NEW ELEMENTS OF THE MITOTIC CONTROL IN

Schizosaccharomyces pombe.

by

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DECLARATION

I declare that this thesis was composed by myself and that the research presented is my own. Due acknowledgement is made within the text for the assistance of others.

Emma Warbrick
September 1990

CONTENTS:

ABBREVIATIONS (i)
ABSTRACT (ii)

CHAPTER 1: INTRODUCTION
1.1: THE EUKARYOTIC CELL CYCLE 1
1.2: GENETIC ANALYSIS OF THE CELL CYCLE IN YEAST 5
1.3: THE CELL CYCLE IN *S.POMBE* 8
1.4: MAJOR GENETIC ELEMENTS OF CELL CYCLE CONTROL IN *S.POMBE* 10
1.5: A UNIVERSAL MITOTIC CONTROL? 14
1.6: GENETIC ELEMENTS OF THE G_2 CONTROL IN *S.POMBE* 15
1.7: AIM AND SCOPE OF THIS PROJECT 22

CHAPTER 2: MATERIALS AND METHODS
2.1: REAGENTS AND COMMONLY USED BUFFERS 24
2.2: NUCLEIC ACID MANIPULATION 25
2.3: METHODS FOR THE MANIPULATION AND HANDLING OF *SCHIZOSACCHAROMYCES POMBE* 36
2.4: METHODS FOR *ESCHERICHIA COLI* 48
2.5: Tn5 TRANSPOSON MUTAGENESIS 53
2.6: DNA SEQUENCING OF *wis1* 26

CHAPTER 3: GENETICS AND PHYSIOLOGY OF win1
3.1: INTRODUCTION 59
3.2: THE NUTRITIONALLY SENSITIVE PHENOTYPE OF THE *wee1.50 cdc25.22 win1.1* TRIPLE MUTANT STRAIN 59
3.3.: INTERACTIONS BETWEEN *win1.1* AND OTHER CELL CYCLE MUTATIONS 67
3.4: GENETIC MAPPING OF THE *win1* LOCUS 79
3.5: DISCUSSION 90

CHAPTER 4: ISOLATION OF PLASMID CAPABLE OF SUPPRESSING A win1.1 CONFERRED PHENOTYPE

4.1: ISOLATION OF PLASMIDS 93

4.2: ANALYSIS OF PLASMIDS ISOLATED FROM GENE LIBRARY SCREENING 96

4.3: DO ANY OF THE ISOLATED PLASMIDS CONTAIN THE *win1* GENE? 106

4.4: DISCUSSION 113

CHAPTER 5: ANALYSIS OF pwis PLASMIDS

5.1: MOLECULAR ANALYSIS 114

5.2: GENETIC ANALYSIS 128

5.3: DISCUSSION 134

CHAPTER 6: GENETIC AND MOLECULAR ANALYSIS OF wis1

6.1: ANALYSIS OF *wis1* TRANSCRIPTION 136

6.2: EFFECTS OF INCREASED *wis1* DOSAGE 139

6.3: EFFECTS OF LOSS OF *wis1* FUNCTION 143

6.4: INTERACTIONS BETWEEN A DELETION ALLELE OF *wis1* AND OTHER CELL CYCLE MUTATIONS 158

6.5: DISCUSSION 161

CHAPTER 7: SEQUENCE ANALYSIS OF wis1

7.1: INTRODUCTION 163

7.2: EXPERIMENTAL STRATEGY 164

7.3: THE *wis1* SEQUENCE 166

7.4: IDENTIFICATION OF THE *wis1* ORF 169

7.5: UPSTREAM ELEMENTS 173

7.6: DOWNSTREAM ELEMENTS 174

7.7: THE *wis1* GENE PRODUCT 179

7.8: HOMOLOGIES BETWEEN *wis1* AND PREVIOUSLY IDENTIFIED GENES 179

7.9: DISCUSSION 189

CHAPTER 8: DISCUSSION

8.1: OVERVIEW 191

8.2: SUMMARY OF RESULTS 192

8.3: PLASMIDS CAPABLE OF SUPPRESSING A *win1.1*-CONFERRED PHENOTYPE 196

8.4: THE ROLES OF *win1* AND *wis1* IN THE CONTROL OF MITOSIS 198

8.5: THE ROLES OF *win1* AND *wis1* IN THE CONTROL OVER ENTRY INTO CONJUGATION AND MEIOSIS 203

8.6: SUMMARY 208

ACKNOWLEDGEMENTS 209

REFERENCES 211

ABBREVIATIONS:

ATP	adenosine triphosphate
BSA	bovine serum albumin
bp	base pair
cAMP	cyclic adenosine monophosphate
cdc	cell division cycle
cM	centiMorgan
DAPI	4,6-diamidine-2-phenylindole dihydrochloride
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetracetic acid
EMM	Edinburgh minimal medium
Fig	Figure
kb	kilobase
kD	kiloDalton
MBC	methyl benzimidazole-2-yl-carbamate
ME	malt extract
MOPS	3-(N-morpholino)propane-sulphonic acid
mRNA	messenger RNA
nt	nucleotide
OD	optical density
ORF	open reading frame
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
TBZ	2-(thiazole-4-yl) benzimidazole
Tris	tris(hydoxymethyl sulphate)methylamine
ts	temperature sensitive
Xgal	5-dibromo4-chloro3-indolylgalactosidase
YE	yeast extract medium
YEPD	yeast extract, peptone, dextrose medium
EtOH	ethanol
KOAc	potassium acetate
NaOAc	sodium acetate

ABSTRACT

This study concerns the analysis of elements involved in the control over entry into mitosis in the fission yeast, *Schizosaccharomyces pombe.* The initial aim was to characterise the role of the *win1* gene in this control system. The *win1.1* mutation shows a strong interaction with *wee1* and *cdc25*, genes which had previously been shown to play an important role in the control over entry into mitosis, probably acting through the *cdc2* protein kinase.

The strategy for the cloning of *win1* was to isolate sequences capable of suppressing the temperature sensitive cdc phenotype arising from the combination of *win1.1* with *wee1.50* and *cdc25.22.* Following the extensive screening of gene libraries, it proved impossible to isolate *win1* using this approach, although five new genes were isolated as multicopy suppressors of this phenotype. None of these sequences correspond to any known mitotic control gene, and therefore identify new genes that affect the control of entry into mitosis. These were named *wis* (win suppressing) 1 to 5.

A molecular analysis was undertaken on the pwis plasmids, and the phenotypes of various cell cycle mutant strains containing the pwis plasmids were also examined. *wis1*$^{+}$ was found to be capable of reducing the cell length at division in a dosage dependent manner, suggesting that *wis1* is involved in a rate limiting step controlling entry into mitosis. A null allele of *wis1* was constructed and found to results in large cells which have poor viability upon entry into stationary phase. DNA sequence analysis of *wis1* predicts a 605 amino acid gene product with a strong homology to serine/threonine protein kinases. Strains lacking in *wis1* function are still sensitive to levels of *wee1* and *cdc25* expression, suggesting that *wis1* acts upstream of these control elements.

The interaction of *win1.1* with other cell cycle mutants was studied and the *win1* locus mapped. The cloning of the closely linked gene *tps19* could provide an alternative strategy for the isolation of *win1.* Both *win1.1* and a *wis1*$^{-}$ allele were found to be capable of suppressing the hypersporulation phenotype of *pat1*^{ts} mutations, suggesting that the *win1* and *wis1* gene products may play a role in the regulation both of mitosis and meiosis.

VLADIMIR: Let us not waste our time in idle discourse!
Let us do something while we have the chance.....

CHAPTER 1

CHAPTER 1: INTRODUCTION

1.1: THE EUKARYOTIC CELL CYCLE

The cell cycle has been defined as the events which take place between the birth of the cell, and its subsequent division into two daughters (Mitchison, 1971). In order to study the mechanisms controlling this complex series of co-ordinated events in eukaryotes, several approaches have been used. In organisms such as yeast, mutations which arrest the cell cycle at specific points have been used, and physiological studies on both embryonic cells and higher eukaryotic cells in culture have been used to study factors controlling progress through the points in the cell cycle at which these cells naturally arrest.

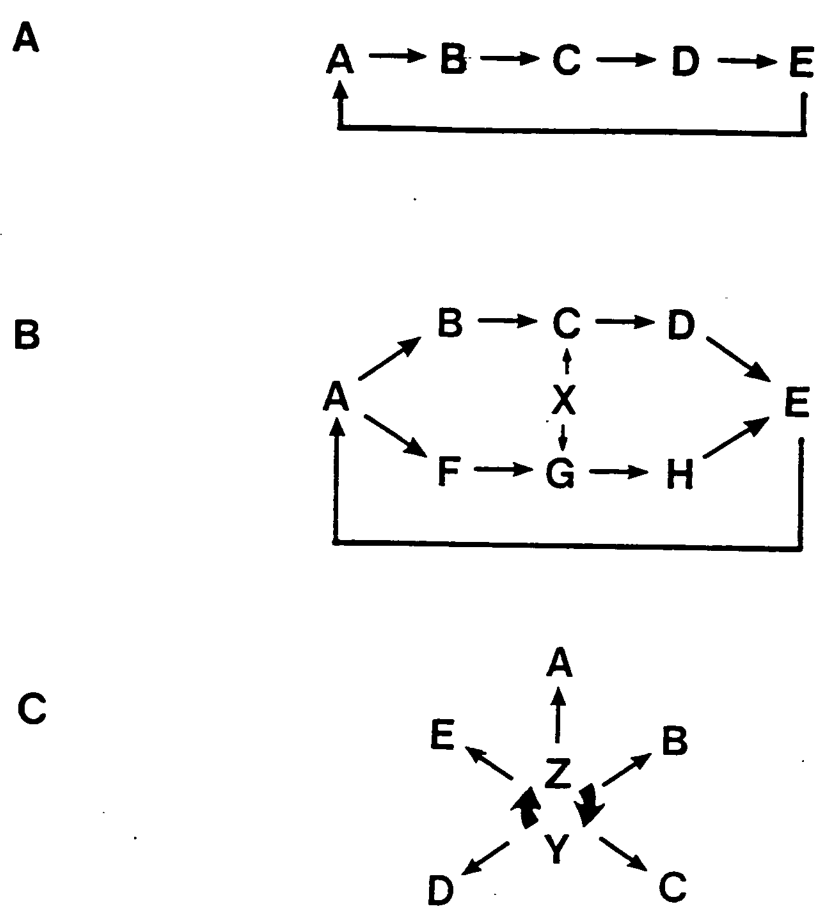
The eukaryotic cell cycle was originally divided into two phases: mitosis, during which the segregation of chromosomes could be observed, and interphase. Interphase may be divided into three phases: G_1, S, and G_2, with mitosis (M) occurring between G_2 and G_1 (Howard and Pelc, 1953). During S phase, chromosome replication takes place: this involves not only the precise replication of the entire DNA content of the nucleus in a semi-conservative manner, but also the assembly of the structural components of the chromosome, such as nucleosomes and scaffold proteins. During mitosis the sister chromatids formed during S phase are partitioned equally to two daughter nuclei. G_1 and G_2 were once viewed merely as "gaps" in the cell cycle, but are now known to be phases during which essential control mechanisms operate.

1.1.1: Temporal controls

The series of events required for the completion of a normal cell cycle take place in a defined temporal order, and several possibilities exist for mechanisms which determine the correct order of events (Fig. 1.1).

One such possibility for the temporal control of cell cycle events is that each event is solely dependent upon the completion of a preceding step. A simple example of such a model would be a series of enzyme catalysed reactions, in which the substrate of each reaction was the product of the preceding one.

Figure 1.1: Models for the control of temporal order



A: Linear dependent sequence: each event (A to E) is dependent upon the successful completion of the previous event.

B: Branched dependent sequence: both pathways are dependent on the completion of steps A and E. Component X ensures the dependency of each pathway upon the other, acting at steps C and G in the two pathways.

C: "Oscillator" model: timing components Z and Y determine the timing of the events A to E.

In many systems, however, it is possible to infer the existence of regulatory control machinery from the existence of a "relief of dependency" when abnormal conditions, such as chemical treatment, or a mutation
within the cell, lead to the relief of dependence of a cycle event upon one which would normally precede it.
Such a result suggests that each step is not merely dependent upon the previous one, and that at least two pathways exist. Under conditions which result in relief of dependency, it is the dependency of one pathway upon another which is relieved, rather than the dependency between single events.

Many examples of such a relief of dependence have been observed (reviewed by Hartwell and Weinert, 1989), suggesting that regulatory controls are common. Evidence such as that derived from of cell cycle mutants in the yeasts *S.pombe* and *S.cerevisiae* suggests that several independent sequences exist which intersect at defined points within the cell cycle to ensure their co-ordination (Pringle and Hartwell, 1981). Under abnormal conditions, these pathways may lose their dependence upon each other, suggesting that specific control mechanisms are involved.

Another possibility for the control of the temporal order of cell cycle events is the existence of a "master oscillator" with a period equal to that of the cycle time. In this system, each event in the cycle would occur independently of other events, and separate pathways could continue entirely independently. Such a model has been used to explain the rapid synchronous divisions in early embryos (Kirschner *et al*, 1985).

1.1.2: The effects of external factors upon the cell cycle

External factors which influence the cell cycle may be divided into those which regulate progress through the cell cycle, and those which regulate a cell's exit from the cycle. In micro-organisms such as yeasts, growth temperature and nutrient availability are the major influencing factors upon growth rate, so for the cells to maintain an approximate mean size at division under varying conditions, cellular growth and cell division must be co-ordinated. In such organisms nutrient availability is also one of the factors involved controlling exit from the cell cycle: cells deprived of essential nutrients will enter a quiescent or stationary phase. Other factors involved in signalling exit from the mitotic cell cycle are those involved in

sexual responses. In multicellular organisms the major factors influencing developmental differentiation and other cell cycle responses are growth factors, such as those in the serum-containing medium used for the culture of mammalian cells.

1.1.3: Control points in the cell cycle

Analysis of proliferation in higher eukaryotic cells has suggested that the major cell cycle control regulating proliferation and differentiation lies in G_1. In culture, untransformed cells require serum, or factors derived from serum, to continue proliferation. Once proliferation ceases, such cells enter a quiescent state from a particular stage of G_1. Such cells differ significantly from G_1 cells in a proliferating population in their physiological state (Baserga, 1985), leading to the idea that they are in a distinct stage in terms of the cell cycle, which has been termed G_0. The existence of such a control point was first hypothesised by Pardee (1974), who observed that cells arrested by a variety of different treatments ceased growth in G_1. The work of Zetterberg and Larsson (1985) supports this hypothesis, and divides G_1 in to two phases: G_1 (post-mitotic) during which they are sensitive to serum concentrations, and G_1 (pre-S). Cells in G_1 (pre-S) have passed the G_1 control point and will go on to progress to division even if incubated in serum free medium. In some organisms this control point appears to be a rate limiting step regulating cell cycle progress in proliferating cells by imposing a minimum size for entry into S phase, although this has not been proved to be the case in mammalian cells.

An analogous rate limiting control point was identified in *S.cerevisiae* by Hartwell (1974) and termed "start". This is the stage in the division cycle of *S.cerevisiae* at which the cell becomes committed either to the mitotic cell cycle, to stationary phase, or to the pathway leading to meiosis and sporulation (diploid) or conjugation (haploids). The point of arrest by mating pheromones, or by loss of *CDC28* function, has been used to define start. Nutritionally arrested cultures of *S.cerevisiae* arrest before the completion of start in an unbudded G_1 state (Pringle and Hartwell, 1981).

In contrast to this, start in *S.pombe* appears not to be rate limiting for cell cycle progress during fast exponential growth (Nurse and Fantes, 1981). Under these conditions, it is a point in G_2 which appears to be rate limiting, though a G_1 control becomes evident in cell size mutants (Nurse, 1975) and at slow growth rates

(Nasmyth, 1985). *S.pombe* cells may arrest in either G_1 or G_2 as a result of nutritional starvation (Costello *et al*, 1986).

1.1.4: The co-ordination of growth and division

A population of cells grown under constant conditions has a mean cell size at division which will remain constant over many generations. Under the influence of changing nutritional availability, cells will regulate their cell division timing, as failure to do so would lead to progressive increases or decreases in cell size. Cell size must be co-ordinated with division rate, so that deviations from the mean cell size are rapidly corrected; this has been demonstrated in several cases (e.g. Prescott, 1956; Fantes, 1977). There are a number of models which can explain the co-ordination of growth with division (Fantes *et al*, 1975). The model supported by most experimental evidence is that passage through a particular control point is regulated by a "sizer" control: the cell must reach a critical cell size before this step may be passed (Nurse and Fantes, 1981). An example of this form of control is observed in *S.cerevisiae*, where progress through start has been shown to dependent upon the attainment of a critical size Johnston *et al*, 1977). In *S.pombe* there is strong evidence for a critical size requirement in G_2 (Fantes and Nurse, 1977), and there is also evidence for such a G_2 control in other lower eukaryotic organisms (reviewed by Berger, 1989).

1.2: GENETIC ANALYSIS OF THE CELL CYCLE IN YEAST

1.2.1: Cell cycle mutants

The genetic analysis of the cell cycle was pioneered by Hartwell and his co-workers who isolated conditional lethal "cdc" mutants of *S.cerevisiae* which were defective in progress through the cell cycle (Hartwell *et al*, 1974). Such mutants have been isolated in several other eukaryotes, but the yeasts *S.cerevisiae* and *S.pombe* have provided most of the information on the genetic control of the eukaryotic cell cycle. Such mutants all define functions which are required for the successful completion of the cell cycle, although only a fraction of these will be involved specifically in control mechanisms.

Mutants in *S.pombe* were classified as "cdc" on the basis of the elongated morphology shown by cells which continue growth without undergoing division (reviewed by

Fantes, 1989). In contrast, the criterion used to classify *S.cerevisiae* mutants as cdc was that of a uniform terminal morphology at the restrictive temperature (Pringle and Hartwell, 1981).

The terminal morphology at the restrictive temperature was used to indicate the stage of cell cycle arrest in each mutant strain. Of particular interest were the "start" mutants which were mating-competent at the restrictive temperature, and arrested as unbudded cells.
Two classes of start mutants were defined on the basis of their terminal phenotypes: class 1 mutants (such as *CDC28*) continued cytoplasmic growth at the restrictive temperature, while class 2 mutants (CDC25, CDC33 and CDC35) arrested as unbudded cells, but did not continue to grow (Reed, 1980).

Genes involved in rate limiting steps controlling the cell cycle may be identified either using cdc mutants which result in a block in cell cycle progress, or by mutations which cause an accelerated passage through a controlling step. In yeasts, such genes have been identified by mutant alleles which result in abnormally small cells. Examples of such genes include *wee1* and *cdc2* in *S.pombe* (Nurse, 1975; Nurse and Thuriaux, 1980; Fantes, 1981), and *CLN3* in *S.cerevisiae* (Nash *et al*, 1988; Cross, 1988).

1.2.2: The use of cell cycle mutants

Several approaches may be used to utilise the properties of cdc mutants in order to examine the control of the cell cycle:
(i) The terminal phenotype of a *cdc* mutant may be informative about the stage of the cell cycle at which the wild type gene product is required by comparing this phenotype to identifiable morphological stages seen as cells progress through the normal cycle.
(ii) The transition point of a *cdc* mutant may be determined by temperature shift experiments, and is defined as the last point in the cycle at which the wild type gene product is required for cell cycle progress.
(iii) The terminal phenotype of double mutants where each of the single mutants has a distinct terminal phenotype may be informative regarding the dependency relationship between the two gene functions.

(iv) If two reversible blocks of the cell cycle may be applied independently, then a dependency relationship between the two blocks may be established by the reciprocal shift method (Hereford and Hartwell, 1974).

(v) It has been possible to identify elements interacting with the *cdc* gene in question by such means as the isolation of chromosomal mutations capable of suppressing the *cdc* defect. These mutations may identify genes which are involved in the same control mechanism as the *cdc* gene.

(vi) Finally, the development of techniques to transform yeast cells with shuttle vectors capable of maintenance both in yeast and *E.coli* has lead to the cloning of many *cdc* genes by complementation of their mutant phenotypes. This has been achieved by the transformation of cells carrying a temperature sensitive *cdc* mutation with a gene library consisting of plasmids containing yeast DNA fragments. Plasmids containing the cdc^{+} gene may be selected by their ability to complement the conditional lethal phenotype of the recipient strain.

The isolation of cell cycle genes may yield information concerning the function of the gene product, and allows *in vitro* manipulation of such sequences, which may be followed by the examination of the effects of such manipulations upon the cell. In yeast, gene transplacement allows the normal copy of a gene to be replaced with a modified version (Rothstein, 1983). The majority of the coding region may be replaced by another marker to investigate the effect of the loss of gene function, or the effect of single base changes may be investigated by *in vitro* mutagenesis. The availability of the cloned gene also allows the analysis of transcript levels, and of elements involved in the regulation of transcription. In a few cases it has been possible to demonstrate evolutionarily conserved cell functions by showing that cloned genes from one species are capable of complementing cell cycle defects in another species: *cdc2* homologues have been isolated from *S.cerevisiae* and human cells which are both capable of complementing *cdc2* mutations in *S.pombe* (Beach *et al*, 1982; Lee and Nurse, 1987).

The predicted amino acid sequence of a gene may be derived from the DNA sequence analysis of cloned genes, and the comparison of such sequences to those of previously characterised proteins may be informative concerning domains involved in protein function and regulation. The sequence of non-coding regions may reveal motifs concerned with transcriptional control and with mRNA processing.

There have been many instances where the cloning of cell cycle genes by complementation has resulted not only in the isolation of the authentic *cdc* gene, but also in the identification of extragenic suppressors which are capable of suppressing the cdc phenotype when present in multiple copies. The isolation of such sequences has proved useful for the identification of other cell genes involved in cell cycle control, such as cyclin homologues identified in *S.cerevisiae* (Hadwiger *et al*, 1989), and the *suc1* gene in *S.pombe* (Hayles *et al*, 1986a).

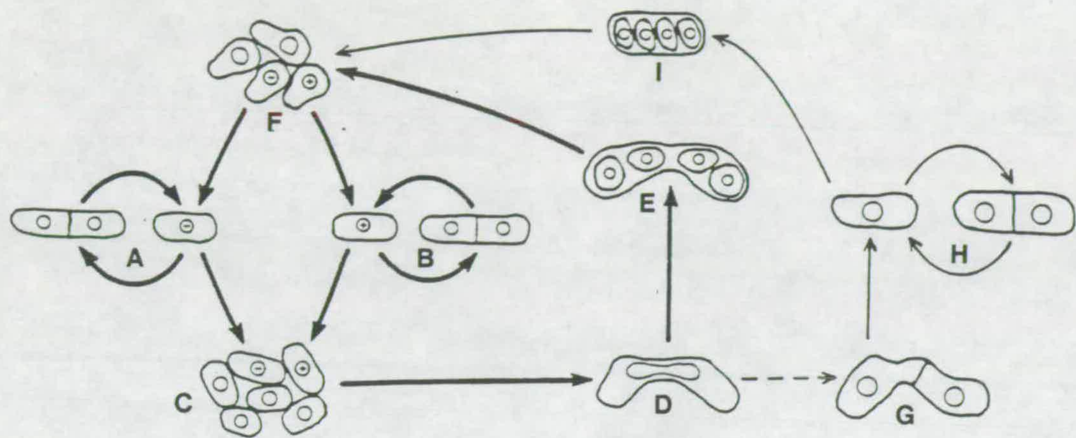
1.3: THE CELL CYCLE IN S.POMBE

The fission yeast *Schizosaccharomyces pombe* was chosen as a model system for cell cycle studies by Mitchison in the 1950s principally because of its linear growth pattern and symmetrical mode of division (Mitchison, 1990). At the same time, Leupold chose *S.pombe* for genetic studies because it was amenable to genetic manipulation, and had a mainly haploid mode of vegetative growth. The life cycle, genetics and molecular biology of *S.pombe* has been extensively reviewed in "Molecular Biology of the Fission Yeast" (A.Nasim, P. Young and B.F. Johnson, eds.) Academic Press, 1989.

Under favourable conditions, *S.pombe* cells reproduce asexually by means of the mitotic cell cycle (Fig. 2). Haploid cells may be of two mating types, plus or minus, and homothallic strains undergo frequent mating type switching, although heterothallic strains with relatively stable mating type may also be isolated. When cells experience starvation, diploid zygotes are formed by pairwise cell fusion between cells of opposite mating type. As sporulation is also induced by starvation, such zygotes usually undergo meiosis immediately, forming four-spored asci. If newly formed diploid zygotes are transferred to fresh medium, some will resume the vegetative cell cycle as diploids, although upon starvation heterozygous diploid cells will enter meiosis, forming azygotic asci.

The cells of *S.pombe* are round ended cylinders of nearly constant diameter which grow by length extension and divide by medial fission. Following the birth of a new cell, growth occurs only at the old end of the cell which existed in the previous cycle; the new end starts to grow at a defined stage termed NETO (New End Take Off) (Mitchison and Nurse, 1985). G_1 is very short in the wild type cell cycle, and DNA replication has already taken place by the time of cell division (Nasmyth, 1979).

Figure 1.2: The S.pombe life cycle.



Haploid cells may be of two mating types, "minus" and "plus", which are relatively stable in heterothallic strains (A and B), though homothallic strains switch mating type frequently. When a mixed culture of cells experiences starvation, they will become agglutinative (C), and diploid zygotes are formed by conjugation and karyogamy (D). Usually meiosis follows immediately, followed by sporulation to give asci (E), which will break down to liberate four haploid spores.

If newly formed zygotes are transferred to fresh growth medium, a proportion will restart the cell cycle as diploid cells (H). Upon starvation, such cells will sporulate, giving rise to an azygotic ascus (I). (Adapted from Egel, 1989)

As well as providing a convenient physiological model for the study of the cell cycle, *S.pombe* is amenable to both molecular and genetic analysis: cells may be propagated as haploids or diploids and the relatively small genome (approximately 15Mbp) means that it has been possible to construct a fairly detailed genetic map (Munz *et al*, 1989). *S.pombe* may be transformed to a high frequency by either linear or circular DNA constructs facilitating the isolation and molecular manipulation of functional DNA sequences.

1.4: MAJOR GENETIC ELEMENTS OF CELL CYCLE CONTROL IN S.POMBE.

1.4.1: Developmental controls

Under conditions of nitrogen starvation, *S.pombe* cells of an appropriate mating type will conjugate to form a diploid zygote. This is normally followed by meiosis, and the formation of four haploid spores. The stage in the cell cycle at which haploid cells make the decision between the mitotic cell cycle, or entry into conjugation followed by sporulation lies in G1, at a point analogous to start in *S.cerevisiae*, and the choice between the meiotic and mitotic pathways for diploid cells may also lie at this point.

The product of the *ran1* gene (also known as *pat1*) appears to be involved not only in controlling conjugation, but also with the regulation of meiosis (Iino and Yamamoto, 1985; Nurse, 1985). Loss of *pat1* function releases the cells from the normal requirements of nutritional starvation and mating type heterozygosity for meiosis and sporulation and releases cells from nutritional, though not from mating type requirements, for conjugation. In contrast, over expression of *pat1* blocks entry into mitosis. The *pat1* gene product was identified as a putative protein kinase on the basis of sequence homologies (McLeod and Beach, 1986), and has been demonstrated to show kinase activity *in vitro* (McLeod and Beach, 1988).

In contrast to *pat1*, mutations in the *mei3* gene block entry into meiosis, while *mei3* overexpression derepresses it (McLeod *et al*, 1987). The *mei3* product has been demonstrated to interact with the *pat1* gene product, and to inhibit *pat1* protein kinase activity *in vitro*, suggesting that the decision between mitosis and meiosis is determined by a balance between *pat11* and *mei3* activities (McLeod and Beach, 1988). The *mei2* gene also appears to be important in this control system: mei2

function is required prior to pre-meiotic DNA synthesis (Bresch *et al*, 1968; Shimoda *et al*, 1985). *mei2* mutants can suppress the meiotic derepression of *pat1* mutants (Iino and Yamamoto, 1985; Beach *et al*, 1985), and transcription of *mei2* is induced under conditions of nitrogen deprivation, although *mei2* overexpression is not sufficient to induce meiosis (Watanabe *et al*, 1988).

Cyclic AMP has been shown to be important in the regulation of many cell signalling processes, not only in *S.pombe* (Levitzki, 1988). High levels of cAMP suppress the lethal phenotype of *pat1*$^-$ strains, and also block the transcriptional induction of *mei2*, and of at least two other mating type genes (Watanabe *et al*, 1988). However, constitutive expression of *mei2* blocks suppression of *pat1* by increased cAMP levels, suggesting that the effect of cAMP upon *pat1* activity may be mediated by changes in *mei2* expression.

The role of cAMP as a cellular signaling mechanism has been extensivley studied in *S.cerevisiae*: a mechanism mediated by the action of cAMP-dependent protein kinases positively regulates cell growth and inhibits differentiating pathways associated with nutrient depletion, such as entry into meiosis and sporulation or entry into stationary phase (Matsumoto *et al*, 1985). Genes involved in a cAMP cascade response have been identified by mutational analysis.

The activity of one of the two *S.cerevisiae RAS* genes is essential for cell growth and for adenylate cyclase activity. *ras1 ras2* double mutant strains, or other mutants which result in a reduced cAMP level, arrest as single unbudded cells, a phenotype similar to that shown by the start mutants *cdc19*, *cdc25*, *cdc33* and *cdc35* at the restrictive temperature (Tatchell, 1986). In contrast, mutations that constitutively raise intracellular cAMP levels, or result in an unregulated kinase activity result in a phenotype characterised by the inability to arrest growth in response to nutrient starvation, a lack of glycogen accumulation, and hypersensitivity to heat shock. *CDC35* (*CYR1*) has been identified as the structural gene for adenylate cyclase (Boutelet *et al*, 1985; Casperson *et al*, 1985), and *CDC25* is believed to act through *ras* proteins as a positive regulator of adenylate cyclase activity (Broek *et al*, 1987). Mutations which lower intracellular cAMP concentrations enable diploid cells to enter meiosis and sporulation in rich media, while mutations activating the cAMP pathway prevent the formation of spores. These observations have lead to the suggestion that in *S.cerevisiae* cAMP levels are involved in controlling the transition from mitotic growth to meiosis and sporulation (Shilo *et al*, 1978; Matsumoto *et al*, 1983). In

S.pombe, the role of the single identified *ras* gene appears to be primarily in developmental control, which also involves cAMP, though the role of cAMP in other aspects of cell cycle control is not yet clear.

