot analysis carried out. DNA probes corresponding to the ORFs of *lacZ*, *rec8*$^+$ and *cdc22*$^+$ were used to probe the membrane (***B***). Samples were taken for flow cytometry analysis every hour for three hours to monitor progression of the culture through S phase (***C***). The Image J programme was used to quantify the northern analysis; the results are normalized to the rRNA levels and are measured in arbitrary units (***D***).

❄ = control RNA samples: M, from asynchronous mitotic GG 677 (pSPΔ178.22MCB2) cells; E, from GG254 cells (pSPΔ178.Empty), 3h meiotic time-course sample; WT, from asynchronous mitotic GG1 cells.

4.3.2.2 pSPΔ178.rec12MCB and pSPΔ178.rec15MCB meioses

Northern blot analysis was next carried out for the pSPΔ178.rec12MCB (GG 675) and pSPΔ178.rec15MCB (GG 672) strains, which were induced to undergo synchronous meioses as before (Section *4.2*). The equal loading of the RNA samples on the northern blots was confirmed by examination of the 18S and 35S rRNA bands (Figure 4.3 ***A***) and the northern membranes for pSPΔ178.rec12MCB and pSPΔ178.rec15MCB were both hybridized with probes for *lacZ* and *cdc22*$^{+}$, as well as *rec12*$^{+}$ and *rec15*$^{+}$ respectively (figure 4.3 ***B***). Again, the cells were judged to have entered a synchronous pre-meiotic S phase using flow cytometry analysis (Figure 4.3 ***C***). However, as was the case with the pSPΔ178.3M construct the *lacZ* northern blots were exceedingly difficult to interpret due to a large amount of background expression.

Quantification of the scanned autoradiographs for the pSPΔ178.rec15 timecourse using Image J software (Figure 4.3 ***D***), showed that RNA profiles, similar to those seen in a wild type diploid meiotic timecourse (Chapter 3, Figure 3.1), were obtained for both *cdc22*$^{+}$ and *rec15*$^{+}$. This confirmed that our haploid strain GG 672 had successfully entered meiosis. The quantification also confirmed the presence of a poorly defined *lacZ* transcript, the periodicity of which mirrored that of the *cdc22*$^{+}$ and *rec15*$^{+}$ genes. This confirms that the native *rec15*$^{+}$ MCB sequence is functional during meiosis and able to confer G1–S periodic expression, albeit weak expression, on to a reporter gene.

However, no distinguishable *lacZ* transcript was detected during the pSPΔ178.rec12MCB timecourse. Taking into account this observation, and the fact that the *lacZ* transcript from the pSPΔ178.rec15MCB timecourse had been very difficult and time consuming to obtain, it was decided that meiotic inductions using the pSPΔ178.rec7MCB and pSPΔ178.rec8MCB containing strains would not be pursued. An improved, more-specific and sensitive reporter system was needed to study these weaker UAS elements.

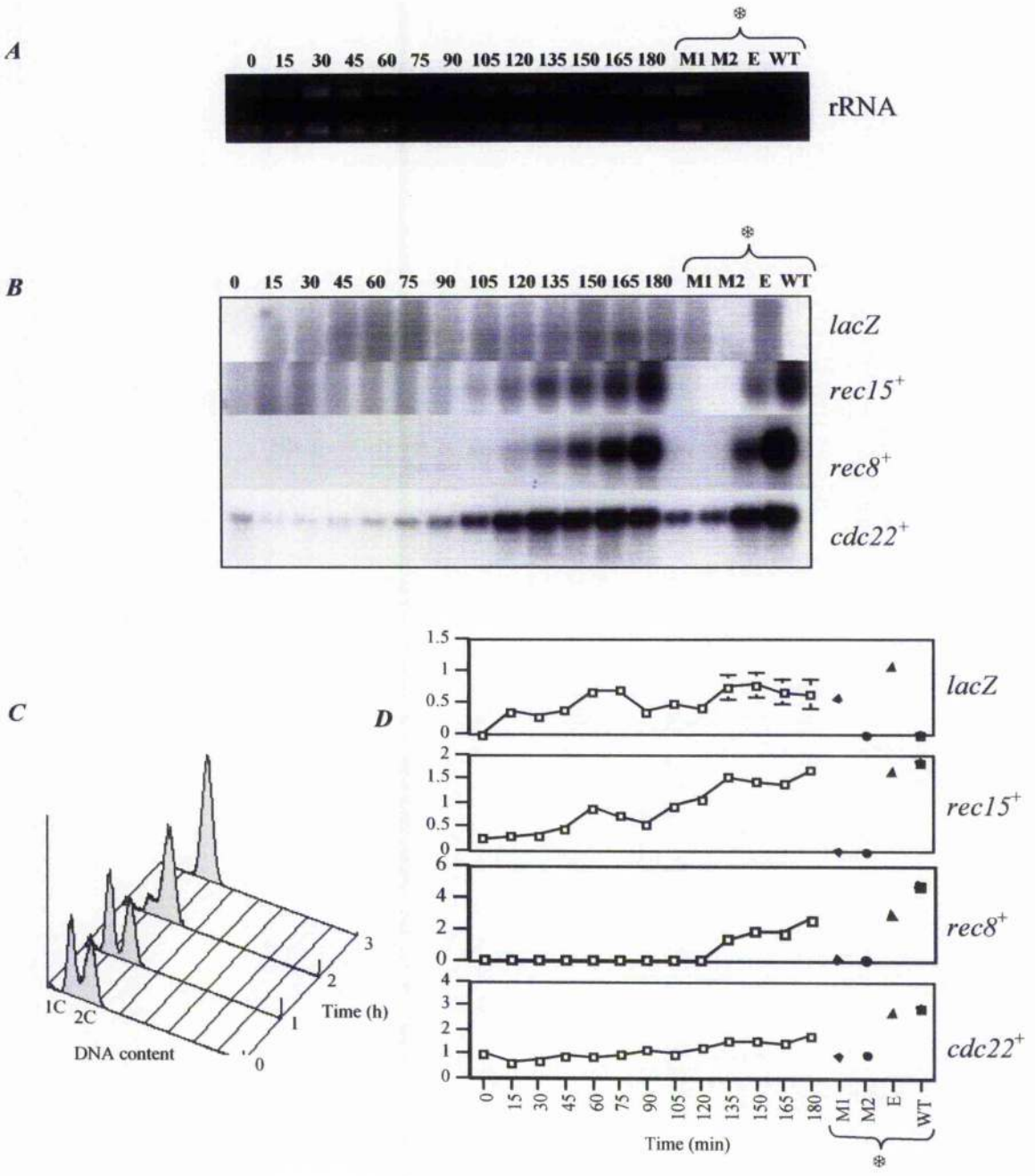


Figure. 4.3. pSPΔ178.rec15MCB synchronous meiosis

A *pat1-114* fission yeast strain containing the pSPΔ178.rec15MCB plasmid (GG 672) was induced to undergo a synchronous meiosis. Samples for RNA extraction were taken every 15 minutes. A denaturing formaldehyde gel was run containing 10 μg of RNA per lane and stained with ethidium bromide to confirm equal loading of samples (***A***). The RNA was transferred to a nitrocellulose membrane and northern blot analysis carried out. DNA probes corresponding to the ORFs of *lacZ*, *rec15*$^+$, *rec8*$^+$ and *cdc22*$^+$ were used to probe the membrane (***B***). Samples were taken for flow cytometry analysis every hour for three hours to monitor progression of the culture through S phase (***C***). The Image J programme was used to quantify the northern analysis; the results are normalized to the rRNA levels and are measured in arbitrary units (***D***).

❄ = control RNA samples: M1, from asynchronous mitotic GG 677 (pSPΔ178.22MCB2) cells; M2 from asynchronous mitotic GG 672 (pSPΔ178.15MCB2) cells; E, from GG254 cells (pSPΔ178.Empty), 3h meiotic time-course sample; WT, from asynchronous mitotic GG1 cells.

The difficulty experienced in obtaining interpretable results using northern blots for the *lacZ* reporter gene in pSPΔ178, prompted a search for an improved reporter vector. A budding yeast, integrating reporter vector, YIPMELβ2, had been designed specifically to overcome the problems experienced with background expression from the *CYC1*ΔUAS–*lacZ* reporter cassette (Melcher *et al.*, 2000). It was therefore decided to adapt this vector for use in fission yeast. To do this the *URA3* budding yeast gene of YIPMELß2 had to be replaced with the *ura4*$^+$ fission yeast gene. The *ura4*$^+$ gene provides nutritional selection for the plasmid (in *ura4*$^-$ yeast strains) as well as the homologous sequences required for homologous recombination and integration into the fission yeast genome at the *ura4*$^+$ locus.

4.4.1 SpIPMELβ2 construction

The integrating plasmid pFY20 (GB 77) was used as a source of a functional *ura4*$^+$ gene. To completely delete the budding yeast *URA3* gene from YIPMELβ2 would have required a complex series of restriction digests. So, instead, *URA3* was disrupted by inserting the fission yeast *ura4*$^+$ gene between the *Nco*I and *Apa*I sites of the *URA3* coding sequence.

Synthetic oligonucleotides were designed to amplify the *ura4*$^+$ plus its promoter region from pFY20 and incorporate *Nco*I and *Apa*I sites on either end. This DNA fragment was then cloned into pBSKS$^+$ (Invitrogen). The *ura4*$^+$ gene was then digested out of pBSKS$^+$ura4 and cloned into the *Nco*I/*Apa*I digested YIPMELβ2 to create SpIPMELβ2. The *ura4*$^+$ gene has a unique *Avr*II restriction site that allowed the linearization of the vector for integration into the fission yeast genome at the *ura4*$^+$ locus. This was done in strains containing a point mutation in the *ura4*$^+$ gene (*ura4-294*), which permitted for the selection of *ura*$^+$ colonies on selective medium.

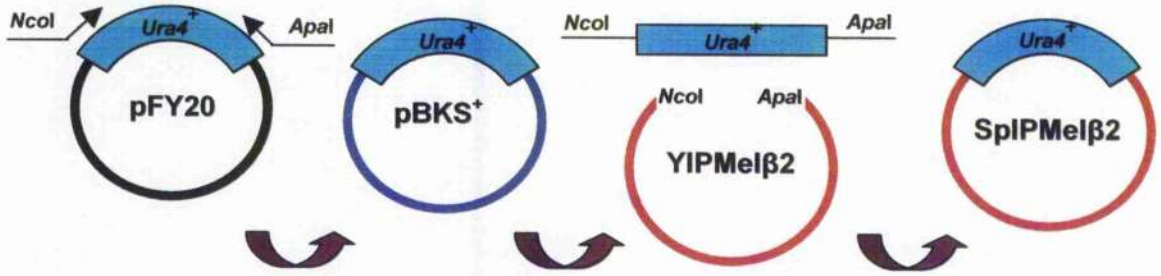


Figure. 4.4. Construction of SpIPMELβ2

The *ura4*$^+$ gene was amplified from pFY20 using *Nco*I and *Apa*I primers and cloned directly into *Sma*I digested pBluescriptKS$^+$. *Nco*I and *Apa*I were then used to digest *ura4*$^+$ out of pBSKS$^+$ and to digest YIPMelβ2. These two DNA fragments were then ligated to produce SpIPMel β2.

4.5 SpIPMELβ2 experiments

The DNA sequences containing the *rec7*$^{+}$ and *rec8*$^{+}$ MCB motifs were cloned into the unique *Xho*I site of SpIPMELß2 to create SpIPMELß2rec7MCB (GB 304) and SpIPMELß2rec8MCB (GB 305). The *rec15*$^{+}$ and *cdc22*$^{+}$-MCB2 motif fragments were digested out of pSPΔ178 (GB 87 and GB 73 respectively) and cloned into SpIPMELβ2 to create SpIPMELβ2rec15MCB (GB 306) and SpIPMELβ2cdc22MCB2 (GB 307) (see Appendix IIIb). These plasmids were then linearised with *Avr*II and integrated into the GG190 genomic *ura4* locus by homologous recombination (GG 681 and GG 680, respectively). Integrated strains were initially identified by selection on medium lacking uracil and subsequently confirmed by PCR using primers GO 521 (5′ GCTTCGTCGGCATCTCTGC 3′) and GO 522 (5′ TTCGCGCGTCCCGCAGCGC 3′)

The fission yeast *pat1-114* strain containing SpIPMELβ2cdc22MCB2 (GG 680) was induced to undergo a synchronous meiosis (Section *4.2*) and samples were taken every 15 minutes for RNA extraction and every hour for flow cytometry analysis. Equal loading of the RNA samples on the northern blot was confirmed by examination of the 18S and 35S rRNA bands (Figure 4.5 ***A***) and the northern blot was hybridized with probes for: *cdc22*$^{+}$, *rec8*$^{+}$ and *lacZ* (Figure 4.5 ***B***). The experiment was judged to have entered a synchronous pre-meiotic S phase using flow cytometry analysis (Figure 4.5 ***C***) and quantification of the scanned autoradiographs was carried out using Image J software (Figure 4.5 ***D***). RNA profiles, similar to those seen in a wild type diploid meiotic timecourse (Chapter 3, Figure 3.1), were obtained for both *cdc22*$^{+}$ and *rec8*$^{+}$, confirming that GG 680 had successfully entered a synchronous meiosis.

The RNA profile obtained using the *lacZ* probe proved much easier to analyse using the SpIPMELβ2 plasmid because, as expected, most of the non-specific, background *lacZ* expression was eliminated. A prominant *lacZ* transcript was detected, the profile of which mirrored those of *cdc22*$^{+}$ and *rec8*$^{+}$. This observation confirmed the pSPA178cdc22MCB2 experiment showing that these native MCB motifs were functional in meiosis and showed that a more sensitive fission yeast UAS reporter vector had been successfully engineered.

Unfortunately, due to time constraints further experiments using the other MCB motif types in this vector were not completed.

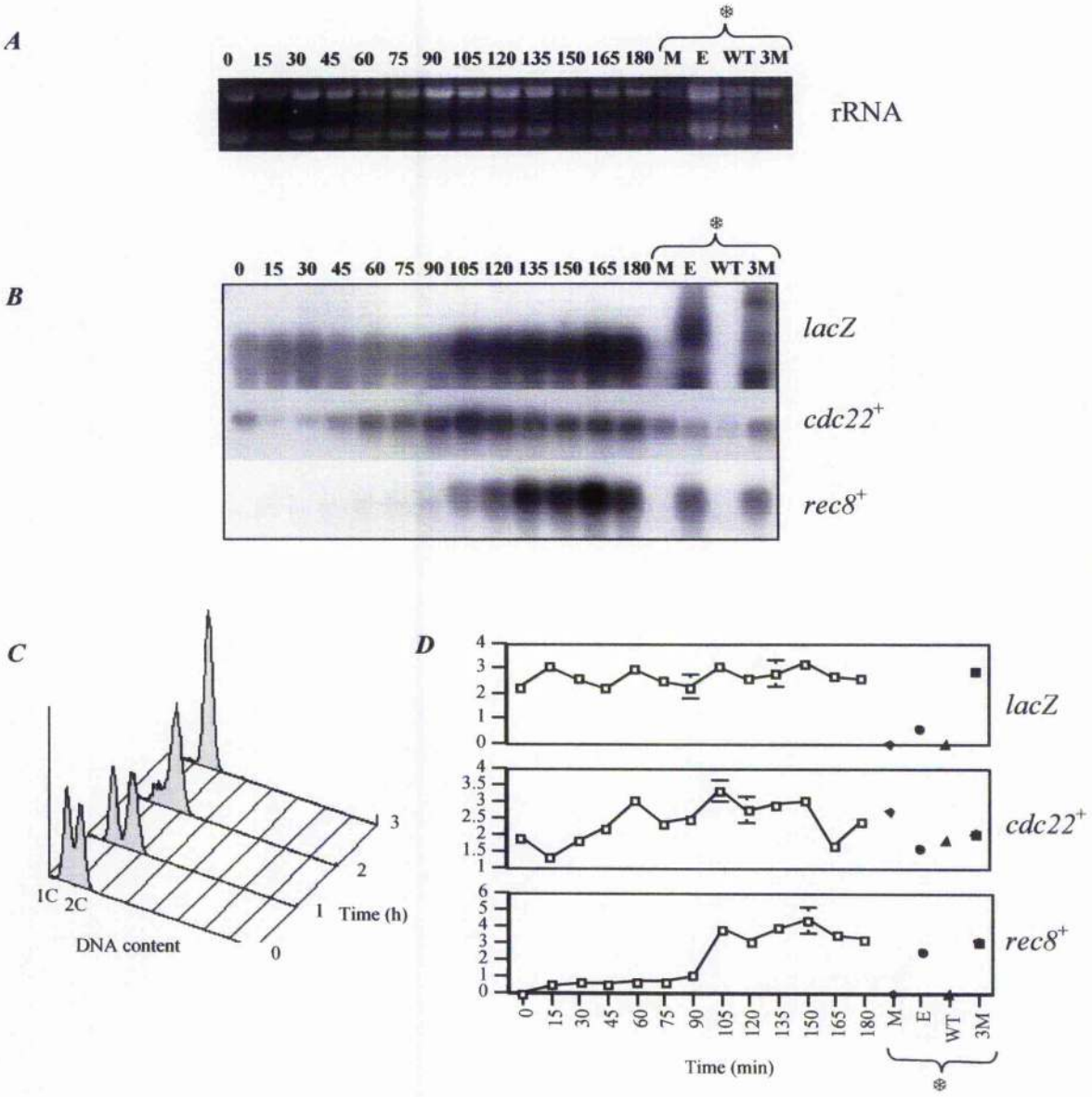


Figure 4.5. SpIPMELβ2.22MCB2 synchronous meiosis.

A *pat1-114* fission yeast strain containing the SpIPMELβ2.22MCB2 plasmid (GG 680) was induced to undergo a synchronous meiosis. Samples for RNA extraction were taken every fifteen minutes. A denaturing formaldehyde gel was run containing 10 μg of RNA per lane and stained with ethidium bromide to confirm equal loading of samples (***A***). The RNA was transferred to a nitrocellulose membrane and northern blot analysis carried out. DNA probes corresponding to the ORFs of *lacZ*, *rec8*$^+$ and *cdc22*$^+$ were used to probe the membrane (***B***).Samples were taken for flow cytometry analysis every hour for three hours to monitor progression of the culture through S phase (***C***). The Image J programme was used to quantify the northern analysis; results were normalized to the rRNA levels and are measured n arbitrary units (***D***).

❄ = control RNA samples: M, from asynchronous mitotic GG 680 (SpIPMELβ2.22MCB2) cells; E, from GG 678 cells (SpIPMELβ2.Empty), 3h meiotic time-course sample; WT, from asynchronous mitotic GG1 cells; 3M, from (pSPΔ178.3M), 3h meiotic time-course sample.

It has been shown in Chapter 3 of this thesis that several fission yeast genes containing MCB motifs in their promoters are periodically and coincidentally expressed at the G1–S boundary of the meiotic cell cycle. So, in this chapter, experiments are described which investigate whether these MCB motifs were functional during meiosis in fission yeast. A synthetic triple MCB motif and native *cdc22*$^{+}$-MCB2 and *rec15*$^{+}$ MCB motifs were all shown to confer G1–S specific transcription on to the *lacZ* reporter gene, confirming that MCB motifs are physiologically relevant during the meiotic cell cycle in fission yeast.

The number and arrangement of individual 5' ACGCGT 3' motifs varies widely between the promoters of different genes, although certain patterns are apparent. The MCB motifs range from the simple, single Type I MCB sequence of the *rec7*$^{+}$ gene, to the complex two-part Type IV MCB motif of *cdc22*$^{+}$. Ideally, in this chapter, the ability of examples of each of these MCB motif-types to induce reporter gene expression during a meiotic timecourse, would have been investigated. This would have provided a more precise insight into exactly what sequences are required to induce meiotic-specific gene expression. Unfortunately, the *CYC1*ΔUAS-*lacZ* reporter cassette of pSPΔ178 produced a large amount of non-specific background transcription and was therefore not sensitive enough to reveal activity, or lack of activity, for the weaker UAS motifs of *rec7*$^{+}$, *rec8*$^{+}$ and *rec12*$^{+}$. A more sensitive reporter system was therefore sought to try and overcome this problem.

The UAS reporter plasmid YIPMELß2 (Melcher *et al.*, 2000) was adapted for use in fission yeast by disrupting the budding yeast *URA3* gene with the fission yeast *ura4*$^{+}$ gene to produce SpIPMELβ2. This provided *ura4*$^{+}$ DNA sequences to allow the homologous recombination of a single copy of the linearized vector into the fission yeast genome. Although several different MCB-motif constructs were made, time limitations meant that only the strain containing SpIPMELβ2.cdc22MCB2 (GG 680) was analysed. SpIPMELβ2.cdc22MCB2 showed a G1–S specific *lacZ* transcript profile, with little background transcription, demonstrating that SpIPMELβ2 works successfully in fission yeast. With more time, the other SpIPMELβ2 constructs that were made could be used to dissect the exact MCB-motif sequences needed to induce meiotic-specific gene expression in fission yeast and allow the refinement of the consensus sequence for MCB motifs.

- Using the fission yeast UAS reporter construct pSPΔ178, a synthetic DNA oligomer containing three consecutive MCB sequences, and the downstream MCB motif of *cdc22*$^{+}$, were shown to confer G1–S periodic gene expression on to a reporter gene during the meiotic cell cycle.
- Using pSPΔ178, the MCB motif of *rec15*$^{+}$ was shown confer meiotic-specific G1–S periodic gene expression on to a reporter gene during the meiotic cell cycle.
- An integrating fission yeast reporter vector (SpIPMELβ2) was developed by modifying a budding yeast UAS reporter vector (YIPMELβ2) to overcome problems experienced with pSPΔ178.
- Using SpIPMELβ2 it was confirmed that the *cdc22*$^{+}$-MCB2 motif cluster conferred G1–S specific gene-expression to *lacZ* during the meiotic cell cycle.
- Combined, these experiments demonstrate that MCB motifs are physiologically relevant during meiosis in fission yeast and control G1–S-specific meiotic transcription in this organism.

Chapter 5

Identification of a meiotic MCB-motif DNA-binding activity in fission yeast

5.1.1 The mitotic MCB-motif binding activity

The DNA-binding activity that interacts with the MCB motifs in the promoters of genes including *cdc22*$^+$, *cdc18*$^+$ and *cdt1*$^+$ — which are expressed specifically at the G1–S period of the fission yeast mitotic cell cycle — was identified as the MBF transcription factor (also known as DSC1; Lowndes *et al.*, 1992; Kelly *et al.*, 1993; Hoffman & Beach 1994). MBF contains the protein components Cdc10p, Res1p, Res2p and Rep2p (Lowndes *et al.*, 1992; Caligiuri & Beach, 1993; Miyamoto *et al.*, 1994; Zhu *et al.*, 1994; Nakashima *et al.*, 1995) of which Cdc10p is absolutely required for cell cycle progress (Nurse & Bissett, 1981), Res1p and Res2p show DNA-binding activity (Caligiuri & Beach 1993; Zhu *et al.*, 1994) and Rep2p has an activating function (Nakashima *et al.*, 1995).

MCB motifs have been shown to confer cell-cycle regulation to a reporter gene during mitosis (Lowndes *et al.*, 1992). However, mutations in *cdc10*$^+$ have differing effects on the transcription of MCB-regulated genes (Fernandez-Sarabia *et al.*, 1993; Kelly *et al.*, 1993; Hofman & Beach, 1994; McInerny *et al.*, 1995; Baum *et al.*, 1997) indicating that the MBF complex has both activating and repressing functions.

5.1.2 Electrophoretic mobility shift assay

It is possible to identify the protein, or protein complex, which binds a specific UAS by the use of an electrophoretic mobility shift assay (EMSA). DNA fragments, containing sequences of interest, are radiolabelled (Section *2.6.1*) and incubated with a sample of protein from cells under investigation. During this incubation, the radiolabelled DNA fragments are able to bind to proteins, or protein complexes, that are present in the protein extract. The protein–DNA mixture is then electrophoresed on an acrylamide gel, which separates the reaction in the gel on the basis of mobility. The gel is then dried and visualized by autoradiography.

Unbound, radiolabelled DNA fragments become separated from the protein in the reaction mixture and appear as a 'free probe' band at the bottom of the gel, as they have a high mobility being small in size. Radiolabelled DNA fragments that have bound to a protein, or a protein complex, are retarded in the gel by the protein complex, and therefore 'shift' the radioactive signal up the gel.

To ensure that the shifted radioactive signal represents a specific interaction between a protein and a DNA fragment, rather than a non-specific general DNA-binding reaction, a 'self-competition' experiment can be undertaken. An increasing ratio of non-radiolabelled DNA, identical to the labelled fragment, is added to successive, otherwise identical, reaction mixtures, which are run on a gel as before. If the binding reaction is specific to the DNA fragment used, the radioactive signal will decrease over the course of the successive reactions as the non-

radiolabelled DNA competes for the binding of the protein. If however, the binding reaction is not specific to that DNA fragment, the radioactive signal will not be competed with by the addition of unlabelled competitor DNA, as the radiolabelled DNA will still be bound. Therefore, it is possible to identify protein complexes that interact specifically with particular DNA sequences.

5.1.3 Summary

In Chapter 3, it was established that MCB motifs are present in the promoters of fission yeast genes that are expressed at the G1–S boundary of the meiotic cell cycle. In Chapter 4 it was shown that MCB motifs from these genes are functional during meiosis and are therefore physiologically relevant in fission yeast during the meiotic cell cycle. In this chapter, therefore, we undertook to identify a binding activity for these meiotic UAS motifs in fission yeast. The MCB1 and MCB2 clusters of the *cdc22*$^{+}$ gene and the MCB clusters of the *rec7*$^{+}$, *rec8*$^{+}$, *rec11*$^{+}$ and *rec15*$^{+}$ genes were used in this investigation.

To discover if the meiotic MCB motifs could bind to an MBF-like complex, EMSAs were carried out using an example of each different type of MCB motif, as identified and characterized in Chapter 3. Initially, cell samples were taken from an asynchronous, mitotic, diploid fission yeast culture (GG 375) and the protein was extracted and stored at –70°C (Section *2.7.2*). DNA fragments to be used in the EMSA were then labelled with radioactive γ-ATP using Klenow DNA polymerase (Promega; see Section *2.7.1*).

