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pol5^{+}, a potential link between cell cycle and cell growth in fission yeast

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Dedicated

To

Abu Gee

Acknowledgements

In the name of Allah most gracious and most merciful.

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Abstract

SpCdc10p is the major component of the cell cycle regulated transcription factor complex DSC1 (DNA synthesis control 1) in fission yeast. DSC1 regulates the periodic transcription of a set of genes at the G_1-S transition, which is required to pass the mitotic G_1 checkpoint termed 'START' and also for S phase progression. The promoters of DSC1 regulated genes contain a common *cis*-acting element called MCB (*Mlu*1 cell cycle box) that binds to DSC1 to confer periodic gene expression. Some of the genes required for S phase include *cdc22*$^+$ (large subunit of ribonuleotide reductase), *cdc18*$^+$ and *cdt1*$^+$ (DNA replication licensing factors) and *cig2*$^+$ (the major S phase cyclin). *cig2*$^+$ interacts cyclically with the cyclin-dependant kinase, SpCdc2p, to pass START and enter mitotic division. Both budding yeast and mammalian cells contain homologous gene expression systems and are controlled by DSC1-like activities called SBF/MBF and E2F in yeast and humans, respectively.

The aim of this study was to isolate novel proteins that interact with SpCdc10p, with particular focus on the 61 amino acid C-terminal region believed to be important for its regulation. Two-hybrid analysis and co-immunoprecipitation assays revealed a novel protein called SpPol5p. The two-hybrid experiment suggested a potentially strong interaction between SpPol5p and the C-terminal of SpCdc10p and the co-immunoprecipitation assays confirmed that the interaction was direct between these two proteins.

Recently, ScPolVp, the budding yeast homologue of SpPol5p was identified and shown to be involved in rRNA synthesis, with a less important role as a DNA polymerase. Relatively little is known about SpPol5p function and it was proposed that it had the same role as ScPolVp due to its amino acid sequence similarities. In this thesis *pol5*$^+$ biology was studied further. Northern blot analysis allowed the mRNA profile of *pol5*$^+$ to be assayed throughout the cell cycle of fission yeast. It was shown to be constitutive and in low abundance. Investigations into the mRNA levels of *pol5*$^+$ in the mutant *cdc10-C4* confirmed that *pol5*$^+$ was not under the transcriptional control of SpCdc10p. Disruption of *pol5*$^+$ revealed it to be an essential gene, as is budding yeast

POLV. Further to understanding the biology of *pol5*$^{+}$, the effect of over-expression of *pol5*$^{+}$ was examined, which was found to have no effect.

Finally, the role of SpPol5p in rRNA synthesis was investigated by performing a series of pulse-chase labelling experiments of rRNA. Localisation studies using GFP-tagged versions of SpPol5p were performed to visualise its distributiion within the cell, to see whether ScPolVp localised to the nucleolus, the site of ribosome assembly. Initial experiments suggested that SpPol5p may have a role in rRNA synthesis as *pol5*$^{+}$ over-expression caused a delay in onset of rRNA production. *pol5*$^{+}$ 'Shut-off' experiments also revealed an effect on synthesis of rRNA, although further investigations are required. GFP-tagged SpPol5p localised to the nucleus. These preliminary experiments provide a foundation from which future experimental work can be continued, with particular attention given to the role of *pol5*$^{+}$ in rRNA production in fission yeast.

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Abbreviations

ade	adenine
AD	activation domain
APC/C	anaphase promoting complex/cyclosome
ARS	autonomously replicating sequence
ATP	adenosine triphosphate
BD	binding domain
bp	base pair
Cdc, CDC	cell division cycle
CDK	cyclin-dependent kinase
ChIP	chromatin immuno-precipitation
cm	centimetre
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
Da	dalton
DDK	Dfp1-dependent kinase
dH_2O	distilled water
DMF	dimethyl formamide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleosidetriphosphate
DP	DRTF1 protein
DPE	downstream promoter element
DRTF1	differentiation-regulated transcription factor 1
DSC1	DNA synthesis control 1
DSE	distal sequence elements
DTT	dithiothreitol
E2F	E2A binding factor
ECL	enhanced chemiluminescence
EDTA	ethylene diamine tetraacetic acid
EMM	Edinburgh minimal media
g	gram
G	guanine

GB	Glasgow collection number for bacteria
GBY	Glasgow collection number for budding yeast
GFP	green fluorescent protein
GG	Glasgow collection number for fission yeast
GO	Glasgow collection number for oligonucleotides
GST	glutathione *S*-transferase
G_0	quiescence
G_1	gap 1
G_2	gap 2
h	hour
His	histidine
HRP	horseradish peroxidase
INR	initiator sequence
I	initiator element
IPTG	isopropyl β-thiogalactopyranoside
kb	kilobasepairs
kDa	kilodalton
kV	kilovolts
l	litre
LB	luria bertani
leu	leucine
M	molar
M (phase)	mitosis
mA	milliamp
mM	millimolar
Mb	megabasepairs
MBF	MCB-binding factor
MCB	*Mlu*I cell cycle box
MCM	mini-chromosome maintenance
MCS	multiple cloning site
mg	milligram
min	minute
ml	millilitre
mRNA	messenger RNA
M_r	relative molecular mass

M	molecular weight marker/ladder
nm	nanometre
nmt	no message in thiamine
OD	optical density
ORC	origin recognition complex
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethyleneglycol
pIC	pre-initiation complex
PMSF	phenylmethanesulfonylfluoride
pRB	retinoblastoma gene product
pre-RC	pre-replication complex
PSE	proximal sequence element
R	restriction point
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
rRNA	ribosomal RNA
S (phase)	synthesis (phase)
SBF	SCB-binding factor
SCB	Swi4-Swi6 cell cycle box
SD	synthetic drop-out media
SDS	sodium dodecyl sulphate
sec	second
snRNA	small nuclear RNA
T	thymine
TAF	TBP associated factor
TBE	Tris/Borate/EDTA buffer
TBP	TATA box binding protein
TCA	tri-chloro acetic acid
TE	Tris/EDTA buffer
TEMED	N, N, N', N'-tetramethylethylenediamine
Thia	thiamine
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
tRNA	transfer RNA

trp	tryptophan
ts	temperature sensitive
U	units
UAF	upstream activating factor
UAS	upstream activating sequence
UBF	upstream binding factor
UV	ultraviolet
µCi	microcuries
µg	microgram
µl	microlitre
µM	micromolar
V	volts
v/v	volume to volume
w/v	weight to volume
YE	yeast extract
°C	degrees Celsius
Δ	deletion

Chapter 1
Introduction

1.1 Introduction

All organisms are made of cells, which divide and multiply. Cells divide as a part of development or to replace injured or dead cells. Cell growth and division follows a specific pattern called the cell cycle (Figure 1A), which is strictly controlled so that the daughter cells produced are exact genetic copies of the mother. It is therefore important that the different phases of the cell cycle are precisely coordinated and that they follow in the correct order.

The overall process of the mitotic cell cycle follows a series of highly complex events, which are tightly controlled and regulated. The mitotic cell cycle comprises four distinct phases called G_1 (gap 1 phase), S (synthesis phase), G_2 (gap 2 phase) and M (mitosis) where G_1, S and G_2 represent interphase and M phase is further subdivided into prophase, metaphase, anaphase, and telophase. During interphase the cell is engaged in metabolic activity and is preparing for mitosis by faithfully duplicating all of its organelles and chromosomes and ensuring the cell is of proper size before cell division occurs. During each gap phase the cell checks the integrity of the genome allowing time for growth and repair. During G_1, environmental conditions influence a cell's decision to enter S phase. Once a cell has entered S phase it is irreversibly committed to complete that round of proliferation until the next G_1 phase. Prior to entry into S phase a cell can undergo different developmental pathways. G_2 phase allows a cell to grow to a certain size whilst also ensuring that the chromosomes have been replicated precisely, before the onset of mitosis. During prophase chromatin in the nucleus begins to condense and the nuclear membrane dissolves. This is followed by metaphase and anaphase, where spindle fibres align the chromosomes along the middle of the cell nucleus and the paired chromosomes separate to opposite sides of the cell, respectively. Finally in telophase chromatids arrive at opposite poles of cell, and new membranes form around the daughter nuclei. Ultimately, two identical daughter cells are formed by cytokinesis.

A vast amount of research has led to the definition of a unified theory of cell cycle control that holds true for all eukaryotes. Perhaps the most outstanding finding is that many of the basic components of the basic cell cycle machinery are both structurally and functionally conserved. In fact, many components of the cell cycle

regulatory machinery from animal cells can substitute for their lower eukaryotic counterparts.

The use of model organisms in biological research has proved to be an important way of elucidating gene function. A model organism is one that is extensively studied to understand particular biological phenomena, with the expectation that discoveries made in the model organism will provide insight into the workings of higher organisms, namely humans. This works because evolution re-uses fundamental biological principles and conserves metabolic and regulatory pathways. There are many model organisms. In eukaryotes, several yeasts, particularly *Saccharomyces cerevisiae* ("baker's" or "budding" yeast) and *Schizosaccharomyces pombe* (*pombe* is Swahili for beer) have been widely studied, largely because they are quick and easy to grow. The cell cycle of yeast is very similar to the cell cycle in humans, and regulated by homologous proteins. Leading the way in cell cycle research are budding, *Saccharomyces cerevisiae*, and fission, *Schizosaccharomyces pombe*, yeasts, where many of the key aspects of cell cycle control were first established (Hartwell 1991, Nurse *et al.* 1998).

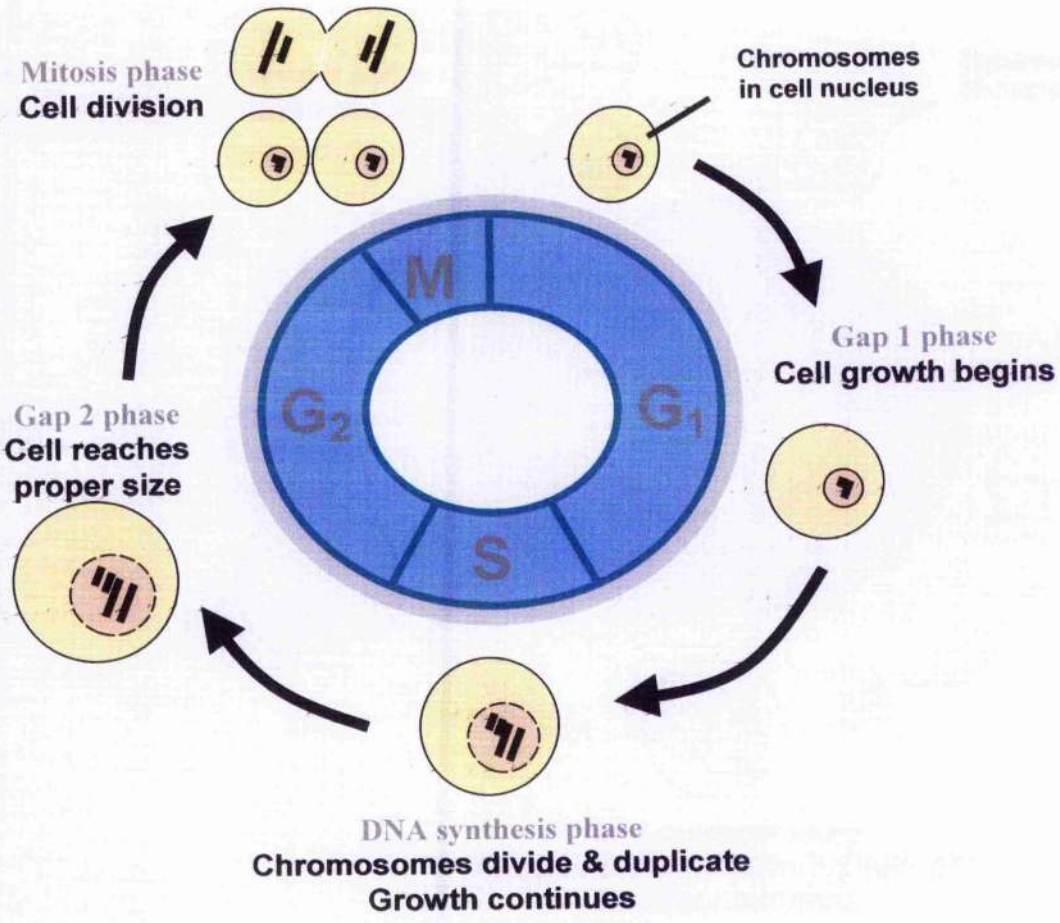


Figure 1A Schematic of the eukaryotic cell cycle. The eukaryotic cell cycle is the orderly passage of four distinct phases: G_1 (gap 1 phase); S phase (DNA synthesis phase); G_2 (gap 2 phase) and finally M phase (mitosis). The completion of the cell cycle is marked by cytokinesis and cell division before cells enter a new round of proliferation.

1.2 The life cycles and genetics of budding and fission yeasts

The two yeast species, budding and fission, which are only distant cousins, have been central to the study of the eukaryotic cell cycle. Although they are only unicellular, they comprise basic features that are common amongst higher eukaryotic systems, which have made them ideal model organisms for cell cycle research and now also for other cell biology investigations (Lew *et al.* 1997, Forsburg & Nurse 1991, Forsburg 1999). It should be noted that both these yeast species are highly divergent in evolution, separated more than 1,000 million years ago, therefore control mechanisms that are found to be conserved between these organisms can be applied to all eukaryotes (Sipiczki 2000, Heckman *et al.* 2001).

1.2.1 Budding yeast life cycle and genetics

The budding yeast is a eukaryotic, unicellular, ascomycete fungus, which has a haploid genome size of 12 Mb. This is divided among 16 chromosomes, varying in size. The budding yeast sequencing project (Goffeau *et al.* 1996) revealed approximately 6,000 genes with an average size of 1.5 kb, of which 5% carry introns. This resource has been invaluable to the genetic analysis of budding yeast providing a vital tool for the comparison of genes and proteins with other eukaryotes.

Cells in budding yeast grow by bud formation in either the haploid or diploid state. Initially, a small bud is formed which continues to grow during the cell cycle before finally pinching off to form two genetically identical daughter cells where, to begin with, one is smaller in size, than the other.

Budding yeast can follow three alternative developmental pathways at G_1, depending on environmental conditions (Figure 1B). In the presence of a plentiful supply of nutrients the cells can continue through the mitotic cell cycle and proliferate. However when the cells are starved they can leave the mitotic cycle at G_1 and enter stationary phase where they become dormant. Otherwise, haploid cells of opposite mating type, "a" and "α", may conjugate to form a diploid cell. Diploids can continue through the mitotic cycle, unless starved, which then prompts meiosis and the formation of four haploid spores.

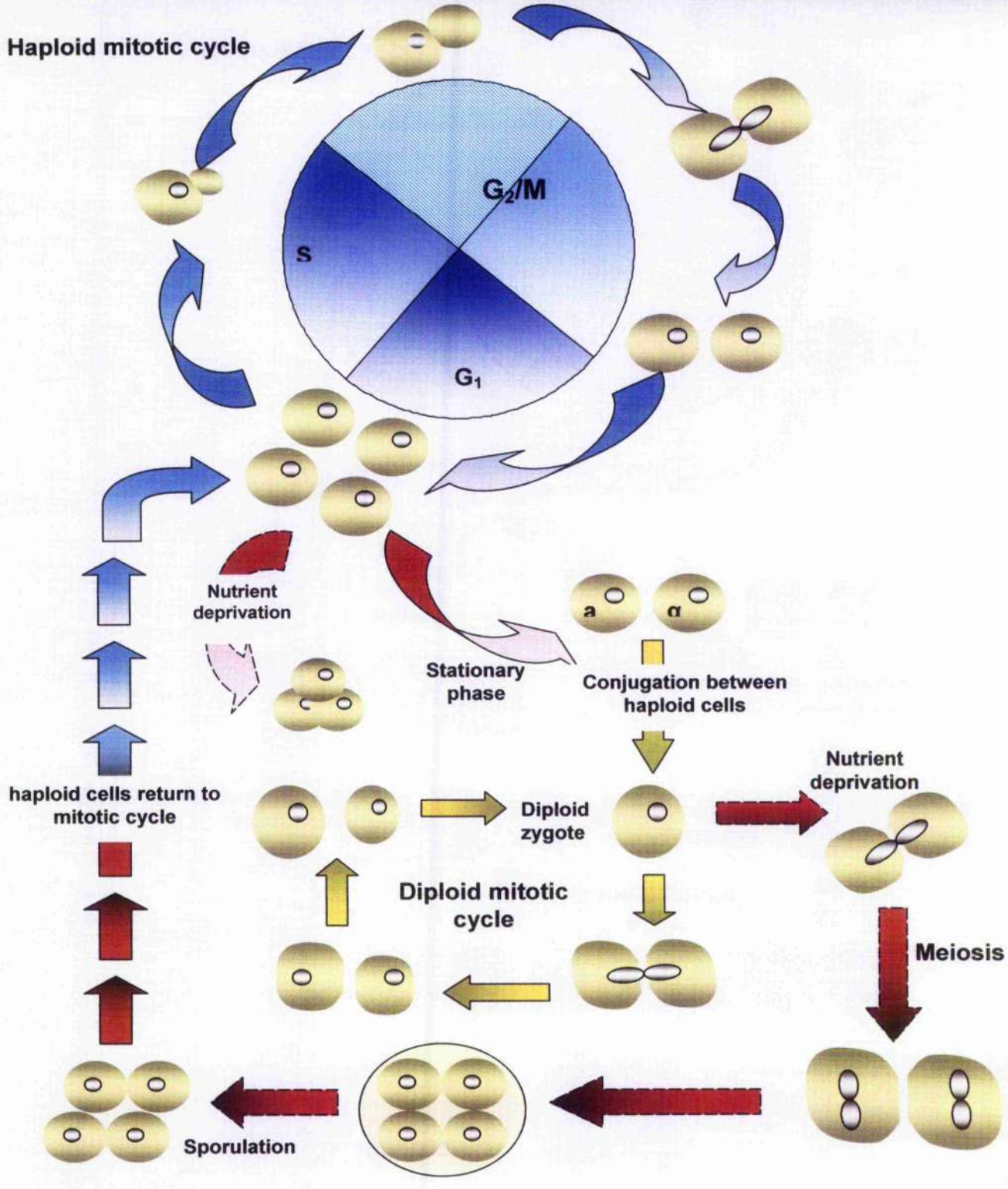


Figure 1B The life cycle of budding yeast, *Saccharomyces cerevisiae*. Budding yeast grows by a process of bud formation in either the haploid or the diploid state. When haploid cells are starved of nutrients they enter stationary phase, unless they are in the presence of cells of the opposite mating type (**a** and α). Cells of opposite mating types conjugate to form diploid cells. If replenished with fresh nutrients, the diploid cells enter into the diploid mitotic cycle. If the diploid cells are also starved, the programme of meiosis and sporulation is entered, resulting in the formation of four haploid spores, which can then enter the mitotic cell cycle in favourable conditions.

1.2.2 Fission yeast life cycle and genetics

The fission yeast is a eukaryotic, unicellular, ascomycete fungus. It is rod shaped at 7–14 μm in length and 3–4 μm in diameter. It grows by apical extension, and divides by septation and medial fission (Forsburg & Nurse 1991, MacNeill & Nurse 1997). Fission yeast normally exists in the haploid state and has a genome size similar to budding yeast, at around 14Mb. This is divided among only 3 chromosomes, 5.7, 4.7 and 3.5 Mb in size. The fission yeast-sequencing project, which has only recently been completed (Wood *et al.* 2002), revealed nearly 5,000 genes. This resource has been invaluable to the genetic analysis of fission yeast providing a vital tool for comparing with budding yeast and other eukaryotes.

Fission yeast can undergo different developmental pathways (Figure 1C). In the presence of nutrients, haploid fission yeast cells enter the mitotic cell cycle. Upon starvation of nutrients several alternative fates can occur. If cells of only single mating type are present, fission yeast will exit mitosis and accumulate in stationary phase. If both mating types, 'h^+' and 'h^-' are present, conjugation occurs, resulting in the formation of a short-lived 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 a fresh supply of nutrients before meiosis has been initiated, a diploid mitotic cycle will be entered (Forsburg & Nurse 1991, Hayles & Nurse 1992).

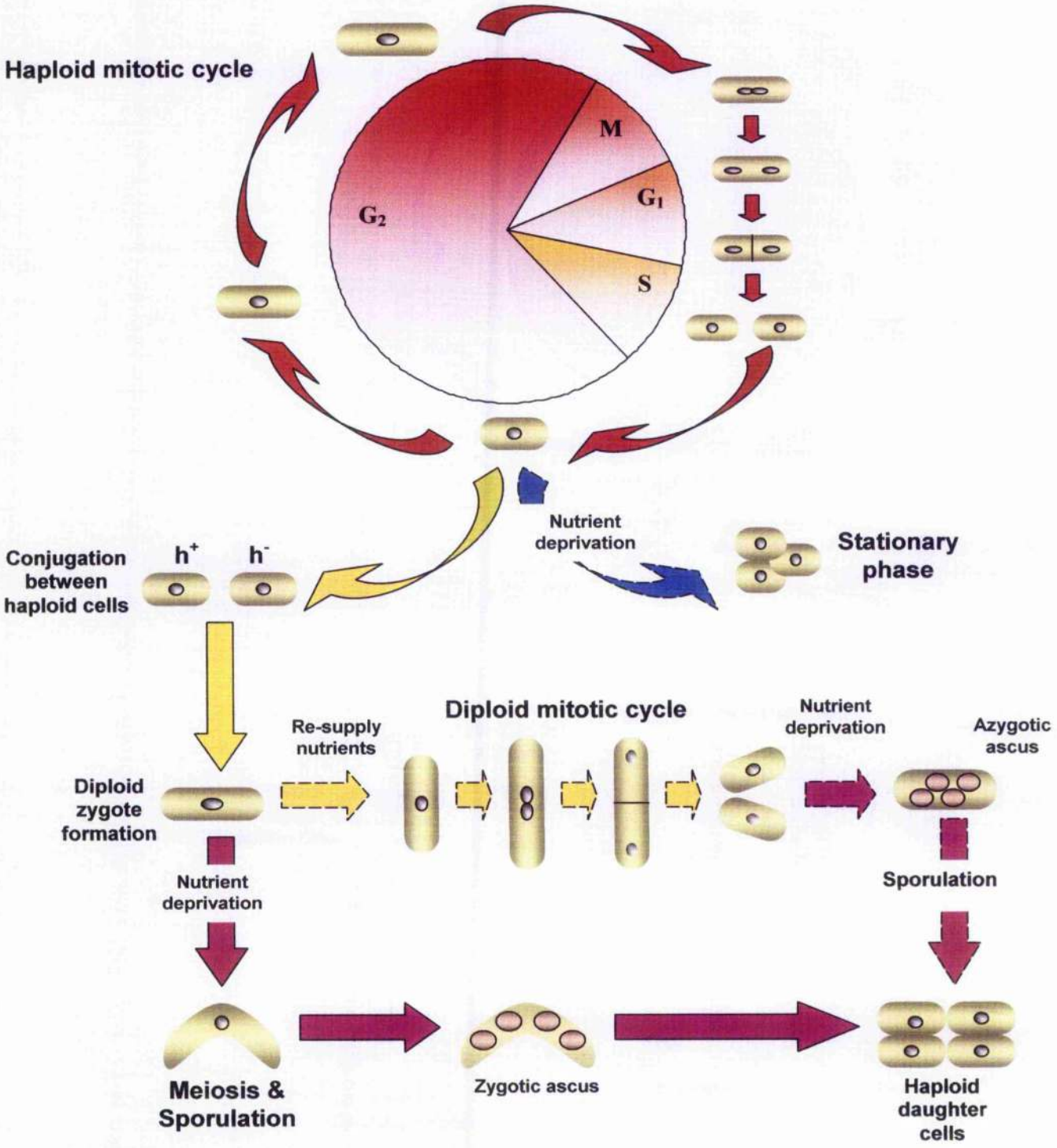


Figure 1C The life cycle of the fission yeast, *Schizosaccharomyces pombe.* Fission yeast cells grow stably as haploids and proliferate by mitotic division. In the absence of nutrients cells can exit the mitotic cycle and enter into stationary phase. Alternatively, in the presence of cells of opposite mating type (h^+ and h^-) conjugation occurs forming a diploid zygote. The diploid state is unstable in fission yeast and proceeds directly into the meiotic cycle, producing a zygotic ascus containing four haploid ascospores. The four haploid spores can again re-enter the mitotic cycle. The temporary diploid state can be maintained if nutrients are re-supplied immediately following conjugation. In this case, the diploid cell can undergo the diploid mitotic cycle until nutrients become limiting. Starved diploid cells proceed into the meiotic cycle producing an azygotic ascus.

1.3 The cell cycle, control points and mutants

The fission yeast has a cell cycle typical of most eukaryotes, with the four distinct phases, G_1, S, G_2 and M (Figure 1C). The budding yeast has regular G_1 and S phases however, mitotic spindle formation and spindle pole body duplication occur very early in the cycle, during S phase, to allow migration of the nucleus to the bud neck. Thus there does not appear to be a clearly defined G_2 phase in budding yeast. Unlike that of higher eukaryotic cells, the nuclear envelope of both yeasts does not break down and the mitotic spindle form inside the nucleus and are attached to spindle pole bodies at their peripheries.

There are two major control points that exist in both budding and fission yeast mitotic cell cycles. These control points allow the cells to monitor the integrity of the genome both before and after replication and also to ensure that the cells reach a critical size before cell division can occur. The first occurs in late G_1 and is termed "START". The second occurs in late G_2, before the onset of mitosis and cell division. As in higher eukaryotes, budding yeast primarily regulates its cell cycle from the G_1-S transition (Hartwell 1974). The control of the fission yeast cell cycle however is essentially at the G_2-M transition. This is because cells have already reached S phase before cytokinesis or cell division is completed. Therefore G_1 is relatively short-lived in fission yeast. This gives fission yeast, which is fundamentally a haploid organism, genetic advantage, as possession of two full copies of the genome may provide protection against DNA damage (Forsburg & Nurse 1991, Humphrey 2000).

In both budding and fission yeasts passage of START and S phase entry requires prior completion of mitosis in the previous cell cycle, DNA damage check and repair and growth to a minimal size. Once START is passed the cell is committed to the mitotic cell cycle, until it returns to G_1 (Forsburg and Nurse 1991). Passage of the G_2 control point and entry into mitosis is also dependent on the cell reaching a certain minimum size, as well as prior completion of S phase and repair of any DNA damage. Size control at G_1 is usually hidden only revealing itself in the absence of nutrients or in cell cycle mutants.

Foremost to the advances made in cell cycle research in both yeasts have been the isolation of cell division cycle mutants (Hartwell *et al.* 1974, Nurse *et al.* 1976). These mutants allow the identification of many of the functional aspects required for

cell cycle progression. Cell division cycle mutants are so-called because they become blocked for cell cycle progression at specific points or display altered regulation of the cell cycle. Mutations in genes required for cell cycle progress are responsible for this phenomenon. These mutants were originally identified by screening for cells that continued to grow but did not divide at non-permissive temperature. In budding yeast, *CDC* mutants are recognised by their uniform bud phenotype (Hartwell *et al.* 1974) and perhaps more strikingly in fission yeast, *cdc* mutants exhibit a highly elongated phenotype compared to the wild type rod-shaped cells (Nurse *et al.* 1976).

The equivalent of START in mammalian cells is termed "Restriction point" (R) and as in yeast this marks an irreversible commitment to complete the entered round of mitotic division (Pardee 1974, Blagosklonny & Pardee 2002). Mitogenic growth factors or negative regulatory growth factors can cause higher eukaryotes to leave mitotic growth and enter G_0 phase. This is dissimilar to stationary phase of yeast where departure from the cell cycle and cell accumulation before the G_1 control point, START, is triggered by nutrient deprivation (Bartlett & Nurse 1990). As a result, mammalian cells remain metabolically active, as they are not starved and are busy carrying out their functions in the organism. e.g., secretion, attacking pathogens (Lodish *et al.* 1995).

1.4 The mitotic cell cycle*

As described earlier in this chapter, the eukaryotic cell cycle comprises four distinct phases, where two vital checkpoints exist in late G_1 and in late G_2. The former of these is termed START/R in yeast and mammalian cells, respectively. The decision to enter a new round of proliferation is conducted at START/R. Progression through the cell division cycle is controlled by a complex network of processes involving two key mechanisms working in conjunction with each other. The first is the cyclic assembly, activation and disassembly of cyclin-CDK complexes that drive forward the different parts of the cell cycle. CDK (cyclin dependant kinases) and cyclins are two families of proteins where CDK molecules activate downstream processes by phosphorylating specific proteins and cyclins bind to the CDK molecules to control their ability to phosphorylate the specific target proteins. Cyclin-CDK complexes act by enabling entry into a key stage such as S phase or mitosis, but also inhibiting exit from that stage. Increase in cyclin-CDK activity facilitates complete passage of a key stage followed by removal of the activity by cyclin degradation, marking the successful completion of the events of that stage of the cell cycle (Koepp *et al.* 1999). The second mechanism which controls cell cycle progression is cell cycle regulated transcription and is discussed in more detail later in this chapter.

In mammals, a succession of kinases (HsCdk1p, HsCdk2p, HsCdk4p and HsCdk6p) are expressed along with a series of cyclins (A, B, D and E) as cells go from G_1 to S to G_2 and finally M. Cyclin D-HsCdk4p/HsCdk6p and cyclin E-HsCdk2p activities are required for G_1-S transition and passage of R. Next the cyclin A-HsCdk2p activity drives S phase and finally cyclin B-HsCdk1p is necessary for entry into M phase. The E2F family of transcription factors control the expression of a number of genes required for G_1-S progress and associate with the G_1-S cyclin-CDK complexes.

In budding yeast, a single CDK provides the kinase activity of both checkpoints. ScCdc28p associates with at least three different cyclins (ScCln1p, ScCln2p and ScCln3p) to drive passage through G_1 and entry into S phase. Similar to

the mammalian E2F family, the MBF and SBF transcription factors regulate the expression of G_1-S phase specific genes whose protein products associate positively with ScCdc28p or in an inhibitory manner with the cyclin-ScCdc28p complexes. ScCdc28p then follows to drive S phase and DNA replication by complexing with ScClb5p and ScClb6p cyclins and finally ScClb1-4p cyclins control entry and progression of mitosis (Nasmyth 1996).

Fission yeast also has only one CDK driving the cell cycle along with a number of cyclins. The homolog of budding yeast MBF/SBF transcription factors in fission yeast is DSC1, which controls expression of a number of genes that are also essential to cell cycle progress. The molecular mechanisms of the mitotic cell cycle in fission yeast are described in detail as follows.

1.4.1 The molecular mechanisms of the fission yeast mitotic cell cycle

1.4.2 G_1-S transition

In fission yeast, the regulation of progress through the cell cycle is controlled by a combination of cell cycle regulated transcription and the activities of different cyclin-CDK complexes. A single CDK, called SpCdc2p, exists in fission yeast and controls both checkpoints in late G_1 and late G_2. SpCdc2p activity is regulated through its association with four different cyclins (SpCig1p, SpCig2p, SpPuc1p and SpCdc13p). Levels of SpCdc2p are detected constantly throughout the cell cycle; therefore its activity is determined by the cyclin it is bound to at each stage of the cell cycle.

During G_1-S phase, the cell cycle transcription factor complex, DSC1, activates the expression of MCB containing genes, which are required for S phase onset. The major S phase cyclin, SpCig2p, associates with SpCdc2p to allow entry into S phase. However, the presence of SpRum1p, which accumulates from the anaphase of the previous mitotic phase, is inhibitory to the activation of SpCig2p-SpCdc2p. SpPuc1p and SpCig1p cyclins, which are insensitive to SpRum1p, associate with SpCdc2p and cause inactivation by phosphorylation and subsequent degradation of SpRum1p. This permits the activity of SpCig2p-SpCdc2p to increase and allows

* In the interest of clarity, in this thesis, proteins from different organisms will be given the following prefixes: Sp (*Schizosaccharomyces pombe*); Sc (*Saccharomyces cerevisiae*); Hs (*Homo sapiens*). Proteins will be written with a capital letter and ended with the letter 'p' for protein.

entry into S phase (Martin-Castellanos *et al.* 1996, Mondesert *et al.* 1996, Benito *et al.* 1998).

SpRum1p also blocks the activity of SpCdc13p-SpCdc2p, which is required for the entry and completion of mitosis, levels of which remain from the previous M phase. SpRum1p binds to SpCdc13p and targets it for ubiquitin-mediated proteolysis by the 26S proteosome, otherwise SpCdc13p-SpCdc2p would remain active and cause premature M phase, before chromosomes are duplicated, resulting in aneuploidy.

Ubiquitin-mediated proteolysis by the 26S proteosome is essential to the accurate ordering of cell cycle events. The anaphase promoting complex/cyclosome (APC/C) catalyses the transfer of ubiquitin to both G_1 and mitotic cyclins, targeting them for proteolysis. The APC/C is a multimeric complex which is cell cycle regulated. The activity of APC/C is triggered by its association with SpSlp1p and SpSte9p. In G_1 the mitotic cyclins, SpCdc13p and SpCig1p are targeted by the APC/C-SpSte9p complex for degradation. SpSte9p is also negatively regulated by SpCdc2p-dependant phosphorylation, which causes SpSte9p to dissociate from APC/C and subsequent degradation.

SpRum1p and APC/C-SpSte9p work in tandem to degrade the remaining SpCdc13p-SpCdc2p from the previous M phase. SpRum1p is then degraded by the action of SpPuc1p and SpCig1p bound to SpCdc2p. This releases the inhibitory effects of SpRum1p on the SpCig2p-SpCdc2p complex thereby promoting entry into S phase.

1.4.3 The onset of S phase (DNA synthesis)

A complete and accurate account of DNA replication is not yet fully understood in eukaryotes, but biochemical and genetic research so far in yeast and higher organisms has painted an overall picture that initiation of DNA replication is conserved in eukaryotes.

1.4.3.1 Origins of replication

The replication of chromosomes in all living organisms initiates at DNA defined sites called the origins of replication. The early steps involved in the recruitment of replication initiator proteins and the establishment of replication forks require origin

replication sequences. In budding yeast these origin of replication sequences are well classified and are called ARSs (autonomously replicating sequences), based on their ability to promote autonomous replication of plasmids (Marahens & Stillman 1992). These *cis*-acting DNA elements are defined by 100–200 bp blocks of DNA, containing an 11 bp, AT-rich, consensus sequence called the ACS (ARS consensus sequence), which is essential for their function (Bell & Stillman 1992, Diffley & Cocker 1992, Marahens & Stillman 1992). Replication origins in fission yeast differ from their budding yeast cousins in several respects. Fission yeast origins can be up to 500-1,000 kb in size and contain one or more sequence blocks of 20-50 bp. These sequences are also AT-rich (Okuno *et al.* 1999, Masukata *et al.* 2003), as in budding yeast, however they do not share a common consensus sequence comparable to the budding yeast ACS replication origins. Studies in eukaryotes imply that long regions of DNA containing discrete blocks of essential sequence are important for initiation of DNA replication.

1.4.3.2 Origin recognition complex

Studies in eukaryotes revealed an activity that bound specifically to ACS replication origins. This activity is a hetero-hexameric complex called the origin recognition complex (ORC) and in fission yeast comprises six proteins, SpOrp1p-SpOrp6p (Moon *et al.* 1999, Chaung *et al.* 2002). Analogous complexes also exist in both budding yeast and higher eukaryotes (Austin *et al.* 1999, Bell & Stillman, 1992, Kelly & Brown 2000). The ORC is believed to bind to DNA via the SpOrp4p, in fission yeast. The ORC serves as the scaffolding for the orderly recruitment of the different components of the DNA replication machinery (Brown & Kelly 2000, Lei & Tye 2001).

1.4.3.3 Minichromosome maintenance complex

The mini-chromosome maintenance complex (MCM) was also first discovered in budding yeast and was initially thought to be a replication initiation factor. More recent data suggests it also has another essential role as a helicase required for replication elongation. In fission yeast a family of six MCM proteins, SpMcm2p-SpMcm7p, share conserved DNA binding domains (Forsburg 2004). The SpMcm2-7p

proteins form a hetero-hexameric complex that in combination with the ORC forms a key component of the pre-recognition (pre-RC) complex. The loading of the MCM complex onto replication origins is orchestrated by cell cycle-dependent protein kinases, which themselves are also tightly regulated (Tye 1999).

1.4.3.4 Assembly of the pre-RC and DNA licensing by SpCdc18p and SpCdt1p

The loading of the MCM complex is assisted by two independent and crucial factors that govern licensing of DNA for replication initiation. SpCdc18p and SpCdt1p are two proteins, both under the regulation of DSC1 complex (Baum *et al.* 1998, Nishitani *et al.* 2000), that are transcribed in late M phase, although SpCdc18p levels are suppressed by SpCdc2-SpCdc13p-dependant phosphorylation and degradation. Upon entry into G_1, SpCdc2p-SpCdc13p activity is degraded by SpRum1p, thereby allowing SpCdc18p to accumulate (Baum *et al.* 1998, Jallepalli *et al.* 1997, Lopez-Girona *et al.* 1998). SpCdc18p and SpCdt1p bind to chromatin independently of each other but also interact with each other. Analogously, both SpCdc18p and SpCdt1p are required for initiating replication but seemingly, SpCdc18p is also necessary for elongation. The importance of SpCdc18p and SpCdt1p has been confirmed by studies in homologues of both proteins in budding yeast and mammalian cells, ScCdc6p/HsCdc6p and ScTah11p/HsCdt1p, respectively (Kelly *et al.* 1993, Hofmann & Beach 1994, Lygerou & Nurse 2000, Tanaka & Diffley 2002). The rapid increase in SpCdc18p levels in late G_1 in concert with SpCdt1p allows the assembly of the pre-RC. SpCdc18p and SpCdt1p bind to chromatin along with the ORC and coordinate the loading of SpMcm2-7p at the origins of replication and in so doing license DNA for replication (Nishitani *et al.* 2000, Lygerou & Nurse 2000).

1.4.3.5 Activation of DNA replication: CDK & DDK

The initiation of DNA replication is considered to be a two-stage process and marks the beginning of S phase. The first is the ordered assembly of the pre-RC at multiple origins of replication in order to license DNA for replication. The second stage requires the action of two separate protein kinases, the CDK SpCdc2p-SpCig2p and the DDK (Dfp1-dependent kinase) SpHsk1p-SpDfp1p (Lei & Tye 2001, Moser & Russell 2000).

The SpCdc2p-SpCig2p kinase is the major S phase promoting CDK in fission yeast but as yet the specific targets of SpCdc2p-SpCig2p are unclear, with respect to replication initiation. As well as having a positive role in activating DNA replication, SpCdc2p-SpCig2p also has a negative role in prevention of re-replication (Jallepalli *et al.* 1997, Lopez-Girona *et al.* 1998). In late G_1 rising levels of SpCdc2p-SpCig2p activity, due to degradation of SpRum1p, phosphorylate SpCdc18p, targeting it for degradation. Whilst being degraded, transcription of *cdc18*$^+$ under the control of DSC1 also declines and as a result only allows a small gateway of opportunity by which SpCdc18p can instigate formation of the pre-RC, thus preventing re-initiation of already fired origins of replication. Transcription of *cdt1*$^+$ is also under DSC1 control and is transcribed in parallel with *cdc18*$^+$ (Hofmann & Beach 1994, Nishitani *et al.* 2000) suggesting that SpCdt1p may also be degraded in a CDK-dependant manner (Nishitani *et al.* 2000). Therefore the prevention of re-replication until the following M phase may be due to both cell cycle-regulated periodic transcription and CDK-dependant proteolysis working synchronously.

The second kinase, SpHsk1p, is a member of the Cdc7p family of protein kinases, and is essential for the initiation of DNA replication (Brown and Kelly 1998). SpHsk1p levels are constant throughout the mitotic cell cycle and have kinase activity but substrate specificity is only conferred by forming a complex with SpDfp1p. SpDfp1p is periodically expressed, peaking at the G_1-S transition thus recruiting the active SpHsk1p-SpDfp1 complex to the pre-RC in late G_1 (Brown & Kelly 1998, Brown & Kelly 1999). SpHsk1p-SpDfp1p can phosphorylate the SpMcm2p-7p complex on the SpMcm2p resulting in a conformational change (Diffley & Labib 2002, Nishitani & Lygerou 2002, Brown & Kelly 1998, Lee *at al.* 2002). The SpMcm2p-7p complex then becomes an active helicase important for unwinding dsDNA at replication forks (Lee & Huwitz 2001, Labib & Diffley 2001). SpHsk1p-SpDfp1p mediated phosphorylation of the SpMcm2p subunit is dependant on the SpCdc23p associating with the DDK. SpCdc23p might have a vital role in recruiting SpHsk1p-SpDfp1p to the pre-RC at origins of replication (Lee *et al.* 2003) and further for the recruitment of SpSna41p (a homologue of ScCdc45p) to origins (Gregan *et al.* 2003).

The recruitment of SpSna41p is thought to be necessary for the loading of DNA polymerase α onto the SpMcm2p-7p complex (Uchiyama *et al.* 2001). The CDK and DDK activities initiate a cascade of events that ultimately lead to the

establishment of functional replication forks (Diffley & Labib 2002). Short primers are synthesised by DNA polymerase α, with the aid of a DNA primase complex, to originate DNA synthesis (MacNeill & Nurse 1997). This is followed by commencement of DNA replication by recruitment of other components of the replication machinery such as DNA polymerase δ and ε (required for elongation of new strands with proofreading activity) (Diffley & Labib 2002, Dahlen *et al.* 2003) and SpDna2p (required to fill the gaps between Okazaki fragments) (Kang *et al.* 2000). Before progressing into G_2 phase the replicated DNA is assembled into chromatin. Cohesin complexes, which mediate the association of the replicated sister chromatids (sister chromatid cohesion) are also loaded on to the DNA during S phase. Termination occurs when two opposing replication forks meet and the nascent DNA from the two forks are ligated together (Diffley & Labib 2002). A model for DNA licensing in fission yeast is depicted in Figure 1D.

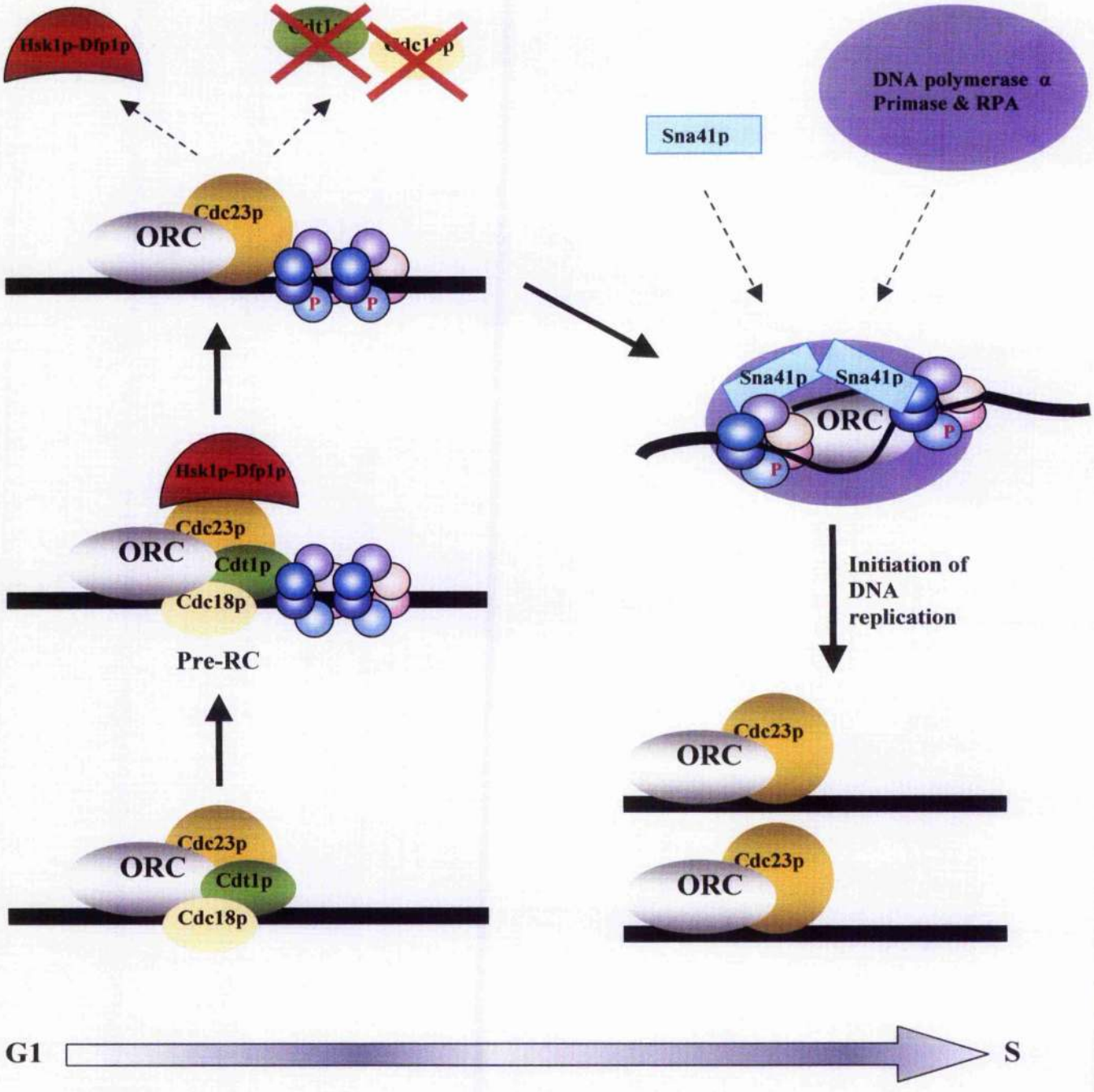


Figure 1D Model for DNA licensing and replication in fission yeast. The ORC complex, consisting of Orc1p–Orc6p, is bound to multiple origins of replication on DNA. Cdc18p and Cdt1p independently associate with chromatin at the origins to which Cdc23p binds. Cdc18p and Cdt1p then recruit the MCM complex consisting of Mcm2p–Mcm7p, thereby forming the pre-RC. This is known as DNA licensing Once the pre-RC is formed Cdc18p and Cdt1p are removed from the complex and Hsk1p–Dfp1p and Cdc2p-Cig2p enter to trigger the initiation of DNA replication. Hsk1p–Dfp1p phosphorylates the MCM complex, which results in the activation of its helicase activity. Hsk1p–Dfp1p then dissociates 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 (single strand DNA binding protein). Adapted from Lei & Tye (2001).

1.4.4 G_2-M transition

Cells must reach a critical size before they can pass the late G_2 checkpoint and enter M phase. This is induced by the raised activity of the SpCdc2p-SpCdc13p kinase complex (Moser & Russell 2000). SpCdc2p-SpCdc13p activity, which is present after passage of START and throughout G_2, is inhibited by phosphorylation on Tyrosine-15 (Y-15) residue by the SpWee1p and SpMik1p kinases (MacNeill & Nurse 1997). SpCdc2p–SpCdc13p is activated by the de-phosphorylation of Y-15, which is promoted by the protein tyrosine phosphatase SpCdc25p and the SpWee1p inhibitory kinases, SpCdr1p and SpCdr2p (MacNeill & Nurse 1997).

The maintenance of low SpCdc2p–SpCdc13p activity coincides with peak expression of the *mik1*$^+$ gene and consequent protein levels, which is under the cell cycle-regulated control of the DSC1 complex (Ng *et al.* 2001, Baber-Furnari *et al.* 2000, Christensen *et al.* 2000). Thus, the elevated levels of SpMik1p are important for preventing premature entry into mitosis by suppressing SpCdc2p-SpCdc13p activity (Baber-Furnari *et al.* 2000, Christensen *et al.* 2000). In comparison, the levels of SpWee1p are constitutive throughout the cell cycle (Moser & Russell 2000). The translation of both SpCdc25p and SpCdc13p is sensitive to reduced translation initiation efficiency (Daga & Jimenez 1999) suggesting in part that the control of SpCdc25p translation initiation might form part of the mechanism coupling cell cycle and cell growth (Daga & Jimenez 1999, Kellogg 2003). A combination of these mitotic activators and inhibitors working in harmony with each other leads to the activation of SpCdc2p-SpCdc13p complex and drives ensuing mitotic events (Su & Yanagida 1997).

1.4.5 Checkpoint controls and DNA integrity

During the cell cycle and particularly during DNA replication, a cell can encounter problems such as DNA damage or a block to replication. And so before entering M phase a cell must ensure that the genome has been fully and faithfully replicated. It is therefore crucial that a cell does not continue onto mitosis before the integrity of the genome is analysed and repaired. Cell cycle checkpoints are the main operators of the monitoring of DNA replication. These checkpoints recognise any problems that occur

due to intrinsic or extrinsic factors and subsequently delay cell cycle progression by inhibiting cell cycle machinery until the problem is fixed (Hartwell & Weinert 1989).

SpCdc2p coupled with SpCdc13p, drives entry into M phase after a lengthy G_2 in fission yeast. DNA damage checkpoints delay entry into mitosis by inhibiting SpCdc2p kinase activity. In fission yeast the two main effectors of the checkpoint pathways are the SpChl1p and SpCds1p protein kinases, which trigger a response to DNA damage and stalled DNA replication signals, respectively (Rhind & Russell 2000, Boddy and Russell 2001). Initially the SpRad proteins sense DNA damage or stalled replication and respond by activating the kinase activities of SpChk1p and SpCds1p by phosphorylation (Rhind & Russell 2000).

The Y-15 phosphorylation status of SpCdc2p prevents mitotic entry and the SpChk1p and SpCds1p indirectly maintain this inhibition. SpChk1p responds to DNA damage signals by negatively regulating SpCdc25p phosphatase activity (Rhind *et al.* 2000). Also SpMik1p levels increase in response to DNA damage and may also function to delay entry into mitosis by suppressing SpCdc2p-SpCdc13p activity (Baber-Furnari *et al.* 2000). It has been proposed that the checkpoint mediated activation of SpMik1p and inhibition of SpCdc25p ensures that the phosphorylation of SpCdc2p is maintained and in doing so prevents mitotic entry, although the exact mechanisms are not fully understood (Rhind *et al.* 2000). SpWee1p, along with SpCdc25p, is also required for Y-15 de-phosphorylation of SpCdc2p but does not seem to have an important role in checkpoint function (Rhind & Russell 2001).

1.4.6 Mitosis and cytokinesis

The co-ordination of mitosis and cytokinesis is a problem that all eukaryotes must overcome to ensure the stable transmission of the genome during cell division and essentially this process is similar in all eukaryotes observed (Simanis 2003, Alberts *et al.* 1994). Errors in the proper choreography of these events can lead to aneuploidy or genetic instability leading to cell death or disease (Nigg 2001).

Entry into mitosis is promoted by CDK-dependant phosphorylation and de-phosphorylation of various proteins kinases. In fission yeast SpCdc2p-SpCdc13p kinase activity is required to reach a critical peak to permit mitosis, controlled at the G_2-M transition by SpCdc25p (MacNeill & Nurse 1997). These events trigger a series of highly complex events that in conjunction achieve chromosome condensation,

segregation and finally cytokinesis. The exit from mitosis depends on inactivating mitotic cyclin-CDK complexes by the action of the APC/C complex in the shape of ubiquitin-mediated proteolysis (Morgan 1999, Harper *et al.* 2002).

Preparation for mitosis (replication and establishment of cohesion): The cell prepares early in the cell cycle for mitosis shortly after DNA replication in S phase, after which the newly replicated sister chromatids are physically connected until anaphase, and the spindle pole body is duplicated (Nasmyth *et al.* 2000). Chromatids are joined together to ensure that premature separation of chromatids does not occur before anaphase begins, important in preventing aneuploidy. The physical connection between sister chromatids is held together by a cohesion complex, which in fission yeast is composed of four proteins called SpPsm1p, SpPsm3p, SpRad21p and SpPsc3p (Hagstrom & Meyer 2003).

Prophase: As cells approach prophase, chromosomes are indistinguishable. By a process called condensation, chromosomes begin a marked change in structure until the loosely packed assortment at interphase becomes fully compact at metaphase. By another process termed resolution, the now visible rod-shaped arms of the chromosomes attach to the mitotic spindle via the centromeres, to allow separation without entanglement. In fission yeast, the condensin complex is composed of the SpCut14p-SpCut3p hetero-dimer complex associated with SpCnd1p, SpCnd2p and SpCnd3p (Hagstrom & Meyer 2003).

Pro-metaphase: This is the stage that is defined by the breakdown of the nuclear envelope, although this does not occur in yeast (Alberts *et al.* 1994). The centromere assembles the kinetochore that mediates the attachment of the mitotic spindle.

Metaphase: A combination of chromosome cohesion and the mitotic spindles, pulling sister chromatids to opposite poles of the cell, cause chromosomes to completely align at the centre of the cell in what is called the metaphase plate. A spindle-checkpoint mechanism operates to ensure correct spindle-chromosome attachment and delays anaphase until all the chromosomes are attached and under tension (Hagstrom & Meyer 2003).

Anaphase: At anaphase the cohesion between the sister chromatids is dissolved and they separate in a burst of movement. The APC/C complex that triggers the proteolysis of target proteins induces the onset of anaphase. In fission yeast APC/C forms a complex with SpSlp1p and aids the separation of sister chromatids by

ubiquitylating a protein called SpCut2p/Securin. Securin is normally found bound to the SpCut1p/separin and is thought to inhibit the proteolyic activity of separin. Ubiquitin-mediated proteolysis of Securin is thought to release the proteolytic potential of separin and thereby drive separation of sister chromatids to opposite poles of the cell by dissolving the cohesin complex (Yanagida 2000).

Telophase & Cytokinesis: In telophase the newly segregated chromosomes de-condense. The exit from mitosis requires the inactivation of M phase cyclin-CDK activity by ubiquitin-mediated proteolysis (Morgan 1999). Two homologous signalling cascades exist in budding and fission yeast called the mitotic exit network (MEN) and the septation initiation network (SIN), respectively. These are essential for the completion of mitosis and cytokinesis along with other mitotic events (McCollum & Gould 2001, Bardin & Amon 2001).

1.5 Transcription

The primary function of a chromosome is to act as a template for the synthesis of RNA molecules in order to express the genetic information stored in the form of genes. RNA synthesis, popularly termed transcription, is a highly selective and complex process. The DNA that is transcribed can form either mature messenger RNA (mRNA) or structural RNAs. The former, mRNAs, are used to translate into proteins and the latter synthesise RNAs that have a structural or catalytic role as part of the protein synthetic machinery.

There are three major RNA polymerases that carry out gene transcription in eukaryotic organisms. RNA polymerase II transcribes mRNA required for protein translation and RNA polymerases I and III transcribe ribosomal RNA (rRNA) and other small RNAs, respectively (Alberts *et al.* 1994, Lewin 1997) and are required in a structural or catalytic role.

1.5.1 Transcription by RNA polymerase II

RNA polymerase II (RNA pol II) makes all of the mRNA precursor molecules and thus determines which proteins a cell will make. Initiation of transcription is quite broad ranging when comparing transcriptional control modules of simple unicellular organisms to metazoan species. The yeast transcriptional unit is composed of a simple

core promoter named the TATA box, and an upstream activator sequence (UAS) and silencer element spaced within 100-200 bp of the TATA box. Conversely, the metazoan transcriptional control module is formed of a complex arrangement of multiple upstream or downstream silencer, enhancer and insulator elements, surrounding a core promoter region consisting of a TATA box, initiator sequences (INR) and downstream promoter elements (DPE) (Figure 1E).

1.5.1.1 RNA polymerase II promoter sequences

Within the core promoter of the unicellular yeast transcription unit, resides a TATA box, which is the binding site for the TATA box binding protein (TBP). The initiation of transcription is marked by an initiator element (I) and is located at varying distances from the TATA box in budding and fission yeast (Struhl 1989, Levine & Tjian 2003, Choi *et al.* 2002). The core promoter and the binding of TBP to the TATA box is the primary event in the selection and activation of gene transcription. The binding of TBP is regulated by activating sequences (UAS) found upstream of the core promoter, which comprise of one or several binding sites for sequence-specific transcription factors. It is likely that there are many forms of the UAS sequences that recruit more than one sequence-specific transcription factor, depending on the gene involved (Struhl 1989, Levine & Tjian 2003).