1.4.2: G_1 controls

Cell cycle controlling steps exist in both G_1 and G_2, although in exponentially growing cells, the G1 control point is cryptic (Nurse and Fantes, 1981). That is, conditions do not normally prevail under which this control is a rate limiting step. In cells which are significantly smaller than would be expected under normal conditions due either to mutation (Nurse, 1975; Nasmyth *et al*, 1979), or to physiological manipulation (Nurse and Thuriaux, 1977; Nasmyth *et al*, 1979), a size requirement for entry into S phase is revealed. This G_1 size control appears to act with the completion of the *cdc10* step at start (Nasmyth, 1979).

Cells arrested by *cdc2* and *cdc10* mutants were shown to be capable of directly entering the meiotic pathway (Nurse and Bisset, 1981), and on this basis *cdc2* and *cdc10* were defined as mutants involved in a G_1 control analogous to Start in *S.cerevisiae*. Novak and Mitchison (1989) have identified the G1 transition point of *cdc2* in growing cells, and found that it was not coincident with the *cdc10* transition point. In *wee1* cells, the *cdc2* transition point is as much as one third of a cycle before that of *cdc10*, and appears to be associated with an early G_1 event, rather than control over entry into S phase. A role for *cdc2* in the determination of the temporal dependency of cell cycle events has been suggested by the observation that certain mutant alleles of *cdc2* affect the dependency relationship between S phase and mitosis (Enoch and Nurse, 1990). *cdc2* is also involved in the control over entry into mitosis, and will be discussed in the following section.

1.4.3: G_2 controls

During exponential growth, the major rate limiting step in the *S.pombe* cell cycle takes place in G_2 and acts to control entry into mitosis. Cells respond to changes in medium or growth rate by changes in the timing of entry into mitosis. This may be explained if a form of size control is in operation controlling entry into mitosis, which may be regulated by growth rate and nutritional conditions (Nurse and Fantes, 1981).

Further evidence for the existence of such a control was provided by the isolation of "wee" mutants, which undergo mitosis and cell division at a reduced size. Two genes gave rise to such a class of mutant: *wee1* and *cdc2* (Thuriaux *et al*, 1978). A requirement had already been shown for *cdc2* in both G_2 and G_1 (Nurse and Bisset, 1981), suggesting that *cdc2* occupied a central role in the control of the cell cycle. In *wee1* mutants, the critical size required for entry into mitosis is reduced, and *wee1* cells show no response upon a shift to nitrogen-depleted medium. This is in contrast to wild type cells, in which the critical size for entry into mitosis is normally dependent upon growth medium components (Fantes and Nurse, 1977), which show a transient stimulation of mitosis and division upon a shift to nitrogen depleted medium (Fantes and Nurse, 1978). These observations suggest a role for *wee1* as a monitor of nutritional status.

The *cdr* mutations were also identified on the basis of their altered response to nutritional shifts: both *cdr1* and *cdr2* mutant strains show a reduced division response upon a shift to nitrogen-depleted medium compared to that shown by wild type cells (Young and Fantes, 1987). *wee1* is epistatic to *cdr1* and *cdr2*, suggesting either that *cdr1* and *cdr2* are required in two separate path ways which each involve *wee1*, or that *cdr1* and *cdr2* act in a single pathway involving *wee1* (Young and Fantes, 1987). *cdr1* is allelic to *nim1* (P. Young, Pers. Comm.), which was identified independently as an extragenic suppressor of *cdc25* (Russell and Nurse, 1987b).

cdc25 was implicated in this control point following the observation that mutations in *wee1* were capable of suppressing the G_2 arrest shown by *cdc25* cells upon a shift to the restrictive temperature (Fantes, 1979, 1981). Subsequent analysis has shown that *wee1* and *cdc25* act independently to control entry into mitosis, possibly by regulating the activity of the *cdc2* gene product (Russell and Nurse, 1986; Russell and Nurse, 1987a).

The *cdc13* gene was originally thought to be required during mitosis, judging from functional dependency analysis (Fantes, 1982), and the terminal phenotype of *cdc13.117* (Nasmyth and Nurse, 1981). More recently, the observations that complete loss of *cdc13* function results in cell cycle arrest with a G2 phenotype (Hagan *et al*, 1988; Booher and Beach, 1988), that *cdc13* and *cdc2* show complex allele specific interactions (Booher and Beach, 1987), and that the *cdc2* and *cdc13* gene products show cytological co-localisation and a physical interaction (Booher *et al*, 1989) have suggested that *cdc13* has a role in the G2/M control (see Section 6.1.c.).

1.5: A UNIVERSAL MITOTIC CONTROL?

Recent molecular and biochemical evidence has indicated that the mechanisms controlling the eukaryotic cell cycle have been evolutionarily conserved among widely divergent species. Homologues of p34, the protein kinase which is the *cdc2* gene product, the interacting protein p13 encoded by *suc1*, and cyclin-like molecules have been implicated in cell cycle controls in a range of organisms (Reviewed by Lewin, 1990).

The original observations that the *S.pombe cdc2* gene product was functionally homologous to the *CDC28* gene product in *S.cerevisiae* (Beach *et al*, 1982) suggested that *cdc2* may be part of fundamental cell cycle control present in all eukaryotic cells. Molecular biologists and biochemists involved in cell cycle studies were able to settle their differences when the *cdc2* protein kinase (p34) was found to be a constituent of maturation promoting factor (MPF) in *Xenopus Laevis* (Dunphy *et al*, 1988; Gautier *et al*, 1988). MPF was originally defined as an activity present in mature amphibian egg cytoplasm that was capable of inducing the meiotic maturation of oocytes in the absence of protein synthesis, and has been the subject of extensive biochemical investigation. A highly purified preparation of MPF was found to consist of two polypeptides of relative molecular mass 34kD and 45kD (Lohka *et al*, 1988). The 34kD polypeptide was identified as a homologue of *cdc2* (Dunphy *et al*, 1988; Gautier *et al*, 1988), and the 45kD subunit was demonstrated to be homologous to a family of proteins known as cyclins (Draetta *et al*, 1989). Cyclins were first identified as proteins which showed accumulation during interphase, and rapid proteolysis during mitosis in early embryos (Evans *et al*, 1983) and exist in two classes, A and B, which are weakly related, but which share a stretch of approximately 150 amino acids known as the cyclin box. The *cdc13* gene in *S.pombe* shows a high degree of homology with class B cyclins (Goebl and Byers, 1988; Solomon *et al*, 1988). Biochemical and genetic evidence suggest a direct physical interaction between the *cdc2* and *cdc13* gene products (Booher and Beach, 1987; Booher *et al*, 1989). Cyclins appear to be necessary for the activation of the *cdc2* protein kinase homologue in many systems, though their accumulation may not be the activating step.

A class of cyclin-like (CLN) genes have been identified in *S.cerevisiae* which are believed to control the G1/S activity of *CDC28*, which is functionally

interchangeable with *cdc2* (Beach *et al*, 1982). These proteins show only weak homology to A or B cyclins, and their roles in the cell cycle have not been elucidated, although *CLN3* function does seem to depend upon an intrinsic instability, as with other cyclin proteins (Nash et al, 1988; Cross, 1988). It has recently been reported that genes with a strong homology to B cyclins have been identified in *S.cerevisiae* by their interaction with as allele of *CDC28* which is specifically defective in G_2 function (Surana *et al*, 1990).

Homologues of other genes involved in the *S.pombe* cell cycle have also been identified in other species. *cdc25* homologues have been identified in both *Drosophila* (Edgar and O'Farrell, 1989) and *S.cerevisiae* (Russell *et al*, 1989) which are functionally interchangeable with the *cdc25* gene. It has also been demonstrated that the *wee1* gene product can delay the initiation of mitosis in *S.cerevisiae*, suggesting a conserved control mechanism.

1.6: GENETIC ELEMENTS OF THE G_2 CONTROL IN S.POMBE

1.6.1: Major genetic elements of mitotic control

Many interacting elements have been identified acting to control entry into mitosis in *S.pombe*. The major elements were identified by mutation, and physiological and classical genetical studies have been followed by the cloning and molecular analysis of the genes involved in this control system. A model for the mechanism by which the mitotic control in *S.pombe* operates is shown in Figure 1.3, and the properties of the individual elements are descibed in the following section.

1.6.1.a: cdc2

Cells carrying temperature sensitive alleles of *cdc2* undergo cell cycle arrest when shifted to the restrictive temperature, becoming blocked in either G_1 or G_2 (Nurse and Bissett, 1981). One cold sensitive *cdc2* allele has been isolated which appears to defective only in progression through G_2 (Booher and Beach, 1987) and dominant "wee" mutations of *cdc2* have been identified which result in an early entry into mitosis and division (Nurse and Thuriaux, 1980; Fantes, 1981). These observations suggest that *cdc2* activity is required both in G_1 and G_2, and is also involved in a rate limiting step controlling progression through the G_2/M control point.

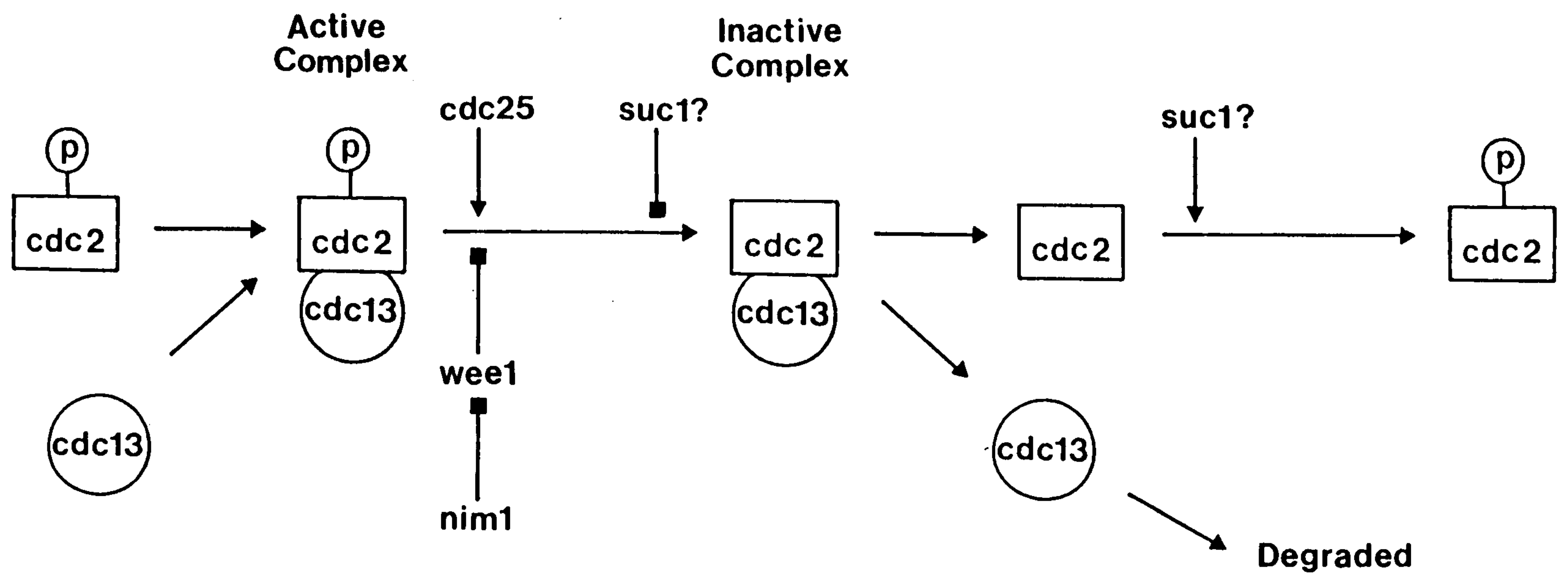


Figure 1.3: A model for the interaction of elements involved in the mitotic control of S.pombe.

(The *cdc13* gene product is also phosphorylated.)

The *cdc2* gene product has been identified as a 34kD phosphoprotein with protein kinase activity (Simanis and Nurse 1986; Draetta *et al*, 1987) Homologues of *cdc2* have been identified by both functional and sequence homologies in a wide range of eukaryotes, including yeast, plants, and mammals (Beach *et al*, 1982; Draetta *et al*, 1987; Lee and Nurse, 1987; Gautier *et al*, 1988; Dunphy *et al*, 1988; Arion *et al*, 1988; Labbe *et al*, 1988; John, *et al*, 1989). In *S.pombe*, entry into M phase is believed to be brought about by the activation of p34 kinase activity, which has been demonstrated to be periodic, reaching a peak at the time of M phase, using an *in vitro* assay system with histone H1 as a susbstrate (Moreno et al, 1989; Booher *et al*, 1989). In cells starved of essential nutrients, protein levels remains unchanged though there is a decrease in the level of p34 phosphorylation, which appears to be associated with loss of kinase activity in cell extracts (Simanis and Nurse, 1986). Regulation of kinase activity in growing cells is not due to changes in either transcript (Durkacz *et al*, 1986) or protein levels during the cell cycle (Simanis and Nurse, 1986), and so is likely to result from post-transcriptional modification (such as phosphorylation) or subunit interactions, both of which appear to be involved.

The major phosphorylated amino acids in the p34 kinase phospho-tyrosine and phospho-threonine. Dephosphorylation of tyrosine and threonine residues, which from site-specific mutagenesis experiments have been implicated in the regulation of p34 activity, occurs as cells enter mitosis. Dephosphorylation of particular residues appears to be coincident with the rise in kinase activity observed prior to M phase (Gould and Nurse, 1989).

Many elements interacting with p34 have been identified, two of which, *nim1* (Russell and Nurse, 1987b) and *wee1* (Russell and Nurse, 1987a) show a strong homology to protein kinases, and one of which (*dis1/bws1*) is homologous to the catalytic subunit of mammalian type-1 phosphatase (Ohkura *et al*, 1989; Booher and Beach, 1989), emphasising the importance of protein phosphorylation in the control over entry into mitosis (reviewed by Witters, 1990). Both the *suc1* and *cdc13* genes show genetic interactions with *cdc2*, and there is strong evidence to suggest thst they regulate *cdc2* activity by direct physical interaction with p34 (reviewed by MacNeill and Nurse, 1990).

1.6.1.b: suc1

A range of allele specific interactions exist between *cdc2* and *suc1*, which was first identified on the basis of its ability to suppress certain temperature sensitive *cdc2* mutants when present on a multicopy plasmid (Hayles *et al*, 1986a). *suc1* mutations have been isolated as extragenic suppressors of $cdc2^{ts}$ mutants (Hayles *et al*, 1986b). These *suc1* mutations are dominant, suggesting that they are due to an alteration, rather than a loss, of *suc1* function. *suc1* levels do not affect *cdc2* transcription, but a direct physical interaction between p34 and the *suc1* gene product (p13) has been demonstrated (Brizuela *et al*, 1986). p13 is not required for the activation of the p34 protein kinase at mitosis, but seems to be required at a later stage in the mitotic process (Moreno *et al*, 1989). Loss of *suc1* function results in cells blocked in the cell cycle containing mitotic spindles, and a high kinase activity, suggesting that p13 may be involved in the post-mitotic inactivation of p34, rather than regulating its activity upon entry into mitosis. Over-expression of *suc1* results in cell elongation, an effect which appears to be specific to G2 rather than G1 (Hayles *et al*, 1986b; Hindley *et al*, 1987), and strains in which *suc1* is highly over-expressed are defective in the second meiotic division (Hayles *et al*, 1986b). These observations suggest that *suc1* also has a role in regulating the timing of nuclear division.

1.6.1.c: cdc13

The *cdc13* gene product is a 56kD protein which shows homologies to type B cyclins (Goebl and Byers, 1988; Solomon *et al*, 1988; Hagan *et al* 1988). As is characteristic of this class of proteins, it is catastrophically degraded at mitosis (Moreno *et al*, 1989). p34 kinase activity appears to be dependent on the presence of p56 in germinating spores, although *cdc13.117* cells retain a high level of kinase activity upon a shift to the restrictive temperature. One explanation for these observations is that p56 is required to regulate p34 kinase activity upon both entry into and exit from mitosis. Immunofluorescence staining of the p34 and p56 proteins has shown that p56 may be acting as a "molecular chaperone" with a role in determining the nuclear localisation of p34 during mitosis (Booher *et al*, 1989). The lack of spindle formation in *cdc13.117* cells at the restrictive temperature (Hagan *et al*, 1988), and the sensitivity of such cells to the antimicrotubule drug thiabendazole (Booher and Beach, 1988) have pointed to a role for the *cdc13* gene product in processes affecting mitotic spindle formation.

1.6.1.d: cdc25

The protein kinase activity of p34 is regulated at the G2/M transition by the antagonistic effects of the *wee1* and *cdc25* gene products. Evidence for this regulation comes from two sources: the allele specific interactions of *cdc2w* alleles with *wee1* and *cdc25*, and the additive effects of varying *wee1* and *cdc25* expression levels (Russell and Nurse, 1987a; Russell and Nurse, 1986).

The *cdc25* gene product is a protein of 67kD which shows no homologies with previously identified sequences in the current data bases (Russell and Nurse, 1986). Upon a shift to the restrictive temperature $cdc25^{ts}$ strains are blocked before the initiation of mitosis (Nurse *et al*, 1976; Fantes, 1979) and contain a low level of p34 kinase activity (Moreno *et al*, 1989), suggesting that *cdc25* function is required for the activation of p34 kinase activity at mitosis. Once blocked $cdc25^{ts}$ strains are returned to the permissive temperature, a peak in kinase activity coincides with chromosome condensation and levels of kinase activity decline at anaphase (Moreno *et al*, 1989). *cdc25* has also been demonstrated to act as a dosage dependent inducer of mitosis (Russell and Nurse, 1986).

cdc25 mutations also result in some alterations with respect to the translational machinery of the cell, as they show allosuppressor activity (Nurse and Thuriaux, 1984). This suggests that *cdc25* may be involved in sensing the nutritional state of the cell, possibly by monitoring growth rate, which would in turn affect tRNA and protein synthesis. Other mutations, such as *sal3* and *cdr* mutations, also show phenotypes which combine allosuppressor activity with an effect upon the cell cycle (Nurse and Thuriaux, 1984; Young and Fantes, 1984).

1.6.1.e: wee1

In contrast to *cdc25*, the *wee1* gene product functions as a dosage dependent inhibitor of mitosis. Inactivation of *wee1* results in cell division at a reduced size (Nurse, 1975), while overexpression causes a delay in the initiation of mitosis (Russell and Nurse, 1987a). Loss of *wee1* function relieves the cell of the requirement for *cdc25* activity for entry into mitosis (Russell and Nurse, 1986). The predicted *wee1* gene product shows sequence similarities with serine/threonine protein kinases, and, although kinase activity has not yet been demonstrated *in vitro*, mutations predicted

to abolish kinase activity have been shown to eliminate *wee1* function *in vivo* (Russell and Nurse, 1987a).

1.6.2: A model for the control of entry into mitosis

The extensive studies carried out on the control elements described here has made it possible to postulate a model for their interactions. In this scheme, *cdc2* activity is required to activate entry into mitosis, and this activity is controlled antagonistically by the actions of *wee1* and *cdc25*. The evidence that *cdc2* activity is regulated independently by the levels of both *wee1* and *cdc25* rests upon the additive effects effects of varying *wee1* and *cdc25* levels, and the interaction of specific *cdc2* alleles with *wee1* and *cdc25*.

Two forms of *cdc2w* mutations which bring about advanced entry into mitosis have been identified, which show allele specific interactions with *wee1* and *cdc25*. Cells containing one type of mutation, typified by *cdc2.1w*, are specifically insensitive to *wee1* expression, though respond essentially normally to *cdc25* levels (Russell and Nurse, 1987a, Thuriaux *et al*, 1978). In contrast, mutants of the type typified by *cdc2.3w* are sensitive to levels of *wee1* expression, but show little response to changes in *cdc25* levels (Russell and Nurse, 1987a). These observations suggest that *wee1* and *cdc25* act independently on *cdc2* to regulate its activity in G_2. This theory is supported by the observation that the effects of *wee1* inactivation and *cdc25* overexpression are additive. Upon a shift to the restrictive temperature, a strain with artificially high levels of *cdc25* which also has temperature sensitive *wee1* activity undergoes what has been termed "mitotic catastrophe" (Russell and Nurse, 1986). This phenotype, which causes in a rapid decrease in viability, is also shown by *cdc2.3w* $wee1^{ts}$ strains at the restrictive temperature (Russell and Nurse, 1987a). This is the result that would be expected if the *cdc2.3w* gene product has the characteristics of p34 constitutively "on" with respect to activation by *cdc25*. The mitotic catastrophe phenotype appears to be due to cells attempting mitosis and division prematurely, and is characterised by a range of mitotic division abnormalities (Russell and Nurse, 1987a).

Since dephosphorylation of *cdc2* is required for its activation, an attractive possiblity for the interaction between *wee1* and *cdc2* is that *wee1* inhibits *cdc2* function by phosphorylation, as *wee1* encodes a putative protein kinase. As *cdc25* acts antagonistically to *wee1*, this simple model would predict that *cdc25* might encoded a

phosphatase. The *cdc25* gene product shows no homologies to protein phosphatases, however, though it may act to control phosphatase activity.

1.6.3: Other elements involved in the mitotic control

1.6.3.a: nim1

This gene was identified as a suppressor of $cdc25^{ts}$ alleles when present in multicopy, and encodes a putative protein kinase (Russell and Nurse, 1987b). Strong overexpression of *nim1* gives a wee phenotype, and deletion results in elongated cells, suggesting that *nim1*, like *cdc25*, is a positive dosage dependent control element in the mitotic control. Loss of *wee1* function renders the cells insensitive to *nim1* expression levels, and overexpression of *nim1* does not bypass the requirement for *cdc2* function. These observations suggest that *nim1* acts through *cdc2*, possibly by regulating *wee1* activity. Changes in the levels of *nim1* expression have no effect upon *wee1* transcript levels, suggesting that their interaction is post-translational. One attractive possibility is that the *nim1* protein kinase regulates the activity of the *wee1* gene product by phosphorylation (Russell and Nurse, 1987b).

1.6.3.b: bws1

This gene was isolated in the form of a sequence which reversed the suppression of *cdc25* by *wee1* when carried on a multicopy plasmid (Booher and Beach, 1989). It seemed likely that the genetic screen from which the *bws1* gene was isolated would identify *wee1*, or possibly other protein kinases which might be capable of acting upon *wee1* substrates. Suprisingly, the predicted *bws1* gene product showed a strong homology to the catalytic subunit of mammalian type-1 protein phosphatase, and was allelic to *dis2* which had been cloned independently (Ohkura *et al*, 1989). *dis2* is one of four *dis* loci in which temperature sensitive mutations block chromosome disjunction (Ohkura *et al*, 1988). *sds21*, which will suppress *dis1* mutations when present in multicopy, also encodes a type-1 protein phosphatase (Ohkura *et al*, 1989). The *bim*G gene in *Aspergillus nidulans* appears to be important for the completion of mitosis (Doonan and Morris, 1989), and phosphatases have been demonstrated to play an important in the cell cycle control of other eukaryotes (Cycer and Thorner, 1989)

1.6.3.c: The mcs genes

The six *mcs* genes were identified because of the ability of mutant alleles to suppress the mitotic catastrophe phenotype resulting from the combination of *wee1*^{ts} and *cdc2.3w*. All *mcs* mutant alleles show a range of phenotypic interactions with different cell cycle mutations, including the *cdc2w* alleles, *wee1.50*, *cdc13.117* and *cdc25.22* (Molz *et al*, 1989). Both the *mcs2* gene and one extragenic suppressor of *mcs2* have been cloned and sequenced, and although the predicted *mcs2* gene product shows no obvious homologies with other identified proteins, its extragenic suppressor shows a strong homology with the protein kinase family (Molz, Pers. Comm.).

1.6.3.d: win1

A mutation defining the *win1* gene was identified by its ability to reverse the suppression of *cdc25* by *wee1*, a similar effect to that resuting from the overexpression of *bws1/dis2* (Ogden and Fantes, 1986). Only one mutant allele of *win1* has been isolated, *win1.1*, which is temperature insensitive, and in most genetic backgrounds results in a slight increase in cell division length. *win1.1* in combination with *wee1.50 cdc25.22* gives a striking phenotype: the cells are phenotypically wee on rich medium, but phenotypically cdc on minimal medium. The interaction is not allele specific to *wee1.50*, and will not reverse the suppression of *cdc25.22* by *cdc2.3w*. Dominance relations imply that *win1.1* is a recessive mutation. Since *win1.1* does not confer a conditional phenotype, it is not possible to determine if *win1* is required at a specific time within the cell cycle. (Ogden and Fantes, 1986)

1.7: Aim and scope of this project.

The initial aim of the work undertaken for this thesis was to characterise the role of *win1* in the control of the cell cycle. The strategy for this project could be divided it into three categories:

(i) The isolation of the *win1* gene. This would make possible manipulation of cloned sequences to examine the effects of *win1* overexpression and the deletion of the *win1* functional region on the c

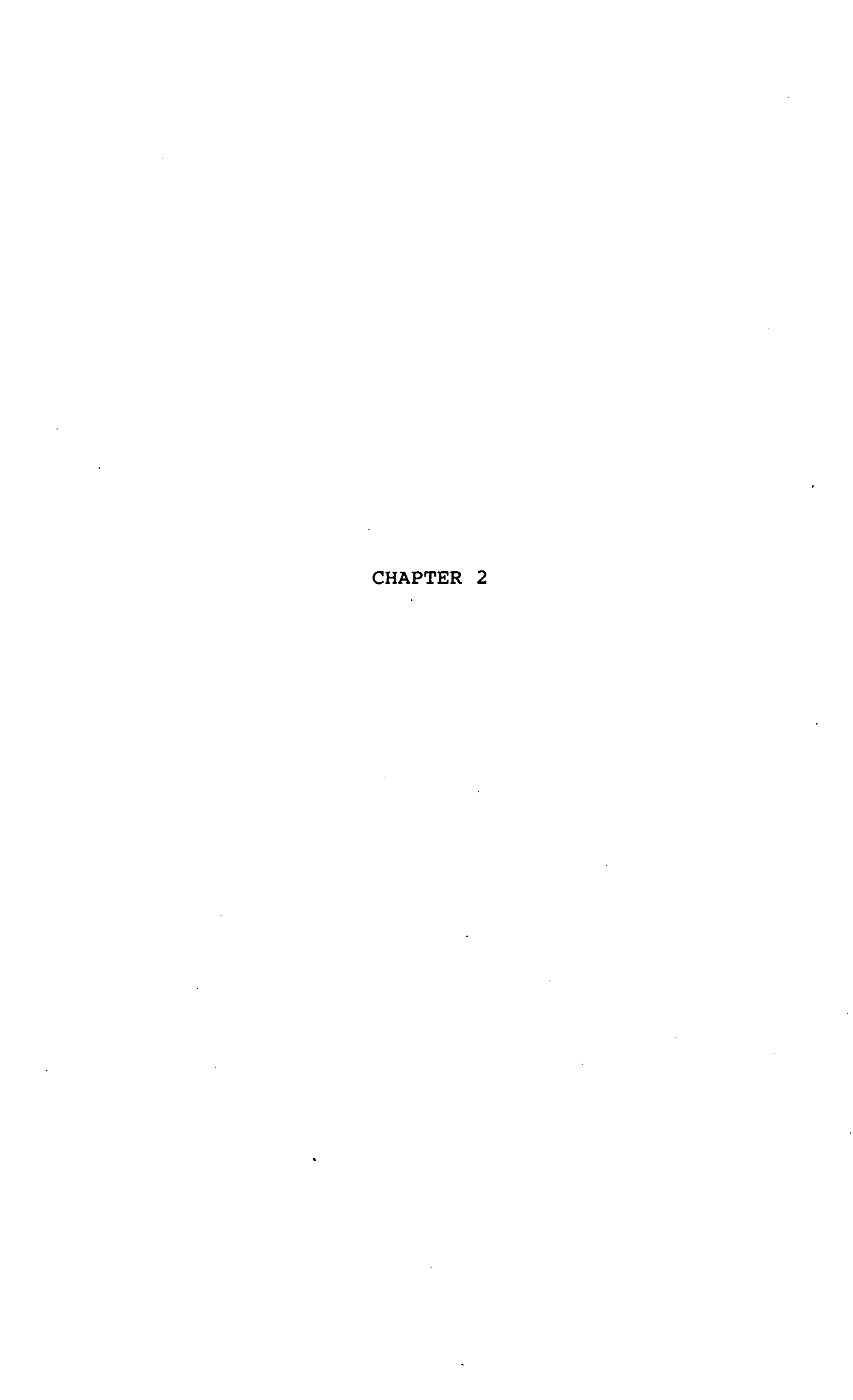
ell. The analysis of *win1* transcription, and the determination of the *win1* DNA sequence could also be undertaken, from which the amino acid sequence of the *win1* gene product could be predicted.

(ii) The study of interactions between *win1* and previously identified cell cycle genes.

(ii) The investigateion of the nutrient dependent phenotype of the *wee1.50 cdc25.22 win1.1* triple mutant strain.

The strategy for cloning *win1* was dependent upon the isolation of sequences capable of suppressing the cdc phenotype arising from the combination of *win1.1* with *wee1.50 cdc25.22.* Following extensive screening of gene libraries, it proved impossible to isolate the authentic *win1* gene using this approach, although 5 new genes presumed to be involved in the mitotic control were isolated as suppressors of this phenotype. These were named *wis* (win suppressing) 1 - 5.

The work described here centres on the the molecular and genetic analysis of one of these suppressors, *wis1*, including the determination of its DNA sequence, and the study of interactions between *win1.1* and previously identified genes involved in the mitotic control.



CHAPTER 2

CHAPTER 2: MATERIALS AND METHODS

2.1: REAGENTS AND COMMONLY USED BUFFERS

All reagents used were of analytical grade, except were indicated. Nucleic acid modifying enzymes were obtained mainly from Amersham, Boehringer Mannheim, Gibco-BRL and Pharmacia, and were used according to the manufacturers' instructions. Many standard methods for buffer preparation and nucleic acid handling were taken from Maniatis *et al*, (1982).