Similar DNA fragments to those used in the UAS reporter vector experiments (Chapter 4) were chosen for use in the EMSA and they represented the MCB1 and MCB2 clusters of the *cdc22*$^{+}$ gene and the MCB clusters of the *rec7*$^{+}$, *rec8*$^{+}$, *rec11*$^{+}$ and *rec15*$^{+}$ genes. Self-competition experiments were carried out for each probe to ensure that any proteins, or protein complexes, identified in these experiments were interacting specifically with the radioactive probes used, and were not merely representing a non-specific DNA-binding complex. Therefore, for each EMSA probe, four reactions were completed for each group and are shown in Figure 5.1.

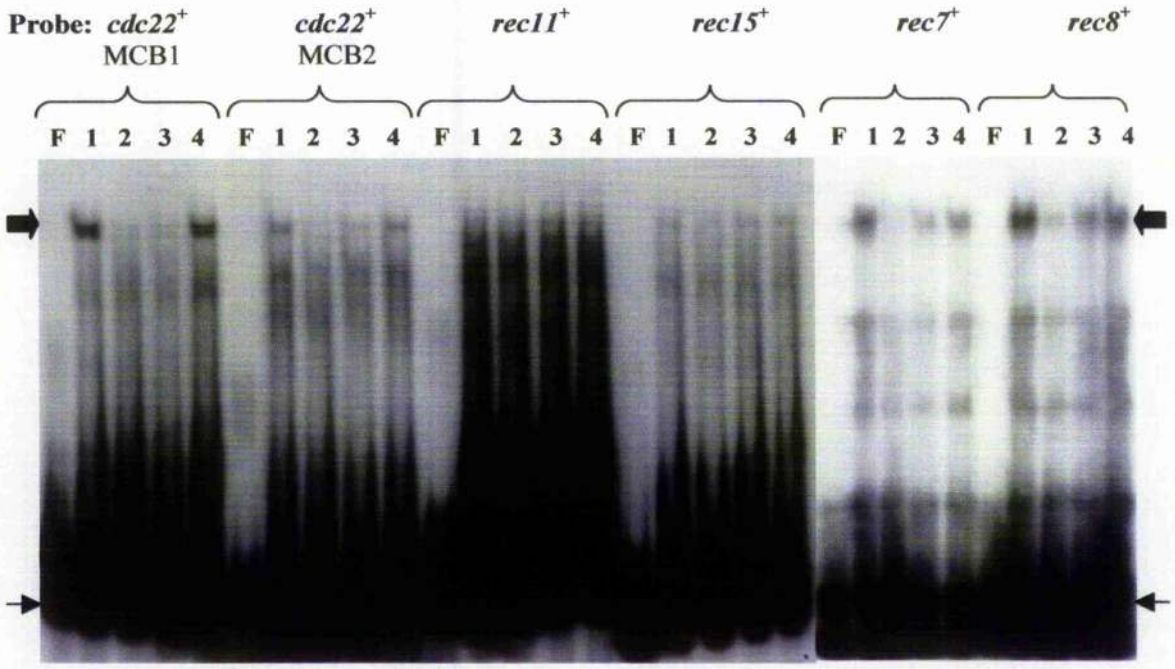
The first lane in each group represents an EMSA reaction containing a 20µl aliquot of the asynchronous mitotic protein and the radioactively labelled MCB probe. The second, third and fourth lanes in each group represent the self-competition reactions. In addition to the reactants used in the first lane, these subsequent reactions contain an excess amount (Figure 5.1: lane 2), a 1 in 10 dilution (Figure 5.1: lane 3), and a 1 in 100 dilution (Figure 5.1: lane 4), of unlabelled competitor DNA, which consists of the same DNA fragment that was used to make the labelled probe.

Identical *cdc22*$^{+}$ MCB1 and MCB2 probes have been used previously in EMSA experiments (Lowndes *et al.*, 1992) and are known to bind the MBF transcription factor, which is responsible for MCB-regulated transcription during mitosis in fission yeast. For each MCB probe used, a binding activity was identified which appeared to have the same mobility as the MBF complex that retards the *cdc22*$^{+}$ MCB1 and *cdc22*$^{+}$ MCB2 probes. Furthermore, in each case, the retarded signal that was visualized by autoradiography was competed with by the addition of excess amounts of the same non-radiolabelled DNA probe. This retarded signal also returned, in each case, when diminishing amounts of the unlabelled competitor DNA were added. In the case of the *rec11*$^{+}$ probe the EMSA was unclear, indicating that the complex binds this DNA fragment weakly.

These experiments show, therefore, that the *rec7*$^{+}$, *rec8*$^{+}$, *rec11*$^{+}$ and *rec15*$^{+}$ MCB motifs bound a protein complex that is present in mitotic cells and is very similar in mobility to the MBF complex that is known to bind the *cdc22*$^{+}$ probes. These *rec*$^{+}$ gene promoter sequences contain much simpler arrangements of MCB motifs than those present in the *cdc22*$^{+}$ probes, but

the sequences are, nevertheless, highly homologous. It is likely, therefore, that the MCB motifs of the *rec7*$^{+}$, *rec8*$^{+}$, *rec11*$^{+}$ and *rec15*$^{+}$ genes bound the same, or a closely related, MBF transcription factor.

Figure 5.1: Meiotic-specific MCB motifs bind to mitotic MBF



Key:
F= free radiolabelled probe
1= radiolabelled probe + protein extract
2= radiolabelled probe + excess unlabelled probe + protein extract
3= radiolabelled probe + 1/10 dilution of unlabelled probe + protein extract
4= radiolabelled probe + 1/100 dilution of unlabelled probe + protein extract

Figure 5.1: Meiotic-specific MCB elements bind to mitotic MBF
An electrophoretic mobility shift assay (EMSA) was performed using protein extracted from asynchronous mitotic cells (GG 375). MCB sequences amplified from genes that are expressed specifically in meiosis (*rec11*$^+$, *rec15*$^+$, *rec7*$^+$, *rec8*$^+$), were prepared as probes and used in comparison with MCB-motif probes that were amplified from genes that are expressed in both mitosis and meiosis (*cdc22*$^+$ MCB1 and *cdc22*$^+$ MCB2). To check the specificity of the shift, excess, a 1/10 dilution and a 1/100 dilution of unlabelled probe were added to lanes designated 2–4 respectively. The upper arrow shows the position of the MBF–DNA complex as determined by the *cdc22*$^+$ MCB1 and *cdc22*$^+$ MCB2 reactions. The lower arrows show the position of the free probe.

5.3 Meiotic-specific MCB motifs compete with the cdc22$^+$ *MCB1 and* cdc22$^+$ *MCB2 EMSA probes for binding of mitotic MBF*

It had been initially established that a protein complex of similar mobility to the MBF complex retarded the MCB probes from the *rec7*$^+$, *rec8*$^+$, *rec11*$^+$ and *rec15*$^+$ promoters, and, the MBF complex is known to interact with the *cdc22*$^+$ MCB1 and MCB2 probes in mitotic cells. To provide further evidence that these UAS motifs were binding the same, or a closely related, protein complex, cross-competition experiments were undertaken. These experiments involved adding non-radiolabelled *rec7*$^+$, *rec8*$^+$, *rec11*$^+$ and *rec15*$^+$ MCB probes to an EMSA reaction that contained the radiolabelled *cdc22*$^+$ MCB1 probe and the appropriate protein extract. If the unlabelled probes were able to bind MBF they would compete with the labelled probes for access to the MBF complex and the retarded radioactive signal on the autoradiograph would disappear.

Figure 5.2 shows such a competition EMSA experiment. Protein extracts from cell samples that were taken from an asynchronous, mitotic, diploid fission yeast culture (GG 375) were used in these reactions. Initially an EMSA reaction containing the *cdc22*$^+$ MCB1 probe alone (Figure 5.2: lane 2) was run in an acrylamide gel alongside subsequent self-competition reactions containing excess amounts (Figure 5.2: lane 3) and a 1 in 10 dilution (Figure 5.2: lane 4) of unlabelled *cdc22*$^+$ MCB1 probe as control reactions.

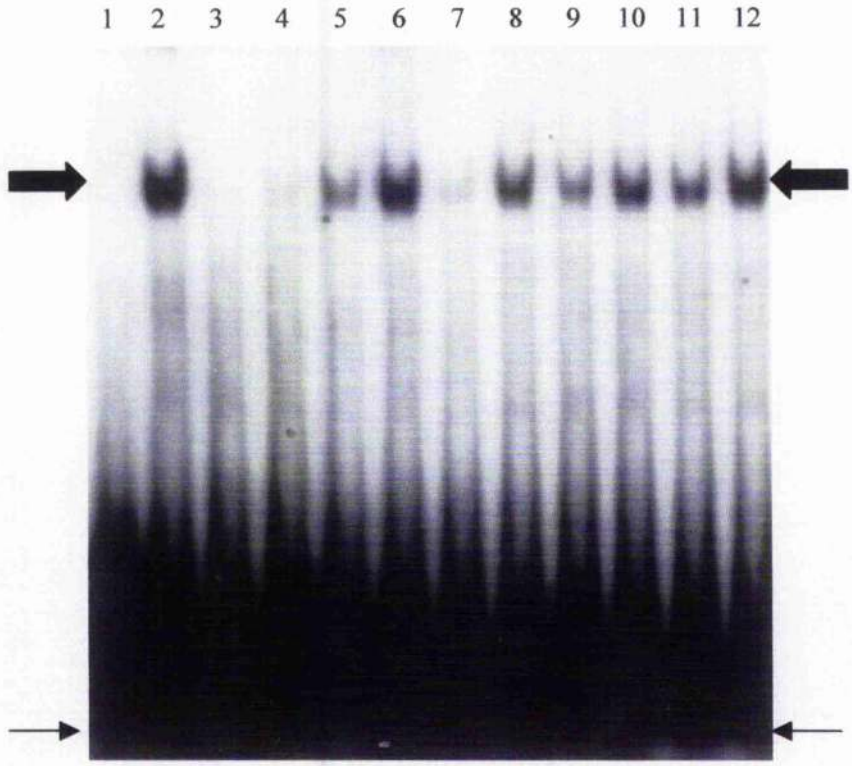
The next lanes represent EMSA reactions containing radiolabelled *cdc22*$^+$ MCB1 probe, mitotic protein extract and additional, competing, unlabelled probes as follows: excess *rec7*$^+$ MCB probe (Figure 5.2: lane 5), 1 in 10 dilution of the *rec7*$^+$ MCB probe (Figure 5.2: lane 6), excess *rec8*$^+$ MCB probe (Figure 5.2: lane 7), 1 in 10 dilution of the *rec8*$^+$ MCB probe (Figure 5.2 lane 8), excess *rec11*$^+$ MCB probe (Figure 5.2: lane 9), 1 in 10 dilution of the *rec11*$^+$ MCB probe (Figure 5.2: lane 10), excess *rec15*$^+$ MCB probe (Figure 5.2: lane 11), 1 in 10 dilution of the *rec15*$^+$ MCB probe (Figure 5.2: lane 12).

The radioactive signals from the retarded DNA–protein complexes showed that, as expected, the *cdc22*$^+$ MCB1 probe competed strongly with itself, and almost no retarded signal was detected in the excess or the diluted competitor lanes (Figure 5.2: lanes 3 and 4). The unlabelled *rec7*$^+$ and *rec8*$^+$ MCB probes both competed well with the radiolabelled *cdc22*$^+$ MCB1 probe for the binding of MBF when added in excess, although a 1 in 10 dilution of the unlabelled *rec*$^-$ MCB probes was not sufficient to compete with the signal produced by the *cdc22*$^+$ MCB1 probe. However, the detected radioactive signal in both these diluted competitor lanes was still weaker than the signal produced with *cdc22*$^+$ MCB1 probe alone. The *rec11*$^+$ and *rec15*$^+$ MCB probes did compete with the *cdc22*$^+$ MCB1 probe for the binding of MBF but to a lesser extent than the *rec7*$^+$ and *rec8*$^+$ MCB probes. Again, although a significant retarded signal was detected from the radiolabelled *cdc22*$^+$ MCB1 probe in the diluted competitor lanes for the

rec11^{+} and *rec15*$^{+}$ MCB probes (Figure 5.2: lanes 10 and 12), it was still not as strong as the signal detected with the *cdc22*$^{+}$ MCB1 probe alone.

Essentially identical results were obtained when the *cdc22*$^{+}$ MCB2 probe was used as the radiolabelled probe (Appendix V). Given that the *cdc22*$^{+}$ MCB1 and MCB2 probes are known to bind the MBF complex in mitotic cells (Lowndes *et al.*, 1992), this EMSA shows that the MCB clusters in all of the *rec*$^{+}$ genes tested were able to directly compete for binding of the mitotic MBF complex.

Figure 5.2: Meiotic-specific MCB motifs compete with the mitotic MBF



Key
1 = free *cdc22*$^+$ MCB1 probe
Lanes 2–12 contain radiolabelled *cdc22*$^+$ MCB1 probe + protein extracted from asynchronous, mitotic, wild-type diploid cells plus:
2 = no competitor
3 = excess unlabelled *cdc22*$^+$ MCB1 probe
4 = 1/10 dilution of *cdc22*$^+$ MCB1 probe
5 = excess unlabelled *rec7*$^+$ probe
6 = 1/10 dilution of *rec7*$^+$ probe
7 = excess unlabelled *rec8*$^+$ probe
8 = 1/10 dilution of *rec8*$^+$ probe
9 = excess unlabelled *rec11*$^+$ probe
10 = 1/10 dilution of *rec11*$^+$ probe
11 = excess unlabelled *rec15*$^+$ probe
12 = 1/10 dilution of *rec15*$^+$ probe

Figure 5.2: Meiotic-specific MCB motifs compete with the mitotic MBF
Asynchronous mitotic protein samples were prepared from wild type haploid cells and an EMSA carried out using radiolabelled *cdc22*$^+$ MCB1 probe and various unlabelled competitor DNA probes taken from the promoters of meiotic-specific genes (see key). The upper arrows show the position of the MBF–DNA complex. The lower arrows show the position of the free probe.

5.4.1 A meiotic MBF-like complex binds to cdc22⁺ MCB motifs

It has been demonstrated in previous studies (Lowndes *et al.*, 1992) that the *cdc22*$^{+}$ MCB clusters bind the MBF complex during mitosis, and it has been shown in this chapter that MCB clusters from meiotically expressed genes compete for MBF binding in mitotic cells. However, we next wanted to investigate whether MCB motifs interacted with a similar complex during meiosis.

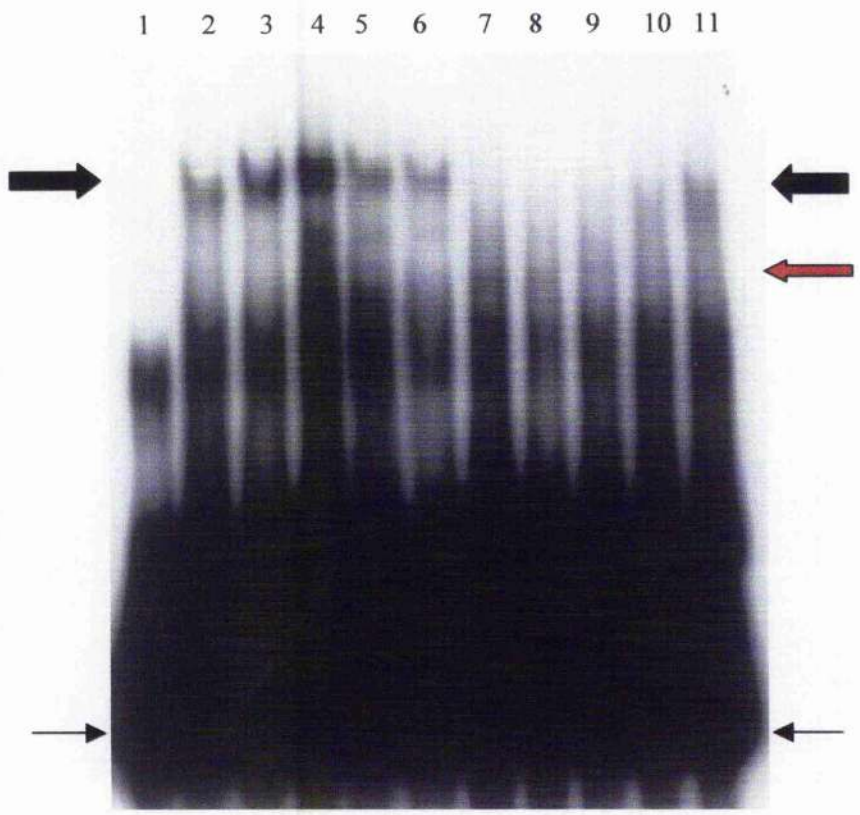
Therefore, two identical asynchronous, diploid, *pat1-114*, mitotic cultures (strain GG 375) were grown at 25°C, to a cell density of between 5 and 8 x 10^{6} cells per ml. One of the cultures was then induced to undergo a synchronous meiosis from asynchronously dividing mitotic cells by shifting the temperature of growth to 36°C. Cell samples were taken for protein extraction and flow cytometry analysis every hour for three hours. The second culture was treated in the same way as in previous meiotic induction experiments (Section *2.8.6*) in that the culture was first transferred into growth medium lacking nitrogen and grown overnight at 25°C. This arrested the cells in G1 phase, effectively synchronizing the culture, before nitrogen and amino acids were re-added and the growth temperature was increased to 36°C to induce meiosis — before cell samples were taken as before. Flow cytometry analysis showed that the nitrogen-starved, synchronized meiotic culture was arrested in G1 phase at the start of the experiment and subsequently passed through a highly synchronous S phase, as determined by a doubling of the DNA content of the cells (Appendix VI). As expected, the asynchronous meiotic culture showed an unchanging DNA-content profile across the course of the experiment (Appendix VI).

Protein was extracted from cell samples taken from both the asynchronous meiotic culture and the nitrogen-starved synchronous meiotic culture, and EMSA experiments were carried out for both sets of protein samples using the *cdc22*$^{+}$ MCB1 DNA fragment as a radiolabelled probe. The results are shown in Figure 5.3. In the asynchronous meiotic culture, as in agreement with previous results (Caligiuri *et al.*, 1997), an MCB-binding complex was detected throughout the course of the experiment. In the synchronized meiotic culture, however, a retarded radioactive signal was detected during the first hour of the experiment that was of much higher mobility than the mitotic MBF complex (compare Figure 5.3: lanes 7, 8 and 9 (red arrow) to the control in lane 2, the mitotic *cdc22*$^{+}$-MCB1–MBF-complex (black arrow)). Following this, in the 3 h time point a second retarded radioactive signal appeared (Figure 5.3: lane 11) which was of similar mobility to the mitotic MBF complex as shown in the control lane (Figure 5.3: lane 2).

The nitrogen starved asynchronous experiment was repeated with the *cdc22*$^{+}$ MCB2 DNA fragment as a radiolabelled probe and identical results were obtained (Figure 5.4).

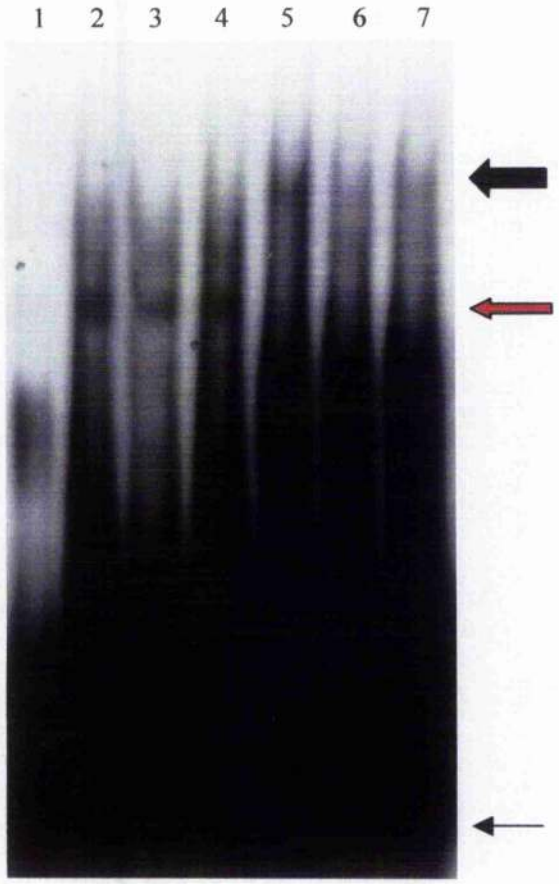
A self-competition experiment was then carried out to determine the specificity of the two MCB-binding complexes of different mobility (Figure 5.4). Protein aliquots from the 1 h cell sample, which contained the high mobility band, and from the 3 h cell sample, which contained the MBF-like band, were run in an EMSA with radiolabelled *cdc22*$^{+}$ MCB1 probe as well as both excess and a 1 in 10 dilution of unlabeled *cdc22*$^{+}$ MCB1 probe. The high-mobility binding activity from the 1 h protein sample was not competed with by the addition of unlabelled probe. However, the radioactive signal from the binding activity in the 3 h protein sample, which was a similar mobility to the mitotic MBF complex, was distinctly diminished after addition of unlabelled probe. This indicated that whilst the high-mobility binding activity was due to a non-specific protein–DNA interaction, the binding activity in the 3 h protein samples represents a specific interaction that is likely to be an MBF-like complex.

These results indicate that the *cdc22*$^{+}$ MCB clusters bind a complex during the meiotic cell cycle that is likely to be similar to MBF. Furthermore, the fact that this complex is detected only after the completion of S phase, as judged by the doubling of the DNA content of the cells (Appendix VI), has implications for the function of the MCB clusters and the complex that binds them (see Section 5.5).



Key
1 = free *cdc22*$^+$ MCB1 probe
Lanes 2–11 contain radiolabelled *cdc22*$^+$ MCB1 probe + protein extracted from:
2 = asynchronous, diploid *pat1-114* cells
3 = *pat1-114* diploid cells shifted to 36°C: 0 h
4 = *pat1-114* diploid cells shifted to 36°C: 1 h
5 = *pat1-114* diploid cells shifted to 36°C: 2 h
6 = *pat1-114* diploid cells shifted to 36°C: 3 h
7 = –N *pat1-114* diploid cells, 25°C
8 = –N *pat1-114* diploid cells, 36°C: 0 h
9 = –N *pat1-114* diploid cells, 36°C: 1 h
10 = –N *pat1-114* diploid cells, 36°C: 2 h
11 = –N *pat1-114* diploid cells, 36°C: 3 h

Figure 5.3: Meiotic MBF bound to* cdc22$^+$ *is undetectable until late S phase
An EMSA was performed using radiolabelled *cdc22*$^+$ MCB1 as a probe. Protein was extracted from diploid *pat1-114* cells, which were thermally induced to enter meiosis, both with (–N) and without prior nitrogen starvation. Samples were taken for protein extraction at 0 h, 1 h, 2 h and 3 h after temperature shift. The upper arrows show the position of the MBF–DNA complex. The lower arrows show the position of the free probe. The red arrow shows the position of a smaller complex in lanes 7 and 8.



Key
1 = free probe
Lanes 2–7 all contain radiolabelled *cdc22*$^+$ MCB2 probe plus:
2 = –N *pat1-114* diploid protein, 36°C: 1 h
3 = –N *pat1-114* diploid protein, 36°C: 1 h + excess unlabelled *cdc22*$^+$ MCB2 probe
4 = –N *pat1-114* diploid protein, 36°C: 1 h + 1/10 of unlabelled *cdc22*$^+$ MCB2 probe
5 = –N *pat1-114* diploid protein, 36°C: 3 h
6 = –N *pat1-114* diploid protein, 36°C: 3 h + excess unlabelled *cdc22*$^+$ MCB2 probe
7 = –N *pat1-114* diploid protein, 36°C: 3 h + 1/10 of unlabelled *cdc22*$^+$ MCB2 probe

Figure 5.4: The meiotic MBF complex binds* cdc22$^+$ *MCB2 specifically
EMSAs were performed using radiolabelled *cdc22*$^+$ MCB2 probes. Protein was extracted from diploid *pat1-114* cells (GG 375), which were thermally induced to enter meiosis after nitrogen starvation (–N). Samples were taken for protein extraction at 1 h and 3 h after temperature shift. The upper arrow shows the position of the complex in lanes 5–7. The red arrow shows the position of a smaller complex in lanes 2, 3 and 4. The lower arrow shows the position of the free probe.