In metazoans, a gene can have several enhancer sequences, approximately 500 bp in length, which are located upstream or downstream of the gene itself. Located within the enhancers are multiple recognition sequences for the binding of sequence-specific transcription factors. Each enhancer is responsible for a subset of the total expression pattern of the gene, governing tissue, cell-type, or cell cycle specific gene expression (Levine & Tjian 2003, Kadanoga 2002). The core promoter, in metazoans, is approximately 60-80 bp in length and includes the transcription initiation site (Inr) (Kadanoga 2002, Levine & Tjian 2003). There are three different sequence elements that can associate with the TBP bound to the transcription factor TFIID (Initiator complex): TATA, initiator element (Inr) and the downstream promoter element (DPE) (Kadanoga 2002, Levine & Tjian 2003). Fission yeast is similar to the metazoan system in the initiation of transcription and occurs within 25 bp of the TATA box, a trait not seen in budding yeast where it occurs at several discrete sites about 40-120 bp from the edge of the TATA box (Choi *et al.* 2002). Finally the insulator sequences

flank these multiple regulatory sites and function to prevent the inappropriate regulation of adjacent genes (Levine & Tjian 2003). A combination of multiple signalling pathways and transcription factors combined with interaction with an intricately organised transcription module allows for the precise control of tissue, cell-type and cell cycle specific gene expression in higher eukaryotes.

1.5.1.2 RNA polymerase II transcription machinery

As mentioned earlier the binding of the initiation complex to the TATA box marks the initiation of transcription. The initiation complex comprises the TFIID bound to TBP and 10-12 TBP associated factors. This complex is recruited to the core promoter where it instigates the assembly of the pre-initiation compex (PIC) (Wasserman & Saur 2001). TFIID has a number of tasks to carry out in order to initiate transcription. Specific DNA sequence elements within the core promoter are recognised by TFIID in an activator-dependant manner (Wasserman & Saur 2001). TFIID also alters the chromatin structure of the core promoter for further assembly of the PIC and the continuation of transcription (Wasserman & Saur 2001). Lastly, TFIID binds with a number of general transcription factors; TFIIA, TFIIB, TFIIE, TFIIF and TFIIH, that interact with RNA polymerase II and facilitates PIC assembly and consequently transcription initiation and elongation (Wasserman & Saur, 2001).

Current research suggests that much of the general RNA polymerase II machinery is conserved amongst eukaryotes where RNA polymerase II itself, which is a heteromeric complex composed of 12 subunits, Rpb1-Rpb12, is conserved in yeast and humans (Myer & Young 1998, Shpakovski *et al.* 2000). Nevertheless, metazoan systems have the added complexity of regulating tissue and cell-specific transcription, lacking in unicellular eukaryotes. With this point noted it is understandable that higher eukaryotes have evolved many more diverse layers of transcriptional control (Levine & Tjian 2003). When examined at the PIC assembly level, there are tissue-specific and TBP-related factors, not present in yeast, that provide an advanced level of control over gene expression in metazoans (Levine & Tjian 2003)

Another feature of transcriptional regulation are the transcription cofactor complexes (Levine & Tjian 2003). The yeast mediator is a multi-subunit co-activator complex and acts as an interface between gene-specific regulatory proteins and the

general transcription machinery (Myers & Kornberg 2000). Whilst yeast has one such complex, metazoans contain several related complexes (Levine & Tjian 2003).

Finally, chromatin remodelling via chromatin remodelling enzymes (eg. SWI/SNF) (Levine & Tjian 2003) and histone modification (via histone acetyl transferases and deacetylases) (Kouzarides 2000) is essential for PIC assembly and the initiation of transcription and correspond to another source of RNA polymerase II transcriptional regulation.

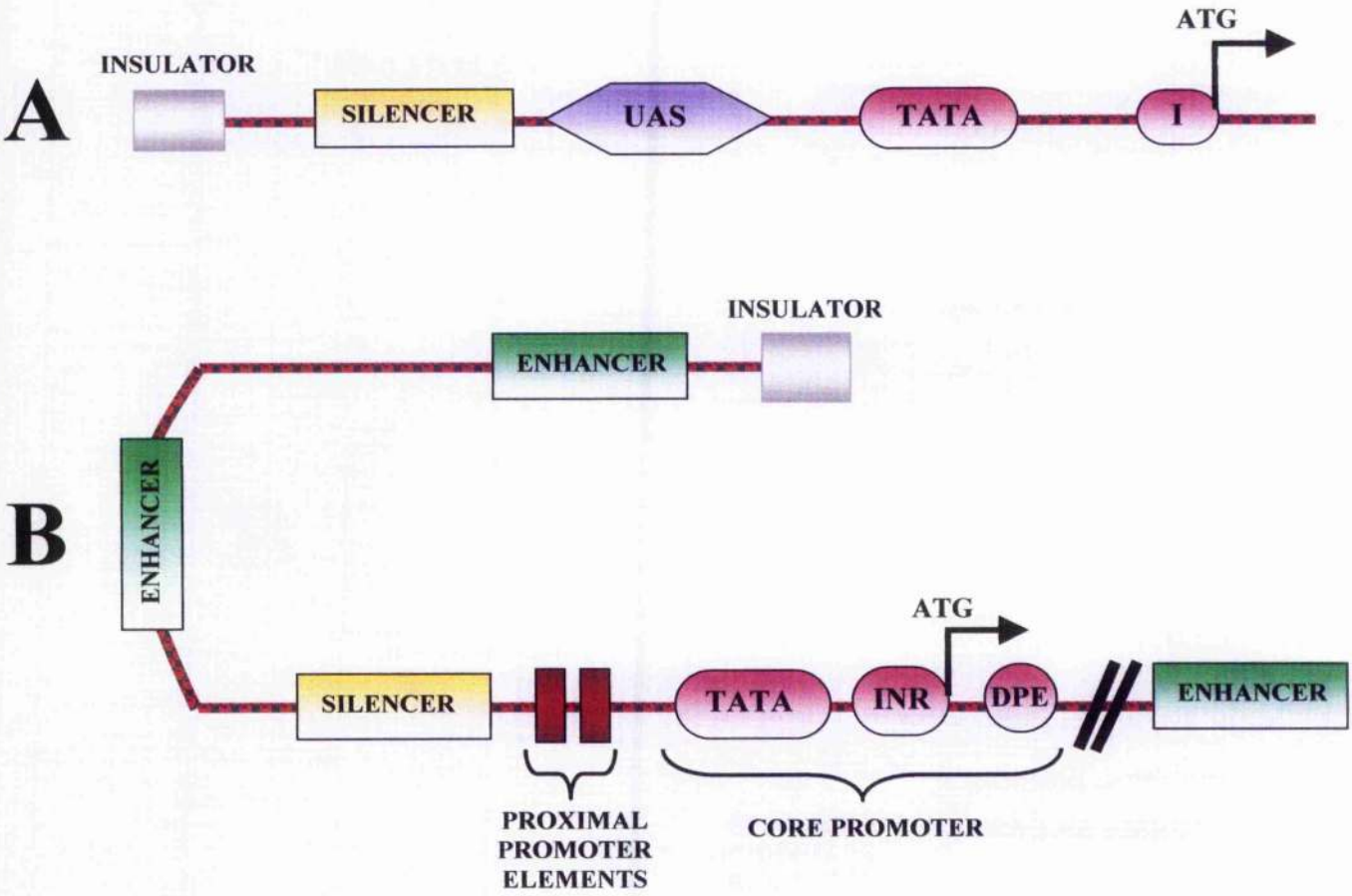


Figure 1E Comparison of Yeast and Metazoan RNA polymerase II promoters.

A. A simple yeast RNA polymerase II promoter module comprising a TATA box, an initiator element (I) and also upstream activating sequences (UAS) and silencer elements, spaced within 100-200 bp of the TATA box. **B.** A complex metazoan transcriptional control module comprising an arrangement of multiple, clustered, enhancer, silencer and initiator elements. The core promoter contains a TATA box, initiator elements (INR) and downstream promoter elements (DPE). Adapted from Levine & Tjian (2003)

1.5.2 Transcription by RNA polymerase I

The continuous transcription of multiple gene copies ensures an adequate supply of rRNAs, which are immediately packaged with ribosomal proteins to form ribosomes (the machinery required to translate mRNA into functional proteins). The packaging occurs in the nucleus in a large, distinct structure called the nucleolus. The nucleolus contains large loops of DNA emanating from chromosomes, each of which contains a cluster of rRNA genes. The rRNA genes are transcribed at rapid rates by RNA polymerase I.

Many individual protein factors are required for the initiation of rRNA gene transcription (Figure 1F). 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 1997, Grummt 2003) (Figure 1F). The TATA-binding protein (TBP) and a second protein complex, Core Factor (CF), are recruited to the downstream core promoter and are themselves required for the recruitment of the functional holoenzyme, comprising RNA polymerase I and RRN3p (Grummt 2003) (Figure 1F). In budding yeast, UAF remains associated with the upstream element acting as a scaffold for reinitiation where other components of the RNA polymerase I transcription machinery cycle on and off the promoter during each successive round of transcription (Aprikian *et al.* 2001).

In mammalian cells, the upstream binding factor (UBF) interacts with the upstream control element and then acts with TIF-IB (TBP-containing promoter selectivity factor) to recruit an assortment of TBP-associated factors (TAFs). These provide promoter-recognition function. The active RNA polymerase I holoenzyme is then recruited by the interaction of UBF and TIF-IB with TIF-IA (homologue of budding yeast RRN3p) (Lewin 1997, Grummt 2003).

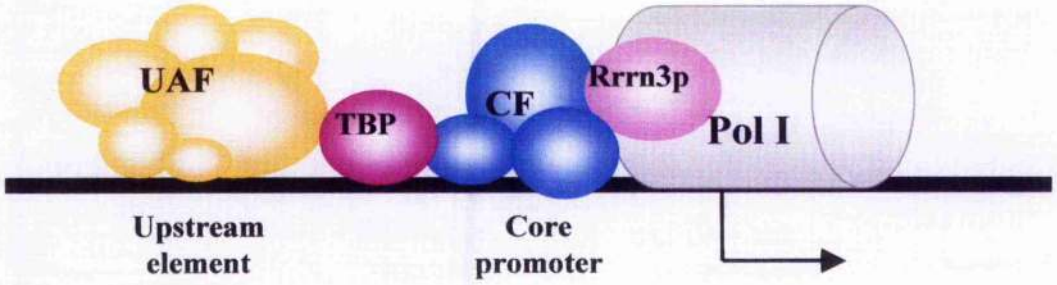


Figure 1F A Schematic view of the components of the yeast RNA polymerase I transcription initiation complex. The black line represents the rDNA promoter sequences and the bent arrow indicates the transcription initiation site. The upstream activating factor (UAF) comprises Rrn5p/9p/10p and histones H3 and H4. Core factor (CF) comprises Rrn6p/7p/11p. Adapted from Grummt (2003).

1.5.2.1 Budding yeast DNA polymerase V

The budding yeast *POLV* has only recently been identified, with relatively little known about its biological function. Very interestingly, however, ScPolVp appears not to have DNA polymerase properties, but instead is required for 35S and 5S rRNA synthesis, through a non-RNA polymerase activity, possibly through interacting with the RNA polymerase I machinery (Shimizu *et al.* 2002, Yang *et al.* 2003). This proposal is based on a number of observations: rRNA synthesis but not tRNA, is severely inhibited in *POLV* mutants; ScPolVp binds near or at the RNA polymerase I enhancer sequence of 35S rRNA-encoding DNA repeating units; ScPolVp localises to the nucleolus; *POLV* is an essential gene; and *polV* mutants do not effect chromosome replication (Shimizu *et al.* 2002). A protein with similar peptide sequence, Myb-binding protein 1a, has also recently been isolated in mice, suggesting ScPolVp-like proteins may be universally present in eukaryotes (Tavner *et al.* 1998, Keough *et al.* 2003).

1.5.2.2 Fission yeast 35S rDNA promoter binding factors

A fission yeast 35S rDNA promoter binding activity has been identified that is believed to regulate rRNA production through binding an upstream promoter domain, and a number of its components identified (Boukhgalter *et al.* 2002, Liu *et al.* 2002). These include Spp27p, SpRm5hp, SpRm7hp and SpRm10hp, which are known to bind to the 35S rDNA promoter as part of the DNA-binding activity, and in some cases they have been shown to stimulate rDNA transcription *in vitro* (Boukhgalter *et al.* 2002, Liu *et al.* 2002).

1.5.3 Transcription by RNA polymerase III

RNA polymerase III of budding yeast and humans has been the most extensively researched and now more recent fission yeast research is also revealing striking similarities and differences between the budding yeast, human and fission yeast systems (Lewin 1997, Huang & Maraia 2001, Paule & White 2000). RNA polymerase III is directed by multi-subunit transcription factors to synthesise a

number of small transcripts such as tRNAs, 5S rRNA and U6 small nuclear RNAs (snRNAs). The type of promoter they contain can classify these genes into different sets.

The first type of promoter is the Type I promoter (Figure 1G) which, unlike the promoters for RNA polymerases I and II, resides within the transcribed region. The type I promoters consist of an A-block, an initiator element (IE) and a C block and are typically found in 5S rRNA genes. In Type I RNA polymerase III promoters, TFIIIA binds to DNA sequences that encompass the C-block and is required for the binding of TFIIIC.

Type II promoters are the most common elements, which are seen in the tRNA genes (Figure 1G). Type II promoters are also internal and consist of two highly conserved sequence blocks, 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. Both Type I and Type II RNA polymerase III transcriptional promoter units contain a conserved terminator sequence (Tn) that is important for the reinitiation of successive rounds of transcription. In Type II RNA polymerase III promoters, TFIIIC recognises the B-block and binds DNA sequences that encompass both the A-block and B-block sequence elements.

TFIIIC binding mediated the further recruitment of the TBP-containing TFIIIB complex in both type I and II promoters. In budding yeast the TFIIIC complex resembles a dumbbell shape and makes contact with both the A and B blocks in different Type II promoters despite the varying distance between the blocks in different Type II genes. Human TFIIIC is composed of two subunits named TFIIIC1 and TFIIIC2. TFIIIC2 recognises the B block and subsequently recruits TFIIIC1 and TFIIIB.

Type III promoters are normally seen in snRNA genes (Figure 1G), although are sometimes found in other RNA genes (eg. Silkworm and *Xenopus* tRNA genes), 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. RNA polymerase III is directed to the initiation site by the binding of TFIIIB and remains stably bound to the DNA for successive rounds of transcription. Type 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. 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 to aid recruitment of TFIIIB to the TATA box, which then goes on to recruit RNAPol III.

The completion of the fission yeast sequencing project has allowed database searches that have found fission yeast homologues of the many RNA polymerases and their accessory factors, across species. However the way these homologues function can be significantly different between systems. For example in human Type III promoters a discrete set of TFIIIB related accessory factors are required but no such factors could be identified in fission yeast. Also, fission yeast absolutely require a TATA box for transcription initiation unlike the human and budding yeast system which employ TATA-less promoters. This suggests that the overall mechanism for the recruitment of RNA polymerase III in fission yeast is divergent compared to budding yeast and humans (Hamada *et al.* 2001).

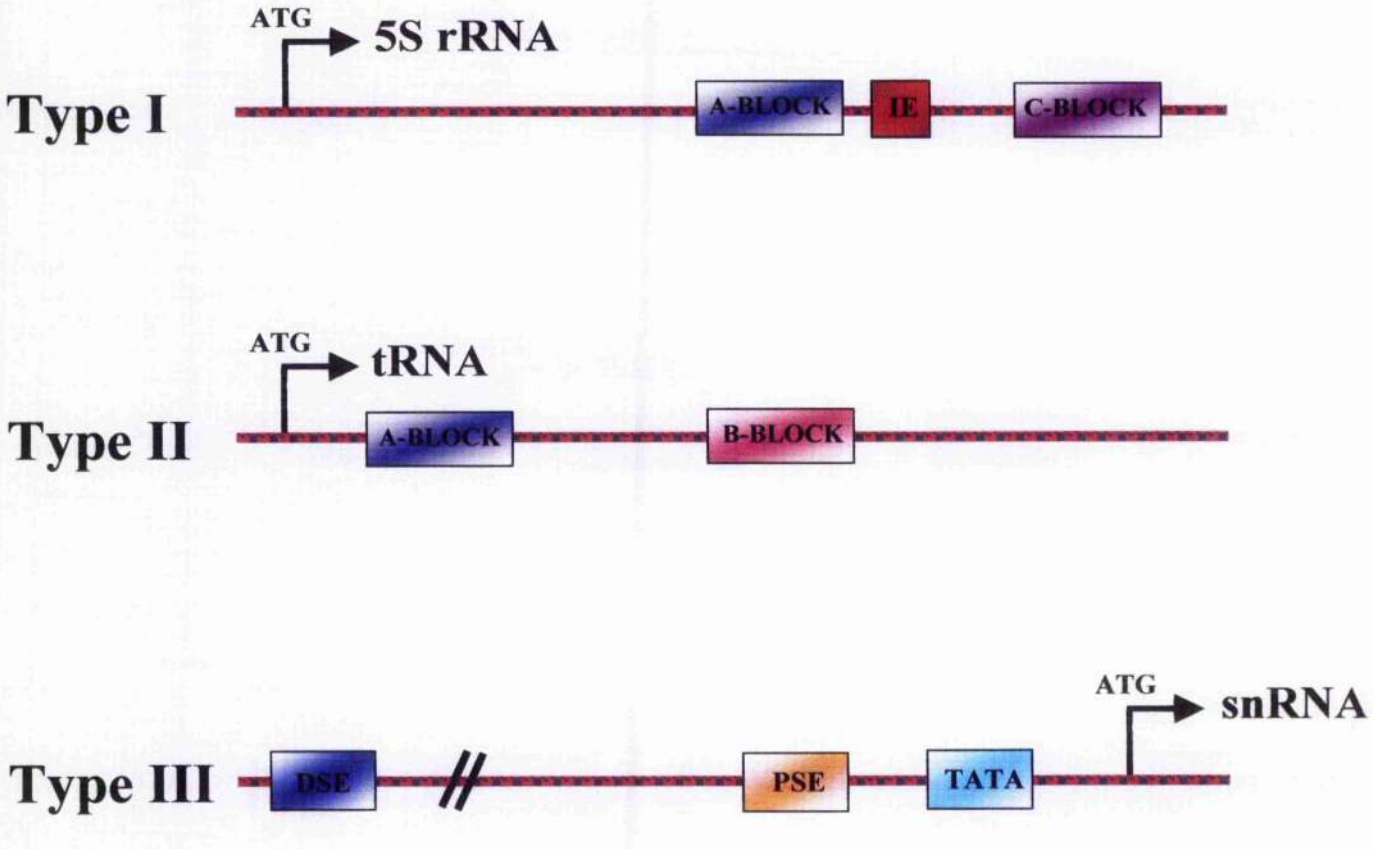


Figure 1G A schematic of three general types of promoter, in eukaryotes, used by RNA polymerase III. The black arrow indicates the transcription initiation site. The various promoter elements are shown boxed, including the intermediate element (IE), the proximal sequence element (PSE) and the distal sequence element (DSE).

1.6 Cell cycle regulated periodic transcription

The precise control of the different events during a cell cycle is controlled by the phase-specific transcription of particular groups of genes. The expression of these genes coincides with the time they are required in the cell cycle (Breeden 2003, McInerny 2004). A cell undertakes efficient and accurate management of the components of cell cycle machinery for reasons that sometimes cannot be explained but for the most it is commonly for economical resourcing. Also, the presence of regulatory proteins outside of their designated period of activity can be detrimental to the cell. For example, cyclins that are expressed outside of their normal time frame can lead to premature passage of START/R or entry in mitosis before DNA replication is complete. Finally, the regulation of orderly passage through checkpoints in the cell cycle is dependant on the cell-cycle-phase-specific expression of key factors (Breeden 2003, McInerny 2004). The conservation of these cell cycle regulated waves of expression is evident in eukaryotes and, therefore, stamps the importance of this feature.

Microarray analysis in both budding and fission yeast have revealed ~800 and ~400 genes, respectively that are cell cycle regulated (Spellman *et al.* 1998, Rustici *et al.* 2004). Similarly, global transcription in humans has been analysed to reveal cell cycle regulated transcription in the region of ~700 genes (Cho *et al.* 2001).

1.6.1 Cell cycle regulated transcription at the G_1-S transition

Along with a plentiful supply of nutrients cells also require the activity of cyclin-CDK complexes in order to progress through the cell cycle. Passage of START/R marks the irreversible commitment to complete the entered round of the mitotic cell cycle until the following S phase. The entry and completion of S phase requires the expression of S phase specific genes of which the most prominent are the G_1-S phase cyclins. G_1-S phase cyclin-CDK complexes promote passage of START/R and activate downstream events, therefore the precise expression of these genes is essential to orderly completion of the cell cycle.

Cell cycle regulated transcription is central to the coordinated expression of G_1-S phase genes and is a phenomenon that has been well documented in yeast and

mammalian cells. In budding yeast the MBF and SBF transcription factor complexes are responsible for the periodic transcription of genes required for S phase (Merrill *et al.* 1992). An MBF-like complex also exists in fission yeast called DSC1* that has an analogous role (Whitehall *et al.* 1999). Similarly the mammalian equivalent of these cell cycle transcription factors is the E2F complex (Dyson 1998). However the similarities between MBF and DSC1 at the primary sequence level are not shared by E2F but the remarkable similarities in function suggest that E2F is the functional homologue of MBF.

1.6.2 G_1-S phase specific transcription in budding yeast

In budding yeast the activity of ScCdc28p coupled with G_1 cyclins is required to pass START. The correct temporal expression of these cyclins, along with many other genes required for S phase, is essential for the orderly completion of DNA replication and S phase. Two different yet related transcription factor complexes exist in budding yeast that control the periodic transcription of genes at the G_1-S boundary.

The first complex is termed SBF (SCB-binding factor) and is a hetero-dimeric complex composed of ScSwi4p and ScSwi6p (Figure 1H). Many genes including the G_1 cyclins, *CLN1*, *CLN2*, *PCL1* and *PCL2* cyclins, contain a common 5'-CACGAAA-3' sequence element in their promoter, known as the ScSwi4p-ScSwi6p cell cycle-box (SCB). The SBF complex associating with SCB elements activates transcripion of genes required for S phase. ScSwi4p is a 123 kDa protein that is the DNA binding subunit of SBF via its N-terminus. It also has a centrally located ankyrin-repeat motif, that is proposed to be involved in protein-protein interactions, and also a C-terminus site that is for heterodimerisation with Swi6p (Figure 1I) (Andrews & Herskowitz 1989, Primig *et al.* 1992, Bork *et al.* 1993, Sedgwick & Smerdon 1999). ScSwi6p is the 92 kDa subunit of SBF and has a regulatory function as it does not confer any DNA binding capabilities (Figure 1I). ScSwi4p and ScSwi6p heterodomerise via their C and N-termini, respectively (Sedgwick *et al* 1998). ScSwi6p also has a centrally located ankyrin-repeat motif and distinct transcriptional

* DSC1 (DNA synthesis control 1) is the transcription factor complex in fission yeast, responsible for expression of G_1-S phase specific genes, that is homologous to budding yeast MBF. DSC1 is also commonly referred to as MBF in fission yeast but for this thesis will be called DSC1 to distinguish between the two yeast species.

activation domains at both the N and C-termini (Sedgwick *et al.* 1998, Breeden & Nasmyth 1987, Primig *et al* 1992, Andrews & Moore 1992, Foord *et al.* 1999).

The second complex is MBF (MCB binding factor) and is also a hetero-dimeric complex composed of ScSwi6p and ScMbp1p (Figure 1H) (Lowndes *et al.* 1991, Lowndes *et al.* 1992b, Dirick *et al.* 1992, Koch *et al.* 1993, Iyer *et al.* 2001). Chromatin immunoprecipitation and microarray hybridization revealed that while SBF activated genes are predominantly involved in membrane and cell wall biosynthesis and budding, MBF activated genes are mostly involved in DNA synthesis and repair pathways (Vishwanath *et al.* 2001, Johnston & Lowndes 1992, Koch & Nasmyth 1994). Included in these genes are the *CLB5* and *CLB6* cyclins, important for cell cycle progression (Epstein & Cross 1992, Schwob & Nasmyth 1993). The promoters of MBF activated genes all have a common *cis*-acting element with the consensus sequence, 5'-ACGCGT-3', which corresponds to the *Mlu*I restriction enzyme recognition site and was therefore named the *Mlu*I cell cycle box (MCB). Similar to the architecture of SBF, the 120 kDa ScMbp1p subunit provides the MCB element mediated DNA binding capability and ScSwi6p again provides the regulatory aspect of the complex (Dirick *et al.* 1992, Koch *et al.* 1993). ScMbp1p is structurally and functionally very similar to ScSwi4p (Figure 1I). It also mediates DNA binding via its N-terminus and heterodimerises via its C terminus, which are separated by the centrally located ankyrin-repeat motif (Koch *et al.* 1993).

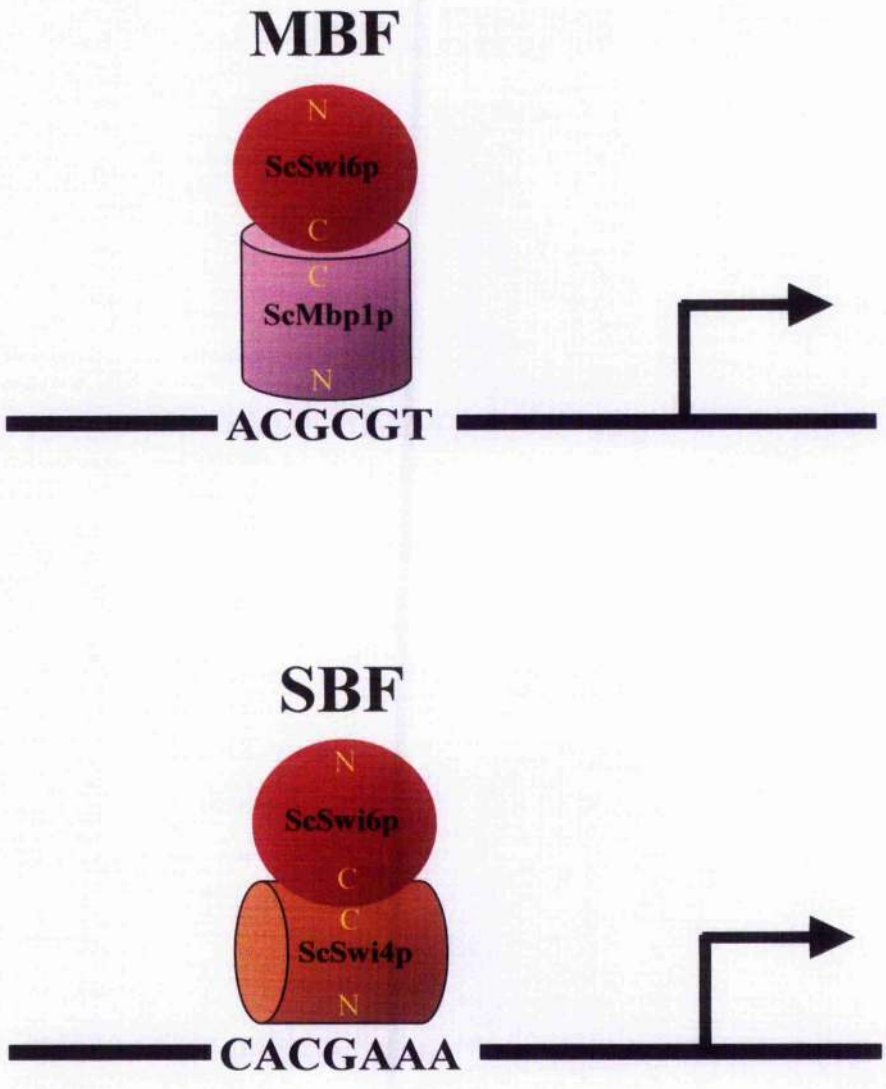


Figure 1H A schematic representation of the MBF and SBF DNA-binding complexes in budding yeast. The MBF (MCB binding factor) and SBF (SCB binding factor) complexes bind to MCB (*Mlu*I cell-cycle box) and SCB (Swi cell-cycle box) promoter sequences, respectively. The N and C-termini of the individual proteins are in yellow.

1.6.2.1 Functional redundancy between SBF and MBF dependant transcription

The overall mechanisms of SBF and MBF dependant gene expression is not yet fully understood and is not as clear cut as described. Studies revealed apparent functional redundancy between the two systems. While *mbp1/swi4* double mutants are lethal, single mutants of either gene remain viable suggesting that the function of at least one complex can suffice for cell cycle progression (Koch *et al.* 1993). Judging by the striking structural similarities between the protein subunits it does not come as a surprise that there is functional overlap between the systems. *In vitro* experiments show that, if present in high enough concentrations, the SCB and MCB elements can cross-compete with each other for complex binding however SBF and MBF show preference to SCB and MCB elements, respectively (Taylor *et al.* 2000). One apparent example of overlapping functional redundancy is documented *in vivo* where G_1-S regulated transcription is mediated by the SBF associating with MCB-like motifs (Partridge *et al.* 1997).

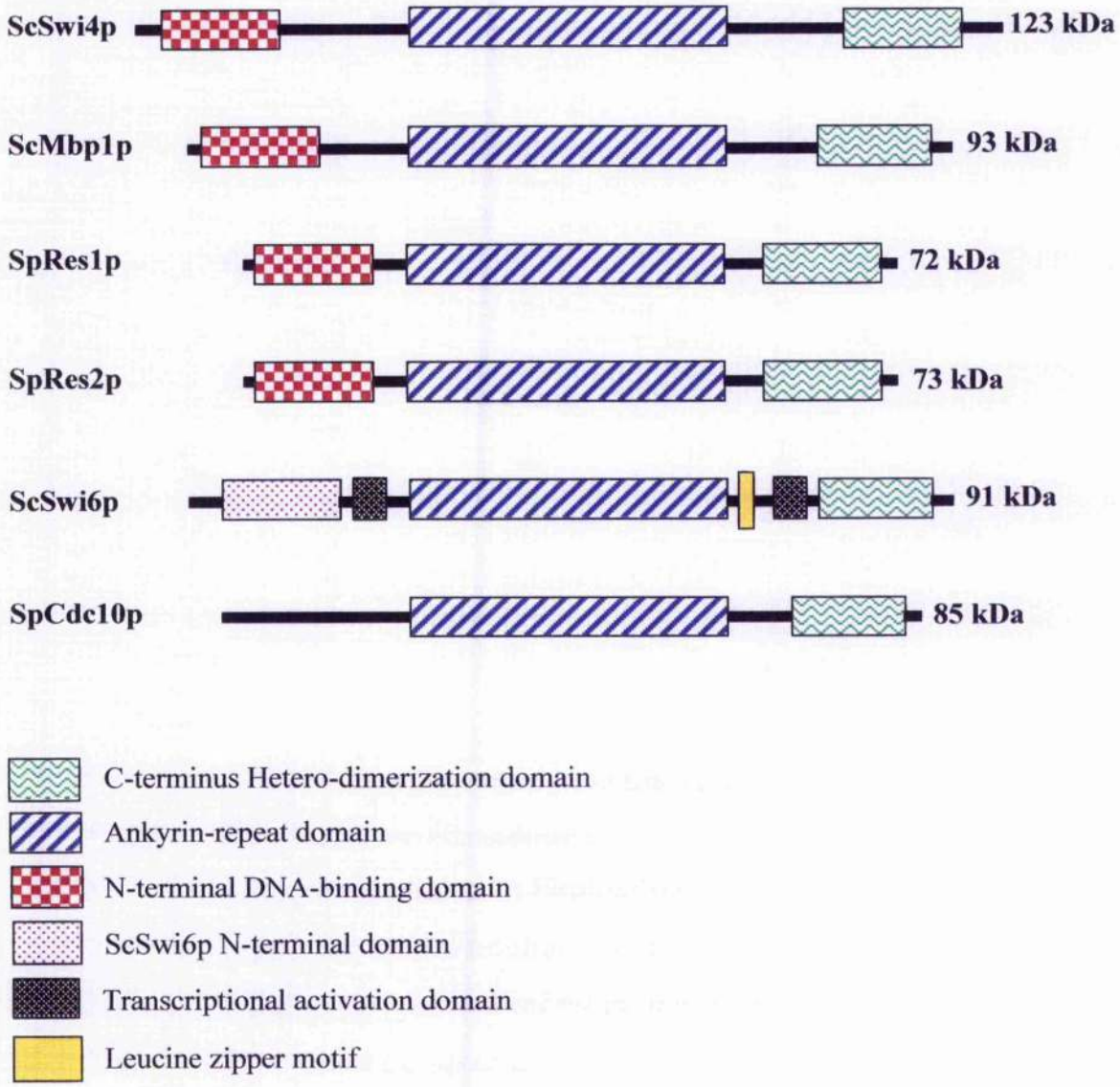


Figure 1I A schematic of the domain architecture of some of the components of MBF, SBF and DSC1. A schematic representation of the major domains in ScSwi4p, ScMbp1p, SpRes1p, SpRes2p, ScSwi6p and SpCdc10p: (note that the N-terminus domain of ScSwi6p and ScCdc10p does not bind to DNA).

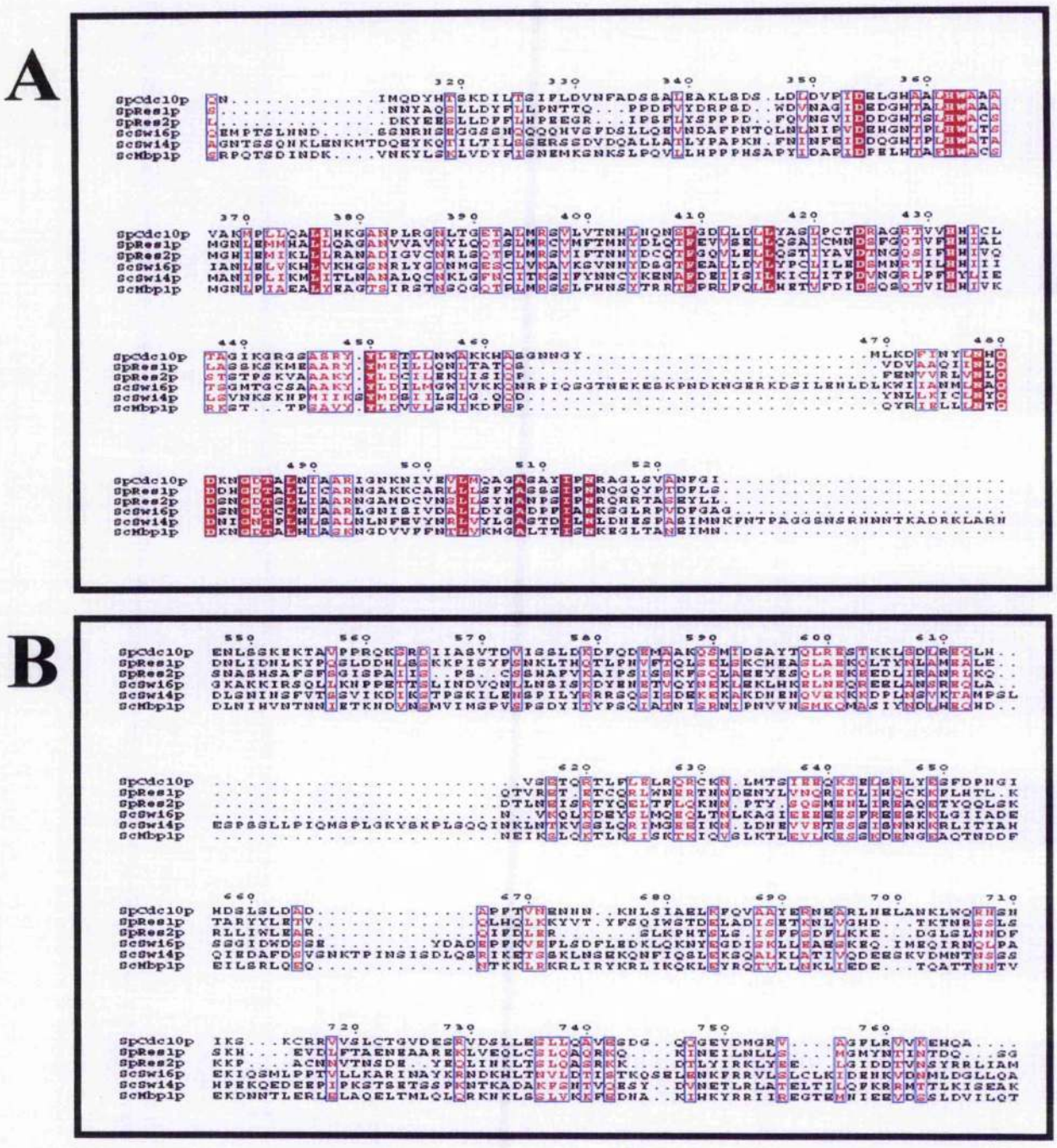


Figure 1J Alignments of homologous regions of the different components of MBF/SBF and DSC1 transcription factor complexes. A. Alignment of SpCdc10p, SpRes1p, SpRes2p, ScSwi6p, ScSwi4p and ScMbp1p, ankyrin-repeat domain and **B.** heterodimerization domain. Identical residues are shown as white characters, boxed in red and similar residues are shown in red characters, boxed in white. This figure was generated using the ESPript program (Gouet *et al.* 1999).

1.6.3 G_1-S phase specific transcription in fission yeast

In fission yeast the activity of SpCdc2p coupled with G_1 cyclins is required to pass START. A complex, functionally related to SBF/MBF in budding yeast, exists in fission yeast called DSC1 (DNA synthesis control 1) that is responsible for the periodic transcription of several genes, including the G_1 cyclins SpPuc1p, SpCig1p and SpCig2p (Fisher & Nurse 1995). Contained within the promoters of these genes are the MCB motifs, equivalent to those found in budding yeast, that bind with the DSC1 transcription factor complex. At least 10 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, Lowndes *et al.* 1992, Ayte *et al.* 2001, Maqbool *et al* 2003) have been directly shown to be under the control of DSC1 during mitosis.

DSC1 comprises at least four proteins; SpRes1p, SpRes2p, SpRep2p and SpCdc10p 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). The SpRes1p and SpRes2p are the MCB-specific DNA binding subunits of DSC1, which interact with the regulatory subunits, SpCdc10p and SpRep2p. Comparison of the amino acid sequences of ScSwi4p and ScSwi6p with SpCdc10p reveals remarkable homology between the proteins (Figure 1J) and proposes a conserved mechanism manages G_1-S specific transcription across both yeast species (Merrill *et al.* 1992).

In addition, another DSC1-like complex has recently been identified that regulates the expression of genes required in meiosis. This complex comprises at least SpRes2p, SpCdc10p and SpRep1p (Cunliffe *et al.* 2004). Microarray anaysis has identified approximately 100 genes that are transcribed specifically during late G_1-S phase in the meiotic cell cycle, which also have MCB enriched promoter regions (Mata *et al.* 2002). The meiotic DSC1 complex controls the transcription of genes that are required for both mitosis and meiosis (e.g. *cdc22*$^+$ and *cdc18*$^+$) and also genes that are exclusively required for meiosis (e.g. the *rec*$^+$ genes – required for recombination) (Cunliffe *et al.* 2004).

1.6.3.1 SpCdc10p

The 85 kDa protein SpCdc10p (Figure 11) (Aves *et al.* 1985) is an essential component of the DSC1 transcription factor complex in both mitosis and meiosis (Baum *et al.* 1998, Beach *et al.* 1985, Lowndes *et al.* 1992, Nurse & Bissett 1981). SpCdc10p shares remarkable homology with ScSwi4p and ScSwi6p (Merrill *et al.* 1992), especially in the ankyrin repeat motif (characteristic of the SpCdc10p/ScSwip family of proteins), but is not able to bind DNA, similar to ScSwi6p, and relies on interactions with the DNA binding proteins SpRes1p and SpRes2p (Aves *et al.* 1985, Breeden & Nasmyth 1987, McInerny *et al.* 1995, Zhu *et al.* 1994). The ankyrin motifs and the C-terminus of SpCdc10p have been shown to be important for normal function (Reymond & Simanis 1993) as the majority of *cdc10* temperature sensitive (*ts*) mutants are affected within the ankyrin-repeat domain, although as yet have an undefined role (Reymond *et al.* 1992). The *ts* mutant *cdc10-C4*, which encodes a protein, truncated of 61 amino acids at the C-terminus by a non-sense mutation in the gene, displays altered regulatory properties at high and low temperatures (McInerny *et al.* 1995). In *cdc10-C4* mutant cells, genes that are under DSC1 control show a loss in periodicity, becoming constitutively transcribed throughout the cell cycle, indicating that SpCdc10p has both positive and negative roles in the control of MCB regulated gene expression (McInerny *et al.* 1995)

1.6.3.2 SpRes1p and SpRes2p

SpRes1p and SpRes2p are 72 kDa and 73 kDa proteins, repectively (Figure 11), and display common structural features associated with general transcription factors. Between themselves, their N-termini are strikingly similar and confer the DNA binding domains of both proteins. The similarities extend to the centrally located ankyrin-repeat motif, and other common architectural features, which affirms their place in the SpCdc10/ScSwip/ScMbp1p family of transcription factors (Tanaka *et al.* 1992, Caligiuri & Beach 1993, Miyamoto *et al.* 1994, Zhu *et al.* 1994, Ayte *et al.* 1995, Zhu *et al.* 1997)

Three separate groups identified SpRes1p, as both a suppresser of the fission yeast *pat1-114* mutation (Tanaka *et al.* 1992, Marks *et al.* 1992, Caligiuri & Beach 1993) and as a Cdc10p interacting partner (Caligiuri & Beach 1993). SpRes1p is non-

essential and has a major role in G_1-S phase specific transcription during the mitotic cell cycle. This is evident in Δ*res1* mutants, where MCB-regulated transcription is low and constant (Baum *et al.* 1998), and also when *res1*$^+$ is overexpressed causing an increase in MCB regulated transcription (Ayte *et al.* 1995, Baum *et al.* 1998). However, SpRes1p is not thought to have a significant role in the meiotic cell cycle, as Δ*res1* mutants show no impairment in their progression through meiosis (Caligiuri & Beach 1993, Tanaka *et al.*, 1992). Also SpRes1p levels decrease during meiosis (Ayté *et al.*, 1997) therefore SpRes1p is thought to inhibit entry into meiosis (Caligiuri & Beach 1993, Tanaka *et al.* 1992).

Subsequently, SpRes2p was identified as a suppressor of the Δ*res1* mutant in a genetic screen designed to identify DNA-binding partners of SpCdc10p (Miyamoto *et al.* 1994, Zhu *et al.* 1994). The demonstration that SpRes2p, together with SpCdc10p, bound specifically to MCB elements both *in vitro* and *in vivo*, confirmed that, like SpRes1p, it was a DNA-binding partner of SpCdc10p (Zhu *et al.* 1994) and is found associated with the DSC1 complex throughout the cell cycle (Ayte *et al.* 1997, Whitehall *et al.* 1999). Although SpRes2p is non-essential, the deletion of *res2*$^+$ exhibits a loss of periodic transcription by MCB controlled genes (Baum *et al.* 1998). Initial suggestions were that SpRes2p was a negative-regulator of SpCdc10p during the cell cycle by binding to the C-terminus of SpCdc10p, although this theory was quashed by showing that SpRes2p associated with the truncated SpCdc10-C4p protein, which confers constitutively high expression of MCB regulated genes (Whitehall *et al.* 1999). Therefore SpRes2p does not simply act as an activator or repressor of transcription but in fact the SpCdc10p-SpRes2p complex is thought to require the association of SpRep2p for mitotic specific trans-activation (Nakashima *et al.* 1995, Tahara *et al.* 1998).

SpRes2p also has an essential role in the meiotic cell cycle as overexpression of *res2*$^+$ enhances meiotic entry (Ayte *et al.* 1997) and Δ*res2* mutants are unable to complete meiosis (Miyamoto *et al.* 1994, Zhu *et al.* 1994). Following entry into meiosis, levels of SpRes2p increase while SpRes1p levels decrease which is in accord with SpRes2p being induced under nitrogen starvation conditions in diploid cells (Miyamoto *et al.* 1994, Ayte *et al.* 1997).

The overall mechanism of the role of SpRes1p and SpRes2p in DSC1 regulated transcription is not yet fully understood but investigations into their function reveals that there is overlapping function between these two DNA binding proteins.

As mentioned SpRes1p and SpRes2p are principally proteins required for mitosis although SpRes2p also has a distinct meiotic role. The double deletion of both *res1*$^+$ and *res2*$^+$ is lethal to fission yeast, however overexpression of *res2*$^+$ rescues the lethality of cells deleted for *res1*$^+$ (Miyamoto *et al.* 1994). Furthermore overexpression of either gene can rescue the conditional lethality of *cdc10-129* mutants suggesting that SpRes2p can compensate for loss of SpRes1p function (Miyamoto *et al.* 1994). Phenotypic analysis of cells deleted for *res1*$^+$ and *res2*$^+$ show that SpRes1p is only required for mitosis whereas SpRes2p is required in both mitosis and meiosis but predominantly in meiosis (Tanaka *et al.* 1992, Caligiuri & Beach 1993, Miyamoto *et al.* 1994, Zhu *et al.* 1994).

1.6.3.3 SpRep1 and SpRep2p

SpRep1p and SpRep2p are 53 kDa and 25 kDa proteins, respectively which share highly homologous C-terminus zinc finger motifs that are essential for their function (Figure 1K). Genetic suppressor analysis indicates that there is functional redundancy between the SpRep1p and SpRep2p however in contrast SpRep1p and SpResp2 proteins have distinctly separate meiotic and mitotic roles, respectively (Sugiyama *et al.* 1994, Nakashima *et al.* 1995).

The deletion of *rep1*$^+$ has no effect on mitotically dividing cells, which coincides with the *rep1*$^+$ gene not being expressed during the mitotic cell cycle (Sugiyama *et al.* 1994). Δ*rep2* cells, during the mitotic cycle, display cold sensitivity (although viable at 30°C) at temperatures below 18°C and become arrested at START yet are unaffected for meiosis with the majority of cells completing the sexual cycle. Conversely, Δ*rep1* cells are unable to initiate pre-meiotic DNA synthesis and are unaffected for the vegetative cycle (Sugiyama *et al.* 1994, Ding & Smith 1998, Cunliffe *et al.* 2004). Overexpression of *rep1*$^+$ rescues the cold sensitive lethality of Δ*rep2* cells, demonstrating that SpRep1p can compensate for loss of SpRep2p mitotic function. Also, overexpression of either *rep1*$^+$ or *rep2*$^+$ rescues the conditional lethality of *cdc10-129* mutants, which is how both proteins were first cloned (Sugiyama *et al.* 1994, Nakashima *et al.* 1995).

Therefore, the proposed roles of both SpRep1 and SpRep2 proteins are as activators of DSC1-mediated transcription in meiosis and mitosis, respectively. In support of this theory is the demonstration that SpRep2p may act as a transcriptional

activator subunit for SpRes2p (Nakashima *et al.* 1995, Tahara *et al.* 1998) possibly through the centrally located SpRes2p binding and C-terminus activation domains of SpRep2p (essential for its function) (Nakashima *et al.* 1995, Tahara *et al.* 1998).

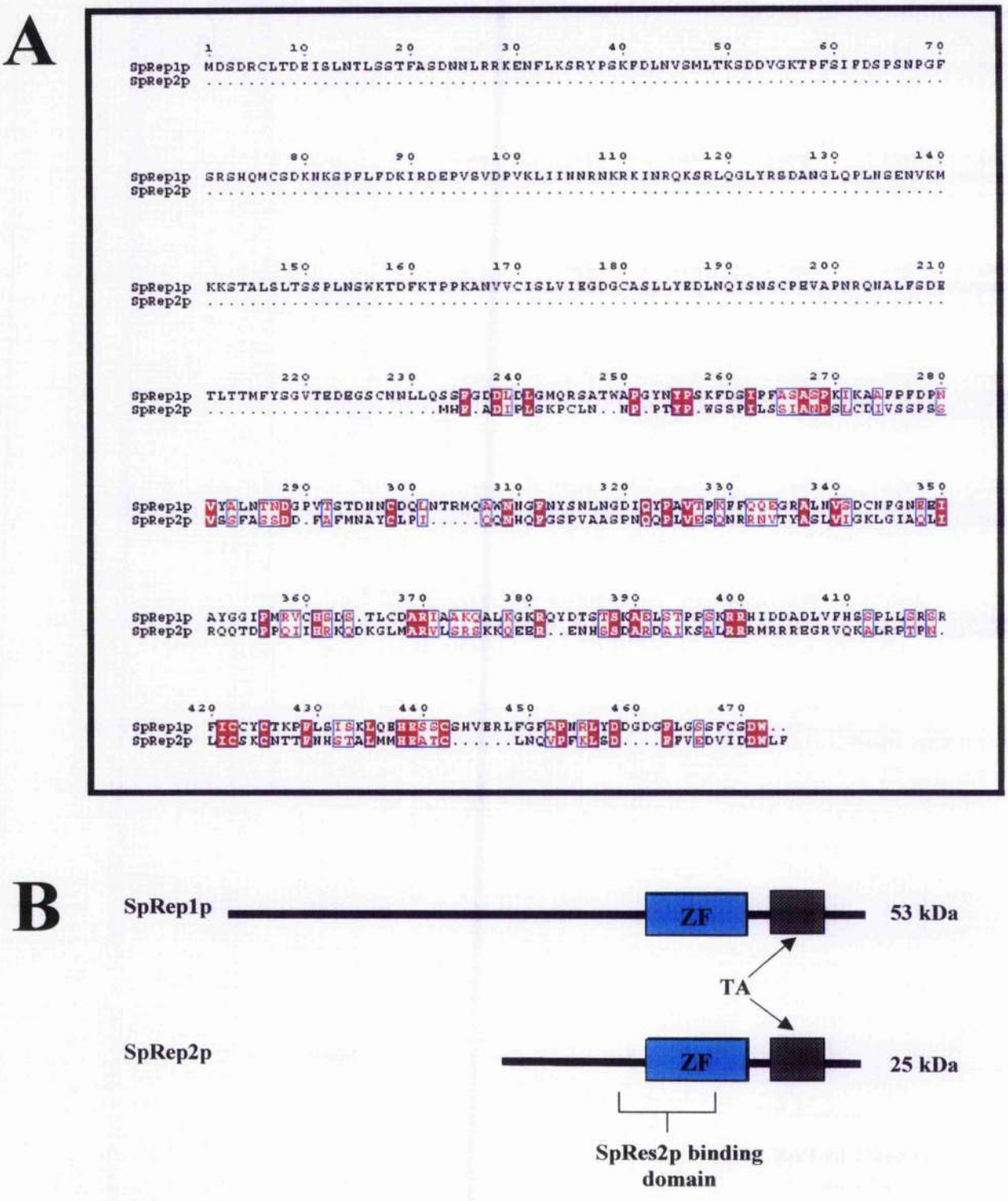


Figure 1K SpRep1p and SpRep2p structural comparison. A. Sequence alignment of SpRep1p and SpRep2p. Identical residues are shown as white characters, boxed in red and similar residues are shown in red characters, boxed in white. This figure was generated using the ESPript program (Gouet *et al.* 1999). **B.** Domain architecture of SpRep1p and SpRep2p schematic representation of the major domains of SpRep1p and SpRep2p (adapted from Nakashima *et al.,* 1995). TA (Transcriptional activation domain), ZF (Zinc finger motif).

1.6.4 Regulation of DSC1-dependant gene expression

The current proposed model for DSC1-dependant gene expression argues that SpRes1p and SpRes2p work together with SpCdc10p to form a single mitotic complex. Evidence supporting this idea comes from electro-mobility shift assays that show that the DSC1 activity produced from fission yeast mitotic extracts contained at least SpRes1p, SpRes2p and SpCdc10p (Ayte *et al.* 1997, Zhu *et al.* 1997). Band-shift activity of DSC1 is also lost in cells deleted for either of the *res* genes, suggesting that both of the proteins are necessary for complex formation (Ayte *et al* 1997, Zhu *et al.* 1997). In addition, SpRes1p and SpRes2p form a heterodimeric complex *in vitro*, dependant on SpCdc10p (Zhu *et al.* 1997).

Further evidence, *in vivo*, confirmed a heterotrimeric complex composed of SpRes1p, SpRes2p and SpCdc10p, existed throughout the mitotic cell cycle (Ayte *et al.* 1997, Reymond *et al.* 1993, Whitehall *et al.* 1999), proposing that the DSC1 complex comprises all three proteins in the control of G_1-S phase specific transcription in fission yeast (Whitehall *et al.* 1999).

Genetic analysis shows that the SpPas1p-SpPef1p kinase has an apparent role in the activation of SpRes2p by means not yet fully understood although it has been suggested that SpPas1p-SpPef1p kinase might directly phosphorylate SpRes2p (Tanaka & Okayama 2000). Conversely, the mechanisms of repression of DSC1 activity are not completely understood, nonetheless, it is partly explained by SpCig2p-SpCdc2p kinase activity, which binds to SpRes2p and phosphorylates SpRes1p, causing repression of DSC1-dependant transcription (Ayte *et al.* 2001). Therefore a negative feedback loop appears to down-regulate DSC1-dependant transcription as SpCig2p is itself a transcriptional target of DSC1 (Ayte *et al.* 2001).

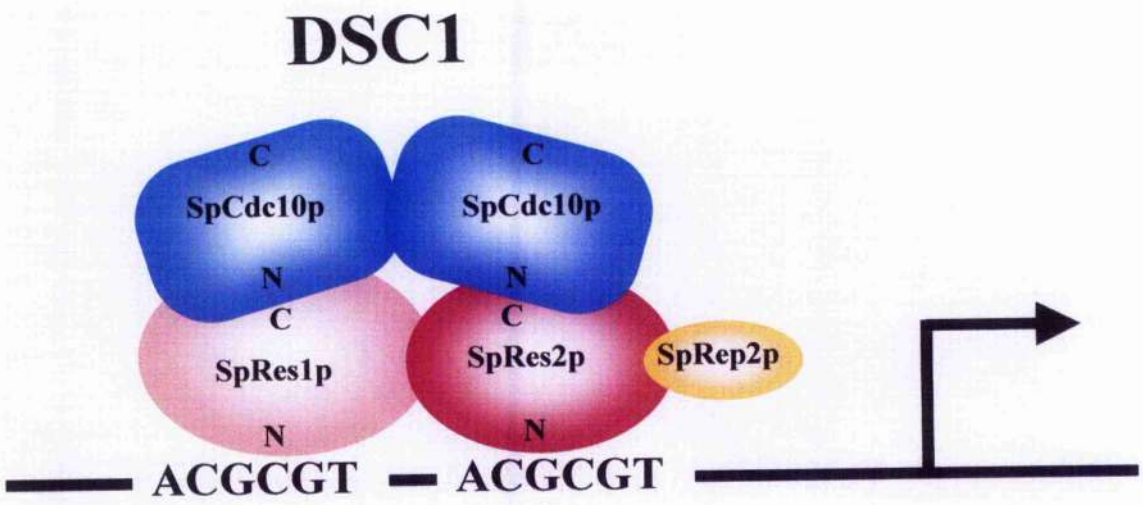


Figure 1L A schematic representation of the DSC1 (DNA synthesis control 1) DNA-binding complex in fission yeast. The DSC1 complex binds to MCB (*Mlu*I cell-cycle box) promoter sequence. The N and C-termini of the individual proteins are in black.

1.6.5 G_1-S phase specific transcription in mammalian cells

In mammalian cells, the passage of Restriction point and entry into S phase is governed by a G_1-S phase specific programme of transcription, which is overseen by the E2F family of transcription factors (Dyson 1998), in a scenario similar to both budding and fission yeasts. This suggests that a homologous G_1-S transcriptional control system exists in higher eukaryotes.

Similar to yeast, genes expressed under the control of the E2F system encode proteins required for S-phase onset and completion of DNA synthesis and replication. A few examples in this vast array of E2F targets are; the HsCdc6p, HsOrc1p and HsMcmp proteins and also key cell cycle regulators such as cyclin A, cyclin E and HsCdk1p (Ren *et al.* 2002).

The promoters of these target genes contain a common consensus sequence, 5'-TTTCGCGC-3', which is bound by E2F, and resembles the MCB/SCB elements (Dyson 1998). Although there are marked similarities between the human and yeast G_1-S transcription systems there is no primary sequence homology between E2F proteins and MBF/SBF or DSC1 of either yeast species, despite being involved in an analogous process.

1.6.5.1 The E2F and DP proteins

E2F functions as a heterodimeric complex that is composed of one subunit from a family of E2F proteins and another subunit from the DP family of proteins. In total seven E2F proteins (E2F-1 to E2F-7) and two DP proteins (DP-1 and DP-2) have been characterised in mammals (Figure 1M) (Trimarchi & Lees 2001, DeBruin *et al.* 2003). Both families of proteins contain highly conserved DNA-binding and dimerization domains. E2Fs 1-5 also contain transactivation and pocket protein binding domains in their C-termini that are absent in E2F 6 and 7 and also the DP proteins (Figure 1M) (Trimarchi & Lees 2002, DeBruin *et al.* 2003). Not a great deal is known about the explicit properties of the individual E2Fs although the DP subunits are believed to activate transcription by enhancing E2F subunit activity. Each E2F protein can be placed into one of three different subtypes based on their structural and functional properties and their ability to activate or repress transcription (Dyson 1998).