2.1.1: Tris-HCl

Tris Base (Tris[hydroxymethyl]aminomethane) was dissolved in water and the pH of the solution adjusted to the required value by the addition of HCl. Water was added to give a 1M stock solution.

2.1.2: EDTA

A stock solution of 0.4M EDTA (Ethylenediaminetetramino acid di-sodium salt) was made by dissolving solid EDTA in water, adjusting the pH to 8.0, and adding to water to the required volume.

2.1.3: TE

A buffered solution consisting of 10mM Tris-HCl and 1mM EDTA (pH7.6). STE buffer consisted of TE buffer containing 0.1M NaCl.

2.1.4: Phenol

Phenol was pre-equilibrated with 1M Tris-HCl, followed by TE buffer (pH 7.6), and contained 0.1% hydroxyquinoline and 0.2% ß-mercaptoethanol to retard oxidation (Maniatis *et al*, 1982).

2.1.5: Chloroform

Chloroform refers to a 1:24 (v/v) mixture of chloroform and isoamyl alcohol unless otherwise stated.

2.1.6: Citrate-phosphate buffer (pH5.6)

0.1M Citric acid monohydrate	42ml
0.2M Na_2HPO_4	58ml

2.2: NUCLEIC ACID MANIPULATION

2.2.1 General methods

2.2.1.a: Extraction with phenol/chloroform

Proteins were removed from solutions containing nucleic acids by extraction with phenol or with a 1:1 mixture of phenol and chloroform. Traces of phenol were removed by a further extraction with chloroform. Extraction was carried out by adding a volume of the organic phase equal to that of the solution of nucleic acid. The phases were mixed to form an emulsion and then separated by centrifugation. The aqueous phase was then transferred to a fresh tube and the nucleic acid recovered by precipitation.

2.2.1.b: Precipitation of nucleic acids

DNA was precipitated by one of three methods:

1: 0.1 volume of 3M NaOAc pH5.2 was added followed by 2 volumes of absolute ethanol.

2: NaCl was added to give a final concentration of 0.1M, followed by 2 volumes of absolute ethanol.

3: 0.4 volumes of NH4OAc were added followed by 2 volumes of isopropanol.

In each case the solution was mixed, cooled at -20°C and the DNA recovered by centrifugation. The pellet was washed in 70% ethanol, dried briefly, and dissolved in an appropriate volume of water or TE buffer.

2.2.2: Plasmid vectors

2.2.2.a: pUC-based plasmids.

The pUC plasmids are used for the manipulation of foreign genes in *E.coli*. They consist of the pBR322-derived ampicillin resistance gene and origin of DNA replication, ligated to a portion of the *LacZ* gene of *E.coli*. pUC8 and pUC9 contain a polylinker in opposite orientations. pUC18/19 plasmids contain a more extensive polylinker than pUC8/9 (Vieira and Messing, 1982; Fig. 2.1). The vectors pTZ18/19 are similar to the

pUC plasmids, but contain in addition the f1 origin of replication (Zoller and Smith, 1983). If the host cell is superinfected with the helper phage M13K07, replication will be initiated at the f1 origin, resulting in the production of single-stranded DNA. pTZ plasmids also contain the bacteriophage T7 promoter adjacent to the polylinker for *in vitro* synthesis of large amounts of specific RNA. (Fig. 2.1)

2.2.2.b: pDB248

The vector pDB248 was derived from the *E.coli* plasmid pBR322 (Bolivar *et al*, 1977) and the *S.cerevisiae* plasmid pJDB248 (Beggs, 1978). It is capable of autonomous replication in *E.coli* and *S.pombe*. The *S.cerevisiae LEU2* gene carried by this plasmid is capable of complementing mutations in the *leu1* gene of *S.pombe* and the *leuB6* gene of *E.coli*. The plasmid also contains antibiotic resistance genes amp^r and tet^r which allow the use of the antibiotics ampicillin and tetracycline for plasmid selection in *E.coli* (Fig. 2.2).

2.2.2.c: pDB262

pDB262 is capable of autonomous replication in *E.coli* and *S.pombe*. Cloning DNA fragments into either the *Hin*dIII or *Bcl*I inactivates the lambda cI repressor gene product, and allows expression of the tetracycline resistance gene which is fused to the bacteriophage lambda P_R promoter. This plasmid is especially useful for gene library construction, since selection for tetracycline resistance ensures that all *E.coli* transformants contain recombinant plasmids (Wright *et al*, 1986; Fig. 2.2).

2.2.2.d: pIRT2

This plasmid was constructed from the vector pUC18 by inserting a 1.2kb *Eco*RI fragment containing ARS1 into the *Eco*R1 site in the polylinker, and a 2.2kb *Hin*dIII fragment containing the *LEU2* gene into the *Hin*dIII polylinker site (Russell, 1989). This plasmid is capable of autonomous replication in *S.pombe* and *E.coli* and allows the use of polylinker sites for the cloning of DNA fragments. However, there is no means of selecting specifically for recombinant plasmids in *E.coli* (Fig. 2.2).

2.2.2.e: pIRTU

This plasmid is similar to pIRT2, the only difference being that pIRTU contains a 1.7kb *Hin*dIII fragment containing the *S.pombe* $ura4^+$ gene in place of the *LEU2* HindIII fragment in pIRT2.

Figure 2.1: pUC and pTZ plasmids.

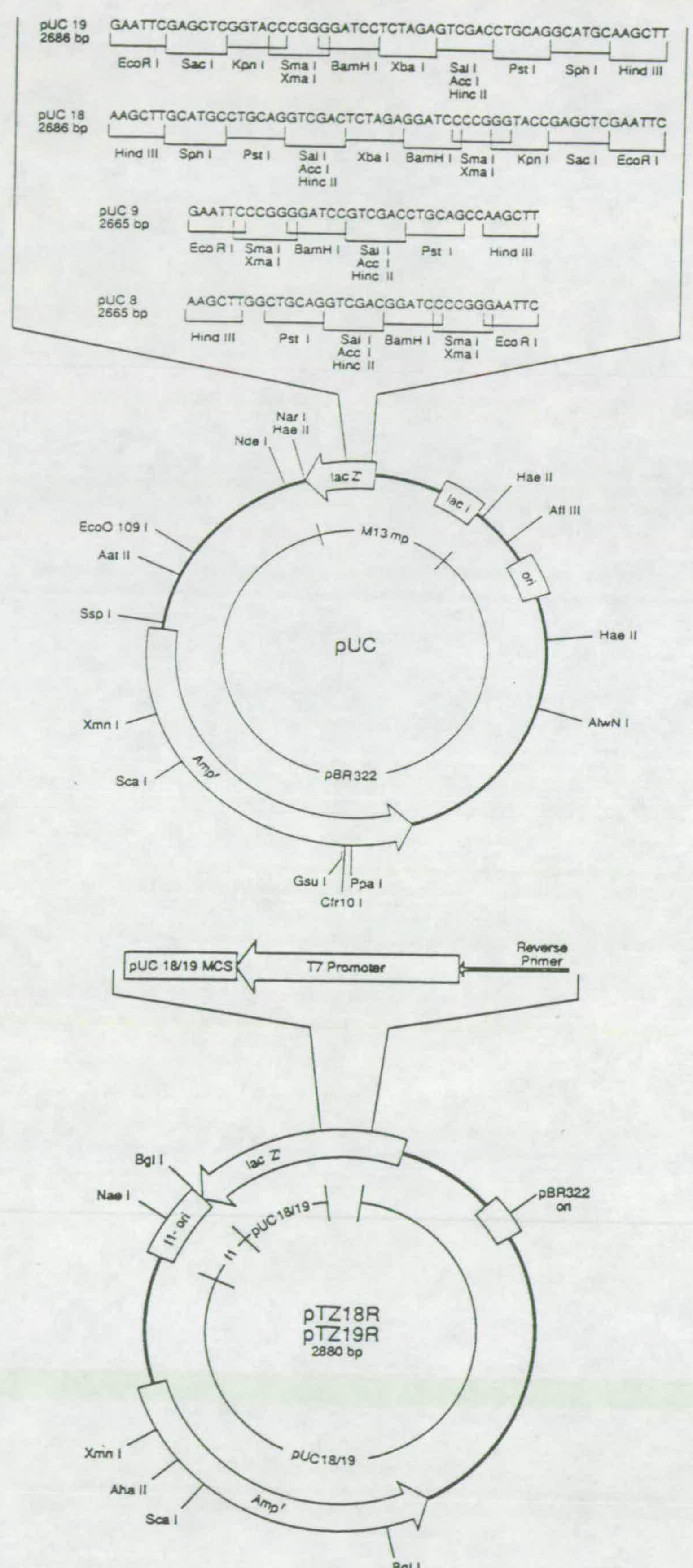
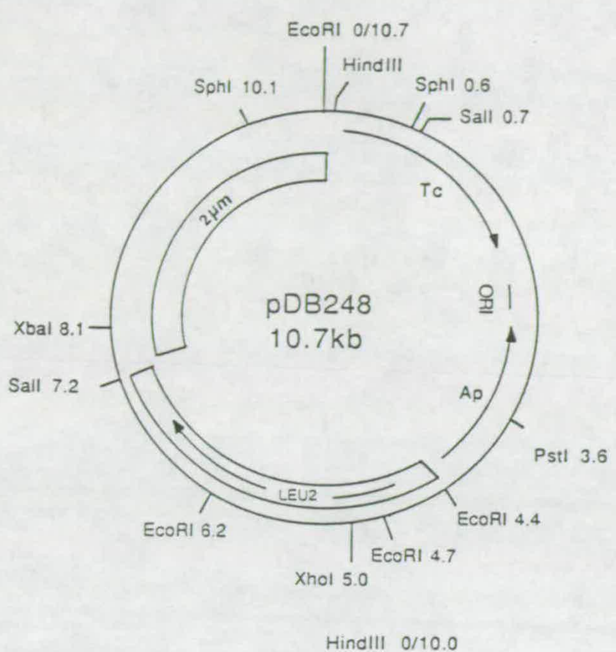
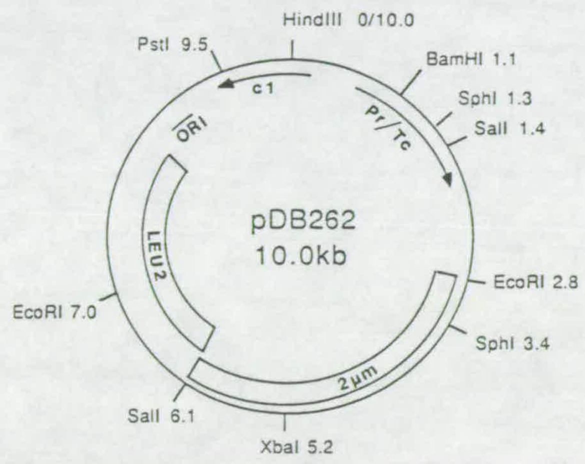
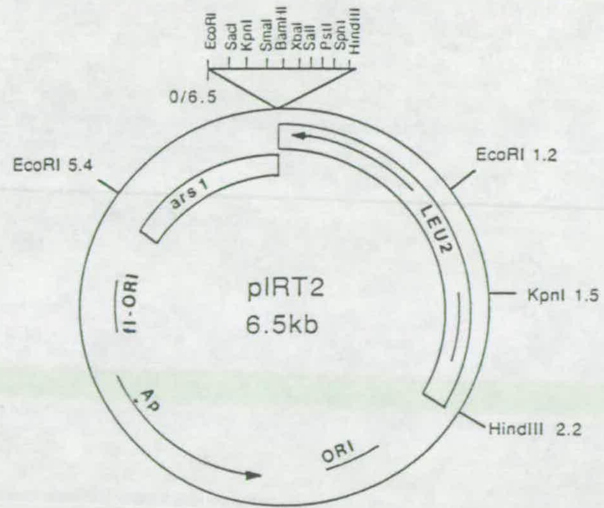


Figure 2.2: pDB248, pDB262 and pIRT2.







2.2.2.f: pWH5

This plasmid was constructed by a modification of pDB262. The truncated ß-lactamase gene of pDB262 was restored by the insertion of a 760bp *Pst*I fragment from pUN121 into the *Pst*I site in pDB262 to allow selection of the plasmid in *E.coli* by ampicillin resistance. Two new restriction sites (*Eco*RI and *Sma*I) were introduced into the cI gene by replacing the 1.1kb *Bcl*I-*Bam*HI fragment with the analogous fragment from pUN121 (Wright *et al*, 1986).

2.2.2.g: pDAM6

The plasmid pDAM6 consists of the 4.0kb *Pst*I fragment containing the *LEU2* gene from the *S.cerevisiae* vector YEp13 inserted into the *Pst*I site of pBR325. Cloning of fragments into the unique *Hin*dIII and *Bam*HI sites within this vector results in insertional inactivation of the tetracycline resistance gene. This plasmid is capable of autonomous replication in *E.coli*, though not in *S.pombe* (Wright *et al*, 1986).

2.2.2.h: pSP100

This vector was derived from the plasmid pFL20 (Losson and Lacroute, 1983) by deletion of the *stb*-containing *Eco*R1 fragment. It contains pBR322, the *S.cerevisiae URA3* gene and the *S.pombe* sequence *ars1*.

2.2.3: Molecular analysis of DNA

2.2.3.a: Restriction enzyme analysis

Restriction endonucleases were used as recommended by the manufacturers in the appropriate buffers supplied with the enzymes. DNA in solution was incubated along with the restriction enzyme at 37°C (unless otherwise recommended) for 1-15 hours. Digests were terminated by the addition of 0.1 volumes of 0.2M EDTA pH 8.0.

2.2.3.b: Treatment with Klenow enzyme for blunt ending

When it was necessary to convert the staggered ends left by some restriction enzymes to blunt ends, the cut DNA (5 - 10 μg) was treated with the Klenow fragment of DNA polymerase I at 25°C in 10 - 20μl of a buffer containing the following:

Potassium phosphate (pH 7.4)	130mM
$MgCl_2$	6.5mM
DTT	1mM
BSA	32μg/ml
dATP/dCTP/dGTP/dTTP	33μM

2.2.3.c: Ligation of DNA

Ligation of restriction enzyme termini was carried out in a solution containing the following:

DNA	1 - 10μg/10 l
Tris-HCl (pH 7.6)	66mM
$MgCL_2$	6.6mM
DTT	1mM
ATP	66 M
T4 DNA ligase	1 - 2 units/10μl

This was incubated for 6-18 hours at 14°C for cohesive termini, and at 25°C for blunt ended termini.

2.2.4: Labelling of DNA fragments with ^{32}P-containing nucleotides

2.2.4.a: Oligonucleotide-primed labelling

50ng of DNA was routinely labelled using the Pharmacia Oligolabelling kit after the method of Feinberg and Vogelstein (1983). Appropriate DNA fragments were isolated by excising bands from 0.8% w/v low melting point agarose ("Seaplaque" made by FMC BioProducts) gels made with TAE electrophoresis buffer (section 2.2.5). The isolated fragment was placed in a pre-weighed microcentrifuge tube, and water added at a ratio of 3ml H_2O/g of gel. The tube was then placed in boiling water for 10 minutes to melt the agarose and denature the DNA.

The labelling reaction was carried out by the addition of the following reagents:

(i) H_2O (to a total volume of 50μl)

(ii) 10μl OLB buffer (see below)

(iii) 2μl of 10mg/ml bovine serum albumin

(iv) 30 - 50ng DNA in agarose (up to 32.4μl total volume)

(v) 5μl of [^{32}P]dCTP at 10 Ci/μl (Amersham)

(vi) 2 units of large fragment of *E.coli* DNA polymerase I (Klenow).

The reaction was incubated at room temperature for 4 - 12 hours, before being stopped by the addition of 200μl of a solution containing 20mM NaCl, 20mM Tris-HCl (pH 7.5), 2mM EDTA, 0.25% SDS, 1μM dCTP.

OLB is made from the following components:

Solution O: 1.25M Tris-HCl, 0.125M $MgCl_2$ at pH 8.0.

Solution A: 1ml solution O + 18µl 2-mercaptoethanol + 5µl of dATP, dTTP and dGTP at a concentration of 0.1M.

Solution B: 2M Hepes, titrated to pH 6.6 with 4M NaOH.

Solution C: Hexadeoxyribonucleotides (Pharmacia) evenly suspended in TE at 90 OD units/ml.

To prepare OLB, solutions A, B and C were mixed in a ratio of 100:250:150, respectively.

2.2.4.b: Production of labelled single stranded probes

Single stranded DNA obtained from pTZ plasmids (Section 2.4.1) was annealed with the reverse sequencing primer, which is a 17 base oligonucleotide complementary to a sequence neighbouring the polylinker in pTZ 18/19, in the following solution:

5µl single stranded DNA (0.5µg)

2µl reverse sequencing primer (5ng)

1µl Klenow enzyme buffer (as supplied by manufacturers)

4.5µl H_2O

The reverse sequencing primer 1. This mixture was heated to 80°C for 10 minutes, and then allowed to cool slowly to room temperature. The following solutions were then added:

1µl of [^{32}P]dCTP at 10 Ci/µl

1µl DTT (0.1M)

1µl of a solution containing 0.5mM dGTP, dATP and dTTP

2 units Klenow enzyme.

This was incubated at room temperature for 1 - 2 hours, before stopping the reaction by adding of 1µl of 0.4M EDTA.

2.2.5: Agarose gel electrophoresis of DNA

2.2.5.a: Electrophoresis buffers

(i) E Buffer

Tris base	36mM
NaH_2PO_4	30mM
EDTA	1mM

(final pH 7.6)

(ii) TAE buffer

Tris-acetate	40mM
EDTA	1mM

(final pH 8.0)

A 5 x concentrated stock was made by dissolving 24.2g of Tris base in water, adding 57.1ml glacial acetic acid and 25ml 0.4M EDTA (pH8.0), and adding water to a final volume if 1l.

2.2.5.b: Methods

For the analysis of cut and ligated DNA preparations, 0.6% agarose gels in a variety of shapes and sizes were used. Agarose (type 2, medium EEO, Sigma) was dissolved in 1 x electrophoresis buffer by brief boiling. Gels were run with an applied voltage of 4-8 volts/cm. Following electrophoresis, the gel was stained with a 1 μg/ml solution of ethidium bromide for 10-30 minutes. The DNA was then visualised with an ultra-violet transilluminator (Ultra Violet Products) and photographs taken with a Polaroid MP4 land camera and Polaroid Type 667 positive film.

2.2.5.c: Isolation of DNA fragments

To isolate DNA fragments from agarose gels, a gel slice containing the appropriate fragment was excised from low gelling temperature agarose in TAE buffer. A volume of TE buffer equal to three times that of the gel slice was added and the agarose melted at 60-65°C for 10 minutes. The mixture was cooled, extracted with phenol and chloroform, and the DNA recovered by precipitation with ethanol. The DNA was finally resuspended in an appropriate volume of water or TE buffer.

2.2.6: Agarose gel electrophoresis of RNA

2.2.6.a Electrophoresis buffer

A 5x buffer stock (pH 7.0) was made to give a final concentration:

25mM MOPS

5mM NaOAc

1mM EDTA

2.2.6.b: Methods

1.2g agarose (type 2, medium EEO, Sigma) was dissolved in 63ml H2O + 16ml 5x buffer by heating, followed by cooling to 60°C. 17ml of 38% w/v solution of formaldehyde

was added, mixed, and the gel poured immediately. Samples for loading were prepared by the addition of 5M NaCl to a final concentration of 0.15M and 2.5 volumes of absolute EtOH, followed by precipitation overnight at -20°C. Following centrifugation, the pellet was redissolved in 30µl of the following buffer:

formamide (deionized) 600µl

formaldehyde (38% w/v) 200µl

5x electrophoresis buffer 240µl

H_2O 160µl.

Formamide was deionised with mixed bed resin (AG 501-X8 D, BioRad Laboratories, as described by Maniatis *et al*, 1982). The RNA samples were incubated at 60°C for 5 minutes followed by the addition of 1µl of 2mg/ml ethidium bromide, and loading into the gel slots. These gels were run under the same conditions as described in Section 2.2.5. One slot was loaded with a small amount of tracking dye (containing bromophenol blue) as described in Section 2.2.5.

2.2.7: Filter hybridisation of DNA

2.2.7.a: Southern blotting.

A modification of the method of Southern (1975) was used to detect specific DNA fragments from plasmids or within the *S.pombe* genome. GeneScreen or GeneScreen Plus nylon membranes (NEN-Du Pont) were used to immobilise the DNA and the procedures for hybridisation taken from the manufacturers' instructions. Southern blotting was carried out either using capillary absorption or by vacuum blotting.

When the capillary method was used the agarose gel was soaked in 0.2N NaOH 0.6 NaCl for 30 minutes in order to denature the DNA, and then neutralised in blotting buffer (0.025M Na_2HPO_4/NaH_2PO_4 pH 6.5) for 1 hour with three changes of buffer. The gel was then placed on a blotting apparatus that allowed blotting buffer to be drawn up through sheets of blotting paper acting as wicks, through the gel, then the nylon membrane, and finally to be absorbed into a stack of paper towels. Transfer was allowed to continue for at least 12 hours.

The vacuum blotting method used apparatus from Pharmacia (LKB 2016 VaCuGene vacuum blotting system). The gel was treated with depurination buffer (0.25N HCl), denaturation buffer (1.5M NaCl 0.5M NaOH), and neutralising buffer (1.0M Tris-HCl pH 5.0 2.0 NaCl) for 3-4 minutes each. These were drawn into the gel by the applied vacuum, and removed by aspiration after the allotted time. Finally transfer was allowed

to proceed for 20-30 minutes in a solution of 20x SSC buffer (3M NaCl 0.3M sodium citrate) through the gel onto the nylon membrane.

Once transfer was complete, the membrane was removed from the gel and washed in water or blotting buffer to remove any residual agarose. The membrane was then air dried and, in the case of GeneScreen membrane, baked under vacuum at 80°C for 3 hours.

2.2.7.b: Hybridisation of filters

Hybridisation of the two types of membrane was carried out according to the manufacturers' instructions, in each case following the preferred protocols described using 50% formamide in the prehybridisation and hybridisation solutions. Hybridisations were carried out at 42°C and were always allowed to proceed for at least 6 hours. The membrane was washed according to manufacturers' instructions in each case, and, after washing, allowed to air dry before being wrapped in Saran Wrap and autoradiographed.

2.2.7.c: Autoradiography

The wrapped filter was placed in an X-ray film cassette adjacent to a preflashed sheet of Kodak X-Omat S type 1 film. An intensifying screen (Du Pont Lighting Plus) was placed next to the film and the sealed cassette placed at -70°C. After a sufficient exposure time, the film was developed in Kodak LX-24 developer for 4 minutes, washed in water, and fixed in Kodak FX-40 fixer for 5 minutes. The film was rinsed extensively in water and air dried.

2.2.7.d: Removal of probe from hybridisation filters.

GeneScreen filters were washed in the following buffer at 65 - 70°C for 1 - 3 hours:

Tris-HCl (pH 8.0)	5mM
EDTA	0.2mM
Sodium pyrophosphate	0.05%
Ficoll	0.002%

GeneScreen Plus filters were washed in 0.4M NaOH at 42°C for 30 minutes, followed by 0.1 x SSC, 0.2M Tris-HCl (pH 7.5) at 42°C for 30 minutes.

2.2.8: Filter hybridisation of RNA

2.2.8.a: Northern blotting

Transfer of RNA samples from gel to membrane was carried out using the capillary method, similar to that described for Southern blotting, although the gel required no pretreatment. Transfer was carried out overnight onto GeneScreen membrane. Once transfer was complete, the membrane was washed in blotting buffer to remove any residual agarose, and baked in a vacuum oven at 80 - 100°C for 2 - 4 hours.

2.2.8.b: Hybridisation

This was carried out as described for Southern blots. Following hybridisation, filters were washed according to the manufacturers instructions for Northern blots.

2.2.8.c: Autoradiography

This was carried out as described for Southern blots.

2.3: METHODS FOR THE MANIPULATION AND HANDLING OF SCHIZOSACCHAROMYCES POMBE

2.3.1: General methods

2.3.1.a: Strains

The wild type and mutant strains of the fission yeast *Schizosaccharomyces Pombe* Lindner were all derived from the heterothallic 972 (mating type h^-) and 975 (mating type h^+) isolates described by Leupold (1950).

Strain list:

(i) *win1.1*-containing strains:

wee1.50 cdc25.22 win1.1 h^+
wee1.50 cdc25.22 win1.1 h^-
wee1.50 cdc25.22 win1.1 leu1.32 h^+
wee1.50 cdc25.22 win1.1 leu1.32 h^-
win1.1 h^+
win1.1 h^-
win1.1 leu1.32 h^-
win1.1 leu1.32 h^+
cdc13.117 win1.1 h^-
wee1.50 win1.1 h^-
cdc2.1w win1.1 h^+
cdc2.1w win1.1 leu1.32 h^-
cdc2.3w win1.1 h^+
cdc2.3w win1.1 leu1.32 h^-
wee1.50 win1.1 h^+
win1.1 ura4.D18 h^+
win1.1 [adh-cdc25:ura4] ura4.D18
wee1.50 win1.1 ura4.D18
wee1.50 win1.1 [adh-cdc25:ura4] ura4.D18
win1.1 mcs3.12 wee1.50 cdc25.22 leu1.32
win1.1 mcs4.13 wee1.50 cdc25.22 leu1.32
win1.1 mcs6.13 wee1.50 cdc25.22 leu1.32
win1.1 mcs4.13

win1.1 ade6.216 ura4.D18 leu1.32 h^-

win1.1 leu1.32 swi5.39 h^- (SW1 and SW2)

win1.1 lys1.131 his6.365 swi5.39 h^+

win1.1 lys1.131 his6.365 ura1.171 swi5.39 h^+

(ii) Cell cycle mutants:

wee1.50 cdc25.22 h^+

wee1.50 cdc25.22 h^-

cdc13.117 h^+

cdc13.117 leu1.32 h^+

cdc13.117 leu1.32 h^-

wee1.50 h^+

wee1.50 leu1.32 h^+

wee1.50 leu1.32 h^-

cdc2.1w leu1.32 h^+

cdc2.1w leu1.32 h^-

cdc2.3w leu1.32 h^+

cdc2.3w leu1.32 h^-

wee1.50 cdc2.1w

wee1.50 cdc2.3w

wee1.50 cdc2.1w h^+

wee1.50 cdc2.1w h^-

wee1.50 cdc2.3w h^+

wee1.50 cdc2.3w h^-

[adh-cdc25:ura4] ura4.D18 leu1.32 h^-

mcs3.12 wee1.50 cdc25.22 leu1.32 h^-

mcs4.13 wee1.50 cdc25.22 leu1.32 h^-

mcs6.13 wee1.50 cdc25.22 leu1.32 h^-

mcs4.13 leu1.32 h^-

cdc25.22 leu1.32 h^-

cdc2.33 ura4.D18 leu1.32 h^-

cdr1.34 leu1.32 h^+

cdr2.69 leu1.32 h^-

(iii) Developmental mutants

pat1.114 ade6.216 leu1.32 h^-

pat1.114 win1.1 h^-

cgs1.1 ade6.216 leu1.32 h^{90}

cgs2.3 ade6.210 h^{90}

(iv) *wis1* disruptant strains:

wis1::LEU2 ade6.216 ura4.D18 leu1.32 h^+ (D4)

wis1::LEU2 wee1.50 ura4.D18 ade6.216 leu1.32 h^+

wis1::LEU2 cdc2.1w ura4.D18 leu1.32 h^-

wis1::LEU2 cdc2.3w ade6.216 leu1.32 h^-

wis1::LEU2 [adh-cdc25:ura4] ura4.D18 leu1.32

wis1::LEU2 pat1.114 ura4.D18 ade6.216 leu1.32 h^+

wis1::LEU2 pat1.114 ura4.D18 ade6.216 leu1.32 h^-

wis1::LEU2 win1.1 ade6.216 ura4.D18 leu1.32 h^+

wis1::LEU2 win1.1 ade6.216 ura4.D18 leu1.32 h^+

(v) Others:

leu1.32 h^+

leu1.32 h^-

ura1.131 lys1.171 ade6.704 mat2.102

ura1.171 his6.365 lys1.131 ade4.31 swi5.39 h^+

swi5.39 h^{90}

ade2.17 swi5.39 h^-

ade2.17 h^-

tps19.17 h^-

ade6.210 ura4.D18 leu1.32 h^+

ade6.210 ura4.D18 leu1.32 h^-

ade6.216 ura4.D18 leu1.32 h^+

ade6.216 ura4.D18 leu1.32 h^-

Notes:

Details of strains containing integrated copies of the pwis plasmids are described in Table 3.4 and 5.4.

Details of strains containing increased copies of *wis1* are described in Section 6.2.2.

All strains with a disrupted allele of *wis1* described above are derived from disruptant strain D4.

Mating types are indicated where known.

2.3.1.b: Media and growth conditions for S.pombe.

Strains of *S.pombe* were routinely cultured on solid YEA complex medium containing per litre:

glucose	30g
yeast extract	5g
adenine	75mg
uracil	75mg

For liquid culture, the complex medium YEPD was used containing per litre:

glucose	30g
yeast extract	5g
Bacto-peptone	5g

The minimal medium used, EMM, was a minor modification of EMM2 (Mitchison, 1970; as modified by Nurse, 1975). It contains the following compounds per litre:

glucose	20g
potassium hydrogen pthalate	3g
disodium hydrogen phosphate (anhydrous)	1.8g
ammonium chloride	5g

Vitamins, salts, and trace minerals were added from sterile stock solutions as described by Mitchison (1970).

EMM-glut contained 3.74g/l monosodium glutamate substituted for NH_4Cl. For solid media, 20g/l agar and 1ml 1N KOH were added. Amino acids, adenine or uracil were added from sterile stock to a final concentration of 7.5mg/ml where appropriate. Phloxin B was added to a final concentration of 20μg/ml when required after autoclaving. This dye aids the detection of colonies containing an increased number of dead cells (Kohli *et al*, 1977). 1.2M sorbitol was included in solid media for the regeneration of osmotically sensitive spheroplasts.