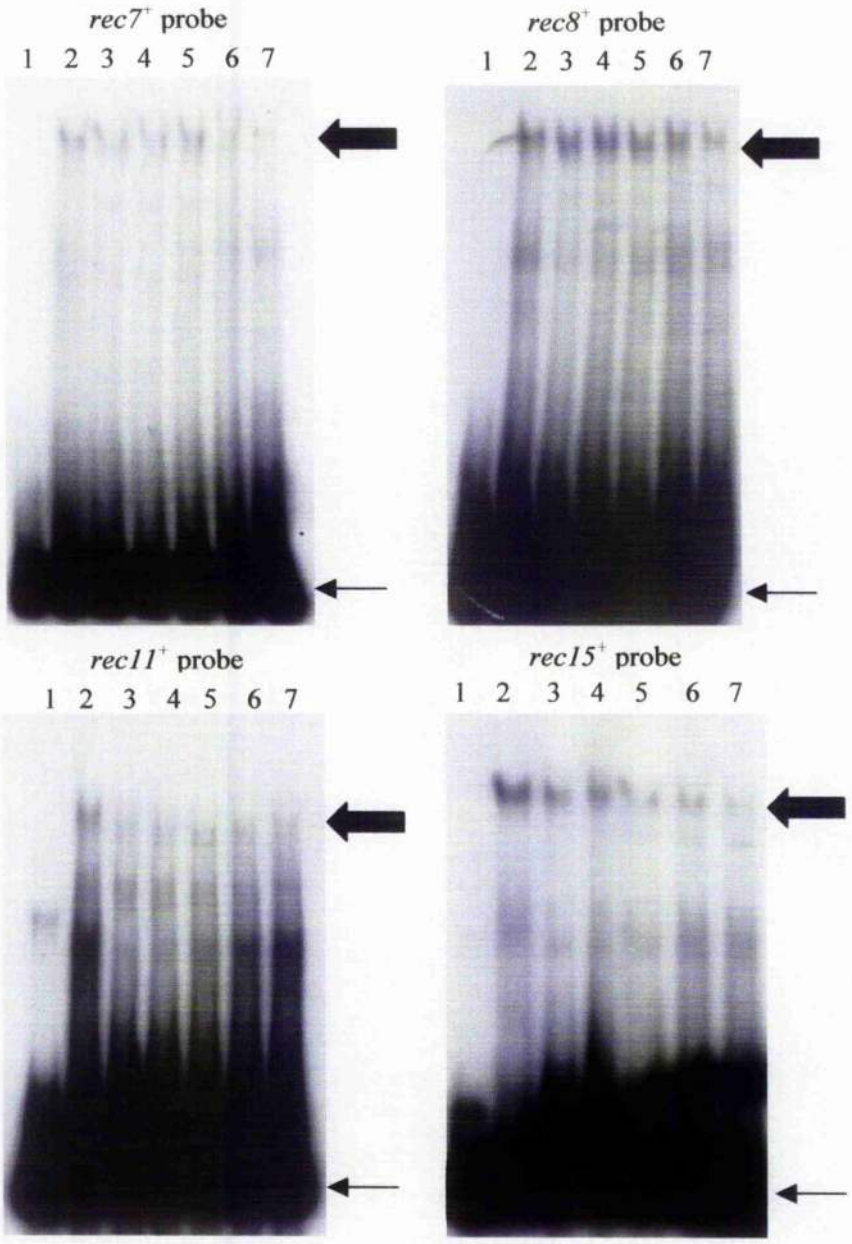
5.4.2 A meiotic MBF-like complex binds to rec$^+$ gene MCB motifs

It has been established that the MCB clusters from *cdc22*$^+$, which is expressed in both the mitotic and meiotic cell cycles, can bind an MBF-like complex during meiosis, but which is only detectable by EMSA after 3 h. It was then decided to investigate the possible protein interactions of the MCB clusters from the *rec*$^+$ genes, which are expressed predominantly during the meiotic cell cycle.

Therefore, aliquots of the same protein samples (asynchronous, meiotic *pat1-114* diploid cells GG 375) that were used in the meiotic *cdc22*$^+$ MCB-binding experiments were employed in EMSAs in which the *rec7*$^+$, *rec8*$^+$, *rec11*$^+$ and *rec15*$^+$ MCB clusters were used as radiolabelled probes (Figure 5.5). In each case it was clear that the *rec*$^+$ gene MCB clusters associated with a DNA binding activity that was of identical mobility to that seen with the *cdc22*$^+$ MCB probe in mitotic protein samples (Figure 5.5: lane 2 in each autoradiograph). Furthermore, unlike the *cdc22*$^+$ MCB probe, this binding activity was detected throughout the synchronous meiosis.

These results indicate that the MCB motifs found in the promoters of *rec*$^+$ genes, which are expressed predominantly during meiosis, bind an MBF-like complex during meiosis. However, the nature of the interaction between meiotic-specific *rec*$^+$ gene MCB motifs with MBF differs from the interaction of the mitotically and meiotically expressed *cdc22*$^+$ MCB motifs with MBF. This may explain how the *rec*$^+$ genes, which have similar UAS sequences to the *cdc22*$^+$ group of genes, are expressed specifically during meiosis.

Figure 5.5: A meiotic MCB-binding complex is detected with rec$^+$ gene probes



Key
1 = free probe
2 = mitotic asynchronous *pat1-114* diploid protein, 25°C
3 = –N *pat1-114* diploid, 25°C
4 = –N *pat1-114* diploid, 36°C: 0 h
5 = –N *pat1-114* diploid, 36°C: 1 h
6 = –N *pat1-114* diploid, 36°C: 2 h
7 = –N *pat1-114* diploid, 36°C: 3 h

Figure 5.5: A meiotic MCB-binding complex is detected with rec$^+$ gene probes
EMSAs were performed using radiolabelled *rec7*$^+$, *rec8*$^+$, *rec11*$^+$ and *rec15*$^+$ MCB probes. Protein was extracted from diploid *pat1-114* cells (GG 375), which were thermally induced to enter meiosis after nitrogen starvation (–N). Samples were taken for protein extraction before and at 1 h, 2 h and 3 h after temperature shift. The upper arrow shows the position of the retarded complex. The lower arrow shows the position of the free probe.

It has been previously established that, during the mitotic cell cycle, MCB UAS motifs interact with the DNA-binding complex known as MBF with the result that the genes they control are expressed periodically at the boundary of the G1 and S phases. EMSA analysis of the mitotic MBF complex has established that it contains Cdc10p, Res1p and Res2p (Lowndes *et al.*, 1992; Caligiuri & Beach, 1993; Miyamoto *et al.*, 1994; Zhu *et al.*, 1994; Nakashima *et al.*, 1995) and requires the action of the Rep2 protein (Nakashima *et al.*, 1995; Tahara *et al.*, 1998).

In this thesis it has been previously established that several genes that are periodically expressed at the G1–S phase boundary, specifically during meiosis, contain MCB elements within their promoter regions. Furthermore, when investigated using a UAS reporter vector, these MCB motifs were shown to be physiologically relevant during meiosis as they could confer meiotic-specific G1–S expression to a reporter gene. It was therefore decided to investigate whether these MCB motifs interacted with a binding activity during meiosis similar to the mitotic MBF complex.

Initially, the ability of MCB motifs from genes expressed specifically during meiosis to bind the mitotic MBF complex was examined. It was shown that Type I and Type II MCB motifs, which are less complex than those from genes expressed in both mitosis and meiosis, did indeed specifically interact with a binding activity that was of similar mobility to the mitotic MBF complex. Further EMSA experiments demonstrated that these MCB motifs could compete with the *cdc22*$^{+}$ MCB clusters for binding of the MBF complex in mitotic cells. Together these results indicate that the *rec*$^{+}$ gene MCB motifs do interact with the mitotic form of the MBF complex.

Subsequent EMSA experiments were carried out to determine the nature of possible protein interactions with MCB motifs during the meiotic cell cycle. First, EMSA experiments using *cdc22*$^{+}$ MCB-motif DNA fragments detected a complex of similar mobility to mitotic MBF, which was only seen after the cells had passed through pre-meiotic S phase. This indicates that a meiotic form of the MBF complex interacts with the MCB motifs in the promoters of the *cdc22*$^{+}$ group of mitotically and meiotically expressed genes. Furthermore, these results imply that — as in the mitotic cell cycle — the meiotic MBF complex does not act simply as an activator of transcription.

Second, EMSA experiments using meiotic-specific (*rec*$^{+}$ gene) MCB-motif DNA fragments detected a complex of similar mobility to mitotic MBF that was seen throughout the course of the meiotic experiment. These results indicate that MBF binds to the meiotic-specific (*rec*$^{+}$ gene) MCB motifs. However, as a complex that is likely to be MBF is detected throughout the meiotic experiment, the interaction between MBF and the *rec*$^{+}$ gene MCB motifs must be different to that seen with MBF and the mitotic and meiotic MCB motifs. This observed

discrepancy in the timing of the MBF–MCB interactions between the two MCB-motif types, implies that there is a difference in the consequences of binding MBF for these two different types of MCB motifs ('meiotic specific' and 'mitotic and meiotic'). This is because, although differing MBF-binding profiles are detected, the two types of genes have very similar mRNA profiles during the meiotic cell cycle.

- Meiotic-specific (*rec*$^{+}$ gene) MCB motifs bind mitotic MBF.
- 'Mitotic and meiotic' (*cdc22*$^{+}$) and 'meiotic-specific' (*rec*$^{+}$ gene) MCB motifs both bind an MBF-like complex during the meiotic cell cycle but the nature of their interaction with this complex is different.

Chapter 6

Study of MBF components during meiosis in fission yeast

In Chapter 3 of this thesis it was shown that several known fission yeast MCB-regulated genes, which are expressed during the G1–S phase transition of the mitotic cell cycle, are also expressed specifically at the G1–S phase transition of the meiotic cell cycle. Furthermore, other genes which also contain MCB motifs in their promoters, and yet are specific to the meiotic cell cycle, are also periodically expressed at the G1–S phase transition. DNA sequences of the promoter regions of these genes were compared and several different MCB 'Types' were identified.

Subsequently, in Chapter 4, MCB elements were shown to be physiologically relevant during meiosis in fission yeast and the MCB motif of the *rec15*$^{+}$ gene was shown to confer meiotic-specific G1–S phase expression to a reporter gene. In Chapter 5, MCB motifs were shown to interact with a binding activity similar to mitotic MBF during meiosis. However, MCB motifs from 'meiotic specific' and 'mitotic and meiotic' genes seemed to interact in different ways with the same, or a similar, MCB-binding activity. These observations, and the fact that meiotic-specific MCB motifs can compete for the binding of the mitotic MBF complex (known to bind *cdc22*$^{+}$ MCBs in mitotic-cell protein extracts; Lowndes *et al.*, 1992), strongly suggest that the meiotic MCB-binding activity is closely related to mitotic MBF.

An investigation into the activities of the MBF components during the meiotic cell cycle was therefore undertaken. Haploid fission yeast *pat1-114* strains that were mutated in the *cdc10*$^{+}$ gene or that carried deletions of the *res1*$^{+}$, *res2*$^{+}$, *rep1*$^{+}$ or *rep2*$^{+}$ genes were used. Similarly, haploid fission yeast *pat1-114* strains that carried one of the MBF components in the pREP1 overexpression plasmid were made for each of the *cdc10*$^{+}$, *res1*$^{+}$, *res2*$^{+}$, *rep1*$^{+}$ and *rep2*$^{+}$ genes. The pREP1 vector allows gene expression to be controlled by the *nmt1*$^{+}$ (no message in thiamine) promoter, which causes expression of the cloned gene to be restricted to conditions in which thiamine is absent. The fission yeast strains used in this chapter were all constructed in our laboratory by Dr. Stewart White.

This chapter describes the examination of these strains where MBF component genes have been manipulated. These proteins are all known to be associated with the MBF complex during the mitotic cell cycle or, in the case of Rep1p, to be associated with MCB-regulated gene expression during meiosis. It was therefore considered likely that a transcription factor complex that was closely related in its component structure to the known MBF complex might be responsible for MCB-regulated gene transcription during the meiotic cell cycle. The aim of experiments in this chapter, therefore, was to establish whether manipulation of these proteins affected MCB-regulated gene expression during the meiotic cell cycle.

6.1.1 The components of MBF: Cdc10p

The fission yeast Cdc10p is absolutely required for passage through START (Nurse & Bissett, 1981) and has been identified as the major component of the MCB-binding activity MBF (also known as DSC1; Lowndes *et al.*, 1992). Fission yeast strains containing the *cdc10-129* or *cdc10-V50* temperature-sensitive point mutations and the *cdc10-C4* temperature-sensitive nonsense mutation (which results in the loss of the C-terminal 61 amino acids of Cdc10p) all arrest in G1 phase of the cell cycle upon shifting the cultures to the restrictive temperature of 36°C (Nurse & Bissett, 1981; Reymond *et al.*, 1992; McInerny *et al.*, 1995).

Such mutations in *cdc10*$^{+}$ have a major effect on the transcription of MCB-regulated genes. The *cdc22*$^{+}$, *cdc18*$^{+}$ and *cdt1*$^{+}$ genes all show much lower levels of mRNA in mitotic *cdc10-129* and *cdc10-V50* fission yeast strains at the restrictive temperature (Fernandez-Sarabia *et al.*, 1993, Kelly *et al.*, 1993; Hoffman & Beach, 1994). However, the *cdc10-C4* mutation, while showing a similar phenotype to the *cdc10-129* and *cdc10-V50* mutations at 36°C (G1 arrest and loss of MCB-regulated gene transcription), displays a second interesting phenotype at temperatures below 25°C (McInerny, *et al.*, 1995). Increasing levels of MCB-regulated gene transcription occur as the *cdc10-C4* strain is shifted to progressively lower temperatures, which is also accompanied by the loss of periodicity of this gene expression (McInerny *et al.*, 1995). The effect of this low temperature *cdc10-C4* phenotype on MCB-regulated gene expression during the meiotic cell cycle will therefore also be investigated in this chapter. Overexpression of *cdc10*$^{+}$ has only a slightly repressive effect on MCB-regulated gene transcription during mitosis (Baum *et al.*, 1997). Therefore, while Cdc10p is clearly a crucial component of the mitotic MBF transcription factor, it might be capable of both activation and repression of MCB-regulated gene transcription.

6.1.2 The components of MBF: Res1p

Res1p was first identified as a multi-copy suppressor of both *cdc10*$^{-}$ and *pat1* mutations (Tanaka *et al.*, 1992) and is also known to be an important component of the mitotic MBF complex (Caligiuri & Beach, 1993; Ayté *et al.*, 1995; Baum *et al.*, 1997; Whitehall *et al.*, 1999).

A *res1* deletion (*res1*Δ) strain grows poorly at 30°C and is severely sensitive to heat and cold, arresting in G1 at 36°C and 23°C. Furthermore, G1 arrested *res1*Δ cells are able to conjugate without prior nitrogen starvation, indicating that Res1p acts as both a positive regulator of the mitotic cell cycle and a negative regulator of the meiotic cell cycle, and, unlike Cdc10p, is not required for pre-meiotic S phase (Caligiuri & Beach, 1993). Levels of MCB-regulated gene mRNA are also low and lose their periodicity in *res1*Δ cells (Baum *et al.*, 1997) whereas overexpression of *res1*$^{+}$ causes overexpression of MBF-regulated genes (Ayté *et al.*, 1995; Baum *et al.*, 1997).

6.1.3 The components of MBF: Res2p

Res2p was isolated as both an extragenic suppressor of a *res1*Δ mutation (Miyamoto *et al.*, 1994) and as a protein product from a fission yeast cDNA library that interacted with Cdc10p to induce MCB-regulated reporter-gene transcription (Zhu *et al.*, 1994).

Res2p is a part of the MBF complex (Miyamoto *et al.*, 1994; Zhu *et al.*, 1994, Ayté *et al.*, 1997; Baum *et al.*, 1997; Zhu *et al.*, 1997; Whitehall *et al.*, 1999) and is present, with Res1p, in MBF throughout the mitotic cell cycle (Ayté *et al.*, 1997; Whitehall *et al.*, 1999). Res2p also interacts with the activating Rep2p subunit of MBF (Nakashima *et al.*, 95, Tahara *et al.*, 1998) and a Pcl-like cyclin, Pas1p (Tanaka *et al.*, 2000). Although it is not essential for mitosis, Res2p has a distinct role in this cell cycle (Miyamoto *et al.*, 1994; Zhu *et al.*, 1994; Baum *et al.*, 1997; Whitehall *et al.*, 1999). A *res2*Δ strain shows elevated levels of MCB-regulated gene mRNA and loss of the periodicity of gene expression during the mitotic cell cycle, although overexpression of *res2*$^{+}$ has little effect on mRNA levels (Ayté *et al.*, 1997; Baum *et al.*, 1997).

However, Res2p is known to have a major role in the meiotic cell cycle of fission yeast (Miyamoto *et al.*, 1994; Zhu *et al.*, 1994; Ayté *et al.*, 1997; Zhu *et al.*, 1997) and, in agreement with this suggestion, is highly induced after nitrogen starvation (Miyamoto *et al.*, 1994). A *res2*Δ strain is unable to complete meiosis (Miyamoto *et al.*, 1994; Zhu *et al.*, 1994; Ayté *et al.*, 1997) whereas over-expressing Res2p enhances entry into meiosis (Ayté *et al.*, 1997).

6.1.4 The components of MBF: Rep2p

Rep2p was also identified as a multi-copy suppressor of a temperature sensitive *cdc10* mutation and has been shown to interact with the MBF complex via a specific contact with Res2p (Nakashima *et al.*, 1995, Tahara *et al.*, 1998). *rep2*$^{+}$ mRNA levels are sensitive to nitrogen starvation and a *rep2*Δ fission yeast strain shows a marked delay in G1 phase during mitotic growth as well as decreased overall levels of MCB-regulated-gene mRNA, although periodicity of MCB-regulated gene expression is retained (Nakashima *et al.*, 1995, Baum *et al.*, 1997). Furthermore, overexpression of *rep2*$^{+}$ during mitosis results in constitutive expression of MCB-regulated genes (White *et al.*, 2001). These observations indicate that Rep2p is a mitotic-specific activator of the MBF complex.

6.1.5 The Rep1 protein

The *rep1*$^{+}$ gene was isolated as a multi-copy suppressor of a temperature-sensitive *cdc10*$^{-}$ mutation and although it is not required for the mitotic cell cycle it has a crucial role in the initiation of pre-meiotic S phase (Sugiyama *et al.*, 1994).

A *rep1*Δ strain passes normally through mitosis but on entry into meiosis fails to induce expression of *res2*$^{+}$ or MCB-regulated genes (Sugiyama *et al.*, 1994). But, overexpression of *rep1*$^{+}$ does not induce the expression of *res2*$^{+}$ during meiosis (Sugiyama *et al.*, 1994). However,

when over-expressed during mitosis, *rep1*$^{+}$ causes loss of periodicity of expression of MCB-regulated genes in a similar fashion to overexpression of *rep2*$^{+}$ (White *et al.*, 2001). Furthermore, *rep1*$^{+}$ mRNA is induced in a *ste11*$^{+}$ dependent fashion during nitrogen starvation and conjugation indicating that *rep1*$^{+}$ has a meiotic-specific role (Sugiyama *et al.*, 1994).

6.1.6 Summary

In this chapter, experiments were undertaken to investigate whether manipulating the expression of genes known to encode components of the MBF complex had an effect on the incidence of G1–S specific MCB-regulated gene expression during the meiotic cell cycle. Separate haploid *pat1-114* fission yeast strains containing mutations or deletions of the *cdc10*$^{+}$, *res1*$^{+}$, *res2*$^{+}$, *rep1*$^{+}$ and *rep2*$^{+}$ genes were obtained for these purposes. Similarly, separate haploid *pat1-114* fission yeast strains containing each MBF component gene in the pREP1 over expression vector were also utilized. The fission yeast strains used in this chapter were all constructed in our laboratory by Dr. Stewart White.

6.2.1 Induction of synchronous meioses

The fission yeast *pat1-114* strains used in this chapter were induced to undergo a synchrounous meiosis using the method described in Section *2.8.6*. Briefly, each strain was grown overnight in EMM at 25°C to a cell density of between 5 and 8 x 10^6 cells per ml. The cultures were then transferred to nitrogen-free EMM overnight to arrest the cells in G1. The following day NH_4Cl was added back to the culture at a concentration of 0.5g l^{-1}, to encourage a more synchronous meiosis (Muramaki & Nurse, 1999). The temperature of the culture was then raised to 36°C and 35 ml samples were taken every 15 min for RNA extraction and 1 ml samples were taken hourly for flow cytometry analysis.

To induce overexpression of the MBF-component genes, fission yeast strains containing pREP1 plasmids in which the MBF-gene was under the control of the *nmt1*$^+$ promoter were grown to mid-exponential phase at 25°C in the presence of thiamine (*nmt1*$^+$ off). The cultures were then shifted to medium lacking both thiamine (*nmt1*$^+$ on) and nitrogen for at least 16 h before the thermal induction of meiosis was undertaken.

This method varied only in the case of the h$^+$ *cdc10-C4 pat1-114* strain. The phenotype of *cdc10-C4* strains is the same as that seen in other temperature sensitive *cdc10* mutant strains when the cultures are grown at 36°C. However, an alternative phenotype is observed if *cdc10-C4* cells are grown at 24°C (McInerny *et al.*, 1995). To observe the effects of this cold-sensitive *cdc10-C4* phenotype on MCB-regulated gene expression, these cultures were therefore raised to 36°C for only 15 min before being returned to the lower temperature of 24°C. Examination of these cells after 10 h showed that this treatment was sufficient to cause induction of meiosis as formation of asci did occur (personal communication, Dr. Stewart White).

6.2.2 Northern-blot analysis of the MBF-component fission yeast strains

Total RNA was extracted from the time course samples for each culture and northern blots carried out as described previously (Section *2.6*). The equal loading of RNA samples on northern blots from the synchronous haploid meioses was confirmed by examination of the 18S and 35S rRNA bands — which were revealed by staining the formaldehyde gel with ethidium bromide prior to the transfer of RNA to the nitrocellulose membrane. The northern membranes for each fission yeast strain were hybridized with probes for MCB-regulated genes representative of the classes described in Chapter 3: *cdc22*$^+$ a Type V MCB-motif gene that is periodically expressed at the G1–S boundary during both mitosis and meiosis; and *rec8*$^+$ and *rec11*$^+$, Type II and Type III MCB genes, respectively, that are both periodically expressed at the G1–S boundary specifically during meiosis. The northern membranes for each strain were

then hybridized with probes for each of the individual components of MBF: *cdc10*$^{+}$, *res1*$^{+}$, *res2*$^{+}$, *rep1*$^{+}$ and *rep2*$^{+}$. The cultures were monitored for progression through pre-meiotic S phase using flow cytometry analysis, which measures the DNA content of the cells.

6.2.3 Notes on interpretation of the MBF-component analysis

Each fission yeast strain used in this chapter was induced to undergo a synchronous meiosis and the resulting cell samples were subsequently subjected to northern-blot analysis. Specific control lanes were included at the end of each formaldehyde gel that was run during these experiments to facilitate the interpretation of the data. First, samples from an otherwise 'wild type' (h^{-}/h^{-} *pat1-114/pat1-114 ade6-M210/ade6M-216*) diploid synchronous meiosis were included. The samples represent the trough (105 min) and peak (180 min) samples of mRNA abundance for the average MCB-regulated gene transcript profile. This allowed a direct comparison to be made between normal wild-type levels of transcript abundance and the levels of transcript abundance seen in the experiment. Thus, a comparison between the experimental 105 min and 180 min samples and the control 105 min and 180 min samples on the same northern membrane is an accurate comparison of mRNA levels between 'wild type' and experimental strains.

Second, an RNA sample from asynchronous mitotic h^{-}/h^{-} *pat1-114/pat1-114 ade6-M210/ade6-M-216* cells (GG 375) was also included in the final lane on the formaldehyde gel so that the presence or absence of each transcript during the mitotic cell cycle could be considered.

Third, in each figure depicting MCB-regulated-gene analysis, examples of autoradiographs of the *cdc22*$^{+}$, *rec8*$^{+}$ and *rec11*$^{+}$ transcript profiles for a normal synchronous meiosis of the h^{-}/h^{-} *pat1-114/pat1-114 ade6-M210/ade6-M-216* strain were also included. In these figures, the autoradiographs are labelled 'Normal meiotic profile'. This allowed a comparison to be made between the RNA abundance profile seen during a normal diploid *pat1-114* synchronous meiosis and the RNA profile seen during a synchronous meiosis of the experimental strains.

Finally, Figure 6.2 represents a normal diploid *pat1-114* synchronous meiosis that was analysed for the expression of MBF components and to which the subsequent MBF component analyses can be compared.

MBF components

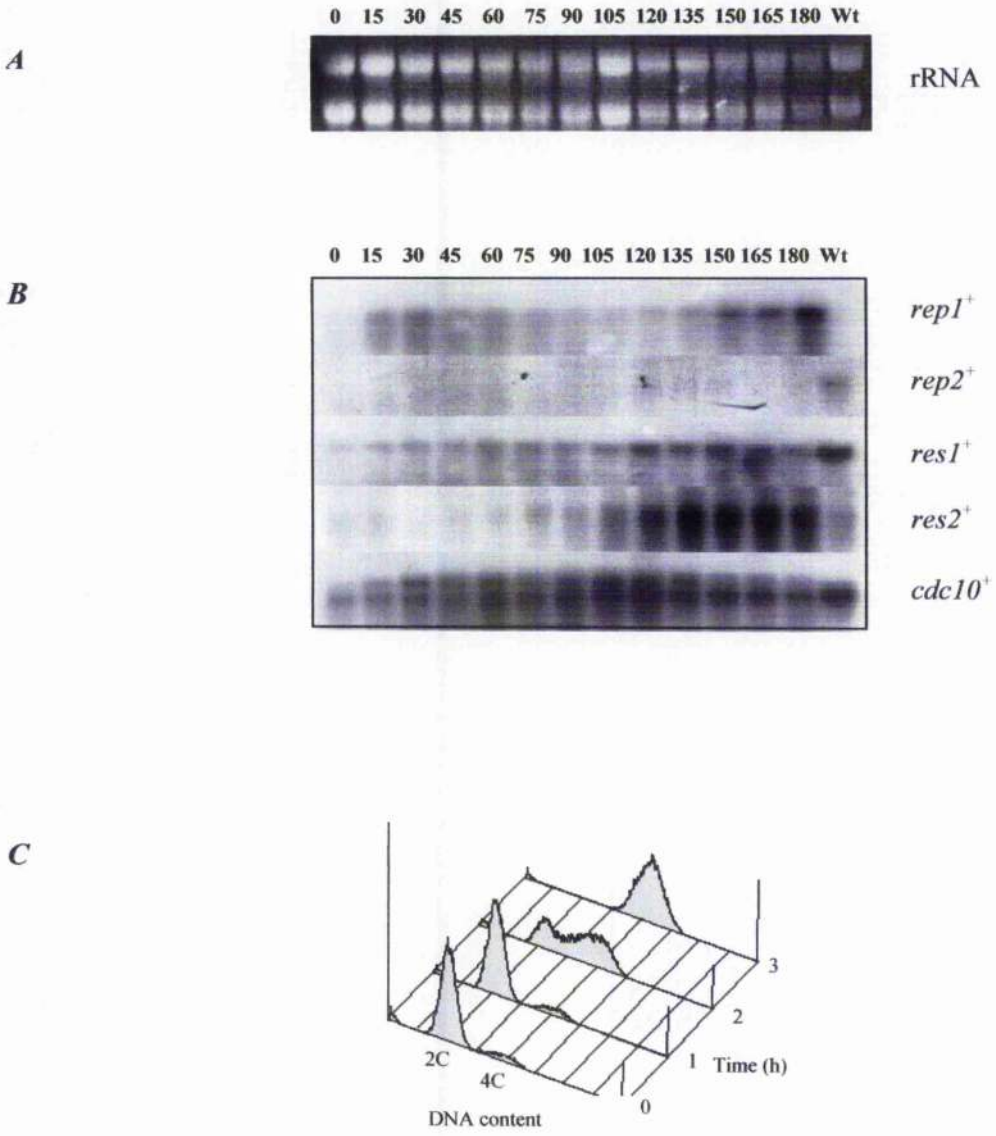


Figure 6.1: Diploid **pat1-114** ***synchronous meiosis***
Diploid *pat1-114* cells (GG 375) were induced to undergo a synchronous meiosis (see Section *2.8.6*) and samples were taken every 15 min. RNA was extracted and northern blots performed to probe for the components of the MBF complex. ***A*** shows the ethidium bromide stained RNA gel. ***B*** shows the autoradiographs for each component of the MBF complex and ***C*** shows the flow cytometry analysis of the synchronous meiosis.