E2Fs 1-3: These proteins promote transcription from E2F responsive genes and drive proliferation, and are thereby placed in the "activating E2Fs" category. Activating E2Fs are cell cycle regulated and are periodically expressed at the G_1-S boundary.

E2Fs-4 and -5: These proteins come under one of the two types of "repressive E2Fs". Unlike E2Fs 1-3, these two proteins are constitutively expressed throughout the cell cycle and are most commonly present in G_0 cells. They associate with pocket proteins and bind to promoters in an inactive form only to be activated by the release of the pocket proteins.

E2Fs-6 and -7: These two proteins come under the second type of transcriptional repressors. However unlike the other E2Fs, these proteins lack the C-terminus transactivation and pocket-binding domains, making them insensitive to the pRB family of pocket proteins. E2F-6 and 7 are thought to repress transcription by binding to promoters and thereby blocking the action of activating E2F complexes. E2F-6 works in this manner and can recruit members of the polycomb chromatin re-modelling family to repress chromatin (Stevens & LaThangue 2003). E2F-7 is slightly different compared to all other E2F and DP family members, in that the dimerisation domain is replaced with a second DNA-binding domain, suggesting it can associate with DNA without the need of a DP partner (DeBruin *et al.* 2003, Logan *et al.* 2004).

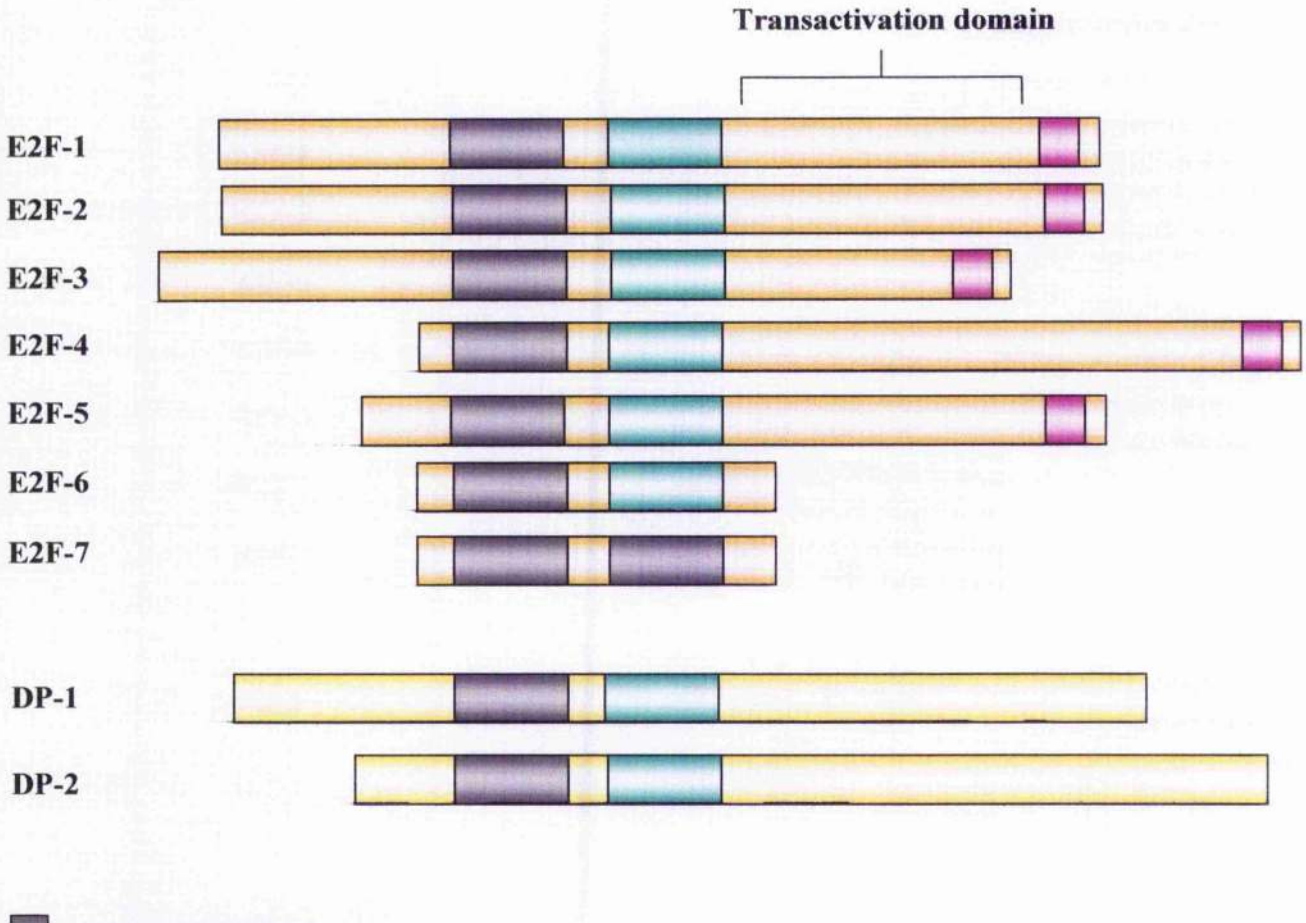


Figure 1M A structural comparison of the E2F and DP family of proteins. E2F and DP proteins share a conserved DNA-binding domain and hetero-dimerization domain. E2Fs 1-5 also have pocket-binding sites. (Note: E2F-7 does not have a dimerization domain but a second DNA-binding domain). This figure was adapted from Stevens & LaThangue (2003).

1.6.5.2 Regulation of E2F-dependant gene expression

Mammalian transcriptional control is a highly complex network of processes that is as yet to be fully comprehended. Central to the control of E2F-dependant transcription is the pRB family of pocket proteins. pRB proteins function to negatively regulate E2F depending on its phosphorylation status. *RB* was the first tumour suppressor gene to be discovered and disruption in pRB function is involved in the majority of all human cancers (Weinberg 1995).

The mechanism by of mammalian G_1-S transcription as it is understood so far is as follows: In G_0 and early G_1 phases, hypophosphorylation of pRB enables it to associate with E2F. Upon the action of both positive and negative regulatory growth signals (Sherr 1996) a signalling cascade is initiated and ultimately leads to the activation of the HsCdk4p/6p-cyclin D kinase activities. The active kinase complexes then phosphorylate pRB, making it hyperphosphorylated and thereby reducing its affinity for E2F. The release of E2F from pRB allows E2F to activate expression of many genes that are required for entry into S phase and DNA replication (Dyson 1998). The association of pRBs to pocket-binding sites of E2Fs are thought to physically block the C-terminus transactivation domain and render E2F inactive. Chromatin-remodelling machinery (e.g. histone deacetylases and histone methyl transferases) can repress transcription through changes in chromatin structure, in a pRB-dependant manner (Stevens & LaThangue 2003).

As described earlier, E2F can bind to promoters and cause activation or repression of transcription depending on its association with pocket proteins. Therefore this divergence in role led to the generation of three groups of E2F complexes (Dyson 1998). (1) "activator E2Fs", where the activation domain of promoter bound E2F is free to promote transcription. (2) "type I repressor E2Fs", where the activation domain of promoter bound E2F is blocked by pRB pocket proteins. (3) "type II repressor E2Fs", where pRB pocket proteins recruit and assemble chromatin-remodelling machinery to repress chromatin.

The precise mechanism by which E2F controls G_1-S transcription is not known. However, the method by which HsCdk2p-cyclin A can both bind to E2F-1 and phosphorylate DP-1, thereby inhibiting DNA-binding activity, is reminiscent of the negative feedback loop of SpCig2p on DSC1 activity in fission yeast, as cyclin A is a transcriptional target of E2F (Stevens & LaThangue 2003).

1.7 Project aims

This project aimed to identify novel proteins that interacted with SpCdc10p with particular focus on the regulatory properties of the C-terminus of SpCdc10p. The identification of SpPol5p prompted a series of experiments to investigate the role of the DNA polymerase SpPol5p and its intriguing association with the cell cycle transcription factor SpCdc10p.

Recently budding yeast ScPolVp has been shown to be involved in the production of rRNA. High sequence homology with ScPolVp suggests that fission yeast SpPol5p may also be involved in the same process. The association of SpPol5p with SpCdc10p suggests that SpPol5p is a missing link between the two separate though linked processes of cell cycle and cell growth. This thesis aims to investigate this hypothesis further.

Chapter 2

Materials & Methods

2.1 Bacterial materials

2.1.1 Bacterial chemicals

All chemicals used were of the highest grade available commercially and the distilled water was of Millipore-Q quality. Ampicillin, kanamycin and NZ amine were from Sigma. One Shot™ ultracompetant InvαF *Escherichia coli (E.coli)* cells were purchased from Invitrogen and the QIAprep® Spin Miniprep Kit was supplied by QIAGEN.

2.1.2 Bacterial Media

All strains of *E.coli* were grown in Luria Broth (LB; 10 g Bacto tryptone, 5 g Bacto yeast extract and 10 g NaCl per litre pH 7.5) or NZY^+ broth (5 g NaCl, 2 g $MgSO_4.7H_2O$, 5 g yeast extract, 10 g NZ amine per litre pH 7.5). LB plates were made by adding 7.5 g bacto agar to 500 ml LB. All media were autoclaved before use and supplemented with ampicillin (50 $\mu g\ ml^{-1}$) or kanamycin (30 $\mu g\ ml^{-1}$) where appropriate.

2.1.3 Bacterial strains and vectors

All bacterial strains and vectors used in this thesis are listed in Appendix I. The annotation GB refers to the Glasgow lab bacteria collection number. One Shot™ InvαF ultracompetant *E.coli* (Invitrogen), *E.coli* DH5α and *E.coli* BL21 CodonPlus(DE3)-RIL (Stratagene) strains were routinely used.

2.2 Bacterial methods

Based on methods devised by Sambrook & Russell (2001)

2.2.1 Preparation of electrocompetent bacterial cells

The appropriate bacterial strain was streaked out onto LB plates overnight at 37°C. A single colony was picked to inoculate a 10 ml LB culture overnight. The following day the culture was transferred to another 200 ml LB culture and grown at 37°C, shaking until the culture reached an optical density (OD) of between 0.5-0.6 at 595 nm. The culture was placed on ice for 30 minutes and then harvested by centrifugation at 3,000 rpm for 10 min at 4°C. The supernatant was poured off and cells were washed by resuspending

the cell pellet in 10 ml of ice-cold sterile dH_2O. Cells were pelleted again at 3,000 rpm for 10 min and this step was repeated. The cells were then resuspended in 10 ml sterile ice-cold 10% glycerol. Cells were divided into 100 µl aliquots and used immediately or stored at -70°C.

2.2.2 Transformation of competent bacterial cells using electroporation

The BIORAD *E.coli* pulser was used in electro-transformation using the method adapted from the BIORAD manual. To 50 µl of competent DH5α *E.coli* cells, 1-2 µl of the required plasmid DNA was added and left on ice for 1 min. The DNA-cell mix was then transferred to a pre-chilled 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 immediately to the cells and transferred to a 1.5 ml screw cap microfuge tube and incubated in a 37°C shaker for 45-60 min. Transformed cells were plated onto LB plates containing appropriate antibiotic and incubated at 37°C overnight.

2.2.3 Transformation of competent bacterial cells using heat-shock

OneShot™ InVαF and BL21 CodonPlus(DE3)RIL *E.coli* cells were used for propagation of recombinant plasmid DNA and for the expression of recombinant proteins respectively, and were routinely transformed using the heat-shock method. To 50 µl of competent cells 1-5 µl of plasmid DNA was added to a pre-chilled microfuge tube. The mixture was kept on ice for 15 min and heat shocked at 42°C for 90 sec and then returned to ice for 2 min. 450 µl of SOC was added and the mixture was incubated in a 37°C shaker for 45-60 min. Transformed cells were plated onto LB plates containing appropriate antibiotic and incubated at 37°C overnight.

2.2.4 Plasmid miniprep

10 ml of LB plus antibiotic was inoculated and grown from a single colony. Plasmid DNA was purified from the culture using the QIAprep® Spin Miniprep Kit using the protocol in the manufacturer's manual. After no more than 16 h incubation at 37°C cells were pelleted at 13,000 rpm for 1 min and then resuspended in 250 µl buffer P1 (50 mM

Tris/HCl pH 8.0, 10 mM EDTA, RnaseA 100 μg ml^{-1}). Cells were then lysed by addition of 250 μl buffer P2 (0.2 M NaOH, 1% SDS) and incubated for 5 min. 350 μl of buffer N3 (2.55 M KOAc pH 4.8) was added and the mixture was then centrifuged at 13,000 rpm for 10 min to pellet cell debris. 2 x 220 μl of supernatant was put through a QIAprep column to allow DNA to bind to the column. The column was washed with 750 μl buffer PE and spun twice to remove all trace of buffer. Finally to elute the DNA, 50 μl of nuclease-free water was added. Agarose gel electrophoresis was used to determine DNA quality and yield.

2.3 DNA materials

2.3.1 DNA chemicals

Specialised chemicals such as Sephadex G-50, [α-P^{32}] dCTP (3,000 Ci/mmol) were obtained from Amersham Pharmacia Biotech. N_6 (random hexanucleotide mixture), Klenow buffer and DNA molecular weight marker X (0.07-12.3 Kb) were obtained from Roche and glass beads from Sigma.

2.3.2 DNA enzymes and kits

All restriction enzymes, T4 DNA ligase, calf intestinal alkaline phosphatase, dNTPs, *Taq* DNA polymerase were obtained from Promega. $Vent_R$ DNA polymerase was obtained from New England Biolabs. Expand High Fidelity PCR system was obtained from Roche. QIAquick® Gel Extraction Kit was supplied by QIAGEN. Ribonuclease A was obtained from Sigma and Superscript® Reverse Transcriptase kit was obtained from Invitrogen.

2.3.3 Oligonucleotides

Oligonucleotides required were synthesised by MWG-AG Biotech and DNA technology A/S. All oligonucleotides used in this thesis are listed in Appendix II. The annotation GO refers to Glasgow lab oligonucleotide collection number. Sequencing service was provided by MWG-Biotech

2.4 DNA methods

2.4.1 Polymerase chain reaction

All PCR reactions were carried out in a MWG-Biotech Primus Thermal Cycler using one of the following polymerises: *Taq* DNA polymerase, Vent$_R$ DNA polymerase, Expand High Fidelity PCR System.

2.4.1.1 PCR using *Taq* DNA polymerase

PCR reactions were carried out using 0.5 ml thin walled PCR tubes and the following were added in a final volume of 100 µl: 10 x Thesit buffer (300 mM Tris-HCl pH 8.5, 20 mM $MgCl_2$, 50 mM β-mercaptoethanol, 1% Thesit (polyoxyethylene-9-lauryl ether), 0.1% gelatin (non-essential) at a 1 x final concentration, 200 µM of each dNTP, 100 ng of each primer, 50 ng of DNA template and finally 2 U of *Taq* DNA polymerase. The reaction mix was placed in the thermal cycler and run using a typical program as follows:

Denaturation	94°C for 5 min
[LOOP 30 x	
Denaturation	94°C for 15 sec
Annealing*	50°C for 30 sec
Extension	72°C for 1 min$^+$
Close LOOP]	
Final Extension	72°C for 5 min
Incubation	4°C forever

(*Annealing temperatures varied depending on oligonucleotides used (Tm calculated using the equation: Tm = 4 x (G + C) + 2 x (A + T)) and $^+$extension time typically 1 min per kb product.)

2.4.1.2 PCR using $Vent_R$ DNA polymerase

The PCR reaction was carried out as described for *Taq* DNA polymerase with the following adjustments. In a 100 µl final volume containing 10 x thermopol buffer (100 mM KCl, 100 mM $(NH_4)_2SO_4$, 200 mM Tris-HCl pH 8.8, 80 mM $MgSO_4$, 1% Triton X-100) at 1 x final concentration, 200 µM of each dNTP, 100 ng each of appropriate oligonucleotides, 50 ng of DNA template and 2 U of $Vent_R$ DNA polymerase.

2.4.1.3 PCR using Expand High Fidelity PCR system

The PCR reaction was carried out as described for *Taq* DNA polymerase with the following adjustments. In a 100 µl final volume containing 10 x Expand High Fidelity buffer with 15 mM $MgCl_2$ at a 1 x final concentration, 200 µM of each dNTP, 100 ng each of appropriate oligonucleotides, 50 ng of DNA template and 2.6 U Expand High Fidelity mix.

2.4.2 Isolation of genomic DNA

10 ml of *Schizosaccharomyces pombe* cells were grown for 2-3 days at permissive temperature until saturation point. Cells were harvested by centrifugation at 3,000 rpm for 5 min. The cells were then resuspended in 0.5 ml dH_2O and transferred to a 1.5 ml screw cap microfuge tube where they were pelleted by a short 5 sec spin. Supernatant was discarded and the cells were resuspended in the residual liquid before adding 0.2 ml solution A (10% Triton, 10 % SDS, 1 M NaOH, 1 M Tris-HCl pH 7.5, 0.5 M EDTA), 0.2 ml phenol:chloroform (1:1) and 0.3 g acid washed glass beads. Cells were lysed using the HYBAID Ribolyser at 3 x 40 sec bursts at setting 4. 400 µl of TE (10 mM Tris-HCl pH 8, 1 mM EDTA) was added followed by centrifugation at 13,000 rpm for 5 min. The aqueous layer only, containing both DNA and RNA, was transferred to a fresh 1.5 ml microfuge tube. 1 ml of 100% ethanol was added to the aqueous phase and gently mixed by inverting to avoid breakage of large DNA fragment. This was then pelleted by centrifugation at 13,000 rpm for 5 min and supernatant discarded. An additional 1 min spin was run to remove excess ethanol. The pellet was resuspended in 400 µl of TE and 3 µl Ribonuclease A (50 µg/ml) and incubated at 37°C for 5 min to digest unwanted

RNA. 1 ml of 100 % ethanol and 8 µl ammonium acetate was then added, gently mixed by inversion and stored at -70°C for 5-10 min. The DNA was pelleted by centrifugation at 13,000 rpm for 10 min and the supernatant discarded. The pellet was air-dried and resuspended in 100 µl TE and the DNA stored at -20°C.

2.4.3 Agarose gel electrophoresis

For the required percentage gel, routinely 1%, the appropriate amount (1 g 100 ml^{-1}) of agarose was dissolved in 1 x TBE (45 mM tris-borate, 1 mM EDTA) then 2-3 µl ethidium bromide was added to give a slab of agarose gel. Samples for analysis were diluted 5 fold by the addition of 6 x loading buffer (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, 0.15% (w/v) Ficoll) before being loaded on the agarose gel. Gels were run at 100 V in 1 x TBE buffer for between 40 min and 1 h depending on product size or until dye was about 1 cm from bottom of gel. Gels were viewed using a UV transilluminator and photographed using E. A. S. Y imaging software.

2.4.4 DNA extraction from agarose gel

The QIAquick Gel Extraction Kit (QIAGEN) was used to purify DNA of 70 bp to 10 kb from agarose gels according to manufacturer's instructions.

Briefly, DNA was excised from the gel using a sterile scalpel. 3 x gel volumes of Buffer QG (supplied) was added to the gel and dissolved at 50°C. The melted agarose was applied to a QIAquick spin column and centrifuged for 1 min. The supernatant was discarded and the DNA was now bound to the column. 750 µl Buffer PE (containing 80% (v/v) ethanol) was added to wash the column. The column was centrifuged for 1 min to remove buffer and then spun for a further minute to remove excess ethanol. Finally DNA was eluted by adding 50 µl Buffer EB (1 mM Tris-HCl pH 8.5) or nuclease-free water and stored at -20°C. The quality and quantity of DNA was analysed by agarose gel electrophoresis.

2.4.5 Restriction Digestion

Prior to ligations or for restriction analysis, PCR products and plasmids are routinely digested as follows: 2-5 µl DNA was digested using 1 µl restriction enzyme for a single digest or 1 µl of each enzyme for a double digest, together with 2 µl of appropriate enzyme buffer in a 20 µl final volume. The reaction was incubated at 37°C for approximately 1 h with some exceptions where manufacturer guidelines were observed.

2.4.6 Dephosphorylation of digested plasmid

Digested plasmid was dephosphorylated by the addition of 1 U calf intestinal alkaline phosphatase and incubated for a further 30 min at 37°C.

2.4.7 Ligations

Digested insert DNA was ligated into digested, dephosphorylated purified plasmid at various ratios (1:3, 1:5, 1:7). A standard ligation reaction was carried out as follows: 1 µl plasmid was mixed with 5 µl insert, 10 x T4 DNA ligase buffer at a 1 x final concentration and 1 U of T4 DNA ligase. The reaction mix was incubated at 14°C overnight and the following day transformed into DH5α competent cells using the standard protocol (Section 2.2.2). The resulting colonies were screened by restriction analysis (Section 2.3.8) to confirm presence of insert DNA.

2.4.8 TA Cloning of blunt ended PCR products

The TA Cloning® Kit (Invitrogen) was used to clone blunt-ended PCR products as described in the manufacturer's manual.

2.4.9 Quantification of nucleic acids by spectrophotometry

DNA and RNA samples were diluted 100-fold in dH_2O and the concentration estimated by measuring the absorbance at 260 nm on a spectrophotometer. The following equation was used to calculate DNA or RNA concentration in μg/μl:

A_{260} of 1.0 = 40 μg/ml RNA *or* 50 μg/ml DNA

Therefore multiply A_{260} by dilution factor (**x 100**)
then **x 40** (RNA) or **x 50** (DNA)
divide by 1,000 = μg/μl

2.4.10 Preparation of radio-labelled probe

Probes for northern blot analysis were prepared by random hexanucleotide (N_6) primed labelling of PCR generated DNA fragments. To a screw-cap microfuge tube the following were added in order: 5 μl probe DNA made up to 11 μl with dH_2O (denatured at 94°C for 5 min then placed on ice for 5 min), 2 μl N_6 (random hexanucleotide mixture), 2 μl Klenow Buffer, 2 μl dCTP and 2 μl [α-P^{32}] dCTP. The mixture was incubated at 37°C for 1-4 h.

To prepare the size-exclusion column, Sephadex G-50 was prepared by adding two volumes of TE and autoclaving. The plunger of a 1 ml syringe (Plastipak) was removed and a small amount of siliconised glass wool was used to plug the end of the syringe before a microfuge tube was placed on the end of the syringe. Both syringe and microfuge tube were placed into a 50 ml centrifuge tube and Sephadex G-50 was added to the syringe and centrifuged for 5 min at 3,000 rpm. TE was removed and the process repeated until 0.7 ml of Sephadex G-50 remained in the syringe. A fresh microfuge tube was then placed on the bottom of the syringe and the radiolabelled probe (made up to 100 μl with dH_2O to increase volume added to column) was added to the Sephadex G-50

column. This was centrifuged at 3,000 rpm for 5 min and the purified probe was collected in the microfuge tube where it was monitored by a Geiger counter to confirm that radioactivity had been incorporated into DNA probe. Purified probe was used in Northern blot analysis.

2.4.11 Flow cytometry

Cells were fixed by pelleting cells by centrifugation at 13,000 rpm for 1 min and discarding the supernatant. Cells were washed in 1 ml of dH_2O then fixed by gradually resuspending, dropwise, in 70% ethanol while vortexing. Fixed cells could then be stored at 4°C.

0.3 ml of fixed cells were pelleted by centrifugation at 13,000 rpm for 1 min and supernatant discarded. 0.5 ml of 50 mM sodium citrate was used to wash cells before pelleting and again cells were resuspended in 1 ml 50 mM sodium citrate. For labelling of DNA, 0.1 mg/ml of Ribonulease A (5 µl of 40 µg/ml stock) was added and 2 µg/ml of propidium iodide (50 µl of 40 µg/ml stock). The samples vortexed then incubated at 37°C for 2-4 h. The cells could be analysed immediately or stored at 4°C for up to 1 week. Samples were analysed using a Becton Dickinson FACScan according to manufacturer's instructions. Data was collected using CellQuest software. 20,000 cells were counted and DNA content (FL2-H) plotted against counts (Moreno *et al.* 1991).

2.4.12 RT-PCR and cDNA synthesis

Following total RNA extraction (Section 2.6.1) and mRNA purification (Section 2.6.2) the following procedure was followed for the genesis of cDNA: A 20 µl reaction volume was used for 1 ng-5 ng of total RNA or 1-500 ng mRNA. The following components were added to a nuclease-free microfuge tube: 1 µl random primers (50-250 ng) or gene-specific primer (GSP), 1-5 ng total RNA or 1-500 ng mRNA, 1 µl dNTP mix (10 mM of each), 12 µl dH_2O. The mixture was heated to 65°C for 5 min and immediately put on ice. The tube was briefly centrifuged and the following added: 4 µl 5 x First-Strand buffer, 2 µl 0.1 M DTT and an optional 1 µl RNaseOUT™. The contents of the tube were mixed gently and incubated at 42°C for 2 min or if using random primers incubate at 25°C for 2 min. 1 µl of Superscript™ II RT was added and mixed by pipetting gently. If

using random primers the mixture was incubated at 25°C for 10 min. Next the mixture was incubated at 42°C for 50 min and finally the reaction was activated by heating to 70°C for 15 min. The cDNA was then used for amplification by PCR using appropriate primers and program for the required gene.

2.5 RNA materials

2.5.1 RNA chemicals

Specialised chemicals such as AquaPhenol were obtained from Qbiogene. Salmon sperm DNA was obtained from Sigma. Dextran sulphate was obtained from Amersham Pharmacia Biotech. Genescreen membrane was purchased from NEN Life Science and Bovine serum albumin (BSA) was bought from Helena Biosciences.

2.5.2 RNA enzymes and kits

Superscript® II Reverse transcriptase kit was obtained from Invitrogen and mRNA purify kit was from Biolabs.

2.5.3 Photographic materials

X-ray film was purchased from Fujifilm. The X-Omat 100 processor was supplied by Kodak.

2.6 RNA methods

2.6.1 Preparation of total RNA

RNA extraction from fission yeast required the growth of a 200 ml culture of cells to mid-exponential phase and harvested at 3,000 rpm for 5 min. Supernatant was discarded and cells could be stored at -70°C at this stage. The cell pellet was resuspended in 1 ml STE (0.32 M sucrose, 20 mM Tris-HCl pH 7.5, 10 mM EDTA pH 8.0) and transferred to a screw cap microfuge tube and centrifuged at 13,000 rpm for 1 min. The cell pellet was resuspended in 200 µl STE followed by 0.3 g acid washed glass beads. 600 µl NTES (100 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, 1% (w/v) SDS) was then added and then

500 µl of water saturated hot phenol at 65°C. Cells were lysed using a Hybaid Ribolyser for 3 x 40 sec bursts at setting 4. After centrifugation at 13,000 rpm for 5 min the upper aqueous phase and protein interface was transferred into a fresh microfuge tube containing 400 µl hot phenol. The mixture was again ribolysed for a 1 x 40 sec burst and again spun for 5 min. The aqueous phase was transferred to 400 µl of phenol:chloroform (1:1) at room temperature and ribolysed. After spinning, the aqueous phase was transferred to a second aliquot of phenol:chloroform and ribolysed. Again a 5 min spin was applied and the aqueous phase transferred to a 300 µl aliquot of phenol:chloroform and ribolysed. After spinning, the aqueous phase was transferred to a 300 µl aliquot of chloroform and ribolysed. Finally after spinning for 5 min the aqueous phase was transferred to a fresh microfuge tube where 3 volumes of 100% ethanol and one tenth volume of 3 M sodium acetate pH 5.2 were added. The RNA was precipitated overnight at -20°C. The following day RNA was pelleted by centrifugation at 13,000 rpm for 10 min and supernatant was discarded. The pellet was washed in 200 µl 70% ethanol in RNase-free dH_2O and centrifuged for 1 min. Ethanol was then carefully removed and the pellet was resuspended in 55 µl RNase-free dH_2O and dissolved by incubating at 65°C with frequent pipetting. 5 µl of RNA was diluted in 500 µl of dH_2O to measure quality and quantity by spectrophotometry (Section 2.4.9).

2.6.2 mRNA purification

mRNA was purified using the mRNA purify kit (Biolabs) following the protocol in the manufacturer's manual.

2.6.3 RNA formaldehyde gels

1 g of agarose was dissolved in 63 ml of dH_2O and 20 ml of 5 x MNE (120 mM MOPS, 25 mM NaOAc, 5 mM EDTA pH 7) by boiling and then cooling to 60°C. 17 ml of formaldehyde was added and gently mixed. The mixture was poured into a horizontal gel mould and left to set for a up to an hour. 15 µl RNA buffer (600 µl formaldehyde, 200 µl formamide, 240 µl 5 x MNE, 160 µl dH_2O) and 1 µl of 0.5 mg/ml ethidium bromide were added to each 10-20 µg RNA sample, incubated at 60°C for 5 min and then loaded

onto the formaldehyde gel. RNA was then separated by electrophoresis at 60 V for 3-4 h in 1 x MNE buffer.

2.6.4 Filter hybridisation of RNA

2.6.4.1 Northern blot

Genescreen membrane was used to transfer RNA by capillary action (Sambrook *et al.* 1989) using manufacturer's guidelines. Two long sheets of Whatman paper were soaked in 0.1 M sodium phosphate buffer pH 6.5 (61 ml 1 M Na_2HPO_4, 39 ml 1 M NaH_2PO_4 made to 1 litre with dH_2O) and placed on a glass plate to form a wick over a reservoir of 0.1 M sodium phosphate buffer. 2 smaller pieces cut to slightly larger than gel size were also soaked and placed on top. The RNA gel was soaked in 0.1 M sodium phosphate buffer to remove excess formaldehyde and then place upside down on Whatman papers with gel spacers placed along the edges of the gel. Genescreen membrane cut to gel size was soaked in dH_2O first then 0.1 M sodium phosphate buffer and placed on top of the gel. Another piece of Whatman paper soaked in buffer was placed on top of the membrane and rolled over with a glass pipette to remove air bubbles. A dry Whatman paper was placed on top followed by a thick bundle of absorbent tissues. This was all topped off with a heavy weight. The transfer was left for 16-24 h at room temperature after which the towels and upper Whatman papers were discarded. The membrane was carefully lifted, marked on the bottom right corner for orientation and washed in 0.1 M sodium phosphate buffer for 20 min with gentle rotation. The membrane was then air dried on Whatman paper and the RNA was fixed to the membrane using a UV crosslinker at 1200 MJ.

2.6.4.2 Hybridisation

The membrane was pre-hybridised prior to addition of radio-labelled probe for 2-4 h in a Techne hybridisation oven in 18 ml of hybridisation buffer (10 ml formamide, 4 ml P Buffer (1% (w/v) BSA, 1% (w/v) pyrolidine, 1% (w/v) ficoll, 250 mM Tris-HCl pH 7.5, 0.25% (w/v) sodium pyrophosphate, 5% (w/v) SDS), 4 ml 50% (w/v) dextran sulphate (10 g dextran sulphate, 20 ml dH_2O), 1.16 g NaCl). The hybridisation buffer was heated

to dissolve salt at 42°C and then 200 µl salmon sperm DNA (after denaturing at 95°C for 5 min then on ice for 5 min) was added. After pre-hybridisation the radio-labelled probe (Section 2.4.10) was denatured at 95°C for 5 min and placed on ice for 5 min, then added directly to the hybridisation buffer. Hybridisation was carried out overnight at 42°C. The following day the hybridisation buffer was poured away and the membrane was first washed twice for 5 min each time in 2 x SSPE (from 20 x SSPE stock: 3 M NaCl, 20 mM NaH_2PO_4, 200 mM EDTA pH 7.4) at 42°C. The membrane was then washed in 2 x SSPE + 0.5% SDS at 65°C for 15 min. The signal to background ratio was monitored using a Geiger counter and if background was still high the 65°C wash was repeated. Finally, the membrane was rinsed in 0.1 x SSPE at room temperature. The membrane was wrapped in cling film before processing for autoradiography.

2.6.4.3 Membrane stripping

Membranes were stripped of radioactive probe by incubating at 70°C for 30 min, or until no radioactivity could be detected, in strip buffer (5 mM Tris-HCl, 0.2 mM EDTA, 0.05% (w/v) sodium pyrophosphate, 0.002% (w/v) pyrolidine, 0.002% (w/v) BSA, 0.002% (w/v) ficoll). The membrane could be re-probed for other genes of interest.

2.7 Protein materials

2.7.1 Protein chemicals and equipment

Ultra pure imidizole and nickel chloride were purchased from MERCK, BDH. Triton X-100 was bought from Fisons, Loughborough. Isopropyl β-thiogalactopyroniside (IPTG) was purchased from Melford Laboratories Ltd, Suffolk. Glutathione Sepharose 4B and Chelating Sepharose™ Fast flow slurry was supplied by Amersham Pharmacia Biotech. Glutathione (reduced), acrylamide:bisacrylamide solution were supplied by Sigma. Bradfords Reagent (BIO-RAD protein assay reagent) was purchased from BIO-RAD.

Nitrocellulose and Hyperfilm were purchased from Amersham Pharmacia Biotech. Kodak supplied the X-Omat 100 processor.

2.7.2 Molecular weight markers

The relative molecular mass (subunit M_r) of proteins on SDS-PAGE was determined by comparison with low M_r markers. Full-Range Rainbow™ molecular weight marker was purchased from Amersham Pharmacia Biotech.

2.8 Protein methods

2.8.1 Bacterial cultures for protein induction

BL21 CododnPlus(DE3)-RIL cells transformed with the required plasmid were picked from a single colony and grown in 10 ml of NZY^+ media plus appropriate antibiotic for no more than 16 h at 37°C with shaking. 1 ml of this pre-culture was used to inoculate another 50 ml of NZY^+ plus antibiotic in a 37°C shaker until A_{600} was between 0.5-1. 500 µl of 0. 1 mM IPTG was added for induction at 37°C for 3 h. Samples were taken at zero time and then every hour after induction to check for overexpression. The individual samples were pelleted by centrifugation at 13,000 rpm for 1 min and pellets were resuspended in Laemmli sample buffer (2% (w/v) SDS, 10% (w/v) sucrose, 62.5 mM Tris-HCl pH 6.8, Pyronin Y dye) using 10 µl per 0.1 A_{600} units. After 3 h induction the cells were spun down at 3,000 rpm for 15 min and the supernatant was discarded. The cells pellet was then resuspended in the appropriate buffer for purification.

2.8.2 Dialysis of protein samples

Visking tubing was prepared by boiling in 10 mM sodium bicarbonate pH 8, 1 mM EDTA for 10 min. This was then rinsed in sterile dH_2O and finally stored in 100% (v/v) ethanol. Prior to use the tubing was thoroughly rinsed in dH_2O. Dialysis of protein took place at 4°C overnight with frequent changes of dialysis buffer.

2.8.3 Determination of protein concentration

The method of Bradford (1976) was routinely applied.

2.8.4 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

The solutions required for SDS-PAGE are described as follows:

Running buffer

25 mM Tris-HCl pH 8.3, 0.25 M glycine, 1% (w/v) SDS.

Acrylamide solution

29.2% (w/v) acrylamide/0.8% (w/v) bis-acrylamide.

Resolving gel buffer

10% (w/v) acrylamide, 0.5 M Tris-HCl pH 8.8, 0.1% (w/v) sodiumdodecyl sulphate(SDS), 0.1% (w/v) ammonium persulphate, 0.1% (v/v) N, N, N^1, N^1-tetramethylethylene diamine (TEMED).

Stacking gel buffer

4.5% (w/v) acrylamide. 0.06 M Tris-HCl pH 6.8, 0.1% (w/v) SDS, 0.1% (w/v) ammonium persulphate, 0.1% (v/v) TEMED.

The Laemmli *et al.* (1970) method of protein resolving under denaturing conditions was adopted. Samples for analysis on SDS-PAGE were resuspended in Laemmli buffer to which 1 M DTT was added to a final concentration of 150 mM, prior to boiling, for 5 min to ensure protein denaturation. 10 µl of each sample was loaded onto the gel alongside 10 µl of Rainbow M_r marker. Gels were run using the Biorad Mini-Protean gel kit system at 120 V for 1 h or until dye front was approximately 1 cm from bottom of the gel. Gels were then stained in 0.1% Coomaassie Brilliant Blue, 10% (v/v) acetic acid, 50% (v/v) methanol for 1 h followed by destaining in 10% (v/v) acetic acid, 10% (v/v) methanol overnight.

2.8.5 Immunoblotting using ECL™ (Enhanced Chemiluminesence)

Anti-HIS (mouse) primary antibody was supplied by Sigma and Anti-GST (Rabbit) primary antibody was supplied by Amersham Pharmacia Biotech. Anti-Mouse secondary HRP-Antibody and Anti-Rabbit secondary HRP-Antibody were supplied by Dako. Western blotting protocols were carried out as detailed in the manufacturer's instructions.

2.8.6 Solutions required for Anti-GST detection

Blocking buffer: 20 mM Tris-HCl pH 7.2, 15 mM NaCl, 5% non-fat milk, 0.2% Tween 20.

Wash buffer: 20 mM Tris-HCl pH 7.2, 15 mM NaCl, 1% non-fat milk.

1° Antibody solution: 20 mM Tris-HCl pH 7.2, 1% non-fat milk, 0.1% Tween 20, 1:1,000 or 1:5,000 dilution of 1° anti-GST antibody (1 µl in 5 ml).

2° Antibody solution: 20 mM Tris-HCl pH 7.2, 150 mM NaCl, 1% non-fat milk, 1:1,000 dilution (10 µl in 10 ml) anti-Rabbit HRP antibody.

High Salt Wash: 20 mM Tris-HCl pH 7.2, 150 mM NaCl.

2.8.7 Solutions required for Anti-HIS detection

Blocking buffer: 20 mM Tris-HCl pH 7.2, 15 mM NaCl, 5% non-fat milk, 0.2% Tween 20.

TBS-Tween: 10 mM Tris-HCl pH 7.5.

TBS-Tween/Triton Buffer: 20 mM Tris-HCl pH 7.5, 500 mM NaCl, 0.05% (v/v) Tween-20, 0.2% (v/v) Triton X-100.

1° Antibody solution: As above with anti–HIS antibody.

2° Antibody solution: As above with anti-mouse HRP antibody.

High Salt wash: As above.

2.8.7 Immunoblotting protocol

Proteins were electrophoretically transferred, after SDS-PAGE (Section 2.8.4), to a nitrocellulose membrane using the Biorad Mini-Protean gel kit system at 50 mA for 2 h in 1 x transfer buffer (10 x transfer buffer per litre: 25 mM Tris-HCl pH 7.2, 192 mM glycine, 0.02% (w/v) SDS, 20% (v/v) methanol) plus 20% (v/v) methanol. The non-fixative dye Ponceau S was used to check protein transfer onto the membrane. This could then be washed off using dH_2O followed by blocking non-specific binding sites by immersing membrane in blocking buffer for 1 h at room temperature on a rotator. The membrane was then washed 3-4 times for 15 min each time in wash buffer for GST detection or 2 x 10 min in TBS-Tween/Triton buffer for HIS detection followed by

incubation in the appropriate primary antibody solution for 1 h at room temperature or overnight at 4°C. The membrane was then washed in wash buffer three times for 15 min each time or TBS-Tween/Triton buffer for 2 x 10 min. The membrane was then incubated for 1 h at room temperature in appropriate secondary antibody. The membrane was given final washes in appropriate wash buffers and then given a final high salt wash rinse before detection. The detection step was carried out as described in the Amersham protocol for ECL™ detection. In the dark room, autoradiography film was placed onto the membrane and exposed for and initial 30 sec before developing the film using a Kodak X-Omat-100 processor. Different exposure times were tried to get the best results.

2.8.8 Preparation of bacterial cell extracts

GST and HIS fusion proteins were expressed as described (Section 2.8.1).

The pellet from a 50 ml bacterial culture was resuspended in 5 ml 1 x PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 pH 7.3) for GST fusion proteins and Buffer A (20 mM KH_2PO_4, 0.5 mM imidizole, 100 mM NaCl pH 7.5) for HIS fusion proteins. The cells were then disrupted under high pressure using a French Pressure cell at 750 psi. 3 passes were usually made. The supernatant was collected after centrifugation at 13,000 rpm for 15 min at 4°C.

2.8.9 Column purification of GST-tagged proteins

Cell protein extract were put through a glutathione sepharose 4B column with a bed volume of 2 ml. The column was washed in 30 bed volumes of 1 x PBS buffer to remove ethanol and finally equilibrated with 1:1 bead and 1 x PBS buffer. The protein was applied to the beads and incubated at 4°C for 1 h. The column was again washed with 5 bed volumes of 1 x PBS and then finally eluted with 1 bed volume of elution buffer (10 mM reduced glutathione in 50 mM Tris-HCl pH 8) for 10 min and the eluate collected in 1 ml fractions. This process was repeated a further 3 times. Collected fractions were analysed using SDS-PAGE along with whole cell extract sample, supernatant sample and pellet sample (all of which were taken after French press). Fractions where protein was detected were pooled and dialysed in Buffer A.

2.8.10 Column purification of HIS-tagged proteins

The column was prepared by loading a bed volume of 2 ml of chelating fast flow slurry and washing with 30 bed volumes of buffer A to remove ethanol. Nickel ions (0.1 M NiCl pH 4.5-5) were loaded onto the column to bind to the imidoacetate binding sites. The column was then washed in Buffer A for 5 bed volumes. Supernatant containing HIS-tagged protein was applied to the column and incubated at 4°C for 1 h before being eluted in 4 ml elution buffer (20 mM KH_2PO_4, 0.5 M imidizole, 100 mM NaCl) and collected in 2 ml fractions. This was repeated a further 3 times. Collected fractions were analysed using SDS-PAGE along with whole cell extract sample, supernatant sample and pellet sample (all samples taken after French press). Fractions where protein was detected were pooled and dialysed in Buffer A.

2.8.11 GST pulldown

To assay for interaction between two proteins a GST pulldown assay was adopted. 500 µl of glutathione sepharose 4B beads were added to a microfuge tube. Beads were washed in 1 ml buffer A (20 mM KH_2PO_4, 0.5 mM Imidizole, 100 mM NaCl pH 7.5), centrifuged and supernatant discarded. The wash process was repeated a further 4 times and on the last wash the beads were equilibrated 1:1 in 500 µl buffer A. 100 µl of prepared beads were taken to a fresh microfuge tube where 1 ml of dialysed GST- tagged protein sample was added to the beads and incubated at 4°C for 1 h on a rotator. Next 1 ml of HIS-tagged dialysed sample was added direct to the microfuge tube and also incubated at 4°C for 1 h on a rotator. The sample was spun for a short light spin in a centrifuge and the supernatant was removed and kept on ice in a separate microfuge tube. The beads were washed three times in 1 ml buffer A and all washes were kept on ice after spinning at 2,000 rpm for 5 min. After the final wash the pellet was resuspended in 150 µl Laemmli sample buffer and 21 µl DTT. The sample was boiled for 10 min and then spun for 10 min at 13,000 rpm. The supernatant was removed and kept on ice. All samples including washes were TCA precipitated and kept on ice.

2.8.12 TCA (tri-chloroacetic acid) precipitation

10 µl of TCA was added to each sample and incubated overnight at 4°C. The following day the samples were centrifuged for 15 min at 13,000 rpm. The supernatant was removed and kept aside. The pellet was washed in 500 µl acetone. The samples spun and acetone removed. The pellets were resuspended in 20 µl laemmli sample buffer and 10 µl DTT. 10 µl of each sample were boiled and loaded onto SDS-PAGE gels followed by Western blot analysis.

2.9 Fission yeast materials

2.9.1 Fission yeast media

Media used for the propagation of fission yeast were as described by Moreno *et al.* (1991)

2.9.2 Fission yeast strains and plasmid vectors

Fission yeast strains used in this thesis are described in Appendix III. The annotation GG refers to the Glasgow lab fission yeast collection number. Fission yeast plasmid vectors were obtained from laboratory stocks described in Appendix I.

2.9.3 Fission yeast chemicals

Specialised fission yeast chemicals such as diamino-2-phenylindole dihydrochloride (DAPI) and poly-L-lysine were purchased from Sigma. Mounting media was Citifluor AF1 and Citiflour AF3 obtained from Citiflour.

2.10 Fission yeast methods

2.10.1 Mating fission yeast cells

To mate yeast of opposite mating type the procedure used in Moreno *et al.* (1991) was adopted.

2.10.2 Preparation of competent yeast cells

100 ml of the appropriate strain of *S. pombe* was grown to exponential phase (2-5 x 10^6 cells/ml) and pelleted at 3,000 rpm for 5 min. The cells were washed in 5 ml of LiAc:TE (100 mM LiAc, 1 x TE) pelleted at 3,000 rpm for 5 min and resuspended in 1 ml LiAc:TE plus 1 ml 2 M sorbitol. Cells were aliquoted into 100 µl and stored at -70°C.

2.10.3 Lithium acetate transformation

The lithium acetate method (Schiestl & Geitz 1989, Geitz *et al.* 1992) was carried out as follows: 1 µg of DNA and 50 µl of carrier salmon sperm DNA (5 µl of 10 mg/ml stock) was added to 100 µl of competent yeast cells in a microfuge tube. 700 µl of PEG mix (40% PEG 3350, 100 mM LiAc, 1 x TE) was added and the mixture was vortexed and incubated at 30°C for 30 min. 43 µl of dimethyl sulphoxide (DMSO) was added before the cells were heat shocked at 42°C for 15 sec and then pelleted for a very light spin. Cells were resuspended in 200 µl sterile dH_2O and spread onto selective plates and left to grow at 30°C for 2-5 days.

2.10.4 Fixing and viewing stained cells

10 ml of yeast cells were grown to exponential phase and fixed by adding 1.1 ml formaldehyde (4% final concentration) and incubated for 1 h at room temperature. Cells were washed 3 times in 1 x PBS and resuspended in the residual liquid. If cell suspension was too thick a little more PBS was added.

To label spore DNA, DAPI was added to a final concentration of 1 µl/ml (0.5 µl of 1 mg/ml stock) and gently mixed. Stained cells could be viewed immediately or stored at 4°C in darkness.

Glass slides for viewing cells were prepared as follows: Glass slides and coverslips were rinsed in 100% ethanol and air-dried. Slides were coated in poly-L-lysine (diluted by a factor of 10 in dH_2O) removing excess poly-L-lysine by rinsing with dH_2O and drying at 80°C. 6 µl of fixed cells were pipetted onto poly-L-lysine coated slide. 5 µl of mounting media AF1 plus 5 µl AF3 were used to cover the cells. Cells were covered with a coverslip and left for 30 min to allow cells to fix to slides. Cells were visualised

under a fluorescence microscope using a Zeiss Axiostar microscope and images were captured using a Sony DS-75 digital camera.

2.11 Budding yeast materials

2.11.1 Budding yeast chemicals

Specialised chemicals such as synthetic drop-out (SD) media (lacking tryptophan, leucine, histadine and adenine) and low-melting point agarose were purchased from GIBCO BRL.

2.11.2 Budding yeast media

Media used for the propagation of budding yeast were as follows: Routinely budding yeast was grown in YPD (2% bacto tryptone, 1% yeast extract, 2% glucose) liquid or YPD (as for liquid YPD plus 2% agar) agar plates. Synthetic drop-out (SD) media consisted of SD drop-out media (minus tryptophan, leucine, histadine, adenine), 0.67% yeast nitrogen base. The SD media was supplemented with appropriate amino acids as required. For SD agar plates 2% agar was added.

2.11.3 Budding yeast strains and plasmid vectors

Saccharomyces cerevisiae strains used in this thesis are described in Appendix IV. The annotation GBY refers to the Glasgow lab budding yeast collection number. *S. cerevisiae* plasmid vectors were obtained from laboratory stocks described in Appendix I.

2.12 Budding yeast methods

2.12.1 Budding yeast transformation

The LiAc transformation method was adopted as described in Section 2.10.5.

2.12.2 X-gal overlay assay

S. cerevisiae cells were grown on SD agar plates plus appropriate amino acids for 2-3 days at 30°C. 10 ml stock solution (0.5 M potassium phospahate buffer (39 ml 1 M

KH_2PO_4, 61 ml 1 M K_2HPO_4 plus 100 ml dH_20), 6% di-methyl formamide (DMF), 0.1% SDS) was mixed with 80 mg low-melting agarose and heated in a microwave for 40 sec. The solution was allowed to cool for approximately 1 min until its temperature was 60°C. 30 µl X-gal (40 mg/ml stock) was added to the solution and using a Pasteur pipette the cells were covered with the warm solution. The agarose was allowed to set and solidified plates were incubated at 30°C. Plates were checked at hourly intervals for blue colour development for about 6 h, after which they were left overnight.

2.12.3 β-galactosidase assay

To quantify the interaction in 2-hybrid x-gal overlay assay the β-galasctosidase assay was carried out as follows: 5 ml of cells were grown in YEPD or SD media to mid-log phase. Cells were centrifuged and resuspended in 5 ml Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$), 50 mM β-mercaptoethanol pH 7 (not autoclaved)) and placed on ice. The OD at 600 nm was measured. Using a straight, or diluted cell mix, 10 x or 20 x (40 or 80 µl brought to 0.8 ml with Z buffer). Using a Pasteur pipette, 1 drop of 0.1% SDS and 2 drops of chloroform was added to each sample and vortexed well for 15 sec. The samples were equilibrated at 30°C for 15 min. 160 µl of 4 mg/ml ONPG (4 mg/ml in 0.1 M potassium phosphate buffer pH 7) was then added and vortexed well for 10 sec. Samples were then incubated at 30°C the duration of incubation was timed. The samples were removed after about 15–20 min (empirically determined by colour). The reaction was quenched by adding 400 µl of 1 M sodium carbonate. Cell debris was spun down and the OD at 420 nm and 550 nm was measured. Units were calculated using the following formula, where vol is volume of culture used in assay in mls and Time is minutes at 30°C:

$$U = 1{,}000 \times [(OD_{420}) - (1.75 \times OD_{550})] / [(Time) \times (Vol) \times OD_{600}]$$

Chapter 3

SpPol5p, a novel interacting partner of SpCdc10p

3.1 Introduction

Schizosaccaromyces pombe has a cell cycle typical of most eukaryotic systems (Nurse 1990). Divided into four distinct phases, the first named G_1, a short gap phase where growth occurs, followed by DNA synthesis (termed S-phase) and then a lengthy G_2 phase where most growth occurs. Upon reaching the required cell size, cells finally enter M-phase or mitosis where replicated chromosomes separate, and cells divide into two new daughter cells.

Central to the coordinated regulation of these discrete cell cycle events are two major control points. The first control point, termed 'START', occurs before the onset of DNA replication at G_1 and the second point is at late G_2 before the cells divide in mitosis, the G_2/M boundary (Nurse & D'Urso 1995, Nurse & Woollard 1995). The control of the early part of the cell cycle begins by the cell having to make a decision as to which developmental pathway to follow. This decision is based on the cells' nutritional and environmental situation at the time. In the presence of nutrients cells enter a new round of proliferation, but upon the exhaustion of nutrients cells become starved where they can enter either the sexual (meiotic) cycle or enter into stationary phase (G_0). Once a cell passes START it is committed to complete mitosis until reaching the next G_1 phase.

Progression through START requires the activity of the cyclin dependant kinase *cdc2*$^+$ (a homologue of *cdc28*$^+$ in budding yeast) and G_1 cyclins, along with transcriptional activation of specific genes whose products are required for DNA replication.

3.2 Transcriptional control of 'START'

Cell cycle regulated periodic transcription is fundamental to the control of the cell cycle (Lowndes *et al.* 1991). A large group of genes has been identified that are periodically expressed in G_1 encoding proteins that are both directly and indirectly required for DNA synthesis such as *cdc22*$^+$, encoding the large subunit of ribonucleotide reductase (Fernandez-Sarabia *et al.* 1993), *cdc18*$^+$, encoding the protein required for replication initiation (Muzi-Falconi *et al.* 1996) and *cig2*$^+$ which encodes a B-type cyclin that complexes with SpCdc2p (Connelly & Beach 1994). The

gene products of *mik1*$^+$ and *cdc18*$^+$ (Kelly *et al.* 1993, Ng *et al.* 2000) are involved in checkpoints that ensure mitosis does not occur until S-phase is complete. These genes have a common *cis*-acting element in their promoter regions called an MCB (*Mlu*1 cell cycle box), the nucleotide consensus sequence of which is 5'-ACGCGT-3'. MCB sequences, which can occur more than once in a gene promoter, are essential for coordinated G_1/S periodic expression, and are recognized by a DNA binding activity called DSC1 (DNA synthesis control 1) in fission yeast (Lowndes *et al.* 1992, Maqbool *et al.* 2003).

A similar transcription factor system exists in *S. cerevisiae* with the difference that two parallel complexes control S-phase gene expression named MBF and SBF. The MBF transcription factor complex comprises ScSwi4p and ScMbp1p (Koch *et al.* 1993) and recognises the consensus sequence 5'-ACGCGN-3' in promoter sequences of genes principally involved in DNA replication and repair. The SBF transcription factor complex comprises ScSwi4p and ScSwi6p (Sidorova & Breeden 1993, Foster *et al.* 1993) and recognises the consensus sequence 5'-CGCGAAAA-3' in promoter sequences of genes mainly involved in budding, cell wall and membrane biosynthesis.

The analogous complex in fission yeast, DSC1, is made up of at least four proteins: SpCdc10p, SpRes1p, SpRes2p and SpRep2p. The SpRes1p-SpCdc10p complex has a major role in the mitotic cycle and a minor role in meiosis, whereas the SpRes2p-SpCdc10p complex has a major role in meiosis and a small role in mitosis (Nakashima *et al.* 1995, Sturm *et al.* 1996). SpRep2p binds to SpRes2p and activates transcription under acceptable nutritional conditions explaining the link between the cell's nutritional status and its tendency to pass START. Accordingly, fission yeast uses overlapping parallel systems to drive pre-mitotic and pre-meiotic START transcription.

The transcription control systems are conserved at the level of primary protein sequence. SpCdc10p, SpRep2p, SpRes2p of fission yeast and ScSwi4p, ScSwi6p and ScMbp1p of budding yeast all contain four copies of highly conserved ankyrin repeat motif (Ewaskow *et al.* 1998). ScSwi4p, ScMbp1p, SpRes1p and SpRes2p also all share highly conserved DNA binding domains.

3.3 The role of SpCdc10p

The *cdc10*$^{+}$ gene encodes a phosphoprotein product of 85 kDa whose steady-state levels remain constant throughout the cell cycle (Aves *et al.* 1985). It is a major component of the DSC1 complex in fission yeast (Lowndes *et al.* 1992). Along with *cdc2*$^{+}$, *cdc10*$^{+}$ was one of the first G_1 *cdc* genes identified and was found to be essential for START progression (Nurse & Bissett 1981). It shares high homology in the ankyrin repeat motifs with budding yeast ScSwi4p and ScSwi6p and mutations in these motifs can abolish function (Reymond *et al.* 1992, Ayté *et al.* 1995). Res1p binds to SpCdc10p via its carboxyl-terminal. The over-expression of this portion of Res1p leads to G_1 cell cycle arrest (Ayte *et al.* 1995) and a similar phenotype is observed when the carboxyl-terminal of SpCdc10p is overproduced (McInerny *et al.* 1995).

In wild type cells, MCB-DSC1 regulated genes are periodically expressed at the G_1-S boundary, but in one *cdc10*$^{+}$ mutant, named *cdc10-C4*, this periodicity is lost and MCB-DSC1 regulated genes are constitutively expressed throughout the cell cycle (McInerny *et al.* 1995). The change in *cdc10-C4* is a non-sense mutation resulting in a 61 amino acid truncation at the C-terminus of SpCdc10p (Reymond *et al.* 1992). This strain is interesting as it displays two different phenotypes at high and low temperatures. At high temperature it behaves like a conventional *cdc* mutant showing a characteristic elongated phenotype as it continues to grow but cannot divide. The other interesting trait of this mutant, however, is seen at low temperature where the cells continue to divide but after an extended G_2 phase, because of a delayed mitosis resulting in a longer cell length at the time of division (McInerny *et al.* 1995). The explanation for this is due to the constitutive expression of *cdc18*$^{+}$ and *mik1*$^{+}$, as it is known that the ectopic over expression of both these genes results in the same phenotype (Kelly *et al.* 1993, Ng *et al.* 2001).

This observation prompts the suggestion that there might be a negative regulator of SpCdc10p interacting through its C-terminus. This activity was thought to be SpRes2p, as Δ*res2* cells also express *cdc18*$^{+}$ at constitutively high levels (Baum *et al.* 1997). However, SpRes2p interacts with SpCdc10-C4p lacking the 61 amino acids at the carboxyl-terminal (Whitehall *et al.* 1999). These findings suggest that the interesting regulatory properties of SpCdc10p, contained within its C-terminus region, are mediated by an unknown regulatory protein that binds to it.