A nitrogen limiting medium ME consisting of 30g/l malt extract and 20g/l agar was used to induce conjugation and sporulation.

2.3.1.c: Storage of S.pombe

Strains of S.pombe were stored for up to a few months on yeast extract plates or slants at 4°C. Long term storage was carried out on silica gels as described by Gutz *et al*, (1974) or in medium containing 30% glycerol at -70°C.

2.3.2: Genetic analysis of S.pombe

2.3.2.a: Crossing strains

The standard genetical procedures of Gutz *et al*, (1974) and Kohli *et al*, (1977) were followed. Strains were crossed by mixing together fresh isolates of two cell types on the surface of an ME plate. The mating mix was incubated at 25°C for 2 to 3 days to allow zygotes and/or asci to form. Crosses were between strains of h^+ and h^- mating types unless otherwise indicated. The progeny of crosses were examined either by random spore analysis or by tetrad analysis.

2.3.2.b: Random Spore Analysis

A loopful of mating mix was resuspended in 1ml of sterile distilled water containing 20 l of a stock solution of the snail gut enzyme Helicase (Suc d'Helix pomatia, Industrie Biologique, France) and incubated overnight at 35°C. The stock is a 1 in 10 dilution of the preparation supplied. The spore concentration was estimated by a haemocytometer count, and an appropriate dilution plated onto solid media .

2.3.2.c: Tetrad analysis

Single asci were isolated from a streak of the mating mix on a YEA plate using a fine glass needle attached to a Leitz micromanipulator. The plate was then incubated at 35°C for approximately 8 hours or overnight at 20°C to allow the ascus wall to break down. The spores were then separated on the surface of the plate with the micromanipulator and allowed to form colonies.

2.3.2.d: Analysis of phenotypes

The phenotypes of the cells within a colony were tested by replica plating or by streaking from a master plate onto EMM plus or minus growth supplements for auxotrophs, and onto YEA or fully supplemented EMM at the restrictive and permissive temperatures for temperature sensitive strains.

2.3.2.e: Diploid construction

Two methods for constructing diploids were used: one involved the *mat2.102* (*mei1.102*) mutation, and the other complementing alleles of *ade6*.

Strains carrying the *mat1.102* mutation at the mating type locus are able to conjugate with either h^+ or h^- strains, but in the case of an h^- partner, the diploid nucleus formed is unable to sporulate. In suitable genetic background, diploid clones may be selected on media on which the haploid parents are unable to grow. The two strains were crossed as described above, but after incubation overnight, the mating mix was streaked onto a suitable selective medium containing Phloxin, and diploid colonies identified by their darker red colour.

If sporulation competent diploids were required, then h^+/h^-strains could be constructed using complementing alleles of *ade6*. The alleles *ade6.210* and *ade6.216* both confer adenine requirement for growth, but heterozygous diploids *ade6.210/ade6.216* are prototrophic. The advantage of this system is that very little recombination occurs between these two loci, so very few prototrophic haploids arise. The diploids produced may be induced to sporulate easily, which is useful for some forms of genetic manipulation, such as deletion of an essential gene. The disadvantage of this system is that the diploids must be continuously kept in growth, for they will sporulate once stationary phase is reached. Strains carrying the alleles were crossed in the normal way, left to conjugate overnight and then streaked onto media that imposed a selection for adenine. Diploid colonies were recognised by their colour on phloxin-containing plates, and their ability to sporulate checked microscopically.

2.3.3: Cell physiology

2.3.3.a: Growth of liquid cultures

A single colony was inoculated into a 10ml EMM or YE preculture and incubated at the permissive temperature until stationary phase was reached. An aliquot of the preculture was inoculated into 200ml of an appropriate liquid medium in a 500ml Erlenmeyer flask and incubated with shaking for 18 - 14 hours at 25°C or 35°C.

2.3.3.b: Determination of cell number

Cell number per ml of culture was determined either by haemocytometer count, or from a 0.1ml sample fixed in a filtered 0.1% formaldehyde, 0.1% sodium chloride solution.

After sonication to ensure that clumps were broken up, the cells were counted electronically with a Coulter counter (Industrial D) as described by Mitchison (1970).

2.3.3.c: Temperature shift experiments

A culture of the appropriate strain was incubated with shaking at the permissive temperature until the early exponential phase of growth was reached. At this point half of the culture was transferred to a fresh flask at the restrictive temperature, with the remainder of the culture remaining at the permissive temperature to act as a control.

2.3.3.d: Cell length measurements

Cells were grown to a density of 1.0 - 5.0 x 10^6 cells/ml in the required liquid medium. The length of at least 24 septated cells were measured using an eyepiece graticule calibrated against a micrometer slide on a Zeiss photomicroscope using a 40 x objective.

2.3.3.e: Staining of S.pombe **to reveal DNA and actin distribution.**

Exponentially growing cells in liquid culture were fixed with 3% formaldehyde, by the addition of $^{1}/_{10}$ $^{vol}/_{vol}$ 30% formaldehyde in PM buffer.

PM buffer (pH 6.5)

KH_2PO_4	40mM
K_2HPO_4	40mM
$MgCl_2$	0.5mM

3g p-formaldehyde was added to approximately 8ml PM buffer and mixed thoroughly. This was heated to 60°C and 1ml 5M NaOH added. More was added if the solution did not clear. This was then made up to 10ml with PM buffer and cooled. Typically cells from 10ml of a log-phase *S.pombe* culture were fixed. The cells were kept suspended during fixation for 30 minutes, after which time they were harvested by centrifugation and washed 3 times with fresh PM buffer. The cells were permeablized by resuspension in PM buffer containing 1% Triton X-100 for 30 seconds, and then washed again with fresh PM buffer 3 times. Following the final wash, the cells were resuspended in 50-100µl PM buffer or rhodamine-phalloidin solution (20µg/ml in 10% methanol, 90% PM buffer) when required. Monolayers of cells were air dried down onto coverslips and inverted onto a drop of 1µg/ml DAPI. The coverslips were sealed with nail varnish and observed.

Stained cells were examined using the 40x (Neofluar) objective on a Zeiss photomicroscope. Epifluorescent illumination (Wotan Mercury lamp HBO-50W) was

used in combination with Zeiss filter set 48 77 15 for rhodamine excitation and 48 77 02 for DAPI excitation.

DAPI will complex preferentially with A-T rich double-stranded DNA molecules (Williamson and Fennel, 1975) Rhodamine-conjugated phalloidin has been shown to be a specific stain for actin (Wehland *et al*, 1980).

2.3.4: Transformation procedures for S.pombe.

2.3.4.a: Protoplast transformation method

This method is similar to that described by Beach and Nurse (1981), and gives transformation frequencies of up to 1×10^4 transformants per μg plasmid DNA.

Solutions:

(i) TF1

Sorbitol	1.2M
Citrate-phosphate (pH5.6)	20mM
ß-mercaptoethanol	1% v/v
EDTA (pH 5.6)	40mM

(ii) TF2

Sorbitol	1.2M
Citrate-phosphate (pH5.6)	20mM
ß-mercaptoethanol	0.2% v/v

(iii) TF3

Sorbitol	1.2M
Tris-HCl (pH7.6)	10mM

(iv) TF4

Polyethylene glycol 4000	20%
Tris-HCl (pH7.6)	10mM
$CaCl_2$	10mM*

(v) TF5

Tris-HCl (pH7.6)	10mM
Yeast extract	0.5mg/ml
Leucine	0.5mg/ml

Uracil 0.5mg/ml

$CaCl_2$ 10mM*

* A sterile 1M solution of $CaCl_2$ was added after autoclaving to the required concentration.

Cells were grown in 200ml of EMM plus any appropriate supplements to a density of approximately 0.5 - 1.0×10^7/ml, and then harvested in a Sorvall RC-5B centrifuge at 7krpm for 5 minutes. The pellet was resuspended in 30ml of TF1 and transferred to a sterile polypropylene tube. The cells were then harvested in a MSE benchtop centrifuge and the pellet suspended in 5ml of filter-sterilised TF2 containing 5mg/ml NovoSP enzyme. Cells were incubated at 30°C and protoplast formation monitored microscopically. When the sample contained approximately 50% spherical protoplasts, the cells were harvested a MSE benchtop centrifuge at half speed. The protoplasts were washed three times in TF3 by centrifugation, and resuspended to a final density of 5×10^8/ml in TF3 containing 10mM $CaCl_2$. Plasmid DNA in a volume of 10µl or less was added to 0.1ml of the protoplast suspension in an Eppendorf tube, and the mixture incubated at room temperature for 15 minutes. 1ml of Tf4 was added and the mixture incubated for a further 15 minutes. The protoplasts were pelleted and resuspended in TF5. After incubation at 25°C for 1 hour, the cells were spread gently onto the surface of an EMM-sorbitol plate and incubated at 28°C until colonies appeared.

2.3.4.b: Lithium thiocyanate transformation procedure

This method is adapted from that described by Keszenman-Pereyra and Hieda (1988) for *S.cerevisiae* and does not require the formation of protoplasts, but gives transformation frequencies of only 2 - 5×10^3 transformants per µg plasmid.

Solutions:

(i) Tris buffer

10mM Tris-HCl (pH 7.6)

(ii) TB (filter sterilised)

200mM lithium thiocyanate

1mM magnesium acetate

0.2mM calcium acetate

10mM Tris-HCl (pH 7.6)

(iii) TBT

1ml TB

0.15ml triacetin (filter sterilised)

(iv) PEG

70g polyethylene glycol 4000

100ml Tris buffer.

Cells were grown to a density of approximately 0.5 - $1.0x10^7$/ml. 10ml of this culture centrifuged in a MSE benchtop centrifuge, the cells resuspended in 10ml sterile water and centrifuged again. The pellet was then resuspended in 0.5ml of freshly made up TBT. For each transformation, 0.115ml of this cell suspension was used, to which was added 10 l of a solution containing 250ng to 2μg plasmid DNA. Two volumes of PEG were then added, mixed well, and the cell suspension incubated at 30°C for 2 hours. The cells were then heat shocked at 42°C for 5 minutes, 1ml of Tris buffer added, and the cells collected by a short period of centrifugation in a microfuge. The pellet was resuspended in 200μl Tris buffer, and the cells plated directly onto selective media.

2.3.5: Preparation of DNA from S.pombe

2.3.5.a: Genomic DNA

Cells were grown to late log phase (1 - $2x10^7$/ml) in 200ml of EMM with appropriate supplements. They were harvested by centrifugation for 5 minutes at 7 krpm in a Sorvall RC-5B centrifuge, and washed once in 50ml 20mM citrate-phosphate (pH5.6). The cells were then harvested by centrifugation in a MSE benchtop centrifuge and the supernatant discarded. They were resuspended in 5ml TF1. A further 5ml of this buffer was added containing 50mg NovoSP enzyme and the cells incubated at 30°C. When more than 80% of the cells had become osmotically sensitive, they were harvested at half speed in a MSE benchtop centrifuge and the pellet resuspended in 4ml 0.15M NaCl, 0.1M EDTA (pH 8.0). 0.2ml Proteinase K (1mg/ml) and 0.15ml 25% SDS was immediately added and the preparation incubated at 45°C for 1 hour, followed by 15 minutes at 70°C, and finally cooled on ice. 1/10 volume of 5.0M KOAc was added, and the sample incubated on ice for 30 minutes or longer. The sample was then centrifuged at 9krpm for 10 minutes, the supernatant transferred to a fresh tube, 0.4ml RNAse (1mg/ml) added and the sample incubated at 37°C for 1 hour. Following cooling to room temperature, the sample was extracted once with chloroform, and 2 volumes of ethanol added to the aqueous phase. The sample was cooled to -20°C and centrifuged to recover the precipitate. The pellet was drained well and resuspended in 4ml TE buffer. The sample was extracted with phenol/chloroform, and the DNA recovered by a second

precipitation with ethanol. The final pellet was resuspended in 1ml TE buffer and the DNA concentration estimated by comparison with known standards on agarose gels.

2.3.5.b: Recovery of plasmid DNA from S.pombe

Cells were grown to a density of 1 - $2x10^7$/ml in a volume of 10ml under selective conditions and harvested in an MSE benchtop centrifuge. The pellet was resuspended in 1.5ml TF1 containing 5mg/ml NovoSP enzyme and incubated at 30°C until the cells were over 90% osmotically sensitive. The cells were harvested at low speed in a microfuge and resuspended in 300μl TE buffer. 35μl 10% SDS (w/v) was added and the tube incubated at 65°C for 10 minutes. 100μl of 5M KOAc was then added, the contents of the tube mixed well, and incubated on ice for 30 minutes. The tube was then spun at high speed at 4°C in a microfuge for 10 minutes. The supernatant was removed to a fresh tube and treated with the GeneClean Kit (Stratech Scientific Ltd.) as follows: 50μl of supernatant was added to 100μl NaI solution along with 5μl of "glassmilk". This was incubated at room temperature for 5 minutes and then spun for 5 seconds to pellet the silica particles. The pellet was washed 3 times with 400μl of "NEW" wash. The DNA was eluted from the silica twice with 10μl of TE at 55°C for 3 minutes each time. 5μl of this solution (equivalent to 250μl of original yeast culture) was then transformed into 100μl of competent E.coli JA226.

2.3.6: Preparation of RNA from S.pombe

A culture of the appropriate strain was grown to late log phase in EMM plus appropriate supplements. Cells from 50ml of this culture were harvested by centrifugation, transferred to an Eppendorf tube, and washed twice in 1ml 0.15M NaCl by centrifugation in a microfuge. The cells were then resuspended in 0.5ml of an RNA extraction buffer, which consisting of the following components:

NaCl	0.5M
Tris-HCl (pH7.6)	0.2M
EDTA	0.01M
SDS	1.0% w/v

The cell suspension was transferred to a glass tube, and an equal volume of acid washed glass beads (0.5mm diameter) added. 0.5ml of a 1:1 mixture of phenol:chloroform was then added, the mixture vortexed for 40 seconds, placed on ice for 60 seconds, and then vortexed for a further 40 seconds. The cells lysate was transferred to an Eppendorf tube and centrifuged for 5 minutes. The aqueous phase was removed and extracted with 1:1 phenol:chloroform, followed with an equal volume of chloroform. 2.5 volumes of ethanol was added, and the RNA precipitated at -20°C for a few hours, followed by

recovery by centrifugation. The pellet was washed once in 70% ethanol, allowed to dry, and resuspended in 100μl TE buffer. The RNA concentration was determined by measuring A_{260nm}, where 1 unit is equivalent to 40μg/ml RNA. The ration of A_{260nm}:A_{260nm} was used to estimate the purity of the RNA sample.

2.4: METHODS FOR Escherichia coli

2.4.1: General methods

Methods used for the routine handling of E.coli were taken from Maniatis *et al* (1982)

2.4.1.a: Strains

The following strains were routinely used:

Strain Genotype

JA221 *recA1 leuB6 trpE5 hsdR- hsdM+ lacY600*

JA226 *recBC leuB6 trpE5 hsdR- hsdM+ lacY600*

DB6656 *pyrF::u trp lacZ hsdR- hsdM+*

JM101 *supE thi D(lac-proAB) (F' traD36 proAB lacIqZDM15)*

BJ5183 *F- recBC- sbcB- endA galK met- strR thi-1 bioT hsdR*

5K *F- thi- thr-*1 *leuB6 lacY*1 *tonA*21 *supE*44 (lambda)$^-$ r_K^- m_K^+

554 *araD*189Δ 7697Δ *lacX*74 *galG*$^-$ *gulK*$^-$ *hsn*$^-$ *hsm*$^+$ *strA recA*13

Mutations in the *leuB* (JA221 and JA226) are complemented by the *S.cerevisiae LEU2* gene, and mutations in *pyrF* are complemented both by the *S.cerevisiae URA3* gene, and by the *S.pombe ura4*$^+$ gene. DB6656 was used specifically for the detection of plasmids containing *ura*$^+$ sequences, and JA221 and JA226 for the detection of *LEU2* sequences. JA226 and BJ5183 were used for the recovery of plasmid from *S.pombe*, and JM101 for the propagation of pUC-based plasmids and recovery of single stranded DNA. 5K was used in the transposon mutagenesis protocol (Section 2.5). Both JA221 and JM101 were used for routine plasmid propagation.

2.4.1.b: Media and growth conditions for E.coli

Strains of *E.coli* were routinely cultured on the rich medium LB consisting of the following:

Bacto-Tryptone	10g/l
NaCl	10g/l
Yeast extract	5g/l

Glucose was added from a sterile stock solution to 1g/l after autoclaving.

For the production of single stranded DNA, cells were grown on 2xYT medium.

Bactotryptone	16g/l
NaCl	10g/l
Yeast extract	10g/l

Two forms of minimal medium were used:

(i) DMM (Davis minimal medium)

K_2HPO_4 (anhydrous)	10.5g/l
KH_2PO_4	4.5g/l
$(NH_4)_2SO_4$	1.0g/l
Sodium citrate	0.5g/l

After autoclaving, the following solutions were added:

20% $MgSO_4$	1ml
1mg/ml thiamine	1ml
3.75mg/ml tryptophan	10ml
40% glucose	5ml

(ii) M9

Na_2HPO_4	6g/l
KH_2PO_4	3g/l
NaCl	0.5g/l
NH_4Cl	1g/l

The pH of this solution was adjusted to 7.4 and then the following components added:

1M $MgSO_4$	2ml
20% glucose	10ml
1M $CaCl_2$	0.1ml

Cells were cultured at 37°C unless otherwise stated. Cell growth was estimated by optical density on a Unicam SP600 spectrophotometer.

2.4.1.c: Antibiotics

(i) Ampicillin

A stock solution of 100mg/ml of the sodium salt was made in 50% ethanol 50% water. This was used at a final concentration of 50 - 100μg/ml.

(ii) Chloramphenicol

Solid chloramphenicol was dissolved in 100% ethanol at 34 mg/ml. This was added to media at a final concentration of 10 μg/ml.

(iii) Kanamycin

Kanamycin sulphate was dissolved in water at 25 mg/ml. This was used at a final concentration of 50 - 70 μg/ml.

(iv) Tetracycline

Tetracycline hydrochloride was dissolved in 50% ethanol 50% water at 12.5mg/ml. This was used at a final concentration of 12.5 - 15μg/ml.

Antibiotic stock solutions were stored at -20°C and were added to autoclaved media cooled to 55°C.

2.4.1.d: X-gal

X-gal (5-bromo-4-chloro-3-indolyl-ß-D-galactoside) stock solution was made in dimethylformamide at 20mg/ml and stored at 4°C. It was used at a final concentration of 20μg/ml.

2.4.1.d: Storage

Strains were stored for up to a month on LB agar plates at 4°C. Long term storage was in medium containing 20% glycerol at -70°C.

2.4.2: Transformation of E.coli

Two methods of preparing competent cells were used: the first method gives cells that could be used the same day or the day after. The second method described here gives competent cells that can be stored for long periods at -70°C before use.

2.4.2.a Calcium chloride procedure

A stationary phase culture of *E.coli* in LB was diluted 1 in 100 into fresh LB medium and incubated with shaking at 37°C. When the culture reached an optical density A_{650} = 0.2 the cells were chilled on ice for 10 minutes, then transferred to sterile McCartney bottles and harvested by centrifugation at 4°C. The cells were resuspended in half the original culture volume of ice cold 0.1M $CaCl_2$ and incubated on ice for 20 minutes. The cells were pelleted again, and resuspended in 1/100th of the original culture volume

of ice-cold 0.1M $CaCl_2$. Aliquots of 0.1ml were dispensed into Eppendorf tubes and DNA added in a volume of 10μl or less. After incubation on ice for a further 30 minutes, the transformation mixture was heat shocked at 42°C for one minute and then returned to ice. 400μl of LB was added and the cells incubated at 37°C for 30 minutes to 1 hour to allow expression of plasmid borne antibiotic resistance. Appropriate aliquots were spread onto LB plates containing the appropriate antibiotic, which were then incubated overnight at 37°C.

2.4.2.b: Competent cells for frozen storage

Solutions:

(i) TfBI

$CaCl_2.6H_2O$	2.2g/l
Glycerol	150g/l
NaOAc	2.86g/l

The volume was made up to 1l, and the pH adjusted to 5.9 by the addition of a few drops of glacial acetic acid. The following were then added in solid form:

RbCl	12g/l
$MnCl_2$	9.9g/l

(ii) TfBII

MOPS	2.09g/l
$RbCl_2$	1.2g/l
$CaCl_2.6H_2O$	16.4g/l
Glycerol	150g/l

The pH was adjusted to 6.8 by the addition of 10N KOH.

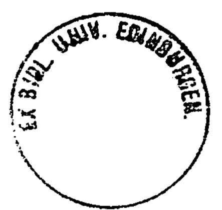
(iii) Psi broth

Tryptone	20g/l
Yeast extract	5g/l

After autoclaving, 20ml/l of the following solution (filter sterilised) was added:

$MgSO_4.7H_2O$	246g/l
NaCl	29g/l
KCl	18.6g/l

0.2ml of a stationary phase culture of *E.coli* in LB was inoculated into 20ml psi broth and grown to A_{550} of 0.48. This culture was cooled briefly on ice, and the cells harvested in a pre-cooled Sorvall RC-5B centifuge. The cells were resuspended in 33ml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of ice cold TfBI and incubated on ice for 15 minutes. The cells were then harvested as previously, and resuspended in 4ml of ice cold TfBII. The cells were incubated on ice for 20 minutes, after which time 0.2ml aliquots were frozen in liquid nitrogen and stored at -70°C. When required, tubes were thawed on ice and 0.1ml volumes of competent cells utilized as described for Method 1.

2.4.3: Isolation of plasmid DNA from E.coli

Plasmid DNA was isolated by the boiling method described below (Maniatis *et al*, 1982) when several small scale preparations were required, or by a modification of the alkaline lysis method of Birnboim and Doly (1979) when larger amounts were required.

2.4.3.a: Small scale plasmid preparations.

5ml of stationary phase culture of *E.coli* grown with shaking in the presence of the appropriate antibiotic were centrifuged in a MSE benchtop centrifuge, and the resulting pellet resuspended in 250μl of STET (8% sucrose, 5% triton-100, 50mM EDTA, 50mM Tris-HCl pH 8.0). 25μl of STET containing 10mg/ml lysozyme was added and the mixture incubated on ice for 10 minutes. The tubes were then plunged into boiling water for 40 seconds and then returned to ice. The tubes were spun in a microfuge for 10 minutes and the resulting gelatinous precipitate removed with a toothpick. One volume of isopropanol was added and the samples incubated at -20°C for 10 minutes, followed by centrifugation in a microfuge for 5 minutes. The pellet was resuspended in 100μl of STE buffer and 2 volumes of ethanol added. Following incubation at -20°C for 1 hour, the tubes were centrifuged as previously, the pellets dried, and resuspended in 50μl of TE or water.

2.4.3.b: Large scale plasmid preparations

250 ml of a stationary phase culture of *E.coli* grown with shaking in the presence of the appropriate antibiotic in LB medium were harvested in a Sorvall RC-5B centrifuge at 7krpm for 10 minutes. The pellet was resuspended in 6ml of the following solution: 10mM EDTA, 15% sucrose, 2mg/ml lysozyme, 25mM Tris-HCl(pH 8.0) and the sample incubated on ice for 20 minutes. 12ml 0.2M NaOH containing 1% SDS was added and mixed carefully by inversion. After incubation on ice for a further 10 minutes, 7.5ml 3M NaOAc pH 4.6 was added, mixed carefully by inversion, and the sample returned to ice for a further 20 minutes.. The sample was the centrifuged at 10krpm for 10 minutes, and the supernatant transferred to a fresh tube. 50μl of RNAse (1mg/ml) was added and the tube incubated at 37°C for 20 minutes. The aqueous phase was extracted twice with

an equal volume of a 1:1 mixture of phenol/chloroform, and 2 volumes of ethanol added to precipitate the DNA. The pellet was resuspended in 1.6ml water, and 0.4ml 4M NaCl added. 2ml 13% PEG 4000 was added and the sample incubated on ice for 1 hour. The plasmid DNA was recovered by centrifugation at 10krpm for 10 minutes, the supernatant removed, and the pellet washed in 70% ethanol before being dried and dissolved in 0.25ml TE Buffer or water.

2.4.5: Production of single stranded DNA

The plasmids pTZ18/19 contain the f1 origin of replication so, on infection with the helper phage M13K07, cells containing these plasmids will synthesize single stranded DNA from this origin, which will be released in the form of phage particles (Section 2.2.2.a). High titre preparations of helper phage were produced by growing a culture of infected *E.coli* (JM101) in 2xYT in the presence of 70 g/ml kanamycin. Cells were removed by repeated centrifugation.

In order to produce single stranded DNA, a colony of the required clone in pTZ18/19 was grown overnight in LB plus ampicillin. This culture was diluted 1 in 20 into 2xYT plus ampicillin and grown to A_{600} = 1.0. 2ml of this culture was infected with M13K07 at a concentration of 10 pfu/cell, and shaken vigorously at 37°C for 1 hour. After this time 400μl of infected cells was added to 10ml of 2xYT plus kanamycin (70μg/ml), and this culture grown overnight with good aeration. The cells were removed by repeated centrifugation until no pellet was produced, after which 1.5ml of supernatant phage suspension was mixed with 0.2ml 27% PEG 4000, 3.3M NaCl. After standing for one hour at room temperature, this was centrifuged for 10 minutes in a microfuge and the supernatant removed. The pellet was resuspended in 0.65ml TE, and 40μl 40% PEG 4000 followed by 80μl 5M NaCl were added. This was mixed well, allowed to stand for 30 minutes at room temperature, and then centrifuged for 10 minutes as above. The supernatant was discarded, the pellet resuspended in 200μl TE, and the resulting suspension extracted twice with an equal volume of 50:50 phenol/chloroform. The DNA was precipitated with EtOH, and resuspended in an appropriate volume of TE. The amount of single stranded DNA produced was estimated by comparison with known standards on agarose gels.

2.5: Tn5 TRANSPOSON MUTAGENESIS

The aim of using this form of mutagenesis was to produce clones of a plasmid species which contained the transposon Tn5 inserted at a different site in each clone. This

method was used to delimit function regions within plasmids containing *S.pombe* sequences. The Tn5-containing clones could be identified by the sequences conferring kanamycin resistance (Kan^R) contained within the transposon.

2.5.1: Preparation of a high titre phage lysate

The phage used as the source of Tn5 was (lambda)cI_{857} O^{am} with the insertion of Tn5 in the *red* gene. The phage was propagated in the strain 5K which is *sup*E, because of the amber mutation in the *O* gene. A 5ml culture of 5K was grown overnight in LB supplemented with 0.2% maltose. An infection was set up with 50µl cells and 100µl phage (10^6pfu/ml) and incubated at room temperature for 10 minutes to allow the phage to adsorb. 6ml LB top agar was prepared, pre-cooled to 45-50°C, and added to the cells. The mixture was poured onto a LB plate, allowed to set for 10 minutes, and the plate incubated at 37°C. After 3-4 hours the plates were inspected for the onset of lysis, and used when nearly confluent lysis had occurred (typically after a further 2-3 hours). The phage were recovered by breaking off the top agar and collecting it in a glass tube, which was centrifuged at 15krpm for 15 minutes. The agar pelleted to about half the total volume, and the aqueous supernatant was collected and stored over CH_3Cl containing no isoamyl alcohol. The phage lysate was titrated against *E.coli* strain 5K at appropriate dilutions. A typical lysate gave at least 10^{10} pfu/ml.

2.5.2: Mutagenesis using (lambda)::Tn5

This procedure depends on infecting a plasmid-containing *E.coli* strain with (lambda)(Tn5) and selecting for kanamycin resistant clones which arise from integration of the transposon into either plasmid or *E.coli* genomic sequences. This is done under conditions were the phage can neither lysogenise (cI857 at 37°C), nor replicate (<u>Oam</u> in a sup^o background), so that Km^R colonies reflect transposition events.

The target plasmid to be mutagenised was first transformed into *E.coli* strain 554 which is *sup*^{o}. A culture of this strain containing the required plasmid was then grown up overnight in LB plus the relevant antibiotic for the selection of plasmid sequences. This culture was diluted 1/100 in LB plus antibiotics plus 0.2% maltose and grown up to OD_{550} = 0.5. 5ml of cells were harvested by centrifugation and resuspended in 2.5ml SM phage buffer (Maniatis *et al*, 1982). 1ml of phage suspension was added to 1ml of this cell suspension and incubated at room temperature for 10 minutes. 4ml of LB was then added and the mix incubated at 42°C for 10 minutes, followed by 37°C for 30

minutes. The cells were then collected by centrifugation, resuspended in a small volume and spread onto LB plates containing kanamycin and any antibiotic resistance markers carried on the target plasmid. This plate was incubated overnight, and gave an almost confluent growth of colonies.

These colonies represent transposition events not only into the target plasmid, but also into the *E.coli* genome. In order to isolate plasmid clones containing integrated transposon sequences, a bulk preparation of plasmid DNA was made by combining the transformants in a cell suspension and using this to inoculate a culture which was grown overnight in LB plus kanamycin. Plasmid DNA was prepared by the miniprep boiling method (2.4.3.a), and this plasmid preparation used to transform 5K to Km^R. Single transformants were then screened for the nature of plasmid::Tn5 they contained. Restriction endonuclease mapping was used to determine the position of integration, and a selection of plasmids used to transform *S.pombe* to determine if they retained the activity for which they had originally been selected.

2.6: DNA SEQUENCING OF wis1

2.6.1: Production of deletions

A library of unidirectional "nested deletions", which consists of a series of plasmid subclones with progressively more of the *wis1* sequence deleted, was prepared as a convenient strategy for DNA sequencing (Henikoff, 1984). These experiments were carried out using the Pharmacia nested deletion kit, following the manufacturers instructions.

In order to construct unidirectional deletions, plasmid DNA is digested with two restriction enzymes with unique sites in the polylinker, one to generate a blunt, or 5'-overhanging end adjacent to the target sequence, and the other to generate a 3'-overhang. This doubly digested DNA is treated with Exonuclease III which results in a progressive removal of nucleotides from one strand of the target sequence, creating a single stranded region which is subsequently removed with S1 nuclease. The 3'-overhang, which is not susceptible to Exo III, protects the vector sequences from digestion. Nested deletions are generated by the removal of aliquots at timed intervals during Exo III digestion. Following S1 treatment, the plasmids are recircularised by treatment with T7 DNA ligase, and preparations transformed into competent *E.coli*. It is

then possible to screen the resulting deletion library by electrophoretic analysis of plasmid preparations from individual transformants.