6.3. **cdc10$^+$** *analysis*

6.3.1 **cdc10$^+$** *is involved in MCB-regulated gene transcription during meiosis*

The *cdc10*$^+$ mutant strains h$^+$ *cdc10-129 pat1-114*, h$^+$ *cdc10-C4 pat1-114* and h$^-$ *cdc10-V50 pat-114*, and the *cdc10* overexpression strain h$^-$ pREP1:*cdc10*$^+$ *pat1-114 leu1-32* were induced to undergo synchronous meioses and subjected to northern-blot analysis as described above (Sections *6.2.1–6.2.3*). Figure 6.2.1 depicts the analysis of MCB-regulated gene transcript abundance in each experimental strain.

In each of the fission yeast *pat1-114 cdc10*$^-$ mutant strains, the transcript abundance of all three MCB-regulated genes tested was dramatically affected over the course of a synchronous meiosis (Figure 6.2.1 *A*) and none of these strains were able to enter S phase, as judged by flow cytometry analysis (Figure 6.2.1 *C*). In fact, in each *cdc10*-mutant strain, expression of the MCB-regulated genes was virtually abolished. Furthermore, when *cdc10*$^+$ was over-expressed during a synchronous meiosis, expression of MCB-regulated genes was dramatically increased over normal wild-type levels. These observations confirm that, *cdc10*$^+$ is required for the expression of MCB-regulated genes during the meiotic cell cycle.

6.3.2 Manipulation of **cdc10$^+$** *affects the expression of* **res2$^+$**

Figure 6.2.1 depicts typical autoradiographs that were obtained when northern membranes for the *cdc10*$^+$ analysis strains were probed for the MBF components *cdc10*$^+$, *res1*$^+$, *res2*$^+$, *rep1*$^+$ and *rep2*$^+$ (Figure 6.2.2).

Expression of the *rep1*$^+$ gene remains unaffected in any of the fission yeast strains in which the *cdc10*$^+$ gene was manipulated.

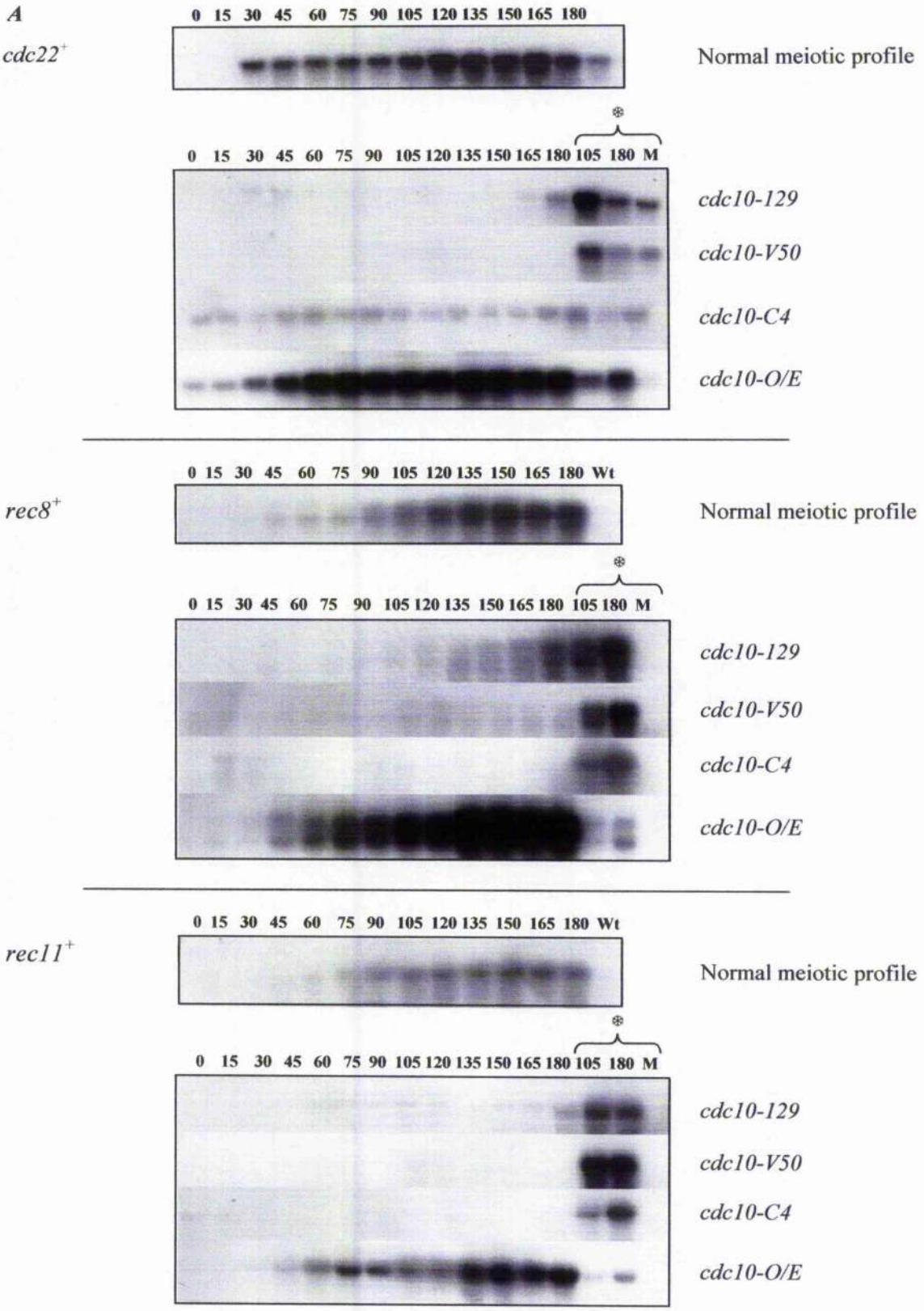
In both the *cdc10-129* and *cdc10-V50* strains there is very little expression of either *rep2*$^+$ or *res1*$^+$ during the synchronous meiosis as compared to the mitotic control samples in the final three lanes (Figure 6.2.2). This was expected as both of these genes are predominantly expressed during mitosis. *res2*$^+$ expression was, however, affected in both of these strains. Instead of the gradual increase in transcript abundance during the second hour of the synchronous meiosis that is normally seen (Figure 6.1), very little *res2*$^+$ transcript was seen throughout either of these mutated *cdc10*$^+$ experiments. Flow cytometry analysis showed that both the *cdc10-129* and the *cdc10-V50* strains are arrested in early G1 phase so it is likely that this cell cycle arrest had a role in the lack of *res2*$^+$ gene expression.

In the *cdc10-C4* experiment it is interesting to note that all of the *rep2*$^+$, *res1*$^+$, *res2*$^+$ and *cdc10*$^+$ genes showed a consistently high level of transcript abundance throughout the experiment. In the case of the *res2*$^+$ gene this was equal to the peak level of expression seen in the 180 min wild-type control lane. The consistent abundance of the *res1*$^+$ and *rep2*$^+$ gene transcripts throughout this experiment is more surprising, however, because neither gene is

normally expressed to a high level during a synchronous meiosis experiment (see 105 min and 180 min control samples and Figure 6.1). There are two possible explanations for this. First, it is possible that the *cdc10-C4* cultures did not enter the meiotic cell cycle due to the cell cycle arrest (observed by flow cytometry analysis). This would mean that the cells were effectively stalled in the mitotic cell cycle, where $res1^+$ and $rep2^+$ gene expression would be expected. Alternatively, however, if these cells had entered the meiotic cell cycle, it is possible that the mutation in the $cdc10^+$ gene caused aberrant expression of these genes. That asci were commonly observed after inducing a synchronous meiosis in *pat1-114 cdc10-C4* cells indicates that the cells did enter the meiotic cell cycle (Dr. Stewart White personal communication). However, it is also possible that the cultures contained a mixed population of cells, some of which were stalled in the mitotic cell cycle (and produced $res1^+$ and $rep2^+$ transcripts), and some of which had entered the meiotic cell cycle (and produced asci). A more detailed analysis of this fission yeast strain would be required to confirm any of these possibilities.

Finally, significant results were obtained in the analysis of the $cdc10^+$ overexpression strain. Normal transcript profiles were obtained in each case when the *cdc10-C4* northern membrane was probed for $rep1^+$, $rep2^+$ or $res1^+$ mRNA (see 105 min and 180 min control lanes and Figure 6.1). However, the $res2^-$ transcript increases significantly in abundance throughout the course of the experiment to levels much greater than those seen in a normal synchronous meiosis experiment (compare to 105 min and 180 min control lanes on the same membrane). As shown in Chapter 3, the $res2^+$ gene does have a close match to an MCB motif in its promoter region, which, in combination with these results, indicates that the $res2^-$ gene might be under the control of Cdc10p, at least during meiosis.

Figure 6.2.1* cdc10^{+} *analysis: MCB-controlled transcripts



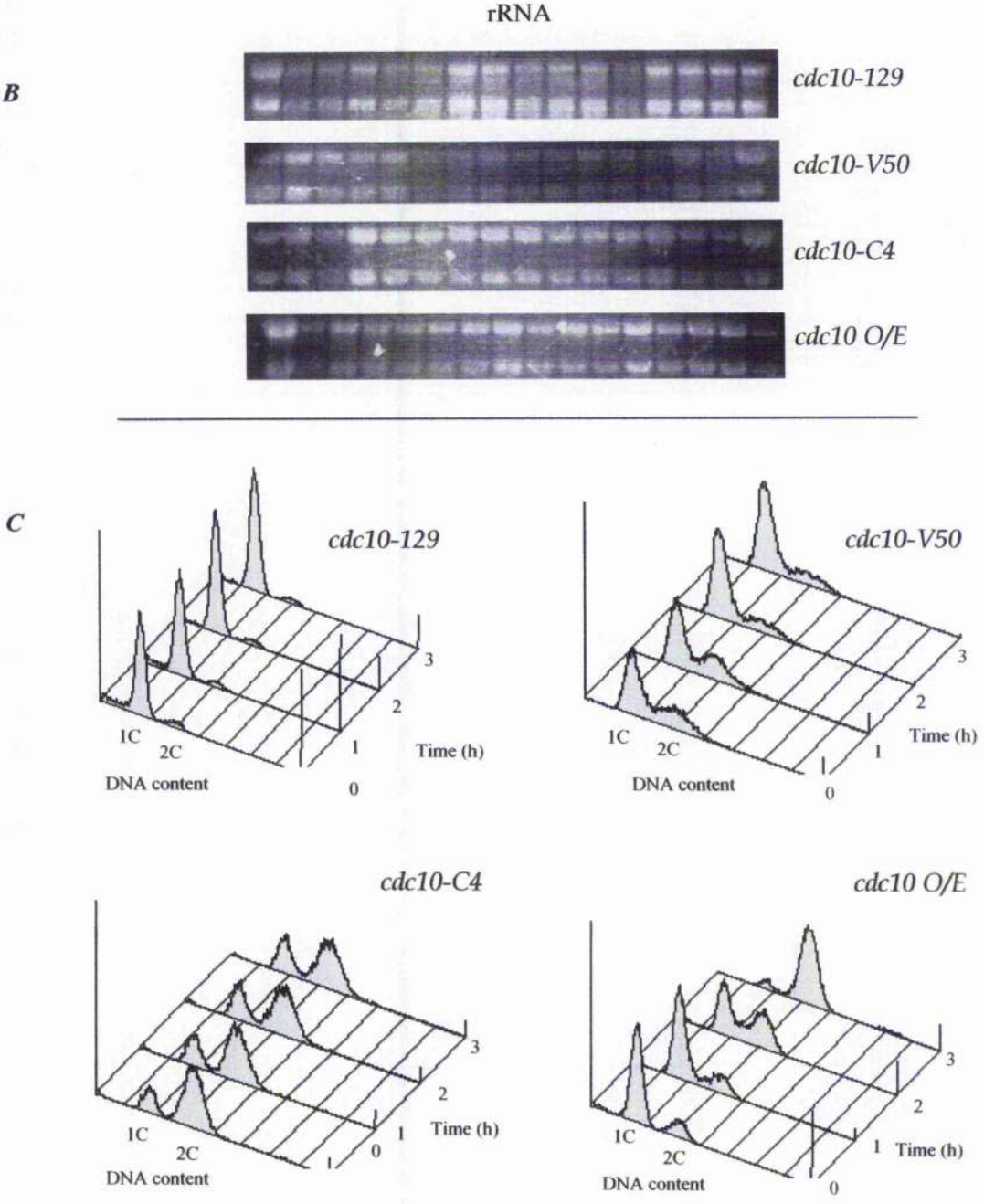
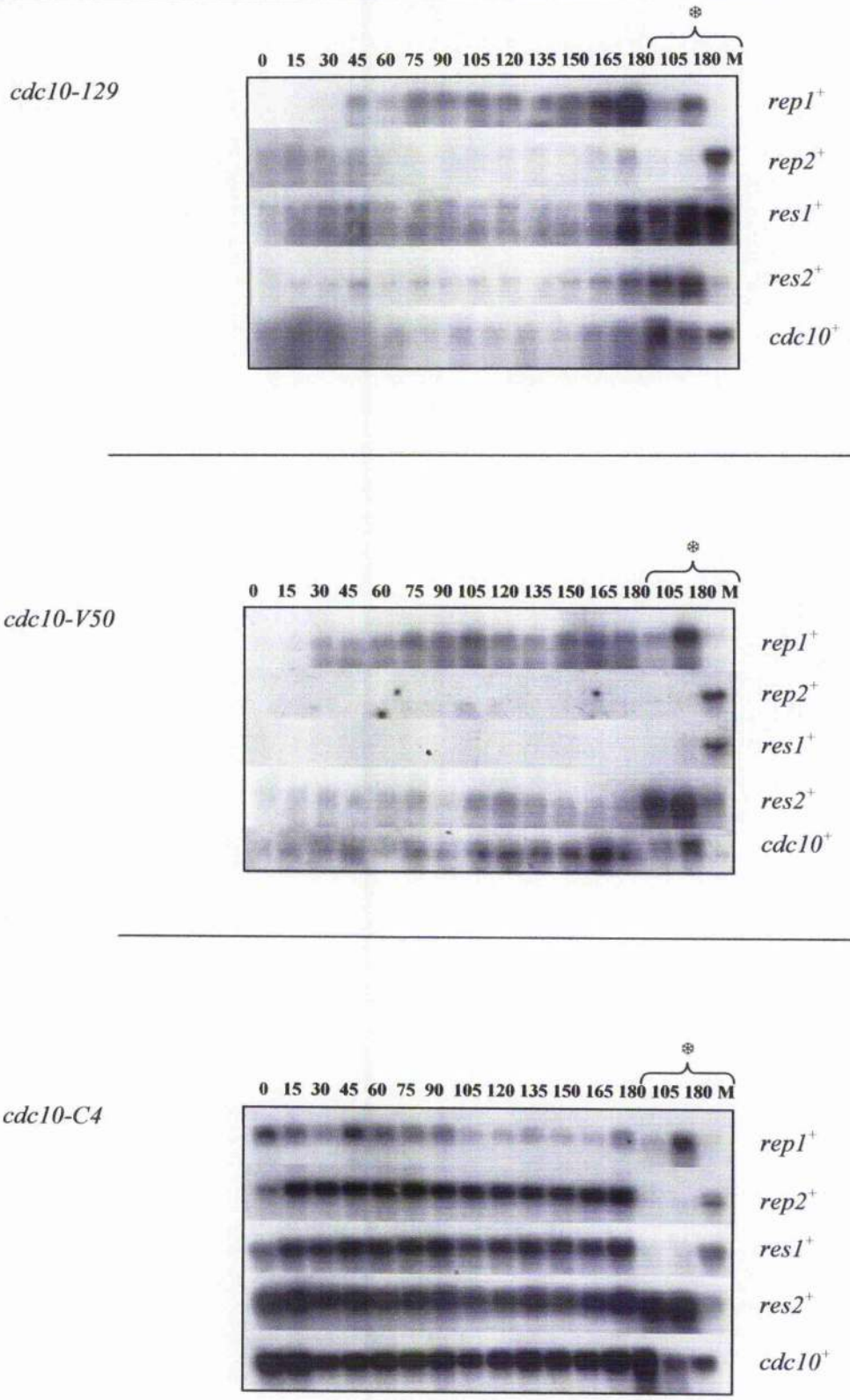


Figure 6.2.1* cdc10^{+} *analysis: MCB-controlled transcripts

Thermally induced *pat1-114*, synchronous meioses were carried out for *cdc10-129*, *cdc10-V50*, *cdc10-C4* and *cdc10-O/E* fission yeast strains and samples were taken every 15 min for RNA extraction and every hour for flow cytometry analysis. Northern-blot analysis was carried out and each membrane was probed for *cdc22*$^{+}$, *rec8*$^{+}$ and *rec11*$^{+}$. ***A*** shows the northern-blot analysis for the MCB-regulated gene probes. Autoradiographs of each separate gene are grouped together so that a direct comparison can be made between the transcript incidence and abundance in each different strain. Similarly, an autoradiograph of each gene taken from a normal *pat1-114* induced diploid synchronous meiosis is included for comparison. ***B*** shows rRNA on the ethidium bromide stained formaldehyde gels for each strain as a measure of RNA loading and ***C*** shows flow cytometry analysis for each strain.

❄ = control RNA samples: M, from asynchronous mitotic GG 375 cells; 105, 105 min sample from a 'wild type' (GG 375) diploid synchronous meiosis; 180, 180 min sample from a 'wild type' (GG 375) diploid synchronous meiosis.

Figure 6.2.2 cdc10^{+} analysis: MBF components



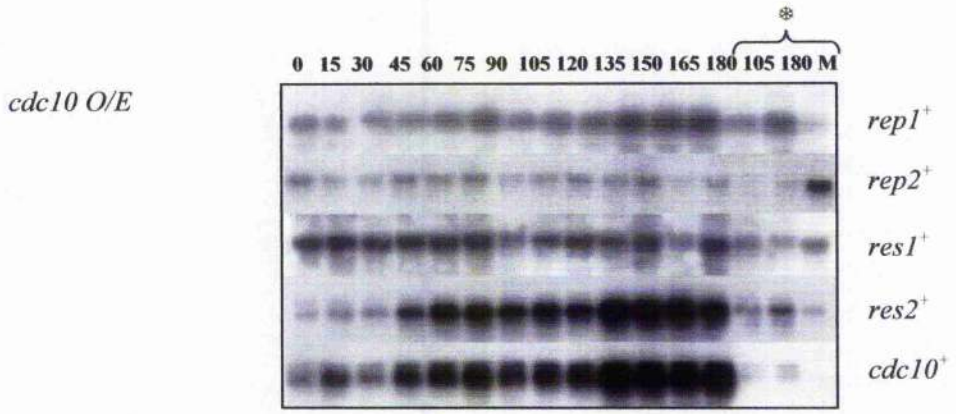


Figure 6.2.2* cdc10$^+$ *analysis: MBF components

This figure shows the autoradiographs resulting from the analysis of the MBF components *cdc10*$^+$, *res1*$^+$, *res2*$^+$ *rep1*$^+$ and *rep2*$^+$ using the fission yeast strains *cdc10-129*, *cdc10-V50*, *cdc10-C4* and *cdc10-O/E* during synchronous meioses. The northern membranes from Figure 6.2.1 were hybridized with probes for the MBF components.

❄ = control RNA samples: M, from asynchronous mitotic GG 375 cells; 105, 105 min sample from a 'wild type' (GG 375) diploid synchronous meiosis; 180, 180 min sample from a 'wild type' (GG 375) diploid synchronous meiosis.

6.4 res1$^+$ *analysis*

6.4.1 res1$^+$ *affects MCB-regulated gene transcription during meiosis*

The fission yeast strains h$^-$ *res1::ura4*$^+$ *pat1-114 ura4-D18* and h$^-$ pREP1:*res1*$^+$ *pat1-114 leu1-32* were induced to undergo synchronous meioses and northern-blot analyses were carried out as before (Sections *6.2.1–6.2.3*). Figure 6.3.1 depicts the analysis of MCB-regulated gene transcript abundance in each experimental strain.

The *cdc22*$^+$, *rec8*$^+$ and *rec11*$^+$ transcript profiles were not greatly affected during a synchronous meiosis in the *res1*Δ mutant and, as judged by flow cytometry, the culture passed through pre-meiotic S phase as normal (Figure 6.3.1 *C*). Apart from the *cdc22*$^+$ transcript beginning to disappear slightly earlier than usual, the first appearance of transcript and the levels of transcript abundance reached, in each case, remained very similar to the transcript profiles seen in a normal *pat1-114* diploid synchronous meiosis. This observation confirms that Res1p does not have a major role in the activation of transcription of MCB-regulated genes during the meiotic cell cycle.

When the *res1*$^+$ gene was overexpressed, however, as well as appearing earlier than normal, the *cdc22*$^+$, *rec8*$^+$ and *rec11*$^+$ transcripts all showed an increase in the level of transcript abundance — particularly in the case of the *cdc22*$^+$ and *rec8*$^+$ genes. This did not have any effect on the passage of this culture through pre-meiotic S phase however (Figure 6.3.1 *C*). Overexpression of *res1*$^+$ has been shown to cause induction of MCB-gene expression during the mitotic cell cycle (Caligiuri *et al.*, 1993), so this result is not unexpected.

6.4.2 Manipulation of res1$^+$ *affects expression of other MBF-component genes*

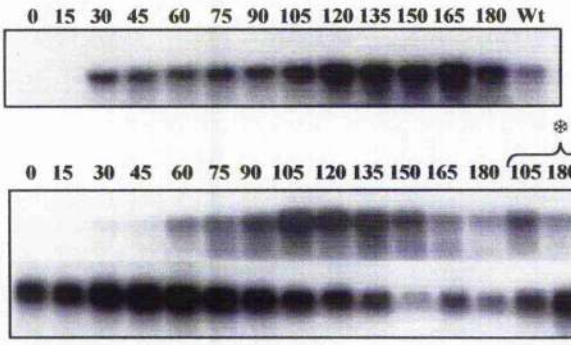
Figure 6.3.2 depicts typical autoradiographs that were obtained when northern membranes for the Res1 analysis strains were probed for the MBF components *cdc10*$^+$, *res1*$^+$, *res2*$^+$, *rep1*$^+$ and *rep2*$^+$. Due to time constraints, results were not obtained for the profile of the *cdc10*$^+$ transcript in a *res1*Δ strain. Deleting *res1*$^+$ had no effect on the genes tested in this experiment.

Overexpressing *res1*$^+$, however, caused the clarity of the MBF-component results in this strain to be quite poor as a double signal appears in the *rep1*$^+$, *rep2*$^+$ and *res2*$^+$ autoradiographs. This is likely to be caused by the cross-hybridization of the northern probe to the extremely abundant overexpressed *res1*$^+$ transcript. However, in the *rep1*$^+$ and *rep2*$^+$ autoradiographs this double signal is relatively constant throughout the experiment. But in the case of the *res2*$^+$ autoradiograph the lower transcript showed a distinct increase in abundance over the course of the synchronous meiosis, providing further evidence to indicate that *res2*$^+$ expression may be under the transcriptional control of the MBF complex.