In this study, therefore, we set out to investigate further the regulatory properties of the C-terminus of SpCdc10p by performing a yeast two-hybrid screen to isolate novel proteins that bind to this part of SpCdc10p.

3.4 The two-Hybrid method identifies novel proteins that bind to proteins of interest

In the past interactions between proteins were studied using biochemical techniques such as co-immunoprecipitation and cross-linking. A novel genetic system devised by Fields & Song (1989) was developed to assay the interaction between two proteins by taking advantage of the ScGal4p protein in *S. cerevisiae* (Figure 3A). In *S. cerevisiae* the native ScGal4p protein contains both a DNA binding domain and a separate activating domain that together are required to induce the transcription of the *GAL1* gene. The yeast two-hybrid system exploits this observation so that protein X is fused in frame to the activation domain (AD) of ScGal4p protein in a reporter plasmid and protein Y is fused in frame to the binding domain (BD) of ScGal4p protein in a separate reporter plasmid. If proteins X and Y interact then the AD and BD of ScGal4p are brought into close proximity causing activation of transcription of *GAL1-lacZ* (Figure 3A). This method was later adapted to screen for novel proteins that interacted with a known protein of interest (Chien *et al.* 1991) by screening a cDNA library ligated into the AD plasmid. In this chapter a two-hybrid screen is described to identify novel binding proteins to the C-terminus of SpCdc10p.

3.4.1 Construction of bait vector pGBT9-C4 (GB 82)

The oligonucleotides GO 140 and GO 141 were used to amplify the 183 bases of the C-terminus of *cdc10*$^{+}$ (referred to in this thesis for clarity as 'C4') introducing an *Eco*RI and *Bam*HI site into the PCR product at 5' and 3' sites, respectively. A plasmid containing wild type *cdc10*$^{+}$ (GB 72) was used as template using a program with 30 cycles of 94°C for 15 s, 60°C for 15 s and 68°C for 1 min. The PCR product was run on a 1% agarose gel and gel extracted using the QIAgen gel extraction kit. This was then cloned into the plasmid pGBT9 (GB 81) (Appendix I) in frame with the ScGal4p

DNA-binding domain, which was subsequently confirmed by sequencing (MWG-Biotech), to create pGBT9-C4 (GB 82).

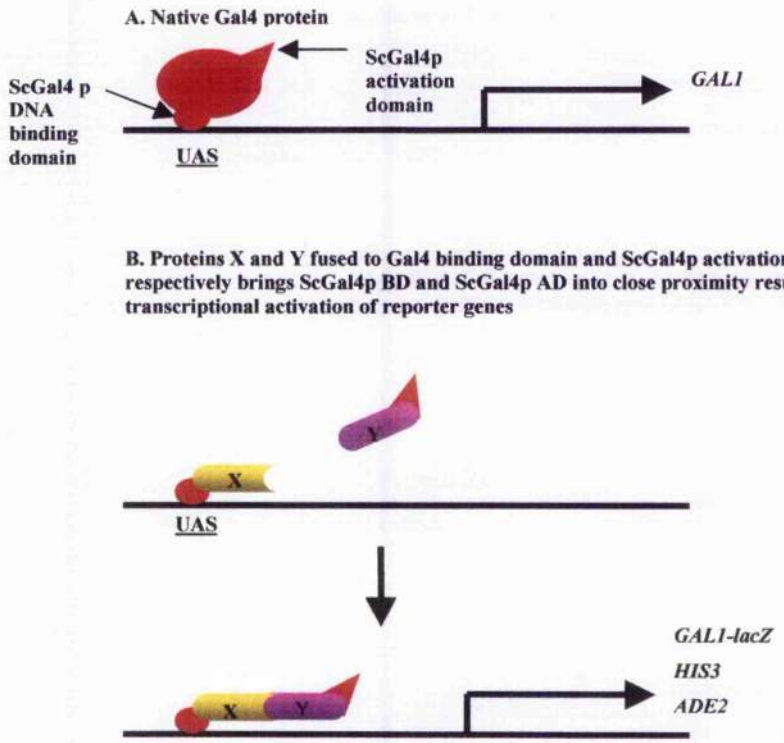


Figure 3A. Schematic diagram of transcriptional activation by ScGal4p in two-hybrid assays. A. The native ScGal4p has a DNA binding domain (BD) and a separate activation domain (AD), which causes transcription *GAL1* genes. **B.** The two-hybrid assay exploits both the BD and AD domains of ScGal4p to test for interactions between proteins. If proteins X and Y interact this brings the AD and BD close together to allow transcription of the reporter genes to take place.

3.4.2 Testing for auto-activation

As SpCdc10p is part of a transcription factor complex it was a vital preliminary control experiment for the two-hybrid screen to test for auto-activation. This would ensure that the C4 fusion protein with the ScGal4p BD did not stimulate transcription

of the reporter genes in the yeast two-hybrid strain (GBY 122) that was to be used for the two-hybrid screen, leading to false positives.

pGBT9-C4 (GB 82) was transformed into the empty yeast two-hybrid strain (GBY 122) and grown in the absence of tryptophan. Transformants were then replica-plated to media lacking tryptophan, histidine and adenine. Growth was not detected, even after a seven day incubation, demonstrating that no auto-activation was taking place as *HIS3* and *ADE2* transcription did not occur (Figure 3.1C). Also, transforming both pGBT9-C4 (GB 82) and the empty library vector pGAD424 (GB 123) into the two-hybrid strain (GBY 122) eliminated the possibility that C4 was interacting directly with the ScGal4p AD. Transformants were grown in the absence of tryptophan and leucine followed by replica plating to media lacking tryptophan, leucine, histidine and adenine. Transformants were not able to grow even after extended incubation periods. These experiments proved that no auto-activation of the reporter genes occurred, thus allowing this construct to be used for the two-hybrid screen.

3.4.3 Positive controls

Two positive controls were included to ensure that the two-hybrid system was working correctly and that the transcription of reporter genes was due to positive interactions and not a defective strain.

The first positive control (GBY 109) consisted of a bait vector (BD) containing the golgi protein GM130 (pFB238) and a prey vector (AD) containing the GRASP55 protein (pFB240). These two proteins are known to interact strongly in a two-hybrid assay(Shorter *et al.* 1999). The two-hybrid strain (GBY 122) was transformed with pFB238 and pFB240 (GBY 109) and tested for stimulation of transcription of reporter genes. Transformants were replica plated on media lacking leucine, tryptophan, histidine and adenine, and growth was detected after 2 days incubation at 30°C. The transformants were then covered with an X-gal overlay solution to test for expression of the β-galactosidase gene and after 30 min-1 h incubation, blue colour formation was observed, showing that the two-hybrid system was functioning properly (Figure 3.2C).

The second positive control (GBY 110) used another set of well known proteins that interact strongly, the SNF1 and SNF4 proteins (Celenza & Carlson

1989), and their interaction tested, as described for GM130 and GRASP55, resulting again in growth and a blue colour formation (Figure 3.2C).

3.4.4 Library screen

After testing that the two-hybrid system was functioning correctly the next stage was to perform the large-scale library screen. The budding yeast two-hybrid strain GBY 122 was transformed with the bait vector pGBT9-C4 (GB 82) and grown on media lacking tryptophan. This strain was then transformed with a second vector pGADGH (Stratagene) containing the fission yeast cDNA-AD library. These transformants were then plated onto 69 large agar plates with media lacking tryptophan and leucine and incubated at 30°C. The number of independent transformants screened was estimated by taking two small aliquots of the transformants mixture and plating onto small agar plates lacking tryptophan and leucine. This was estimated at approximately 6 million transformants, of which 111 colonies that grew contained putative interacters. These colonies were restreaked onto fresh media lacking tryptophan, leucine, histidine and adenine. 100 of these failed to re-grow and were discarded. The remaining 11 colonies were tested for expression of β-galactosidase.

An X-gal overlay assay was carried out as a semi-quantitative method of measuring the degrees of interaction of the 11 isolates with C4. This method proved useful in further reducing the number of false positive colonies and also allowed the colonies to be ordered into levels of strengths of interactions. Of the 11 isolates the strongest interaction where blue colour developed after 90 min was seen in the colony named pAD11 (GBY 121). The next strongest blue tone was seen in pAD4 (GBY 114) and pAD8 (GBY 118) after 6 h incubation. After an overnight incubation pAD2 (GBY 112), pAD3 (GBY 113), pAD6 (GBY 116), pAD7 (GBY 117), pAD9 (GBY 119) and pAD10 (GBY 120) showed weak blue formation and the remaining two pAD1 (GBY 111) and pAD5 (GBY 115) did not change colour at all (Figure 3B). Compared to the positive controls, which began to change colour after around 30 min, pAD11 (GBY 111) showed a very strong interaction. The next level of interaction strength was seen on pAD4 (GBY 114) and pAD8 (GBY 118) followed by pAD2 (GBY112), pAD3 (GBY 113), pAD6 (GBY 116), pAD7 (GBY 117), pAD9 (GBY 119) and pAD10 (GBY 120). These 9 plasmids were extracted from yeast and sent for

sequence analysis (MWG Biotech) to determine which genes were present in the library vectors.

3.4.5 Plasmid rescue and sequencing

After plasmid miniprep from the yeast two-hybrid strain (GBY 122) the plasmids of interest were rescued by means of selection. As the yeast strains contained two plasmids both containing the ampicillin resistance gene the ampicillin resistance method could not be adopted to select specifically for the library vector, and therefore another method had to be used. The only difference between the two vectors that could be exploited was the nutritional marker *TRP1* on the bait vector containing the C4 fragment and *LEU2* on the library vector. In this case the bacterial strain KC-8 that has tryptophan and leucine auxotrophic requirements was used as it can be complemented with *S. cerevisiae TRP1* and *LEU2* genes, respectively. Hence, transforming KC-8 with yeast miniprep DNA and growing the cells on M9 minimal media plates plus ampicillin and tryptophan and lacking leucine should select for the library vector. The plates were incubated for 36 h after which single colonies for each of the 9 isolates were picked and used to inoculate 10 ml of LB plus ampicillin. Plasmid minipreps were performed, using the QIAgen miniprep kit, on each of the 9 samples and subsequently sent for sequencing using the sequencing oligonucleotide GO 158. Of the 9 samples sent for sequencing only 2 returned useful sequences. The plasmid pAD9 (GBY 119) contained the N-terminus of phosphoribosylaminoimidizole (AIR) carboxylase, which is the product of the *ade6* gene (Wood *et al.* 2002) but as this was one of the weaker interactions therefore attention was focussed towards pAD11 (GBY 121).

The most interesting of the results was the sequence returned for pAD11, as it was the C-terminus of DNA polymerase 5 ($pol5^{+}$) (SPBC14C8.14c, Wood *et al.* 2002). This finding was surprising, as it was not expected that a cell cycle transcription factor might interact with a DNA polymerase. The name DNA polymerase 5 was given to this gene as a result of the fission yeast sequencing project where the sequence of $pol5^{+}$ was found to show high sequence similarities to budding yeast *POLV*; it also contained consensus sequences that are common to most RNA polymerases (Shimizu *et al.* 2002, Yang *et al.* 2003).

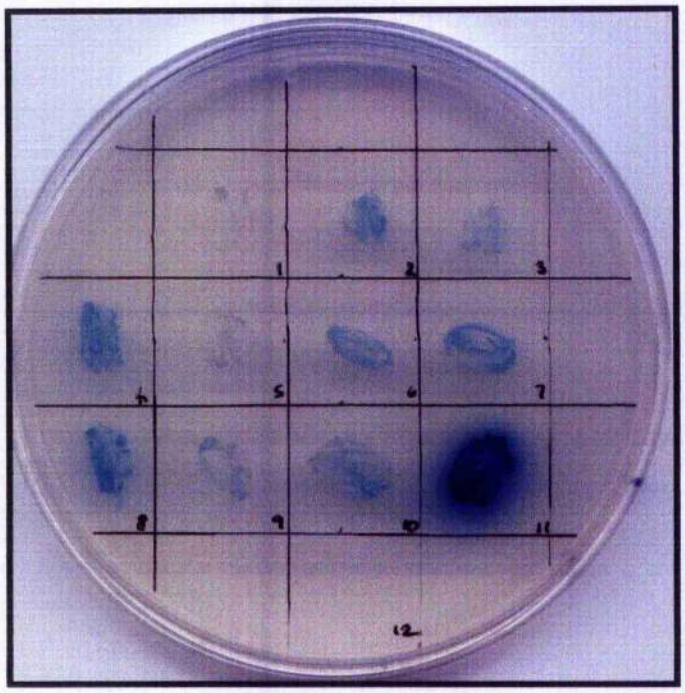


Figure 3B. Two-hybrid screen revealing 11 potential interacting proteins with the C-terminus of SpCdc10p. Isolates were grown at 30°C for 3 nights then covered with X-gal agarose. Isolates 1 and 5 produced no colour change even after overnight incubation at 30°C. Isolates 2, 3, 6, 7, 9 and 10 produced a faint blue colour after an overnight incubation. Colonies 4 and 8 changed blue after 6 h incubation and finally colony 11 produced the fastest colour production after only 90 min incubation. Numbers 1 - 11 correspond to the pAD plasmid (i.e. 1 = pAD1, 2 = pAD2 etc.).

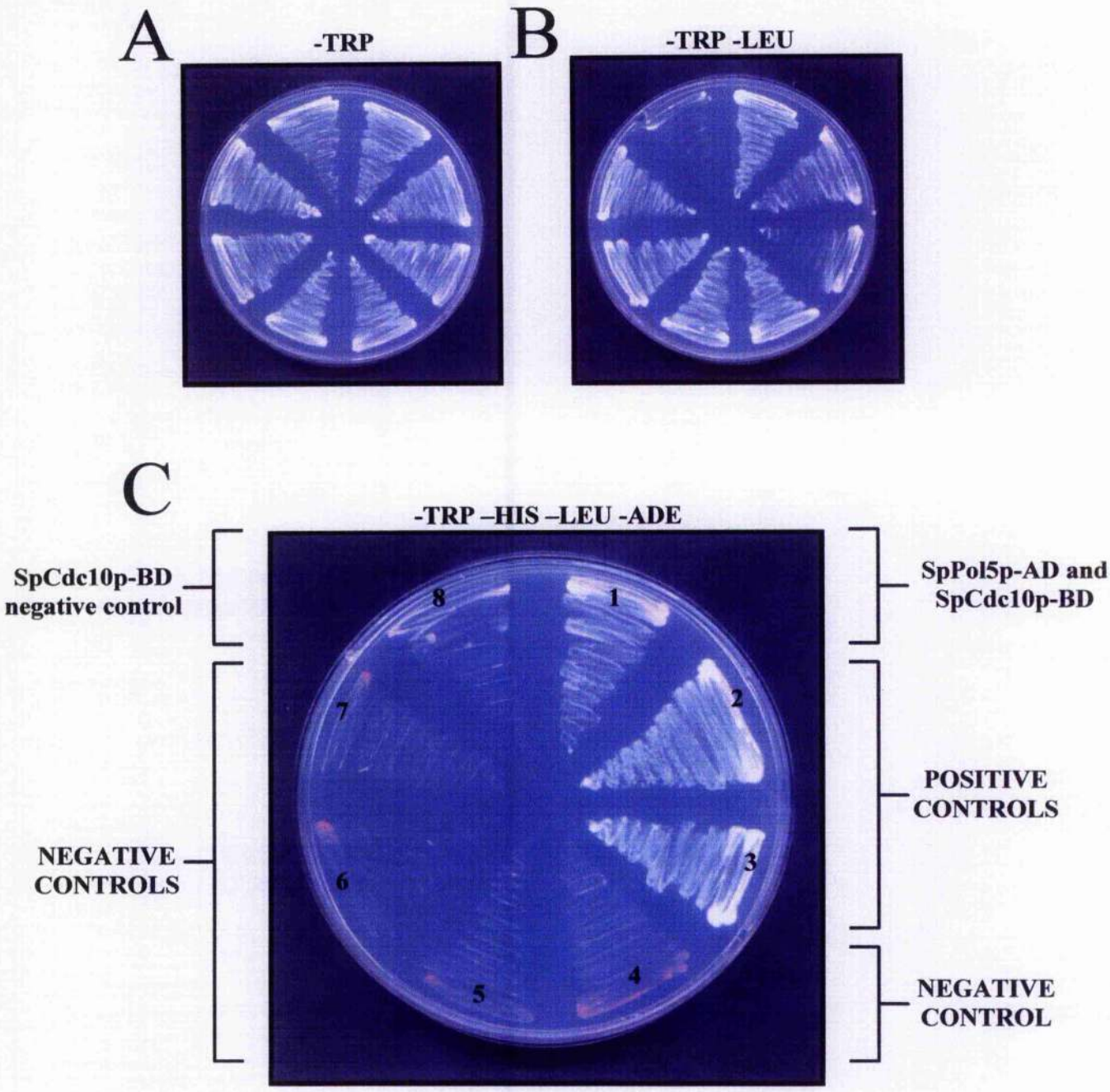


Figure 3.1C. Confirmation that the C-terminus of SpCdc10p interacts with SpPol5p using two-hybrid analysis. A, SD media with leucine, histidine and adenine added allowing all isolates to grow. **B,** SD media plus histidine and adenine allowing all but the SpCdc10p-BD to grow. **C,** Quadruple dropout plate lacking all four nutrients showing growth only occurs where there is interaction of the two proteins causing expression of reporter genes so allowing growth in the absence of all four nutrients. Isolate 1 is the SpCdc10p-BD plus SpPol5p-AD. Isolates 2 and 3 are positive controls, GM130-BD plus GRASP55-AD and SNF1-BD plus SNF4-AD, respectively. Isolates 4-7 are negative controls, SpPol5p-AD with lamin-BD, p53-BD, cdk-BD and SNF1-BD, respectively. Isolate 8 is SpCdc10p-BD by itself.

The interaction between SpCdc10p and SpPol5p was confirmed by retransforming the isolated pAD11 (GBY 121) plasmid into the yeast two-hybrid strain (GBY 122) containing pGBT9-C4 (GB 82). Also, negative controls were completed to show this was a specific interaction and was not an effect of either unusual activity in the strain GBY 122, or by autoactivation by SpPol5p. The two-hybrid strain (GBY 122) plus the *pol5*$^{+}$ AD vector was transformed with four other random genes. These genes were lamin (GBY 174), a member of a family of intermediate filament proteins (Nagle 1988), p53 (GBY 173), a tumour suppressor gene, cdk1 (GBY 175), a cyclin dependent kinase (Koff *et al.* 1992, Elledge & Spottswood 1991), and SNF1 (GBY 172), a protein kinase that associates with SNF4 (Celenza & Carlson 1989). All were kindly supplied, precloned into the pAS vector, by Dr. E. Warbrick (University of Dundee). After transformation the newly retransformed *pol5*$^{+}$ AD plasmid along with the positive and negative controls were replica plated onto dropout media lacking all four nutrients (Figure 3.1C). The positive controls grew as expected, as did the re-transformed SpPol5p and SpCdc10p. The negative control vectors containing lamin, p53, cdk1 and SNF1 were unable to grow in the absence of all four nutrients, showing there was no interaction between SpPol5p and these four different random proteins. This demonstrated that there was no autoactivation taking place through SpPol5p, and also that the interaction between SpPol5p and SpCdc10p was specific. A separate negative control with the SpCdc10p BD vector on its own was also placed alongside the other isolates, to demonstrate further that no autoactivation was taking place.

Next, the isolates were grown on dropout media lacking tryptophan and leucine to allow the various control strains to grow. The plate was covered in X-gal agarose to measure the strengths of interaction of all the different isolates (Figure 3.2C). The X-gal overlay assay permits qualitative visualisation of the strengths of interaction between the two proteins, but the β-galactosidase assay quantifies these interactions to provide a numerical comparison of the two-hybrid assay. The β-galactosidase assay quantifies these interactions (Table 3A) using the equation below.

$$U = \frac{1{,}000 \times [(OD\ 420) - (1.75 \times OD\ 550)]}{[(TIME) \times (Vol) \times OD\ 600]}$$

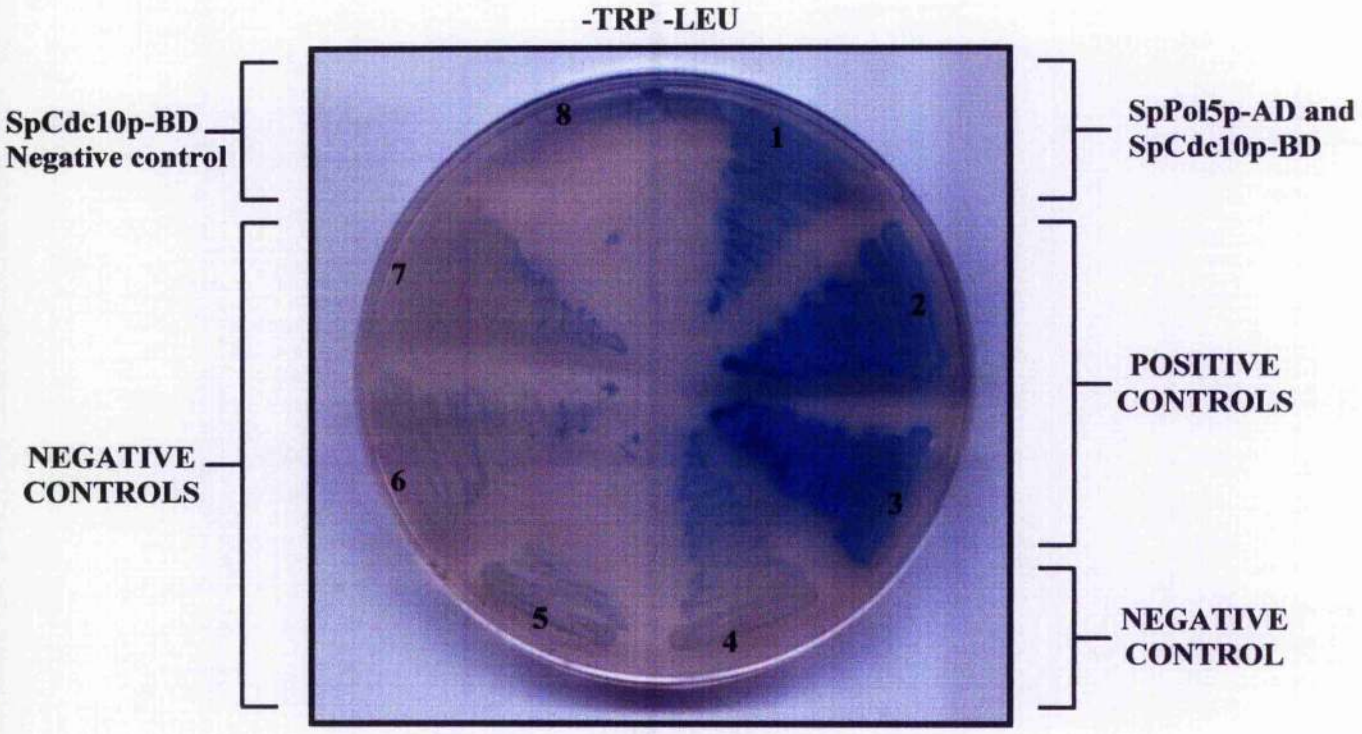
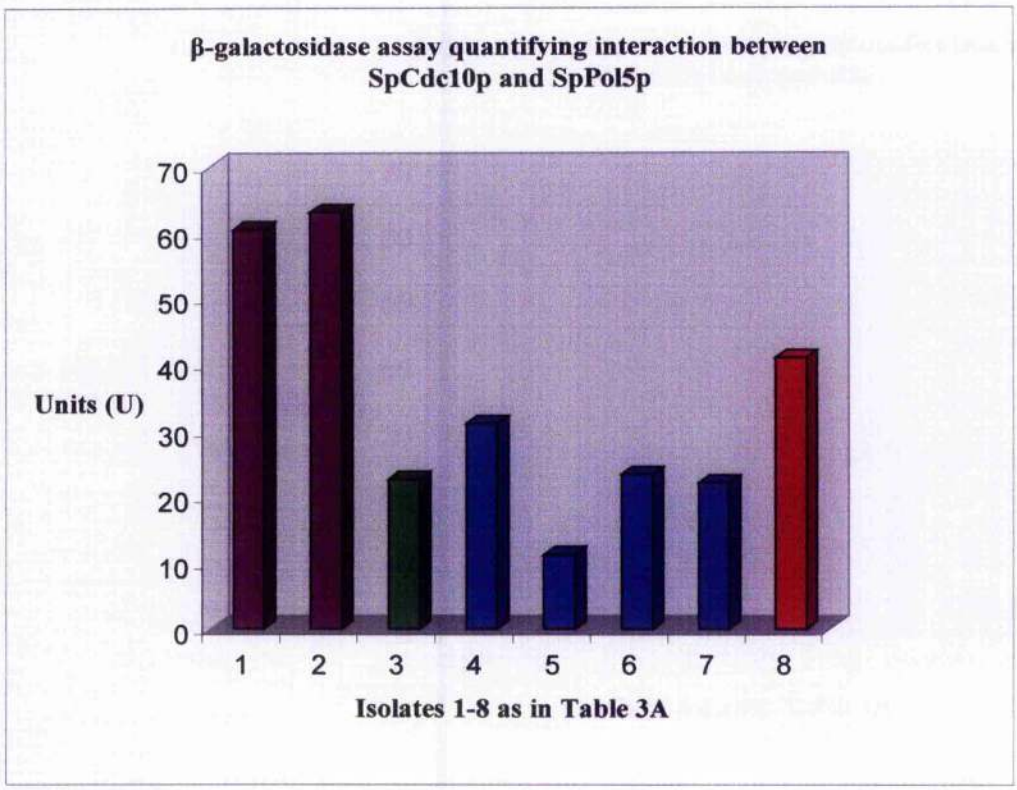


Figure 3.2C. Confirmation of interaction between the C-terminus of SpCdc10p and SpPol5p by two-hybrid X-gal overlay assay. 1, SpCdc10p-BD vector plus SpPol5p-AD vector. **2 and 3,** positive controls, GM130-BD plus GRASP55-AD and SNF1-BD plus SNF4-AD, respectively. **4-7,** negative controls, SpPol5p-AD with lamin-BD, p53-BD, cdk-BD and SNF1-BD, respectively. **8.** SpCdc10p-BD by itself. Only the two positive controls and SpCdc10p-BD plus SpPol5p-AD were able to produce rapid blue colour development demonstrating a true and strong interaction. Isolates 4-7 show faint blue colour but only after overnight incubation whereas isolate 1 changed colour in 1.5 h and isolates 2 and 3 after only 30 min. Isolate 8 is the SpCdc10p-BD vector alone and was unable to grow on SD media lacking tryptophan and leucine as it also required adenine to grow.



		OD at 420 nm	OD at 550 nm	OD at 600 nm	Units (U)
1. GM130-BD and GRASP55-AD	GBY 109	0.04	-0.06	0.959	**60.4**
2. SNF1-BD and SNF4-AD	GBY 110	0.21	-0.06	2	**63.0**
3. SpCdc10p-BD	GBY 127	0.027	-0.069	2.6	**22.6**
4. SNF1-BD and SpPol5p-AD	GBY 132	0.029	-0.043	1.341	**31.0**
5. lamin-BD and SpPol5p-AD	GBY 133	0.011	-0.024	1.881	**11.3**
6. cdk-BD and SpPol5p-AD	GBY 134	0.031	-0.041	1.73	**23.4**
7. p53-BD and SpPol5p-AD	GBY 135	0.022	-0.032	1.411	**22.1**
8. SpCdc10p-BD and SpPol5p-AD	GBY 136	0.072	-0.065	1.821	**40.9**

Table 3A. β-galactosidase assay quantifying the interaction between SpPol5p and C-terminus of SpCdc10p in the two-hybrid assay. The degree of interaction between the two proteins is measured in units (U). In agreement with the visualised X-gal overlay assay, the two positive controls display the strongest interaction with values above 60 U. The interaction between SpCdc10p and SpPol5p is similarly strong with a value of 40.9 U. The negative controls give much lower values confirming weak or no interaction.

The two-hybrid analysis suggests that the C-terminus of SpCdc10p and the C-terminus of SpPol5p interact. We next used a different technique, an *in vitro* GST pull-down assay, to confirm this interaction.

3.5 GST 'pull-down' assay

The GST (Glutathione S-transferase) pull-down method, as with the two-hybrid method, is routinely applied to investigate interactions between proteins. This method uses bacterially expressed tagged versions of genes, which are subsequently purified and used to attempt to 'pull-down' each other *in vitro* to show interaction.

3.5.1 Preparation of tagged genes

The C-terminus of *cdc10*$^{+}$ and *pol5*$^{+}$ were cloned into the pGEX-KG (GB 159) and pET28-a (GB 12) vectors, respectively.

The pGEX-KG vector is a bacterial expression vector that upon stimulation with IPTG induces the over-expression of the cloned gene. The gene of interest is cloned in-frame, upstream of GST resulting in a GST tagged version. In this case the C-terminus of *cdc10*$^{+}$ was amplified by PCR using the oligonucleotides GO 234 and GO 479. The PCR product was cloned in-frame with the GST sequence of the pGEX-KG vector to create GST-*cdc10*$^{+}$. This vector was confirmed by sequencing (MWG Biotech), followed by transformation into the *E. coli* BL21 CodonPlus bacterial strain.

pET28-a is another bacterial expression vector, which upon addition of IPTG induces over-expression of the cloned gene. The difference is that the pET vectors carry a 6HIS tag. The C-terminus of *pol5*$^{+}$ was amplified by PCR using the oligonucleotides GO 236 and GO 237 and was cloned in-frame, upstream of the 6HIS sequence of pET-28a vector resulting in a 6HIS tagged version of *pol5*$^{+}$. The 6HIS-*pol5*$^{+}$ vector was confirmed by sequencing (MWG Biotech) and then transformed into the *E. coli* BL21 CodonPlus bacterial strain.

3.5.2 Induction of bacterially expressed genes

Once the plasmids were transformed into the bacterial expression strain the genes were over-expressed to produce large amounts of tagged proteins to be used for pull-down experiments. A single colony from each plate, containing either GST-*cdc10*$^{+}$ or 6HIS-*pol5*$^{+}$, was picked and used to inoculate 10 ml of NZY^{+} broth plus appropriate antibiotic and grown overnight at 37°C shaking for no more than 16 h. The following day a 1 ml sample of each culture was used to inoculate 50 ml of NZY^{+} plus appropriate antibiotic. This was grown at 37°C whilst shaking until the OD at 600 nm reached between 0.5-0.6. IPTG was added to induce over-expression of tagged SpCdc10p and SpPol5p for 3 h. A sample of culture at zero time was taken before IPTG induction, and then every hour for analysis on an SDS-PAGE gel and also by western blot. The results for the induction of protein can be seen in Figure 3D.

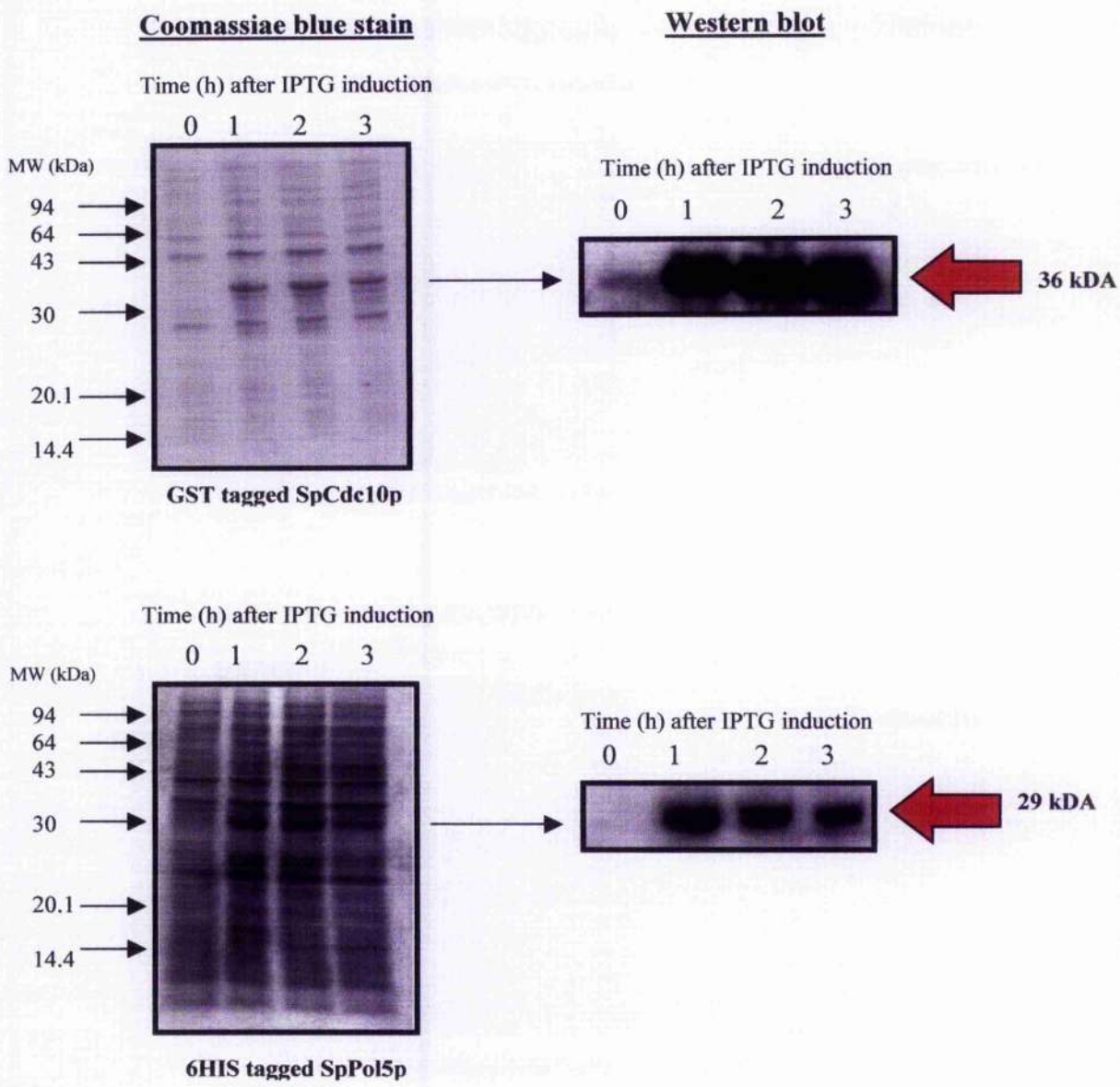


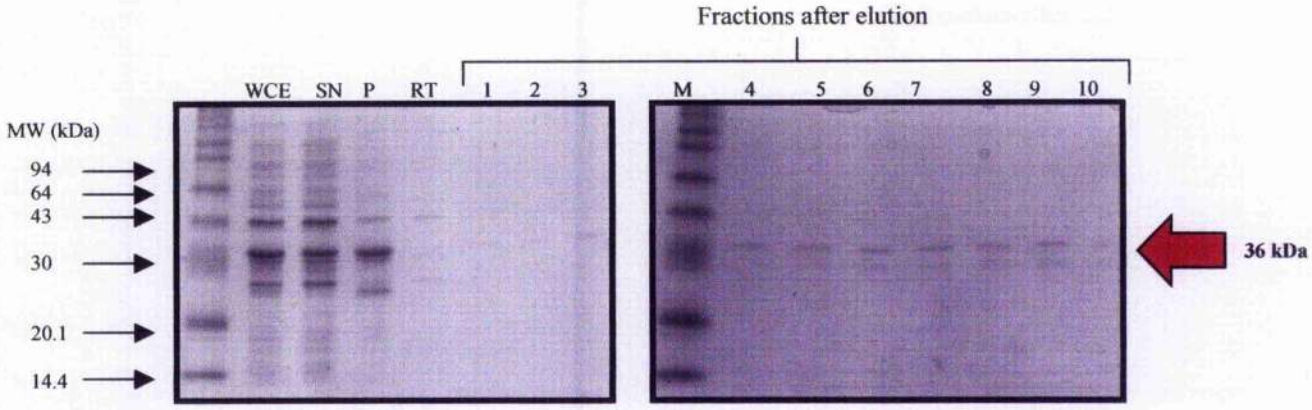
Figure 3D. Induction of successful expression of GST-SpCdc10p and 6HIS-SpPol5p in bacteria. Both GST-SpCdc10p and 6HIS-SpPol5p were over-expressed at 37°C before samples were taken at zero time before IPTG (1mM) induction and then every hour for 3 hours thereafter. Protein was visualised by coomassiae blue stain and then by western blot analysis using anti-GST antibody for SpCdc10p and anti-HIS antibody for SpPol5p. Molecular weight marker (MW) bands are shown for each gel as a guide for the size of proteins.

3.5.3 Protein purification and GST pull-down assay

Once conditions had been optimised for protein induction, protein was over-expressed and purified in preparation for the GST pull-down assay. GST-SpCdc10p and 6HIS-SpPol5p were purified, after induction, by collecting cells by centrifugation followed by cell lysis using a French Press. GST-SpCdc10p was purified using the glutathione sepharose 4B column and 6HIS-SpPol5p was purified using a divalent metal ion affinity column packed with nickel ions bound to fast flow slurry. 1 ml fractions were collected, analysed by SDS-PAGE (Figure 3E), and samples containing protein were pooled for dialysis. Once proteins were purified, dialysis was carried out before GST pull-down experiments were performed. 1 ml of tagged SpCdc10p was added to glutathione sepharose beads and allowed to bind for 1 h. Tagged SpPol5p was then added to the same microfuge tube and incubated for a further hour to allow interaction to take place between the two proteins. All samples, including washes, were analysed by western blot using antibodies against GST and 6HIS to detect SpCdc10p and SpPol5p, respectively. The results of these purifications are shown in Figure 3F.

The results from the GST pulldown experiment confirmed the two-hybrid interaction. When GST-SpCdc10p was pulled-down, SpPol5p was detected in the same fraction. A control experiment was carried out in parallel where GST was induced by itself using the empty pGEX vector (Figure 3F). GST protein was purified and mixed with purified 6HIS tagged SpPol5p. SpPol5p was unable to bind to GST on its own and was detected in the washes, so the interaction with SpCdc10p was specific and not as a result of SpPol5p binding directly to GST. Therefore the combination of two-hybrid and pull-down data confirms that the C-terminus of SpCdc10p binds to SpPol5p in fission yeast.

GST-SpCdc10p purification



6HIS-SpPol5p purification

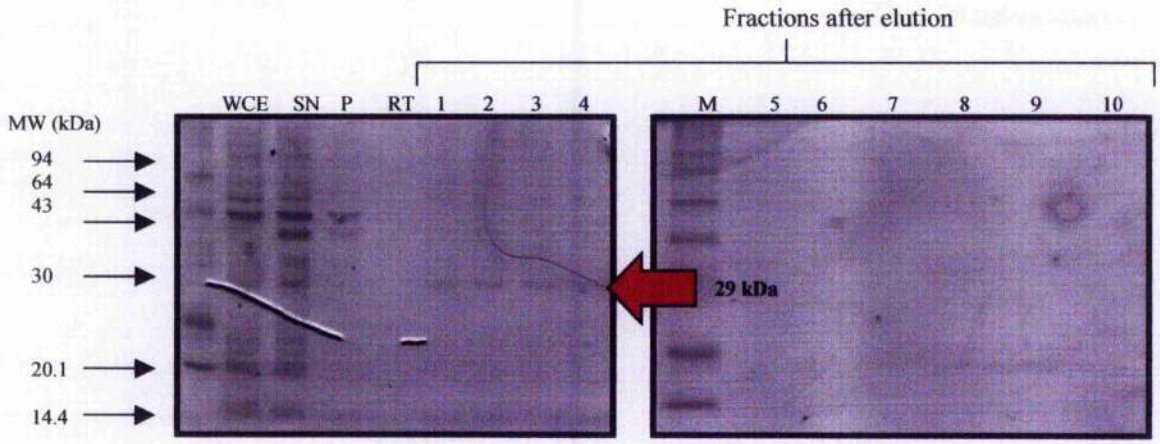


Figure 3E. Column purification of bacterially expressed tagged proteins. GST-SpCdc10p was purified using glutathione sepharose beads and ten 1 ml fractions were collected and analysed by SDS-gel coomassaie blue staining. Elution fractions 4-9 were pooled for dialysis and used in the GST pull-down. 6HIS-SpPol5p was purified using a divalent metal cation affinity column using fast flow slurry to pack the column. Elution fractions 1-4 were pooled for dialysis and used in the GST pull-down. Abbreviations: WCE, whole cell extract; SN, supernatant; P, pellet; RT, run-through; molecular weight marker (MW) bands are shown for each gel as a guide for the size of proteins.

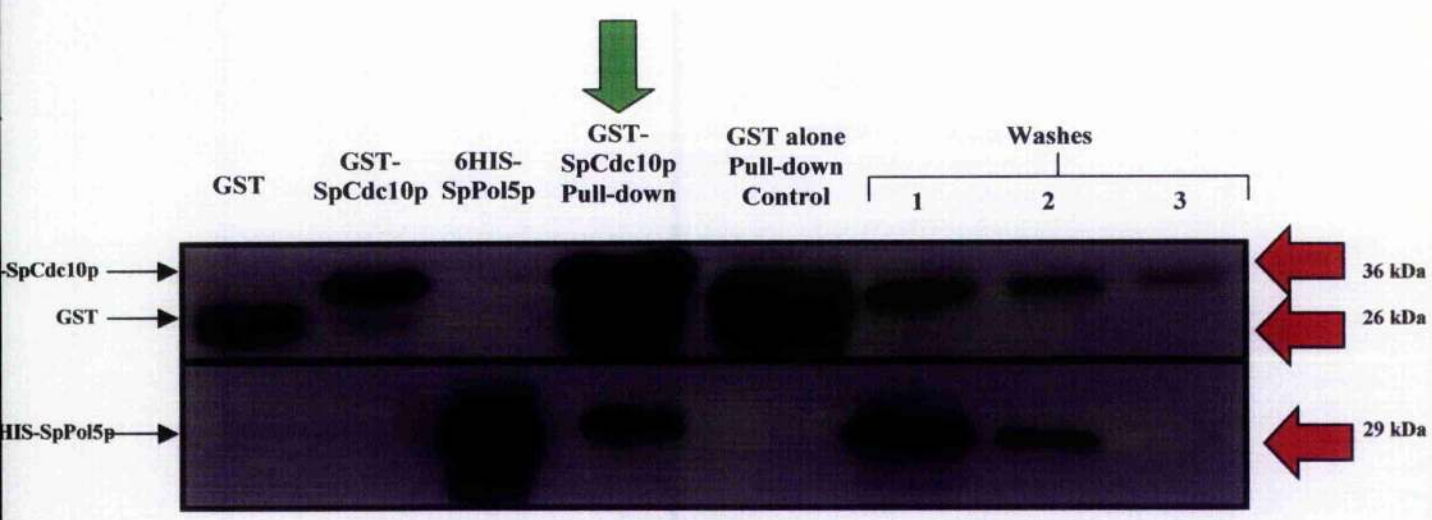


Figure 3F. *In vitro* GST pull-down assay confirming the interaction between SpPol5p and SpCdc10p. GST expressed by itself displays a protein of 26 kDa, and GST-SpCdc10p is 36 kDa. 6HIS-SpPol5p is 29 kDa. SpCdc10p and SpPol5p interact as 6HIS-SpPol5p is detected in the same pulldown fraction as GST-SpCdc10p. In contrast, GST alone was unable to pull-down SpPol5p, which instead was detected in the wash lanes 1-3.

3.6 Budding yeast *POLV* - RNA polymerase

One of the most important components of a cell are DNA polymerases. There are many different types of polymerases that have been identified in both prokaryotes and eukaryotes, involved in major processes such as DNA replication, repair and recombination (Burgers 1998, Cox & Lehman 1987). The genome sequence of *S. cerevisiae* (Goffeau et al. 1997) revealed *YEL055C,* and because of its resemblance to many of the B-type polymerases it was named *POLV* (Argos 1988). Experiments to test the DNA polymerase activity of this gene were carried out by Shimizu *et al.* (2002) by generating mutants of *POLV* in the presumed polymerase active-site, thought to be common amongst all B type polymerases. Firstly, the DNA polymerase activity in wild type cells was assayed and was demonstrated to have weak polymerase activity. Next, mutant SpPol5p was assayed for polymerase function and was found to be impaired, confirming that *POLV* was indeed a polypeptide with DNA polymerase activity.

However, further investigation into the role of *POLV* complicated matters. When *POLV* null mutants were generated they were found to be inviable. B type polymerases are thought to be required for DNA damage checkpoint control repair mechanisms and therefore are normally non-essential genes. As *POLV* is an essential gene this implies that *POLV* has other roles. Further analysis of the *POLV* sequence suggested an alternative function (Yang *et al.* 2003). Previous studies suggested SpPol5p was a B type polymerase because it contains six DNA polymerase domains found in all B type polymerases, but this new analysis of specific regions of SpPol5p showed that it was more similar to the animal protein MybBP1A, which is an apparent rDNA transcription regulator that interacts with MYB-containing transcription factors (Figure 3Ga) (Tavner *et al.* 1998, Shimizu *et al.* 2002, Yang *et al.* 2003). The traditional view of the secondary structure of all DNA and RNA polymerases is that they have α-β composition with conserved core β-sheets. This is different to the structure of MybBP1A, which is predominantly α-helical in structure. Therefore, it was suggested that the weak polymerase activity associated with *POLV* should be re-evaluated.

The creation of temperature sensitive *pol5* mutants (Shimizu *et al.* 2002) allowed the manipulation of *POLV* function to investigate its role further. Experiments showed that cells lacking *POLV* function severely inhibited the production of rRNAs, and also increased the copy numbers of the rRNA encoding DNA (rDNA) repeating unit on chromosome XII. These data suggest that *POLV* is involved in processes outside of DNA polymerase activity and instead is linked to rRNA production (Shimizu *et al.* 2002).

3.7 Fission yeast *pol5*$^{+}$ - required for rRNA production?

Sequence alignments show *S. pombe pol5*$^{+}$ is highly similar to *S. cerevisiae POLV.* There is strong homology between Pol5p of both yeast species and MYB proteins of metazoan species including human and mouse. Figure 3Gb aligns SpPol5p with various other DNA Polymerases, as this was originally thought to be the function of SpPol5p, and demonstrates a lower degree of homology than with MybBP1 proteins.

The protein consensus sequence "YslpRL.pu.ussc" (s = small residues, l – aliphatic residues, p = polar residues, u = tiny residues and c = charged residues), important for MYB protein function, was also found in both fission and budding yeast SpPol5p. As seen in Figures 3Ga and 3Gb, there are many more highly conserved regions, of both budding and fission yeast species, related to MYB proteins than for class B DNA polymerases. These similarities prompt additional exploration into the role *pol5*$^{+}$ has to play in fission yeast and whether it plays the analogous role to budding yeast *POLV* in rRNA production.

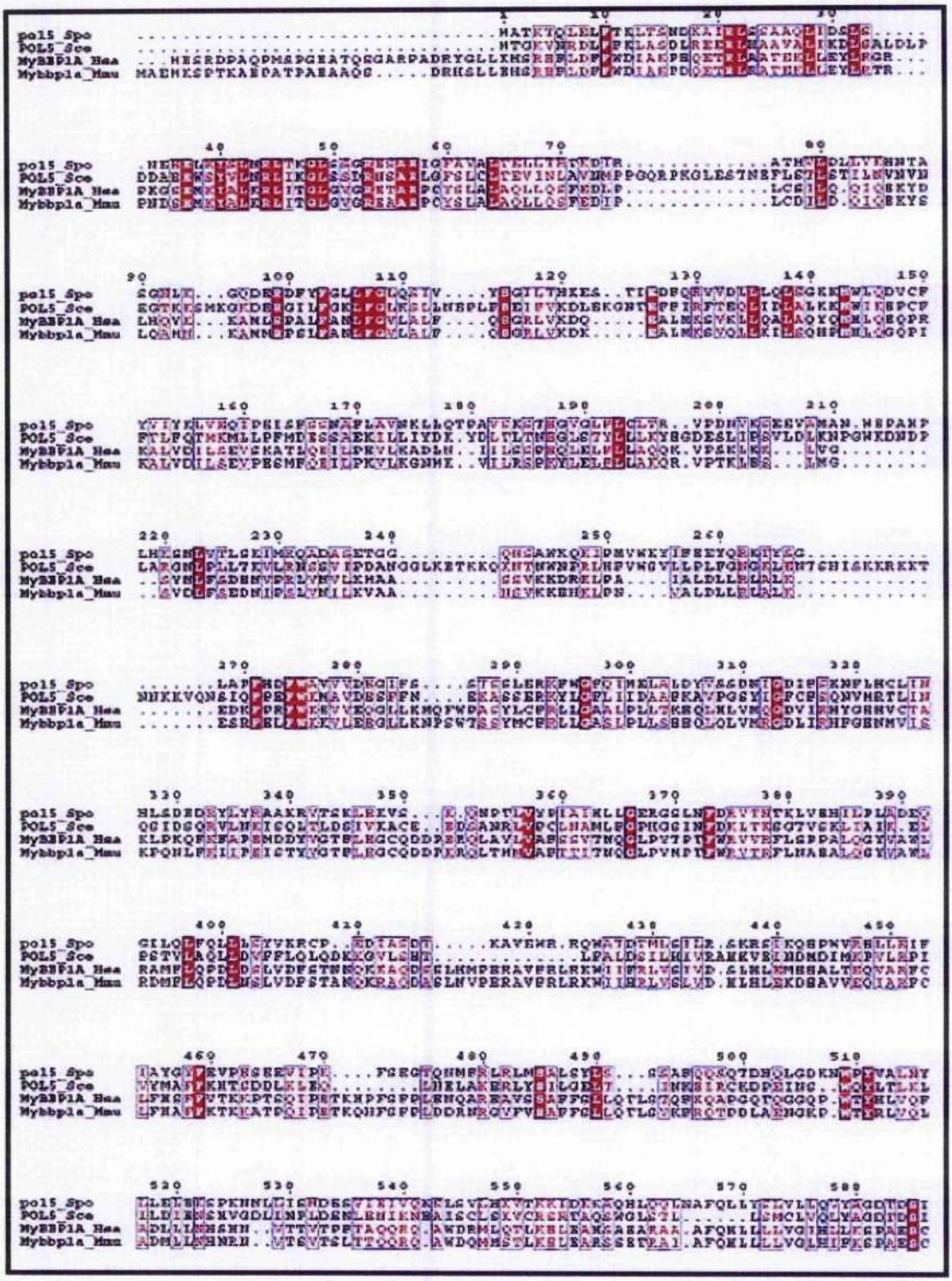


Figure 3Ga. Multiple protein sequence alignment of SpPol5p of Sce, *Saccharomyces cerevisiae* and Spo, *Schizosaccharomyces pombe* with MybBP1A protein of Hsa, *Homo sapiens* and Mmu, *Mus musculus*. Regions shaded in red show identical matches for amino acid residues and boxed areas indicate strong homology between proteins.

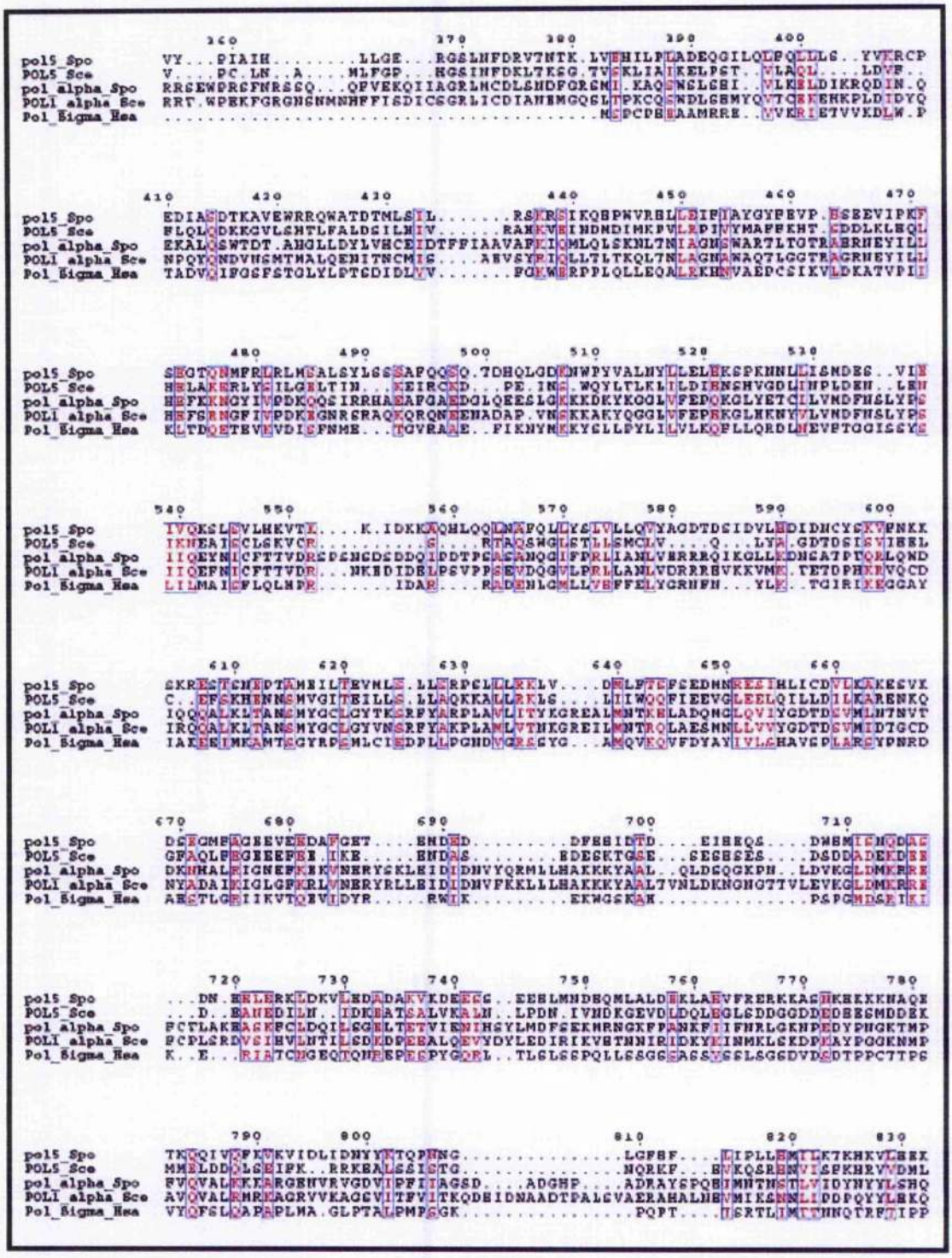


Figure 3Gb. Multiple protein sequence alignment of SpPol5p of Sce, *Saccharomyces cerevisiae* and Spo, *Schizosaccharomyces pombe* with a selection of other class B DNA polymerases of *S. pombe*, *S. cerevisiae* and *homo sapiens*. Boxed areas indicate strong homology between proteins.

3.8 Summary

In this study SpPol5p was discovered as a result of a two-hybrid screen to be a novel binding partner of SpCdc10p. SpCdc10p is a well characterised major component of a cell cycle transcription factor complex that operates at the G_1-S boundary of the fission yeast cell cycle to control expression of genes required for DNA replication. It was therefore surprising to discover that a potential rRNA production activity should interact with a cell cycle specific transcription factor, as rRNA production is not directly required for cell cycle progress and instead is important for cell growth. Cell cycle and cell growth are known to be two separate though linked processes (Shirodkar *et al.* 1992, Bartek *et al.* 1996). Could it be that SpPol5p is a missing link between these two separate processes? The next chapter examines this hypothesis further.

Chapter 4

Investigations into the biology of SpPol5p

4.1 Introduction

In Chapter 3, a two-hybrid experiment was described that discovered a novel protein in fission yeast named DNA polymerase 5 (SpPol5p). The experiments revealed that SpPol5p could interact with the cell cycle transcription factor SpCdc10p. Sequence analysis showed SpPol5p to be strongly related to ScPolVp of budding yeast (Shimizu *et al.* 2002). Further sequence analysis of budding yeast ScPolVp and fission yeast SpPol5p revealed that they were very similar to the MYB family of proteins (Yang *et al.* 2003), which are involved in ribosomal DNA (rDNA) regulation. Studies in budding yeast suggest that ScPolVp is also involved in rRNA synthesis (Shimizu *et al.* 2002) and therefore due to the high homology between the polymerases of the two yeast species, SpPol5p of fission yeast could be predicted to be involved in the same process.

pol5^{+} is a relatively large gene at 3 kb in size, unusual for fission yeast, and contains one short intron. There is little information available about the structure and function of the protein, apart from its similarities to budding yeast ScPolVp.