Digestion of *wis1*-containing plasmids to generate appropriate 3'- and 5'-overhanging ends was performed with the following enzymes:

	Enzymes	
Plasmid	blunt/3'-overhang	5'-overhang
pX2	*Sma*I	*Sph*I
pBX2	*Sma*I	*Bam*HI
pX3	*Sma*I	*Sph*I
pBX3	*Sma*I	*Bam*HI

2.6.2: Sequencing reactions

Dideoxy sequencing reactions using T7 DNA polymerase were performed with the Pharmacia T7 sequencing kit, according to the manufacturers' instructions.

This sequencing method depends upon base-specific termination of enzyme catalysed primer extension reactions (Sanger *et al*, 1977). Four reactions are performed, all containing primer, template, and four deoxynucleotides, but each including a different chain-terminating dideoxynucleotide. This leads to a mixture of fragments, each terminated with the particular dideoxynucleotide present in the reaction. When the products of the four reactions are electrophoresed side by side, the sequence in which nucleotides are added to the primer may be deduced from the sequence in which successively larger fragments occur in the four lanes. The positions of the separated fragments are detected by virtue of radioactive label (in this case [Á-^{35}S]dATPÁS) introduced before the primer extension reactions.

The first stage in the sequencing reaction procedure was the annealing of primer to template. 1.5 - 2µg of a single stranded DNA preparation from a deletion clone, or other plasmid, was incubated in a buffered solution containing 8.88ng of reverse sequencing primer at 80°C for 10 minutes. This was followed by slow cooling to room temperature to allow annealing. The next stage was the labelling reaction. To the tube containing the annealed primer and template, 10µCi of [Á-^{35}S]dATPÁS was added, along

with 3 units of T7 DNA polymerase, and a labelling mix which contained dCTP, dGTP and dTTP. This reaction mix was incubated at room temperature for 5 minutes. The final stage was the termination reactions. Equal aliquots from the labelling reaction mix were transferred to microcentrifuge tubes prewarmed to 37°C, each containing different termination mixes. Each termination mix contains a different variety of dideoxynucleotide along with all four deoxynucleotides. Following 5 minutes incubation at 37°C, a stop solution was added to the reactions. Prior to acrylamide gel electrophoresis, an aliquot of each stopped reaction was transferred to a MicroSample plate (Pharmacia) and heated at 75 - 80°C before loading onto the prepared gel.

2.6.3: Acrylamide gel electrophoresis

Electrophoresis was carried out using the BRL Model S2 Sequencing gel electrophoresis system, following the manufacturers' instructions.

The glass plates were cleaned thoroughly, and treated with dimethylchlorosilane, before being assembled, separated by "wedge" spacers, and sealed with tape. The following solutions were prepared:

(i) 40% acrylamide stock, containing 380g/l acrylamide and 20g/l bisacrylamide dissolved in 1l H_2O and filtered through Whatman 3MM paper.
(ii) 10% ammonium persulphate made up freshly in H_2O.
(iii) 10x Tris-borate-EDTA buffer (TBE), containing 121.1g/l Tris base, 55g/l boric acid and 7.4g/l EDTA dissolved in 1l H_2O.
A standard 6% polyacrylamide/urea gel was prepared as follows:

40% acrylamide stock	15ml
Urea	50g
10x TBE	10ml
H_2O	35ml
TOTAL	99ml

1ml of 10% ammonium persulphate and 20μl of TEMED were added to this mixture to initiate polymerisation, and the gel poured immediately. Loading slots were formed with "sharkstooth" combs, following the manufacturers' instructions.
Electrophoresis was carried out at approximately 1,500V (30-45mA) for a total of 6 hours, with a duplicate set of samples added halfway through each run.

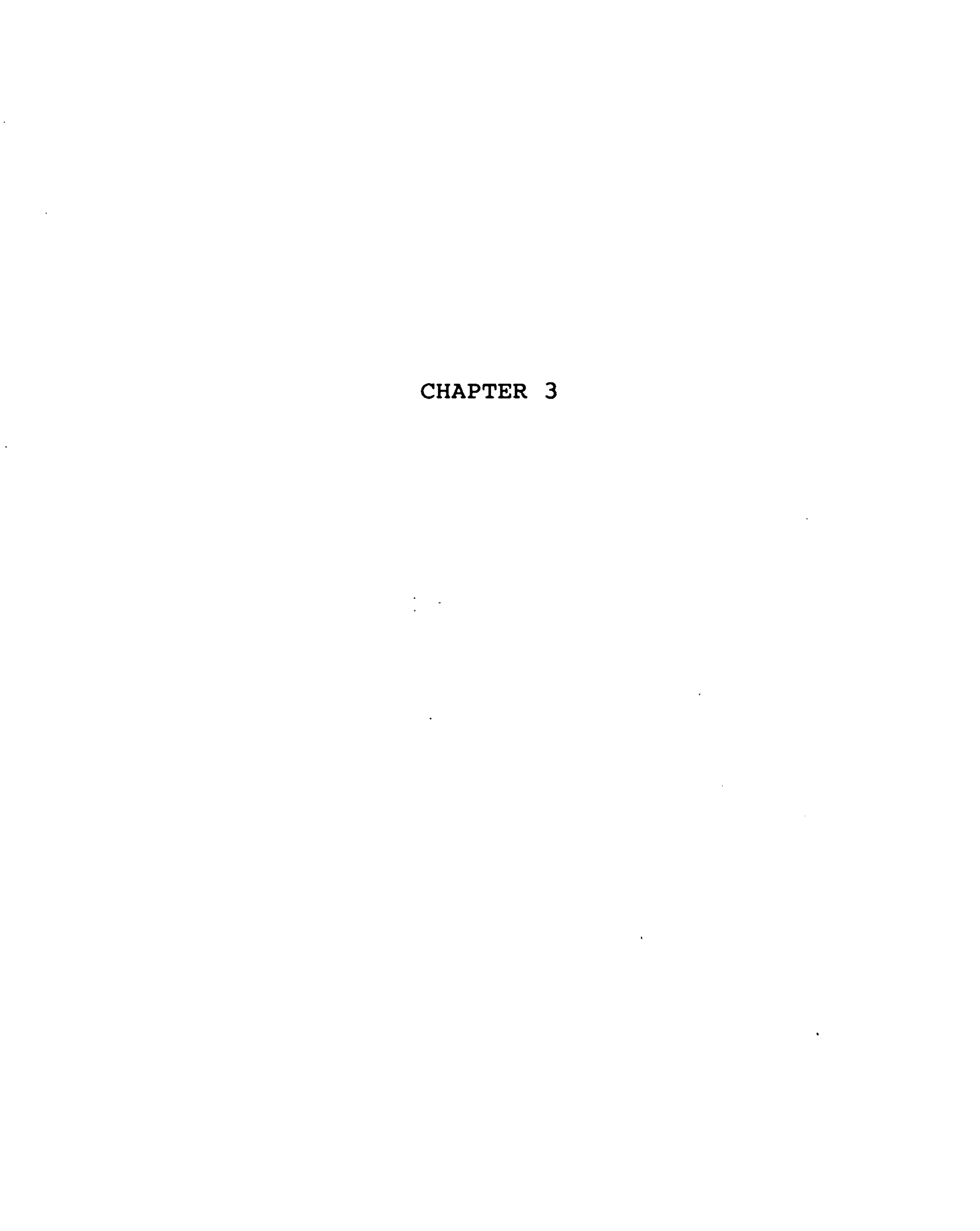
2.6.5: Autoradiography

The glass plates sandwiching the gel were separated so that the gel remained attached to one plate. The gel/plate was then soaked in 10% methanol/10% acetic acid for 20 minutes to fix the gel. The gel was transferred to a sheet of filter paper, covered with plastic wrap, and dried on a vacuum gel drier. Once dry, the gel on its filter paper support was exposed to X-ray film in a cassette overnight, followed by developing and fixing as described in Section 2.7.

2.6.6: Sequence analysis

The polylinker derived from pTZ plasmid sequences was used to identify the start of the *wis1* sequence. The sequence was deduced from the sequence in which successively larger fragments were present in each of the four lanes resulting from the termination reactions. The sequence was analysed using the UWGCG package available from the Seqnet VAX facility at Daresbury.

CHAPTER 3



CHAPTER 3: GENETICS AND PHYSIOLOGY OF win1

3.1: INTRODUCTION

One mutant allele of *win1* (*win1.1*) has been isolated (Ogden and Fantes, 1986). This chapter describes the examination of the phenotype shown by *wee1.50 cdc25.22 win1.1* strains, the investigation of the interaction of *win1.1* with other cell cycle genes, and the mapping of the *win1* locus.

3.2: THE NUTRITIONALLY SENSITIVE PHENOTYPE OF THE wee1.50 cdc25.22 win1.1 TRIPLE MUTANT STRAIN

3.2.1: Introduction

A particularly interesting aspect of the *win1.1* mutation is the nutritionally sensitive nature of its interaction with *wee1* and *cdc25* (Ogden and Fantes, 1986). Cells of the genotype *wee1.50 cdc25.22 win1.1* show a predominantly cdc phenotype on EMM at 35°C, while cells grown on rich medium such as YE are much shorter, and are able to grow and divide. Cell length is heterogeneous in both cases, and the cdc phenotype appears to be affected by plating density, as a significant amount of bulk growth occurs in areas of high cell density on EMM at 35°C.

3.2.2: Investigation of growth medium effects

In order to investigate the nature of the nutritionally sensitive phenotype of *wee1.50 cdc25.22 win1.1* strains, cell growth was examined on various media at 25°C and 35°C, and compared to that of the corresponding *win1*$^{+}$ strain. The heterogeneity in cell length shown by these strains made the evaluation of cell length phenotypes by the measurement of cell length very difficult. In the experiments described here, cell growth on plates was examined after 24 - 48 hours, and the ability of each strain to form colonies in sparsely plated areas was assessed. Examination of the strains described here was undertaken either on streaked plates, or on plates upon which a dilute suspension of cells had been uniformly spread.

One possible explanation for the heterogeneous phenotype shown by *wee1.50 cdc25.22 win1.1* strains at 35°C on EMM was that growth resulted from an incomplete loss of *cdc25* function. If this were the case, then it might be expected that incubation at 37°C

would reduce the temperature sensitive activity of *cdc25.22*, and thus lead to a more extreme cdc phenotype. It was observed that *wee1.50 cdc25.22 win1.1* cells did not show a markedly stronger cdc phenotype on EMM or YE at 37°C, compared to that at 35°C, suggesting that this was not the case.

The colony forming abilities of *wee1.50 cdc25.22 win1.1* strains compared with those of *wee1.50 cdc25.22* strains on YE and EMM were investigated to confirm that the two phenotypes could be easily distinguished on EMM. This was undertaken as preliminary to the gene library screening experiments aimed at cloning *win1*, which are described in detail in Chapter 4.

Small cultures of the strains *wee1.50 cdc25.22 win1.1* and *wee1.50 cdc25.22* in YE were grown to stationary phase at 25°C. Plate inoculated with various dilutions of these cultures incubated at 35°C and at 25°C, and after 4 days the number of colonies/plate was counted (Table 3.1). From these observations it was concluded that it would be possible to distinguish *win1.1* and *win1*$^+$ phenotypes in a *wee1.50 cdc25.22* genetic background on the basis of their colony forming ability on EMM at 35°C. It was also possible to conclude that the presence of 1.2M sorbitol (which would be present in plates used for the regeneration of protoplasts following transformation with gene libraries) had no effect upon the cdc phenotype of these cells. Some cdc mutants have been shown to be suppressed by high osmotic strength.

In further experiments, cells of the genotype *wee1.50 cdc25.22 win1.1* were grown at 35°C upon various media, and their phenotypes compared to those of the same strain grown at 25°C, and a control *win1*$^+$ strain. The media examined are listed in Table 3.2. Casamino acids (Oxoid) is an acid hydrolysate of casein, which is rich in amino acids (not including tryptophan) and also contains various trace elements. Various substances were added to either 20g/l glucose, or to "EMM base", which consisted of EMM minus NH_4Cl.

The observations from these experiments may be summarised as follows: the substitution of Casamino acids and YE for NH_4Cl in the EMM formula resulted in *wee1.50 cdc25.22 win1.1* cells with the shortest lengths, and also gave rise to the largest colonies. The phenotype of cells grown on the glutamate-containing medium was one of shorter cell length than those on EMM (NH_4Cl), though colony forming ability was only slightly better. Cells grown on EMM with both YE and NH_4Cl

Table 3.1: Results of experiments testing the colony forming abilities of win1.1 and win1$^+$ strains.

25°C

Growth medium:	YE	EMM	EMMS*
Genotype:			
wee1.50 cdc25.22	58%	63%	63%
wee1.50 cdc25.22 win1.1	36%	49%	47%

35°C

Growth medium:	YE	EMM	EMMS*
Genotype:			
wee1.50 cdc25.22	73%	64%	63%
wee1.50 cdc25.22 win1.1	7%	0.5%	1%

*EMMS denotes EMM containing 1.2M sorbitol.

Table 3.2: Media used for the examination of the wee1.50 cdc25.22 win1.1 phenotype.

Basis of medium	Nitrogen source (5g/l)
EMM	NH_4Cl
EMM	NH_4Cl (1.2M sorbitol)
EMM	NH_4Cl and YE
EMM	YE
EMM	Sodium glutamate*
EMM	Casamino Acids (Oxoid)
Glucose	YE
Glucose	Casamino Acids (Oxoid)

"EMM" as the basis of medium indicates the components of EMM with no NH_4CL. "Glucose" indicates 20g/l glucose to which the appropriate supplement was added. All media described here contained 20g/l agar.

* Sodium glutamate was added at 3.72g/l

as nitrogen sources were longer than those growing on medium consisting of an EMM base with YE as a nitrogen source. This suggests that the presence of 5g/l NH_4Cl is exerting an over-riding effect upon the *wee1.50 cdc25.22 win1.1* phenotype. Cells grown upon the medium containing both NH_4Cl and YE, however, were still able to form colonies, suggesting that a balance of effects is involved.

In order to make a closer examination of the effects described above, cells of the phenotype *wee1.50 cdc25.22 win1.1* and *wee1.50 cdc25.22* were streaked out upon solid EMM-based media containing either NH_4Cl or Casamino acids as nitrogen source at both 25°C and 35°C. The phenotypes of such strains were examined after 24 - 48 hours, and the numbers of cells falling into various classes recorded (Table 3.3)

The observations described suggest that nitrogen source is an important factor in deciding the temperature sensitive phenotype of *wee1.50 cdc25.22 win1.1* cells. Rich media result in cells with a phenotype similar to the corresponding *win1*$^{+}$ strain at 35°C, while those containing NH_4Cl as a nitrogen source give rise cells with predominantly cdc^{-} phenotype. To test the possibility that the presence of one amino acid might have been responsible for the effects described above, the growth of *wee1.50 cdc25.22 win1.1* cells was observed upon EMM supplemented with individual amino acids. Each amino acid was present in the same concentration as in medium supplemented with Casamino acids. It was not possible to draw any clear cut conclusions from these experiments.

In order to determine the effect of carbon source of the *wee1.50 cdc25.22 win1.1* phenotype, cells were grown on EMM with glycerol substituted for the glucose present in the standard formula. There was little growth of either *wee1.50 cdc25.22 win1.1* or *wee1.50 cdc25.22* strains at 35°C, and growth at 32°C was so poor that it was impossible to interpret the results of examination of the cells.

3.2.3: Temperature shifts

The roles of *wee1* and *cdc25* in the cell cycle have been examined extensively (reviewed by Fantes, 1989), and have been shown to be involved in the control over entry into mitosis. The loss of *cdc25* function results in a G_2 arrest, prior to entry into mitosis, which is relieved by loss of *wee1* function. One possible explanation for the cdc phenotype shown by *wee1.50 cdc25.22 win1.1* cells is that *win1.1* directly reverses the effect of *wee1.50*. If this were the case, then *wee1.50 cdc25.22 win1.1* would be

Table 3.3: Results from the microscopic examination of wee1.50 cdc25.22 win1.1 and wee1.50 cdc25.22 cells grown on media containing Casamino acids or NH_4Cl as nitrogen source.

Nitrogen source: NH_4Cl

	(25°C)		(35°C)	
	win1$^+$	*win1*$^-$	*win1*$^+$	*win1*$^-$
Colony forming:	83	73	76	1
cdc-:	2	4	1	83
No growth:	15	24	23	15

Nitrogen source: CAA

	(25°C)		(35°C)	
	win1$^+$	*win1*$^-$	*win1*$^+$	*win1*$^-$
Colony forming:	85	76	76	34
cdc$^-$:	2	3	1	36
No growth:	13	21	20	33

Cells in sparely plated areas were examined microscopically following 24 - 48 hours incubation at 35°C. The cells were divided into three classes: "colony forming" - groups of growing cells, "cdc$^-$" - isolated cells with an elongated morphology, and "no growth" isolated cells stained red with Phloxin B showing no signs of growth or division.

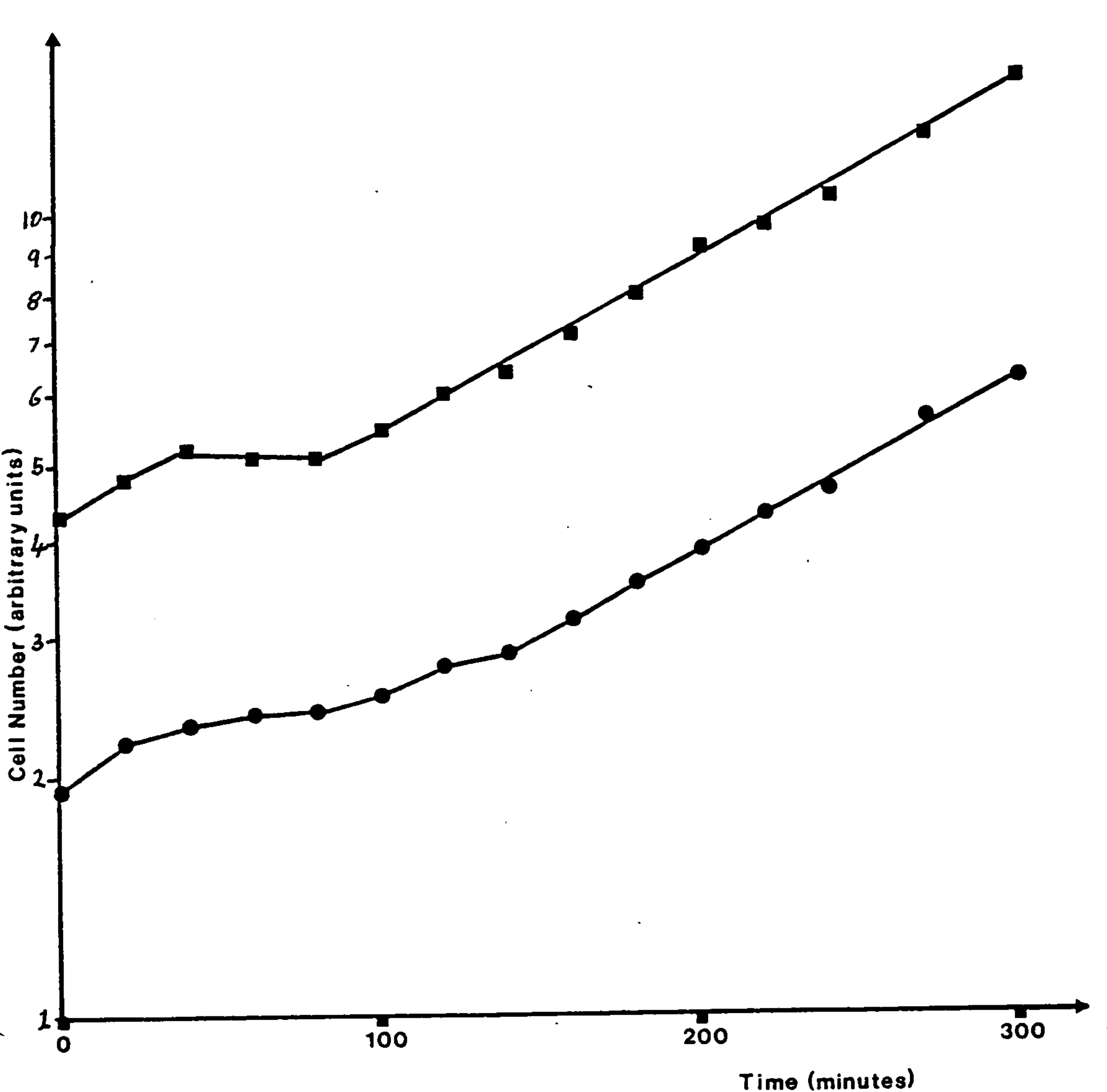
expected to show a first cycle arrest in the G_2 phase of the cell cycle upon a shift to the restrictive temperature.

In order to test this possibility, temperature shift experiments were performed with the *wee1.50 cdc25.22 win1.1* and *wee1.50 cdc25.22* strains on minimal medium. Cells were grown at 25°C in EMM liquid culture to a density of approximately 5 x 10^6 cells/ml and shifted to 35°C. Cell samples were taken periodically for microscopic examination and to determine cell density (Fig. 3.1).

These experiments indicate that the combination of *win1.1* with *wee1.50* and *cdc25.22* does not result in a first cell cycle block upon a shift to the restrictive temperature. The patterns of cell number changes in both *win1.1* and *win1*$^+$ strains are very similar, although there is a slightly longer plateau in cell number in the *wee1.50 cdc25.22 win1.1* strain before the onset of logarithmic growth. Microscopic examination of *wee1.50 cdc25.22 win1.1* cells following a shift to 35°C revealed a very heterogeneous population, rather than uniformly elongated cells. After 4 hours' incubation at 35°C it was possible to detect some cells containing multiple septa, and after 6 hours the population contained a high proportion of cell abnormalities, including multiple septa, eccentric septa, bent and branched cells, and multinucleate cells. These observations suggest an effect upon the spatial distribution of the processes involved in mitosis and cell division in these strains.

The observations described in this section show a contrast in the long and short term effects of *win1* in a *wee1.50 cdc25.22* background following a shift to 35°C. Following incubation at 35°C on solid EMM for 18 - 24 hours, the phenotype of a *wee1.50 cdc25.22 win1.1* is cdc, although examination of the short term effects of such a shift shows that this phenotype is not due to a first cell cycle arrest. It seems likely that some form of cumulative effect gives rise to the cdc phenotype shown by *wee1.50 cdc25.22 win1.1* strains.

Figure 3.1: Increases in cell number of the strains wee1.50 cdc25.22 and wee1.50 cdc25.22 win1.1 following a shift to 35°C.



Cells were grown in EMM to a density of 3-7x10^6 cells/ml at 25°C. Following a shift to 35°C, cell densities from each culture were determined at 20 minute intervals.
by constructing double mutants and examining their phenotypes.

■ wee1.50 cdc25.22

● wee1.50 cdc25.22 win1.1

3.3: INTERACTIONS BETWEEN win1.1 AND OTHER CELL CYCLE MUTATIONS

3.3.1: Experimental strategy

Genetic techniques are a very powerful tool for the analysis of the role of a gene and its product in the cell. Interactions between mutant alleles of the gene in question, and alleles of other genes of interest may be investigated
The *win1.1* mutation was first isolated on the basis of its striking interaction with the mutant alleles *wee1.50* and *cdc25.22*, and its lack of interaction with certain other mutations known to affect mitosis had already been investigated (Ogden and Fantes, 1986). Double mutant strains combining *win1.1* with mutations in each of the cell cycle genes *cdc1*, *cdc2*, *cdc13*, and to *cdr1* and *cdr2*, which have an altered mitotic response to nutritional starvation, were examined. Comparison of the phenotypes of the double mutant strains with those of the corresponding *win1*$^{+}$ strains showed no noticeable differences, apart from a slight increase in cell length.

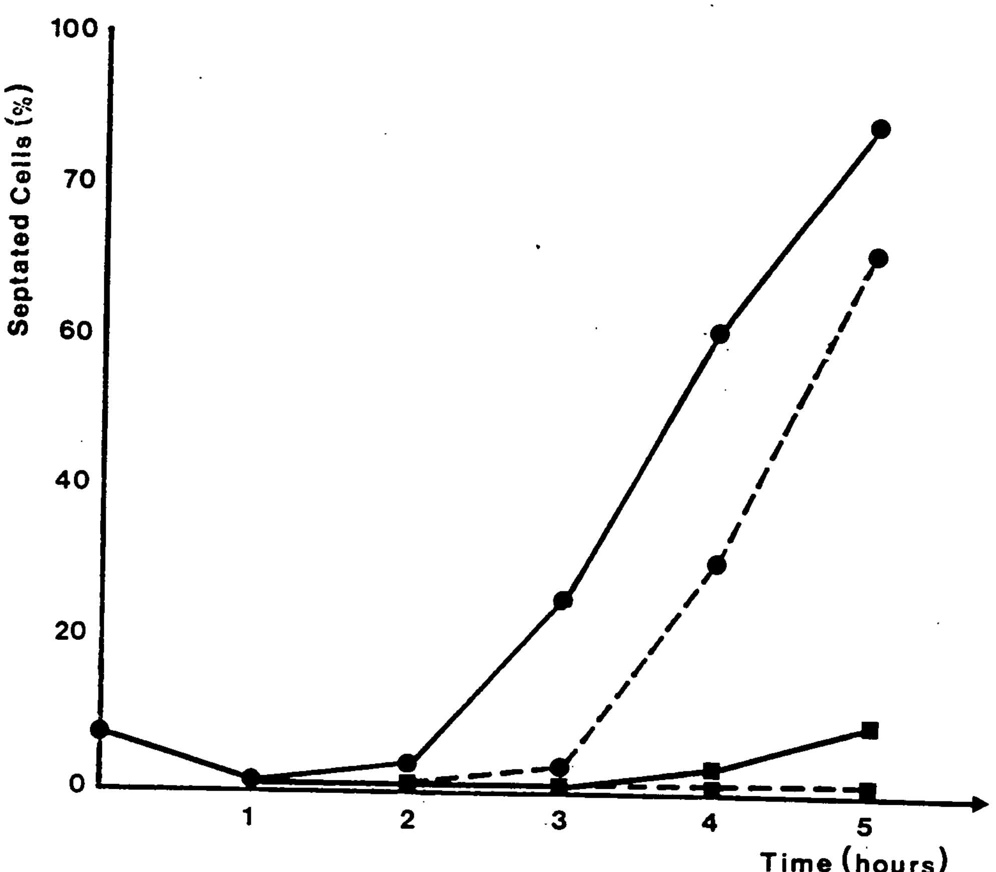
This form of analysis was applied to re-examine the interactions of *win1.1* with *cdc13*, various alleles of *cdc2*, and several of the *mcs* genes. A genetic analysis was also performed to determine if *win1.1* would affect phenotypes resulting from the interactions of *cdc25* and *cdc2.3w* with *wee1.50* (Russell and Nurse, 1986; 1987a).

3.3.2: Interactions between win1.1 and cdc13.117

The cross between the strains *cdc13.117 leu1.32* and *win1.1* was subjected to tetrad analysis. This cross resulted in a spore viability of less than 50%, though it was possible to distinguish progeny with cells of a different phenotype from either of the parental strains at the restrictive temperature (35°C). The phenotype of these presumed double mutant strains was cdc, but the cells appeared longer, and lacked the septa characteristic of *cdc13.117* (Nasmyth and Nurse, 1981). Two of these strains were backcrossed to a wild type strain, and the phenotypes of the resulting progeny examined. Four phenotypic classes were observed: one corresponding to wild type cells, two to the parental strains *win1.1* and *cdc13.117*, and the fourth to the strain under analysis, which was deduced to be the double mutant strain *cdc13.117 win1.1*.

In order to examine the morphology of the double mutant strain more closely, temperature shift experiments were carried out, with the strain *cdc13.117* as a control, and samples taken periodically for analysis. The cells were examined to determine the fraction of cells with septa (Fig.

Figure 3.2: Proportions of septated cells in cultures of the strains cdc13.117 and win1.1 cdc13.117, following a shift to 35°C.



Cells were grown in EMM containing 0.5% glucose to a density of approximately 1×10^6 cells/ml prior to the shift. 200 cells of each strain were examined at hourly intervals, and the proportion of cells containing single, or multiple septa calculated.

Circles indicate *cdc13.117* and squares *win1.1 cdc13.117*.

Dotted lines indicate multiple septa, and solid lines total septated cells.

3.2), and were fixed to allow their staining with DAPI and rhodamine-phalloidin.

After three to four hours it was possible to see condensed chromosomes under DAPI staining in the *win1*$^+$ strain, though this was not observed in the double mutant strain, where staining revealed a round, somewhat diffuse nucleus. After four hours incubation at 35°C, more than half the cells from the *cdc13.117* strain contained condensed chromosomes. In contrast, not more than 3% showed this phenotype in the *win1.1 cdc13.117* strain. In the *win1*$^+$ strain, rhodamine-phalloidin staining revealed actin rings which are laid down prior to the formation of the septum (Marks and Hyams, 1985). In the *win1.1* strain these were not present, and the actin distribution observed was more characteristic of interphase cells, with dots visible at each end of the cell. This evidence suggests that the win1.1 mutation is preventing the "leak-through" into the form of "mitotic" terminal phenotype normally observed in cdc13.117 mutant cells at the restrictive temperature of 35°C.

cdc13.117 strains show a hypersensitivity to the anti-microtubule drug TBZ: at a TBZ concentration of 15 g/ml wild type cells are capable of forming colonies on agar plates, whereas *cdc13.117* strains are not (Booher and Beach, 1988). Similar experiments were performed to compare *win1.1* with wild type cells on plates containing various concentrations of TBZ, but no differences were found between the two strains.

3.3.3: Interactions between win1.1 and mutant allele combinations showing the mitotic catastrophe phenotype

3.3.3.a: Mitotic catastrophe caused by the combination of wee1.50 and cdc2.3w.