Figure 6.3.1* res1$^+$ *analysis: MCB-controlled transcripts

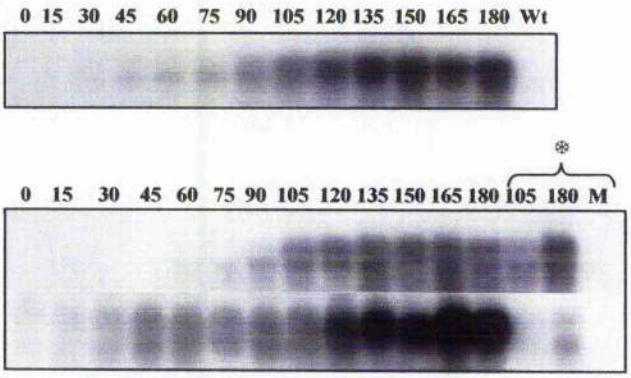
A

cdc22$^+$



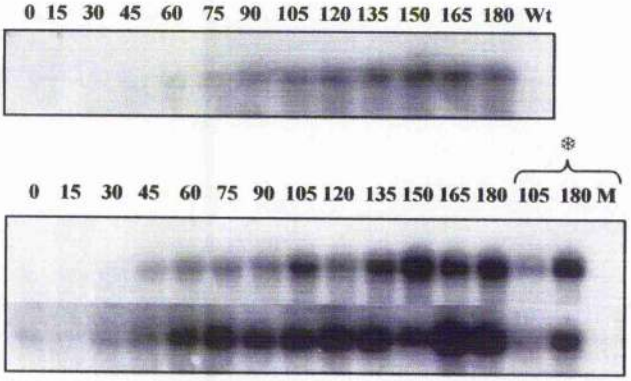
Normal meiotic profile

rec8$^+$



Normal meiotic profile

rec11$^+$



Normal meiotic profile

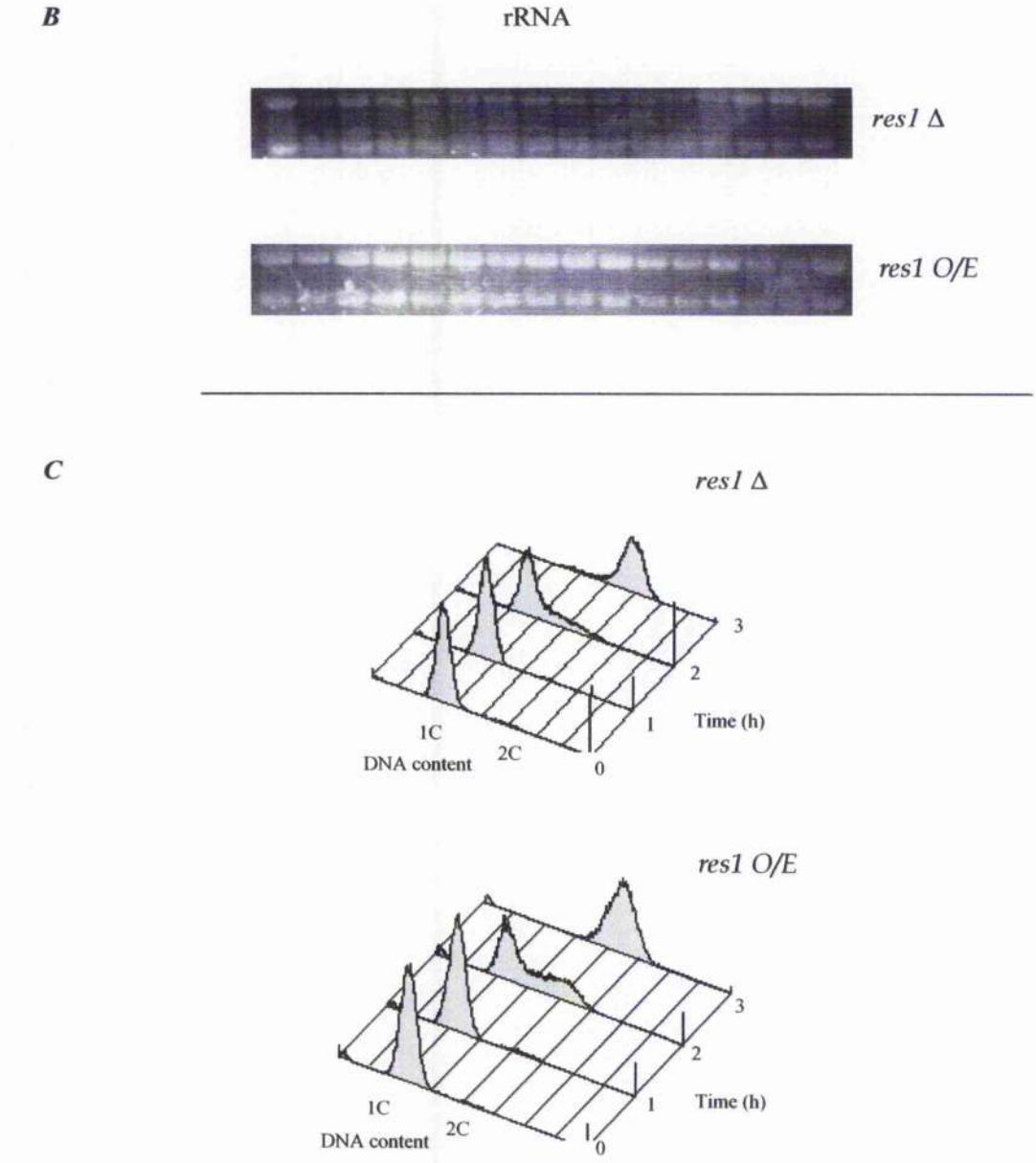


Figure 6.3.1* res1$^+$ *analysis: MCB-controlled transcripts

Thermally induced *pat1-114*, synchronous meioses were carried out for *res1*Δ and *res1-O/E* fission yeast strains and samples were taken every 15 min for RNA extraction and every hour for flow cytometry analysis. Northern-blot analysis was carried out and each membrane was probed for *cdc22*$^+$, *rec8*$^+$ and *rec11*$^+$. ***A*** shows the northern-blot analysis for the MCB-regulated gene probes. Autoradiographs of each separate gene are grouped together so that a comparison can be made between the transcript incidence and abundance in each different strain. Similarly, an autoradiograph of each gene taken from a normal *pat1-114* induced diploid synchronous meiosis is included for comparison. ***B*** shows the rRNA on the ethidium bromide stained formaldehyde gels for each strain as a measure of RNA loading and ***C*** shows the flow cytometry analysis for each strain.

❄ = control RNA samples: M, from asynchronous mitotic GG 375 cells; 105, 105 min sample from a 'wild type' (GG 375) diploid synchronous meiosis; 180, 180 min sample from a 'wild type' (GG 375) diploid synchronous meiosis.

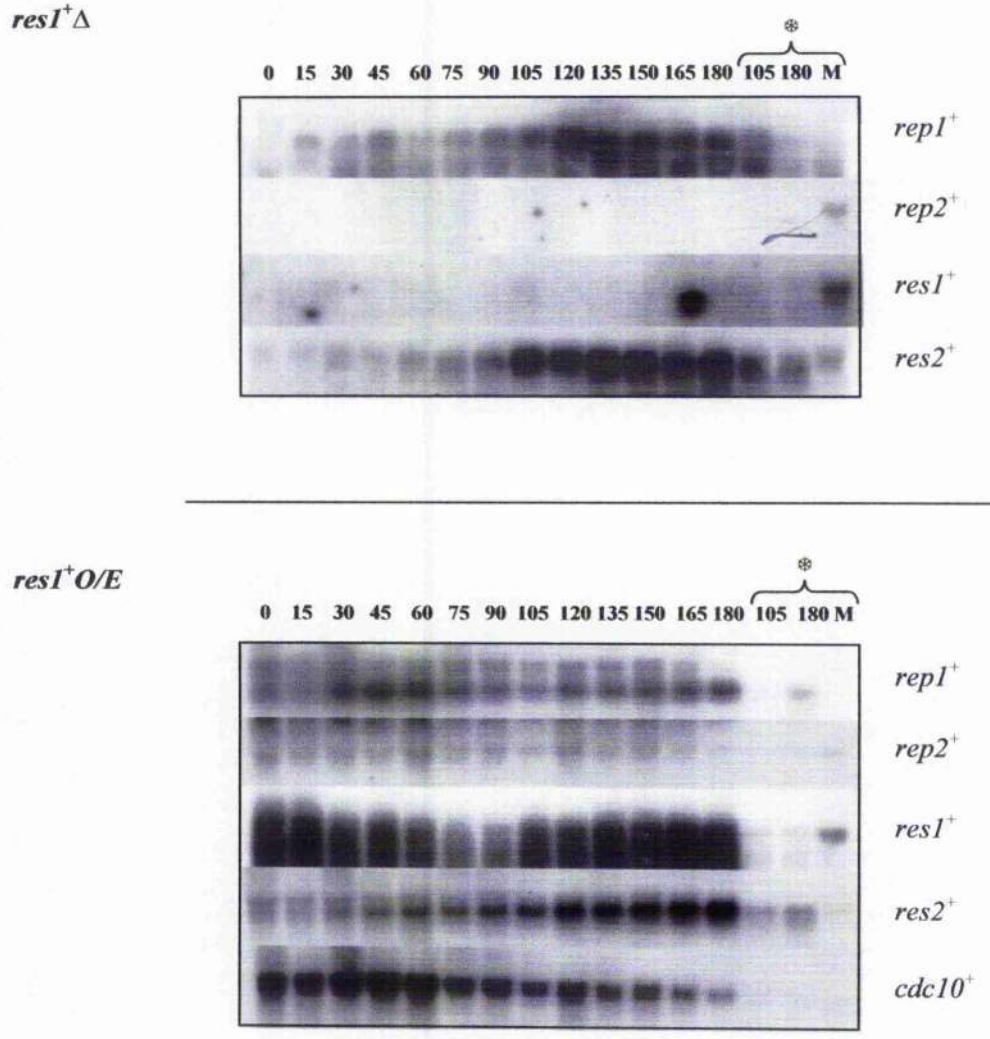


Figure 6.3.2* res1$^+$ *analysis: MBF-component transcripts

This figure shows the autoradiographs resulting from the analysis of the MBF components *cdc10$^+$*, *res1$^+$*, *res2$^+$* *rep1$^+$* and *rep2$^+$* using the fission yeast strains *res1*Δ and *res1-O/E* during synchronous meiosis. The northern membranes from Figure 6.2.1 were hybridized with probes for the MBF components.

❄ = control RNA samples: M, from asynchronous mitotic GG 375 cells; 105, 105 min sample from a 'wild type' (GG 375) diploid synchronous meiosis; 180, 180 min sample from a 'wild type' (GG 375) diploid synchronous meiosis.

6.5 res2$^+$ *analysis*

6.5.1 res2$^+$ *affects MCB-regulated gene transcription during meiosis*

The fission yeast strains h$^+$ *res2::ura4*$^+$ *pat1-114 ura4-D18* and h$^-$ pREP1:*res2*$^+$ *pat1-114 leu1-32* were induced to undergo synchronous meioses and northern-blot analyses were carried out as before (Sections *6.2.1–6.2.3*). Figure 6.4 depicts the analysis of MCB-regulated gene transcript abundance in each experimental strain.

The *cdc22*$^+$, *rec8*$^+$ and *rec11*$^+$ transcript profiles were significantly affected during a synchronous meiosis in the *res2*Δ mutant. The *cdc22*$^+$ transcript was completely absent during the course of a synchronous meiosis when *res2*$^+$ was deleted. RNA from the *rec8*$^+$ and *rec11*$^+$ genes was detected during this experiment although the transcript levels did not even equal those seen in the 105 min control sample. Furthermore, as noted previously (Miyamoto *et al.*, 1992; Zhu *et al.*, 1994), the *res2*Δ strain was unable to pass through pre-meiotic S phase (Figure 6.4 *C*). These observations confirm previous results showing that Res2 is necessary for pre-meiotic S phase (Miyamoto *et al.*, 1994, Zhu *et al.*, 1994, Zhu *et al.*, 1997, Ayté *et al.*, 1997) and also indicate that *res2*$^+$ might be involved in the transcription of the MCB-regulated genes during the meiotic cell cycle.

When the *res2*$^+$ gene was over-expressed, however, the *cdc22*$^+$, *rec8*$^+$ and *rec11*$^+$ transcript profiles all appeared relatively normal over the course of the synchronous meiosis. In each case, the transcripts appeared at similar time points and reached similar levels of transcript abundance as is seen in normal diploid *pat1-114* synchronous meioses and this strain entered pre-meiotic S phase as normal (Figure 6.4 *A*).

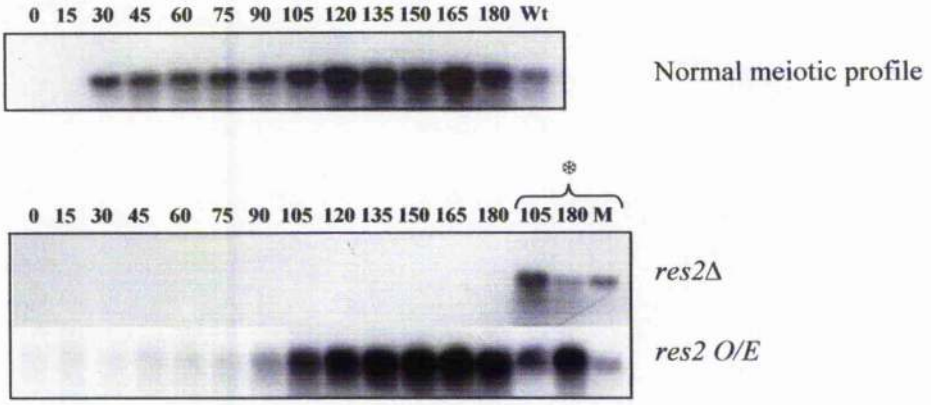
6.5.2 res2$^+$ *does not affect expression of other MBF-component genes*

Figure 6.4 depicts typical autoradiographs that were obtained when a northern membrane for the *res2*Δ strain was probed for the MBF components *cdc10*$^+$, *res1*$^+$, *res2*$^+$, *rep1*$^+$ and *rep2*$^+$. Apart from the obvious lack of a *res2*$^+$ transcript very little effect on any of the other transcripts tested is seen in a *res2*Δ synchronous meiosis. Due to time constraints results were not obtained for the pREP1:*res2*$^+$ overexpression strain.

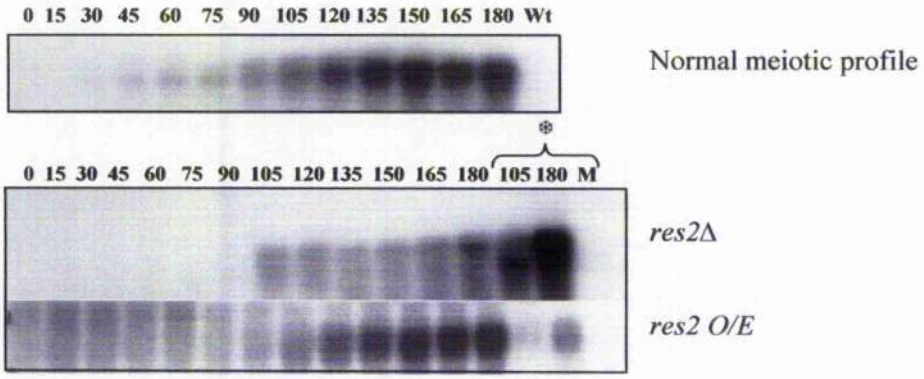
Figure 6.4* res2$^+$ *analysis

A

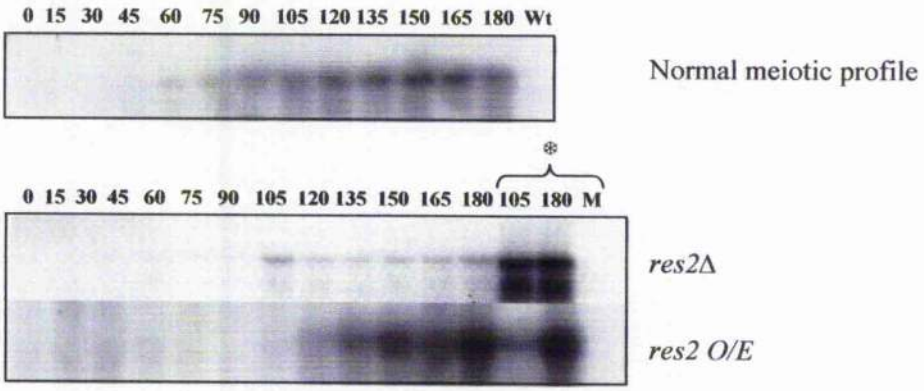
cdc22$^+$

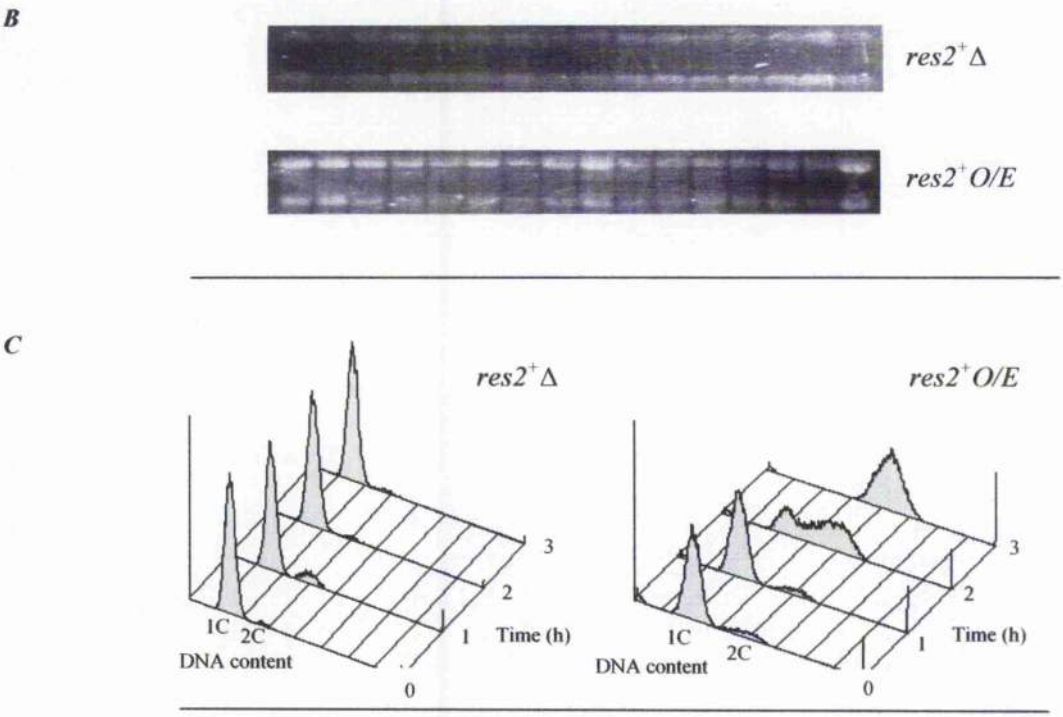


rec8$^+$



rec11$^+$





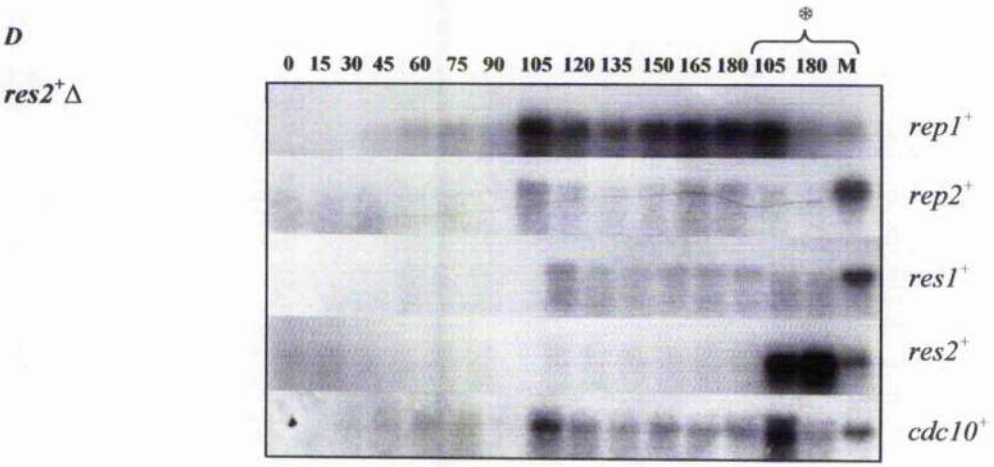


Figure 6.4* res2$^+$ *analysis

Thermally induced *pat1-114*, synchronous meioses were carried out for *res1*Δ and *res1-O/E* fission yeast strains and samples were taken every 15 min for RNA extraction and every hour for flow cytometry analysis. Northern-blot analysis was carried out and each membrane was probed for *cdc22*$^+$, *rec8*$^+$ and *rec11*$^+$. ***A*** shows the northern-blot analysis for the MCB-regulated gene probes. Autoradiographs of each separate gene are grouped together so that a comparison can be made between the transcript incidence and abundance in each different strain. Similarly, an autoradiograph of each gene taken from a normal *pat1-114* induced diploid synchronous meiosis is included for direct comparison. ***B*** shows the rRNA on the ethidium bromide stained formaldehyde gels for each strain as a measure of RNA loading and ***C*** shows the flow cytometry analysis for each strain. ***D*** shows the autoradiographs resulting from the analysis of the MBF components *cdc10*$^+$, *res1*$^+$, *res2*$^+$ *rep1*$^+$and *rep2*$^+$ using the fission yeast *res1*Δ strain. In ***D*** the northern membrane from ***A*** was hybridized with probes for the MBF components.

❄ = control RNA samples: M, from asynchronous mitotic GG 375 cells; 105, 105 min sample from a 'wild type' (GG 375) diploid synchronous meiosis; 180, 180 min sample from a 'wild type' (GG 375) diploid synchronous meiosis.

6.6 rep2^{+} *analysis*

6.6.1 rep2^{+} *affects MCB-regulated gene transcription during meiosis*

The fission yeast strains h^{+} *rep2::ura4*$^{+}$ *pat1-114 ura4-D18* and h^{-} pREP1:*rep2*$^{+}$ *pat1-114 leu1-32* were induced to undergo synchronous meioses and northern-blot analyses were carried out as before (Sections *6.2.1–6.2.3*). Figure 6.5 depicts the analysis of MCB-regulated gene transcript abundance in each experimental strain.

The *cdc22*$^{+}$, *rec8*$^{+}$ and *rec11*$^{+}$ transcripts were all overexpressed during the course of a synchronous meiosis when *rep2*$^{+}$ was deleted. Each MCB-regulated transcript was detected at the 15–30 min time points, which is unusual in the cases of *rec8*$^{+}$ and *rec11*$^{+}$, as these genes are normally not detected until the 45–60 min time points. Furthermore, in each case, the levels of transcript abundance began to peak in the 90 min time points — this would normally not occur until 120 min (see Figure 6.5 'Normal meiotic profiles'). The peak of transcript abundance also appeared to be greater than those seen in the 'wild type' control lanes at the end of the membranes. This result is unusual, as Rep2 was not thought to have a role in the meiotic cell cycle. However, the *rep2Δ* strain did pass through pre-meiotic S phase as normal (Figure 6.5 *C*).

When the *rep2*$^{+}$ gene was overexpressed, however, further significant results were obtained. The *cdc22*$^{+}$ transcript profile remained relatively normal, the first instance of transcript detection was delayed from the usual 35–40 min, until 75–90 min, but the peak of transcription and the abundance of the transcript remained normal. The *rec8*$^{+}$ and *rec11*$^{+}$ transcript profiles, however, were severely affected by *rep2*$^{+}$ overexpression. The transcription of both these meiotic-specific genes appeared to be virtually abolished over the course of the synchronous meiosis. Similar to the *rep1*$^{+}$ overexpression strain, overexpressing *rep2*$^{+}$ also appears to delay S phase entry by approximately one hour (Figure 6.5 *C*). Therefore, overexpressing *rep2*$^{+}$ had differing effects on the Type V, *cdc22*$^{+}$, and the Type II and Type III, *rec8*$^{+}$ and *rec11*$^{+}$, genes.

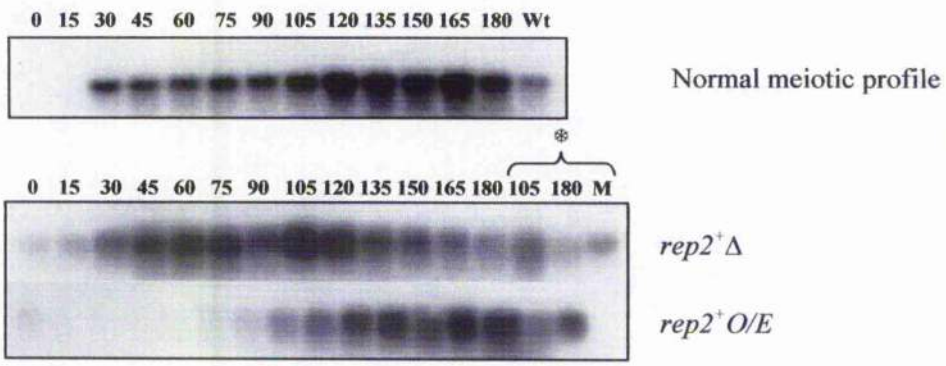
6.6.2 Deletion of rep2^{+} *affects the expression of other MBF-component genes*

Figure 6.5 depicts typical autoradiographs that were obtained when a northern membrane for the *rep2Δ* strain was probed for the MBF components *cdc10*$^{+}$, *res1*$^{+}$, *res2*$^{+}$, *rep1*$^{+}$ and *rep2*$^{+}$. The *res1*$^{+}$, transcript profile was not significantly affected during a synchronous meiosis in a *rep2Δ* strain as very little, or no *res1*$^{+}$ transcript was detected. However, the *res2*$^{+}$ transcript was detected earlier (0 min instead of at 90 min) and appears more abundant during this experiment than in either the control lanes on the same membrane or in a normal diploid *pat1-114* synchronous meiosis. The *cdc10*$^{+}$ transcript similarly appeared to be unusually abundant during this experiment. An increase in *rep1*$^{+}$ transcript levels between 0–75 min was also detected. Due to time constraints, results were not obtained for the pREP1:*rep2*$^{+}$ overexpression strain.