We have established that SpPol5p can interact directly with SpCdc10p. SpCdc10p is a protein required for cell cycle progression and in contrast, SpPol5p might be an RNA polymerase, involved in cell growth. Cell cycle and cell growth are two separate though linked processes. SpPol5p may therefore be a link between these processes. In this chapter we aimed to investigate further the role of *pol5*$^{+}$ in fission yeast.

4.2 Transcription of *pol5*$^{+}$

Initially, the levels of *pol5*$^{+}$ RNA were examined in both asynchronous and synchronous cell cultures, to understand its transcription profile.

We observed *pol5*$^{+}$ mRNA levels in both wild type cells and a mutant of *cdc10*$^{+}$, *cdc10-C4*. SpCdc10p is the major component of the cell cycle transcription factor complex, DSC1, and controls transcription of many genes, including *cdc22*$^{+}$, in a cell cycle dependant manner (Lowndes *et al.* 1992). The strain *cdc10-C4* is a temperature-sensitive mutant that has abnormal regulatory properties at high and low temperatures (McInerny *et al.* 1995). This is as a result of a non-sense mutation at the

C-terminus of SpCdc10p. At low temperature SpCdc10p controlled genes are constitutively expressed throughout the cell cycle resulting in higher levels of expression as compared to wild type cells. Thus this mutant is a convenient way to discover if a gene is under SpCdc10p control.

4.2.1 Transcription of *pol5*$^{+}$ in asynchronous cells

Both *cdc22*$^{+}$ and *pol5*$^{+}$ mRNA were quantified in asynchronous, wild type and *cdc10-C4* cells. Using northern blot analysis (Materials & Methods, Section 2.6.4.1), levels of *cdc22*$^{+}$ and *pol5*$^{+}$ transcripts were examined to determine if *pol5*$^{+}$ was transcribed and whether it was under the control of SpCdc10p. Cells were grown to mid-log phase and then harvested after which RNA was extracted. Equal amounts of RNA was separated by electrophoresis and transferred to a membrane (Materials & Methods, Section 2.6.4). The membrane was probed for *pol5*$^{+}$ and *cdc22*$^{+}$ mRNA by hybridisation, using radiolabelled [α-P^{32}] DNA (Materials & Methods, Section 2.4.10). The results obtained are shown in Figure 4A.

cdc22^{+} transcript was detectable after an overnight exposure, whereas *pol5*$^{+}$ took one week to be detected. As the two probes were of similar labelled activity, this suggests that *pol5*$^{+}$ mRNA is present in lower abundance than *cdc22*$^{+}$, which implies it is transcribed at low levels.

cdc22^{+} is over-expressed in the mutant *cdc10-C4* (Figure 4A). As previously reported, (McInerny *et al.* 1995) this is because *cdc22*$^{+}$ is expressed throughout the cell cycle in this mutant, as *cdc22*$^{+}$ is under SpCdc10p control (Lowndes *et al.* 1992, Maqbool *et al.* 2003). In contrast, *pol5*$^{+}$ mRNA levels are not affected in *cdc10-C4* cells suggesting that *pol5*$^{+}$ is not under SpCdc10p control (Figure 4A).

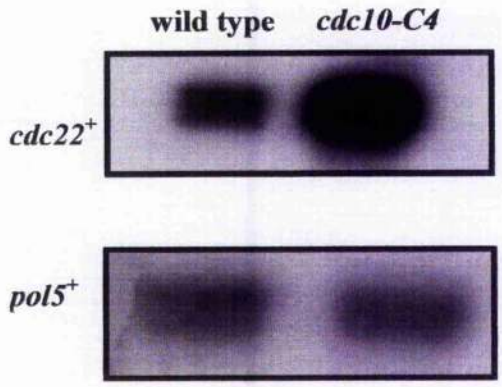


Figure 4A. mRNA levels of *pol5*$^+$ and *cdc22*$^+$ in asynchronous wild type and *cdc10-C4* cells show that *pol5*$^+$ transcription is not under SpCdc10p control. Northern blot analysis of RNA extracted from wild type and *cdc10-C4* asynchronous cells. Cell cultures were grown to mid-log phase at 25°C followed by RNA extraction. RNA was transferred onto GeneScreen membrane where *cdc22*$^+$ and *pol5*$^+$ were probed with [α-P^{32}] radioactively labelled DNA.

4.2.2 Transcription of *pol5*$^+$ in synchronous mitotic cells

The mRNA profile of *pol5*$^+$ was also examined in synchronous mitotic cell cultures using the *cdc25-22* temperature sensitive mutant. Using northern blot analysis, the mRNA profiles of *cdc22*$^+$ and *pol5*$^+$ were examined to determine *pol5*$^+$ transcription through the cell cycle. This experiment would reveal how abundant the *pol5*$^+$ transcript is in the mitotic cycle and it would also show if the transcription of the gene is constitutive or periodic.

A 500 ml culture of YE was inoculated with 5 ml of *cdc25-22* cells taken from a pre-culture grown overnight at 25°C. Cells were grown to mid-log phase at 25°C and then transferred to 36°C for 4 hours, allowing cells to cell cycle arrest. The culture was then put back to permissive temperature (25°C) where cells commenced dividing in a synchronous manner. Samples were taken at regular intervals for northern blot analysis to assay *cdc22*$^+$ and *pol5*$^+$ mRNA levels. The results obtained are shown in Figure 4B.

cdc22$^+$ transcript was detectable after an overnight exposure whereas *pol5*$^+$ transcript took a week to be detected. This correlated with the data shown in Figure 4A, which suggested that *pol5*$^+$ mRNA levels were low as compared to *cdc22*$^+$ and therefore, may also be transcribed in low amounts.

Genes that are under SpCdc10p control contain MCB (*Mlu*I cell cycle box) elements in their promoter regions that confer periodic levels of mRNA throughout the cell cycle (McInerny 2004). Previous results (Figure 4A) showed that *pol5*$^+$ was not under SpCdc10p control. This experiment revealed that *pol5*$^+$ mRNA is present throughout the mitotic cell cycle and the absence of MCB motifs in the promoter area of *pol5*$^+$ (Wood *et al.* 2002) confirm it to be unlikely to be under SpCdc10p control.

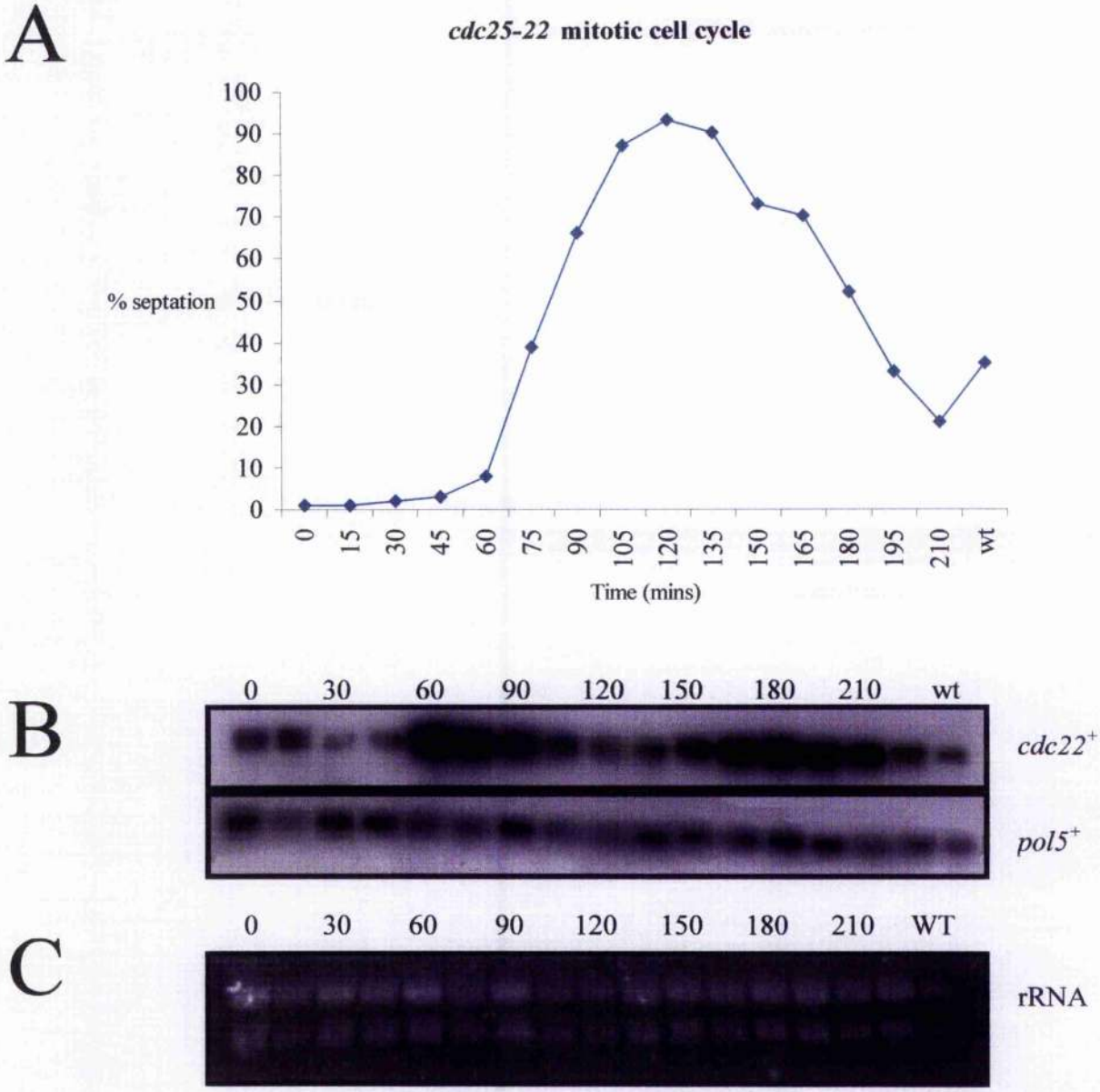


Figure 4B. Transcript profile of *pol5*$^+$ and *cdc22*$^+$ during a *cdc25-22* synchronised mitotic cell cycle. Northern blot analysis was performed on RNA samples prepared at 15-minute intervals following release from restrictive (36°C) temperature to permissive (25°C). **A.** The septation index indicates the synchrony of the cell population. **B.** Northern blot analysis showing mRNA levels of *cdc22*$^+$ and *pol5*$^+$. The control lane is an RNA sample taken from a wild type (WT) asynchronous cell culture. **C.** That the RNA was equally loaded was confirmed by ethidium bromide labeling of ribosomal RNA (rRNA).

4.2.3 Transcription of *pol5*$^+$ in synchronous meiotic cells

The transcript profile of *pol5*$^+$ was also examined in synchronous cell cultures in meiosis using the *pat1-114* temperature sensitive mutant (Bahler *et al.* 1991). This mutant was chosen because cells, when released from nitrogen starvation, will enter a highly synchronous meiotic cell cycle from G_1, mimicking wild type cells as closely as possible. Using northern blot analysis, the mRNA profiles of *cdc22*$^+$ and *pol5*$^+$ were examined to determine the abundance of *pol5*$^+$ transcript in the meiotic cycle and also if the transcription of the gene is constitutive or periodic.

A 500 ml culture of EMM was inoculated with 5 ml of *pat1-114* cells taken from a pre-culture grown overnight at 25°C. This culture was grown overnight at 25°C after which cells were collected by centrifugation and added to a 500 ml culture of nitrogen free EMM. Cells were starved of nitrogen for 16-24 h at 25°C where they became arrested at G_1. They were then transferred to 36°C with the addition of nitrogen (NH_4Cl) where synchronous entry in meiosis was induced. As described before, samples were periodically taken for RNA extraction and northern blot analysis, to examine levels of *cdc22*$^+$ and *pol5*$^+$ mRNA. The results obtained are shown in Figure 4C.

As with the mitotic experiment (Figure 4B), *cdc22*$^+$ transcript was detected after a one night exposure whereas *pol5*$^+$ took one week to be detected. This implies that *pol5*$^+$ is also a rare meiotic transcript. Furthermore, as previously reported, *cdc22*$^+$ is induced periodically during meiosis because it is under SpCdc10p control (Cunliffe *et al.* 2004), whereas *pol5*$^+$ is not as it is constitutively expressed. These results further support the contention that *pol5*$^+$ is not under DSC1 control.

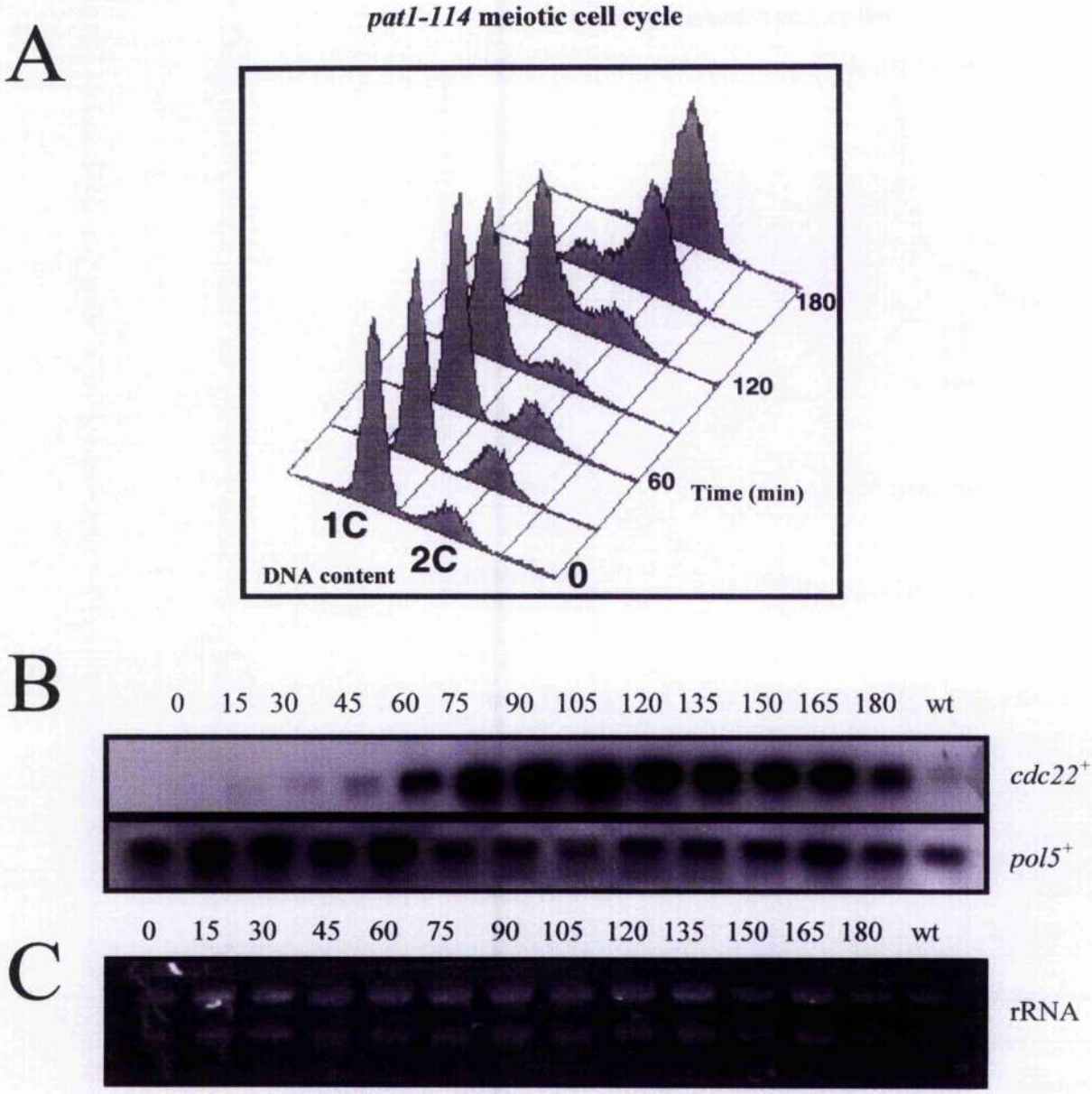


Figure 4C. Transcript profile of *pol5*$^+$ and *cdc22*$^+$ during a *pat1-114* synchronised meiotic cell cycle. Cells were arrested by nitrogen starvation for 16 h at 25°C. They were then transferred to restrictive temperature (36°C) where synchronous entry into meiosis was induced. Cells were harvested at 15 min intervals **A**. Cells were stained with propidium iodide for analysis by flow cytometry to display the synchrony of the cell population. **B**. Northern blot analysis showing mRNA levels of *cdc22*$^+$ and *pol5*$^+$. The control lane is an RNA sample taken from a wild type (WT) asynchronous cell culture. **C**. RNA loading was confirmed by ethidium bromide labeling of ribosomal RNA (rRNA).

4.3 Disruption of *pol5*$^+$

An important way of studying the function of a fission yeast gene is to delete the chromosomal copy of the gene in an otherwise wild type strain. By examining the effect this has on cell growth and division this may allow insights into the possible roles of the gene. The deletion of various genes has different effects, from no effect at all "non-essential", to defects in morphology, to severe cases where cell death occurs. In the latter case the gene is termed as being "essential".

4.3.1 Creation of a diploid strain

The disruption of a novel gene in fission yeast is performed in a diploid strain, as it is not known whether the gene is essential or non-essential.

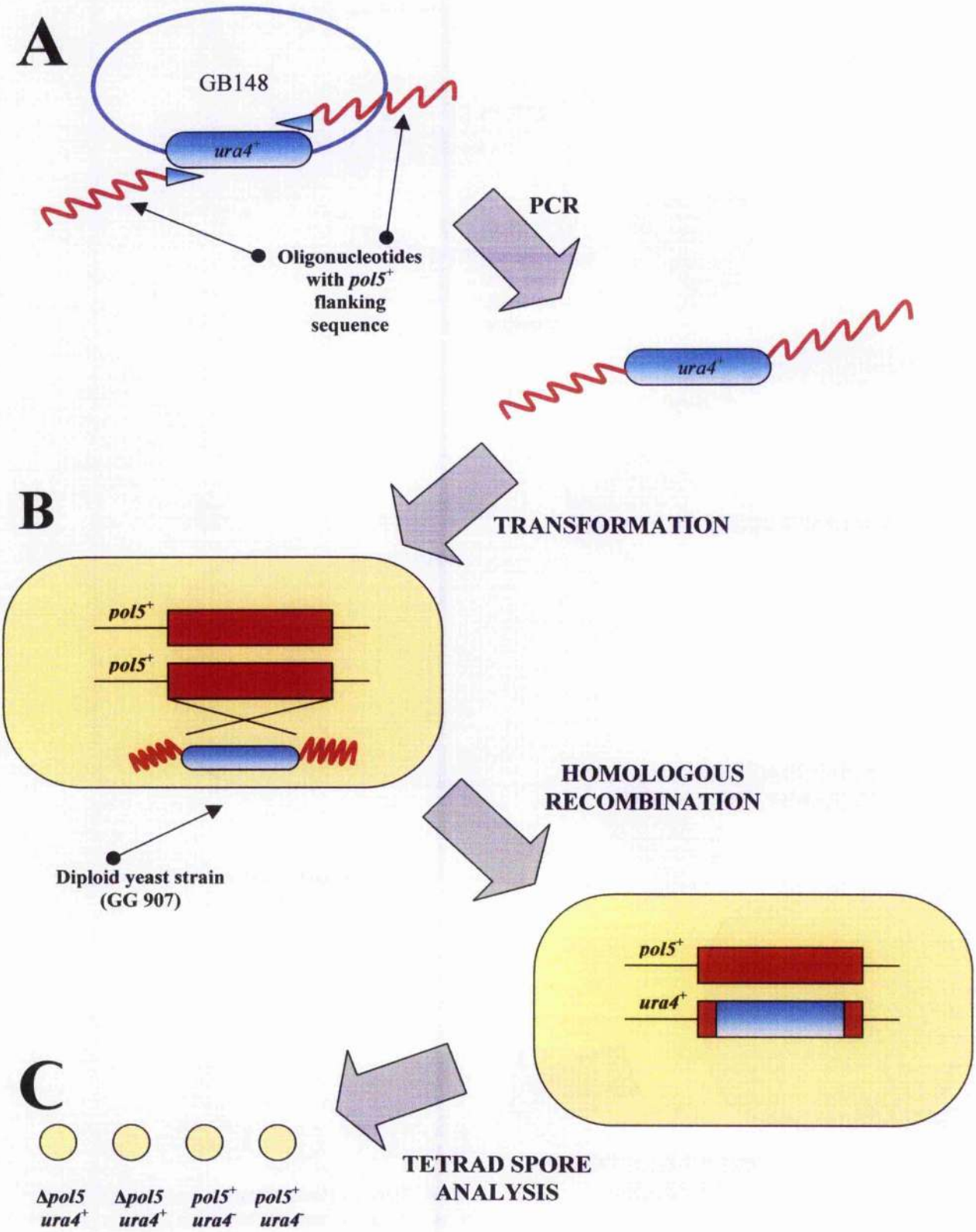
To create the diploid strain, two haploid fission yeast strains of opposite mating type with the following genotype were mated: h$^+$ *ade6-210*, *leu1-32*, *ura4-D18* (GG 376) and h$^-$ *ade6-216*, *leu1-32*, *ura4-D18* (GG 379). When these strains mate they form the following genotype: h$^+$/h$^-$ *ade6-210/ade6-216*, *leu1-32/leu1-32*, *ura4-D18/ura4-D18* (GG 907), where the two adenine mutations complement each other to allow selection for the diploid on ade$^-$ plates. The strains were allowed to mate on an ME plate at 25°C overnight. The following day samples of cells were taken throughout the day and streaked onto EMM plates plus leucine and uracil and incubated at 30°C for 1-2 days. The colonies that arose were again streaked to single colonies on EMM plus leucine and uracil to confirm that they were diploids. A YE masterplate of the new strain was created, as fission yeast diploid strains remain stable on YE and not on EMM where they sporulate. Diploids were frozen down and stored at –70°C in YE.

4.3.2 *pol5*$^+$ is disrupted using a heterologous module for PCR-based gene targetting

The PCR-based gene targetting method devised by Bahler *et al.* (1998) was used to disrupt *pol5*$^+$ (Figure 4D). The plasmid template (GB 148), carrying the *ura4*$^+$ gene in the plasmid pBluescript, was used to amplify the product required for *pol5*$^+$ gene disruption. Oligonucleotides were designed (GO 403 and GO 404) containing the forward and reverse sequences of *ura4*$^+$ with flanking sequences of *pol5*$^+$, approximately 75 base pairs, added to each oligonucleotide. The product was amplified using the High Fidelity PCR system (Materials & Methods, Section 2.4.1.3). 5 µl of the reaction was analysed on a 1% agarose gel stained with ethidium bromide revealing a correct sized product of 2.8 kb.

Diploid cells were transformed with the *pol5*$^+$-*ura4*$^+$ DNA construct using the lithium acetate procedure. Cells were spread onto an EMM plus leucine plate to select for *pol5*$^+$/Δ*pol5*::*ura4*$^+$ diploid colonies that had incorporated the *ura4*$^+$ gene. Potential positives were confirmed by PCR and tetrad spore analysis.

Figure 4D. A Schematic representation of the disruption of *pol5*$^+$ with the *ura4*$^+$ gene using a PCR-based gene targetting method (Bahler *et al.* 1998). **A.** Oligonucleotides were designed with *pol5*$^+$ flanking sequence in order to PCR amplify the *ura4*$^+$ gene, using the template GB148 (*ura4*$^+$ cloned into pBluescript). **B.** The PCR product was transformed into the diploid fission yeast strain (GG 907) where homologous recombination took place, resulting in a diploid strain with one wild type copy of *pol5*$^+$ and one disrupted copy, replacing it with *ura4*$^+$. **C.** Diploids were subsequently analysed by tetrad dissection to test if the disruption of *pol5*$^+$ was lethal to cells.


A
GB148
ura4+
Oligonucleotides with pol5+ flanking sequence
PCR
ura4+
B
TRANSFORMATION
pol5+
pol5+
Diploid yeast strain (GG 907)
HOMOLOGOUS RECOMBINATION
pol5+
ura4+
C
TETRAD SPORE ANALYSIS
Δpol5 ura4+
Δpol5 ura4+
pol5+ ura4-
pol5+ ura4-

4.3.3 PCR analysis of potential *pol5*$^+$/Δ*pol5* diploid isolates

Potential diploids were screened to ensure that *ura4*$^+$ had integrated into the *pol5*$^+$ locus by homologous recombination and not by random, non-homologous, recombination. This was done by PCR using one oligonucleotide within the *ura4*$^+$ gene at the C-terminus and another oligonucleotide outside of the *pol5*$^+$ flanking region that was used for the *pol5*$^+$ disruption experiment (Figure 4E). If *ura4*$^+$ had correctly integrated into the *pol5*$^+$ locus then a product of approximately 500 base pairs was generated. The oligonucleotides GO 474 and GO 475 were used for the PCR reaction with genomic DNA template prepared from each isolate and *Taq* polymerase. Seven positive diploids were identified which all gave products of 500 bp (Figure 4E). Three heterozygous diploids were chosen at random for subsequent tetrad spore analysis.

4.3.4 Tetrad dissection and spore analysis

The three diploid isolates chosen were dissected using a Singer tetrad dissector. Individual tetrads were isolated to a grid position and incubated at 30°C for 2-4 h to allow the cell wall to break open. Once the cell wall had broken down individual spores were separated into four seperate grid positions in the same row. Approximately 60 tetrads were dissected and separated and then left to grow for three days at 30°C on YE medium. All four spores were expected to grow on rich media if the gene was non-essential. If *pol5*$^+$ were essential then only two spores, which were both *pol5*$^+$ and ura$^-$, would be able to grow. No more than 2 spores grew to viable colonies in any of the individual tetrads dissected and all of these were ura$^-$, showing that *pol5*$^+$ is an essential gene (Figure 4F and Table 4A).

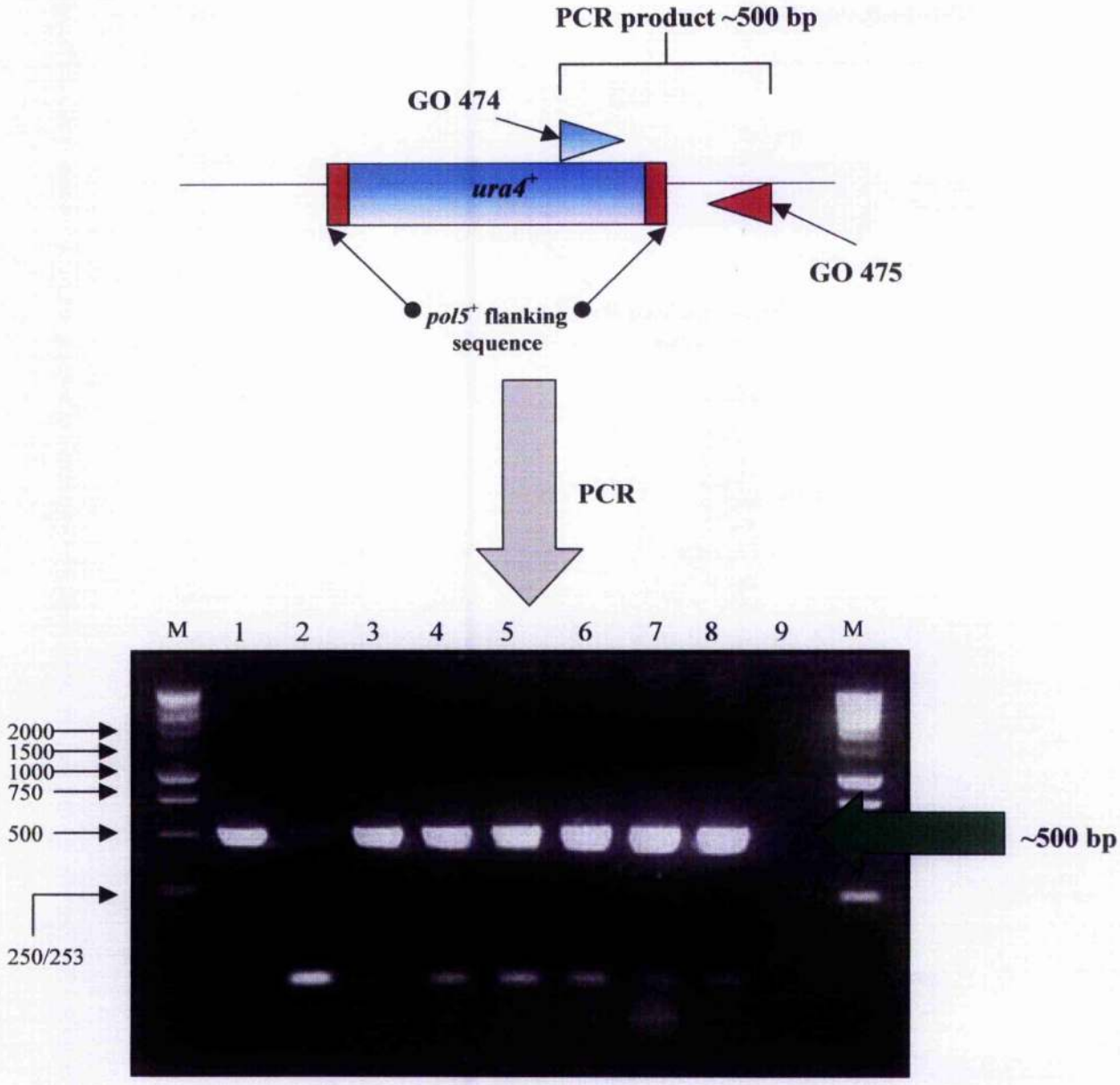


Figure 4E. PCR analysis to confirm the incorporation of *ura4*$^+$ into the *pol5*$^+$ locus by homologous recombination. The oligonucleotides GO 474 and GO 475 were used to amplify a PCR product of approximately 500 base pairs (bp), from genomic DNA prepared from diploid isolates, only if *ura4*$^+$ had correctly incorporated into the *pol5*$^+$ locus. Samples of isolates 1-9 were analysed on a 1% agarose gel stained with ethidium bromide and viewed under a UV illuminator. 1 kb DNA ladder (M) is shown with band sizes indicated in base pairs. The arrow shows the expected PCR product of ~500 bp confirming *ura4*$^+$ had incorporated into the correct gene locus in all isolates, except 2 and 9.

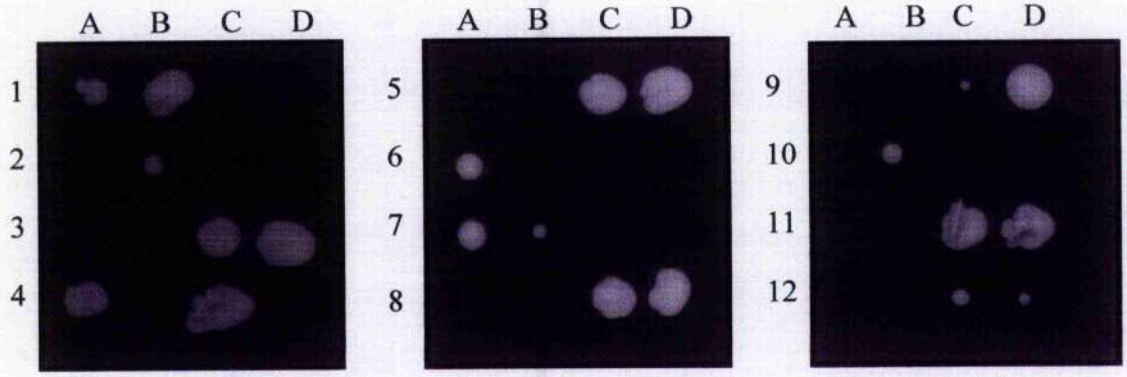


Figure 4F. Tetrad analysis showing that the disruption of *pol5*$^+$ is lethal to fission yeast. 60 tetrads were dissected using a Singer tetrad dissector. Numbers 1-12 are the 12 different tetrads dissected. Spore analysis was carried out on YE plates to allow all spores to grow. Of the 60 tetrads dissected, a selection of which is shown above, no more than 2 spores grew, which were all *ura4*$^-$. Therefore *pol5*$^+$ is an essential gene in fission yeast.

	A	B	C	D	Number of valid spores
1	⬭	⬭			2
2		⬭			1
3			⬭	⬭	2
4	⬭		⬭		2
5			⬭	⬭	2
6	⬭				1
7	⬭	⬭			2
8			⬭	⬭	2
9			⬭	⬭	2
10		⬭			1
11			⬭	⬭	2
12			⬭	⬭	2

Table 4A. Summary of a selection of the tetrads dissected displaying viable spores. No more than 2 spores grew for all tetrads dissected. The spores that grew were all *ura4*$^-$. The spores that were unable to grow were therefore disrupted for *pol5*$^+$, being *ura4*$^+$, confirming that *pol5*$^+$ is an essential gene.

4.4 Over-expression of *pol5*$^+$ by the fission yeast expression vector pREP3x

Another way of analysing the function of a gene is to examine the effects of over-expressing it in cells. As described earlier, *pol5*$^+$ is believed to be transcribed at low levels and is essential, and so it was thought that producing very large amounts of Pol5p might be detrimental to fission yeast and so reveals functions about the gene.

For the over-expression of genes in fission yeast the thiamine-repressible expression vector pREP3x was used. The control of expression of genes is under the control of the thiamine repressible promoter, *nmt* (*not made* in *thiamine*) (Maundrell, 1992). Genes that are cloned into pREP3x are over-expressed in the absence of thiamine and repressed in the presence of thiamine. However the expression of genes is not always completely repressed by these constructs even with the addition of thiamine. The repressed levels of expression depend on the gene that is cloned and can vary from complete repression to quite high levels of over-expression (Maundrell 1993, Forsburg 1993). For varying degrees of expression, different pREP vectors are available that confer varying degrees of control in the presence and absence of thiamine. pREP3x is the strongest of these expression vectors with 100-fold increase in expression in the absence of thiamine but in the absence of thiamine there is still a 20-fold increase in expression of genes. Therefore caution must be taken when choosing suitable vectors to over-express or repress transcription of a gene.

To assay the effects on cell cycle parameters, *pol5*$^+$ was cloned downstream of the thiamine repressible *nmt*3x promoter in the strongest of the expression vectors, pREP3x (GB 340), to induce high over-expression of SpPol5p in a wild type yeast strain (GG 214). The following experiments examine the effect of over-expression of *pol5*$^+$, by the vector pREP3x, on fission yeast.

4.4.1 Cloning *pol5*$^+$ into pREP3x

To permit cloning into the *nmt* vector pREP3x (GB 340) (Figure 4G), two oligonucleotides (GO 505 and GO 506), containing the restriction sites *Sal*I and *Bam*HI, respectively, were designed to PCR amplify the complete sequence of *pol5*$^+$, including start and stop codons. The High Fidelity PCR system (Materials & Methods, Section 2.4.1.3) was used for amplifications and the DNA products were analyzed on a 1% agarose gel and gel purified using the QIAgen gel extraction kit (QIAgen).

Genomic DNA was extracted from wild type fission yeast (GG 217) and used as template for the PCR reaction. A cDNA of *pol5*$^+$ would have been preferable as *pol5*$^+$ contains an intron at the N-terminus region and this may inhibit over-expression of *nmt* by overwhelming the splicesome machinery of the cell. Cell death may occur, as essential proteins are no longer being processed because the splicesome is occupied with the over-expressed gene. Attempts were made to amplify the cDNA of *pol5*$^+$ from a cDNA library but were unsuccessful. This may have been due to *pol5*$^+$ being a rare transcript (Figure 4A, 4B & 4C) and so was under-represented in the cDNA library. In contrast, the PCR of genomic DNA generated a product of approximately 3 kb in size, *pol5*$^+$. As a pre-requisite step, the gene was cloned into the pCR 2.1 vector using the TA cloning kit (Invitrogen) as this facilitated the final cloning stage into pREP3x (Material & Methods, Section 2.4.8) Successful cloning of *pol5*$^+$ was confirmed by restriction digestion and by sequencing (MWG-Biotech).

A number of oligonucleotides were generated to allow sequencing with 95 % coverage of *pol5*$^+$ (Figure 4G & 4H). The sequencing data indicated that six mutations were present, of which three mutations altered the amino acid sequence (Figure 4H). Two mutations occurred within the intron close to the 5' splice site (consensus sequences for the splice sites are indicated in boxes in Figure 4H) and may have affected the splicing of the pre-mRNA of *pol5*$^+$, leading to the translation of a non-functioning protein. Also the mutations within the coding region of the gene may affect the folding and activity of the translated protein leading to the over-expression of a non-functional protein.

The above construct was amplified in *E. coli*, followed by plasmid miniprep. The *pol5*$^+$ insert was removed by restriction digest with *Sal*I and *Bam*HI. pREP3x

was also digested with *Sal*I and *Bam*HI. The insert was sub-cloned into the expression vector with T4 DNA ligase. The pREP3x/*pol5*$^{+}$ (GG 778/779) construct was confirmed by restriction digestion.

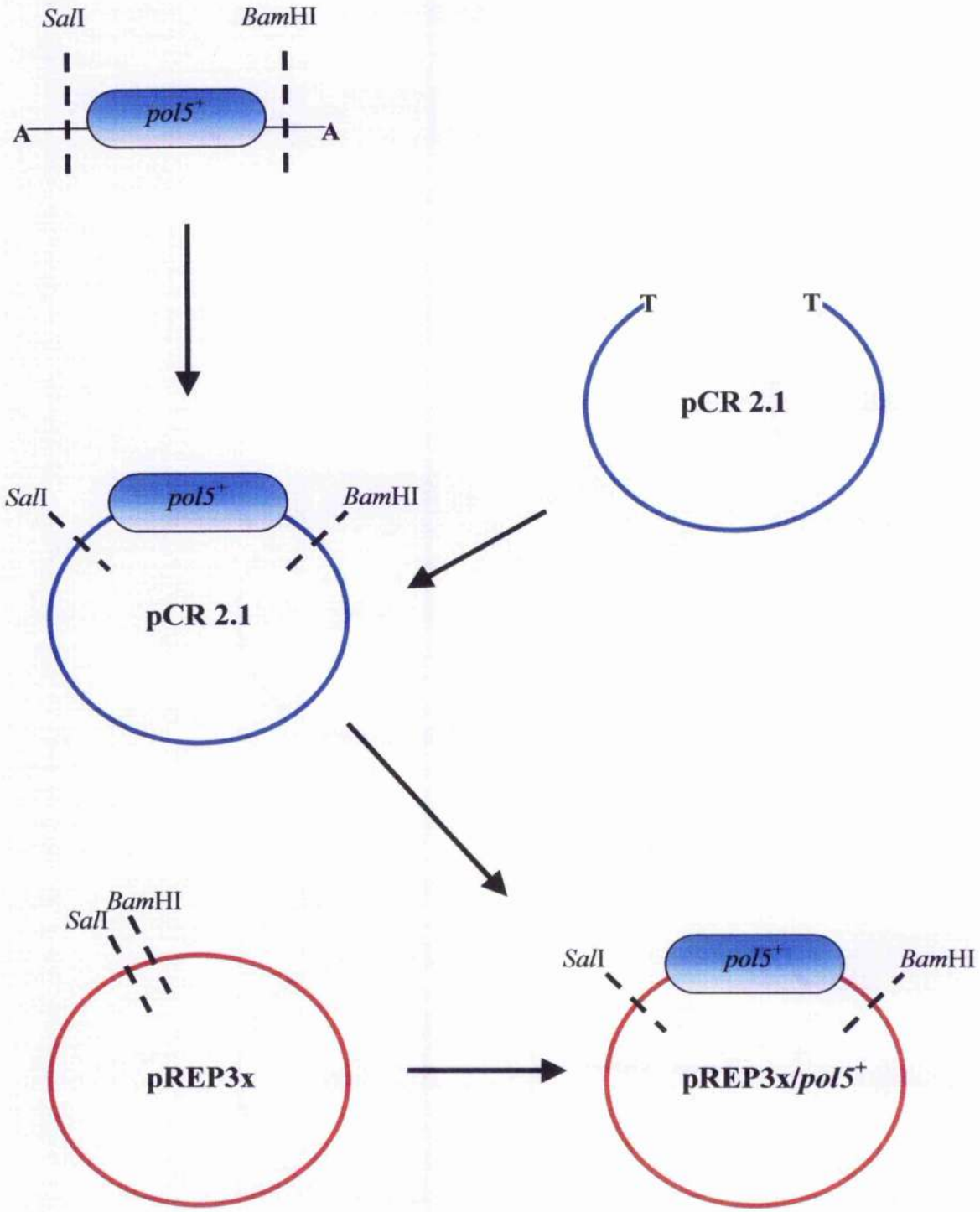


Figure 4G Cloning *pol5*$^+$ into the *nmt* expression vector pREP3x (GB 340). *pol5*$^+$ was PCR amplified and cloned into pCR 2.1 (Invitrogen). Using the restriction enzymes *Sal*I and *Bam*HI, *pol5*$^+$ was cloned into pREP3x, which was also cut with the same restriction enzymes. The ligation mix was transformed into DH5α *E. coli* cells. Diagnostic restriction digests confirmed constructs.

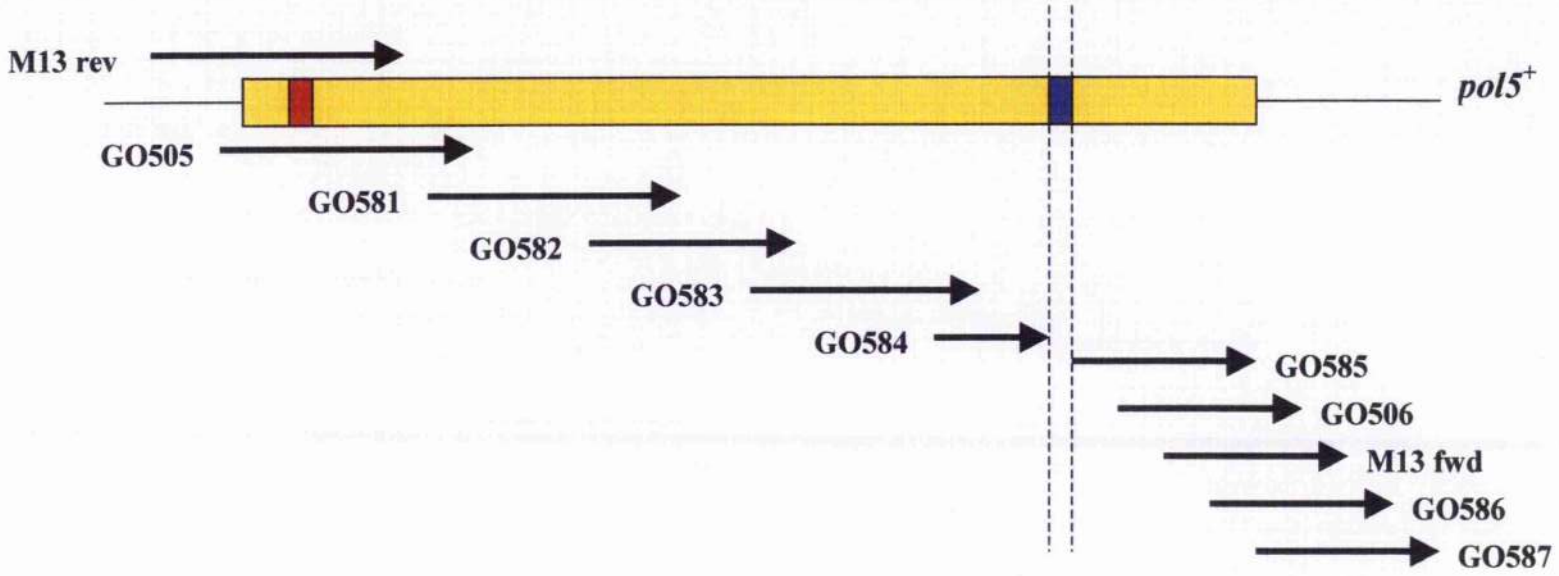
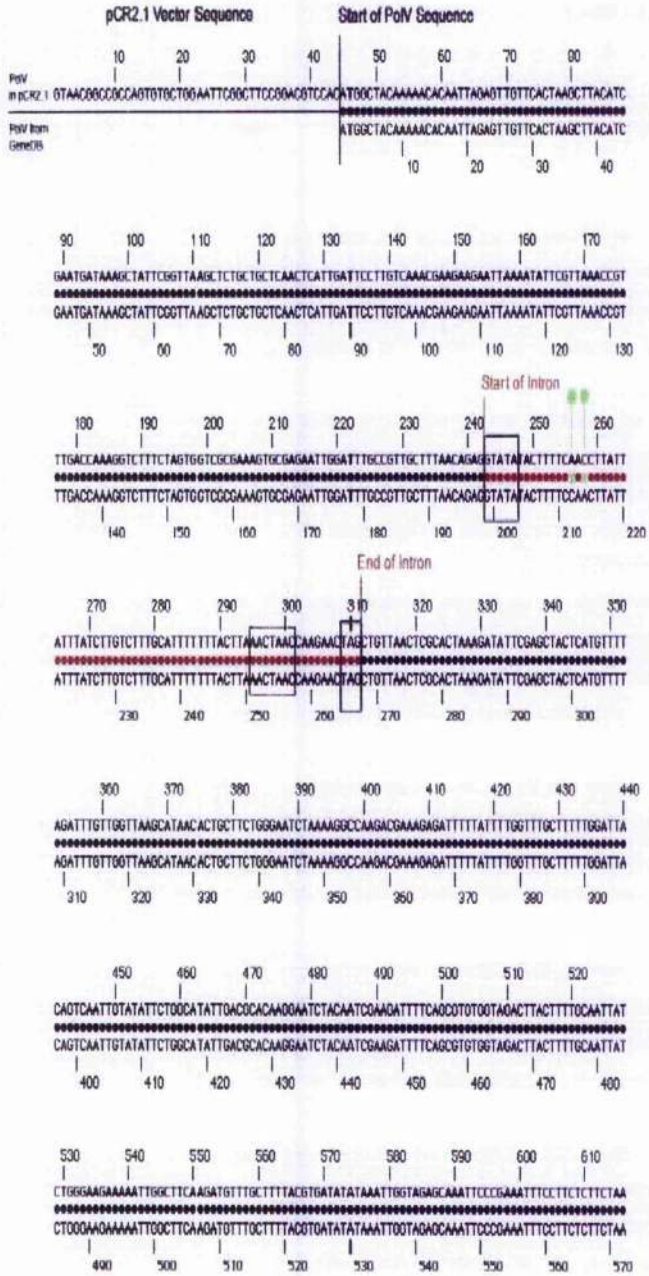
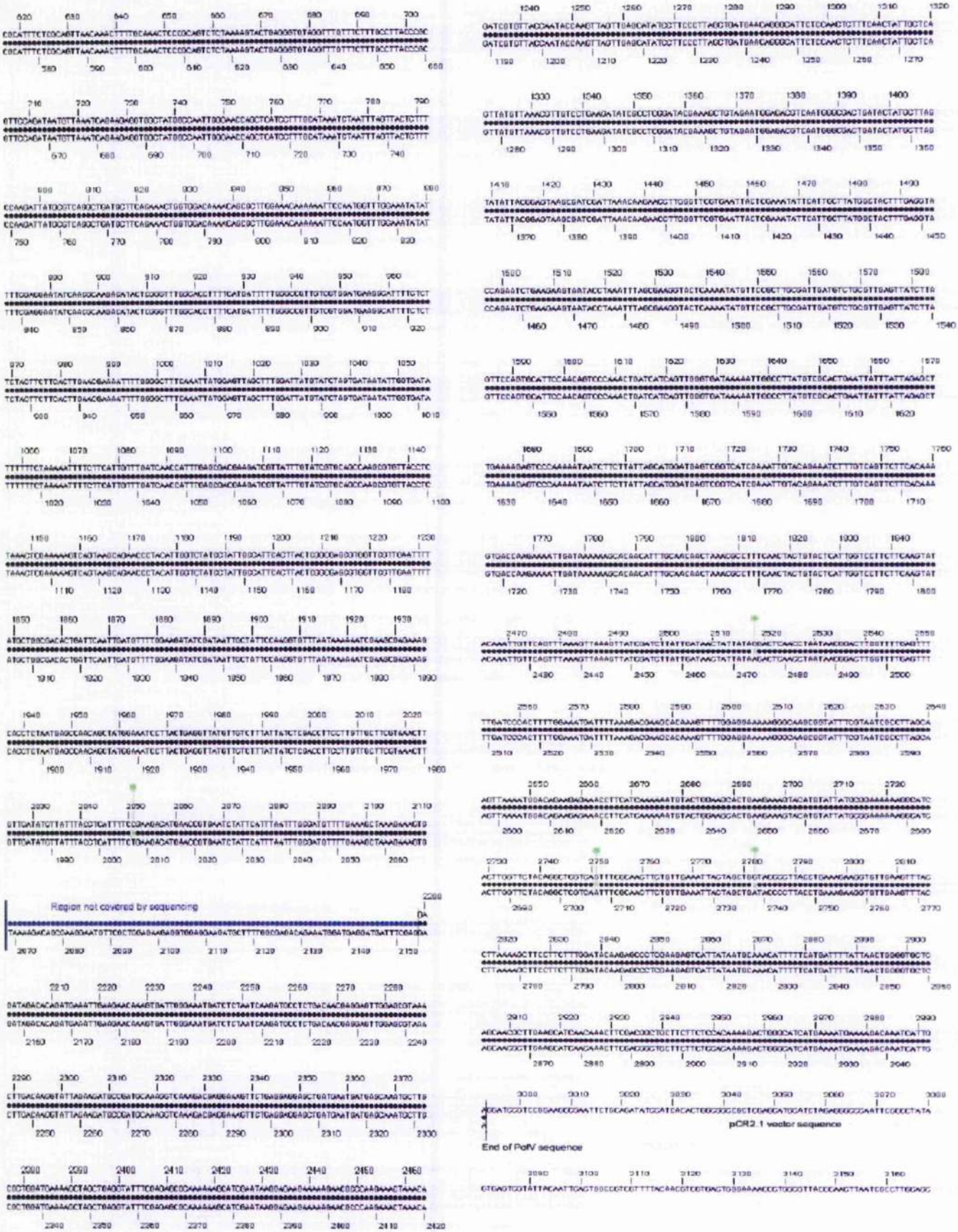


Figure 4H. A schematic representation of *pol5*$^+$ showing area covered by sequencing primers. The red area depicts the *pol5*$^+$ intron. Each arrow represents the area each sequencing primer covers (Appendix II). The blue area and the dotted line show the region that the primers failed to sequence.

Figure 4I. Sequence alignment of *pol5*$^{+}$ from the pombe gene database against *pol5*$^{+}$ in pCR 2.1 (Invitrogen). The red area indicates the single intron in the gene. Boxed areas depict probable splice and branch consensus sequences. The unsequenced area is shaded in blue for the gene database sequence of *pol5*$^{+}$. Point mutations are marked in green.




Region not covered by sequencing
pCR2.1 vector sequence
End of PolV sequence

4.4.2 Confirmation of over-expression of *pol5*$^+$ by pREP3x/*pol5*$^+$ in wild type fission yeast

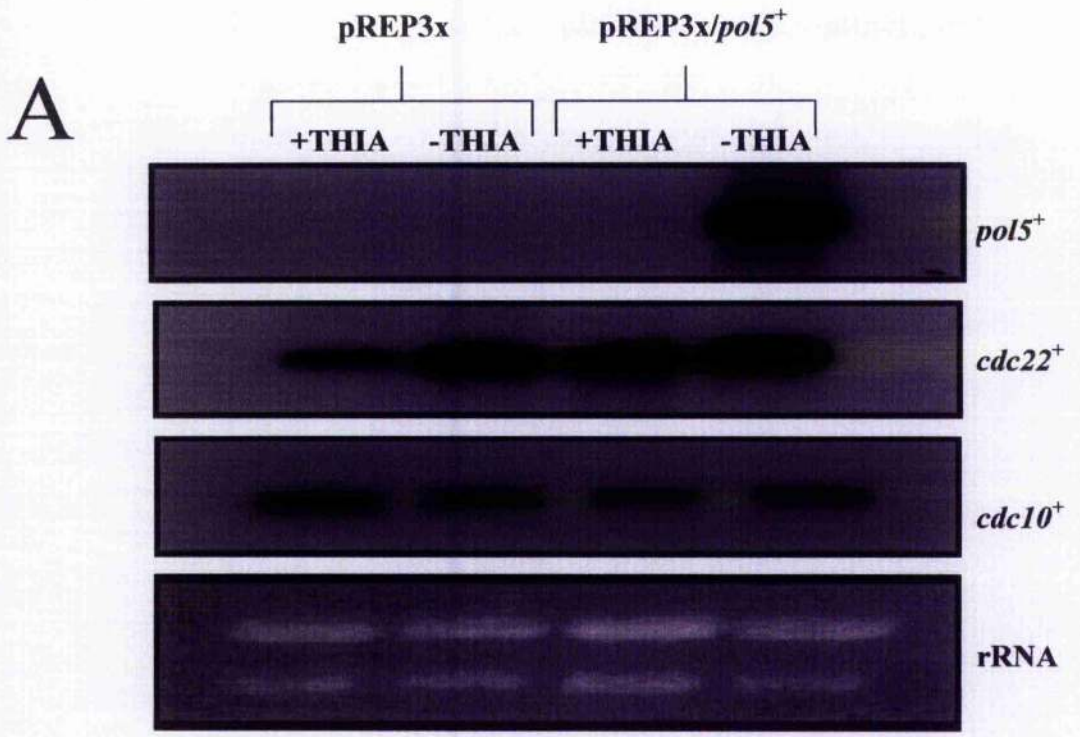
The over-expression construct pREP3x/*pol5*$^+$ (GG 778/779) was transformed into a wild type fission yeast strain (GG 214). At the same time the empty pREP3x vector was also transformed into the wild type strain, to serve as a control (GG 786).

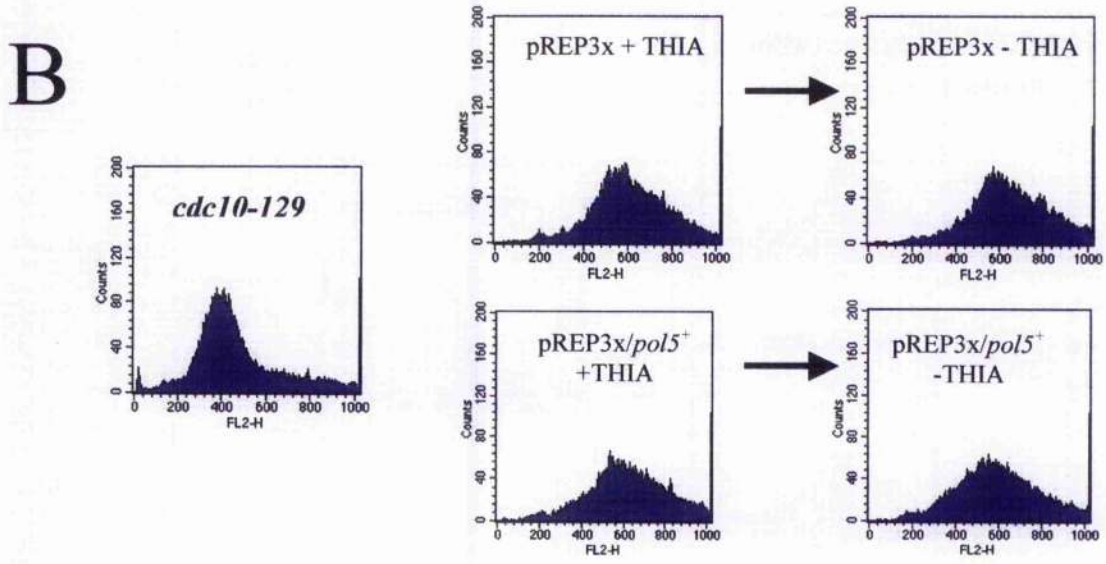
Before examining the effect of over-expression of *pol5*$^+$ *in vivo*, it had to be confirmed that the pREP3x/*pol5*$^+$ (GG 778/779) construct was behaving as expected. This was done by northern blot analysis.