Strains of the genotype *wee1.50*^{ts} *cdc2.3w* have a lethal phenotype at the restrictive temperature which is thought to result from cells attempting to undergo premature mitosis and division. This phenotype is characterised by aberrant division which takes place at a very small cell size and has been termed "mitotic catastrophe" (Russell and Nurse, 1987a). *win1.1* shares many characteristics with the *mcs* mutations, which are capable of suppressing the mitotic catastrophe phenotype resulting from the combination of *wee1.50* and *cdc2.3w* (Molz *et al*, 1989). Experiments were performed to determine if *win1.1* was capable of suppressing the mitotic catastrophe phenotype.

Strains of the genotype *cdc2.1w* and *cdc2.3w* were crossed to *wee1.50* mutant strains, and tetrad analysis carried out. Tetrads of non-parental ditype were selected, and the

putative *wee1.50 cdc2w* double mutant strains backcrossed to wild type strains to confirm their genotypes. Sixteen segregants were examined at 25°C and 35°C in order to differentiate between the phenotypes of *wee1.50* and the *cdc2w* alleles. Strains which on backcrossing gave rise to progeny with two classes of wee phenotype (temperature sensitive and non-temperature sensitive) were deduced to be of genotype *wee1.50 cdc2w.*

The phenotypes of these double mutant strains were examined at 35°C. The results of this analysis agreed with those previously reported (Russell and Nurse, 1987a): the genotype *wee1.50 cdc2.3w* was lethal under these conditions, in contrast to that of *wee1.50 cdc2.1w* which merely resulted in a wee phenotype. Tetrad analysis was then performed upon a cross between the strains *wee1.50 cdc2.3w* and *wee1.50 win1.1.* One tetrad (C) which appeared to be a tetratype was chosen for further analysis, and cells examined at 25°C and 35°C (Table 3.4). In order to confirm the genotypes of the segregants in this tetrad, they were backcrossed to a wild type strain, and the progeny examined by random spore analysis (Table 3.5).

The conclusions that may be drawn from this data are as follows:
1) *win1.1* does not suppress the mitotic catastrophe phenotype resulting from the combination of *wee1.50* and *cdc2.3w*. Thus, *win1.1* is not a mcs-type mutation.
2) At 25°C, the *win1.1* phenotype of slightly elongated cells is not completely suppressed by the presence of *cdc2.3w* (See section 3.3.4).

3.3.3.b: Mitotic catastrophe caused by the combination of wee1.50 and a high level of expression of cdc25⁺

A second form of mitotic catastrophe is observed in cells with an artificially high level of the *cdc25* gene product in a *wee1.50* background at 35°C (Russell and Nurse, 1986). The *cdc25* gene is over-expressed to give a high level of product by linking the *cdc25* coding region to promoter sequences from the *S.pombe adh* (alcohol dehydrogenase) gene (Russell and Hall, 1983). A strain containing such a construct (which leads to a wee phenotype) integrated within the genome was supplied by Paul Russell (Pers. Comm.). This contained an *adh-cdc25* construct including *ura4*$^+$ sequences integrated at the *cdc25* locus in a *ura4.D18* background. A series of crosses were performed to determine if *win1.1* was capable of suppressing this second form of mitotic catastrophe.

A cross between the strains *win1.1 ura4.D18 h*$^+$ and [*adh-cdc25:ura4*] *ura4.D18 leu1.32 h*$^-$ was examined by tetrad analysis. The presence of the *adh-cdc25* construct could be

Table 3.4: Phenotypes of progeny in tetrad C resulting from a cross between the strains *wee1.50 win1.1* and *wee1.50 cdc2.3w*.

Temp.:	25°C	35°C	
Strain	Phenotype	Phenotype	Deduced genotype
C1	wee	MC*	*wee1.50 cdc2.3w*
C2	wee/wild type	MC*	*wee1.50 cdc2.3w win1.1*
C3	win1^{-}	wee	*wee1.50 win1.1*
C4	wild type	wee	*wee1.50*

*MC = mitotic catastrophe

Table 3.5: Results of backcrossing to wild type the strains C1, C2 and C3 which resulted form the cross of wee1.50 win1.1 and wee1.50 cdc2.3w.

Mutation	*wee1.50*	*cdc2.3w*	*win1.1*
Strain			
C1	+	+	-
C2	+	+	+
C3	+	-	+

The symbols + and - indicate the presence or absence of progeny with phenotypes corresponding to those of the named mutant alleles

Table 3.6: Tetrads resulting from the cross between strains win1.1 ura4.D18 h^+ and [adh-cdc25:ura4] ura4.D18 leu1.32 h^-

	a		b		c		d		
Phenotype	ura	win	ura	win	ura	win	ura	win	Class
Tetrad									
1	-	-	+		+		-		T
2	+		-	+	-	+	+		NPD
3	+		+		-	+	-	+	NPD
4	-	+	+		-		+	+	PD
5	+		-	+	+		-	+	NPD

All cells with a ura4+ phenotype showed a wee phenotype.

The abbreviations for class of tetrad are as follows:

T - tetratype, PD - parental ditype, NPD - non-parental ditype. *win1* could not be scored unambiguously in a *ura4*$^+$ strain (see text).

followed by the wee phenotype and its close linkage to the integrated *ura4+* marker. *win1.1* was followed its cell elongation phenotype, though this could only be determined with certainty in a *ura4⁻* background, as it was not possible to predict the phenotype of a [*adh-cdc25:ura4*] *ura4.D18*
win1.1 strain. Five tetrads were examined, and the results shown in Table 3.6.

Two strains from tetrad 3 (3a and 3b) were chosen for further examination, since they were presumed to have the genotype *win1.1* [*adh-cdc25:ura4*] *ura4.D18*, given that the other two members of that tetrad were *win1+ ura4⁻*. The phenotype of the strains was wee, indicating that the presence of the *win1.1* mutation had little or no effect upon cells containing the *adh-cdc25* construct. These two strains were then crossed to a *wee1.50 win1.1 ura4.D18* and tetrad analysis performed (Table 3.7). All the progeny of this cross carry *win1.1*. Those carrying *wee1.50* were identified by the temperature sensitive wee phenotype and those strains with the *adh-cdc25* construct were distinguished by their *ura4+* phenotype. Segregants 1c and 2b were deduced to be of the genotype *wee1.50 win1.1* [*adh-cdc25:ura4.D18* (Table 3.7). These results indicate that *win1.1* is not capable of suppressing the mitotic catastrophe phenotype caused by the overexpression of *cdc25+* in a *wee1.50* mutant background.

3.3.4: Interactions with cdc2w alleles

In order to produce the *win1.1 cdc2w* double mutant strains a *win1.1* strain was crossed to strains *cdc2.1w* and *cdc2.3w*. The cross to *cdc2.1w* gave poor spore viability (50%), compared to that in the cross to *cdc2.3w* (70%). Because of this, it was difficult to use tetrads to analyse these crosses genetically. However, several strains appeared to have darker red colour previously noted to be associated with *win1.1* strains when grown on Phloxin B plates, though without the phenotype of increased cell length characteristic of *win1.1*. Two putative double mutants were picked from each cross and backcrossed to a wild type strain. Random spore analysis of these crosses revealed progeny with phenotypes corresponding to those of *win1.1* and the *cdc2w* mutants, confirming that they were double mutant *win1.1 cdc2w* strains. At this stage it was noted that the cells of genotype *win1.1 cdc2.3w* were longer than those of genotype *win1.1 cdc2.1w*. The cell lengths at division of these strains were determined (Table 3.8).

These results indicate that while the phenotype of increased cell length conferred by *win1.1* is strongly suppressed by *cdc2.1w*, *cdc2.3w* has very little effect. The cell

Table 3.7: Phenotypes of tetrads arising from the cross:

win1.1 wee1.50 ura4.D18

x

win1.1 [adh-cdc25.22 ura4] ura4.D18.

Tetrad 1:

	a	b	c	d
Phenotype				
ura4	-	+	+	-
25°C	win	win	*	win
35°C	win	win	MC	wee

Tetrad 2:

	a	b	c	d
Phenotype				
ura	+	+	-	-
25°C	win	*	win	win
35°C	win	MC	win	wee

MC = mitotic catastrophe

* = cells too ill to determine their phenotype in terms of cell length.

Table 3.8: Cell length at division of strains with combinations of win1.1 with cdc2w alleles.

Temperature:	25°C	35°C
Genotype		
wild type	14.17 (0.43)	
cdc2.1w	8.65 (0.64)	
cdc2.3w	9.05 (0.51)	
win1.1	16.65 (0.70)	
win1.1 cdc2.1w	9.83 (0.76)	10.10 (0.84)
win1.1 cdc2.3w	14.86 (1.61)	14.29 (1.35)

Cells were grown in EMM at the temperature indicated. Cell lengths are given in μm, and Figures in brackets indicate standard deviation.

length at division of the *win1.1 cdc2.3w* double mutant strain is slightly smaller than that a of *win1.1* strain.

3.3.5: Interactions with mcs mutations

3.3.5.a: Description of mcs conferred phenotypes

The six *mcs* genes were identified as a result of the ability of mutant alleles to suppress the mitotic catastrophe phenotype caused by the combination of *wee1.50* and *cdc2.3w* (Molz *et al*, 1989). The only *mcs* mutant alleles to show any recognisable phenotype in an otherwise wild type background are *mcs1.77* and *mcs4.13*. The phenotypes shown by these mutations, (both of increased cell length at division) are suppressed by *wee1.50*. All *mcs* mutant alleles show a range of interactions with different cell cycle mutations, including the *cdc2w* alleles, *wee1.50* and *cdc13.117* (Molz *et al*, 1989).

Of the most immediate interest was the ability of mutations in *mcs3.12*, *mcs4.13*, and *mcs6.13* to reverse the suppression of *cdc25.22* by *wee1.50*, similar to *win1.1*. The mutation *mcs4.13* shows the strongest similarity to *win1.1*, in that its interaction with *wee1.50 cdc25.22* is growth medium specific, and in an otherwise wild type background its phenotype is one of slight cell elongation. These observations raised the possibility that *mcs3*, *mcs6*, or, in particular, *mcs4* might be allelic to *win1*, despite the fact that *win1.1* had already been shown to be incapable of suppressing the mitotic catastrophe phenotype. It was possible that this difference in phenotype between *win1.1* and *mcs3.12*, *mcs4.13* and *mcs6.13* might be due to allele specific effects.

A series of crosses were performed to determine if *win1* was allelic to *mcs3*, *mcs4*, or *mcs6*, using strains supplied by Lisa Molz and David Beach. The strain *wee1.50 cdc25.22 mcs3.12* showed a striking cdc$^-$ phenotype on both YE and EMM. The strain *wee1.50 cdc25.22 mcs4.13* showed a nutritionally sensitive phenotype very similar to that of *wee1.50 cdc25.22 win1.1*: elongated cells which are unable to form colonies at 35°C on EMM, and shorter, colony forming cells on YE. *mcs6.13* has a very weak interaction with *wee1.50 cdc25.22*, which results in slightly elongated cells when compared with the equivalent *mcs6*$^+$ strain. The crosses described below were performed in a *wee1.50 cdc25.22* genetic background which made tetrad analysis difficult, due to a high proportion of two spored asci.

3.3.5.b: Interaction with mcs3

Tetrad analysis of the cross between strains of the genotypes *wee1.50 cdc25.22 win1.1 leu1.32 h*$^+$ and *wee1.50 cdc25.22 mcs3.12 leu1.32 h*$^-$ gave one tetratype tetrad which was selected for further analysis. One segregant was identifiable as *win1*$^+$ *mcs3*$^+$, and two segregants were similar to the parental strains. The fourth segregant appeared darker red on phloxin medium at 25°C and displayed a much stronger cdc phenotype than either parental strain at 35°C on both YE and EMM. (It was possible to distinguish between the two parental strains on the basis of growth on EMM and YE at 35°C.) This putative *win1.1 mcs3.12* strain was backcrossed to a strain of genotype *wee1.50 cdc25.22 leu1.32 h*$^+$ and the cross subjected to random spore analysis. Of the segregants from this cross showing cdc$^-$ phenotype, two classes were observed, one of which was nutritionally sensitive, the other not. On the basis of this data it was deduced that *win1* and *mcs3* were not allelic, and that the phenotype of the mutant strain *wee1.50 cdc25.22 mcs3.12 win1.1* showed a stronger similarity to that of *wee1.50 cdc25.22 mcs3.12* than to that of *wee1.50 cdc25.22 win1.1*.

3.3.5.c: Interaction with mcs4

A similar analysis to that described above was carried out with *mcs4*. From the cross between the strains *wee1.50 cdc25.22 win1.1 leu1.32 h*$^+$ and *wee1.50 cdc25.22 mcs4.13 leu1.32 h*$^-$ two tetratypes were examined which contained one segregant with a *wee1.50 cdc25.22* phenotype. The remaining three segregants in each tetrad were indistinguishable, so to determine which was of the genotype *wee1.50 cdc25.22 win1.1 mcs4.13*, three such segregants from one tetrad were backcrossed to strains of the genotype *wee1.50 cdc25.22*. Backcrosses of one of these segregants resulted in tetrads with either a 2:2 or a 3:1 segregation of cdc$^-$ to cdc$^+$, suggesting this segregant had the genotype *wee1.50 cdc25.22 win1.1 mcs4.13*. The other two segregants, on back crossing, gave a 2:2 segregation pattern of cdc$^-$ to cdc$^+$, indicating that they had the genotypes of the original parent strains.

A further cross was made between strains of genotypes *win1.1 leu1.32 h*$^-$ and *mcs4.13 leu1.32 h*$^+$ which was subjected to tetrad analysis. Putative double mutants were identified by their slightly increased cell length in comparison with the parental strains, and their genotype was confirmed by backcrossing to a wild type strain.

These results demonstrate that *win1* is not allelic to mcs4, and, despite their phenotypic similarities, the effects of these mutations are not additive.

3.3.5.d: Interaction with mcs6

From the cross between the strains *wee1.50 cdc25.22 win1.1 leu1.32 h$^+$* and *wee1.50 cdc25.22 mcs6.13 leu1.32 h$^-$* one tetratype was chosen for analysis. The presence of a segregant with a *wee1.50 cdc25.22* phenotype immediately suggested that the two mutations were not allelic. The two parental strains were easily distinguished on the basis of their phenotypes on EMM at 35°C. The fourth segregant in this tetrad showed a phenotype distinct from those of the parental strains: the cells were very elongated at the restrictive temperature when grown on EMM or YE. This was a much stronger cdc-phenotype than that shown by a *wee1.50 cdc25.22 win1.1* strain on EMM at this temperature. This segregant was backcrossed to a strain of genotype *wee1.50 cdc25.22*, a cross whose asci were almost entirely two spored asci. In some dyads both segregants were sporulation competent due to heterozygosity at the mating type locus. These diploids were themselves subjected to tetrad analysis to confirm that the genotype of this segregant was *wee1.50 cdc25.22 win1.1 mcs6.13.* These results indicate that *win1* in not allelic to *mcs6*, and that *win1.1* and *mcs6.13* show a strong interaction in the *wee1.50 cdc25.22* genetic background.

3.3.6: Interaction with pat1.114

Mutant alleles of *pat1* were isolated (Nurse, 1985) as mutations which released the cells from the normal requirements for sporulation necessary in wild type cells. In an independent investigation, *pat1* chromosomal mutations were isolated as suppressors of *mat2.102* (Iino and Yamamoto, 1985), which also showed a phenotype of hypersporulation similar to that of the *pat1* mutant alleles. *ran1* and *pat1* were later shown to be allelic, and the locus is now designated *pat1* (Kohli, 1987). *pat1* mutations allow strains of opposite mating types to conjugate, and *h$^+$/h$^-$* diploids to sporulate, in rich medium. They also allow haploid cells of either *h$^+$* or *h$^-$* mating type to undergo aberrant meiosis and sporulation.

A cross between the strains *win1.1 leu1.32 h+* and *pat1.114 ade6.M216 leu1.32 h$^-$* (supplied by Maureen McLeod) was subjected to tetrad analysis. There was no detectable linkage between the two markers *pat1* and *win1.* One putative *pat1.114 win1.1* double mutant strain was selected from a tetratype tetrad for further analysis.

The phenotype of this strain was examined microscopically on YE and supplemented EMM at 28°C, 32°C and 35°C with the parental *pat1.114* strain as a control. At the permissive temperature of 28°C the two strains appeared similar, with the *win1.1 pat1.114* double mutant showing slightly longer cells at division. Following one or two days growth at either 32°C or 35°C, the double mutant strain showed a much lower level of sporulation than the *pat1.114* single mutant strain. The cells from both strains ceased growth and became very swollen, though the cells of the double mutant remained longer than those of the *pat1.114* strain. After prolonged incubation at 32°C and 35°C some very abnormal cell morphologies were seen in the double mutant strain, including branched cells and multiple septa. These experiments indicate that the combination of *win1.1* with *pat1.114* prevents the hypersporulation phenotype of shown by $pat1^{ts}$ strains at the restrictive temperature, though win1.1 does not suppress their growth defect.

Mutant alleles of the genes *cgs1* and *cgs2* were isolated on the basis of their ability to suppress the temperature sensitive phenotype of *pat1.114* (Maureen McLeod, Pers. Comm.). In order to determine if either *cgs* mutation was allelic to *win1*, crosses was made between *win1.1 leu1.32* $h^{-}$and the strains *cgs1.1 ade6.216 leu1.32* h^{90} and *cgs2.3 ade6.210* h^{90} and subjected to tetrad analysis. These crosses showed that *win1* showed no significant linkage to either *cgs1* or *cgs2*, indicating that they were not allelic (data not shown).

The predicted *cgs1* product shows a high homology with the regulatory subunit of cAMP-dependent protein kinase from *S.cerevisiae* and other eukaryotes (McLeod and Beach, 1989), and elevated levels of intracellular cAMP have been shown to suppress the phenotype of $pat1^{ts}$ strains (Beach et al, 1985). In order to examine the effect of increased cAMP levels on *win1.1* strains, a comparison was made between *win1.1* and wild type cells grown on plates containing various concentrations of caffeine (trimethylxanthine), which is an inhibitor of cAMP phosphodiesterase. *win1.1* strains showed a slightly increased sensitivity to caffeine compared to that of wild type cells when grown on either YE or EMM-glutamate. Both strains were capable of forming colonies at a concentration of 15mM caffeine, whereas only $win1^{+}$ strains were colony forming at 20mM.

3.4: GENETIC MAPPING OF THE win1 LOCUS

3.4.1 Mapping strategy

The strategy used for the mapping of the *win1* locus was first to allocate the gene to one of the three *S.pombe* chromosomes by mitotic haploidisation (Chapter 2, Kohli *et al*, 1977). The second step was to locate the gene within the chromosome using the *swi5* system which allows genetic mapping over relatively large distances due to reduced meiotic recombination in homozygous *swi5*$^-$ strains (Schmidt *et al*, 1987). The third step was to find genetic markers closely linked to the *win1* locus using classical genetic mapping methods.

3.4.2: Chromosome allocation

An induced haploidisation procedure similar to that described by Kohli *et al* (1977) was used to allocate *win1* to one of the three *S.pombe* chromosomes. Stable diploid strains were constructed by utilising the ability of *mat2.102* strains to conjugate with *h*$^-$ strains giving rise to diploids which are unable to undergo meiosis and sporulation. In an appropriate genetic background, selective medium will only allow growth of diploid cells. In practice, a mating mix of *mat2.102* and *h*$^-$ strains are incubated overnight on ME at 25°C, before streaking out onto selective medium containing phloxin.

The diploid strains used for induced haploidisation were of the genotype *ura1.131/+ lys1.171/+ ade6.704/+ win1.1/+ leu1.32/+ mat2.102/h*$^-$ (Table 3.9). Three independent diploid strains were constructed as described above. Haploidisation was induced by growth overnight at 25°C on YE plates containing the anti-microtubule drug MBC at 10 g/ml. Following drug treatment, the cells were suspended in water and several dilutions plated onto YE-phloxin medium. These plates were then incubated at 28°C to allow colonies to form. Haploid colonies were picked to YE master plates which were replicated to appropriate selective media for the scoring of auxotrophic markers, along with fully supplemented medium. The *win1.1* phenotype was scored by microscopic examination of cells growing on fully supplemented medium on the basis of cell length. One hundred colonies from each of the three independent diploid strains were examined and, of these, about half were haploid. The phenotypes of the haploid segregants were scored, and the segregation pattern of *win1.1* with respect to each of the auxotrophic markers analysed (Table 3.9).

Table 3.9: Results of mitotic haploidisation experiments.

Phenotype of haploids	Diploid strain No.1	No.2	No.3
win1$^-$ ade4$^-$	12	13	18
win1$^-$ ade4$^+$	17	10	22
win1$^+$ ade4$^-$	13	11	16
win1$^+$ ade4$^+$	13	9	8
win1$^-$ leu1$^-$	12	10	13
win1$^-$ leu1$^+$	17	13	27
win1$^+$ leu1$^-$	17	10	9
win1$^+$ leu1$^+$	9	10	15
win1$^-$ ura1$^-$	0	0	0
win1$^-$ ura1$^+$	29	23	40
win1$^+$ ura1$^-$	24	20	24
win1$^+$ ura1$^+$	2	0	0
win1- lys1-	0	0	0
win1- lys1+	29	23	40
win1+ lys1-	25	20	24
win1+ lys1+	1	0	0

Diploid construct used for mitotic haploidisation:

Chromosome:	I	II	III	?
	ura1.131 lys1.171	*mat2.102* +	*ade6.704*	+
	+ +	h^- *leu1.32*	+	*win1.1*

The distribution of the markers *win1, ura1* and *lys1* showed a very high proportion of the parental combinations, as opposed to the random assortment shown by *win1, leu1* and *ade6*. This data strongly suggests that *win1* is located on chromosome I, although it gives no indication of the position with respect to other genetic markers of the gene within this chromosome.

3.4.3: Location of win1 within a region of chromosome I

The second stage in the mapping procedure was to allocate *win1* to a region of chromosome I. In order to do this, crosses were made involving *win1.1* in a *swi5* genetic background. Loss of *swi5* function reduces intra- and intergenic recombination by a factor of approximately ten, which makes it possible to undertake genetic mapping over long distances (Schmidt *et al*, 1987). A *win1.1 swi5.39* h^- strain was crossed to a *win1*$^+$ *swi5*.39 strain carrying several auxotrophic markers distributed throughout chromosome I, and the recombination frequencies between *win1.1* and the auxotrophic markers used to estimate the position of the *win1* locus. Such a marked strain (HE564) of genotype *ura1.171 his6.365 lys1.131 ade4.31 swi5.39* h^+ was kindly supplied by Henning Schmidt, along with a strain of genotype *swi5.39* h^{90}.

Before any crosses could be made with the aim of mapping *win1*, it was necessary to construct a strain of genotype *win1.1 swi5.39* h^-. The effect of *swi5* upon mating type switching was used to identify *swi5.39* strains: *swi5.39* h^{90} strains grown on ME medium show a mottled staining pattern when exposed to iodine vapours due to reduced mating type switching, in contrast to the homogeneous staining of *swi5*$^+$ h^{90} strains. This is the most easily recognisable phenotype of *swi5.39*.

In order to construct a strain of genotype *win1.1 swi5.39* h^+/h^-, the following cross was carried out: *swi5.39* h^{90} x *win1.1 leu1.32* h^-. The heterothallic h^- strain was present in a three-fold excess in the mating mix, in order to lower the proportion of asci resulting from conjugation of the homothallic strain with itself. Following tetrad analysis, it was possible to exclude tetrads resulting from homothallic conjugation by testing the progeny for leucine auxotrophy, as these tetrads were entirely leu$^-$. The remaining tetrads were replicated to ME plates, and after a few days exposed to iodine vapours. NPD tetrads were then identified since they have two homogeneous iodine positive colonies of the genotype *swi5*$^+$ h^{90}, and two iodine negative colonies of the genotype *swi5.39* h^-. These heterothallic

Table 3.10: Crosses of HE564 to win1.1 and win1.1 swi5.39:

Crosses 1 and 2: *win1.1 leu1.32 swi5.39 h⁻*

x

ura1.171 his6.365 lys1.131 ade4.31 swi5.39 h⁻

Cross 3: *win1.1 leu1.32 h-*

x

ura1.171 his6.365 lys1.131 ade4.31 swi5.39 h⁻

10a: Analysis of linkage between auxotrophic markers.

Phenotype	Cross 1	Cross 2	Cross 3
$his6^-$ $ura1^-$	33	52	19
$his6^-$ $ura1^+$	7	6	19
$his6^+$ $ura1^-$	11	6	24
$his6^+$ $ura1^+$	43	32	33
$his6^-$ $lys1^-$	32	45	18
$his6^-$ $lys1^+$	10	13	20
$his6^+$ $lys1^-$	5	5	29
$his6^+$ $lys1^+$	49	33	29
$ade4^-$ $lys1^-$	28	32	22
$ade4^-$ $lys1^+$	23	19	29
$ade4^+$ $lys1^-$	9	18	24
$ade4^+$ $lys1^+$	36	27	20

10b: Analysis of linkage between win1 and auxotrophic markers (See Table 3.7a).

	Cross		
	1	2	3
Phenotype			
win1⁻ ade4-	7	14	19
win1⁻ ade4⁺	40	38	21
win1⁺ ade4⁻	40	35	32
win1⁺ ade4⁺	5	7	23
win1⁻ his6⁻	18	25	15
win1⁻ his6⁺	29	27	26
win1⁺ his6⁻	24	31	23
win1⁺ his6⁺	25	11	32
win1⁻ lys1⁻	10	20	21
win1⁻ lys1⁺	37	32	20
win1⁺ lys1⁻	27	28	26
win1⁺ lys1⁺	22	14	29
win1⁻ ura1⁻	23	27	16
win1- ura1⁺	24	25	24
win1⁺ ura1⁻	23	39	27
win1⁺ ura1⁺	26	13	28

Figure 3.3: Genetic map of S.pombe.

From Munz *et al*, 1989.

Highlighted markers are those used for the mapping of *win1*.

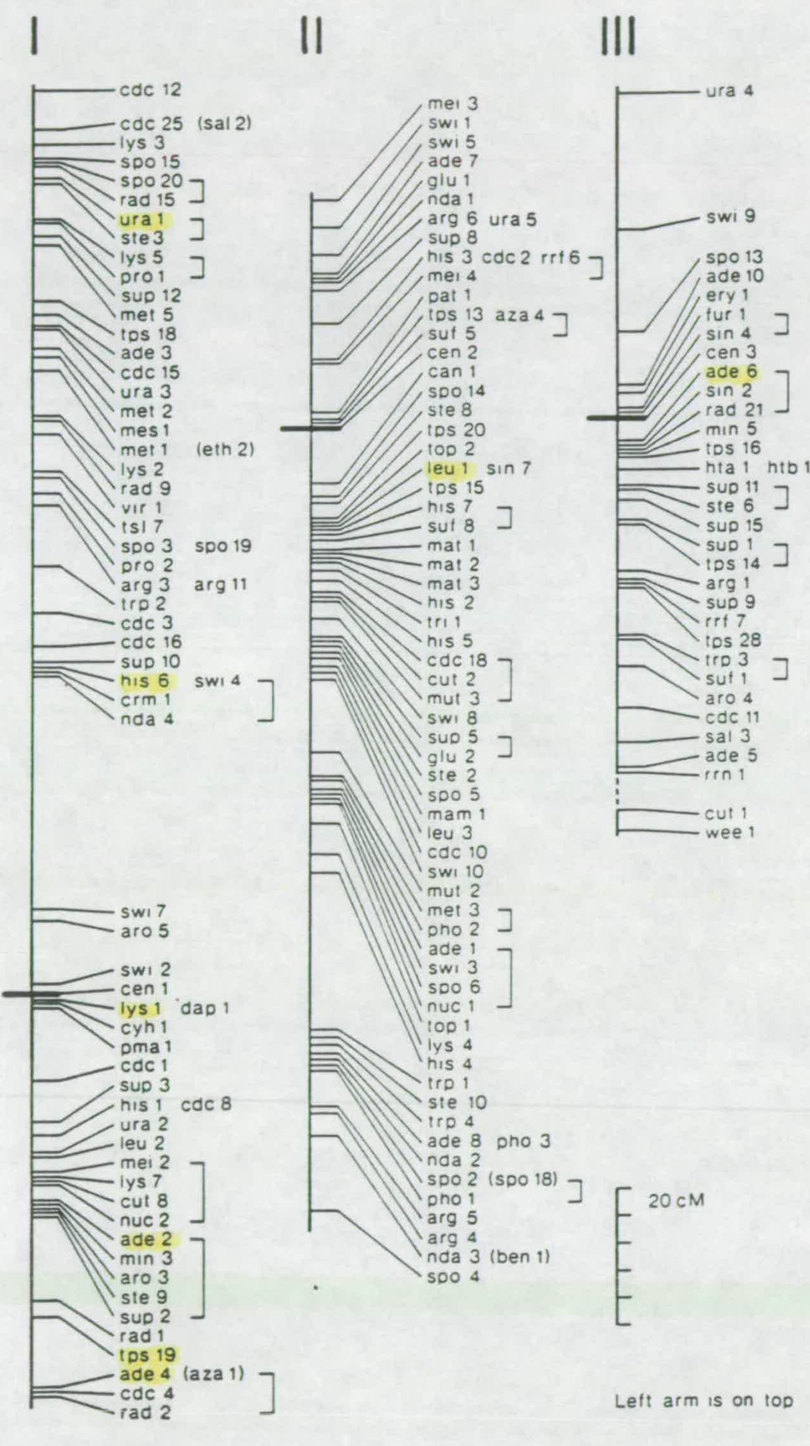
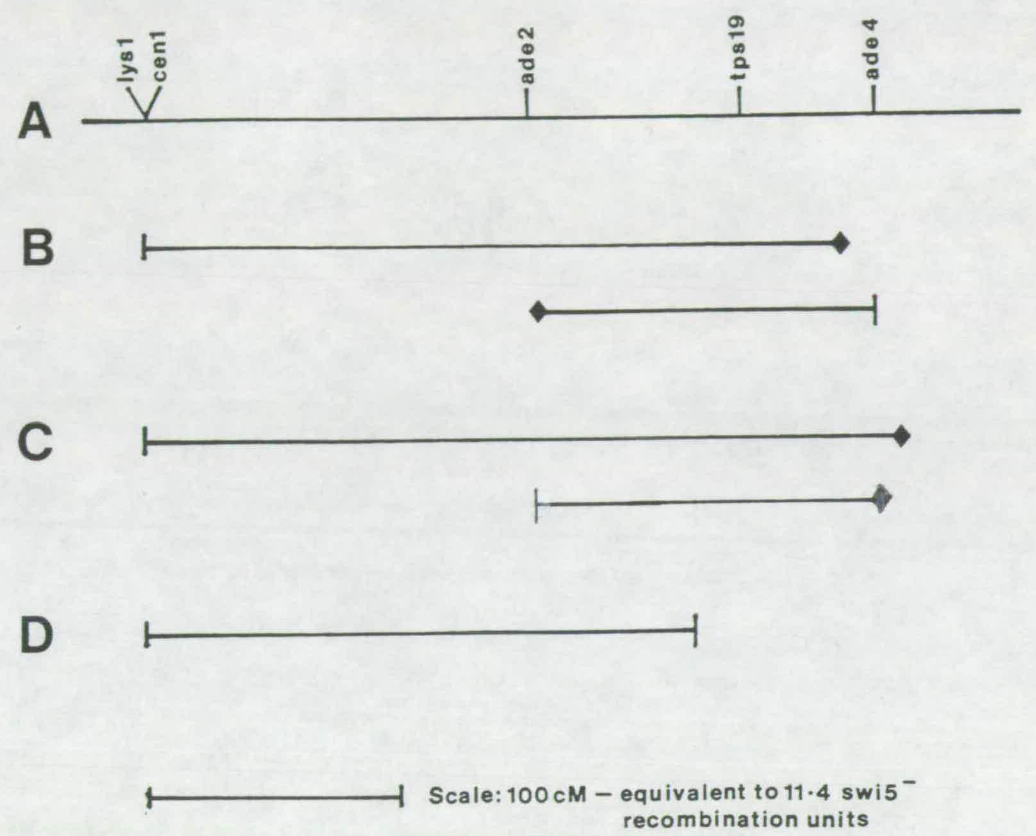


Figure 3.4: Results of mapping crosses in a swi5.39 genetic background.