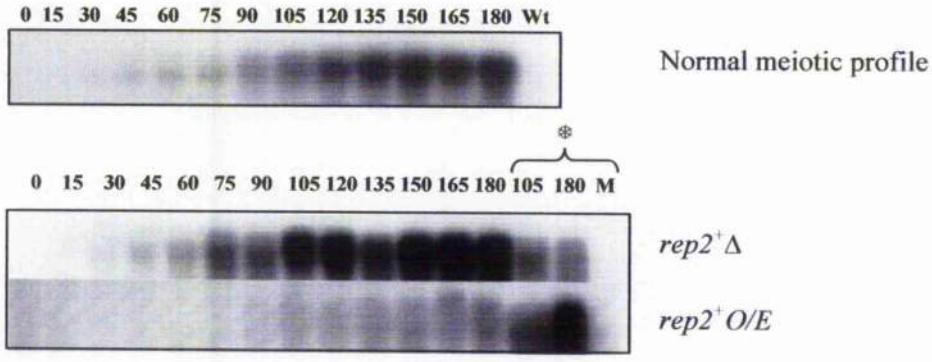
Figure 6.5* rep2^{+} *analysis

A

cdc22^{+}



rec8^{+}



rec11^{+}

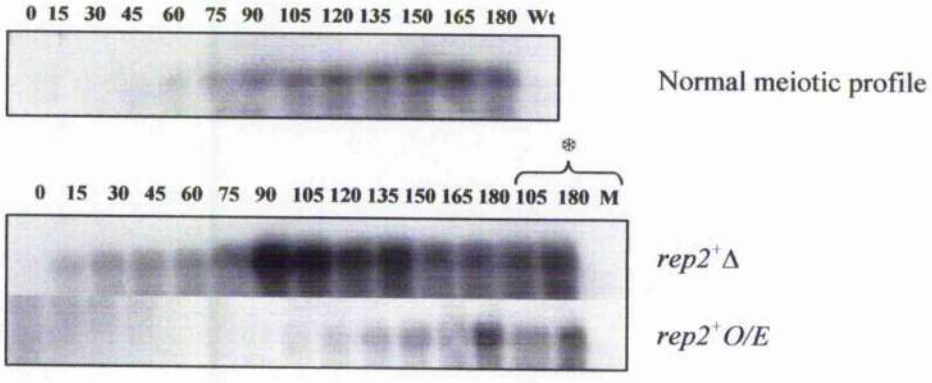


Figure 6.5* rep2$^+$ *analysis: cont'd

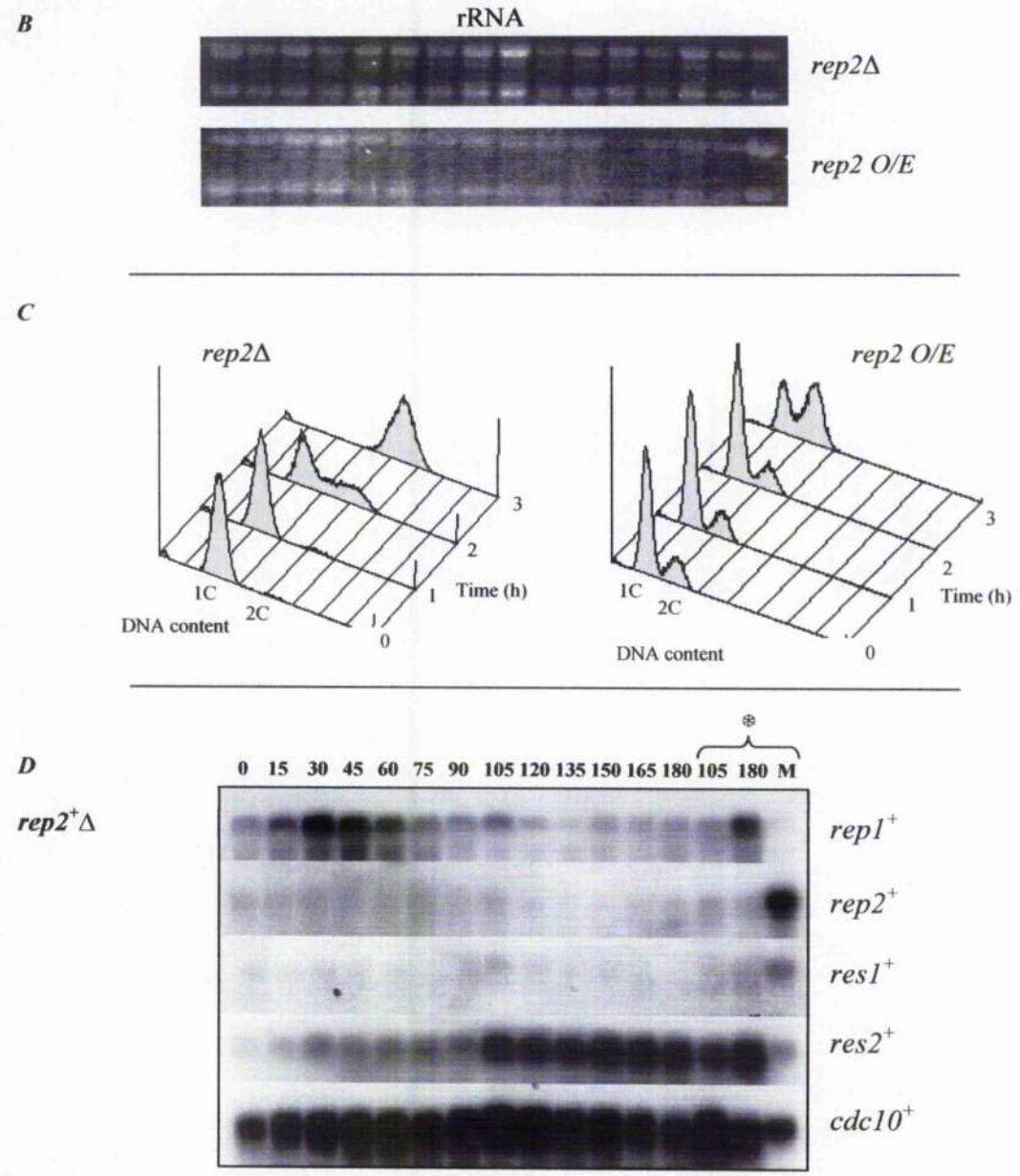


Figure 6.5* rep2$^+$ *analysis

Thermally induced *pat1-114* synchronous meioses were carried out for *rep2*Δ and *rep2-O/E* fission yeast strains and samples were taken every 15 min for RNA extraction and every hour for flow cytometry analysis. Northern-blot analysis was carried out and each membrane was probed for *cdc22*$^+$, *rec8*$^+$ and *rec11*$^+$. ***A*** shows the northern-blot analysis for the MCB-regulated gene probes. Autoradiographs of each separate gene are grouped together so that a comparison can be made between the transcript incidence and abundance in each different strain. Similarly, an autoradiograph of each gene taken from a normal *pat1-114* induced diploid synchronous meiosis is included for comparison. ***B*** shows the rRNA on the ethidium bromide stained formaldehyde gels for each strain as a measure of RNA loading and ***C*** shows the flow cytometry analysis for each strain. ***D*** shows the autoradiographs resulting from the analysis of the MBF components *cdc10*$^+$, *res1*$^+$, *res2*$^+$ *rep1*$^+$and *rep2*$^+$ using the fission yeast *rep2*Δ strain. In ***D*** a northern membrane from ***A*** was hybridized with probes for the MBF components.

❄ = control RNA samples: M, from asynchronous mitotic GG 375 cells; 105, 105 min sample from a 'wild type' (GG 375) diploid synchronous meiosis; 180, 180 min sample from a 'wild type' (GG 375) diploid synchronous meiosis.

6.7 rep1$^+$ *analysis*

6.7.1 rep1$^+$ *affects MCB-regulated gene transcription during meiosis*

The fission yeast strains h$^+$ *rep1::ura4*$^+$ *pat1-114 ura4-D18* and h$^-$ pREP1:*rep1*$^+$ *pat1-114 leu1-32* were induced to undergo synchronous meioses and northern-blot analyses were carried out as before (Sections *6.2.1–6.2.3*). Figure 6.6 depicts the analysis of MCB-regulated gene transcript abundance in each experimental strain.

The *cdc22*$^+$, *rec8*$^+$ and *rec11*$^+$ transcripts were all completely absent during the course of a synchronous meiosis when *rep1*$^+$ was defeted. Furthermore, as noted previously, the *rep1*Δ strain was unable to pass through pre-meiotic S phase (Figure 6.6 ***C***). These observations confirm previous results showing that Rep1 is necessary for pre-meiotic S phase and also indicate that *rep1*$^+$ might be involved in the transcription of the MCB-regulated genes during the meiotic cell cycle (Ding & Smith, 1998).

When the *rep1*$^+$ gene was overexpressed, however, the *rec8*$^+$ and *rec11*$^+$ transcript profiles both appeared to be slightly elevated over the course of the synchronous meiosis in comparison with the 105 min and 180 min control lanes. This strain also gave unusual flow cytometry results, which indicate that overexpressing *rep1*$^+$ delays S phase entry by approximately one hour (Figure 6.6 ***C***).

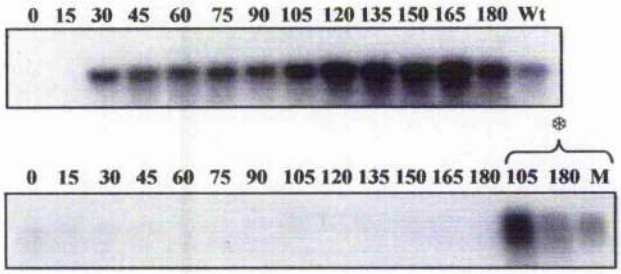
6.7.2 Deletion of rep1$^+$ *affects the expression of* res2$^+$

Figure 6.6 ***D*** depicts typical autoradiographs that were obtained when a northern membrane for the *rep1*Δ strain was probed for the MBF components *cdc10*$^+$, *res1*$^+$, *res2*$^+$, *rep1*$^+$ and *rep2*$^+$. The *rep2*$^+$, *res1*$^+$ and *cdc10*$^+$ transcript profiles do not seem to be significantly affected during a synchronous meiosis in a *rep1*Δ strain. However, the *res2*$^+$ transcript appears significantly less abundant during this experiment than in either the control lanes on the same membrane or in a normal diploid *pat1-114* synchronous meiosis. This agrees with previous observations that *res2*$^+$ is not induced in a *rep2*Δ strain (Sugiyama *et al.*, 1994). Due to time constraints, results were not obtained for the pREP1:*rep1*$^+$ overexpression strain.

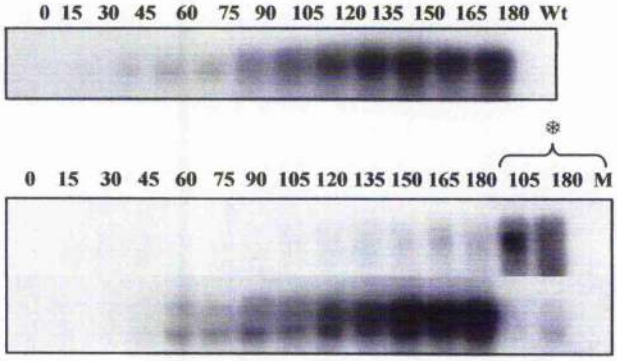
Figure 6.6* rep1^{+} *analysis

A

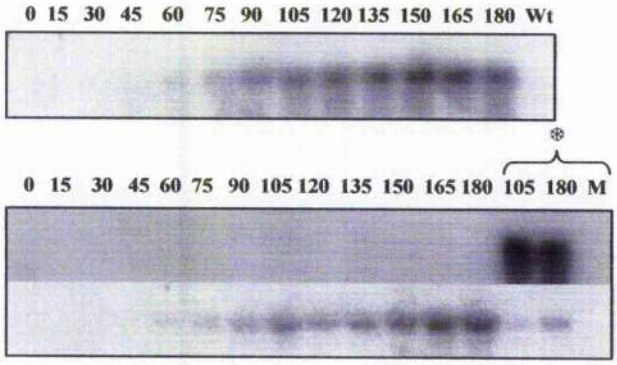
cdc22^{+}



rec8^{+}



rec11^{+}



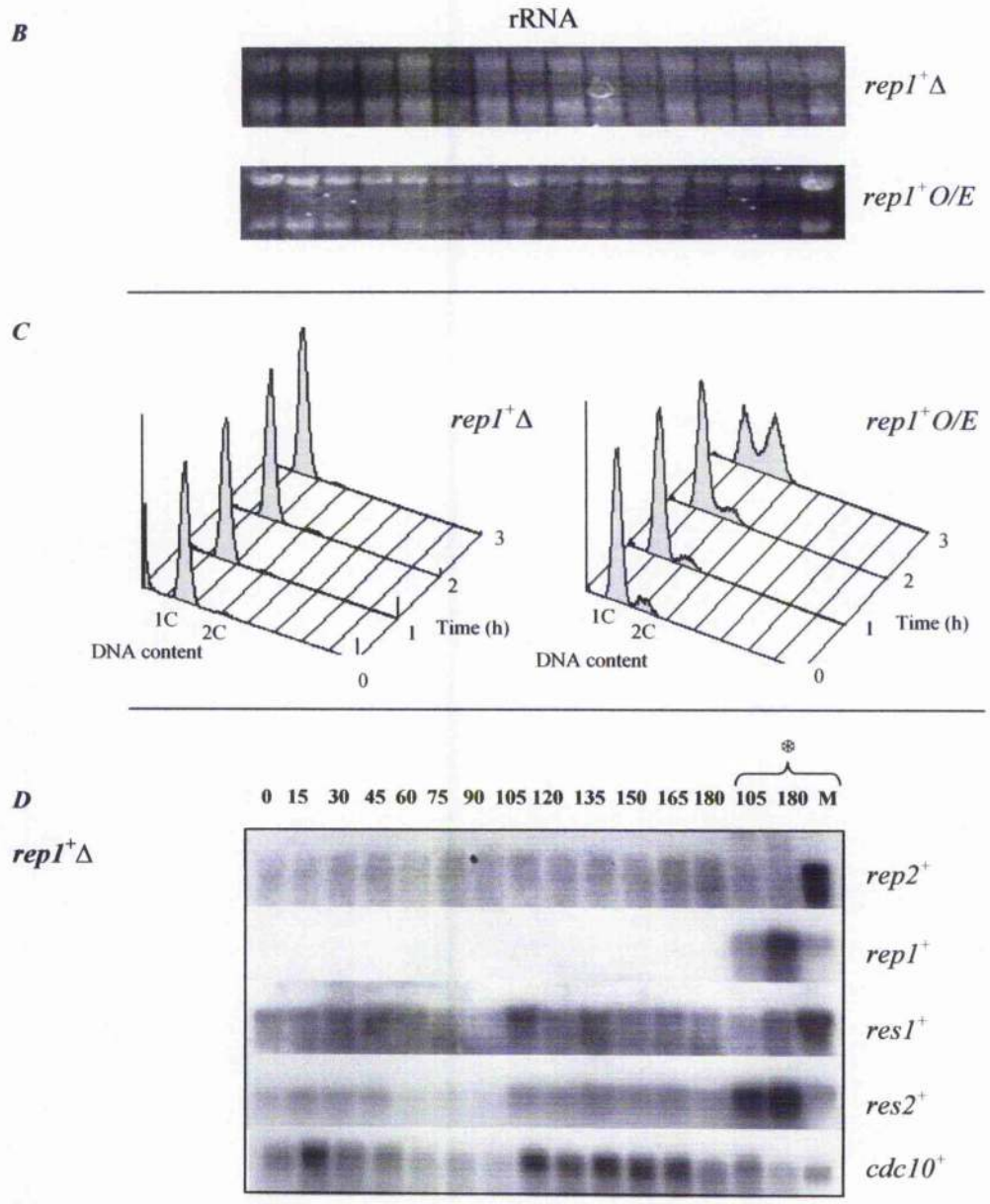


Figure 6.6* rep1$^+$ *analysis

Thermally induced *pat1-114*, synchronous meioses were carried out for *rep2*Δ and *rep2-O/E* fission yeast strains and samples were taken every 15 min for RNA extraction and every hour for flow cytometry analysis. Northern-blot analysis was carried out and each membrane was probed for *cdc22*$^+$, *rec8*$^+$ and *rec11*$^+$. ***A*** shows the northern-blot analysis for the MCB-regulated gene probes. Autoradiographs of each separate gene are grouped together so that a comparison can be made between the transcript incidence and abundance in each different strain. Similarly, an autoradiograph of each gene taken from a normal *pat1-114* induced diploid synchronous meiosis is included for comparison. ***B*** shows the rRNA on the ethidium bromide stained formaldehyde gels for each strain as a measure of RNA loading and ***C*** shows the flow cytometry analysis for each strain. ***D*** shows the autoradiographs resulting from the analysis of the MBF components *cdc10*$^+$, *res1*$^+$, *res2*$^+$ *rep1*$^+$and *rep2*$^+$ using the fission yeast *rep2*Δ strain. In ***D*** a northern membrane from ***A*** was hybridized with probes for the MBF components.

❄ = control RNA samples: M, from asynchronous mitotic GG 375 cells; 105, 105 min sample from a 'wild type' (GG 375) diploid synchronous meiosis; 180, 180 min sample from a 'wild type' (GG 375) diploid synchronous meiosis.

In this thesis an investigation into the functionality of MCB elements and the MBF complex during meiosis has been undertaken. In previous chapters it was established that MCB elements are functional during meiosis and that MCB motifs from certain promoters are capable of inducing the meiotic-specific G1–S expression of a reporter gene. Furthermore, MCB motifs were shown to bind a complex of similar mobility to the mitotic MBF complex in protein extracts taken from cells that were in both the mitotic and the meiotic cell cycles. In this chapter therefore, the expression of the different components of mitotic MBF (*cdc10*$^{+}$, *res1*$^{+}$, *res2*$^{+}$ and *rep2*$^{+}$) and the known meiotic regulator *rep1*$^{+}$ was manipulated to see if there was any effect on the expression of MCB-regulated genes. This would then help establish whether genes which contain MCB motifs in their promoters are indeed regulated by the MCB–MBF system during the meiotic cell cycle.

6.8.1 MCB-regulated gene expression

cdc10^{+}. Deletion of the *cdc10*$^{+}$ gene is lethal to fission yeast cells, so studies were undertaken using the temperature sensitive *cdc10-129*, *cdc10-V50* and *cdc10-C4* mutant *pat1-114* fission yeast cells, as well as the pREP1: *cdc10*$^{+}$ overexpression strain. Cdc10p is known to be required for passage through both pre-mitotic and pre-meiotic S phase (Nurse & Bissett, 1981, Beach *et al.*, 1985) and as the control of transcription of MCB-regulated genes is the function of Cdc10p during mitosis, it is likely that Cdc10p performs a similar function during meiosis. The experiments described in this chapter show that both mutating and overexpressing *cdc10*$^{+}$ during a synchronous meiosis has a dramatic effect on MCB-gene transcription. None of the *cdc10*$^{+}$ mutant strains were able to pass through pre-meiotic S phase, as judged by flow cytometry analysis. However, it is possible that these strains did not pass far enough through the meiotic cell cycle for a real reflection of the state of MCB-regulated gene transcription to be observed. MCB genes are not expressed until 30–45 min at the restrictive temperature in a 'normal' *pat1-114* synchronous fission yeast meiosis. So, if the *cdc10*$^{+}$ mutations caused a cell cycle arrest before this point, MCB-regulated gene expression would not be seen. However, the *cdc10*$^{+}$ overexpression strain passes normally through pre-meiotic S phase and causes a distinct overexpression of the MCB-regulated genes. This indicates that Cdc10p does affect MCB-regulated gene expression during the meiotic cell cycle and implies that the effects on MCB-regulated gene transcription seen in the *cdc10* mutant strains are therefore as much due to the malfunctioning Cdc10p as they are due to the concurrent cell cycle arrest.

res1^{+}. Manipulation of the expression of *res1*$^{+}$ did not have a significant effect on the expression of MCB-regulated genes during a synchronous meiosis, which is not surprising, as Res1p is known to function primarily during the mitotic cell cycle and is not required for the meiotic cell cycle. However, that overexpressing the *res1*$^{+}$ gene causes overexpression of the MCB-regulated genes during meiosis reinforces the suggestion that the MCB–MBF system is responsible for MCB-regulated gene transcription during the meiotic cell cycle.

res2^{+}. Deleting the *res2*$^{+}$ gene, however, had a significant effect on the expression of MCB-regulated genes during a synchronous meiosis. The *res2*Δ strain was unable to pass through pre-meiotic S phase and also showed a complete absence of *cdc22*$^{+}$ transcript and a severe reduction in the levels of both *rec8*$^{+}$ and *rec11*$^{+}$ transcripts. Again, as this strain becomes arrested in G1 phase it is possible that the cells did not pass far enough through the meiotic cell cycle to allow any possible effects on MCB-regulated gene transcription to be seen. However, there are two main arguments against this explanation.

First, the dramatic lack of any detectable *cdc22*$^{+}$ transcript indicates that this is specifically due to the deletion of the *res2*$^{+}$ gene rather than a symptom of the cell cycle arrest, as some detectable *cdc22*$^{+}$ transcript is normally visible even in the very early time points. Second, some *rec8*$^{+}$ and *rec11*$^{+}$ transcripts were detected in the later time points of this synchronous meiosis, This observation indicates that the cells must have passed at least 45–60 min into the meiotic cell cycle before becoming arrested, as this is the time when the *rec8*$^{+}$ and *rec11*$^{+}$ transcripts are normally detected. These observations indicate that the deletion of the *res2*$^{+}$ gene specifically perturbs the expression of *cdc22*$^{+}$, *rec8*$^{+}$ and *rec11*$^{+}$ leading to the inference that the cell cycle arrest is perhaps caused, in part, by the lack of MCB-regulated gene expression. That overexpressing *res2*$^{+}$ had very little effect on MCB-regulated gene expression might indicate that while Res2p has a crucial role in MCB-regulated gene transcription during the meiotic cell cycle, it does not act as the specific activator of this transcription.

rep2^{+}. That manipulating the expression of the *rep2*$^{+}$ gene affects MCB-regulated gene expression during meiosis is quite surprising as this protein was thought to be a mitotic-specific activator of the MBF complex. However, the levels of *cdc22*$^{+}$, *rec8*$^{+}$ and *rec11*$^{+}$ transcript in a *rep2*Δ synchronous meiosis are clearly increased over 'wild type' levels. This can possibly be explained by the suggestion that in normal wild type cells which are entering the meiotic cell cycle there might be a residual amount of the mitotic activator Rep2p left in the cells which is able to exert a mildly repressing effect on meiotic MCB-regulated gene transcription. In *rep2*Δ cells that are passing into the meiotic cell cycle this repressing effect is removed, so more MCB-regulated gene transcript is detected. This explanation is supported by the observation that the

detection of the *cdc22*$^+$ transcript is slightly delayed in cells that are overexpressing the *rep2*$^+$ gene during an induced synchronous meiosis.

That overexpressing the *rep2*$^+$ gene had a dramatic effect on the meiotic-specific *rec8*$^+$ and *rec11*$^+$ transcripts, while not effecting *cdc22*$^+$ transcription, is especially interesting. This indicates that Rep2 might be involved in the mechanism which causes a distinction between 'meiotic specific' and 'mitotic and meiotic' MCB-regulated transcription.

rep1$^+$. Deleting the *rep1*$^+$ gene had a similar effect to deleting the *res2*$^+$ gene; expression of the *cdc22*$^+$, *rec8*$^+$ and *rec11*$^+$ genes was abolished during a synchronous meiosis. Also, overexpressing the *rep1*$^+$ gene caused some overexpression of the *rec8*$^+$ and *rec11*$^+$ genes, although results were unfortunately not obtained for *cdc22*$^+$ due to time constraints.

Previous studies have suggested that Rep1p is required for the initiation of pre-meiotic DNA synthesis (Sugiyama *et al.*, 1994) and the induction of *rec*$^+$ gene transcription during the meiotic cell cycle (Ding & Smith, 1998). The observations seen in this study are in agreement with these previous results and indicate that Rep1p fulfils these two roles through its direct requirement for the expression of all MCB-regulated genes, including *cdc22*$^+$, during the meiotic cell cycle and that it has an activating function.

6.8.2 MBF-component gene expression

To further clarify the interactions between components of the MBF complex it was decided to examine the effects that mutating or overexpressing each MBF component might have on the expression of the other MBF components. Therefore, the northern membranes for each strain that was used in the analysis of the MCB-regulated gene transcripts were also probed for *cdc10*$^+$, *res1*$^+$, *res2*$^+$, *rep2*$^+$ and *rep1*$^+$. One significant observation arose from these experiments, which was that the *res2*$^+$ transcript appears to be under MCB control. Mirroring the effects seen on MCB-regulated genes, the *res2*$^+$ transcript was elevated in the *rep2*Δ and *cdc10-C4* mutation strains as well as in *cdc10*$^-$ and *res1*$^+$ overexpression strains. And, as noted previously (Sugiyama *et al.*, 1994), the *res2*$^+$ transcript was also diminished in a *rep1*Δ strain. These results indicate that Res2p might act in a feedback loop to control the periodic expression of the MCB–MBF system during meiosis.

- Using the separate approaches of overexpressing and mutating or deleting each different component of the MBF complex, an investigation into the possible function of the MBF complex during the meiotic cell cycle was undertaken.

- *cdc10*$^{+}$, *res2*$^{+}$ and *rep1*$^{+}$ were found to be crucial for the expression of both 'meiotic and mitotic' and 'meiotic specific' MCB-regulated genes during the meiotic cell cycle. Surprisingly, *rep1*$^{+}$ was also seen to have an effect.
  - *cdc10*$^{+}$, known to be involved in passage through pre-meiotic S phase (Beach *et al.*, 1985), was shown in this study to specifically affect the transcription of MCB-regulated genes during the meiotic cell cycle.
  - *res2*$^{+}$, known to be crucial for the progression of the meiotic cell cycle and to affect the expression of *cdc22*$^{+}$ and *cdc18*$^{+}$ during the meiotic cell cycle (Ayté *et al.*, 1997), was shown in this study to also be involved in the transcription of meiotic-specific MCB-regulated genes and to be itself under the influence of the MBF transcriptional complex.
  - *rep2*$^{+}$ overexpression causes a significant decrease in the amount of meiotic-specific *rec8*$^{+}$ and *rec11*$^{+}$ transcripts that are detected during a synchronous meiosis but has little effect on the expression of *cdc22*$^{+}$. This indicates that Rep2p might be involved in the mechanism that allows a distinction between mitotic and meiotic and meiotic-specific MCB motifs during transcriptional activation in the two different life cycles.
  - Rep1p, known to be crucial for the progression of the meiotic cell cycle and *res2*$^{+}$ expression (Sugiyama *et al.*, 1994), and thought to be involved in the control of meiotic-specific *rec*$^{+}$ gene transcription (Ding & Smith, 1998), was shown in this study to be crucial for the expression of both 'meiotic and mitotic' as well as 'meiotic specific' MCB-regulated genes during the meiotic cell cycle.

- These results combine to extend previous observations suggesting roles for the components of the MBF complex during the meiotic cell cycle and specifically show that a meiotic form of the MBF complex is responsible for MCB-regulated gene transcription during the meiotic cell cycle.

Chapter 7

Discussion

In fission yeast, it had been established that a protein complex known as MBF — which contains the Cdc10p, Res1p, Res2p and Rep2p protein components — binds to MCB sequences in the promoters of various genes during the mitotic cell cycle, causing G1–S-specific transcription (Lowndes *et al.*, 1992; Caligiuri & Beach, 1993; Fernandez-Sarabia, 1993; Kelly *et al.*, 1993; Hoffman & Beach, 1994; Miyamoto *et al.*, 1994; Zhu *et al.*, 1994; Nakashima *et al.*, 1995; Ayté *et al.*, 1995; Baum *et al.*, 1997; Tahara *et al.*, 1998; Baum *et al.*, 1997; Whitehall *et al.*, 1999). Furthermore, the MBF components Cdc10p and Res2p had been implicated in the progression of fission yeast cells through the G1 and S phases of the meiotic cell cycle (Beach *et al.*, 1985, Miyamoto *et al.*, 1994; Zhu *et al.*, 1994; Zhu *et al.*, 1997; Ayté *et al.*, 1997). A fifth potential component of the MBF complex, Rep1, had also been identified as being essential for meiotic progression (Sugiyama *et al.*, 1994) and is known to affect the expression of both *res2*$^{+}$ (Sugiyama *et al.*, 1994) and a family of meiotic-specific recombination (*rec*$^{+}$) genes (Li & Smith, 97, Ding & Smith, 1998). It had also been noted that several of the meiotic specific *rec*$^{+}$ genes contained MCB elements in their promoter regions (Ding and Smith 98; Mata *et al.*, 2002).