To allow over-expression of *pol5*$^+$ by *nmt*3x the following growth protocol was followed: pREP3x/*pol5*$^+$ (GG 778/779) and empty vector, pREP3x (GG 786), strains were grown in 10 ml of EMM plus thiamine overnight at 30°C. The following morning 5 ml of each of the pre-cultures was used to inoculate another 50 ml of EMM plus thiamine at 25°C. After 8 hours, cells were collected by centrifugation and washed three times in EMM. A sample of cells was taken before washes for flow cytometry (FACs). Each strain was divided between two flasks, one containing 50 ml of EMM plus thiamine and the other with 50 ml of only EMM. These cultures were left to grow overnight at 25°C. The next day samples of cells were taken for flow cytometry followed by harvesting each of the cultures for northern blot analysis to detect *cdc22*$^+$ and *cdc10*$^+$ mRNA levels. The results are shown in Figure 4J.

mRNA levels of *pol5*$^+$ were high in wild type cells containing the pREP3x/*pol5*$^+$ construct in the absence of thiamine, and undetectable at short exposure times in the presence of thiamine (Figure 4J). Therefore, the *pol5*$^+$ over-expression vector was functioning properly. The transcription of *cdc22*$^+$ and *cdc10*$^+$, in both the empty vector strain (GG 786) and pREP3x/*pol5*$^+$ (GG 778/779) strains, remains constant in the presence and absence of thiamine (Figure 4J). *cdc22*$^+$ was exposed overnight in order to see transcript and *cdc10*$^+$ had to be left for one week to detect mRNA. FACs analysis reveals no effect on cell cycle as the profile is the same as for that of the empty vector samples.

Figure 4J. Confirmation of over-expression of *pol5*$^{+}$ by the expression vector pREP3x/*pol5*$^{+}$. A. Fission yeast cells containing empty pREP3x vector (GG 786) were used as a control and were grown in the presence and absence of thiamine. Fission yeast cells containing pREP3x/*pol5*$^{+}$ (GG 778/779) were grown in the presence of thiamine and then in the absence of thiamine, to induce over-expression of *pol5*$^{+}$. RNA was prepared and *pol5*$^{+}$, *cdc22*$^{+}$ and *cdc10*$^{-}$ mRNA levels were examined by northern blot analysis. Equal loading was confirmed by staining ribosomal RNA (rRNA) with ethidium bromide. **B.** FACS (flow cytometry) analysis was performed on cells, stained with propidium iodide. Samples were taken in the presence and absence of thiamine. DNA content of the mutant *cdc10-129* is shown to indicate a peak displaying a 1C DNA content.


A
pREP3x
pREP3x/pol5+
+THIA
-THIA
+THIA
-THIA
pol5+
cdc22+
cdc10+
rRNA


B
cdc10-129
pREP3x + THIA
pREP3x - THIA
pREP3x/pol5+
+THIA
pREP3x/pol5+
-THIA
Counts
FL2-H

4.4.3 The effect of over-expression of *pol5*$^+$ in fission yeast

The effect of over-expressing *pol5*$^+$ *in vivo* was examined in wild type cells. pREP3x/*pol5*$^+$ (GG 778/779) was transformed into a wild type strain (GG 214) and grown on EMM plus thiamine plates overnight at 25°C (Figure 4K). To induce over-expression of *pol5*$^+$, cells were replica-plated, three times, onto EMM plates without thiamine (Figure 4K). Cells were also grown in liquid media, in the presence and absence of thiamine, to see if there was any difference in growth observed in liquid as compared to on solid media (Figure 4L).

No effect on growth by the over-expression of *pol5*$^+$, was observed in wild type cells. The rate of growth remained the same when *pol5*$^+$ was over-expressed as compared to when it was repressed. Strains were also compared to wild type cells and wild type cells carrying the empty expression vector. No differences were observed in rate of growth, on plates, between the *pol5*$^+$ over-expression strain and the control strains.

Samples of cells for the different strains were grown in liquid media and visualised under a light microscope. Figure 4L shows cells in media plus and minus thiamine. Again there was no difference in the morphology of the cells plus or minus thiamine or between the control strains. The reason for this may have been due to the presence of the intron, effecting the splicing machinery of the cell, resulting in defective processing of *pol5*$^+$ mRNA and in turn producing non-functional protein. The mutations within the construct could have affected proper folding of SpPol5p through the changes in amino acid sequence and in effect produced a non-functional protein. Transforming the pREP3x/*pol5*$^+$ construct into a *pol5*$^+$ deletion strain, to see if the mutated version of *pol5*$^+$ rescued the deletion, tested these defects.

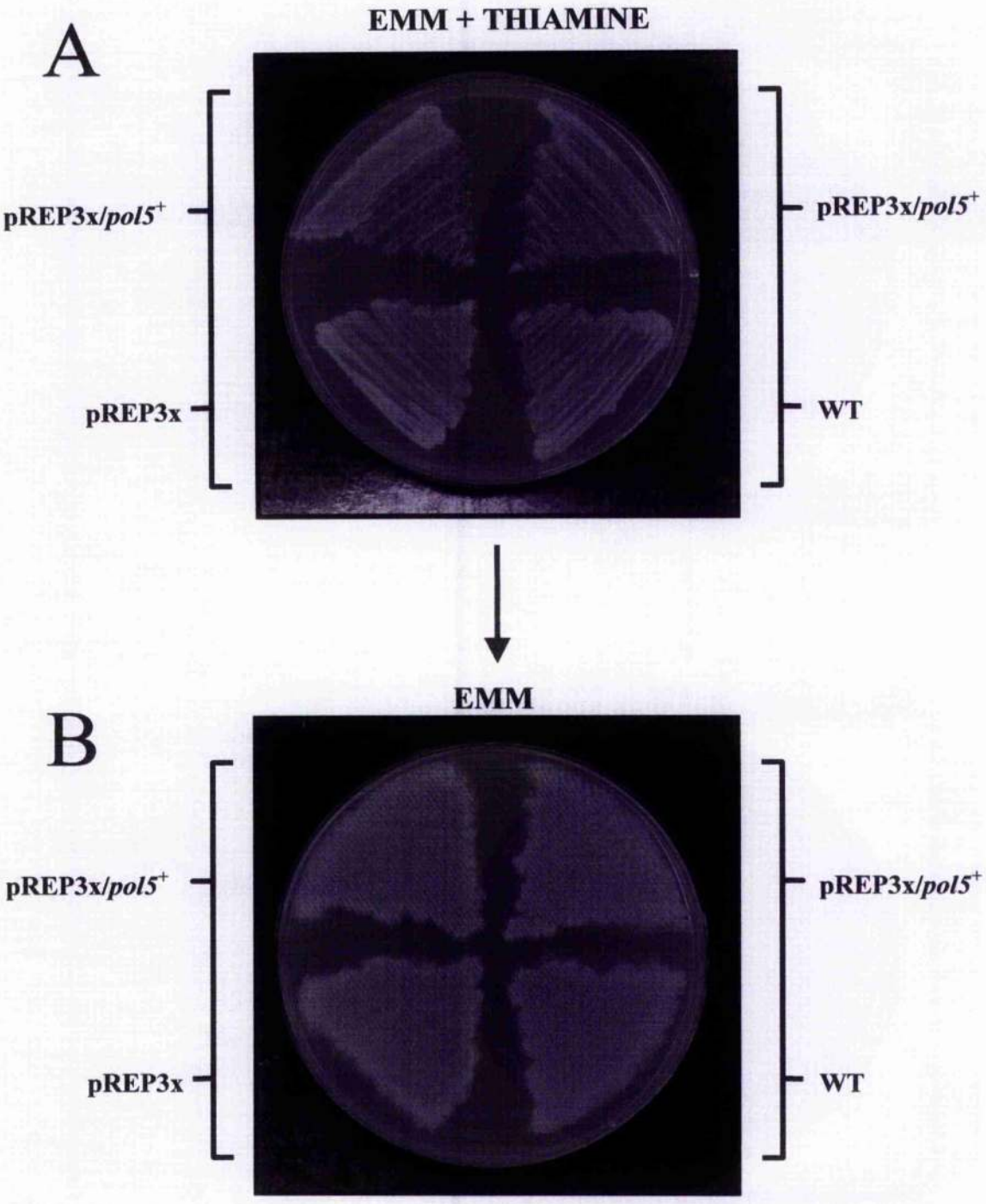
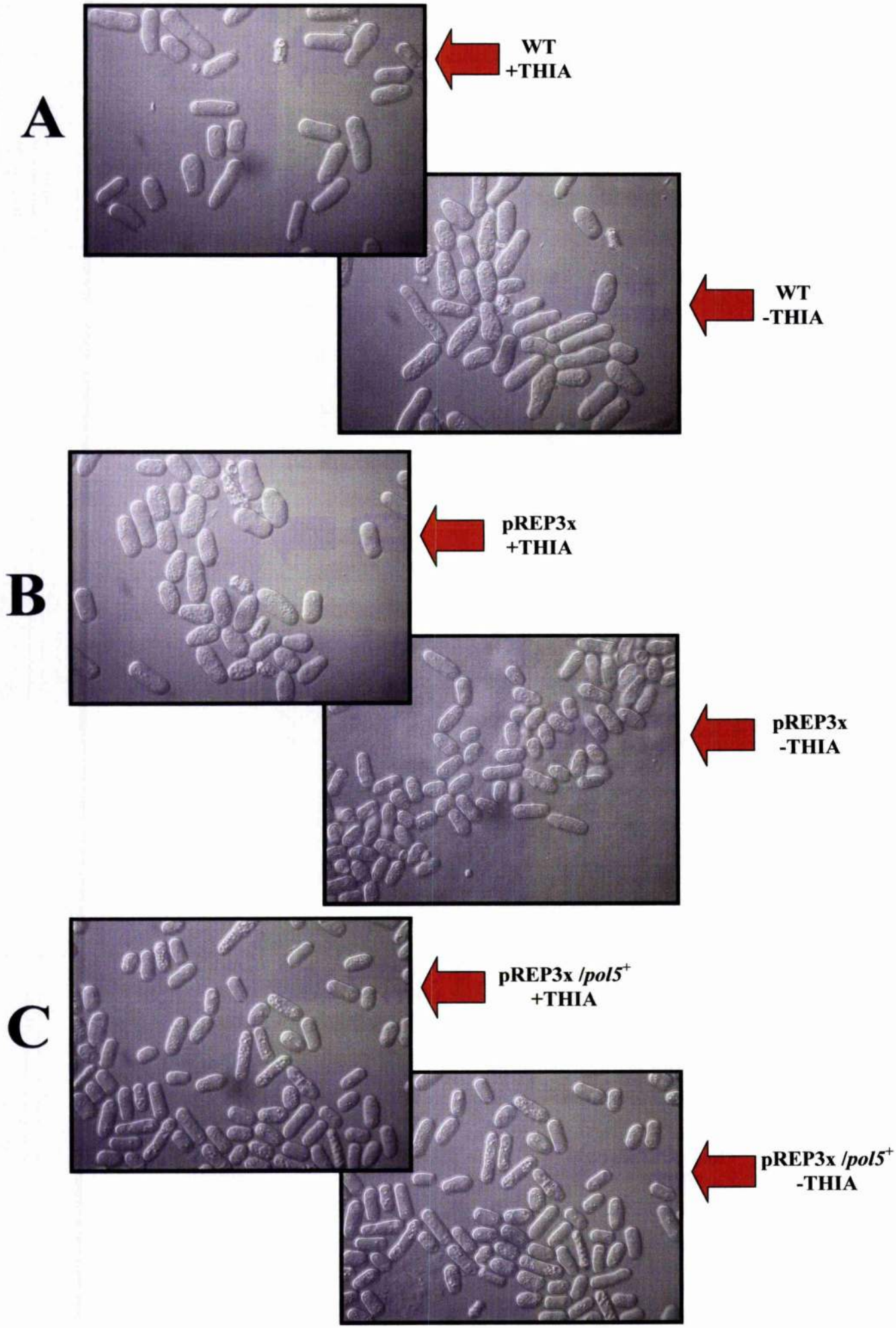


Figure 4K. Over-expressing *pol5*$^+$ has no effect on growth of fission yeast. A. pREP3x/*pol5*$^+$ (GG 778/779), wild type (WT) (GG 217) and pREP3x (GG 786) strains were grown on EMM plus thiamine plates at 25°C overnight. **B.** Cells were replica-plated three times onto EMM plates and grown at 25°C. The growth of all the strains, including controls, appeared the next day at the same time


A
WT
+THIA
WT
-THIA
B
pREP3x
+THIA
pREP3x
-THIA
C
pREP3x /pol5+
+THIA
pREP3x /pol5+
-THIA

D

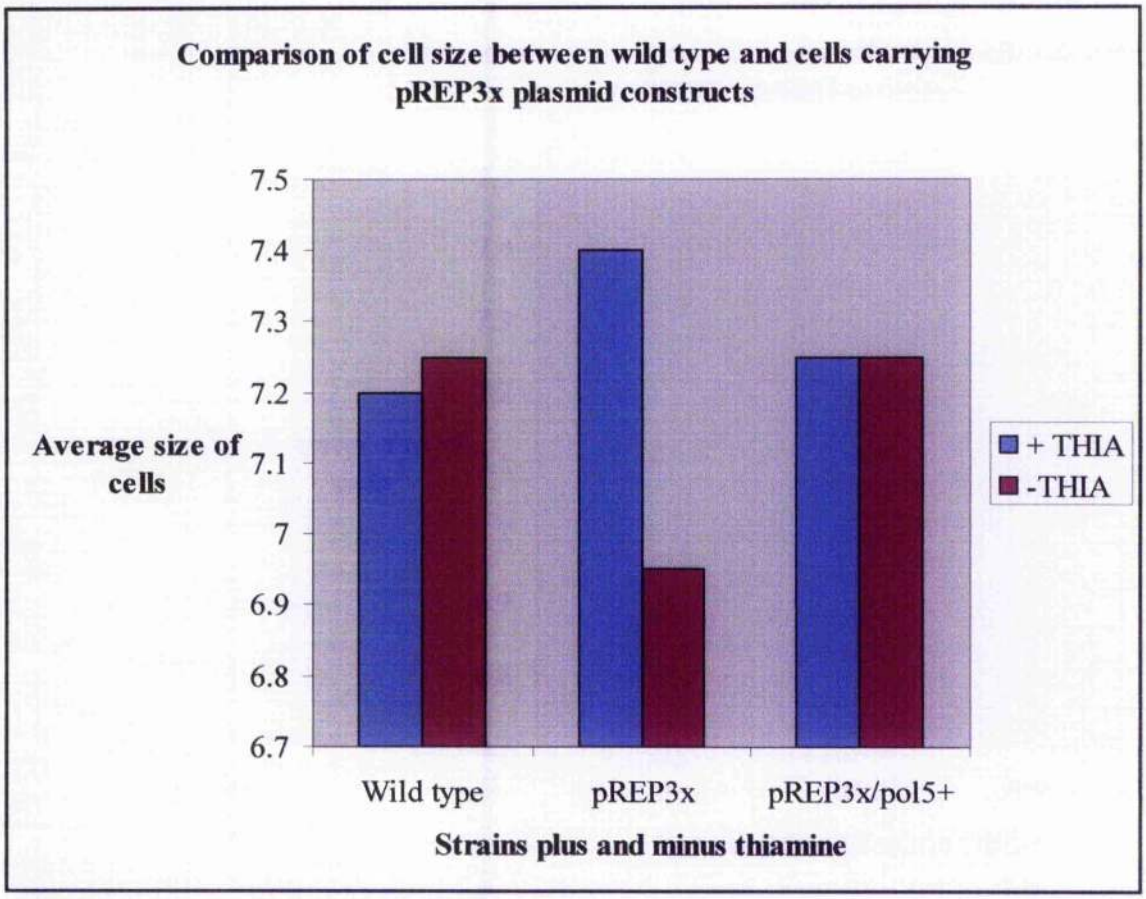


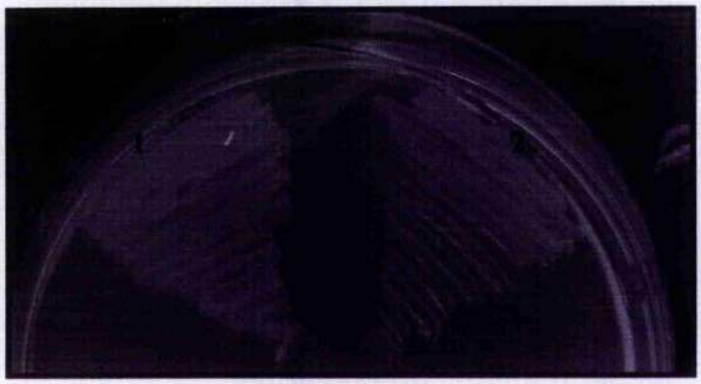
Figure 4L. Micrographs of fission yeast cells over-expressing *pol5*$^{+}$. A. Wild type (WT) (GG 217) cells in the presence and absence of thiamine. **B.** Empty expression vector, pREP3x (GG 786) transformed into wild type cells, grown in the presence and absence of thiamine. **C.** pREP3x/*pol5*$^{+}$ expression vector (GG 778) transformed into wild type cells. Cells were first grown in EMM plus thiamine and then in the absence of thiamine to induce over-expression. **D.** Graph displaying a comparison of the sizes of cells in wild type, pREP3x, and pREP3x/*pol5*$^{+}$ strains.

4.4.4 Rescuing the lethality of Δ*pol5* with pREP3x/*pol5*$^+$

To test whether the presence of the intron and the mutations within the cloned *pol5*$^+$ DNA was affecting the results of the over-expression experiment, the pREP3x/*pol5*$^+$ construct (GB 340) was transformed into a diploid *pol5:ura4*$^+$/*pol5*$^+$ strain (GG 840). As the deletion of *pol5*$^+$ is lethal to fission yeast the experiment had to be done in a diploid strain containing one copy of *pol5*$^+$. Once cells were transformed with the construct, random spore analysis was performed.

A diploid strain *pol5:ura4*$^+$/*pol5*$^+$ (GG 840) was transformed with pREP3x/*pol5*$^+$. Transformed diploids cells were grown on EMM plates to select for cells that had taken up the pREP3x/*pol5*$^+$ construct, and to induce sporulation. Tetrads were glucylase treated to break down the cell wall to release the spores, and were then spread onto EMM plus adenine plates and left to incubate at 30°C. Samples of cells were taken at various times throughout the day and spread onto EMM plus adenine plates, to select for colonies that had taken up the plasmid construct and were producing SpPol5p. Individual colonies were picked from the various plates and a master-plate was formed on EMM plus adenine plates. Many of the colonies grew on the master-plate but this was not conclusive proof that the construct had rescued the deletion. Colonies had to be viewed on phloxin plates to confirm that they were haploids. When analysed on phloxin plates it revealed that many of the colonies isolated were diploids and only a few were haploids. This could have been due to inefficient degradation of the cell wall by the glucylase treatment. Two haploid isolates were tested further on EMM plus adenine and thiamine plates and also on EMM plus adenine plates (Figure 4M). Viable colonies were produced for the *pol5*$^+$ deleted strain in the presence of the pREP3x/*pol5*$^+$ over-expression construct, on plates lacking thiamine thus, inducing *pol5*$^+$ expression. Surprisingly colonies also formed on media containing thiamine. The explanation for this was that even when thiamine was repressing expression there was still significant production of protein by pREP3x to rescue the deletion. The pREP3x vector is the strongest of the pREP expression vectors, but unfortunately does not completely repress transcription in the presence of thiamine.

EMM + ADE



EMM + ADE + THIA

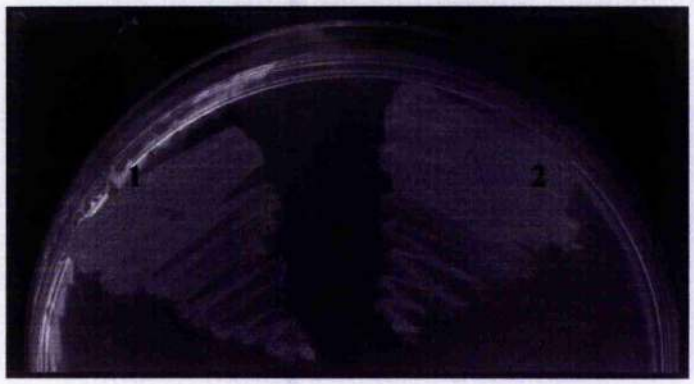


Figure 4M. pREP3x/*pol5*$^+$ rescues the lethality of Δ*pol5*. The construct, pREP3x/*pol5*$^+$ (GB 340) was transformed into a *pol5*$^+$/Δ*pol5* diploid strain. Colonies that were positive for the plasmid and also carried the *ura4*$^+$ selectable marker, were chosen. Viable haploid colonies that grew and were *ura4*$^+$, for the deletion, and *leu*$^+$ for the plasmid, respectively, were rescued for Δ*pol5*. Colonies were then grown on thiamine minus plates to induce over-expression of *pol5*$^+$. Colonies 1 and 2 were mutant haploids deleted for *pol5*$^+$ that were rescued with the pREP3x/*pol5*$^+$ vector. Colonies were also able to grow in the presence of thiamine suggesting the construct was still 'leaky' for expression and was not completely "shut-off".

4.5 Summary

In this chapter a number of experiments were performed to examine the role of *pol5*$^+$. Northern blot analysis of RNA from mitotic and meiotic fission yeast cells examined *pol5*$^+$ transcription. Also the effect of deleting *pol5*$^+$ from the cells was observed. In contrast, *pol5*$^+$ was also over-expressed in wild type cells to study the effect of producing abnormally large amounts of SpPol5p.

Using asynchronous mitotic cell cultures, the mRNA profile of *pol5*$^+$ was analysed using northern blot analysis. Levels of *cdc22*$^+$ and *pol5*$^+$ transcript were detected in both wild type cells and in the temperature sensitive mutant *cdc10-C4*. As *cdc22*$^+$ is under the control of SpCdc10p, through the cell cycle transcription factor complex, DSC1 (Lowndes *et al.* 1992), the amount of *cdc22*$^+$ transcript is periodic throughout the cell cycle. However *cdc22*$^+$ mRNA is elevated in the mutant *cdc10-C4* (McInerny *et al.* 1995) as compared to wild type cells (Figure 4A). This is as a result of a non-functional SpCdc10p, which can no longer confer periodic control over DSC1 controlled genes (McInerny *et al.* 1995). The levels of *pol5*$^+$ in both wild type and *cdc10-C4* are equal. Therefore *pol5*$^+$ is unlikely to be under the control of SpCdc10p. This coincides with the absence of MCB elements in the promoter region of *pol5*$^+$, which are always found in genes that are controlled by DSC1. This observation was confirmed by performing cell cycle experiments in synchronous cell cultures.

Synchronous cells were generated using temperature sensitive mutants for both mitosis and meiosis. The mutant *cdc25-22* was used to measure transcription of *pol5*$^+$ in mitosis. As mentioned, *cdc22*$^+$ is a periodically transcribed gene and is expressed at G_1 before the onset of DNA replication (Gordon & Fantes 1986) (Figure 4B). Analysing *pol5*$^+$ transcription in synchronous cells revealed *pol5*$^+$ as a mitotic gene that is not periodically transcribed (Figure 4B) and therefore not under SpCdc10p control. These experiments also showed that *pol5*$^+$ is a rare transcript as it took one week for mRNA to be detected by autoradiography, compared to overnight for *cdc22*$^+$ suggesting that *pol5*$^+$ is transcribed in low levels.

Cell cycle experiments were also performed to examine *pol5*$^+$ mRNA levels during meiosis. The mutant *pat1-114* was starved of nitrogen to arrest the cells at G_1. Shifting the cells to 36°C and supplementing them with nitrogen (NH_4Cl) allowed

synchronous entry into the meiotic cycle. The experiment revealed that *pol5*$^{+}$ is also a rare, meiotic transcript also and was not periodically transcribed (Figure 4C), which again indicated that SpCdc10p does not control *pol5*$^{+}$ transcription.

Next, a disruption experiment was carried out to see the effect of removing *pol5*$^{+}$ on fission yeast. The method, devised by Bahler *et al.* (1998), allowed incorporation of the *ura4*$^{+}$ nutritional marker in place of *pol5*$^{+}$ (Figure 4D). Through tetrad analysis it revealed that *pol5*$^{+}$ was an essential gene (Figure 4E). This would be consistent with the proposed role of *pol5*$^{+}$ in rRNA production. The inability to produce the "machinery" (ribosomes) that makes proteins would indeed lead to cell death, as essential proteins are no longer being synthesised. As described in Chapter 3, *POL5* in budding yeast is also an essential gene and is thought to be involved in rRNA production. This provides further support to the proposal that fission yeast *pol5*$^{+}$ may have the analogous role as budding yeast *POL5*.

To further our understanding of *pol5*$^{+}$, over-expression studies were next performed. As *pol5*$^{+}$ is a rare transcript it was predicted that the over-expression of this gene might affect cells and so reveal function for the gene. The expression vector pREP3x (as described in Section 4.4) was used to over-express *pol5*$^{+}$ under the control of the thiamine-repressible *nmt*3x promoter. The pREP3x */pol5*$^{+}$ construct was transformed into wild type fission yeast cells and grown in the absence of thiamine to induce over-expression of SpPol5p. Superficially, this had no effect on the cell growth therefore cells were also viewed under a light microscope to see if there was any difference in growth in liquid and whether there was any effect on the morphology of individual cells. There was no difference noted between wild type and over-expressed cells. As mentioned in this chapter, there were a number of problems with this construct. Firstly, due to difficulties amplifying the cDNA of *pol5*$^{+}$ it had to be made from genomic DNA and therefore contained an intron. This could have affected the splicesome machinery of the cell leading to inefficient processing of *pol5*$^{+}$ pre-mRNA resulting in an in-active SpPol5p. Secondly a number of mutations were present in the pREP3x */pol5*$^{+}$ construct, some of which caused amino acid changes and some which may have altered the splice consensus sequences. This also could have resulted in incorrect folding of SpPol5p or failure to remove the intron, therefore inactivating the protein.

To test the functionality of the mutated form of *pol5*$^{+}$, we attempted to rescue the lethality of a *pol5*$^{+}$ deleted diploid strain, by transforming it with this construct.

Upon random spore analysis, haploids that were deleted for *pol5*$^{+}$ and contained the pREP3x /*pol5*$^{+}$ construct were isolated using nutritional selection. This confirmed that active and functional SpPol5p was being made as it rescued a haploid Δ*pol5* strain, so the mutations and the intron were not affecting protein folding and the splicesome machinery, respectively.

Therefore, we could conclude that the over-expression of *pol5*$^{+}$ did not have any effect on fission yeast but there still may have been an effect in the cell at a nuclear level. The following chapter aims to investigate further the biochemistry of SpPol5p by performing pulse-chase labelling experiments and localisation studies.

Chapter 5

The role of SpPol5p in rRNA production and cellular localisation studies

5.1 Introduction

In Chapter 4, several experiments were described that revealed some aspects of *pol5*$^{+}$ biology. The results showed that *pol5*$^{+}$ mRNA is present in low levels, throughout mitosis and meiosis and that *pol5*$^{+}$is not under the control of DSC1. *pol5*$^{+}$ was also shown to be an essential gene. Because *pol5*$^{+}$ mRNA exists in such small amounts it was predicted that the over-expression of *pol5*$^{+}$ might affect cell function. However this was not the case, and analysis of cells both on solid and in liquid media revealed no effect on cell phenotype or growth.

This chapter describes a series of pulse-chase labelling experiments that explore the role of *pol5*$^{+}$ in the synthesis of ribosomal RNA (rRNA). The over-expression experiments in the previous chapter could not capture the effects at a nuclear level, therefore these experiments were designed to analyse the effect on rRNA by the over-expression and repression of *pol5*$^{+}$ *in vivo*.

Pulse-chase labelling experiments were performed in a number of different strains. Firstly, the construct made in Chapter 4, pREP3x/*pol5*$^{+}$ (GG 778/779), was used to over-express *pol5*$^{+}$ to measure its effect on rRNA production. Secondly, various *cdc10* mutants were tested for effects on rRNA production, as SpCdc10p interacts with SpPol5p. Thirdly, a number of "shut-off" constructs, using the thiamine-repressible *nmt* promoter, were generated to assay the effect of removing *pol5*$^{+}$ on rRNA synthesis, and also to examine the transcripts of some other genes, by northern blot, when *pol5*$^{+}$ is "shut–off".

Finally a separate experiment was performed to assess the cellular localisation of SpPol5p. GFP-tagged versions of SpPol5p were created and visualised to determine if SpPol5p localises to the nucleolus, the area in the cell where rRNA is produced.

5.2 Pulse-chase labelling by radioactive [^{3}H] uridine

In order to analyse the synthesis of rRNA by fission yeast, [^{3}H] uridine was used to label ribonucleic acids. Radioactive [^{3}H] uridine labels newly synthesised nucleic acids. Therefore the production of new rRNA can be measured by adding radioactive [^{3}H] uridine to a culture of cells and incubating for 15-20 min to allow incorporation of uridine into the RNA. The increment in new rRNA can be detected by labelling with [^{3}H] uridine at regular time intervals.

5.3 The effect of over-expression of *pol5*$^+$ on rRNA production

The first in the series of pulse-chase labelling experiments was to examine the effect of over-expressing *pol5*$^+$ on the synthesis of rRNA. The construct pREP3x/*pol5*$^+$, described in Chapter 4, was used to over-express *pol5*$^+$ in a wild type yeast strain. To analyse the production of rRNA, incorporation of radioactive [^{3}H] uridine was measured.

5.3.1 Pulse-chase labelling of the over-expression of *pol5*$^+$

A 10 ml pre-culture of pREP3x/*pol5*$^+$ (GG 778) and a separate pre-culture of pREP3x (GG 786) were grown overnight in EMM plus thiamine at 25°C. The next day 1 ml of each pre-culture was used to inoculate another 100 ml of EMM plus thiamine and grown at 25°C overnight with shaking. Cells were then washed three times in EMM and re-suspended in the residual liquid after which 1 ml was used to inoculate a 100 ml culture of EMM and 1 ml for a 100 ml culture of EMM plus thiamine. Flasks were left to grow at 25°C for at least 16 h with constant shaking. The next day, 60 ml of liquid was removed from each of the four conical flasks and discarded. 30 µl of [^{3}H] uridine was added to each conical flask and incubated for 15 min, after which 10 ml of each culture was removed and spun down in a centrifuge at 3,000 rpm for 5 min. Cells could be frozen down at –70°C at this stage. [^{3}H] uridine was added to each culture at 3 h intervals a further two times. Once all the samples had been collected, RNA was extracted from the cells. The amount of RNA present in each sample was

quantified by spectrophotometry at 260 nm. Equal amounts of RNA were separated by electrophoresis. RNA was transferred onto a membrane and left to expose at –70°C for 6-8 weeks after which the x-ray film was developed to view the labelled RNA bands representing newly synthesised rRNA. The results are shown Figure 5A.

In the control experiment, the effect of the strain carrying the empty expression vector, pREP3x, on rRNA production was analysed both with and without thiamine. The pulse labelling data revealed no effect on ribosomal RNA synthesis by the empty vector. The pREP3x/*pol5*$^+$ strain was then analysed in the presence and absence of thiamine. When compared with samples with thiamine, the removal of thiamine, to induce over-expression of *pol5*$^+$, did not have any effect on rRNA synthesis. However when compared to the empty vector data, there was a slight decrease in the amount of rRNA produced in the samples 1 and 2 but then in the last sample 3, levels of rRNA were restored. An explanation for this might be that although no difference was observed between the samples with and without thiamine, for pREP3x/*pol5*$^+$, the variation seen between the empty vector and pREP3x/*pol5*$^+$ strains was due to over-expression of *pol5*$^+$. pREP3x is the strongest of the *nmt* expression vectors and carries a flaw in that it does not completely repress expression when thiamine is present. Therefore samples with and without thiamine, either over-expressing *pol5*$^+$ or massively over-expressing *pol5*$^+$ has the same affect as slight over-expression. These results correlated with the data for the over-expression of *pol5*$^+$ in Chapter 4, where there was no difference noted between cells induced for over-expression or suppression of *pol5*$^+$. Nonetheless, there was no difference in growth between cells containing empty vector and cells carrying the pREP3x/*pol5*$^+$ construct in Chapter 4, which is in contrast to this pulse-chase experiment, which shows an effect on rRNA synthesis.

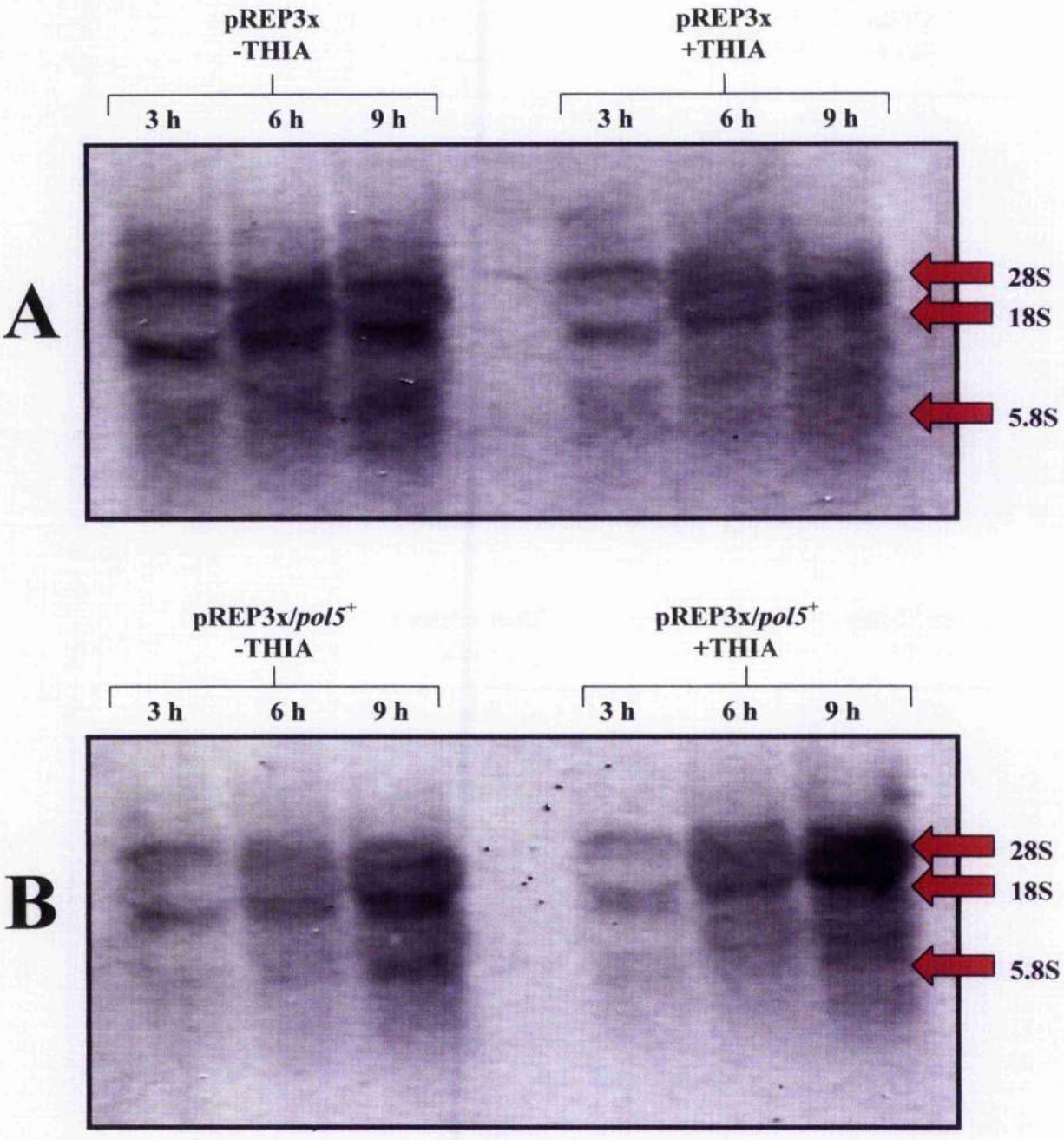


Figure 5A. Pulse-chase labelling experiment measuring the effect of over-expression of *pol5*$^+$ on ribosomal RNA (rRNA) production. A. The pREP3x strain (GG 786) was grown in the presence and absence of thiamine (THIA) and pulsed with radioactive [^{3}H] uridine, for every 3 hourly sample, to label newly synthesised rRNA. **B.** The pREP3x/*pol5*$^+$ strain (GG778/779) was also grown in the presence and absence of thiamine and pulsed with radioactive [^{3}H] uridine, for every 3 hourly sample. RNA was extracted from cells grown at 3 h, 6 h and 9 h and separated by electrophoresis. The red arrows indicate the sizes of the different ribosomal bands.

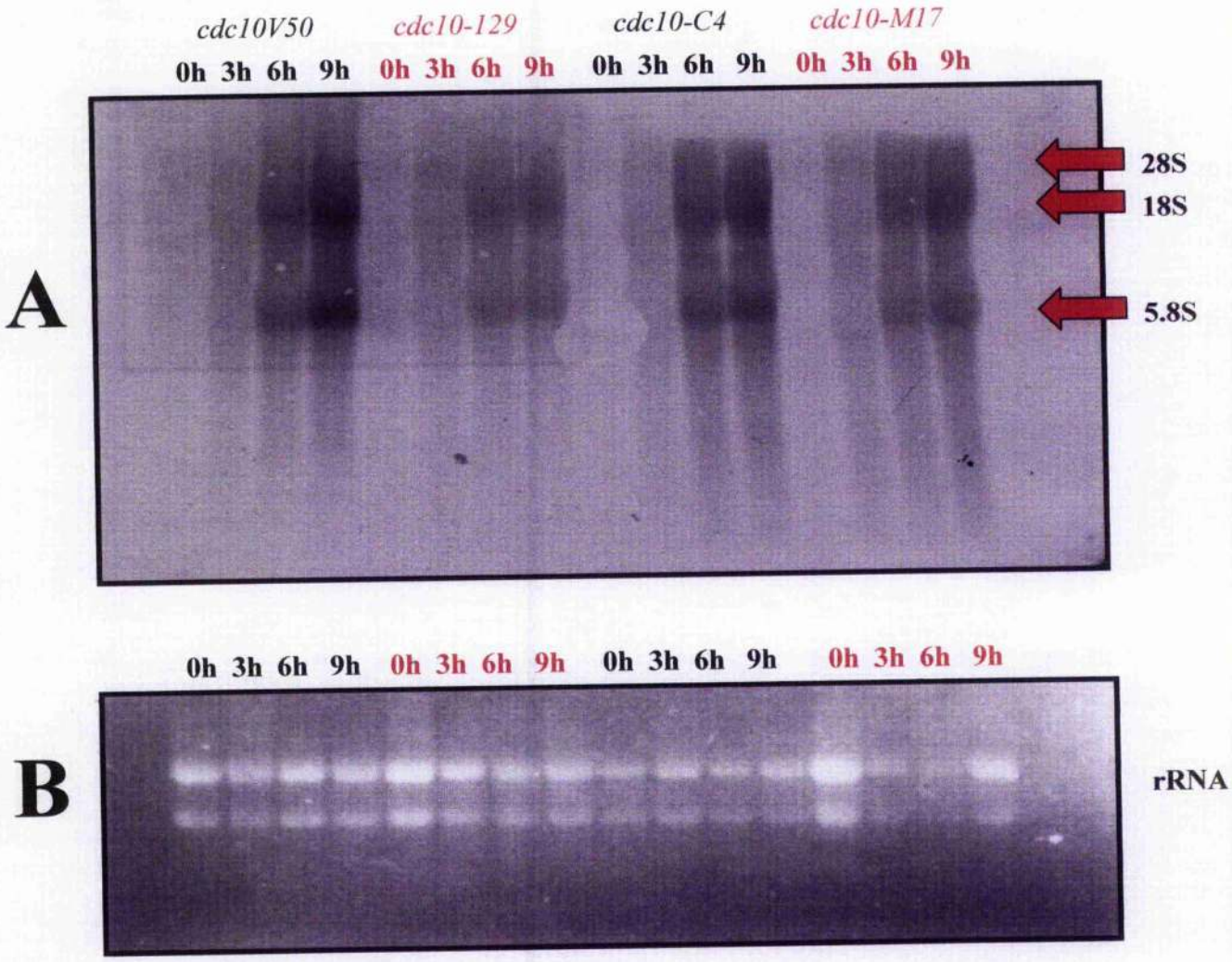
5.4 Examining SpCdc10p function on rRNA synthesis

As described in Chapter 3, SpPol5p was discovered as a novel binding partner of SpCdc10p, through a two-hybrid study, which was then confirmed as being a direct interaction by *in vitro* GST pull-down assays. As SpPol5p is thought to be involved in ribosomal RNA production it was then investigated whether SpCdc10p was also linked to the same process. Performing a series of pulse-chase rRNA labelling experiments using various temperature sensitive *cdc10* mutants was another way to test this hypothesis. The mutants used for this experiment were obtained from laboratory stocks and were *cdc10-129* (GG 28), *cdc10-C4* (GG 96), *cdc10V50* (GG 167) and *cdc10-M17* (GG 202).

5.4.1 Pulse-chase labelling experiments using *cdc10* mutants

Four separate 10 ml pre-cultures of YE containing each of the *cdc10* mutant strains was grown overnight at 25°C. The next day 1 ml of this culture was used to inoculate another 100 ml of YE and grown overnight at 25°C until cells were in mid-log phase. The following day 60 ml was removed from each culture and 30 µl of [^{3}H] uridine was added and left to incubate for 15 min, to allow the labelled activity to incorporate. A 10 ml sample of cells was taken from each flask and centrifuged for 5 min at 3,000 rpm. Meanwhile, the remaining cultures were shifted to restrictive temperature (36°C) and allowed to grow for 3 h. The cells that had been harvested could be frozen and stored at –70°C at this stage. After growth for 3 h at 36°C, another 30 µl of [^{3}H] uridine was added to each culture and incubated for 15 min. 10 ml of culture was taken and cells were harvested by centrifugation. Cultures were grown and pulse-labelled a further two times at 6 h and 9 h. When all the samples were collected, RNA were then extracted from the cells and quantified. Equal amounts of RNA was separated by electrophoresis and transferred onto a membrane. The membrane was left to expose at –70°C for 8 weeks after which the x-ray film was developed to view the labelled rRNA. The results are shown in Figure 5B.

Figure 5B Pulse-chase labelling experiment of newly synthesised rRNA in temperature sensitive *cdc10* mutants. A. All four mutants were grown at permissive temperature (25°C) to mid-log phase where 30 µl of [^{3}H] uridine was added and incubated for 15 min. A sample of cells was taken at time zero (0h). Cells were then shifted to restrictive temperature (36°C) and 3 more samples were taken at 3 h intervals (3h, 6h and 9h). Each time 30 µl of [^{3}H] uridine was added for 15 min. The red arrows indicate the sizes of the ribosomal bands. **B.** rRNA bands were stained with ethidium bromide as a measure of equal loading of RNA. **C.** FACs analysis of *cdc10*$^+$ mutants. The purple peaks show a 2C DNA content of wild type (GG 217) cells. The green peaks show the DNA content of the various mutants at 3 hour intervals.


A
cdc10V50
cdc10-129
cdc10-C4
cdc10-M17
0h 3h 6h 9h 0h 3h 6h 9h 0h 3h 6h 9h 0h 3h 6h 9h
28S
18S
5.8S
B
0h 3h 6h 9h 0h 3h 6h 9h 0h 3h 6h 9h 0h 3h 6h 9h
rRNA
C
cdc10V50
cdc10-129
cdc10-C4
cdc10-M17
WT
0
3
6
9
(h)

Examining the data produced from the pulse-chase experiments revealed that impairing SpCdc10p function did not have any effect on the production of rRNA, as it was still being synthesised and levels of newly synthesised rRNA were increasing with time in all four of the *cdc10*$^{+}$ mutant strains.

5.5 *pol5*$^{+}$ "shut-off"

After examining the effect of over-expression of *pol5*$^{+}$ and the role of SpCdc10p in rRNA synthesis, the next step was to see the effect of loss of *pol5*$^{+}$ function. Deletion experiments of *pol5*$^{+}$, already described in Chapter 4, showed that it was an essential gene. To further our understanding of *pol5*$^{+}$ biochemistry, shut-off experiments were performed to study the effect of removing SpPol5p from a cell. A series of constructs were made to test this, using both plasmid vectors and integrating vectors, in a Δ*pol5* background strain.

Ideally, the shut-off experiment should have been carried out, using the method devised by Bahler *et al.* (1998), by replacing the genomic *pol5*$^{+}$ promoter with the thiamine-repressible *nmt* promoter in its native chromosome context. Attempts to do this were unsuccessful; therefore a different approach was adopted by using various *nmt* plasmid constructs.

5.5.1 Making *pol5*$^{+}$ "shut-off" constructs using the strongest *nmt* vector, pREP3x

From previous experiments (Chapter 4) it was shown that the pREP3x/*pol5*$^{+}$ construct (GB 340) could rescue a Δ*pol5* strain. Another outcome from this experiment was that when *pol5*$^{+}$ was shut-off in the Δ*pol5* strain the cells were still viable. This was because the pREP3x expression vector was not completely repressing *pol5*$^{+}$ transcription, the presence of thiamine. As this plasmid existed in multi-copy this amount of SpPol5p expression was sufficient for fission yeast to survive. A way to overcome this was to use an integrating vector to incorporate *pol5*$^{+}$ in single copy, into the genome of a Δ*pol5* strain. This was carried out by cloning *pol5*$^{+}$ from pREP3x/*pol5*$^{+}$ (GB 340), including the *nmt* promoter and terminating sequences, into the integration vector pJK148 (GB 186) (Keeney & Boeke 1994) (Figure 5C).

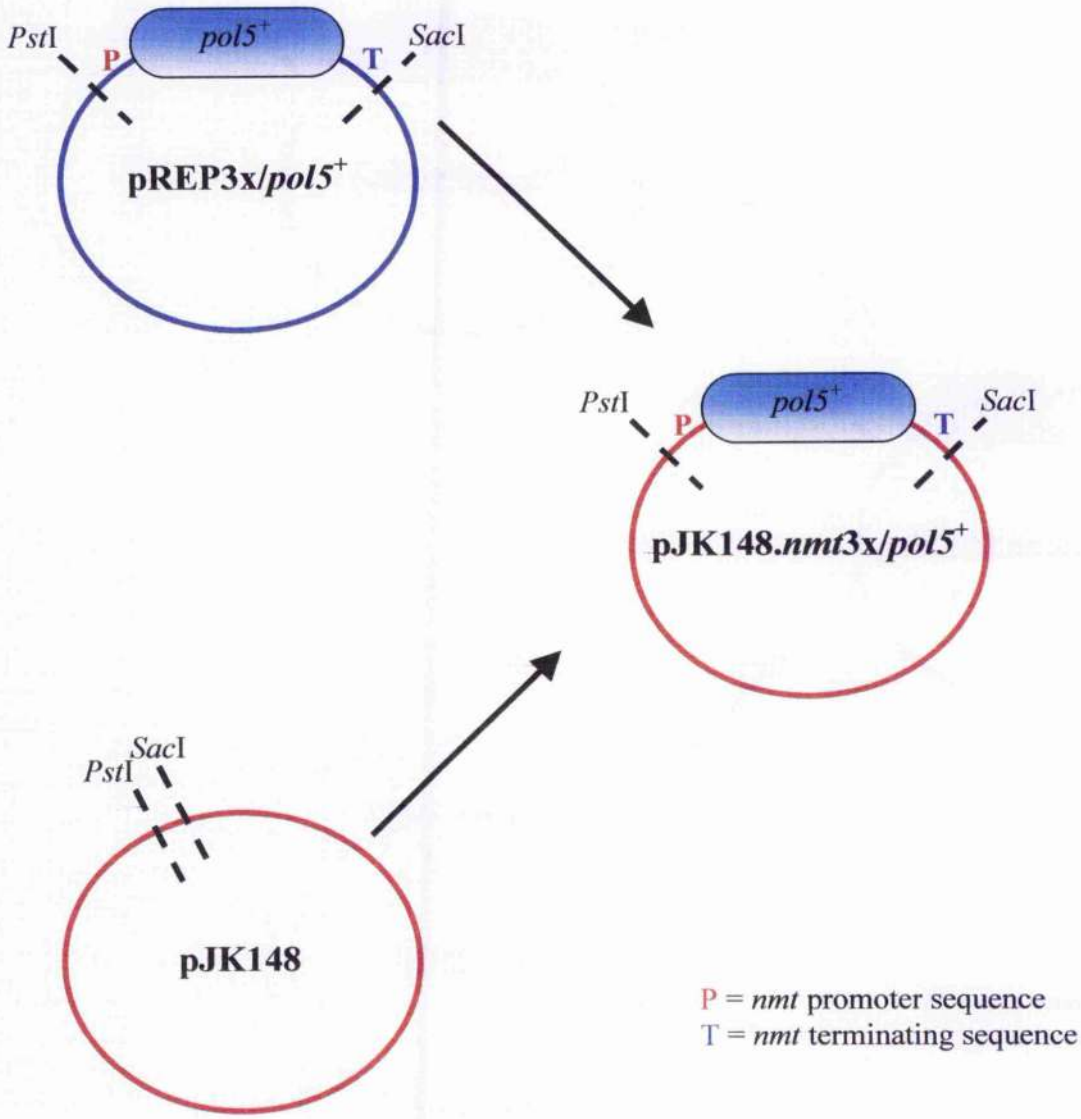


Figure 5C Cloning *pol5*$^+$ from the *nmt* expression vector pREP3x/*pol5*$^+$ (GB 340) into the integration vector pJK148 (GB 186). Using the restriction enzymes *Pst*I and *Sac*I, *pol5*$^+$ was cut from pREP3x/*pol5*$^+$ (GB 340) and cloned into pJK148 (GB 186), which was also cut with the same restriction enzymes. The ligation mix was transformed into DH5α *E. coli* cells. Diagnostic restriction digests confirmed constructs.

pREP3x/*pol5*$^+$ (GB 340) was double digested with *Pst*I and *Sac*I restriction enzymes to release *pol5*$^+$ with the *nmt*, promoter and terminating, sequences on either end. At the same time pJK148 (GB 186) was also digested with *Pst*I and *Sac*I. A ligation reaction was set up using T4 DNA ligase and incubated overnight at 14°C. Next, 1-2 µl of the ligation mix was used to transform into DH5α *E. coli* cells. Individual colonies carrying the pJK148.*nmt*3x/*pol5*$^+$ construct were confirmed by diagnostic restriction digests. Once the new construct was confirmed it was digested with *Nde*I to linearize the plasmid within the leucine gene. *pol5*$^+$/Δ*pol5* diploid cells were transformed with the linearised plasmid by the lithium acetate method (Materials & Methods, Section 2.10.3). The DNA was expected to incorporate into the leucine locus within the genome by homologous recombination. The diploids, which were originally homozygous leu$^-$, were now heterozygous leu$^+$. The transformation mix was allowed to grow on EMM plates to select for cells, which had incorporated leucine and consequently also *pol5*$^+$, which was under the control of the *nmt* promoter. Diploid cells formed tetrads on minimal media (EMM) where they were selected and treated with glucylase, to break down the cell wall, in order to release the spores. Spores were then spread onto EMM plus adenine plates to select for haploids that had incorporated the leucine gene and were now also carrying the *pol5*$^+$ gene under *nmt* control. Cells that grew also had to be *ura*$^+$ in order to grow therefore they were deleted for the wild type copy of *pol5*$^+$ and this was confirmed by PCR (Figure 5Da). The oligonucleotides (GO 474 and GO 475) used for the PCR were the same as the ones used in Chapter 4, which confirmed the replacement of *pol5*$^+$ with *ura4*$^+$ (Figure 5Da). The PCR confirmed that the strains carrying pREP3x/*pol5*$^+$ (GG 831) and also the strain carrying the integrated *pol5*$^+$ (GG 847) were deleted for the wild type copy of *pol5*$^+$. One isolate for each strain was chosen for subsequent experiments.

The next step was to test for an effect on cell viability when *pol5*$^+$ was "shut-off" in fission yeast. This was done by growing the isolates on EMM plus adenine, followed by replica-plating them onto EMM plus adenine and thiamine to repress transcription of *pol5*$^+$.

Frustratingly, there was still no effect on cell viability when *pol5*$^+$ transcription was repressed by thiamine (Figure 5E). This was likely to be because the strongest *nmt* expression vector pREP3x (GB 340) was used and therefore expression of *pol5*$^+$, in the presence of thiamine, was still sufficient to rescue Δ*pol5* haploid cells.

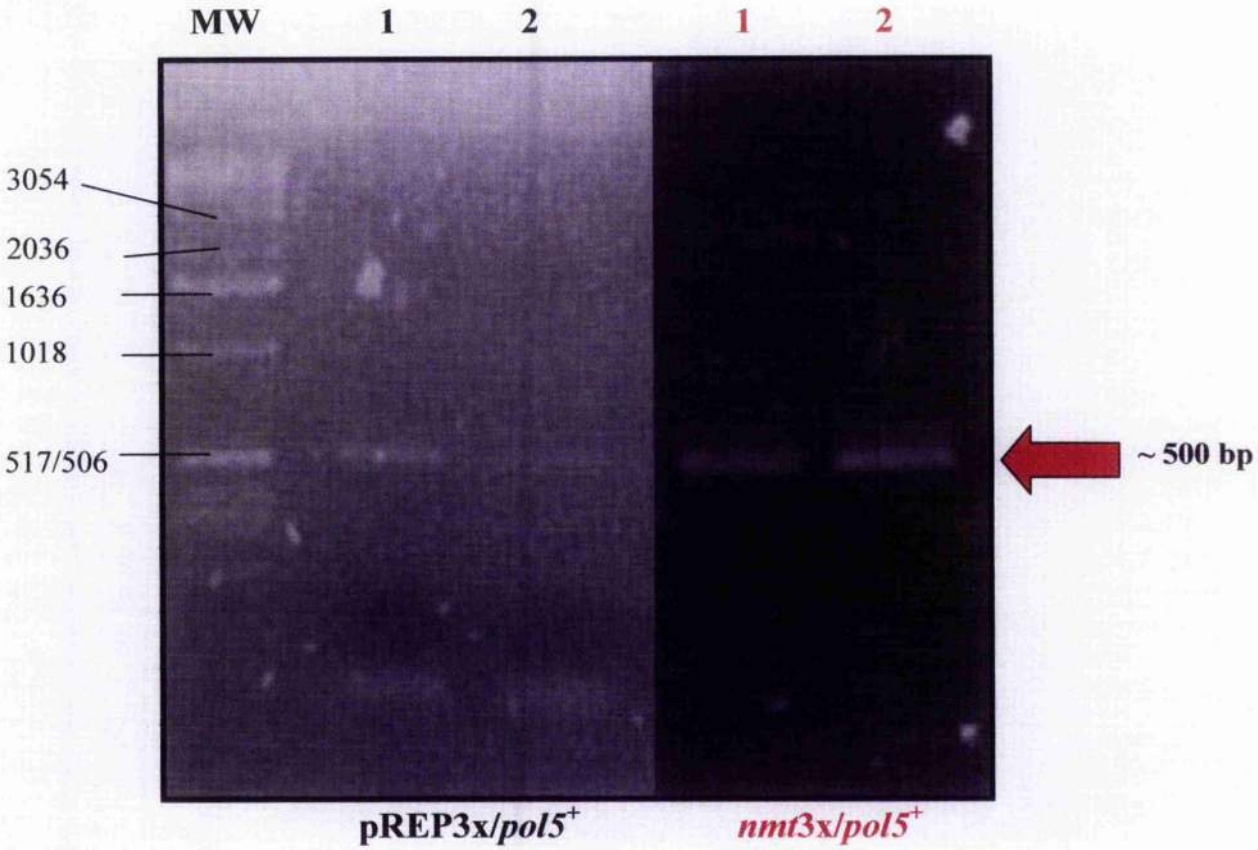


Figure 5Da PCR analysis confirming that the strains used for the *pol5*$^+$ "shut-off" experiment, carrying either plasmid *pol5*$^+$ or integrated *pol5*$^+$, under the control of the strongest *nmt*3x promoter, are both Δ*pol5*. The oligonucleotides, GO 474 and GO 475 were used to confirm that the strains carrying either pREP3x/*pol5*$^+$ plasmid or integrated *nmt*3x/*pol5*$^+$ were deleted for wild type *pol5*$^+$. Two isolates, 1 and 2, were tested for each strain. Isolate 1 was chosen for pREP3x/*pol5*$^+$ (GG 831) and isolate 2 (GG 847) for *nmt*3x/*pol5*$^+$. 1 kb molecular weight ladder (MW) is shown to indicate the size of the DNA products. The red arrow shows a correct sized PCR product of 500 base pairs (bp).