A: The short arm of chromosome I, with distances taken from Figure 3.3.

Distances shown in panels B, C and D are the averages of results from duplicate crosses in a *swi5.39* genetic background. The figures shown in this diagram were calculated as a fraction of the *lys1* - *ade4* linkage in a *swi5.39* genetic background (33 recombination units). Diamonds indicate the predicted position of *win1* with respect to the auxotrophic markers used for mapping.

B: Predicted position of *win1* with respect to *lys1* and *ade4*

C: Predicted position of *win1* with respect to *lys1* and *ade2*.

D: Distance between *lys1* and *ade2*.

Table 3.11: Linkage between markers in crosses involving SW1, SW2, win1.1 leu1.32 and HE564 (see Table 3.10 for details of crosses).

Markers	Crosses 1	2	3	*
	% Recombination			
*his6-ura*1	18.7	12.5	45.3	20.5
*his6-lys*1	15.6	18.7	51.0	16.6
*ade4-lys*1	33.3	38.5	55.8	32.6
*win1-ade*4	12.5	22.3	44.2	
*win1-his*6	44.8	38.3	48.9	
*win1-lys*1	33.3	36.2	51.9	
*win1-ura*1	51.0	42.5	46.3	

Distances were calculated from the data given in Tables 3.7a and 3.7b. "*" refers to figures supplied by Henning Schmidt (Pers. Comm.) for crosses in a *swi5.39* genetic background.

strains were then scored for *win1.1* phenotype by examining cell length in freshly growing patches.

Two independent isolates of the genotype *win1.1 leu1.32 swi5.39* h^- (SW1 and SW2) were crossed to the marked strain HE564. A *win1.1 leu1.32* h^- strain was also crossed to HE564 as a control. These crosses were subjected to random spore analysis and 96 progeny from each cross scored (Tables 3.10a and 3.10b).

The linkage data from these crosses indicate that *win1* is located on the short arm of chromosome I, and, in a *swi5*.39 genetic background, between 12 and 22 recombination units from the *ade4* locus (Table 3.11 and Fig. 3.3). However, the linkage data are not additive, in particular the sum of the *ade4* - *win1* and *lys1* - *win1* linkage is greater than the *ade4* - *lys1* linkage, so there was some uncertainty about the exact position of *win1*. The data from the control cross (3) which was in a *swi5* heterozygous background show no strong linkage between *ade4* and *win1*, which indicates that *win1* is at least 40cM from *ade4*.

In order to locate *win1* more precisely within this region, crosses were performed involving *win1.1* and an *ade2* mutant allele. *ade2* is located approximately midway between *ade4* and *lys1* (Fig. 3.3). A strain of genotype *ade2.17 swi5.39* h^- was made using a similar method to that described for the

construction of the *win1.1 swi5.39* strain. Strains of the genotypes *ade2.17* h^- and *ade2.17 swi5.39* h^- were crossed to the strains SW1.1 (*win1.1 lys1.131 his6.365 swi5.39* h^+) and SW1.4 (*win1.1 lys1.131 his6.365 ura1.171 swi5.39* h^+). SW1.1 and SW1.4 were derived from the cross of SW1 to HE564. The crosses were subjected to free spore analysis and 96 progeny scored in each case (Table 3.12).

The data from the crosses carried out in a *swi5.39* genetic background give a recombinant fraction of *ade2* to *lys1* of 22-28%, which is slightly more than would be expected on the basis of previously published mapping data (Munz *et al*, 1989). In a *swi5.39* genetic background, the recombinant fraction between *win1 and ade2* is 16-18%, which confirms that the *win1* locus is close to that of *ade4* (Fig. 3.4).

The nearest identified marker to *ade4*, excluding *cdc4* and *rad2* which are very tightly linked to *ade4*, is *tps19*. Two independent crosses were analysed between the strains *win1.1*

Table 3.12: Results of crosses between ade2.17 and win1.1 in a swi5.39 genetic background.

Cross 4: *ade2.17 swi5.39 h⁻*

x

win1.1 lys1.31 his6.365 swi5.39 h⁺

Cross 5: *ade2.17 swi5.39 h⁻*

x

win1.1 lys1.31 his6.365 ura1.171 swi5.39 h⁺

Cross 6: ade2.17 h⁻

x

win1.1 lys1.31 his6.365 swi5.39 h⁺

	Crosses		
Phenotype	4	5	6
ade2⁻ win1⁻	9	9	14
ade2⁻ win1⁺	44	29	30
ade2⁺ win1⁻	37	48	29
ade2⁺ win1⁺	6	8	23
lys1⁻ win1⁻	29	37	
lys1⁻ win1⁺	15	16	
lys1⁺ win1⁻	17	20	
lys1⁺ win1⁺	35	21	
ade2⁻ lys1⁻	11	12	
ade2⁻ lys1⁺	42	26	
ade2⁺ lys1⁻	33	43	
ade2⁺ lys1⁺	10	15	

Table 3.13: Results of the crosses between win1.1 leu1.32 h+ and tps19.17 h$^-$.

Phenotype	Number of segregants	
win1$^-$ tps19$^-$	3	2
win1$^-$ tps19$^+$	50	43
win1$^+$ tps19$^-$	40	47
win1$^+$ tps19$^+$	2	2

leu1.32 h^+ and *tps19.17* h^-, the results of which are shown in Table 3.13. These data indicate that *win1* is 4-5 cM from *tps19*.

3.5: DISCUSSION

This chapter describes various genetic interactions between *win1.1* and other cell cycle mutations. The aim of these experiments was to investigate, by examining genetic interactions, the possible role of *win1* within the cell. The terminal phenotype of a *win1.1 cdc13.117* double mutant upon a shift to the restrictive temperature of 35°C was that of an arrest of in G2, similar to that shown by *cdc25*. This is contrast to that of a *cdc13.117* strain, which shows a cdc phenotype, but with many characteristics of a mitotic cell (Nasmyth and Nurse, 1981). One possible explanation for the double mutant phenotype is that *win1.1* causes the reduction of a residual level of *cdc13* activity present in *cdc13.117* cells at the restrictive temperature. The phenotype of cells completely lacking *cdc13* function has been shown to be one of G2 cdc arrest (Hagan *et al*, 1988; Booher and Beach, 1988), i.e. similar to that of a *win1.1 cdc13.117* double mutant strain.

The interactions between *win1.1* and chromosomal mutations or artificial constructs involving *cdc25* which result in a wee phenotype were investigated. The wee mutations *wee1.50* and *cdc2.1w* and the over-expression of *cdc25*$^+$ were all capable of suppressing the cell length phenotype of *win1.1*. However, the combination of *win1.1* with *cdc2.3w* resulted in a phenotype more similar to that of *win1.1*. These observations show that *win1.1* cell are still sensitive to *wee1* and *cdc25* expression levels, and suggest an allele specific interaction between *win1.1* and *cdc2w* alleles.

Neither of the two forms of mitotic catastrophe phenotype, which are caused by the combination of either *cdc2.3w* or over-expressed *cdc25*$^+$ with *wee1.50*, is suppressed by *win1.1*.
win1.1 is not allelic to *mcs3.12*, *mcs4.13* or *mcs6.13*, which are mitotic catastrophe suppressing mutations.

Like *win1.1*, *mcs3.12*, *mcs4.13* and *mcs6.13* are capable of reversing the suppression of *cdc25* by *wee1*. The closest phenotypic similarity with *win1.1* is shown by *mcs4.13*: both mutations have a phenotype of increased cell length, and show a nutritionally sensitive phenotype when combined with wee1.50 and cdc25.22. In order to investigate interactions between these mutations, the phenotypes resulting from the combination of *win1.1* with these three *mcs* mutant alleles in a *wee1.50 cdc25.22* genetic background was

examined. The results of these experiments are difficult to interpret, as the resulting strains contain combinations of four cell cycle mutations. However, one possible interpretation of these results is that *mcs4* and *win1* may lie in the same pathway, not only from the evidence of their similar phenotypes, but from their mutual epistasis when combined in a *wee1.50 cdc25.22* genetic background.

The combination of *win1.1* with *pat1.114* results in a suppression of the hypersporulation phenotype normally shown by *pat1^{ts}* strains at the restrictive temperature, although cells of the double mutant strains do not continue to grow and divide. *win1* is not allelic to *cgs1* or *cgs2*, mutant alleles of which are also capable of suppressing the hypersporulation of *pat1.114*. Elevated levels of cAMP have been shown to suppress the phenotypes of both *pat1.114* and a *pat1* null allele (Beach *et al*, 1985). In *S.cerevisiae*, loss of the regulatory subunit of cAMP-dependent protein kinase has been shown to inhibit meiosis (Matsumoto 1983). The situation appears to be analogous in *S.pombe*, as *cgs1*, which was identified as a chromosomal mutation capable of suppressing *pat1.114*, is highly homologous to the regulatory subunit of cAMP-dependent protein kinase in other eukaryotes. *win1.1* strains show an increased sensitivity to caffeine, which is an inhibitor of cAMP phosphodiesterase, suggesting that these strains have an altered response to intracellular cAMP levels, or an intrinsically higher level. These observations are difficult to interpret without further details of the interactions between *win1* and *pat1*, although one possible explanation is that *win1* is involved in some from of signalling mechanism, possibly nutritionally based, which is involved in sporulation.

The final part of this chapter describes the genetic mapping of *win1*. The *win1* locus was first mapped to chromosome I by a mitotic haploidisation procedure, followed by allocation to a region of that chromosome by a series of crosses in a *swi5.39* mutant background. One problem in using this system to map *win1* was that there appeared to be an increase in meiotic recombination involving *win1.1*, compared with that between other markers. From the data described here, it is difficult to identify any specific causes for this effect, although it seems likely that it is because the crosses were carried out in *swi5.39* background, in which recombination is obviously altered (Henning Schmidt, Pers. Comm.)

Finally *win1* was mapped using conventional techniques to a locus 4 - 5cM from *tps19*. One possible application of this data is to design an alternative strategy for cloning *win1*, which would involve the cloning of *tps19* by complementation, followed by chromosome walking, initially using *tps19* sequences as a probe, to isolate *win1*. Plasmid clones have

been identified which are capable of suppressing the *tps19.17* phenotype (Maria-Victoria Zarate, Pers. Comm.)

CHAPTER 4

CHAPTER 4: ISOLATION OF PLASMID CAPABLE OF SUPPRESSING A win1.1 CONFERRED PHENOTYPE

4.1: ISOLATION OF PLASMIDS

4.1.1: Cloning strategy

The initial aim of these experiments was the isolation of the *win1* gene. The strategy used was based on the initial observation that the triple mutant strain *wee1.50 cdc25.22 win1.1* showed a very low colony forming ability when plated on EMM at the restrictive temperature of 35°C (Ogden and Fantes, 1986). This phenomenon was examined more closely by plating out cell suspensions at varying concentrations onto plates consisting of various solid media.

The results of these experiments (presented in Chapter 3) showed that it would be possible to discriminate between cells with *win1*$^{+}$ and *win1*$^{-}$ genotypes in this genetic background on the basis of their ability to form colonies on EMM at 35°C. They also confirmed that the presence of 1.2M sorbitol (which would be present in solid medium used for the regeneration of protoplasts following the transformation procedure) would not suppress the temperature sensitive phenotype of strains of genotype *wee1.50 cdc25.22 win1.1*.

The approach taken was to transform a strain of the genotype *wee1.50 cdc25.22 win1.1 leu1.32* with various gene libraries consisting of random *S.pombe* genomic sequences contained within autonomously replicating plasmid vectors. The presence of plasmid could be selected for by means of the *S.cerevisiae LEU2* gene present in the plasmid vectors, which is capable of complementing the leu^{-} phenotype of *leu1.32* strains. It was hoped that the presence of plasmid-borne *win1*$^{+}$ sequences could be detected by selecting for growth of the cells on EMM at 35°C.

4.1.2: Gene library screening

Several gene libraries were screened in the course of these experiments (Table 4.1). Following the transformation procedure, protoplasts were allowed to regenerate at 28°C, until small colonies could be observed on the plates (3 - 5 days). Two procedures were used to select for cells able to grow at 35°C: the EMM-sorbitol plates were either replicated

Table 4.1: Gene libraries.

Library	Vector	Insert DNA	Source
A	pDB262	*Hin*dIII*	Ogden
B	pDB262	*Hin*DIII partial	Ogden
C	pDB262	*Hin*DIII	Nurse
D	pDB262	*Hin*DIII	Nurse
E	pDB248	*Sau*3A partial	Ogden and Fantes
F	pDB248	*Sau*3A partial	Ogden and Fantes
G	pDB248	*Sau*3A partial	Beach
H	pWH5	*Sau*3A partial	Young
I	pWH5	*Hin*DIII partial	Young

*DNA prepared from a strain of genotype *wee1.6*

directly to EMM-Phloxin at 35°C, or the cells were scraped off the sorbitol containing plates, resuspended in a small volume of water and immediately replated onto EMM-Phloxin plates at a density of approximately 10,000 cells per plate. In each experiment, a number of cells equal to more that ten times the original number of transformants was plated, to ensure plasmid bearing cells representative of each original transformant would be present. Cells were replated because the strain *wee1.50 cdc25.22 win1.1 leu1.32* shows significant bulk growth on EMM at 35°C. The direct replication of the original EMM-sorbitol plates resulted in large numbers of cells being transferred to the EMM-Phloxin plate at 35°C. This made growing colonies arising from plasmid-borne complementation of the temperature sensitive defect difficult to distinguish from background growth.

4.1.3: Analysis of transformants

For each gene library, a total of at least 5000 to 10,000 transformants were screened in two separate experiments. All colonies that formed at 35°C were picked and grown up at 25°C for further examination. The cells were streaked out on EMM at 35°C in order to confirm their ability to form colonies, and to examine cell size microscopically. Cells of the genotype *wee1.50 cdc25.22* have a cell length less than that of wild type cells grown under these conditions. In contrast, cells of the genotype *wee1.50 cdc25.22 win1.1* show a very heterogeneous cell length, with a high proportion of very long cells.

Strains that showed a high colony forming ability at low plating densities were selected for further analysis to determine if their change in phenotype was due to plasmid borne sequences. Plasmid sequences will not show a high mitotic stability once selection for plasmid-borne markers is relaxed. Growth of cells on non-selective medium will result in many losing plasmid. It is then possible to determine if two markers are unstable, and if they co-segregate, by replicating colonies representative of the cell population to appropriate selective media. If two markers are shown both to be unstable and to co-segregate in such a test, then it is highly likely that both are plasmid-borne.

The transformants isolated from the screen described above were grown on YE at the permissive temperature for 24 hours in order to relax selection for both the leu^+ and the $win1^+$ markers. Cells were then streaked out and grown for several days under the same conditions. Single colonies were isolated and analysed to determine if the $win1^+$ phenotype cosegregated with the leu^+ phenotype by replicating to EMM at 25°C (to test for *leu1* phenotype) and EMM plus leucine at 35°C (to test for *win1* phenotype). In the cases of strains which showed co-segregation of the two markers, a leu^+ colony was selected and plasmid isolated from the cells.

4.2: ANALYSIS OF PLASMIDS ISOLATED FROM GENE LIBRARY SCREENING

4.2.1 Confirmation of win1 suppression

Plasmid DNA isolated from the primary transformants was re-introduced into a *wee1.50 cdc25.22 win1.1 leu1.32* strain in order to confirm that these plasmids were the ones responsible for the cdc^{+} phenotype of the primary transformants. Each plasmid recovered was found to be capable of suppressing the cdc phenotype of this strain as judged by cell size viewed microscopically, and the transformants' ability to form colonies when streaked out on EMM-Phloxin at 35°C.

4.2.2 Comparison of S.pombe insert sequences

The various plasmids described were isolated from several transformation experiments carried out over a period of time. When new plasmids were isolated, one of the first stages in their analysis was to determine if they shared insert sequences with any plasmids already isolated in this screen. This was done by a combination of restriction site mapping and Southern blot analysis. In many cases it was possible to show that various plasmids isolated from one library were all of one species by comparing the sizes of restriction enzyme digest fragments. Where there was any uncertainty, Southern blot analysis was used to determine if there was any homology between the *S.pombe* genomic sequences carried by the different plasmids.

4.2.3: Plasmids resulting from gene library screening

In the first screening experiment (involving gene libraries A - F; see Table 4.1 and Table 4.3) only two transformants, both from gene library A, were identified as containing plasmid sequences capable of complementing the temperature sensitive phenotype of the strain *wee1.50 cdc25.22 win1.1 leu1.32.* Plasmids isolated from these transformants were judged to be of the same species from restriction enzyme analysis (Fig 4.1). One was chosen for further analysis and named pKb.

In the second screening experiment involving gene libraries A - G, four transformant colonies resulting from transformation with gene library A were isolated, three colonies from gene library E, and two colonies from gene library G (Table 4.1 and 4.3). Plasmid DNA was isolated from these transformants, and subject to restriction analysis. The

four transformants from gene library A yielded plasmids identical to pKb. Of the three transformants from gene library E, two gave rise to identical plasmids. These were named pC1. The third yielded a plasmid which was clearly related to pC1, but had a slightly different pattern of restriction sites (Fig. 4.1). This (named pC3) was shown by restriction mapping to contain a slightly larger *S.pombe* genomic insert than pC1 which includes a *Bgl*II and a *Hind*III site (Fig. 4.2). The two transformants from gene library G gave rise to two plasmids with identical restriction fragment patterns. This plasmid was named pH3.

In order to determine if the three plasmids described above contained shared *S.pombe* sequences, ^{32}P labelled samples of plasmids pC3 and pH3 were used to probe a Southern blot which carried samples of the plasmids pKb, pC1, pC3, pH3, p25.27 (which carries a 6.0kb fragment of the sequences encoding *nim1* (Russell and Nurse, 1987b)), and pDB248, all of which were digested with *Hind*III (Fig. 4.3). The libraries from which pC1, pC3 and pH3 were constructed consisted of sized *S.pombe* genomic fragments resulting from partial digestion with the enzyme *Sau*3A, which gives DNA termini compatible with ligation to *Bam*H1 termini. The presence of a *Hind*III site in the vector used (pDB248) means that *Hind*III digest of such plasmids will result in two restriction fragments containing vector sequences. In contrast, the construction of the gene library from which pKb was derived involved the insertion of *S.pombe Hind*III fragments into the *Hind*III site in the vector pDB262. *Hind*III digestion of plasmids made in this way results in only one DNA fragment containing vector sequences.

In experiments designed to determine if plasmids contain shared sequences, it can be more informative to use the entire plasmid as a probe in order to ensure that the full extent of the insert sequences are tested for hybridisation. However, this does result in hybridisation between vector sequences which must be taken into consideration.

Labelled pC3 sequences showed no hybridization with the *S.pombe* sequences contained in p25.27 or pKb (Fig. 4.3). There was hybridisation between pC3 and the two *Hind*III fragments of pH3, due to the presence of vector sequences in both fragments. There was also strong hybridisation with all

Figure 4.1: pKb and pLa plasmid isolates, and restriction analysis of pwis2-C1, pwis2-C2, pwis2-C3, and pwis3-1.

Plasmids were digested with *Hind*III and subject to agarose gel electrophoresis.

A:

Lane 1: pkb

Lane 2: pkb

Lane 3: pla

Lane 4: pla

Lane 5: λ *Hind*III (molecular weight markers)

B:

Lane 1: λ *Hind*III (molecular weight markers)

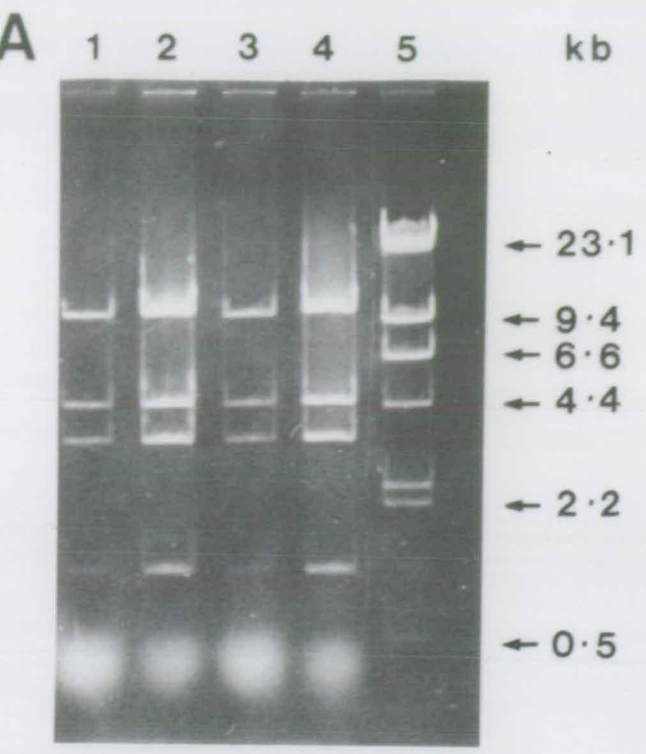
Lane 2: pkb

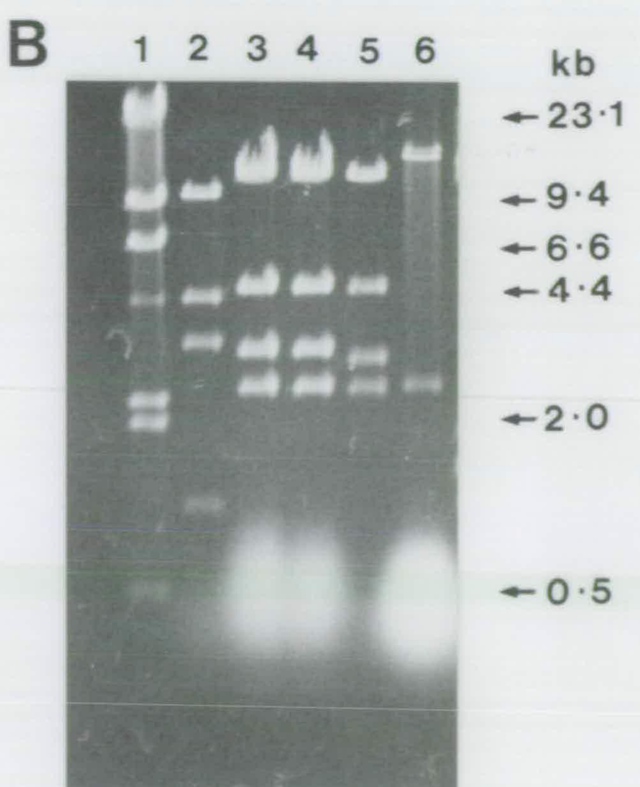
Lane 3: pc1

Lane 4: pc2

Lane 5: pc3

Lane 6: pH3


A
1
2
3
4
5
kb
23·1
9·4
6·6
4·4
2·2
0·5


B
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2·0
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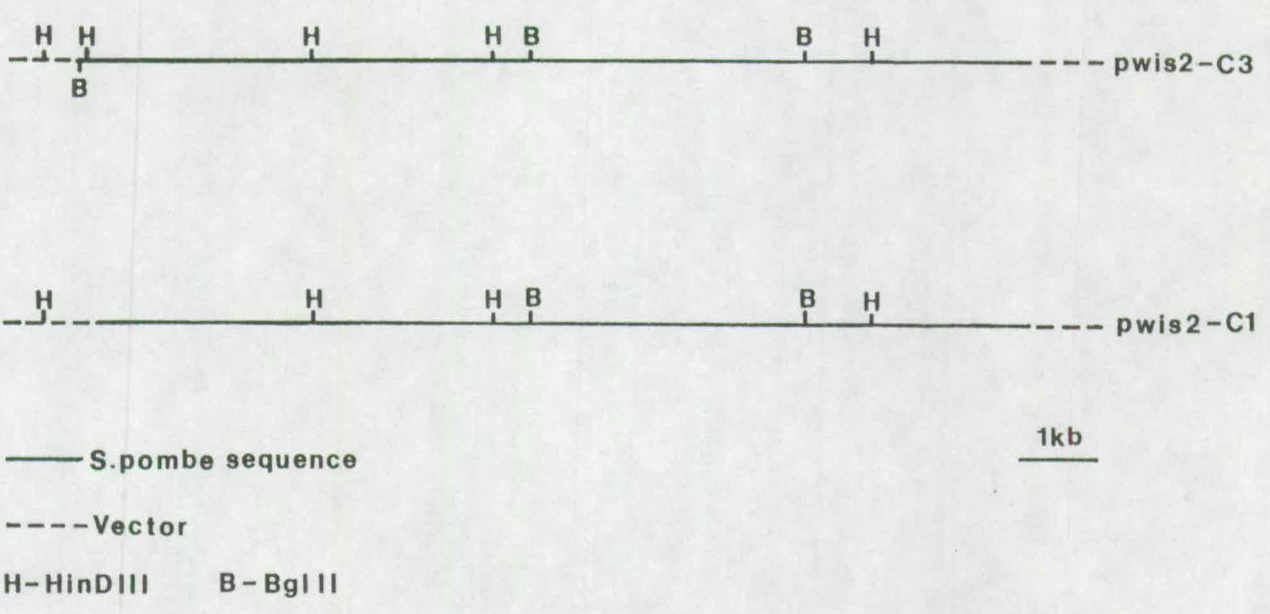


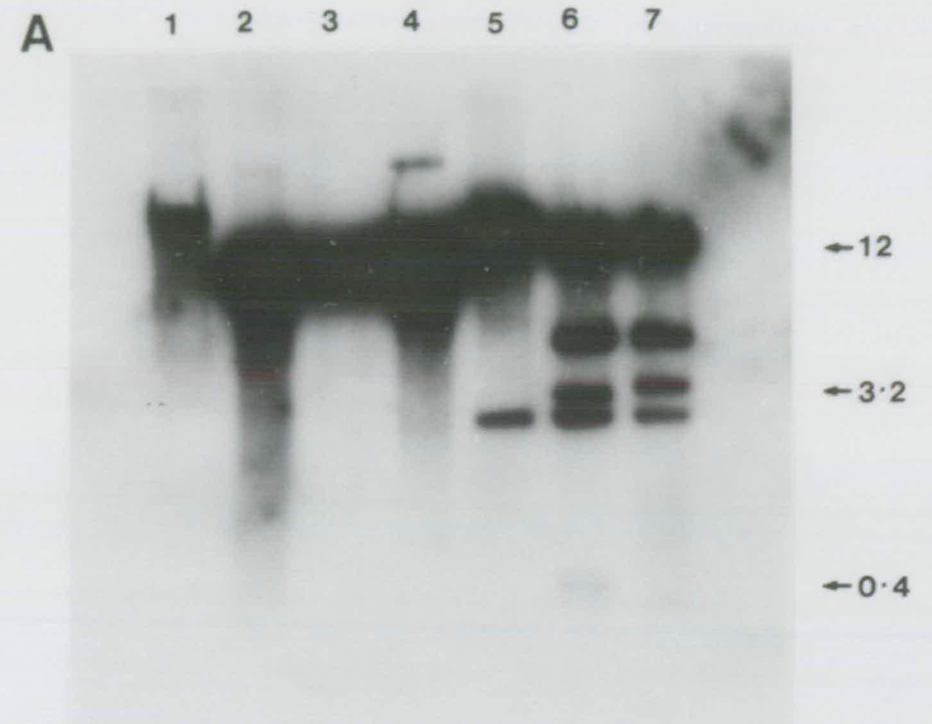
Figure 4.2: Preliminary restriction analysis of pwis2-C1 and pwis2-C3.

Junctions between vector and insert sequences were formed by the ligation of *Sau*3A and *Bam*HI restriction digest termini.

Figure 4.3: Southern blot analysis of pwis1, pwis2 and pwis3 plasmids.

Plasmids were digested with *Hind*III, separated by agarose gel electrophoresis, Southern blotted and probed with pc3 (panel A) and pH3 (panel B). p25-27 contains a *Hind*III fragment of the *nim1* gene.