Thus, an intriguing combination of facts had been uncovered. A known, and well-studied, transcription factor from the mitotic cell cycle (MBF) had been implicated as having a similar role in the meiotic cell cycle. However, a group of genes that were specifically transcribed during meiosis, and not expressed during mitosis, had been shown to contain the MBF recognition sequences within their promoters. The question remained, therefore whether MBF, or a meiotic form of the MBF complex, was responsible for *rec*$^{+}$ gene transcription during the meiotic cell cycle, and, by what mechanism was the expression of the *rec*$^{+}$ genes confined to meiosis? MCB motifs have been shown to confer G1–S-specific periodic transcription on to a reporter gene during the mitotic cell cycle. So, what causes this meiotic-specific gene expression to occur? Is it specific arrangements of MCB motifs in the *rec*$^{+}$ gene promoters, or a meiotic form of the MBF complex, or both, which controls meiotic-specific gene expression at the start of S phase in fission yeast?

7.2 Summary of the aims of this thesis

To address these questions in this thesis, an investigation into the functions of both the MCB motifs and the components of the MBF complex during the meiotic cell cycle was undertaken.

It was initially hypothesized that differences in the number or arrangement of MCB motifs within the promoters of the *rec*$^{+}$ genes might be responsible for designating them as meiotic specific genes. In Chapter 3, therefore, we undertook to show that, during the meiotic

cell cycle, *rec*$^{+}$ genes were expressed at the G1–S phase boundary with a periodicity that was similar to other genes known to contain MCB motifs in their promoters (i.e. *cdc22*$^{+}$ *et al.*). All of these genes were therefore likely to be under the same transcriptional control. Second, we surveyed the promoters of various genes that were expressed at the G1–S boundary specifically during the mitotic cell cycle, during both the mitotic and meiotic cell cycles, or specifically during just the meiotic cell cycle, to see if any distinct pattern arose in the number, sequence or arrangement of MCB motifs within these promoters.

In Chapter 4 of this thesis we undertook to show that MCB elements were physiologically relevant during the meiotic cell cycle. UAS reporter vectors were used to show that both synthetic and exogenous native MCB sequences were able to confer G1–S transcription on to a reporter gene during the meiotic cell cycle.

Given that MCB elements were shown to be functional during meiosis, Chapter 5 describes experiments to identify the DNA-binding activity that interacted with MCB DNA sequences during the meiotic cell cycle. EMSA experiments using meiotic-specific MCB elements as probes and both mitotic and meiotic protein extracts were undertaken.

Finally, in Chapter 6, having established that MCB motifs were functional during the meiotic cell cycle and interacted with a DNA-binding activity of similar mobility to the mitotic MBF complex, experiments to examine the functions of known MBF components during meiosis were described. Fission yeast strains that were overexpressing, or that contained mutations or deletions of, the MBF-component genes (*cdc10*$^{+}$, *res1*$^{+}$, *res2*$^{+}$, *rep2*$^{+}$ and *rep1*$^{+}$) were examined for their effects on MCB-regulated gene transcription during the meiotic cell cycle.

7.3 Periodic gene expression during meiosis in fission yeast: Chapter 3

Many of the *rec*$^{+}$ genes of fission yeast are expressed exclusively during the G1–S phase transition of the meiotic cell cycle (Fox & Smith, 1998). These genes also have MCB motifs within their promoters and it was suggested that they might be under the control of the MBF (or an MBF-like) complex (Ding & Smith, 1998), as the MCB–MBF system is known to control the expression of G1–S-specific genes during the mitotic cell cycle. We were, therefore, interested to address the question of meiotic specific MCB-controlled gene transcription during meiosis.

The G1–S-specific transcription of several MCB containing genes was examined over the course of a diploid *pat1-114* induced synchronous meiosis. Using northern analysis, the transcript abundance of several of the *rec*$^{+}$ genes was found to gradually increase approximately 45 min after the induction of a synchronous meiosis by temperature shift. These *rec*$^{+}$ gene mRNA profiles closely mirrored those seen for several other genes, including *cdc22*$^{+}$, *cdc18*$^{+}$

and *cdt1*$^{+}$, the transcription of which is known to depend on the MBF complex during mitosis (Fernandez-Sarabia *et al.*, 1993, Kelly *et al.*, 1993, Hoffman & Beach, 1994). These results agree with previous work on meiotic gene expression (Li & Smith, 1997, Mata *et al.*, 2002).

There is a strong precedent for groups of genes being under the control of the same transcription factor complex if they are expressed with similar periodicity and have similar UAS motifs in their promoters (Struhl, 1989). An example of this is the control of the mating type switching genes by *ste11*$^{+}$ (Sugimoto *et al.*, 1991). Thus, it was considered likely that both the 'mitotic and meiotic' and the 'meiotic specific' genes were under the control of the same transcription factor during the meiotic cell cycle.

7.3.1 MCB-motif structure

A survey of the promoters of a selection of genes known to be transcribed at the G1–S boundary of the cell cycle was undertaken to analyze the specific DNA sequences and incidence of MCB motifs within their promoters. Recent comprehensive studies of gene transcription during meiosis have revealed that around 100 genes are transcribed transiently at the G1–S boundary of the meiotic cell cycle (Mata *et al.*, 2002), and that several of these genes are specific to meiosis (Ding & Smith, 1998; Watanabe *et al.*, 2001). A large number of these genes are also known to contain MCB motifs within their promoters (Mata *et al.*, 2002). These meiotically expressed MCB-containing genes fell into two categories: those expressed specifically during meiosis, and those expressed during both mitosis and meiosis. Promoters of genes transcribed with G1–S periodicity specifically during the mitotic cell cycle were also considered.

This analysis revealed that MCB motifs could be classified into distinct 'Types' according to the number and arrangement of individual *Mlu*I sequences within their promoters. Type I/ Type II MCB motifs are simple in nature and appear to confer meiotic-specific gene expression. Type III MCB motifs include both mitotic specific genes and meiotically transcribed genes. Type IV MCB motifs are complex in nature and confer G1–S-specific expression during both the mitotic and the meiotic cell cycles. This clear distinction in the complexity of MCB-motifs is intriguing: simpler MCB motifs seem to confer meiotic specific G1–S gene expression and more complex motifs seem to confer periodic gene expression during both the mitotic and meiotic cell cycles. Furthermore, of the genes studied, their protein functions can also be similarly grouped. The genes containing Type I and Type II MCB motifs encoded meiotically expressed recombination and cohesin proteins whereas the complex Type IV MCB genes produced proteins crucial for S-phase progression which were required in both pre-meiotic and pre-mitotic S phases.

7.3.2 Future work

It is clear from this study and from the comprehensive microarray analysis that was recently undertaken (Mata *et al.*, 2002) that several genes, the promoters of which are enriched for MCB motifs, are transcribed specifically at the G1–S phase boundary of the meiotic cell cycle. Now that this complete data set is available a survey of a greater number of the individual MCB motifs from genes encoding a wider range of protein functions, would help to confirm and extend the observations made in Chapter 3 of this thesis. A larger study would help to clarify whether the different Types of MCB motifs do have distinct roles and would possibly provide sufficient data for the definition of consensus sequences for the different classes of MCB motif — that is the 'mitotic specific', 'meiotic specific' and 'mitotically and meiotically' expressed MCB-regulated genes. Ultimately, such information would enhance the understanding of how MBF and its meiotic counterpart interact with, and distinguish between, these genes to produce the G1–S-specific expression of two related but distinct sets of genes in the mitotic and meiotic life cycles.

7.4 MCB motifs are functional during meiosis in fission yeast: Chapter 4

Although MCB motifs were known to be present in the promoters of several genes expressed at the G1–S phase boundary of the meiotic cell cycle, it had not been established whether these UAS motifs were functional during the meiotic cell cycle. Therefore, experiments were undertaken using UAS reporter plasmids to investigate MCB-motif activity during meiosis.

A synthetic DNA fragment, containing three consecutive MCB motifs, induced G1–S specific expression of a reporter gene during a synchronous meiosis. The transcript profile of the reporter gene followed the same pattern as the transcript profiles of the *cdc22*$^{+}$ and *rec8*$^{+}$ genes. Similar results were obtained when a native MCB-motif cluster from the *cdc22*$^{+}$ promoter was used in this same reporter construct. Significantly, when a DNA fragment containing the MCB motifs from the *rec15*$^{+}$ promoter was used in the same reporter construct, meiotic-specific induction of the reporter gene was observed.

The results obtained for the *lacZ* reporter gene were difficult to interpret, particularly when weaker UAS sequences were used, as a significant amount of non-specific background *lacZ* expression was produced from the pSPΔ178 vector. This technical problem is well recognized in the use of yeast extra chromosomal multi-copy reporter plasmids containing the *CYC1–lacZ* reporter cassette (Griggs & Johnston, 1993; Melcher *et al.*, 2000). As a result of this, Melcher and colleagues (2000) produced a series of plasmids for use in budding yeast which were specifically designed to overcome this problem. The plasmid YIPMELβ2 is an integrating plasmid that uses the tightly regulated *MEL1* budding yeast promoter as the minimal reporter-gene promoter; both of these properties help overcome the problem of background reporter-gene expression. We adapted this plasmid for use in fission yeast by replacing the

budding yeast *URA3* nutritional marker sequences for the fission yeast *ura4* nutritional marker sequences, to produce the plasmid SpIPMELβ2. This vector was then able to integrate in single copy into the fission yeast genome at the *ura4*$^{+}$ locus. Several SpIPMELβ2 reporter constructs were made containing different MCB motif DNA fragments, but due to time constraints results were only obtained for SpIPMELβ2.cdc22MCB2. Far less background expression and therefore a much clearer signal from the specific UAS-induced *lacZ* transcript were obtained using this vector. These results also agreed with those obtained using the pSPΔ178.cdc22MCB2 construct.

Both the *cdc22*$^{+}$ MCB cluster and the *rec15*$^{+}$ MCB motif induce periodic reporter gene expression during a synchronous meiosis, which confirmed that the G1–S-specific expression of both the 'mitotic and meiotic' genes (*cdc22*$^{+}$ *et al.*) and the meiotic specific genes (*rec15*$^{+}$ *et al.*) can be ascribed to the presence of MCB motifs within their promoters. These observations show, therefore, that MCB motifs are physiologically relevant during the fission yeast meiotic cell cycle. Furthermore, as the *rec15*$^{+}$ MCB motif appears to confer meiotic-specific expression on the *lacZ* reporter gene, these results imply that the specific sequence or arrangement of the MCB motifs within the promoters of meiotic specific genes, such as the *rec*$^{+}$ genes, is responsible for this specificity of expression.

Experiments have also been conducted in our laboratory by Tracy Riddell in which the *rec8*$^{+}$ meiotic specific promoter sequences and the *rad21*$^{+}$ mitotic specific promoter sequences have been interchanged. This resulted in switching the specificity of *rec8*$^{+}$ expression to mitosis and *rad21*$^{+}$ expression to meiosis. These observations further confirm that, in the case of MCB-regulated genes, it is the promoter regions that determine life-cycle specificity of gene expression.

7.4.1 Future work

Several fission yeast strains have been produced in the course of this work that would allow the analysis of different Types of MCB motif during synchronous meiosis experiments, but which were not used due to time constraints. However, it is anticipated that an examination of the capability of these different Types of MCB motifs to induce either mitotic-specific, mitotic and meiotic or meiotic-specific reporter gene expression would further our understanding of the mechanism by which these UAS motifs confer life cycle specific and periodic gene expression. Indeed it is intended that these constructs be used to help refine the MCB consensus motif, and explore the possibility of designating consensus MCB motifs that are capable of inducing life-cycle-specific gene expression. Designing synthetic DNA fragments containing variations of MCB-motif sequences for use in these reporter constructs is another approach we intend to use to help achieve this goal. The manipulation of MCB promoter sequences *in situ* using a PCR-based method of DNA replacement (Bahler et al., 1998) would also reinforce and extend our

observations. This would allow us to monitor the effects of mutating MCB sequences *in vivo* in the context of the genome.

7.5 Identification of a meiotic MCB-motif binding-activity in fission yeast: Chapter 5

Various experiments — including EMSA analysis using *cdc22*$^{+}$ promoter fragments — have shown that, during the mitotic cell cycle, MCB motifs interact with the MBF transcription factor complex, which causes the G1–S-specific expression of several genes that contain MCB motifs within their promoters (Lowndes *et al.*, 1992; Baum *et al.*, 1997). So, having shown that MCB motifs were functional during the meiotic cell cycle, we wanted to investigate the possibility that MCB motifs might interact with a similar transcription factor complex during this alternative life cycle.

EMSA analysis showed that MCB motifs from the meiotic specific *rec*$^{+}$ genes interacted with the mitotic MBF complex. Not only did *rec*$^{+}$ gene MCB motifs bind a protein complex of similar mobility to MBF in mitotic protein extracts, but they were able to compete with the *cdc22*$^{+}$ EMSA probe for the binding of MBF. Furthermore, MCB motifs from both *cdc22*$^{+}$ and *rec*$^{+}$ gene promoters were shown to interact with a DNA-binding activity from meiotic protein extracts that was of similar mobility to mitotic MBF. Interestingly, the *cdc22*$^{+}$ and *rec*$^{+}$ gene promoter fragments interacted with this binding activity in different ways. When the *cdc22*$^{+}$ probe was used the DNA-binding activity was not detected until the 3 h time point, which coincides with the repression of MCB-gene expression. This is similar to observations made in mitotic cells in which the MBF complex was only detected in G2 arrested cells (Baum *et al.*, 1997). However, using the same meiotic protein extracts the *rec*$^{+}$ gene DNA probes were shown to interact with the DNA binding activity throughout the duration of the synchronous meiosis.

These observations and the fact that *rec*$^{+}$ gene MCB motifs have been shown to confer meiotic specificity on to a reporter gene, imply that the MBF complex (or an MBF-like complex) interacts with the different Types of MCB motifs in different ways. This explains the observation that, in some cases (*cdc22*$^{+}$ *et al.*), MCB motifs confer mitotic and meiotic gene expression onto the genes under their control and in others (*rec15*$^{+}$ *et al.*) they confer meiotic-specific gene expression.

7.5.1 Future work

As DNA-binding activities of identical mobility to the mitotic-MBF complex were found to interact with MCB-motif probes during a synchronous meiosis, and these same probes competed for the binding of the mitotic-MBF complex, it is highly likely that the meiotic MCB-motif binding activity is closely related to mitotic MBF. However to unequivocally identify the meiotic MCB-motif binding activity and the protein components that constitute it, supershift EMSAs need to be carried out. To 'supershift' a DNA-binding activity in an EMSA, an

antibody against a specific protein is added to the reaction mixture before electrophoresis. If the antibody is specific to a protein found in the DNA-binding complex the antibody would bind that protein, hence increasing the overall size of the complex and retarding its migration through the gel. Thus, the protein constituents of a DNA-binding activity can be identified on the basis of the ability of various specific antibodies to bind the protein–DNA complex and retard it in an EMSA. Unfortunately, antibodies specific to components of MBF (i.e. Cdc10p, Res1p, Res2p, Rep1p or Rep2p) were not available to us during the course of this work. However, obtaining such antibodies in the future would allow the direct identification or elimination of the MBF components as being constituents of the meiotic MCB-binding activity.

Furthermore, as the MCB-binding activity identified during the synchronous meiosis showed a different interaction with the *cdc22*$^{+}$ and the *rec*^{1} gene MCBs it would be interesting to look at the behaviour of the MBF complex during a synchronous mitosis experiment. Extrapolating from the results observed in Chapter 5, it would not be surprising if different patterns of MBF–MCB binding were detected for the 'meiotic specific' and the 'mitotic and meiotic' promoter probes during the mitotic cell cycle.

Another technique that could be used to detect the protein components that bind to MCB motifs is chromatin immunoprecipitation analysis (CHIP). This technique uses formaldehyde to fix cells by crosslinking intimately associated molecules together. This maintains the localization of proteins on DNA sequences. Extraction of the chromatin followed by immunoprecipitation of a protein of interest will then specifically isolate any DNA sequences that are attached to that protein. The DNA can then be analysed by PCR to determine the sequences involved (Orlando *et al.*, 1997; Ekwall and Partridge, 1999). This technique could be used with immunoprecipitation of one of the known MBF components, such as Cdc10p, to shed light on the DNA sequences and possibly other proteins that associate with it during meiosis.

7.6 Study of MBF components during meiosis in fission yeast

Having shown that MCB motifs were functional during the meiotic cell cycle and that they interacted with a DNA-binding activity that was likely to be closely related to the mitotic MBF complex, we wanted to study the known components of MBF in the meiotic cell cycle. To this end *pat1-114* fission yeast strains containing mutations of *cdc10*$^{+}$ or deletions of *res1*$^{+}$, *res2*$^{+}$, *rep1*$^{+}$ and *rep2*$^{+}$, as well as strains containing a pREP1 overexpression vector for each component were induced to undergo synchronous meioses. The effects on MCB-regulated gene transcription were monitored by the use of northern blots.

7.6.1 cdc10^{+} *manipulation*

Cdc10p is known to be crucial for passage through START during the mitotic cell cycle and has been suggested to be required in a similar manner during the meiotic cell cycle (Beach

et al., 1985). In this study we have shown that *pat1-114* fission yeast strains containing mutations in the *cdc10*$^{+}$ gene are, in the case of *cdc10-129* and *cdc10-V50* fission yeast strains, unable to induce expression of MCB-regulated genes. As both these strains underwent a cell cycle arrest early in G1 phase it could be argued that the true effect of these mutations on MCB gene expression would not be seen, as transcript from MCB-regulated genes only begins to increase in abundance about 30–40 min after the induction of meiosis by temperature shift. However, as *cdc22*$^{+}$ and rec8^{+} transcript was almost completely absent in these strains this would suggest it is directly attributable to the *cdc10*$^{-}$ mutation, as at 0–30 min some *cdc22*$^{+}$ transcript is normally seen. The absence of both *res1*$^{+}$ and *rep2*$^{+}$ transcript and the presence of *rep1*$^{+}$ transcript in both of these strains indicate that they had entered the meiotic cell cycle.

Interestingly, during the mitotic cell cycle, fission yeast cells carrying the *cdc10-C4* mutation show constitutively high levels of MCB-regulated gene expression in which the periodicity of expression is lost. In contrast, during the meiotic cell cycle, both *rec8*$^{+}$ and *rec11*$^{+}$ transcripts were undetectable and *cdc22*$^{+}$ transcript levels were constitutively low. This result requires further investigation however, as, in this strain, constitutive, high levels of *res1*$^{+}$ and *rep2*$^{+}$ transcript were detected, and the expression of these genes is normally specific to the mitotic cell cycle. This might indicate that the *cdc10-C4 pat1-114* strain was unable to enter the meiotic cell cycle after the 15 min temperature shift that was used in this experiment. Three observations argue against this possibility, however. First, asci were detected after following this method of meiotic induction in a *cdc10-C4 pat1-114* strain (Dr. S. White personal communication); second, expression of *rep1*$^{+}$, a meiotic-specific gene, was induced; and third, MCB-gene expression differed from that seen in a *cdc10-C4* strain during mitosis. However, a more detailed analysis of this strain would be useful to clarify these results. Nevertheless, these observations support the view that the mechanism of regulation of MCB-regulated gene expression during the meiotic cell cycle differs from that used during pre-mitotic G1 and S phase and also indicate that the C terminal portion of Cdc10p that is missing in Cdc10-C4p has an important role in this mechanism.

Interestingly, overexpressing *cdc10*$^{+}$ during a synchronous meiosis also causes the MCB-regulated genes to be overexpressed. This is different to results observed during mitotic experiments in which overexpression of *cdc10*$^{+}$ had no effect on MCB-regulated-gene transcript levels (Baum *et al.*, 1997; White *et al.*, 2001). These results suggest that Cdc10p may be a limiting factor for expression during meiosis but not during mitosis.

7.6.2 res1^{+} *manipulation*

Although *res1*$^{+}$ is normally only expressed during mitosis, overexpressing *res1*$^{+}$ induces a significant increase in the abundance of MCB-regulated gene transcripts during a synchronous meiosis. Res1p is known to induce high levels of MCB-regulated gene expression when it is

overexpressed during the mitotic cell cycle so it is not surprising that it has a similar effect during a synchronous meiosis. This observation provides further evidence that the system used to induce G1–S-specific gene expression during the meiotic cell cycle is closely related to the mitotic MCB–MBF system.

7.6.3 res2$^+$ *manipulation*

Res2p is known to have a significant role in the progression of the meiotic cell cycle as it is required for pre-meiotic S-phase and is known to control transcription of the *cdc22*$^+$ and *cdc18*$^+$ MCB-regulated genes. In this study we show that Res2p is also required for the normal regulation of transcription of the meiotic specific *rec*$^+$ genes. It is interesting to note, however, that while the *cdc22*$^+$ transcript was undetectable in a *res2*Δ strain, a small amount of *rec8*$^+$ and *rec11*$^+$ transcript was still detected. As the *res2*Δ strain becomes arrested in G1 it is possible that the amount of *rec8*$^+$ and *rec11*$^+$ transcript that is detected was a result of unperturbed transcription of these genes and that the depleted levels of transcript observed are due to the stage at which the cells have been arrested in G1. That other known MBF components — including Rep2p, which is known to mediate its effects through Res2p — affect *rec*$^+$ gene expression during a synchronous meiosis indicates that the effects seen on *rec8*$^+$/*rec11*$^+$ transcription are directly due to the absence of Res2p.

7.6.4 rep2$^+$ *manipulation*

Even more surprising, however, was the observation that manipulating the *rep2*$^+$ gene had a significant effect on MCB-regulated gene expression during the meiotic cell cycle. *rep2*$^+$ is only expressed during mitosis and is thought to be an activator of MCB-regulated gene transcription through its actions on Res2p (Nakeshima *et al.*, 1995). In *rep2*Δ strains however, a moderate activating effect on MCB-regulated transcription was seen during meiosis; the peak of transcript abundance occurred slightly earlier than normal and the levels were moderately increased over the 'wild type' control samples. Furthermore, when Rep2p was overexpressed during meiosis, while the *cdc22*$^+$ transcript profile appeared normal, the *rec8*$^+$ and *rec11*$^+$ transcripts were severely depleted. Thus, a clear distinction was seen between the effects on *cdc22*$^+$ transcript abundance and *rec8*$^+$/*rec11*$^+$ transcript abundance.

Rep2p is required for the activation of MCB-regulated gene transcription during mitosis; it is known that the Res2p–Cdc10p complex can bind MCB motifs but is not able to activate transcription in the absence of Rep2p (Nakeshima *et al.*, 1995). Secondly, certain genes (such as *rec8*$^+$ and *rec11*$^+$), which appear to be under MCB-motif regulation, are expressed during the meiotic cell cycle but are not expressed during the mitotic cell cycle. This indicates that there must be a mechanism for preventing the transcription of these meiotic genes during the mitotic cell cycle. We suggest that Rep2p, therefore, not only activates MCB-regulated gene

transcription during mitosis but is also involved (via its interaction with the 'meiotic regulator' Res2p) in the mechanism that causes the MBF complex to distinguish between 'meiotic specific' and 'mitotic and meiotic' MCB motifs in its activation of transcription. Therefore, we propose that Rep2p represses the transcription of meiotic-specific genes during mitosis. That deleting *rep2*$^{+}$ has a moderate activating effect on meiotic MCB-regulated gene transcription supports this view — it is likely that if Rep2p acts as a repressor of meiotic-specific gene transcription during mitosis, under normal circumstances, remaining Rep2p might have a slightly repressive effect on transcription during the meiotic cell cycle.

7.6.5 rep1^{+} *manipulation*

Rep1p has been shown to be specific to meiosis and to act as a global regulator of the transcription of the meiotic specific *rec*$^{+}$ genes as well as affecting the expression of *res2*$^{+}$. These observations prompted the suggestion that Rep1p might act through an association with a meiotic Cdc10p–Res2p complex (Ding & Smith, 1998). In this study we have shown that manipulating the expression of *rep1*$^{+}$ affects the expression of both *cdc22*$^{+}$ and the *rec*$^{+}$ genes during a synchronous meiosis. Deleting *rep1*$^{+}$ abolishes transcription of both *cdc22*$^{+}$ and *rec8*$^{+}$/*rec11*$^{+}$, which is similar to the effects seen in a *res2*Δ strain. Furthermore, in agreement with previous results (Ding & Smith, 1998), overexpression of *rep1*$^{+}$ results in induction of *rec8*$^{+}$/*rec11*$^{+}$ transcription.

7.6.6 Expression of MBF component genes

Finally, the effect of manipulating the expression of individual MBF components on the expression of the other MBF components was investigated during a synchronous meiosis. Except in the case of the *cdc10-C4* strain which requires further investigation, the expression of *cdc10*$^{+}$, *res1*$^{+}$, *rep1*$^{+}$ and *rep2*$^{+}$ were all largely unaffected by the manipulation of the expression of any other MBF components. Interestingly, however, *res2*$^{+}$ transcript abundance was affected similarly to *rec8*$^{+}$/*rec11*$^{+}$ transcript abundance in all the fission yeast strains tested. The *res2*$^{+}$ promoter contains a close match to a single MCB motif so this result is not entirely surprising. However, this regulation of *res2*$^{+}$ expression by the transcription factor of which Res2p is an integral part, implies that a feedback loop mechanism might be operating to control periodic MCB-regulated gene expression during meiosis.