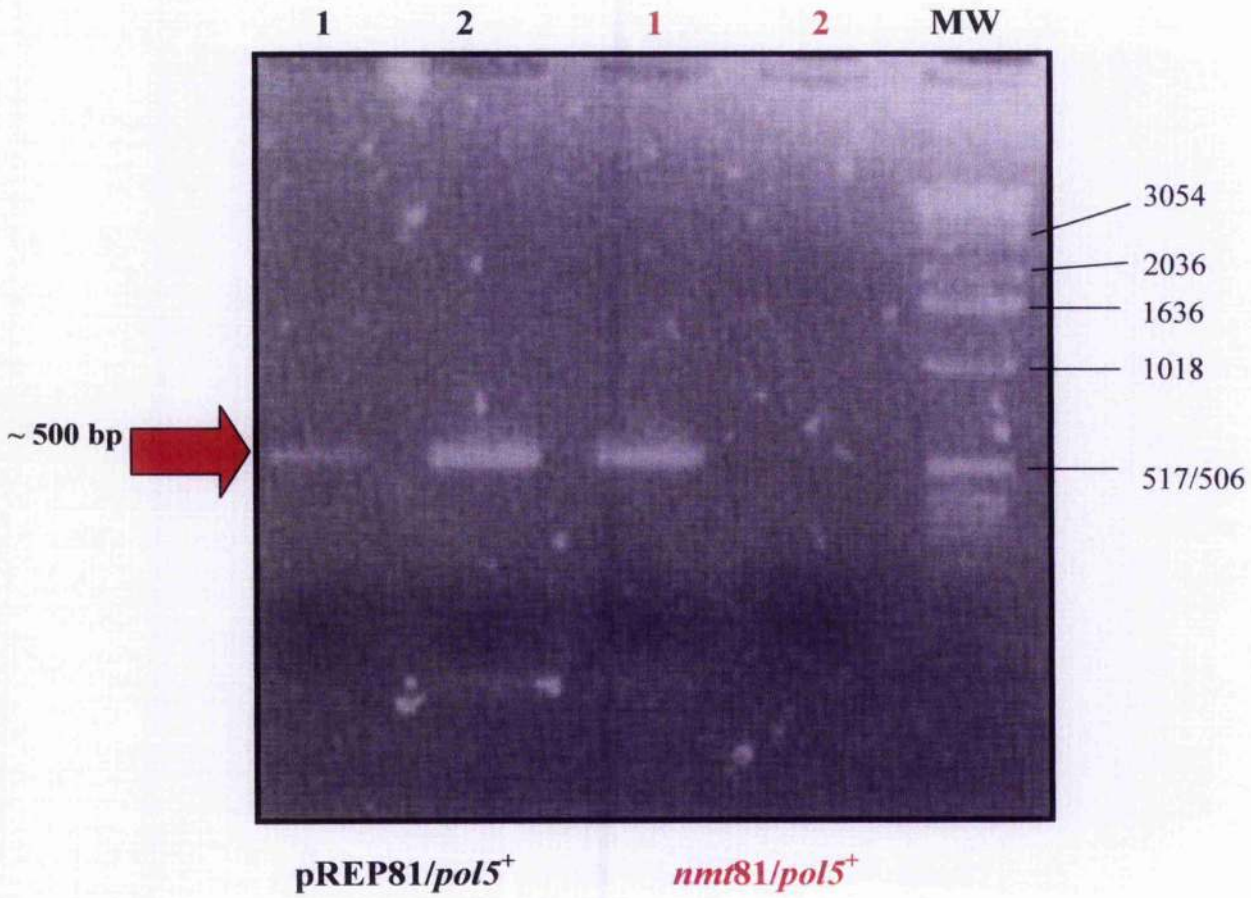
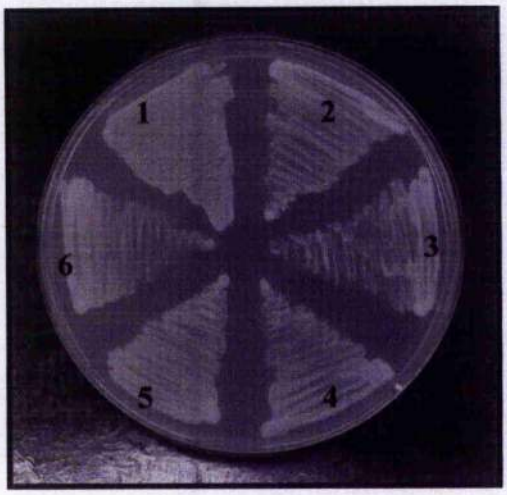


Figure 5Db PCR analysis confirming that the strains used for the *pol5*$^+$ "shut-off" experiment, carrying either plasmid *pol5*$^+$ or integrated *pol5*$^+$, under the control of the weakest *nmt*81 promoter, are both Δ*pol5*. The oligonucleotides, GO 474 and GO 475 were used to confirm that the strains carrying either pREP81/*pol5*$^+$ plasmid or integrated *nmt*81/*pol5*$^+$, using the plasmid pJK148 (GB 186), were deleted for wild type *pol5*$^+$. Two isolates, 1 and 2, were tested for each strain. Isolate 2 (GG 863) was chosen for pREP81/*pol5*$^+$ and isolate 1 (GG 856) for *nmt*81/*pol5*$^+$. 1 kb molecular weight ladder (MW) is shown to indicate the size of the PCR products. The red arrow shows a correct sized PCR product of 500 base pairs (bp).

EMM + ADE



EMM + ADE + THIA

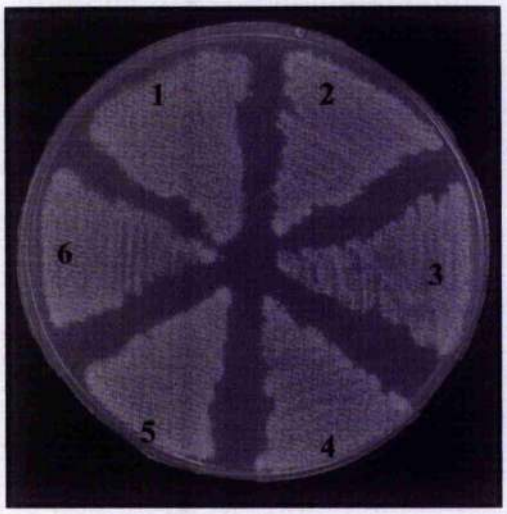


Figure 5E *pol5*$^+$ under varying strengths of *nmt* promoter, rescues a Δ*pol5* strain even when the *nmt* promoter is "shut-off" by addition of thiamine. 1. Wild type (GG 214) fission yeast. **2.** Wild type fission yeast carrying the empty pREP3x plasmid (GG 786). **3.** A Δ*pol5* strain carrying the pREP3x/*pol5*$^+$ (GG 831) plasmid. **4.** A Δ*pol5* strain with integrated *nmt*3x/*pol5*$^+$ (GG 847). **5.** A Δ*pol5* strain carrying the pREP81/*pol5*$^+$ (GG 863) plasmid. **6.** A Δ*pol5* strain with integrated *nmt*81/*pol5*$^+$ (GG 856). Cells were grown on EMM plus adenine to induce over-expression of *pol5*$^+$, after which they were replica-plated onto EMM plus adenine and thiamine to repress the *nmt* promoter and shut-off *pol5*$^+$ transcription. In all the cases the Δ*pol5* haploid strains are still able to survive, as the promoters for all four constructs are not completely shut-off.

5.5.2 Making *pol5*$^+$ "shut-off" constructs using the weakest *nmt*81 vector, pREP81

Integrating *pol5*$^+$, in single copy, under the control of the strongest thiamine-repressible *nmt* promoter failed to permit complete repression of *pol5*$^+$ transcription. Therefore, we cloned *pol5*$^+$ into the weakest expression vector, pREP81 (GB 346).

pREP3x/*pol5*$^+$ was double digested with *Sal*I and *Bam*HI to remove *pol5*$^+$. In parallel, pREP81 was also digested with *Sal*I and *Bam*HI. The two fragments were ligated together (Figure 5F) using T4 DNA ligase and incubated at 14°C overnight. 1-2 µl of ligation mix was used to transform into DH5α *E. coli* cells. Individual colonies carrying the pREP81/*pol5*$^+$ construct were confirmed by diagnostic restriction digests were performed, using the *Sal*I and *Bam*HI restriction enzymes. This construct (GB 365) was used later for pulse-chase labelling experiments.

After *pol5*$^+$ was cloned into the weaker *nmt*81 vector (pREP81), it was then cloned into the integration vector, pJK148. *Pst*I and *Sac*I were used to excise the *nmt*81/*pol5*$^+$ fragment from pREP81/*pol5*$^+$ (GB 365). pJK148 was also digested with the same enzymes followed by a ligation reaction (Figure 5F). Once the new construct was confirmed it was digested with *Nde*I to linearize the plasmid within the leucine marker. Δ*pol5* diploid cells were transformed with the linearised DNA. The transformation mix was allowed to grow on EMM plates to select for cells which had incorporated the *nmt*81 promoter. Diploid cells formed tetrads on minimal media (EMM) where they were selected and treated with glucylase. Spores were then spread onto EMM plus adenine plates to select for haploids that had incorporated the leucine gene and were now also carrying the new *pol5*$^+$ gene under *nmt*81 control. Cells also had to be *ura*$^-$ in order to grow therefore they were deleted for the wild type copy of *pol5*$^+$ and this was confirmed by PCR (Figure 5Db). The oligonucleotides used for the PCR (GO 474 and GO 475) were the same as the ones used in Chapter 4, which confirmed the replacement of *pol5*$^+$ with *ura4*$^+$ (Figure 5Db). The PCR verified that the haploid strains, carrying the plasmid pREP81/*pol5*$^+$ (GG 863) and also the strain carrying integrated *nmt*81/*pol5*$^+$ (GG 856), were deleted for the wild type copy of *pol5*$^+$.

The next step was to test for an affect on cell viability when *pol5*$^+$ was "shut-off" in fission yeast. This was done by growing fission yeast cells either containing the integrated construct *nmt*81/*pol5*$^+$ or the weak expression vector pREP81/*pol5*$^+$ on

EMM plus adenine plates, followed by replica-plating them onto EMM plus adenine and thiamine to repress transcription of *pol5*$^{+}$.

Once again, there was no effect on cell viability even when *pol5*$^{+}$ transcription was being repressed by thiamine. This was likely to be because the *nmt*81 expression vector pREP81 was still expressing sufficient amounts of SpPol5p to allow the cells to survive even when repressed. As described in Chapter 4, *pol5*$^{+}$ is a rare transcript. This implies cells require small amounts of protein. The survival of Δ*pol5* cells with the integrated *nmt*81/*pol5*$^{+}$ construct or pREP81/*pol5*$^{+}$, even when transcription is being repressed by thiamine, suggests that the small amount of *pol5*$^{+}$ transcription occurring is sufficient (Figure 5E).

Nevertheless, the four strains generated; plasmid pREP3x/*pol5*$^{+}$ (GG 831), integrated *nmt*3x/*pol5*$^{+}$ (GG 847), plasmid pREP81/*pol5*$^{+}$ (GG 863) and integrated *nmt*81/*pol5*$^{+}$ (GG 856), were used for pulse-chase experiments to analyse the effect on rRNA synthesis when *pol5*$^{+}$ mRNA levels are being manipulated.

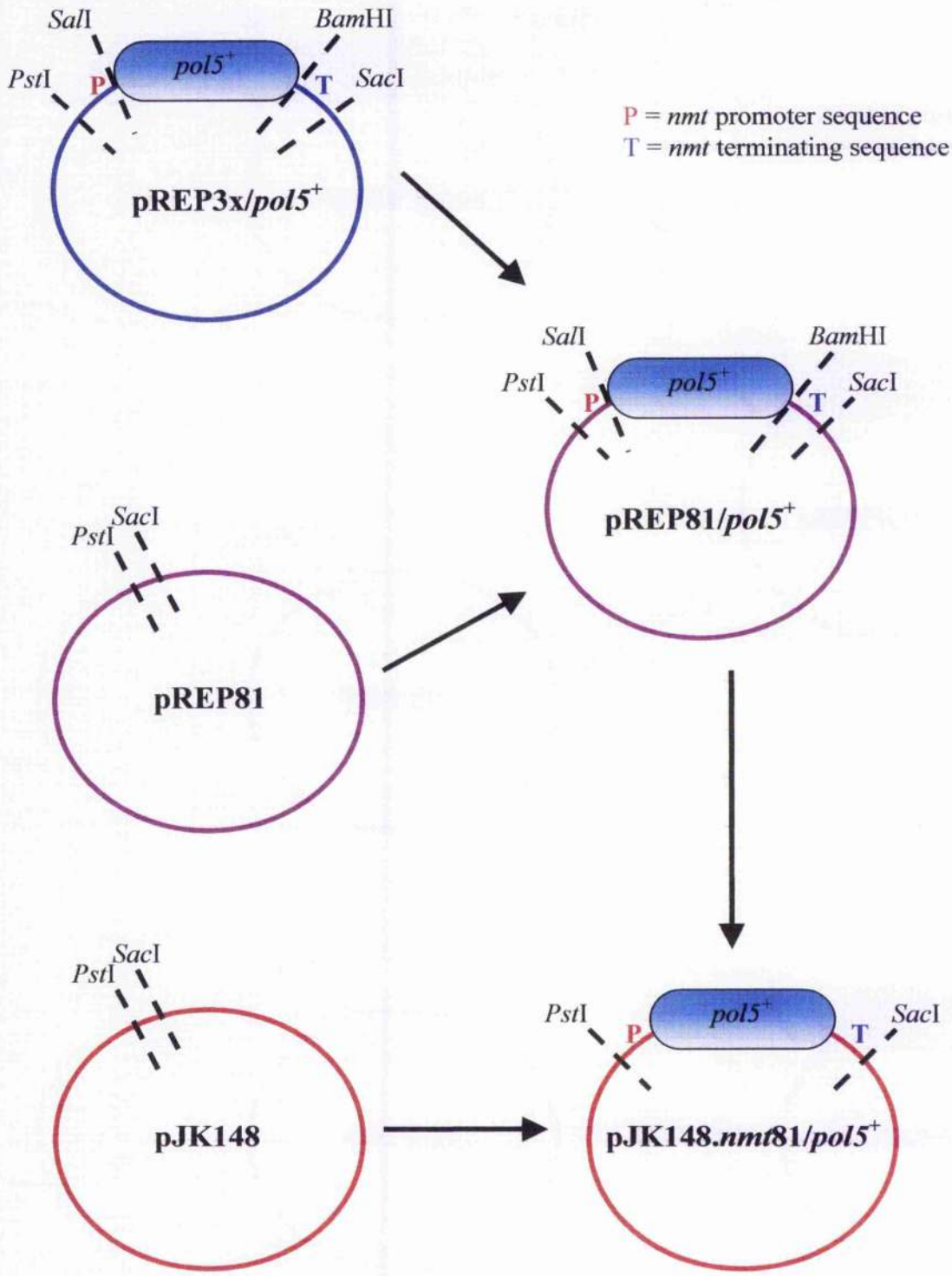


Figure 5F Cloning *pol5*$^+$ from the *nmt* expression vector pREP81 (GB 346) into the integration vector pJK148 (GB 186). *Sal*I and *Bam*HI restriction enzymes were used to cut both pREP3x/*pol5*$^+$ (GB 340), to excise *pol5*$^+$, and pREP81 (GB 346), which were then ligated to form pREP81/*pol5*$^+$ (GB 365). Next pJK148 (GB 186) and pREP81/*pol5*$^+$ were digested with *Pst*I and *Sac*I. The *pol5*$^+$ insert was then cloned into pJK148 to form pJK148.*nmt*81/*pol5*$^+$.

5.5.3 Efficiency of *nmt* "shut-off" observed by northern blot analysis

Before the pulse-chase experiments were performed, northern blot analysis was carried out to examine how cleanly *pol5*$^+$ was being shut-off in the four different shut-off strains. Two isolates from each of the four strains were taken and grown in 10 ml of EMM plus adenine overnight at 30°C. The next day 1-2 ml of each of the eight pre-cultures was used to inoculate one flask with 50 ml of EMM plus adenine and another flask with 50 ml EMM plus adenine and thiamine. In total sixteen flasks were left to grow overnight at 30°C. The following day the cells in each flask were harvested by centrifugation and the RNA was extracted. RNA was separated by electrophoresis and transferred onto a membrane, which was then probed with [α-P^{32}] radioactively labelled *pol5*$^+$ DNA. The membrane was left to expose for various lengths of time to get the best exposure. The results for the varying degrees of strengths of the different *nmt* promoters are shown in Figure 5G. The same membrane was also probed for *cdc10*$^+$ (the major protein of the cell-cycle transcription factor complex, DSC1), and *cdc22*$^+$ (a gene under the control of SpCdc10p) (Figure 5G).

In parallel, the samples where all the strains were "shut-off", i.e. grown in thiamine, were taken and separated on another gel. Levels of *pol5*$^+$ were expected to be much lower in these samples and were therefore left for a longer period of time to expose. The results for this northern experiment are shown in Figure 5H.

pol5$^+$ was being over-expressed in all four *nmt* strains in the absence of thiamine. As expected, the highest over-expression was detected using the strongest expression vector pREP3x/*pol5*$^+$ (Figure 5G). However, the next strongest over-expression of *pol5*$^+$ was seen in the weakest integrated strain, *nmt*81/*pol5*$^+$ (Figure 5G). This was unexpected as this strain was intended to produce the least amount of *pol5*$^+$. Upon examining *pol5*$^+$ expression when the *nmt* promoters were repressed, there were still significant amounts of transcript in both the pREP3x/*pol5*$^+$ and *nmt*81/*pol5*$^+$strains (Figure 5H).

The next level of over-expression was seen in the integrated, *nmt*3x/*pol5*$^+$, strain followed by the strain carrying the pREP81/*pol5*$^+$construct (Figure 5G). These strains also repressed *pol5*$^+$ transcription with the greater efficiency in the presence of thiamine (Figure 5H).

Figure 5G shows that the transcription of some other genes is affected when *pol5*$^+$ is shut-off. In the pREP3x/*pol5*$^+$ strain, levels of *cdc22*$^+$ and *cdc10*$^+$ mRNA

remain the same as wild type. In the three other strains, *cdc22*$^{+}$ and *cdc10*$^{+}$ mRNA levels are significantly reduced when *pol5*$^{+}$ is being over-expressed or repressed.

From these data (Figure 5G & 5H), the effect of over-expressing or repressing *pol5*$^{+}$ transcription in a Δ*pol5* background is disrupting the normal transcription of *cdc22*$^{+}$ and *cdc10*$^{+}$. This might be because of disruption to the normal synthesis or processing of ribosomal RNA. To investigate if repressing or over-expressing *pol5*$^{+}$ has an overall affect on transcription, a random assortment of genes must be probed to see if transcription of genes is affected.

Figure 5G Northern blot experiment analysing *pol5*$^+$, *cdc10*$^+$ and *cdc22*$^+$ transcripts, in four different *nmt/pol5*$^+$ strains, deleted for wild type *pol5*$^+$, when transcription was ON (-thiamine) or OFF (+thiamine). Duplicate isolates were loaded for each strain. The same membrane was also probed for *cdc22*$^+$, *cdc10*$^+$. rRNA bands are shown as a loading control of equal amounts of RNA. The membrane was left to expose overnight to detect the *cdc22*$^+$ and over-expressed *pol5*$^+$ transcripts. *cdc10*$^+$ was detected after a week. **1/3 & 2/4** are pREP3x/*pol5*$^+$ ON & OFF, respectively. **5/7 & 6/8** are *nmt*3x/*pol5*$^+$ ON & OFF, respectively. **9/11 & 10/12** are pREP81/*pol5*$^+$ ON & OFF, respectively. **13/15 & 15/16** are *nmt*81/*pol5*$^+$ ON & OFF, respectively. **WT.** Wild type.

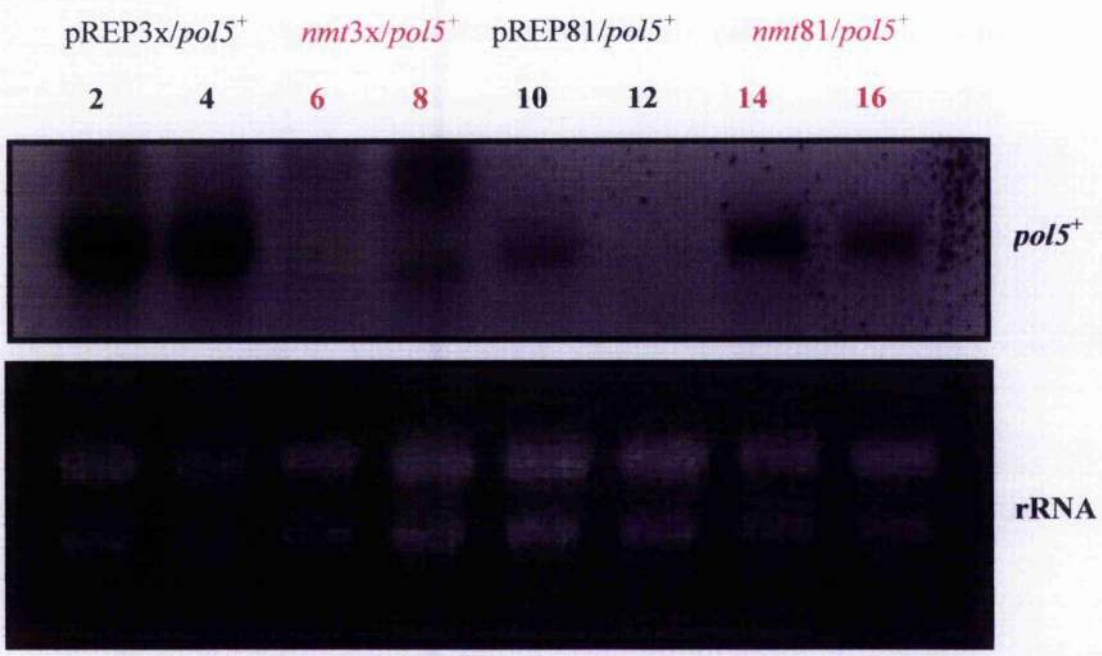


Figure 5H *pol5*$^+$ mRNA levels in four different *nmt/pol5*$^+$ strains, deleted for wild type *pol5*$^+$, when transcription was repressed (+thiamine). rRNA bands are shown as a loading control of equal amounts of RNA. The membrane was left to expose for 2 days. **2 & 4** are pREP3x/*pol5*$^+$. **6 & 8** are *nmt*3x/*pol5*$^+$. **10 & 12** are pREP81/*pol5*$^+$. **14 & 16** are *nmt*81/*pol5*$^+$.

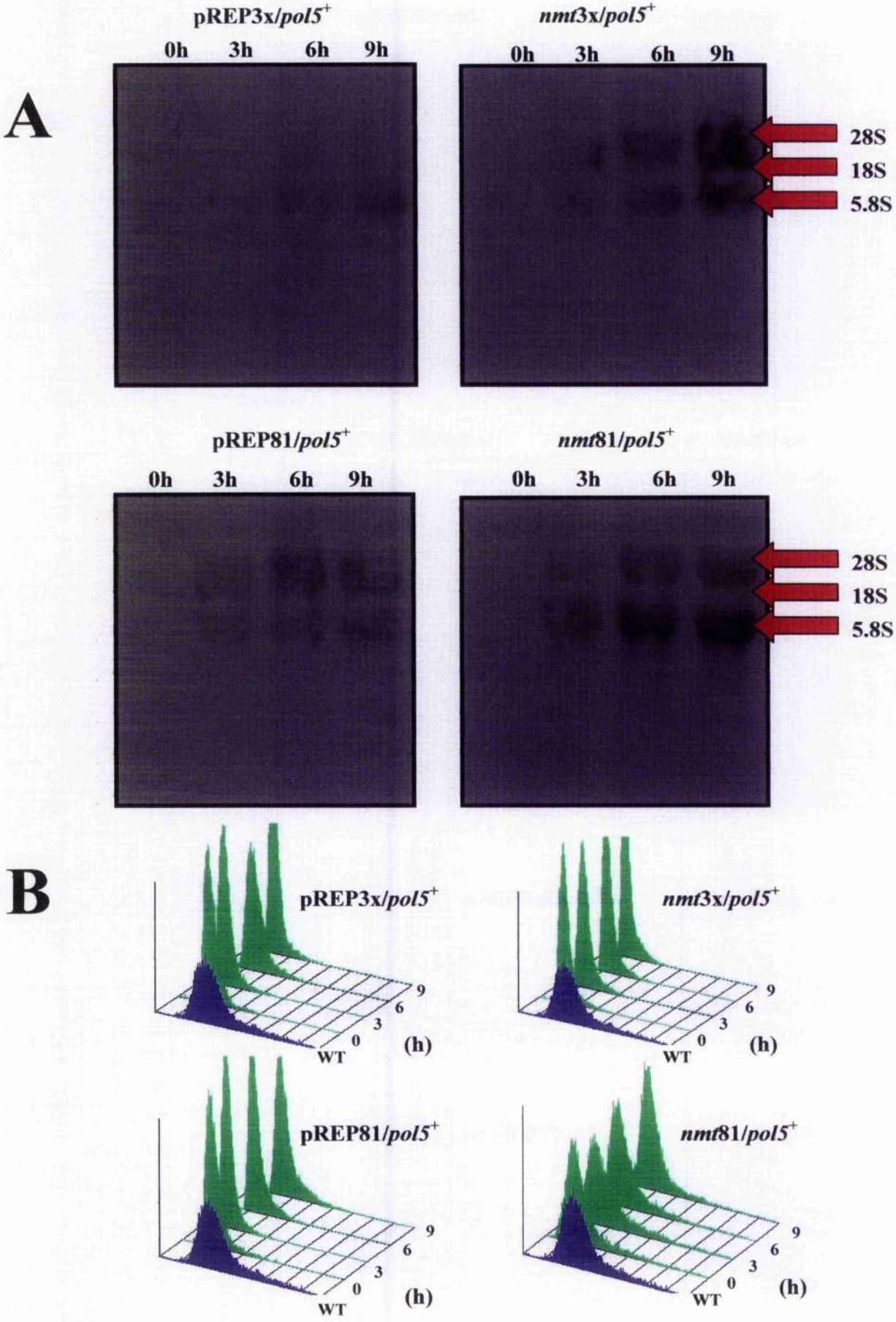
5.4 Pulse-chase labelling experiments using *pol5*$^+$ "shut-off" strains

After the *nmt/pol5*$^+$ shut-off strains were examined by northern blot analysis, one isolate for each strain was selected to perform a pulse-chase labelling experiment.

10 ml pre-cultures of each of the four different *nmt/pol5*$^+$ strain, in EMM plus adenine, were grown overnight at 30°C. The next day, 1-2 ml of each of the pre-cultures was used to inoculate another 100 ml of EMM plus adenine. The four flasks were left to grow overnight, shaking at 30°C. The following morning, 60 ml of each culture was removed and discarded. 30 µl of [^{3}H] uridine was added to each culture and incubated for 15 min. A 10 ml sample of each culture was taken and centrifuged. The cells were frozen down at –70°C and stored for RNA extraction later. Thiamine was then added to the cultures, to shut-off *pol5*$^+$ transcription, and was left shaking at 30°C for 3 h. 30µl of [^{3}H] uridine was added for 15 min after which a 10 ml sample was taken and centrifuged. This process was repeated a further two times at 3 h intervals. Once all the samples were collected, the RNA was extracted and separated by electrophoresis.. RNA was then transferred onto a membrane, which was left to expose at –70°C for 8 weeks after which the x-ray film was developed to view the rRNA bands. The results are shown in Figure 5J.

Figure 5J shows differences between the rRNA profiles of each of the shut-off strains even though equal strengths of labelled activity ([^{3}H] uridine) was added to each sample. The most affected strain was pREP3x/*pol5*$^+$, which shows dramatically reduced amounts of rRNA being synthesised, compared to the other strains. The next most affected strain is the strain carrying the pREP81/*pol5*$^+$ plasmid, which again shows much reduced amounts of rRNA production. The two integrated strains are less affected by *pol5*$^+$ shut-off and show normal rRNA synthesis. These results are puzzling, as they do not correlate with the results of the northern blot experiments (Figure 5G & 5H). These strains must be analysed further to understand their odd behaviour when *pol5*$^+$ transcription is being over-expressed or repressed. Ideally a *pol5*$^+$ shut-off strain must be created where the chromosomal *pol5*$^+$ promoter is replaced with the *nmt* promoter, for cleaner, more efficient, repression of *pol5*$^+$ transcription. The effect of carrying either plasmid *pol5*$^+$ constructs or disrupting the genome, elsewhere, with integrated *pol5*$^+$ might be causing adverse affects on the normal function of fission yeast.

Figure 5J Pulse-chase labelling experiment examining the effect of "shutting-off" *pol5*$^+$ transcription on rRNA synthesis. A. The four strains pREP3x/*pol5*$^+$ (GG 831), *nmt*3x/*pol5*$^+$ (GG 847), pREP81/*pol5*$^+$ (GG 863) and *nmt*81/*pol5*$^+$ (GG 856), were grown in the absence of thiamine to mid-log phase and then pulse-labelled with 30 µl of [^{3}H] uridine for 15 min. A 10 ml sample of cells was taken at zero time (0h). Thiamine was then added to repress transcription of *pol5*$^+$, after which 30 µl of [^{3}H] uridine was added for 15 min, for every 3 hour sample (3h, 6h, 9h). RNA was extracted from cells and separated by electrophoresis. The red arrows indicate the sizes of the different ribosomal bands. **B.** FACs analysis of strains carrying *nmt* constructs. The purple peaks show a 2C DNA content of wild type (GG 217) cells. The green peaks show the DNA content of the various mutants at 3 hour intervals.


A
pREP3x/pol5+
0h 3h 6h 9h
nmt3x/pol5+
0h 3h 6h 9h
28S
18S
5.8S
pREP81/pol5+
0h 3h 6h 9h
nmt81/pol5+
0h 3h 6h 9h
28S
18S
5.8S
B
pREP3x/pol5+
nmt3x/pol5+
pREP81/pol5+
nmt81/pol5+
9
6
3
0
WT
(h)

5.6 Localisation of SpPol5p

The cellular localisation of SpPol5p was studied by generating GFP (Green Fluorescent Protein) tagged versions of the protein.

As described in this thesis, SpPol5p is a strong homologue of budding yeast ScPolVp, and studies in budding yeast ScPolVp strongly suggest it to be involved in ribosomal RNA synthesis (Shimizu *et al.* 2002). rRNA is synthesised within the nuclear structure called the nucleolus and it was confirmed in budding yeast that ScPolVp localises to it (Shimizu *et al.* 2002). The aim of the following experiment was to test if SpPol5p of fission yeast also localised to the nucleolus by studying GFP-tagged SpPol5p.

5.6.1 Cloning of *pol5*$^{+}$ into GFP vectors for N and C-terminus tagging

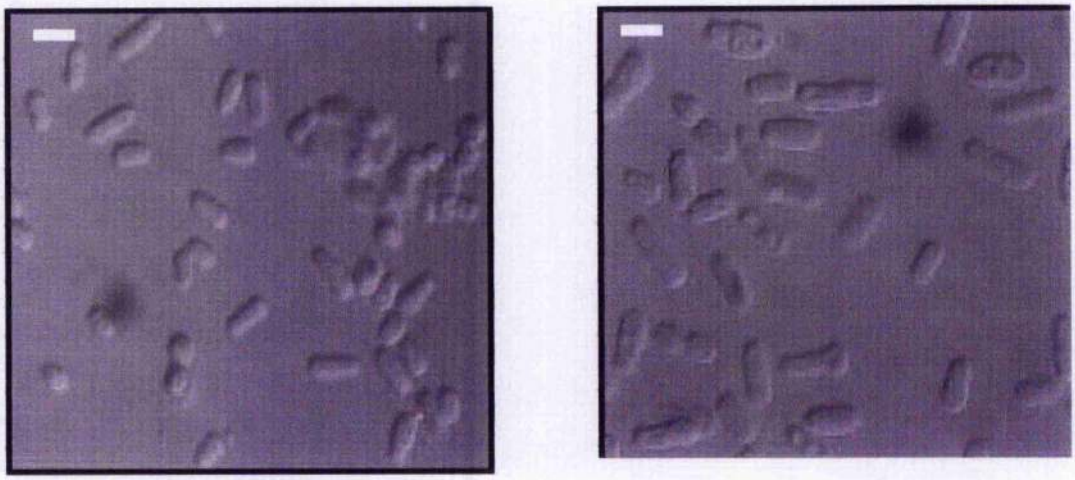
pol5^{+} was PCR amplified from pREP3x/*pol5*$^{+}$ (GB 340) and cloned into pCR2.1 (Invitrogen) to create GB 379 (N-terminus insert) and GB 383 (C-terminus insert). *pol5*$^{+}$ was then sub-cloned into pREP41/42GFP/EGP-N (GB 393) or pREP41/42GFP/EGP-C (GB 394) (Craven *et al.* 1998) using *Sal*I/*Bam*HI to create the constructs GB 393 and GB 395, respectively. These constructs were confirmed by restriction digest and sequencing (MWG Biotech).

5.6.2 Expression of GFP-tagged SpPol5p

The N and C-terminus GFP *pol5*$^{+}$ plasmids (GB393 and GB395, respectively) were transformed into heterozygous diploid Δ*pol5* fission yeast strain (GG 840). Random spore analysis was used to identify and confirm haploid transformants. Colonies that grew on EMM plus adenine plates but did not grow on EMM plates were identified as haploids deleted for *pol5*$^{+}$ that were rescued by the *pol5*$^{+}$/GFP constructs. This was further confirmed by the light pink appearance of haploid colonies on YE phloxin plates. The haploid colonies were cultured overnight and used for microscopic analysis.

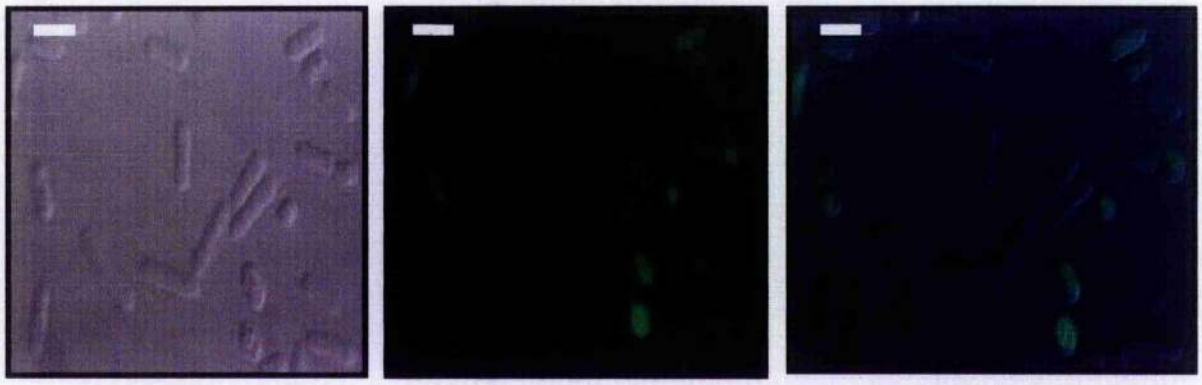
5.6.3 Localisation of GFP-tagged SpPol5p

Haploid transformants expressing GFP-tagged SpPol5p (C-terminus-GG 903) were used for microscope analysis of the cellular localisation of SpPol5p. Figure 5L shows fluorescence GFP staining of the cells expressing GFP-tagged SpPol5p. Comparison of the nomaski images of wild type (GG 217) strain and the haploid transformants (Figure 5K) showed that some transformed cells were granular and longer. The granular and longer cells showed more intense fluorescence for GFP. In many of the cells GFP staining was identified in the nuclear region. DAPI staining of cells in the same field (Figure 5M) clearly shows that in the majority of cells the GFP-tagged SpPol5p is localised to nucleus. The staining appears to be central and smaller than the nuclear region and therefore SpPol5p could be localised in the nucleolus.



Wild type **Haploid transformants**

Figure 5K Cellular morphology of wild type (GG 217) and haploid transformants of GFP/*pol5*$^+$ (GG 903). A comparison of the Nomaski images wild type (GG 217) and GFP/ *pol5*$^+$ transformants (GG 903). White bar = 10 μm.



Nomaski **GFP** **Nomaski/GFP overlay**

Figure 5L GFP staining of cells expressing C-terminus, GFP-tagged SpPol5p. Images for Nomaski and GFP staining were of the same field and the overlay of the nomasky and GFP images shows intense GFP staining in smaller cells. White bar = 10 μm.

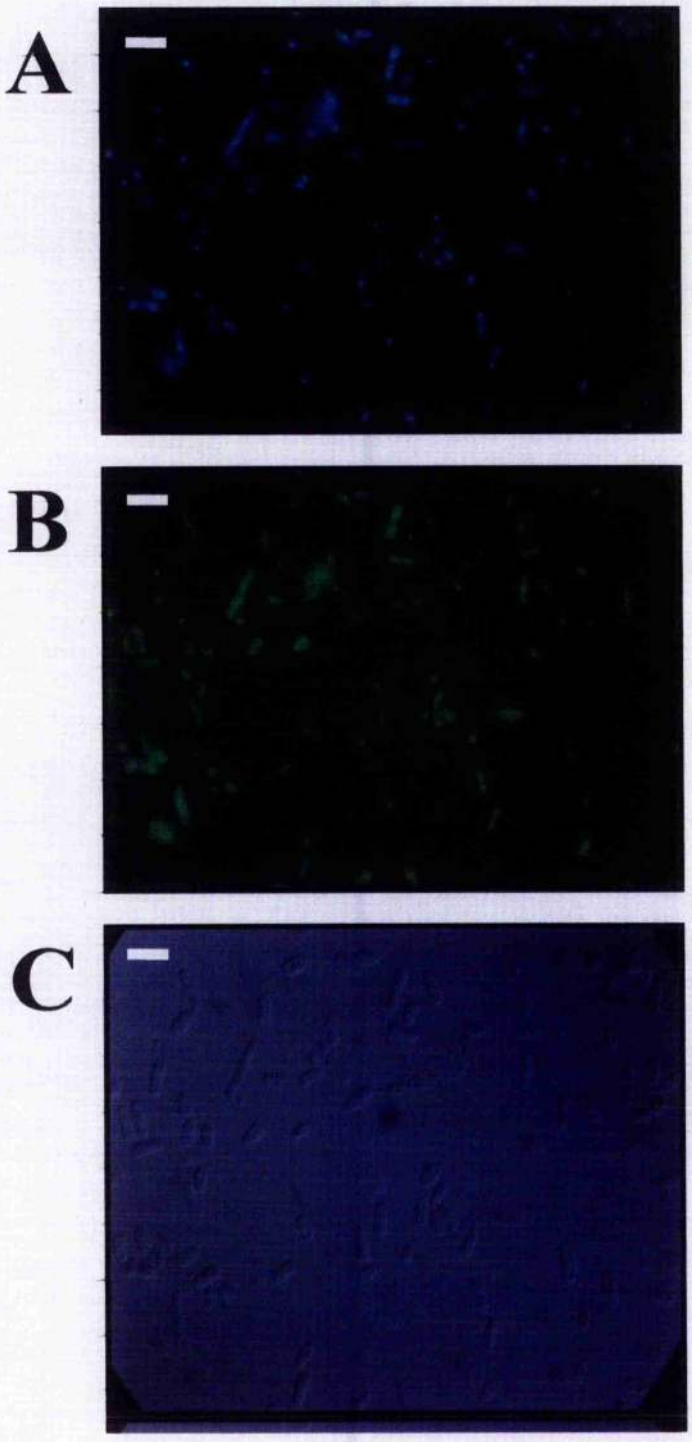
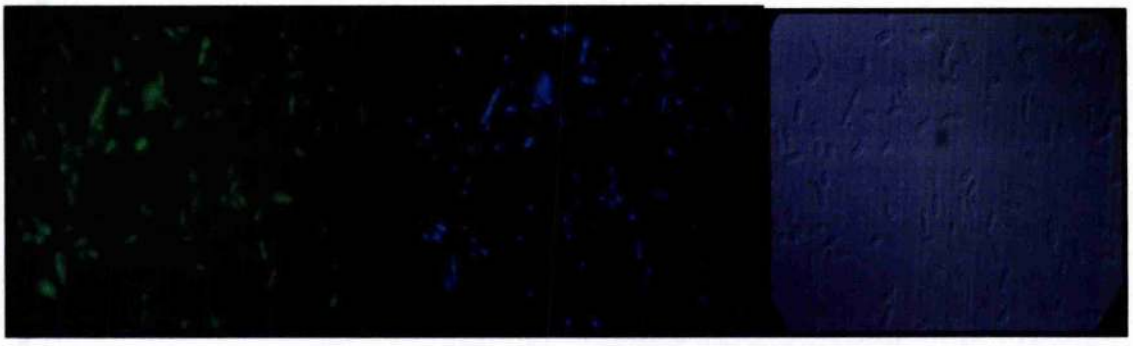


Figure 5M Localisation of GFP-tagged SpPol5p to the nucleus. Images for **A.** DAPI **B.** GFP and **C.** Nomaski, were all of the same field, at 40X magnification, displaying GFP in the nuclear region. White bar = 10 μm.

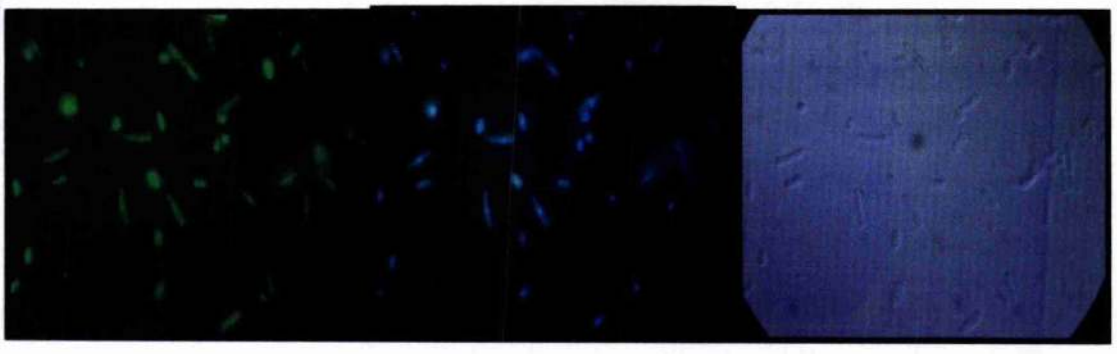
A



GFP **DAPI** **Nomaski**

Cell cycle stage	Early G2	Mid G2	Late G2	M-G_1/S
% Cells	38	35	22	5
GFP staining	1	1	4	4

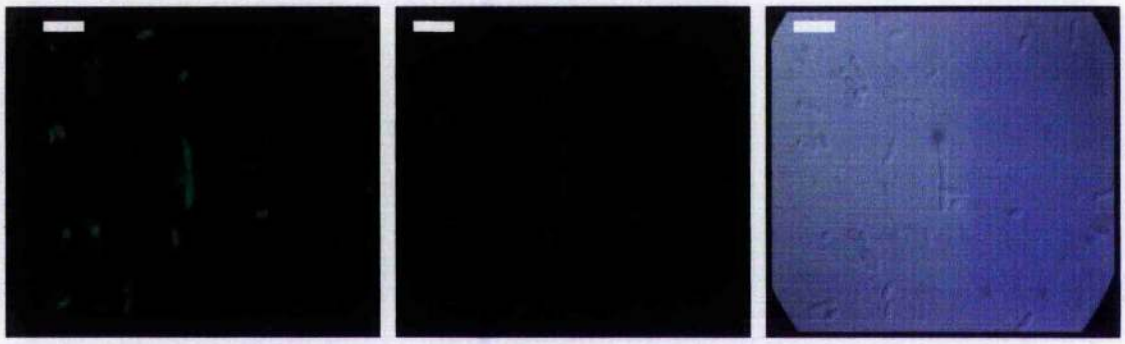
B



GFP **DAPI** **Nomaski**

Cell cycle stage	Early G2	Mid G2	Late G2	M-G_1/S
% Cells	50	46	4	0
GFP staining	5	1	3	-

C



GFP **DAPI** **Nomaski**

Cell cycle stage	Early G2	Mid G2	Late G2	M-G_1/S
% Cells	52	10	44	4
GFP staining	5	2	1	4

Figure 5N Cell cycle distribution of GFP, DAPI and nomaski images of asynchronously dividing cells. A, B and C. Pictures for three different fields showing cell cycle distribution. The number of cells in each stage was counted and GFP staining was scored on a scale of 1-5. White bar = 10 μm.

5.6.4 Cell cycle distribution of GFP-tagged SpPol5p

Asynchronously dividing cells, expressing the GFP-tagged SpPol5p (C-terminus) were analysed for cell cycle distribution of SpPol5p. Nomaski images and DAPI staining were used to correlate cell cycle stages with cell size. These were compared with GFP staining to look at cell cycle distribution of GFP-tagged SpPol5p. Figure 5N shows that some of the smaller cells in early G_2 phase show intense staining. The very long cells, probably in late G_2 or M-G_1/S phase, also show increased staining for GFP. In these cells GFP staining does not appear to be localised in one area of the cell but is distributed over the cell surface. Whereas the majority of cells in the mid G_2 phase show uniform localisation of GFP staining in the nuclear area. GFP staining in the cells was scored on scale of 1-5 and plotted on the graph (Figure 5N) to quantify the staining observed. Figure 5N indicates that the cells in early G_2 or in later G_2 and M phase show increased GFP staining.

5.7 Summary

In this chapter various experiments are described to investigate the biochemistry of SpPol5p. The effect of over-expressing *pol5*$^+$ on rRNA was tested by [^{3}H] uridine pulse-chase labelling. Next, a number of *cdc10*$^+$ mutant strains were examined for an effect on rRNA production, also by pulse labelling. Various *pol5*$^+$ over-expression constructs were then generated, using varying strengths of *nmt*, to examine rRNA synthesis when *pol5*$^+$ was repressed. Northern blot analysis was also performed for each of these constructs to analyse an effect on transcription of *cdc10*$^+$ and *cdc22*$^+$ when *pol5*$^+$ is repressed. Finally, localisation studies were performed using GFP-tagged versions of SpPol5p.

Over-expression of *pol5*$^+$ was analysed in Chapter 4 to reveal that it had no affect on cell growth or viability as well as the rate of proliferation. Budding yeast ScPolVp is believed to be involved in rRNA production (Shimizu *et al.* 2003) and so in this chapter experiments were performed to examine the role of fission yeast Pol5p in the synthesis of rRNA. Pulse-chase labelling using radioactive [^{3}H] uridine was performed on cells over-expressing *pol5*$^+$ through the strongest expression vector, pREP3x. The experiment revealed that over-expressing *pol5*$^+$ caused a delay in production of rRNA as compared to wild type cells (Figure 5A).

In Chapter 3, two-hybrid and GST pull-down experiments revealed that SpPol5p interacted with SpCdc10p. Therefore, four different *cdc10*$^+$ mutant strains were investigated to see if they affected rRNA synthesis. *cdc10-129*, *cdc10V50*, *cdc10-C4* and *cdc10-M17* are temperature sensitive mutants whose Cdc10p function was impaired when grown at restrictive temperature (36°C). Cells were pulsed with [^{3}H] uridine to label rRNA at 3 hourly intervals after transfer to 36°C. Figure 5B showed the synthesis of rRNA bands in the four mutants. All the *cdc10* mutants showed similar results in that, impairing SpCdc10p function had no gross effect on rRNA production.

For the "shut-off" experiment, firstly the original *nmt* construct from Chapter 4, pREP3x/*pol5*$^+$, was used to rescue a *pol5*$^+$ deleted strain. This was exploited to repress *pol5*$^+$ transcription by the addition of thiamine. This still rescued the lethality of Δ*pol5*, as the *nmt* promoter was not completely repressed. As the vector pREP3x/*pol5*$^+$ exists in multi-copy within cells, we used an integrating vector

(pJK148) to incorporate *pol5*$^{+}$, within the leucine locus of the genome, in single copy. This aimed to reduce the amount of *pol5*$^{+}$ being expressed under *nmt*3x control. Also, *pol5*$^{+}$ was cloned into the weakest of the *nmt* expression vectors, pREP81, and then sub-cloned into the integrating vector pJK148. In total four separate strains were made to investigate *pol5*$^{+}$ shut-off on rRNA production. The experiment revealed that the two strains carrying plasmid constructs were impaired for rRNA production. This correlated with the results for the over-expression of Pol5p where over-expressing *pol5*$^{+}$ caused a delay in rRNA production. However, the results did not correlate with the northern blot data (Figure 5G & 5H), which showed that the least amount of repression of *pol5*$^{+}$ transcription was seen by the plasmid pREP3x/*pol5*$^{+}$. This might be explained if repressing *pol5*$^{+}$ has no affect on rRNA synthesis but over-expressing it causes reduced amounts or a delay in rRNA production.

The effect on transcription of other genes has to be investigated further as the data from the northern and pulse-chase experiments are contradictory. This might be because fission yeast is being adversely affected by the plasmid and integrated constructs and therefore ideally the native *pol5*$^{+}$ promoter needs to be replaced by the *nmt* promoter.

Finally, we investigated the cellular localisation of SpPol5p in fission yeast in order to understand its role in rRNA synthesis and cell growth. DNA constructs with a GFP-tag at the C-terminus of SpPol5p were produced and transformed into a Δ*pol5* heterozygous diploid strain. GFP-tagged SpPol5p was able to complement the Δ*pol5* strain and rescue the haploid spores. Haploid transformants expressing GFP-tagged Pol5p were identified using nutritional selection.

Using fluorescence microscopy we investigated the localisation of GFP-tagged SpPol5p (C-terminus) in haploid transformants. DAPI (4'-6-Diamidino-2-phenylindole) forms fluorescent complexes with double stranded DNA and can be used to identify staining of the nucleus. Comparison of GFP and DAPI staining indicated that in majority of cells the staining was in the nucleus. The GFP staining of the nucleus could indicate that the SpPol5p is localising to the nucleolus. However this needs to be confirmed with immunofluorescence using antibodies against other proteins known to localise to the nucleolus. Recently it has been shown in *S. pombe* that the *nuc1*$^{+}$ gene product localises to the nucleolus (Hirano *et al.* 1989) and anti-SpNuc1p antibody could be used to confirm the localisation of GFP-tagged SpPol5p in the nucleolus.

The mitotic cell cycle of fission yeast comprises a relatively short G_1 and S phase and a lengthy G_2 phase before entry into mitosis (M-phase). There are major checkpoints during these stages to co-ordinate cell size and cell division and so examining the distribution of SpPol5p during the cell cycle stages is important in understanding its role in cell division and growth. During the cell cycle, fission yeast cells grow by apical extension therefore, the length of the cells can give an indication of the cell cycle stages. We analysed the distribution of GFP-tagged SpPol5p in asynchronously dividing cells of different sizes. The results show that SpPol5p accumulates more in cells of smaller size or as they are about to divide. However, many of the intensely stained smaller cells were granular and also the longer cells appeared very long and granular compared to wild type cells. This could indicate that the cell cycle regulation in these cells was altered and the increased SpPol5p accumulation, by the GFP-*pol5*$^+$ construct, in these cells may have caused an irregular distribution of SpPol5p.

The results for localisation of SpPol5p were obtained only using the C-terminus GFP-*pol5*$^+$ construct. These need to be verified by localisation of N-terminus GFP-SpPol5p constructs. Furthermore, GFP SpPol5p fusion protein may not act like its wild type counterpart and localisation of SpPol5p cannot be confirmed only by using the fusion proteins. Immunofluorescence with antibodies produced against bacterially produced SpPol5p will permit indirect immunofluorescence studies as an alternative way to confirm GFP localisation. Moreover the cells used to look at the localisation of GFP-tagged SpPol5p were fixed and therefore these results cannot be taken as results for *in vivo* localisation of SpPol5p. The same constructs need to be used with methods for looking at GFP localisation in living cells.

Chapter 6

General summary

6.1 Introduction

In Chapter 3, a two-hybrid screen revealed a novel protein in fission yeast called SpPol5p. It was discovered as a direct interacting partner of SpCdc10p through both, two-hybrid analysis and co-immuno-precipitation assays. SpCdc10p is the major component of the DSC1 cell cycle transcription factor complex in fission yeast, which is composed of at least two other proteins, SpRes1p and SpRes2p. The DSC1 complex operates at the G_1-S boundary and regulates transcription of genes that are required for passage of START and completion of S phase.

SpPol5p was named due to its high sequence homology with budding yeast ScPolVp. Investigations into ScPolVp established it to be potentially involved in the synthesis of rRNA (Shimizu *et al.* 2002). Further sequence analysis of both ScPolVp and SpPol5p showed some similarities with the MYB family of proteins, which are thought to be involved in the transcriptional regulation of rDNA (Tavner *et al.* 1998, Shimizu *et al.* 2002, Yang *et al.* 2003). Consequently, SpPol5p is also proposed to be involved in the synthesis of rRNA.

Cell cycle regulation and cell growth are separate though linked processes (Shirodkar *et al.* 1992, Bartek *et al.* 1996), which in fission yeast is more clearly defined at the G_2-M transition, where a size control mechanism, partly formed by SpCdc25p translation initiation, operates to ensure cells reach appropriate size before they pass the G_2 checkpoint and enter mitosis (Daga & Jimenez 1999). However, the mechanism coupling cell cycle and cell growth at the G_1-S transition is far less understood.

Interestingly, the mechanism coupling cell growth and cell cycle is better understood in mammalian cells where E2F, the mammalian functional homologue of MBF/SBF and DSC1, controls G_1-S cell cycle regulated transcription and is coupled to a growth regulator, pRB. pRB serves as a suppressor of E2F activity and consequently inhibits cell proliferation, but also mediates growth suppression via RNA polymerases I and III (White 1997). Therefore, the discovery that a potential rRNA activity, SpPol5p, interacts with the G_1-S cell-cycle specific transcription factor, SpCdc10p, suggests that SpPol5p might be the missing link between these two separate processes in fission yeast.

6.2 Aim of this study

The purpose of this study was to identify novel proteins that interacted with SpCdc10p, which lead to the discovery of SpPol5p. In addition, further experiments were designed to investigate the biological and biochemical properties of SpPol5p to provide further insight into the possible role it has to play.

Chapter 3 described a two-hybrid screen that isolated a novel protein, SpPol5p, which interacted with SpCdc10p. This was confirmed by performing GST pull-down assays using GST-tagged and HIS-tagged fusion proteins of SpCdc10p and SpPol5p, respectively, in *E. coli*. Chapter 4 described various experiments designed to elaborate the biology of SpPol5p, in the form of disruption and over-expression experiments and the analysis of mRNA levels of *pol5*$^{+}$. Finally, Chapter 5 further investigated the biochemistry of SpPol5p, by investigating its possible role in rRNA synthesis. Various "shut-off" constructs were made where *pol5*$^{+}$ was placed under the control of the thiamine-repressible *nmt* promoter and levels of rRNA were measured by pulse-chase radiolabelling with [^{3}H] uridine. GFP fusion proteins of SpPol5p were also generated to examine the localisation of SpPol5p to see whether it correlated with the recognized site of rRNA production in the nucleolus.

6.3 Summary of results

6.3.1 Chapter 3: Two-hybrid analysis and GST "pull-down"

In Chapter 3, experiments were described that identified SpPol5p as a direct interacting partner of SpCdc10p. Initially the two-hybrid screen showed a possible interaction between the two proteins that was subsequently confirmed by an *in vitro* GST pull-down assay.

At the outset, the C-terminal of *cdc10*$^{-}$ was cloned into a binding domain (BD) vector (pGBT9) in order to perform a large-scale two-hybrid screen, in budding yeast, using a fission yeast cDNA-AD (activation domain) library vector (pGADGH). This resulted in the identification of 11 putative interactors (Figure 3B) of which the strongest was a novel protein called SpPol5p that interacted with the C-terminal of SpCdc10p. This was confirmed by re-transforming the *pol5*$^{+}$ AD vector into the budding yeast strain carrying the C-terminal of *cdc10*$^{+}$, BD vector. This experiment revealed a potentially strong interaction between the two proteins (Figures 3Ca and 3Cb).

As the two-hybrid technique does not substantiate a direct interaction a different method was employed to confirm that SpPol5p and SpCdc10p interact directly with one another. N-terminal tagged GST and HIS fusion proteins of SpCdc10p and SpPol5p, respectively, were created to facilitate purification by affinity chromatography (Figure 3E). Purified fusion proteins were subsequently used to perform GST pulldown assays. Proteins were detected by SDS-PAGE followed by western blot analysis using anti-HIS and anti GST antibodies. HIS-tagged SpPol5p was detected in the same sample along with the GST-tagged SpCdc10p, confirming the two proteins directly interact (Figure 3F).

6.3.2 Chapter 4: Exploration into the biology of *pol5*$^{+}$

Chapter 4 describes a series of experiments designed to investigate the possible role of *pol5*$^{+}$. First, northern blot analysis was performed on asynchronous and synchronous cells to examine the mRNA levels of *pol5*$^{+}$during the cell cycle to

provide insight into the transcription of *pol5*$^+$. Next, a disruption experiment was carried out to observe what effects deleting the chromosomal copy of *pol5*$^+$ would have. Finally, a cloning strategy was adopted to achieve the over-expression of *pol5*$^+$ to survey the effect this would have on the cell.

Asychronous wild type and temperature-sensitive *cdc10-C4* cells were used to examine the mRNA levels of *cdc22*$^+$ and *pol5*$^+$. Northern blot analysis performed on both strains revealed much higher mRNA levels of *cdc22*$^+$ in *cdc10-C4* cells than in wild type (Figure 4A). This is because MCB-containing genes are constitutively over-expressed in the mutant *cdc10-C4* as they are no longer under the periodic control of SpCdc10p (McInerny *et al.* 1995). In comparison, *pol5*$^+$ mRNA levels remained the same in both wild type and *cdc10-C4* cells (Figure 4A). This showed that the transcription of *pol5*$^+$ is not under the control of SpCdc10p.

The mRNA profile of *pol5*$^+$ was also examined in synchronous mitotic and meiotic cell cultures using the *cdc25-22* and *pat1-114* temperature sensitive mutants, respectively (Figure 4B and 4C). *pol5*$^+$ mRNA levels were found to be constitutive and in low amounts throughout the cell cycle which correlated with the results of the previous experiment that showed that *pol5*$^+$ transcription was not under the control of SpCdc10p. This was further supported by the examination of the promoter region of *pol5*$^+$, showing that there were no MCB motifs present (Wood *et al.* 2002), which are always present in promoters of genes that are recognised by the DSC1 cell cycle transcription factor complex.

Examination of the effect of disrupting the wild type copy of a gene, allows insight into its possible roles in cell growth and division. The PCR-based gene targeting method (Figure 4D) (Bahler *et al.* 1998) was employed for the disruption of *pol5*$^+$. Tetrad dissection and spore analysis revealed that *pol5*$^+$ is an essential gene (Figure 4E and 4F). Unfortunately, the disruption of essential genes does not reveal a great deal of its function however, investigations into the budding yeast homologue of *pol5*$^+$ showed that it is also an essential gene (Shimizu *et al.* 2002). As mentioned budding yeast *polV*$^+$ is thought to be involved in the synthesis of rRNA (Shimizu *et al.* 2002) and therefore, not surprisingly, is essential for survival as the inability to produce the "machinery" (ribosomes) that makes proteins would indeed lead to cell death because essential proteins are no longer being synthesised. Therefore judging by the sequence homology between the polymerases of both yeast species, fission yeast *pol5*$^+$ is also thought to be involved in the same process.

Conversely, the over-expression of a gene can also allow in its function to be revealed. *pol5*$^{+}$ was cloned into the *nmt* pREP3x vector (Figure 4G) to allow it to be over-expressed in a wild type yeast background. Unfortunately, *pol5*$^{+}$ was amplified from genomic DNA, which resulted in a product containing a small intron and some mutations within the coding region (Figure 4H and 4I). Due to the extreme difficulty in cloning *pol5*$^{+}$ from genomic or cDNA, a mutated form of *pol5*$^{+}$ was used for the over-expression study. The experiment revealed that over-expression of *pol5*$^{+}$ had no effect on fission yeast (Figure 4J, 4K and 4L) though there was a chance that this was due to non-functional protein being translated. For that reason, it was used to rescue the lethality of a Δ*pol5* strain. The mutated form of *pol5*$^{+}$ was able to rescue a haploid Δ*pol5* strain confirming that active and functional SpPol5p was being synthesised (Figure 4M). Thus, over-expression of *pol5*$^{+}$ does not have an effect on fission yeast growth and division.

6.3.3 Chapter 5: Role of SpPol5p in the synthesis of rRNA

Chapter 5 aimed to investigate the possible role of SpPol5p in rRNA synthesis and whether SpCdc10p is also linked to the same process. This was explored by performing a succession of *in vivo* pulse-chase radiolabelling experiments. To begin with the construct made in Chapter 4 was used to measure the effect of over-expression of *pol5*$^{+}$ at a nuclear level, particularly in the synthesis of rRNA by pulse-chase labelling. Next, four different temperature-sensitive conditional lethal mutants of *cdc10* were tested for rRNA synthesis defects by shifting to restrictive temperature and pulse-chase labelling rRNA at regular intervals. Next, a number of "shut-off" strains were generated where *pol5*$^{+}$ was placed under varying strengths of the thiamine-repressible *nmt* promoter, in a Δ*pol5* background, to examine the effects of removing *pol5*$^{+}$ on rRNA production. In addition to the shut-off experiment was the examination of the effect on transcription of other genes by northern blot analysis, especially *cdc10*$^{+}$ and *cdc22*$^{+}$, when *pol5*$^{+}$ transcription was shut-off. Finally, *in vivo* localisation of SpPol5p was examined by generating GFP tagged versions of the protein to monitor its distribution within the cell.

The over-expression of *pol5*$^{+}$, as described in Chapter 4, had no effect on cell growth or division but pulse-chase labelling established that there was a slight delay in onset of rRNA production by massively over producing SpPol5p (Figure 5A). This

result may have been influenced by the presence of an intron and a number of mutations in the cloned *pol5*$^{+}$ gene, however this seems unlikely as the validity of the construct was confirmed in Chapter 4 by its ability to rescue a Δ*pol5* strain.

Reductions in rRNA production were predicted in mutants of *cdc10*, which were grown separately at permissive temperature (25°C) then shifted to the restrictive temperature (36°C) after which newly synthesised rRNA was measured by incorporation of [^{3}H] uridine. All four mutants displayed no dramatic impairment to rRNA synthesis (Figure 5B) suggesting that SpCdc10p is not directly or indirectly involved in rRNA production but associates with SpPol5p in processes outside of growth regulation. This supports the hypothesis that SpPol5p may be a link between cell cycle and cell growth as SpCdc10p is a major cell cycle transcription factor protein.

Northern blot analysis in Chapter 3 and 4 demonstrated that *pol5*$^{+}$ is a rare transcript and is also essential for survival, respectively. The budding yeast homologue *polV*^{1} is thought to be involved in rRNA production (Shimizu *et al.* 2002, Yang *et al.* 2003), therefore it was predicted that inhibiting *pol5*$^{+}$ transcription would lead to either cell death or disruption of rRNA synthesis in fission yeast. Four different strains were generated, all conferring varying degrees of expression and repression by *nmt*, to measure *pol5*$^{+}$ shut-off on the production of rRNA. The experiment revealed that two of the strains carrying strong and weak *nmt* plasmid constructs were impaired for rRNA production (Figure 5J). However this was not corroborated when northern blot analysis revealed that the integrated constructs were best at repressing *pol5*$^{+}$ (Figures 5G and 5H) transcription and should therefore have been most affected for rRNA synthesis. Nevertheless, the results did agree with data from the over-expression experiment that agreed that increased levels of *pol5*$^{+}$ impaired the production of rRNA. Unfortunately all the *nmt* shut-off strains were still "leaky" for transcription and did not completely shut-off *pol5*$^{+}$ expression (Figure 5H) and since *pol5*$^{+}$ is only ever present in low levels throughout the cell cycle this explains why rRNA synthesis was intact in all four strains strains.

The effect on transcription of other genes needs to be investigated further due to contradicting results from the pulse-chase and northern experiments. Fission yeast may have been unfavourably affected by the presence of the plasmid or integrated constructs therefore, ideally the native *pol5*$^{+}$ promoter should be replaced with the *nmt* promoter for more efficient shut-off of transcription.

Finally, *in vivo* localisation of SpPol5p was measured by following the distribution of GFP-tagged versions of the protein. Due to time constraints only C-terminally tagged protein was analysed therefore further work is required to investigate whether SpPol5p activity and distribution is affected by N or C-terminal tags. Comparatively, the localisation of GFP-SpPol5p was the same as DAPI staining of the nucleus therefore it was concluded that SpPol5p localises to at least the nucleus in fission yeast (Figures 5L and 5M). Investigation into ScPolVp localisation revealed it to be prevalent in the nucleolus, which is the organelle in which the majority of the steps in ribosome synthesis occur. This needs to be confirmed with immunofluorescence using antibodies against other proteins known to localise to the nucleolus (e.g. anti-SpNuc1p antibodies (Hirano *et al.* 1989)) as a direct comparison. Additionally, GFP-SpPol5p fusion protein may not act like its wild type counterpart therefore immunofluorescence with antibodies produced against bacterially produced SpPol5p will permit indirect immunofluorescence studies as an alternative way to confirm GFP localisation.

6.4 Future experimental work

The findings in this thesis aimed to provide evidence into the role of SpPol5p. The limited information available on SpPol5p, only through sequence similarity with ScPolVp, means there is much work to be done in order to gain a clear picture as to its complete role in fission yeast. Though in the space of time available for this project, many steps were taken to achieve this goal, a growing list of issues remain to be addressed and require further investigation.

Foremost is the study of the involvement of SpPol5p in rRNA production. Already performed was the effect of repressing *pol5*$^{+}$ expression on rRNA synthesis, however, due to a combination of time constraints and experimental difficulties an improved technique could not be carried out. This was to replace the native *pol5*$^{+}$ promoter with the *nmt* promoter by the heterologous PCR-based gene targeting method (Bahler *et al.* 1998) in order to achieve complete shut-off of *pol5*$^{+}$ transcription. This *in vivo* approach can also be complemented by an *in vitro* assessment to allow measurement of the ability of bacterially expressed and purified SpPol5p to directly stimulate 35S rDNA transcription through RNA polymerase I.

This *in vitro* transcription assay (Chen *et al.* 1997) anticipates that SpPol5p has a direct role in rDNA transcription and so predicts it will stimulate the production of 35S rRNAs. This experiment is prompted on the basis of published observations that budding yeast ScPolVp binds to the 35S rDNA promoter region (Shimizu *et al.* 2002).

Of equal importance is to address the question: does SpPol5p bind to promoters of 35S rDNA to stimulate rRNA production? This is one way that SpPol5p might control rRNA synthesis and indeed, chromosome immunoprecipitation (ChIP) experiments in budding yeast suggest this may be the case (Shimizu *et al.* 2002). Complementary *in vivo* and *in vitro* binding assays can be implemented to test if SpPol5p binds to rDNA promoter regions. Sufficient amounts of bacterially expressed and purified SpPol5p can provide the reagents to complete electrophoretic mobility shift assays (EMSA) for binding studies. Interestingly, EMSA has recently been used to study other 35S rDNA promoter binding proteins in fission yeast (Chapter 1, Section 1.5.2.1) (Boukhgalter *et al.* 2002, Liu *et al.* 2002). For this binding assay, various upstream fragments of rDNA promoter can be amplified and tested for their ability to bind bacterially expressed SpPol5p. Because small fragments of DNA sequence are used, this gives the advantage of characterising specific DNA sequences as binding regions. Mutational studies can map these sequences and thus, importantly, the *in vitro* assay should be tested primarily as this will greatly assist in completing the potentially difficult *in vivo* ChIP assays. *In vivo* ChIP analysis will extend the EMSA studies (if successful binding is detected). In brief, this can be performed by tagging native SpPol5p with either HA or myc to allow immuno-precipitation of protein cross-linked to DNA. The presence of rDNA promoter regions will be detected by PCR using oligonucleotides specific to these DNA regions.

It is possible that SpPol5p may not directly associate with DNA and instead interact with other factors, whether it is upstream of a signalling pathway or direct interaction with other DNA binding proteins. In fission yeast, a 35S rDNA promoter binding activity has been identified that might regulate rRNA production and a number of its components have been identified (Chapter 1, Section 1.5.2.1) (Boukhgalter *et al.* 2002, Liu *et al.* 2002). The ability of SpPol5p to interact with any of the polypeptide components can be tested in several ways. Firstly, the yeast two-hybrid constructs used in Chapter 3 can be exploited to examine if *in vivo* interactions can be detected. This stands to reason as originally this method was employed to identify Spp27p by its two-hybrid interaction with SpRm10hp (Liu *et al.* 2002). Also,

equivalent to the technique described in Chapter 3, co-immuno-precipitation can support results obtained from the two-hybrid analysis by testing the ability of tagged version of SpPol5p and the rDNA binding proteins to immuno-precipitate with each other *in vitro*. Proteomics can also be utilised to identify interacting proteins of SpPol5p. The tagged versions of SpPol5p can again be exploited to isolate proteins that specifically bind to it. Matrix-assisted laser desorption ionisation-time of flight (MALDI-TOF) mass spectroscopy can allow the identification of these proteins (facilitated by the completion of the fission yeast sequencing project). This technique may identify the 37S rDNA promoter binding proteins but also, interestingly, it may also isolate novel proteins which may include components of the RNA polymerase I or III machinery or their associated factors.

Studies in budding yeast have determined the exclusive localisation of ScPolVp to the nucleolus, the organelle where most ribosome synthesis is carried out. As described in Chapter 5, SpPol5p:GFP constructs were used to determine SpPol5p localisation to the nucleus. Further work is required to confirm SpPol5p localisation to the nucleolus by immunofluorescence using antibodies against other proteins known to localise to the nucleolus. Also, the chromosomal GFP tagging method (Bahler *et al.* 1998) can be utilised to moniter the cellular distribution of SpPol5p in living cells, as it will be expressed from its native promoter and will therefore be expressed at normal levels. Indirect immunofluorescence can be achieved by raising polyclonal antibodies against bacterially produced SpPol5p. This can provide another independent method to confirm SpPol5p localisation and also support the GFP experiments.

The interaction between SpCdc10p and SpPol5p can be examined by *in vivo* co-localisation studies. This can be accomplished by using the recently developed GFP variant, bimolecular multicolour fluorescence complementation analysis (Multicolour BiFC) (Hu & Kerppola 2003). In this method the two potentially interacting proteins are tagged with different fragments of GFP that only fluoresce when brought together. Thus, *in vivo* interactions between SpCdc10p and SpPol5p, in specific locations within living cells, can be analysed. In addition the SpPol5p:GFP construct can be placed into the four *cdc10* mutant backgrounds to examine the effect of defective *cdc10* function on SpPol5p distribution.

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Appendices

Appendix I: Bacterial strains

Gene	Plasmid	Purpose	Origin	Glasgow collection number
-	pET28-a	Bacterial expression vector (N-terminal His-tag)	Novagen	GB 12
-	pGEX-KG	Bacterial expression vector (N-terminal GST-tag)	Lab stock	GB 159
-	pREP3x	Fission yeast expression vector (strongest *nmt* control)	Lab stock	GB 28
-	pREP81	Fission yeast expression vector (weakest *nmt* control)	Lab stock	GB 346
-	pCR 2.1	TA cloning vector	Invitrogen	-
-	pJK148	Fission yeast integrating vector with *leu*$^+$	Lab stock	GB 186
-	pGBT9	2-Hybrid binding-domain vector (bait vector)	Clontech	GB 81
-	pBlueScript (pBCKS)	High copy number cloning vector	Stratagene	GB 6
cdc10$^-$	pGBT9	2-hybrid bait vector containing C-terminus of *cdc10*$^+$	This study	GB 82
cdc10$^-$	pREP1	wild type *cdc10*$^+$ used as PCR template	Lab stock	GB 72
cdc10$^+$	pGEX-KG	Bacterial expression of GST *cdc10*$^+$ (N-terminus)	This study	GB 273
pol5$^+$	pREP81	Fission yeast over-expression of *pol5*$^-$ (weakest *nmt*)	This study	GB 365
pol5$^+$	pCR 2.1	Intermediate cloning step	This study	GB 379
pol5$^+$	pCR 2.1	Intermediate cloning step	This study	GB 382
pol5$^+$	pREP81EGFP-C	Fission yeast expression of GFP-tagged Pol5p (C-terminus)	This study	GB 383
pol5$^+$	pREP81EGFP-N	Fission yeast expression of GFP-tagged Pol5p (N-terminus)	This study	GB 393
pol5$^+$	pREP41EGFP-C	Fission yeast expression of GFP-tagged Pol5p (C-terminus)	This study	GB 394
pol5$^+$	pREP3x	Fission yeast over-expression of *pol5*$^-$ (strongest *nmt*)	This study	GB 340
pol5$^+$	pCR 2.1	Intermediate cloning step	This study	GB 339
pol5$^+$	pJK148	Fission yeast integration of *pol5*$^+$ under *nmt*3x control	This study	GB 353
pol5$^+$	pJK148	Fission yeast integration of *pol5*$^+$ under *nmt*81 control	This study	GB 368
pol5$^+$	pET28-a	Bacterial expression of His *pol5*$^+$ (N-terminus)	This study	GB 265
ura4$^-$	KS-ura4$^-$	Template for PCR-based gene targetting method (Bahler *et al.* 1998)	Lab stock	GB 148

Appendix II: Oligonucleotides

Gene	Purpose	Sequence	Glasgow collection number
cdc10^{+} forward	PCR of C-terminal of *cdc10*$^{+}$ to clone into the 2-Hybrid Binding domain vector pGBT9	GCG CGC GAA TTC CGA AAT TCC AAC ATA AAA TCA AA	GB 140
cdc10^{+} reverse	PCR of C-terminal of *cdc10*$^{+}$ to clone into the 2-Hybrid Binding domain vector pGBT9	GCG CGC GGA TCC GCA ATA TTA TGC TTG ATG TTC TT	GB 141
-	2-Hybrid Activation Domain vector sequencing primer	TAC CAC TAC AAT GGA TG	GB 158
-	Sequencing pCR2.1 plasmids	M13 forward and reverse	MWG-Biotech
-	Sequencing pET-28a plasmids	T7 forward and reverse	MWG-Biotech
cdc10^{+} forward	PCR of C-terminal end of *cdc10*$^{+}$ to facilitate cloning into pGEX-KG	GCG CGC GGA TTC CGA AAT TCC AAC ATA AAA TCA	GB 234
cdc10^{+} reverse	PCR of C-terminal end of *cdc10*$^{+}$ to facilitate cloning into pGEX-KG	GCG CGC GAA TTC GCA ATA TTA TGC TTG ATG TTC TT	GB 479
pol5^{+} forward	PCR of C-terminal fragment of *pol5*$^{+}$ to facilitate cloning into pET28-a	GCG CGC GGA TCC GCC GAT GCA AAG GTC AAA GAC CA	GB 236
pol5^{+} reverse	PCR of C-terminal fragment of *pol5*$^{+}$ to facilitate cloning into pET28-a	GCG CGC GAA TTC ATG CAT TCA ATG ATT TGT CTT TT	GB 237
ura4^{+} forward	PCR of *ura4*$^{-}$ with 5' *pol5*$^{+}$ flanking sequence	CGA AGT TTG ACT TAG ACT CCC CTA CAA CCT CGA TTG TGA GTA GTA GTA CCA AAC ATG GCT ACA AAA ACA CAA TTA CGC CAG GGT TTT CCC AGT CAC GAC	GB 403
ura4^{-} reverse	PCR of *ura4*$^{-}$ with 3' *pol5*$^{+}$ flanking sequence	CAT TCA ATG ATT TGT CTT TTC ATT TTC ATG ATG CCC AGT CTT TTG TGG AGA AGA AGC AGC CGT CGA AGT TTG TTG AGC GGA TAA CAA TTT CAC ACA GGA	GB 404
ura4^{-}	*ura4*$^{+}$ forward sequence at 3' of *ura4*$^{-}$ to check *pol5*$^{+}$ disruption	TCC TGT GTG AAA TTG TTA TCC GCT	GB 474
pol5^{+}	*pol5*$^{+}$ reverse flanking region ~500bp outside of ORF to check disruption	CCT TGG GCT TGG TAG AAT ACA CAC	GB 475
pol5^{+} forward	PCR of *pol5*$^{+}$ to facilitate cloning into pREP3x	CCG GAC GTC GAC ATG GCT ACA AAA ACA CAA TTA GAG TTG TTC	GB 505
pol5^{+} reverse	PCR of *pol5*$^{+}$ to facilitate cloning into pREP3x	CCG GAC GGA TCC TCA ATG ATT TGT CTT TTC ATT TTC ATG ATG	GB 506
pol5^{+}	Sequencing *pol5*$^{+}$ 5' to 3'	GGATTACAGT CAATTGTATA TTCTGGCA	GB 581
pol5^{+}	Sequencing *pol5*$^{+}$ 5' to 3'	ACAGCGCTTG GAAACAAAAA ATTCCAAT	GB 582
pol5^{+}	Sequencing *pol5*$^{+}$ 5' to 3'	ATACCAAGTT AGTTGAGCAT ATCCTTCCCT	GB 583
pol5^{+}	Sequencing *pol5*$^{+}$ 5' to 3'	GTCGCACTGA ATTATTTATT AGAGCCCA	GB 584
pol5^{+}	Sequencing *pol5*$^{+}$ 5' to 3'	TCTGAAGACA TGAACCGTGA ATCT	GB 585
pol5^{+}	Sequencing *pol5*$^{+}$ 5' to 3'	ATCGAATAAG GAGAAGAAAA AGAACGCCCA	GB 586
pol5^{+}	Sequencing *pol5*$^{+}$ 5' to 3'	GATTTTAAA GACGAAGCAC AAAGTTTTGG AGGA	GB 587

Appendix III: Fission yeast strains

Genotype	Origin	Glasgow collection number
h+ *ade6-210 leu1-32 ura4-D18*	Lab stock	GG 376
h- *ade6-210 leu1-32 ura4-D18*	Lab stock	GG 379
h+/h- *ade6-210/ade6-216 leu1-32/leu1-32 ura4-D18/ura4-D18*	This study	GG 907
h- *leu1-32*	Lab stock	GG 214
972 h-	Lab stock	GG 217
pREP3x:*pol5*$^{+}$ *h- leu1-32*	This study	GG 778
pREP3x:*pol5*$^{+}$ *h- leu1-32*	This study	GG 779
pREP3x h- *leu1-32*	This study	GG 786
h+/h- *pol5:ura4*$^{+}$/*pol5*$^{-}$ *ade6-210/ade6-216 leu1-32/leu1-32 ura4-D18/ura4-D18*	This study	GG 840
h+ *cdc10-129*	Lab stock	GG 29
h+ *cdc10-C4*	Lab stock	GG 96
h- *cdc10V50*	Lab stock	GG 167
h+ *cdc10-M17*	Lab stock	GG 202
pREP3x:*pol5*$^{+}$ *h- pol5:ura4*$^{-}$ *leu1-32 ura4-D18 ade6-210*	This study	GG 831
pJK148: *nmt3x:pol5*$^{+}$ *h- leu1-32 ura4-D18 ade6-210*	This study	GG 847
pREP81:*pol5*$^{+}$ *h- pol5:ura4*$^{+}$ *leu1-32 ura4-D18 ade6-210*	This study	GG 862
pJK148: *nmt81:pol5*$^{+}$ *h- leu1-32 ura4-D18 ade6-210*	This study	GG 856
pREP41EGFP-C *pol5*$^{-}$ *h- pol5:ura4*$^{-}$ *leu1-32 ura4-D18 ade6-210*	This study	GG 903

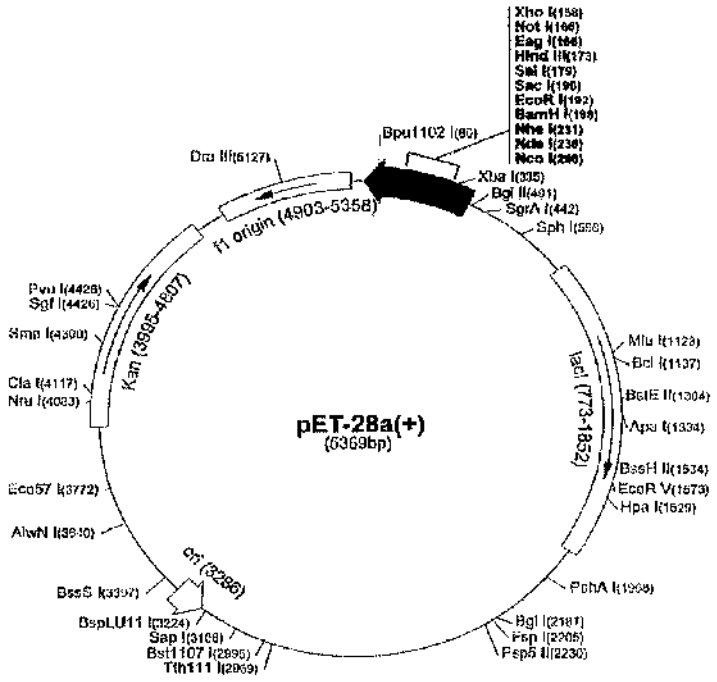
Appendix IV: Budding yeast strains

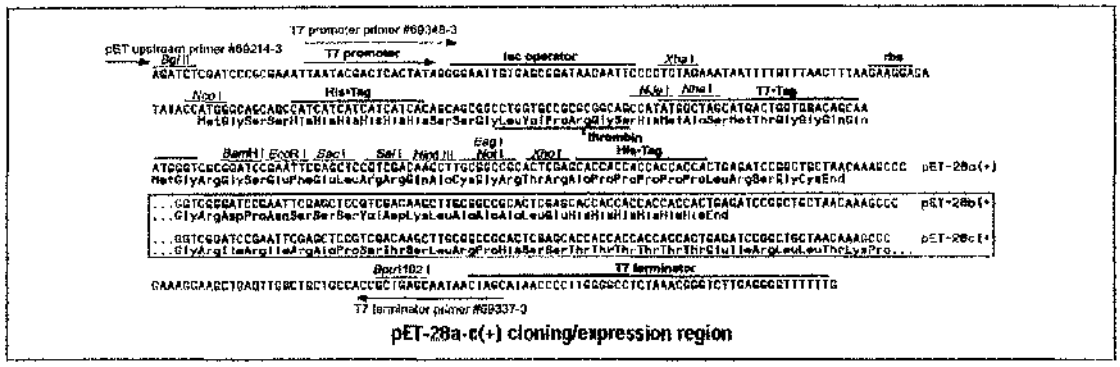
Genotype	Origin	Glasgow collection number
MATa, *gal4, gal80, his3-D200, leu2-3, leu2-112, trp1-D901,LYS2::GAL1-HIS3, ade2::GAL2-ADE2, met2::GAL7-lac, Pj69-4A*	Lab stock	GBY 122
2-hybrid positive control containing GBY 122 + pFB238 (GM 130) + pFB240 (GRASP 56)	Lab stock	GBY 109
2-hybrid positive control containing GBY 122 + SNF1 + SNF4	Lab stock	GBY 110
GBY 127 + pAD11 (*S.pombe* DNA *pol5*$^+$ homologue C-terminus)	This study	GBY 121
GBY 127 + pAD4	This study	GBY 114
GBY 127 + pAD8	This study	GBY 118
GBY 127 + pAD2	This study	GBY 112
GBY 127 + pAD3	This study	GBY 113
GBY 127 + pAD6	This study	GBY 116
GBY 127 + pAD7	This study	GBY 117
GBY 127 + pAD9	This study	GBY 119
GBY 127 + pAD10	This study	GBY 120
GBY 127 + pAD1	This study	GBY 111
GBY 127 + pAD5	This study	GBY 115
GBY 122 + GB 82, 2-hybrid screen. Yeast containing cdc10-C4 terminus bait vector	This study	GBY 127
GBY 128 + GB 123, 2-Hybrid isolate. Yeast containing both pAS-SNF1+ bait vector and *pol5*$^+$ prey vector (pAD11)	This study	GBY 132
GBY 129 + GB 123, 2-Hybrid isolate. Yeast containing both pAS-cdk bait vector and *pol5*$^+$ prey vector (pAD11)	This study	GBY 133
GBY 130 + GB 123, 2-Hybrid isolate. Yeast containing both pAS-p53 bait vector and *pol5*$^+$ prey vector (pAD11)	This study	GBY 134
GBY 131 + GB 123, , 2-Hybrid isolate. Yeast containing both pAS-lamin bait vector and *pol5*$^+$ prey vector (pAD11)	This study	GBY 135
GBY 127 + GB 82 + GB 123, 2-Hybrid isolate. Yeast containing both *cdc10-C4* terminus bait vector and *pol5*$^+$ prey vector (pAD11)	This study	GBY 136
GBY 122 + GB 172, 2-Hybrid screen. Yeast containing pAS-SNF1$^+$ bait vector	Lab stock	GBY 128
GBY 122 + GB 175, 2-Hybrid screen. Yeast containing pAS-Cdk2 bait vector	Lab stock	GBY 129
GBY 122 + GB 173, 2-Hybrid screen. Yeast containing pAS-p53 bait vector	Lab stock	GBY 130
GBY 123 + GB 174, 2-Hybrid screen. Yeast containing pAS-lamin bait vector	Lab stock	GBY 131

Appendix V: Plasmid maps

pET-28a(+) sequence landmarks	
T7 promoter	370-386
T7 transcription start	369
His•Tag coding sequence	270-287
T7•Tag coding sequence	207-239
Multiple cloning sites (*BamH* I - *Xho* I)	158-203
His•Tag coding sequence	140-157
T7 terminator	26-72
lacI coding sequence	773-1852
pBR322 origin	3286
Kan coding sequence	3905-4807
f1 origin	4903-5358

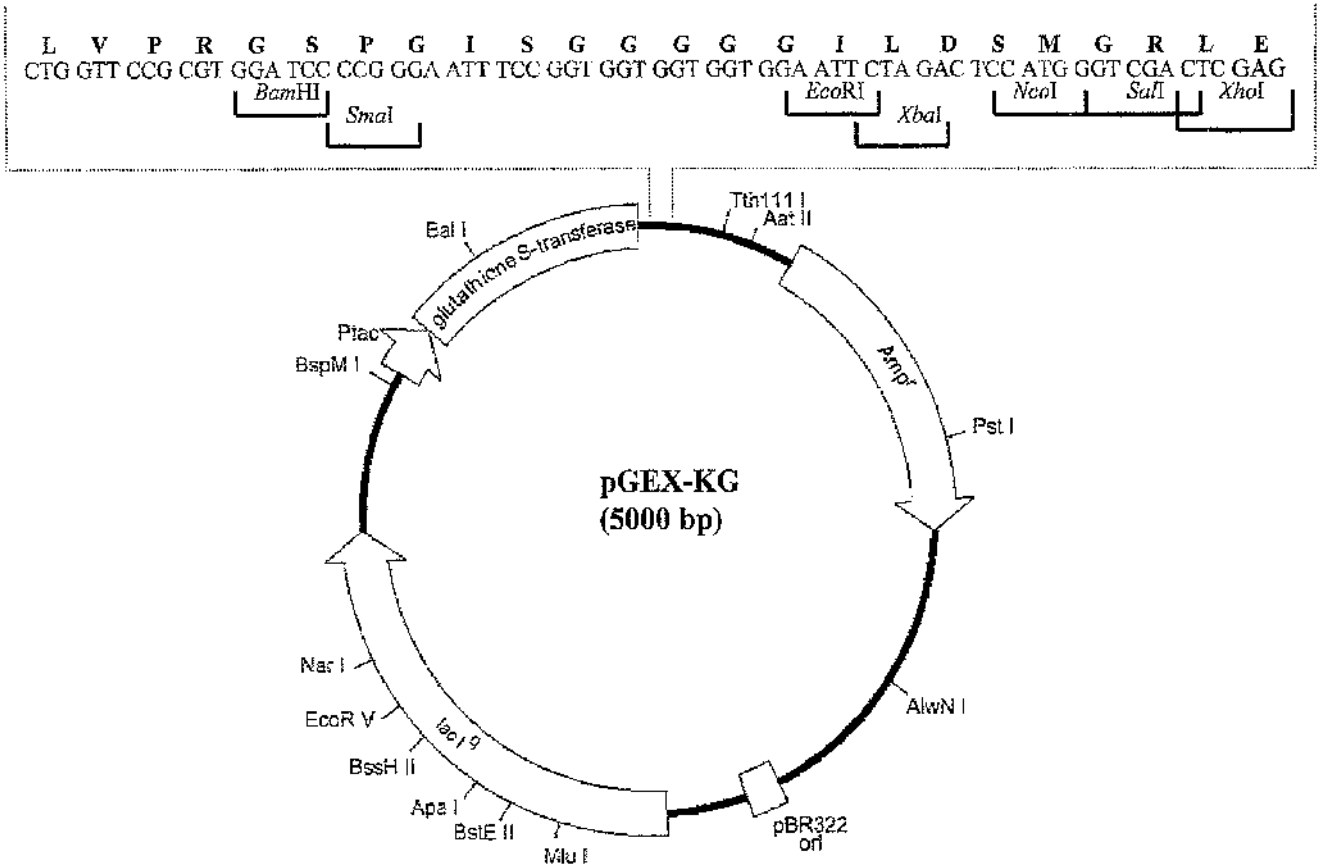
The maps for pET-28b(+) and pET-28c(+) are the same as pET-28a(+) (shown) with the following exceptions: pET-28b(+) is a 5368bp plasmid; subtract 1bp from each site beyond *BamH* I at 198. pET-28c(+) is a 5367bp plasmid; subtract 2bp from each site beyond *BamH* I at 198.





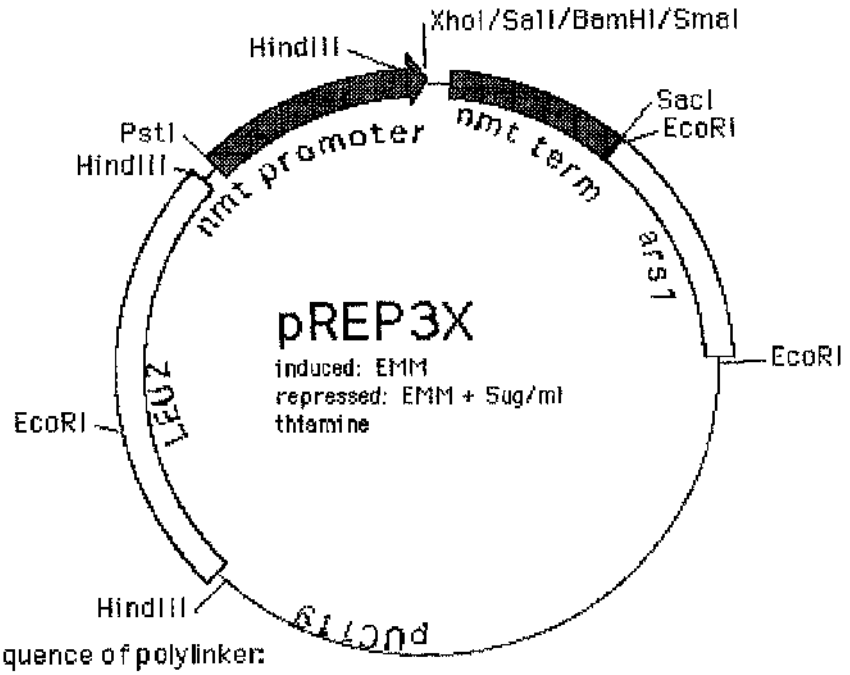
Appendix Va: Plasmid map of the pET-28a-c(+) bacterial expression vector (GB 12).

The pET-28a-c (+) vectors carry an N-terminal His-Tag®/thrombin/T7-Tag® configuration plus an optional C-terminal His-Tag sequence. Unique restriction sites are shown on the circle map. The cloning/expression region of the coding strand transcribed by T7 RNA polymerase is shown in more detail in the box (adapted from www.novagen.com).



Appendix Vb: Plasmid map of the pGEX-KG bacterial expression vector (GB 159).

The pGEX-KG vector carries an N-terminal GST-tag sequence followed by a thrombin cleavage site and unique restriction sites are shown on the circle map. The single-letter symbols of amino acids in-frame with the GST-tag are indicated above the nucleotide sequence together with unique restriction sites within the multiple cloning site. The locations of genes for ampicillin resistance (Amp^r) and Lac repressor ($LacI^q$) are indicated (adapted from Guan & Dixon 1991).



predicted sequence of polylinker:
TGGCCTCGAGGTCGACTCTAGAGGATCCCCGGG

NOTE: Xba is not unique; there are two XbaI sites in the ars.
REPs 41X/42X/81X/82X have no Sal site in the polylinker;
predicted sequence is CATAC CTCGAGGGATCCCCGGG

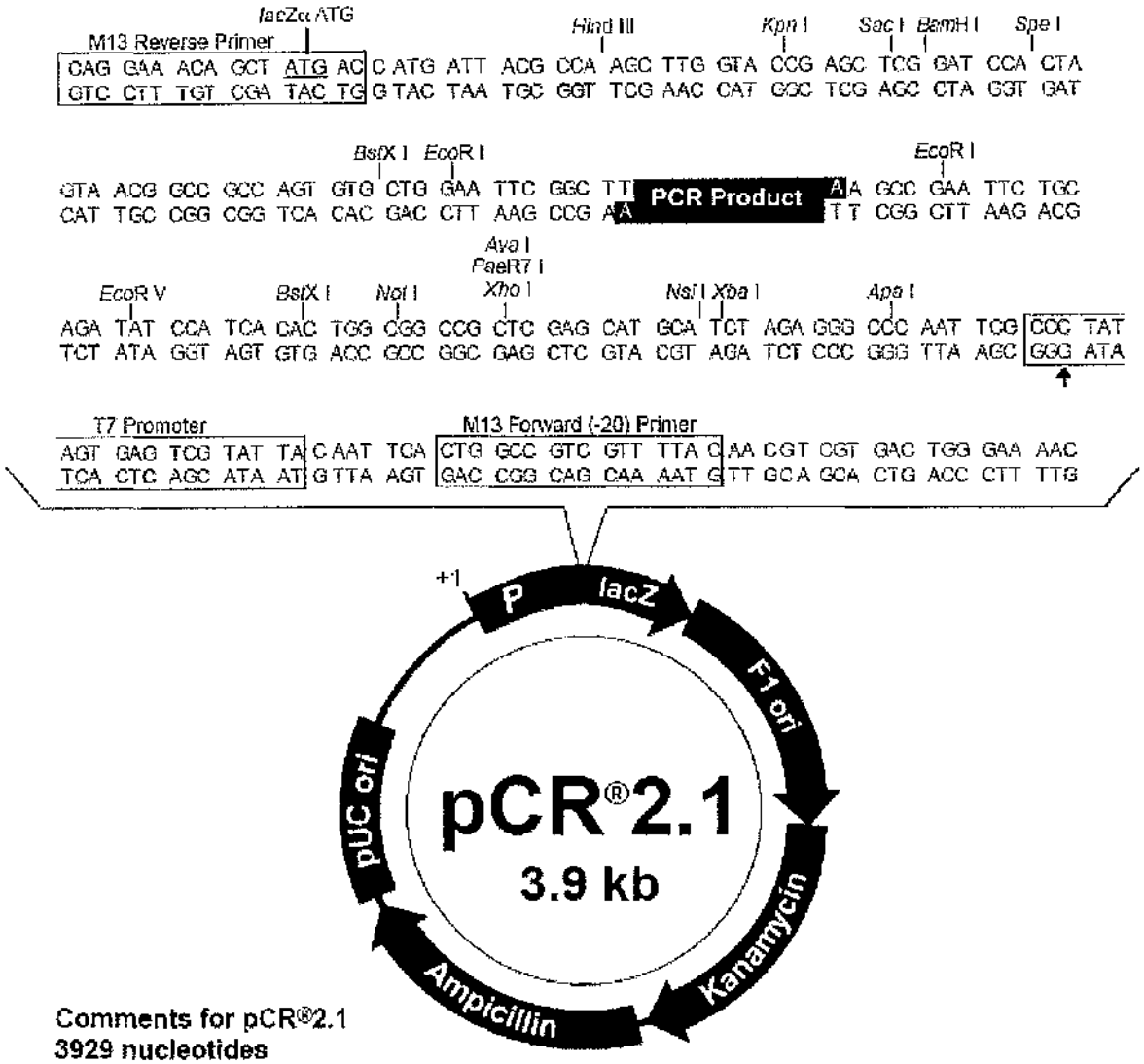
REP3X is derived from original REP3 series by addition of a Xho linker between BalI and SalI; this deletes the ATG within the polylinker, destroys BalI, and recreates SalI. Weaker REPXs (41,81,etc) were constructed by putting the Xho linker between BamHI and NdeI; the NdeI, SalI anmd XbaI sites are lost but BamHI is recreated.

Derivatives of the REP family:
RIP3, RIP3X (no ars, LEU2); RIP4, RIP4X (no ars, ura4+)
REP41 and REP41X (weaker promoter, LEU2)
REP81 and REP81X (weakest promoter, LEU2)
REP42 and 42X (weaker, ura4+); REP82 and 82X (weakest, ura4+)
Polylinker for 41X/81X is Xho/Bam/Sma
Polylinker for 41/81 is Nde/Sal/Bam/Sma

Reporter constructs: REP3X-lacZ, REP41X-lacZ, REP81X-lacZ

References:
REP3, parent vectors Maundrell (1993) Gene 123:127
REP41/42/81/82: basi et al (1993) Gene 123:131
REPX constructs: Forsburg (1993) NAR 21:2955

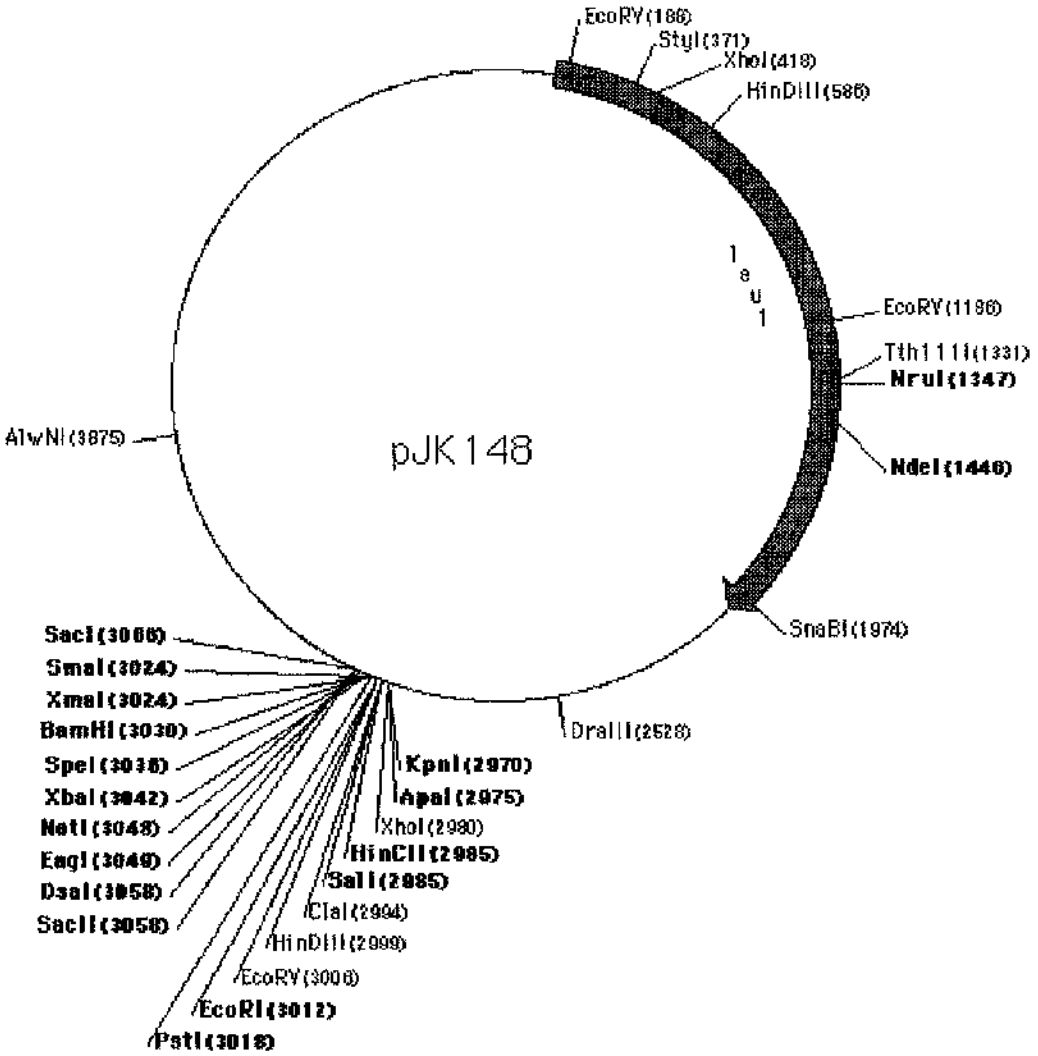
Appendix Vc: Plasmid map of the pREP3x/81x fission yeast over-expression vectors. (GB 28/346). The pREP3x and pREP81x vectors are derived from the original pREP3 series by addition of a *Xho*I polylinker between the *Bal*I and *Sal*I sites; this deletes the ATG within the polylinker, destroys *Bal*I and recreates *Sal*I (adapted from www-rcf.usc.edu/~forsburg).



Comments for pCR®2.1
3929 nucleotides

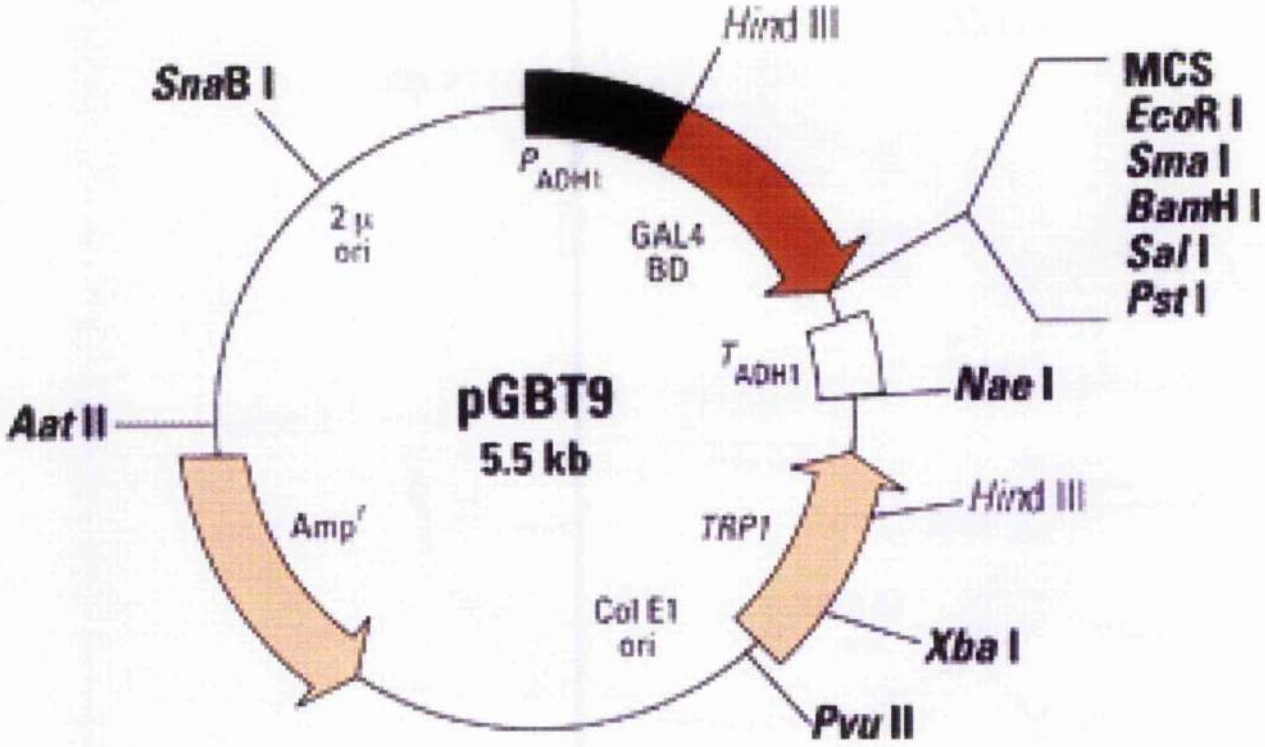
LacZα gene: bases 1-545
M13 Reverse priming site: bases 205-221
T7 promoter: bases 362-381
M13 (-20) Forward priming site: bases 389-404
f1 origin: bases 546-983
Kanamycin resistance ORF: bases 1317-2111
Ampicillin resistance ORF: bases 2129-2989
pUC origin: bases 3134-3807

Appendix Vd: Plasmid map of the cloning vector pCR 2.1. The vector allows the cloning of PCR products. The TA Cloning® technology (pCR 2.1) is designed to clone PCR products produced using *Taq* polymerase (and certain polymerase mixtures). It takes advantage of the terminal transferase activity of these polymerases, which adds a single 3′-A overhang to each end of the PCR product. Unique restriction sites are shown on the map. The plasmid also confers dual antibiotic resistance to ampicillin and kanamycin (adapted from www.invitrogen.com).

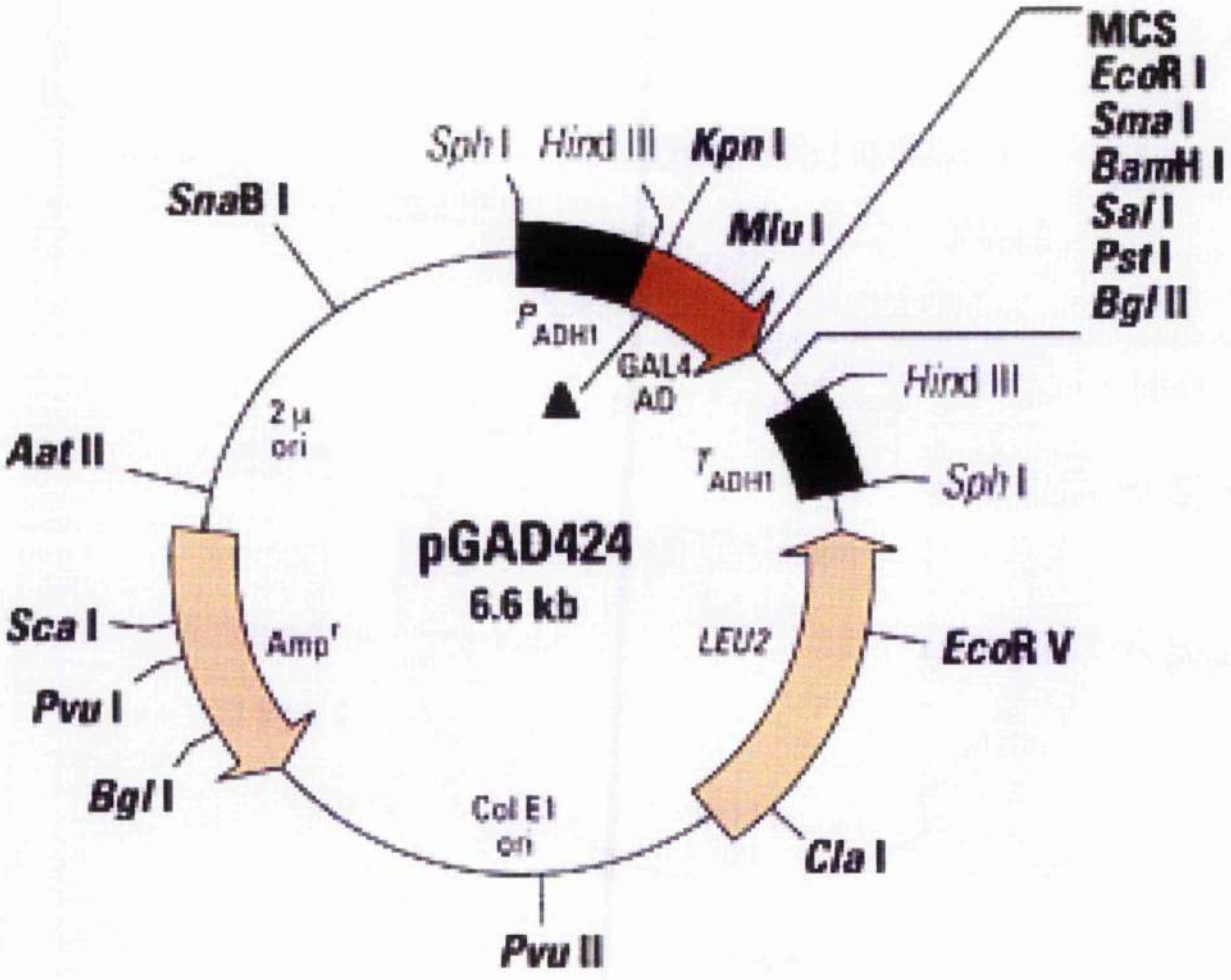


Appendix Ve: Plasmid map of the fission yeast intergration vector pJK148 (GB 186).

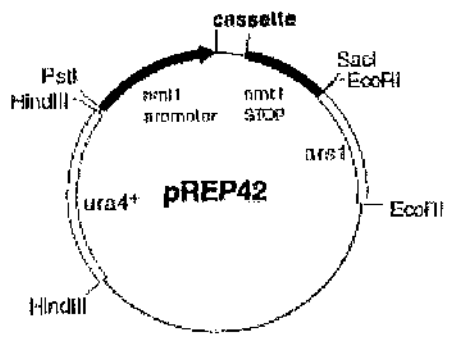
The vector pJK148 carries unique *Nru*I and *Nde*I sites within the *leu1* gene. Upon linearisation this allows integration of the vector into the fission yeast genome at the *leu1*$^{+}$ locus. Unique restriction sites are shown on the circle map. (adapted from www-ref.usc.edu/~forsburg).

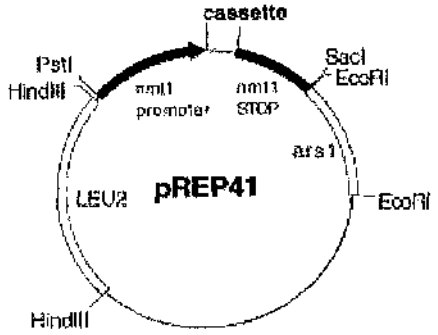


Appendix Vf: Plasmid map of the two-hybrid binding domain vector (GB 81). pGBT9 generates a hybrid protein that contains the sequence of the GAL4 DNA-Binding domain (DNA-BD). For the construction of a hybrid protein, the gene encoding the protein of interest is ligated into the MCS (Multiple cloning site) in the correct orientation and in the correct reading frame such that a fusion protein is generated. The fusion protein is expressed in yeast host cells from the constitutive *ADH1* promoter; transcription is terminated at the *ADH1* transcription signal. The hybrid protein is targeted to the yeast nucleus by nuclear localization sequences that are an intrinsic part of the GAL4 DNA-BD. pGBT9 is a shuttle vector that replicates autonomously in both *E. coli* and *S. cerevisiae*. It carries ampicillin resistance in *E. coli* and the *TRP1* nutritional marker that allow yeast auxotrophs carrying pGBT9 to grow on limiting synthetic medium lacking *trptophan* (adapted from BD Biosciences).

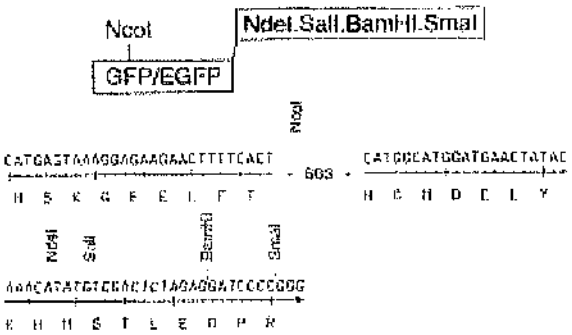


Appendix Vg: Plasmid map of the two-hybrid activation domain vector. pGAD424 generates a hybrid protein that contains the sequences for the GAL4 activation domain. pGAD424 has unique restriction sites located in the MCS region at the 3' end of the open reading frame for the activation domain sequence. For the construction of a hybrid protein, the gene encoding the protein of interest (or a collection of cDNAs in a library vector) is ligated into the MCS in the correct orientation and with the correct reading frame such that a fusion protein is generated. The fusion protein is expressed at high levels in yeast host cells from the constitutive *ADH1* promoter; transcription is terminated at the *ADH1* transcription termination signal. pGAD424 is a shuttle vector that replicates autonomously in both *E. coli* and *S. cerevisiae*. It carries ampicillin resistance in *E. coli* and the *LEU2* nutritional marker that allows yeast auxotrophs carrying pGAD424 to grow on limiting synthetic medium lacking *leucine* (adapted from BD Biosciences).

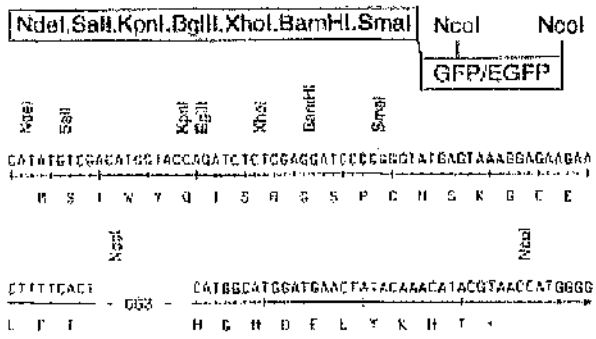




A pREP41/42GFP/EGFP N



B pREP41/42GFP/EGFP C



Appendix Vh: Plasmid maps of the fission yeast GFP-tagging vectors (GB 383 and GB 393). These vectors are for the expression of GFP/EGFP-tagged proteins in fission yeast. The sequences of the cassettes assembled in pREP42/41 to facilitate expression of proteins tagged at their amino (A) and carboxyl (B) terminus with GFP/EGFP are shown (adapted from Craven *et al.* 1998).