7.7 Summary

- In this thesis we have shown that, during a synchronous meiosis, *rec*$^{+}$ genes are expressed with a similar periodicity to the *cdc22*$^{+}$ group of genes, which are known to be under MBF control during the mitotic cell cycle.
- Sequence analysis of the promoter regions of several genes known to be expressed with G1–S specificity during the meiotic cell cycle was undertaken. This revealed that distinct types of MCB motifs are present in the promoters of 'mitotic and meiotic' and 'meiotic specific' genes, supporting the hypothesis that these UAS motifs might determine the life-cycle specificity of the genes under their control.
- UAS reporter plasmids were used to demonstrate that MCB motifs are physiologically relevant during the meiotic cell cycle. Furthermore, these results have shown that the *rec15*$^{+}$ MCB motif confers meiotic-specific gene expression on a reporter gene, also indicating that the MCB motif Type is important in determining the life-cycle-specific expression of MCB-regulated genes.
- EMSA experiments revealed that MCB motifs from meiotic-specific genes can interact with the mitotic MBF complex, and, a meiotic MCB-binding activity was identified which is of similar mobility to the mitotic MBF complex. Furthermore, the meiotic MCB-binding activity shows a different pattern of binding with the 'mitotic and meiotic' *cdc22*$^{+}$ MCB motif and the 'meiotic specific' *rec*$^{+}$ gene MCB motifs during the course of a synchronous meiosis. These observations support the hypothesis that an MBF-related transcription factor is responsible for the control of MCB-regulated gene expression during the meiotic cell cycle and indicate that 'meiotic and mitotic' and 'meiotic specific' genes interact differently with this complex.
- The MBF components Cdc10p and Res2p have been shown to have roles in the progression of the meiotic cell cycle. We have shown that Res2p is important for the expression of *cdc22*$^{+}$ during a synchronous meiosis and also that it is required for *rec*$^{+}$ gene expression. Cdc10p was also shown to be required for MCB-regulated gene expression during the meiotic cell cycle.
- Similarly, Rep1p had previously been shown to be required for the progression of the meiotic cell cycle and to be important for *rec*$^{+}$ gene expression (Sugiyama *et al.*, 1994; Ding & Smith, 1998). From this it was inferred that Rep1p might be involved in MCB-regulated gene expression as a possible component of the MBF complex (Ding & Smith, 1998). In this study we show that manipulation of *rep1*$^{+}$ expression affects *cdc22*$^{+}$ and *rec8*$^{+}$/*rec11*$^{+}$ transcript levels as well as *res2*$^{+}$ transcript levels during a synchronous meiosis.

7.8 A model for the control of MCB-regulated gene transcription

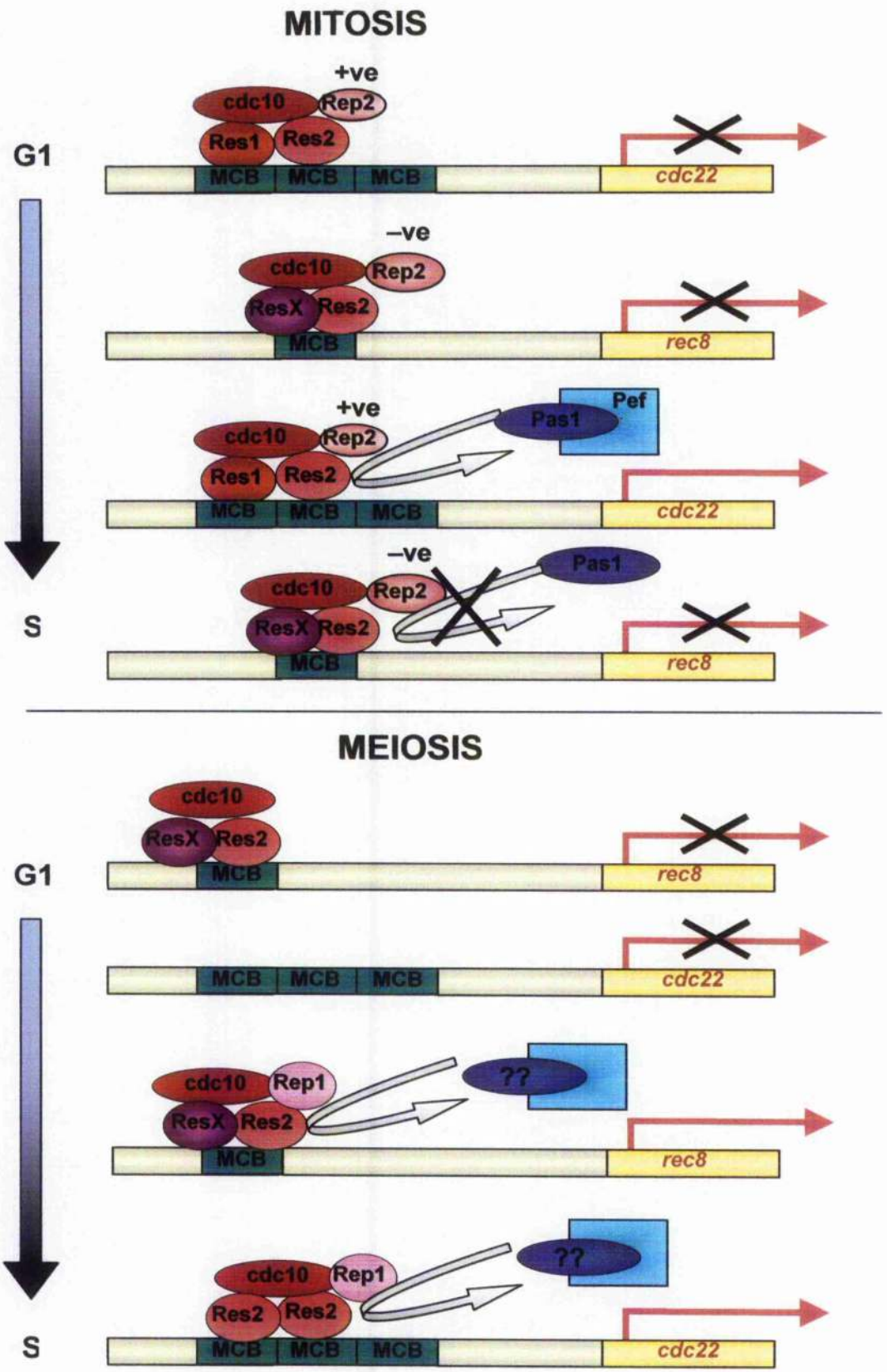
It has previously been proposed that a Res1p–Res2p–Cdc10p complex is required for G1–S periodic transcription of MCB-regulated genes during the mitotic cell cycle (Whitehall *et al.*, 1999). Also, the Rep2p–Cdc10p complex has been shown to require the Rep2p activator in order to instigate MCB-regulated gene expression (Tahara *et al.*, 1998). Finally, the Pas1 cyclin has been shown to activate the Res2p–Cdc10p complex independently of Rep2p during the mitotic cell cycle and to also inhibit mating-pheromone signalling.

In this thesis it has been shown that G1–S transcribed genes are under the control of MCB motifs and that 'mitotic and meiotic' MCB-regulated genes have a sequence pattern that is distinct from 'meiotic-specific' MCB-regulated genes. During meiosis, deletion of Res2p abolishes 'mitotic and meiotic' transcription but some 'meiotic-specific' transcript is still detected. Also, inappropriate expression of Rep2p during a synchronous meiosis seems to downregulate the expression of MCB-regulated *rec*$^{+}$ genes but not MCB-regulated *cdc22*$^{+}$ and might thus be the component of MBF that promotes the distinction between 'mitotic and meiotic' and 'meiotic-specific' MCB motifs. Furthermore, during a synchronous meiosis the transcript levels of *res2*$^{+}$ seem to be under the control of the MBF complex in a similar manner to 'meiotic-specific' MCB-regulated genes. We therefore propose the following model for MCB-regulated gene transcription during the two different life cycles.

Figure 7.1: A model for the control of MCB-regulated gene transcription

1) In mitosis, 'mitotic and meiotic' MCB motifs are bound by Cdc10p–Res1p–Res2p complexes. The Res2p subunit of this complex acts to downregulate transcription in early G1, but MBF is rendered 'activation competent' by Rep2p interaction with the inhibitory Res2p subunit. The periodicity of activation is conferred by subsequent phosphorylation of Res2p by the Pas1–Pef1 cyclin–CDK complex. So, the abundance of the complex is less rate-limiting for transcription than is the activation of the complex by Rep2p/Pas1p.
2) In mitosis, 'meiotic-specific' MCB motifs are bound by Res2p–Cdc10p complexes. Rep2p does not render these complexes transcription competent — possibly due to an interaction with a further as-yet-unknown component of the MBF complex, which binds meiotic-specific MCB elements along with Res2p. This component will be referred to as ResXp.
3) At the beginning of the meiotic cell cycle, 'mitotic and meiotic' MCB motifs become dissociated from the Res1p–Res2p–Cdc10p complex as Res1p is not produced during meiosis. They now rely on a complex containing only Res2p–Cdc10p to activate their transcription during the meiotic cell cycle.
4) Meiotic-specific MCB motifs remain bound by the Res2p–ResXp–Cdc10p complex, which is now made transcription competent by the meiosis-specific production of Rep1p. However transcription does not occur in the meiotic cell cycle until this complex is

activated — possibly by the action of an as yet unknown meiotic-specific cyclin–CDK complex.



7.8.1 A possible candidate for ResX?

When considering the possible mechanism of transcription activation that is used by the MBF transcription factor the available evidence seems to imply that there must be other protein–protein interactions or even MBF components that remain to be discovered. The model we have suggested postulates that there may be another component of MBF that is instrumental in distinguishing between 'mitotic and meiotic' and 'meiotic-specific' MCB motifs, and which, for argument's sake, we have named ResXp. In an attempt to identify any possible candidates for ResXp, the Sanger Centre fission yeast gene database was examined. Interestingly, three of the known components of the fission yeast MBF complex — Cdc10p, Res1p and Res2p — contain a protein domain called APSES, which is a DNA-binding domain that is characteristic of a number of transcription factors in various yeast species (Lakshminarayan *et al.*, 2002). In fact, the ScMbp1p and ScSwi4p budding yeast proteins, which are components of the MBF-related transcription factor from budding yeast (SBF), also contain this domain. There is only one other protein that has been identified by the fission yeast database as containing this APSES domain — SPBC19C7.10. So, it is tempting to speculate that this related protein would be a possible candidate for ResX. Meiotic expression data for SPBC19C7.10 shows that its expression is highly induced on entry into meiosis and it has a second lesser peak of expression during S phase of meiosis, so it is expressed at the correct cell-cycle time to interact with MBF. Blast searches of the fission yeast proteins database using the Res2p fission yeast protein revealed ????? for SPBC19C7.10, which is presumably due to their shared domain but is not indicative of this protein being a true 'Res homologue'. Nevertheless, as it is one of only four proteins in the whole fission yeast genome that shares this domain it would be interesting to study this protein further as the possibility that it may interact with the other APSES domain proteins Res1p, Res2p or Cdc10p seems quite strong. Creating an SPBC19C7.10-deletion strain would allow

Appendix I: Recipes

YE (H. Gutz, H. Heslot, U. Leupold and N. Loprieno (1974). *Handbook of Genetics* (R. C. King ed.), Vol. 1, pg 395. Plenum, New York.

	1l
Glucose	30 g
Yeast Extract	5 g
Adenine	225 mg/l
Uracil	225 mg/ll

For solid media, use 8 g of Difco Bacto agar/ 400 ml.

Edinburgh minimal media
Mitchison (1970) *Methods in cell physiology*. D. M. Prescott ed. Vol 4, pg 131. Academic Press New York; P. Nurse(1975). *Nature* **256**, 547.

		1l
Glucose		20 g
Ammonium Chloride		5 g
Magnesium Chloride		1 g
Sodium sulphate		0.1 g
Calcium Chloride		15 mg
Potassium Hydrogen Phthalate	3 g	
Di-sodium Hydrogen Orthophosphate	1.8 g	
VITAMINS		1 ml
TRACE		100 µl

For nitrogen-free EMM omit Ammonium Chloride.
For solid media, use 8 g of Difco Bacto agar/ 400 ml.

Vitamins (x1000)

Inositol	10 g/l
Nicoti nic acid	10 g/l
Calcium pantothenate	1 g/l
Biotin	10 mg/l

Trace	**200ml**
Boric Acid	1 g
Manganese Sulphate	1.04 g
Zinc Sulphate	0.8 g
Ferric Chloride	0.4 g
Molybdic Acid	0.288 g
Copper Sulphate	80 mg
Citric Acid	2 g
Potassium Iodide	20 mg

Bacterial culture media

Luria-Bertini culture media	20 g/l

For solid media, use 6 g of Micro agar/ 400ml

Protoplast fusion solutions

SP1

1.2 M sorbitol	109.32 g
50 mM sodium citrate	7.4 g
50 mM sodium phosphate	3.5 g
40 mM EDTA	7.4 g
dH_2O	to 500 ml

pH to 5.6 then autoclave.

SP2

1.2 M sorbitol	109.32 g
50 mM sodium citrate	7.4 g
50 mM sodium phosphate	3.5 g
dH_2O	to 500 **ml**

pH to 5.6 then autoclave.

SP3

1.2 M sorbitol	109.32 g
10 mM Tris Cl	5 ml of 1 M stock
dH_2O	to 500 ml

pH to 7.2 then autoclave.

SP4

1.2 M sorbitol	109.32 g
10 mM Tris Cl	5 ml of 1M stock
10 mM $CaCl_2$	0.74 g
dH_2O	to 500 ml

pH to 7.6 then autoclave.

SP5

20% PEG*	100 g
10 mM Tris Cl	5 ml of 1 M stock
10 mM $CaCl_2$	0.74 g
dH_2O	to 500 **ml**

pH to 7.6 then filter sterilise.

* polyethylene glycol 3350 (Sigma, P-3640)

Appendix II: Fission yeast strains used in this study.

Strain	Genotype	Purpose
GG 190	*h⁺ pat1-114, his1-102, leu1-32, ura4-294*	Haploid background strain for transformation with yeast plasmids. *pat1-114* mutation allows temperature-sensitive control of entry into meiosis
GG 164	*h⁻ leu1-32, ade6-M216*	Crossed with GG190 to produce GG335 and GG338
GG 335	*h⁻ pat1-114 leu1-32 ura4-294*	Produced from GG190: GG164 cross in order to produce GG(A65)
GG 338	*h⁻ pat1-114, leu1-32, ura4-294, ade6-M216*	h⁻ haploid strain for production of *pat1* diploid
GG 337	*h⁺ pat1-114, leu1-32, ura4-294, ade6-M210*	h⁺ haploid strain for production of *pat1* diploid
GG 375	*h⁻ /h⁻ , pat1-114/ pat1-114,leu1-32/ leu1-32, ura4-294ura4-294, ade6-M210,ade6-M216*	Diploid background strain for transformation with yeast plasmids
GG 165	*h⁺ leu1-32, ade6 M210*	Crossed with GG335 To produce GG 337

Appendix IIIa: Basic plasmids used in this study

Fission yeast collection Number	Plasmid collection number	Plasmid	Purpose
-	GB6	*p-Bluescript* KS+	Blunt-ended cloning vector for capture of PCR fragments prior to sub-cloning
	GB10	pSPΔ178	High copy *S. pombe* UAS activity analysis vector. Contains *Xho1* cloning site upstream of a LacZ reporter gene under the control of a *CYC1* minimal promoter
-	GB91	YIPMelβ2	*S. cerevisiae* integrating UAS activity analysis vector. Contains multiple cloning site upstream of a LacZ reporter gene under the control of a Mel1 minimal promoter. (Kind gift of Karsten Melcher)
GG 678	GB169	SPIPMelβ2	*S. pombe* derivative of YIPMelβ2constructed for this study

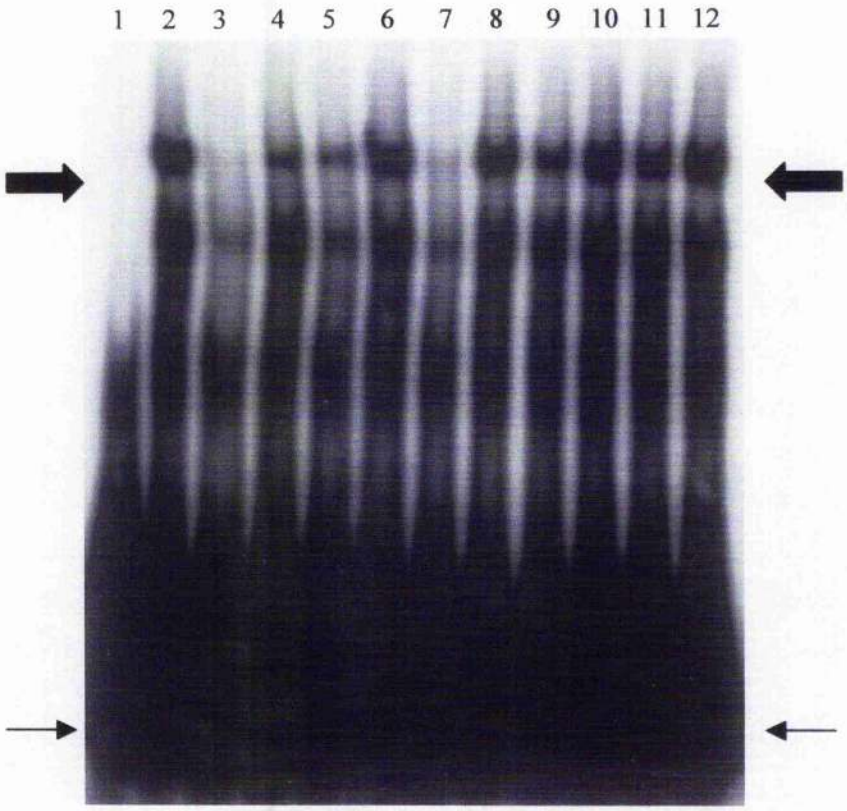
Appendix IIIb: Plasmid inserts

The high copy number plasmid pSPΔ178 and the integrating plasmid SPIPMelβ2 were used as two different approaches for the analysis of the UAS activity of MCB elements. The promoter fragments containing MCB elements for each gene studied were therefore inserted into the *Xho1* cloning site of both vectors.
Promoter fragments were obtained by PCR with appropriate sets of primers followed by blunt-ended cloning into pBscrKS+ and sub-cloning into the UAS analysis vectors. Alternatively, if the MCB cluster was short enough, complementary 5'phosphate synthetic DNA sequences were ordered (MWG) which contained the MCB sequences of the promoter in question flanked by *Xho1* sites. These synthetic DNA sequences were then directly cloned into both UAS analysis vectors (these sequences are represented by a *in the table below).

Fission yeast collection number	Plasmid collection number	Plasmid	Primers used to produce insert. F - forward primer, R - reverse primer, * directly cloned
GG 206 GG 679	GB 41 GB 303	pSPΔ178.3M SPIPMelβ2.3M	TCGATACGCGTTAGATCTACGCGTAGATCTACGCGT A * (palindromic)
-	Not used	pSPΔ178.rec7	F - * AAATGAGGTTCAGTCTAACGCGTCTTCTCATATTCA AACA
	GB 304	SPIPMelβ2.rec7	R - * TGTTTGAATATGAGAAGACGCGTTAGACTGAACCT CATTT
-	Not used	pSPΔ178.rec8	F - * TTTGACGCGTTTAATAAGCTATCTGGTGAACTAACG CGTTCCT
	GB 305	SPIPMelβ2.rec8	R - * AGGAACGCGTTAGTTCACCAGATAGCTTATTAAAC GCGTCAAA
-	Not entered-reverse orientation	pSPΔ178.rec11 SPIPMelβ2.rec11	F – TCGAGACGCGTTCTTATTCATAAATTAGTTACTTTA *
			R – TCGAGACGAGTCGCGTCAGTCAGTCATTAATTATTT *
GG675 -	GB 86 Not used	pSPΔ178.rec12 SPIPMelβ2.rec12	F -GCTTATTTGAATTAAGCTATTG R -GTTGTTAGATCATGGATTGAGC
GG 672 GG 681	GB 87 GB 306	pSPΔ178.rec15 SPIPMelβ2.rec15	F –TAGTATGAATGGGCATCCAA (GO98) R – AATTAAGCGTTACGGGAA (GO99)
GG 676	GB 73	pSPΔ178.cdc22.MCB1	F-GCGCCTCGAGGTAGTTCAATCTCATAGA (GO 36) R- GCGCCTCGAGCTCTGTTTACGACTGAATG (GO37)
GG 677 GG 680	GB 57 GB 307	pSPΔ178.cdc22.MCB2 SPIPMelβ2.cdc22.MCB2	F- GCGCCTCGAGCATTGATCAACATGACTTAAAG (GO 44) R- GCGCCTCGAGGGTGGTAAATACCGGGAA (GO 42)
- -	GB 308	PBSKS^{+}ura4	F- AATTGGGCCCAGCTTAGCTACAAATCC (GO559) R- TACACCATGGAGCTTGTGATATTGACG (GO560)

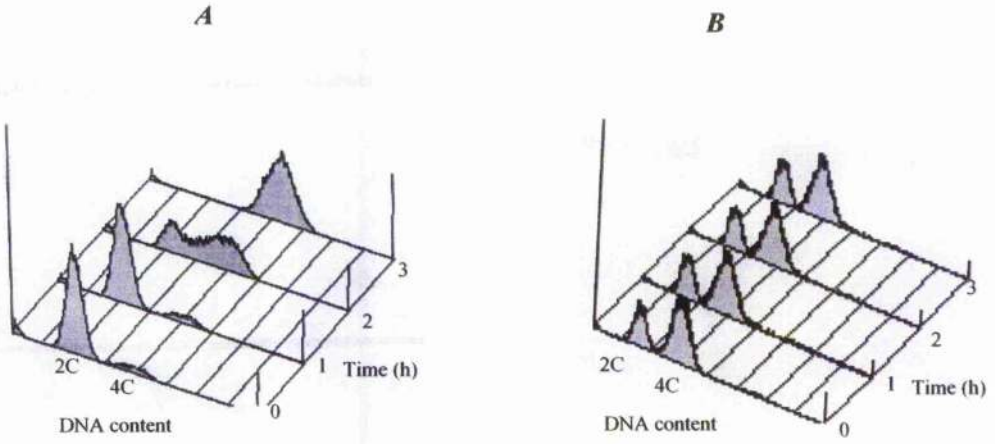
Appendix IV: DNA Probes used

Probe	Primers used	GO number
rec6^{+}	F- TCATAAAGAGCTCTCTTCTG	GO 14
	R- TACTCAGTCTGCAGCCGAAA	GO 15
rec7^{+}	F- GAATCTGTAGTATACGAATC	GO 1
	R- TAACGTCTCGATTGCTTCAA	GO 2
rec8^{+}	F- TGACATGAGTTCTTTCACTC	GO 17
	R- CGTGGTTTGATTGTGATACT	GO 18
rec11^{+}	F- GTCAgGGAAAAGCCCGAAAT	GO 21
	R- AACGAATACGCACCGTTTTA	GO 22
rec12^{+}	F- TGGAATTGGAGTCCGGAGTTC	GO 110
	R- CGGGACAGTAATTACTGCTAC	GO 111
rec15^{+}	F- AGCTCACTGAAGAAGGAATC	GO 108
	R- CGTCATCTGCTAAGAAATCA	GO 109
cdc22^{+}	F- ACACGCGGTCCAACCCGA	GO 30
	R- AGCGGAACTTTGATGTTC	GO 31
cdc10^{+}	F- GGACACGCCGCTCTGCACTGGGCAGCA	GO 112
	R- ATAGACTGTTTTGCAGCCATTTCATCC	GO 113
res1^{+}	F- AGGGATAGCAGAGGTCTTTAA	GO 239
	R- GTTCTCTACGAAGTACCGTAA	GO 240
res2^{+}	F- TTTGCCTGGTGCAGAGGAGCA	GO 241
	R- ACAGAACGAACTCTGCTAAGC	GO 242
rep1^{+}	F- CGTACATATGGATTCTGATCGTTGTTTAACAGACGA	GO 168
	R- GGCCAGGATCCTTACCAATCACTGCAAAAACTCGAACC	GO 169
rep2^{+}	F- CGTACATATGCATTTTGCAGACATTCCTCTTAGCAAGCCA	GO102
	R- GGCCAGGATCCTTAAAAGAGCCAGTCATCTATCACGT	GO 103



Key
1 = free *cdc22*$^+$ MCB1 probe
Lanes 2-12 contain radio-labelled *cdc22*$^+$MCB1probe + protein extracted from asynchronous, mitotic, wild type diploid cells plus:
2 = no competitor
3 = excess unlabelled *cdc22*$^+$ MCB1 probe
4 = 1/10 dilution of *cdc22*$^+$ MCB1 probe
5 = excess unlabelled *rec7*$^+$ probe
6 = 1/10 dilution of *rec7*$^+$ probe
7 = excess unlabelled *rec8*$^+$ probe
8 = 1/10 dilution of *rec8*$^+$ probe
9 = excess unlabelled *rec11*$^+$ probe
10 = 1/10 dilution of *rec11*$^+$ probe
11 = excess unlabelled *rec15*$^+$ probe
12 = 1/10 dilution of *rec15*$^+$ probe

***Appendix V: Meiotic specific MCB motifs compete with the* cdc22$^+$-*MBF bandshift*.**
Asynchronous mitotic protein samples were prepared from wild type haploid cells and an EMSA carried out using radio-labelled *cdc22*$^+$ MCB1 probe and various unlabelled competitor DNA probes taken from the promoters of meiotic specific genes (see key). The upper arrows show the position of the MBF-DNA complex. The lower arrows show the position of the free probe.



Appendix VI: Flow cytometry analysis of cultures used for protein extraction in Chapter 5

Two identical asynchronous, diploid, *pat1-114*, mitotic cultures (strain GG 375) were grown at 25°C, to a cell density of between 5 and 8 x 10^6 cells per ml. ***A*** shows the results of flow cytometry analysis taken from culture 1: the cells were induced to undergo a synchronous meiosis from asynchronously dividing mitotic cells by shifting the temperature of growth to 36°C. Cell samples were taken for protein extraction and flow cytometry analysis every hour for three hours. ***B*** shows the results of flow cytometry analysis taken from culture 2: the cells were treated in the same way as in previous meiotic induction experiments (Section *2.8.6*) in that the culture was first transferred into growth medium lacking nitrogen and grown overnight at 25°C. This arrested the cells in G1 phase, effectively synchronizing the culture, before nitrogen and amino acids were re-added and the growth temperature was increased to 36°C to induce meiosis — before cell samples were taken as before.

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