Lane 1: wild type DNA (undigested)
Lane 2: pDB248
Lane 3: pkb
Lane 4: p25-27
Lane 5: pH3
Lane 6: pc3
Lane 7: pc2


A
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4
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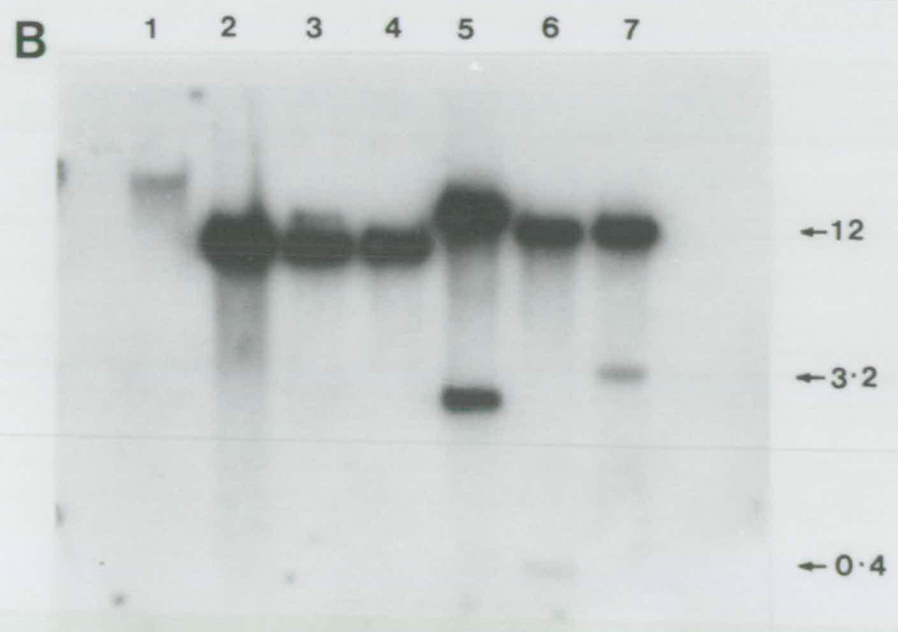
6
7
←12
←3·2
←0·4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the *Hin*dIII fragments of pC1, showing that they share the same insert sequences.

Labelled pH3 sequences also showed no hybridization with the *S.pombe* sequences contained in p25.27 or pKb (Fig. 4.3). There was very weak hybridization between pH3 sequences and the 3.1kb fragment of pC1 and the 0.4kb fragment of pC3. This was consistent with the fact that these *Hin*dIII fragments contain a small section of vector sequence. There is no hybridization other than between vector sequences, implying that pH3 shares no insert sequences with pC1 or pC3.

The following conclusions may be drawn from this data:

1) None of the plasmids pKb, pC3 and pH3 share *S.pombe* insert sequences.

2) pC1 and pC3 share a large portion of insert sequences. One margin of the insert is the same in both cases, judging from restriction analysis, but at the other margin, pC3 contains slightly more *S.pombe* sequence. Further experiments were carried out involving only *S.pombe*-derived sequences as probes at a later stage (Table 4.2). The plasmids were then re-named: pKb as pwis1-1, pC1 and pC3 as pwis2-C1 and pwis2-C3, respectively, and pH3 as pwis3-1 (wis denotes win-suppressing).

In the third screening experiment involving gene libraries G, H and I, four cdc^{+} transformants were recovered, two from gene library H, and two from gene library I (Table 4.1 and Table 4.3). Restriction analysis of the plasmids recovered from these transformants (pN1, pN2, pN3 and pN4) showed that they were all closely related. The plasmids derived from each gene library (pN1 and pN2 from gene library I and pN3 and pN4 from gene library H) were identical to each other, as judged from size of restriction fragments (Fig. 4.4). The two species of plasmids contained similar insert sequences as they gave similar patterns of restriction fragments upon digestion with other enzymes, although those derived from library H contained a slightly larger *S.pombe* insert (data not shown). The two species of plasmid were named pwis4-1 (from gene library H) and pwis4-2 (from gene library I). Neither of these two newly isolated plasmids contained a *Bgl*II site. It therefore seemed probable that they did not contain the same functional sequences as the previously described plasmids, which in subsequent analysis had all been shown to contain a *Bgl*II site within their functional sequences.

The final screening experiment carried out also involved gene libraries G, H and I. Four cdc^{+} transformants were recovered from gene library G, six from gene library H, and fourteen from gene library I (Table 4.1 and Table 4.3). The four transformant

Table 4.2: Pattern of Hybridisation between insert sequences of the pwis plasmids.

	Probe derived from:			
Plasmid	pwis1-1	pwis2-C3	pwis3-1	pwis4-1
pwis1-1	+	-	-	+
pwis2-C3	-	+	-	-
pwis3-1	-	-	+	-
pwis4-1	-	-	-	+
pDa	+	-	-	-
pSf	-	-	-	-
pSk	+	-	-	-

- = no hybridisation + = hybridisation

Probes: 4.1kb *Xba*I fragment of pwis1-1

3.8kb *Bgl*II fragment of pwis2-C3

4.0kb *Bgl*II fragment of pwis3-1

5.0kb *Bam*HI - PvuII fragment of pwis4-1

The plasmids tested were digested with *Hind*III and transferred to GeneScreen Plus membrane.

Table 4.3: Summary of plasmids isolated in gene library screening experiments.

Plasmid	Gene Library	Number Isolated	Library Form
pwis1-1 (pKb)	A	6	*Hind*III
pwis1-2 (pH)	I	14	*Hind*III
pwis1-3 (pDa)	G	4	*Sau*3A
pwis1-4 (pSf)	H	3	*Sau*3A
pwis2-C1 (pC1)	E	2	*Sau*3A
pwis2-C3 (pC3)	E	1	*Sau*3A
pwis3-1 (pH3)	G	2	*Sau*3A
pwis4-1 (pN4)	H	2	*Sau*3A
pwis4-2 (pN2)	I	2	*Hind*III
pwis5-1 (pSk)	H	3	*Sau*3A

The initial name given to plasmid isolates is given in brackets. The name given is that of the particular isolate chosen for further analysis. The restriction enzyme names listed under "library form" are those of the restriction enzyme used in the construction of the library to digest the *S.pombe* genomic DNA

the same plasmid species as judged from restriction analysis which was named pDa (data not shown).

Of the six transformants from gene library H, three yielded one class of plasmid (pSf) and the other three a second class (pSk) (Fig. 4.4). The transformants resulting from gene library I all yielded the same species of plasmid (pH). This library was constructed by the ligation of a sample of partially *Hin*dIII digested *S.pombe* genomic DNA into the vector pWH5. Upon digestion with *Hin*dIII, the pH plasmids gave three fragments, one corresponding to the vector, and the others identical in size to the two larger insert *Hin*dIII fragments in pwis1-1 (data not shown). Upon digestion with *Xba*I and *Bgl*II, the pH plasmids gave fragments that were also identical in size to those derived from the *S.pombe* sequences contained within pwis1-1 (data not shown). From this evidence it was decided that the pH plasmids contained the same functional sequences as pwis1-1, so this plasmid species was named pwis1-2 and no further analysis carried out.

In order to determine if the other newly isolated plasmids contained *S.pombe* sequences already identified in previously isolated plasmids, Southern blot analysis was carried out with filters carrying *Hin*dIII-digested samples of the following plasmids: pwis1-1, pwis2-C3, pwis3-1, pwis4-1, pDa, pSf and pSk. These filters were probed with restriction fragments from pwis1-1, pwis2-C3, pwis3-1 and pwis4-1 which consisted entirely of *S.pombe* insert sequences (Table 4.2). The use of probes consisting entirely of *S.pombe* sequences avoids the problem of hybridisation between vector sequences.

These results show that pDa and pSk share insert sequences with pwis1-1, and presumably the same functional sequences. These plasmids were named pwis1-3 and pwis1-4, respectively. Of the plasmids recovered from this screen, only pSf contained previously unidentified *S.pombe* sequences. This plasmid was then re-named pwis5-1.

Preliminary restriction site analysis of these plasmids indicated that none of them contained the *cdc25* gene. This was later confirmed by further investigations (see Chapter 5).

In order to investigate the phenotype of cells of the genotype *wee1.50 cdc25.22 win1.1* containing *cdc25* sequences carried on multicopy plasmids, the plasmid pcdc25-1 was transformed into such a strain. This plasmid contains *cdc25* sequences in the form of three HindIII fragments in the

Figure 4.4: pN (pwis4), pSk (pwis1-4) and pSf (pwis5-1) isolates .

Plasmids were digested with *Hin*DIII and subject to agarose gel electrophoresis.

A:

Lane 1: pN2

Lane 2: pc3

Lane 3: pkb

Lane 4: pSk

Lane 5: pSf

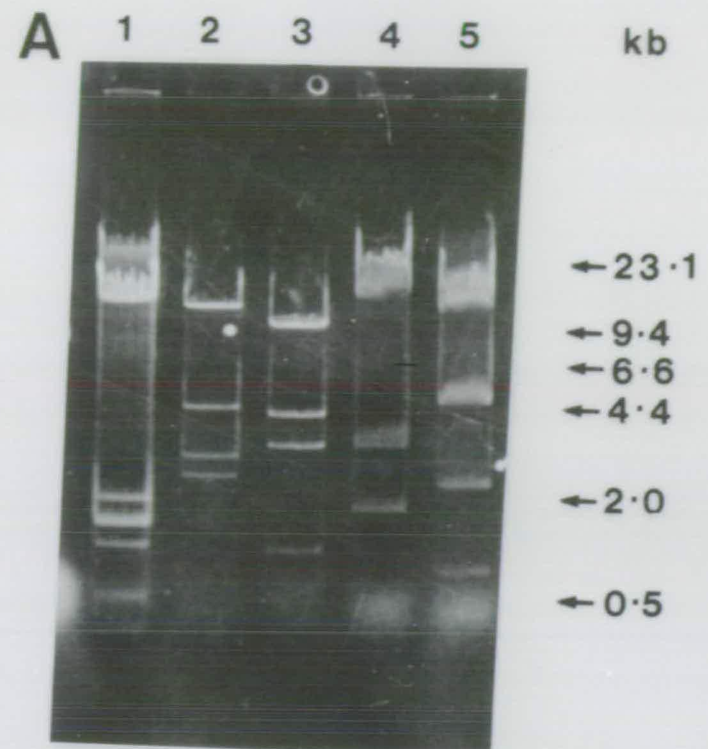
B:

Lane 1: pN2

Lane 2: pN3

Lane 3: pN4

Lane 4: λ *Hin*DIII (molecular weight markers)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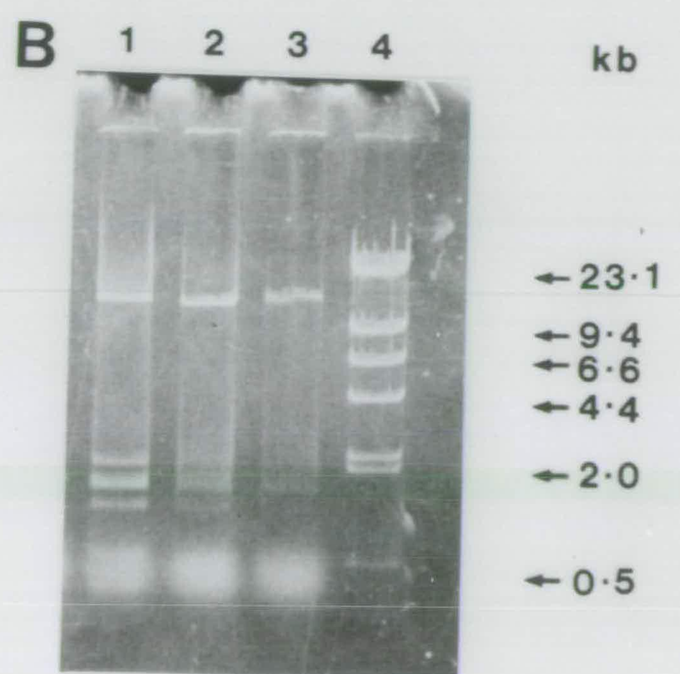
B
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4
kb
23·1
9·4
6·6
4·4
2·0
0·5

vector pWH5 (Young, Pers. Comm.). The phenotypes of such transformants were tested by streaking them out thinly on EMM at 35°C, and examining the cells microscopically after a varying periods of growth. The cells showed a phenotype of very small length at division, with many aberrant divisions. They also showed a very low growth rate with respect to colony forming ability, which was presumably due to a high level of *cdc25⁺* expression in a *wee1⁻* background (Russell and Nurse, 1986).

In all, five independent sequences were isolated that were capable of suppressing the temperature sensitive phenotype of the strain *wee1.50 cdc25.22 win1.1 leu1.32* when carried on multicopy plasmids (Table 4.3).

4.3: DO ANY OF THE ISOLATED PLASMIDS CONTAIN THE win1 GENE?

4.3.1: Strategy for analysis

The next stage in the analysis of these plasmids was to determine if they contained the authentic *win1* gene. In order to do this, integrant strains were made which resulted from homologous recombination between plasmid-borne sequences and genomic sequences using each of the plasmid species described above. Once the integration event had been shown to have occurred by homologous recombination, the integrants were crossed to a strain of genotype *win1.1 leu1.32*. It was then possible to determine if the plasmid-contained *S.pombe* sequences (as followed by the plasmid-borne leu+ phenotype) were closely linked to the *win1* locus, which could be followed by the *win1.1* conferred phenotype of slightly elongated cells. Close linkage between the two would indicate that the plasmid-contained sequences were derived from the *win1* locus, and were very likely to contain the *win1* gene.

4.3.2: Production of integrant strains

A preliminary restriction analysis was performed upon each of the plasmids to find an enzyme which would cut once or twice exclusively within the *S.pombe* insert sequences. Plasmids linearized in this way show a higher frequency of homologous recombination with the genome compared to circular plasmids, as described in *S.cerevisiae* by Orr-Weaver *et al*, (1983). Approximately 1 - 5 g of linearized plasmid was used to transform a *leu1.32 h⁻* strain using the standard transformation procedure. Transformants were then subjected to stability analysis: colonies were picked to YE plates and grown for 24 hours non-selectively, after which time they were streaked out for single colonies upon

non-selective (YE) plates. At least 20 colonies from these plates were picked to a YE master plate, and this plate replicated to EMM. When all the colonies derived from one transformant were capable of growth, it was taken to imply that this leu$^+$ phenotype was due to recombination between plasmid and genomic sequences resulting in the integration of one or more copies of the plasmid.

4.3.3: Molecular analysis of integrant strains

Using molecular techniques, it is possible to determine if the *S.pombe* sequences contained within plasmids isolated from gene libraries are colinear within the *S.pombe* genome, rather that resulting from a ligation artefact. It is also possible to determine if integrant strains have resulted from homologous recombination between plasmid and genomic sequences.

In order to determine if the insert sequences are co-linear within the genome, wild type DNA is digested with a restriction enzyme with no sites in the plasmid under analysis, and subjected to Southern blot analysis. If only one band shows hybridisation to labelled plasmid sequences, then the insert sequences are very likely to be colinear within the genome. If the hybridising band is shifted to a higher molecular weight in DNA samples from integrant strains digested with the same restriction enzyme, this is strong evidence that these strains have resulted from plasmid integration by homologous recombination.

The results of the Southern blot analysis of wild type and integrant strains with the plasmid pwis1-1 is shown in Figure 4.5. Samples of genomic DNA from a wild type strain, and integrant strains Int3 and Int5 were digested with *Mlu*I, which has no sites within pwis1-1. These samples were then subjected to Southern blot analysis, probed with all the sequences contained within pwis1-1 (Fig. 4.5). Wild type DNA shows only one hybridising band, indicating that the insert sequences contained within pwis1-1 are colinear within the *S.pombe* genome. DNA samples from the integrant strains also show a single hybridising band, but one which is of a higher molecular weight compared to that in the wild type samples. This indicates that plasmid integration has taken place by homologous recombination.

Table 4.4: Restriction enzymes used for the linearisation of plasmids, and the resulting integrant strains.

Plasmid	Enzyme	Integrant species
pwis1-1	*Pvu*II	Int2, Int3
pwis2-c3	*Sac*I	C3A, C3B
pwis3-1	*Bgl*II	HC, HD
pwis4-1	*Sst*I	N2I1
pwis4-2	*Xho*I	N4I1, N4I2, N4I3
pwis5-1	*Xho*I	W5I1, W5I2

Further experiments involving wild type strains and those listed in Table 4.4 showed that in the case of each plasmid the insert sequences were colinear within the genome. They also demonstrated that each integrant had resulted from homologous recombination between plasmid and genomic sequences (data not shown).

4.3.4: Genetic analysis of integrant strains

The integrant strains described above were then used in a genetic analysis to determine if the sequences carried by the pwis plasmids contained the authentic *win1* gene. In these integrant strains, the locus from which the plasmid-borne sequences were derived is tagged with the LEU2 marker in a *leu1*$^-$ background. If this were the *win1* locus, then when such integrants are crossed to a strain of the genotype *win1.1 leu1.32*, there should be little or no recombination between the *win1* locus and the LEU2-tagged locus from which the plasmid sequences were derived.

The results of such crosses between the integrant strains derived from the pwis plasmids and *win1.1 leu1.32 h*$^+$ are shown in Tables 4.5 to 4.9. Each cross was subjected to free spore analysis, and the phenotypes of approximately 50 colonies scored in each case. The leu$^{+/-}$ phenotype was scored by testing for growth on minimal medium, and the *win1* phenotype scored by examination of cell length microscopically.

The results of these experiments showed that, in four cases out of five, there was no linkage between the plasmid contained insert sequences and the *win1* locus. In the case of the remaining plasmid, the insert sequences showed a loose linkage to the *win1* locus, but not the figure that would be expected if the plasmid contained the authentic *win1* gene.

These results from this genetic analysis show that the *S.pombe* genomic sequences contained within the pwis plasmids do not contain the authentic *win1* gene, as there is no close genetic linkage between *win1* and the loci from which the inserts were derived. However, several points of interest are raised by the results of these crosses. In the case of the pwis2-C3 integrants, the recombination between the site of integration and the *win1.1* locus was 12%. This indicates that pwis2-C3 does not contain the authentic *win1* gene, but contains sequences which are genetically linked. More interestingly, microscopic examination of strains containing integrated copies of pwis1-1 showed that their cell length at division shorter than that of wild type. This effect is discussed in detail in Chapter 6.

Table 4.5: Genetic Analysis of Crosses Involving pwis1 Integrant Strains.

Phenotype	Integrant strains Int2	Int3
win$^-$ leu$^-$	10 (21%)	12 (26%)
win$^-$ leu$^+$	12 (25%)	15 (32%)
win$^+$ leu$^-$	13 (27%)	6 (13%)
win$^+$ leu$^+$	13 (27%)	14 (30%)

Table 4.6: Genetic Analysis of Crosses Involving pwis2 Integrant Strains.

phenotype	Integrant strains C3A	C3B
win$^-$ leu$^-$	22 (45%)	23 (47%)
win$^-$ leu$^+$	2 (4%)	3 (6%)
win$^+$ leu$^-$	4 (8%)	3 (6%)
win$^+$ leu$^+$	21 (43%)	20 (41%)

Table 4.7: Genetic Analysis of Crosses Involving pwis3 Integrant Strains.

phenotype	Integrant strains HC	HD
win$^-$ leu$^-$	18 (40%)	18 (35%)
win$^-$ leu$^+$	13 (29%)	6 (12%)
win$^+$ leu$^-$	4 (9%)	13 (25%)
win$^+$ leu$^+$	10 (22%)	15 (29%)

Table 4.8: Genetic Analysis of Crosses Involving pwis4 Integrant Strains.

phenotype	Integrant strains N2I1	N4I1	N4I2	N4I3
win^- leu^-	11 (23%)	12 (25%)	14 (29%)	5 (11%)
win^- leu^+	15 (31%)	12 (25%)	12 (25%)	11 (23%)
win^+ leu^-	12 (25%)	10 (21%)	8 (17%)	20 (43%)
win^+ leu^+	10 (21%)	14 (29%)	14 (29%)	11 (23%)

Table 4.9: Genetic Analysis of Crosses Involving pwis5 Integrant Strains.

phenotype	Integrant strains W5I1	W5I2
win^- leu^-	23 (34%)	9 (12%)
win^- leu^+	16 (24%)	13 (17%)
win^+ leu^-	18 (26%)	32 (41%)
win^+ leu^+	11 (16%)	24 (31%)

Figure 4.5: Southern blot analysis of pwis1-1 integrant strains.

S.pombe genomic DNA was digested with *Mlu*I, separated by agarose gel electrophoresis, Southern blotted, and probed with labelled plasmid pkb.

Lane 1: wild type

Lane 2: Int 3

Lane 3: Int 5

Lane 4: pkb digested with *Hin*DIII

1 2 3 4 kb

←18

←4·4

←3·2

←1·2

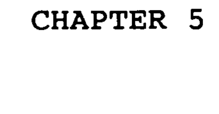
4.4: DISCUSSION

This chapter describes the extensive screening of gene libraries with the aim of isolating sequences encoding the *win1* gene. Five independent sequences have been isolated which are capable of suppressing the temperature sensitive phenotype of strains of the genotype *wee1.50 cdc25.22 win1.1*, although none of them represent the authentic *win1* gene, as demonstrated by integration and genetic mapping. One shows loose linkage with the *win1* locus.

There remain two questions arising from these experiments: why *cdc25*$^+$ sequences were not identified, and why the authentic *win1* gene was not isolated in the gene library screening. If *cdc25* in multicopy were capable of suppressing the temperature sensitive phenotype of the strain *wee1.50 cdc25.22 win1.1*, then the fact that such sequences were not isolated might indicate that the gene libraries described here had not been thoroughly screened. In order to investigate the phenotype of such transformants, a reconstruction experiment was performed involving the transformation of such a strain with *cdc25*$^+$ sequences. Very high levels of *cdc25* expression resulting from stimulation of transcription with the *ad* promotor have been shown to be lethal in a wee1.50 genetic background (Russell and Nurse, 1986). Multiple copies of *cdc25* in such a *wee1.50 win1.1* background were shown not to be lethal, but are evidently detrimental to the cell, which may explain why such transformants were not isolated in the gene library screen described.

A possible reason to explain why *win1* was not isolated is that the effect of multiple *win1* copies in a *wee1*$^-$ genetic background has a similar effect to that seen in the case of *cdc25*. This effect may be lethal, or so detrimental to the cell that transformants are impossible to recognise or recover.

After extensive screening without the isolation of *win1*, work was directed towards alternative protocols for the isolation of *win1*, and towards the study of the sequences isolated as suppressors.



CHAPTER 5

CHAPTER 5: ANALYSIS OF PWIS PLASMIDS

5.1: MOLECULAR ANALYSIS

5.1.1: Analysis of pwis1-1

5.1.1.a: Delimitation of pwis1-1 functional sequences by subcloning

The plasmid pwis1-1 consists of an 8.8kb *S.pombe* genomic insert in the vector pDB262. The library from which it was isolated was constructed by partial digestion of *S.pombe* genomic DNA with *Hin*dIII, followed by ligation with pDB262. The *S.pombe* insert in pwis1-1 consists of three *Hin*dIII fragments of sizes 4.4kb, 3.2 kb and 1.2kb (Fig. 5.1). The first stage in the delimitation of the functional sequences contained within pwis1-1 consisted of various subcloning experiments: each of the three *Hin*dIII fragments was subcloned into pDB262. In addition, a construct was made which lacked the 1.2 *Bgl*II fragment by total digestion of pwis1-1 with *Bgl*II, followed by religation (Fig. 5.1).
Each of these constructs was assayed for wis activity by transformation into the strain *wee1.50 cdc25.22 win1.1 leu1.32* and the phenotype of the transformants examined at the restrictive temperature. None of the plasmids described showed any activity in this assay, so it could be concluded that one or more of both the *Hin*dIII and *Bgl*II sites were within the functional sequence of pwis1-1.

5.1.1.b: Transposon analysis of pwis1-1

The next stage in the analysis was the use of transposon mutagenesis. The strategy behind these experiments was to treat a plasmid sample in such a way as to give a library of plasmid clones, each containing a single transposon integrated at a random site within the *S.pombe* insert. These transposon-containing plasmids could then be assayed for wis activity in the same way as that described for the subclones above. The effect upon plasmid function of individual transposon integrations could then be used to delimit the functional sequence contained within the plasmid in question.

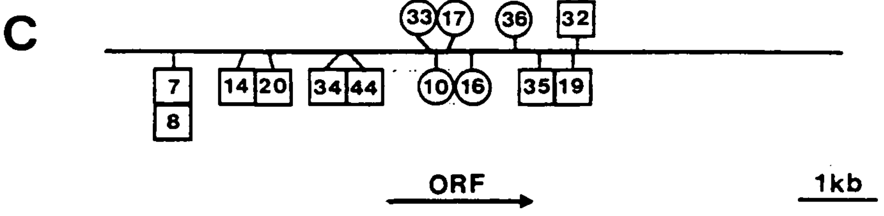
An *E.coli* transformant strain containing pwis1-1 was treated with a bacteriophage lambda isolate containing the transposon Tn5, as described in Chapter 2, and a library of plasmids containing random insertions of the transposon prepared.

Figure 5.1: The molecular analysis of wis1.

A: Restriction map of *S.pombe* insert in pwis1-1.

B: Subclones of pwis1-1, and their *wis*1 activity.

C: Results of transposon mutagenesis. Numbers indicate individual transposon clones. Circles indicate the position of integration of transposons affecting *wis1* activity, and squares the positions of those not affecting *wis1* activity.



The position of transposon integration in each case was determined by restriction analysis. In the case of pwisl-1, plasmids were initially subject to restriction analysis with *Hin*dIII to determine which of the four *Hin*dIII fragments contained the transposon (Fig. 5.1). Tn5 contains two *Hin*dIII sites, symmetrically placed 1.0kb from the termini of the transposon (Fig. 5.2). The pattern of *Hin*dIII fragment sizes from a transposon-treated pwisl-1 clone was different from that of pwisl-1, with one *Hin*dIII fragment disappearing to be replaced with three. Two of these fragments corresponded to the two parts of the original *Hin*dIII fragment with an extra 1.0kb in each consisting of transposon sequences. The third *Hin*dIII fragment consisted entirely of Tn5 sequences, and was present in all transposon containing plasmids. The largest *Hin*dIII fragment of pwisl-1 contains the entire pDB262 vector sequence, so from the examination of *Hin*dIII digests it was possible to exclude from further analyis those plasmids containing a transposon within the vector.

Of those plasmids containing transposons within the *S.pombe* insert sequences, it was normally possible to allocate the transposon integration site to one of two positions on the basis of *Hin*dIII fragment size data. Further restriction analysis, with the restriction enzyme *Bam*HI which has one site within the transposon (Fig. 5.2), was used to determine the position of integration unequivocally.

A number of plasmids were selected and transformed into the strain *wee1.50 cdc25.22 win1.1 leu1.32* in order to determine if the transposon integration had affected plasmid function. The sites of the transposon integrations in the plasmids were examined and the functional regions of the insert determined from their positions.

5.1.1.c: Delimitation of wis1 functional region by comparison of the S.pombe insert sequences contained within three pwisl plasmid isolates

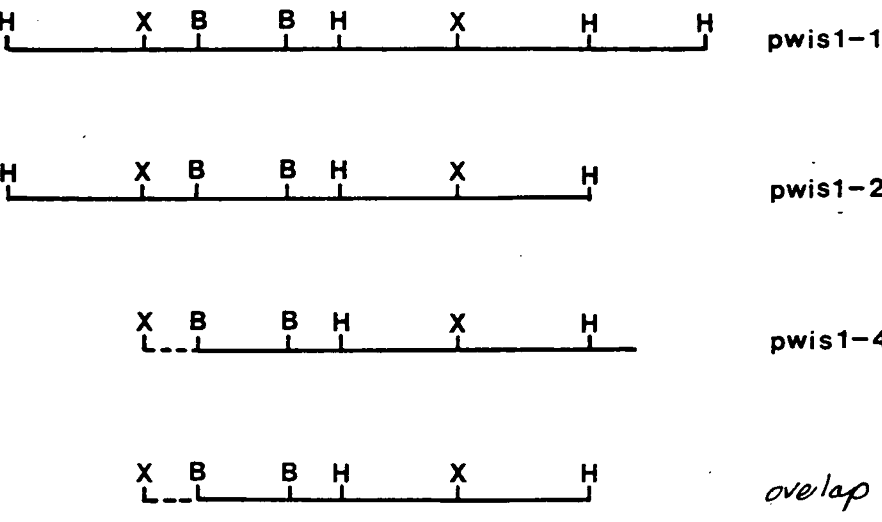
It was also possible to use information concerning the *S.pombe* genomic inserts in pwisl-2 and pwisl-3 to delimit the functional sequence of *wis1*. Restriction maps of the inserts within the plasmids were made, and the extent of overlap between the three plasmids determined (Fig. 5.3). The functional sequence could be assigned to this overlap region, though it was not possible to include pwisl-4 in this analysis, as the extent of the insert sequences could not be determined by restriction mapping.

Figure 5.2: Restriction map of the transposon Tn5.

(From: Jorgenson *et al*, 1979.)

XhoI HinDIII PvuII BglII SmaI SalI XhoI BamHI BglII HinDIII XhoI

Figure 5.3: **S.pombe genomic sequences contained within pwis1 plasmids isolated from various gene libraries.**



1kb

B - Bgl II H - HinD III X - Xba I

—— S.pombe sequences

--- Margin of insert not defined

This was presumably due to plasmid re-arrangement, or some form of ligation artefact. From this data, it was possible to delimit the functional region of *wis1* to a 5.8kb region between the left-hand *Xba*I site and the *Hin*dIII site at the right hand side of the 3.2kb *Hin*dIII fragment (Fig. 5.4).

5.1.1.d: Isolation of a 4.1kb fragment containing the functional region of pwis1.1

The data from the transposon mutagenesis experiments suggested that a subclone of pwis1-1 containing only the internal 4.1kb *Xba*I fragment should contain the *wis1* functional region. This fragment was subcloned into pTZ18 in both orientations to give plasmids pX2 and pX3 (Fig. 5.4). In order to construct the plasmid pIRT-X3, this 4.1kb *Xba*I fragment was subcloned into the plasmid pIRT2 using the flanking sites, *Sph*I and *Sac*I, from the polylinker in pTZ18 (Fig. 5.3). pIRT-X3 was shown to have activity in suppressing the cdc phenotype of the triple mutant strain *wee1.50 cdc25.22 win1.1*, and in suppressing the cell length phenotype of a *win1.1* strain.

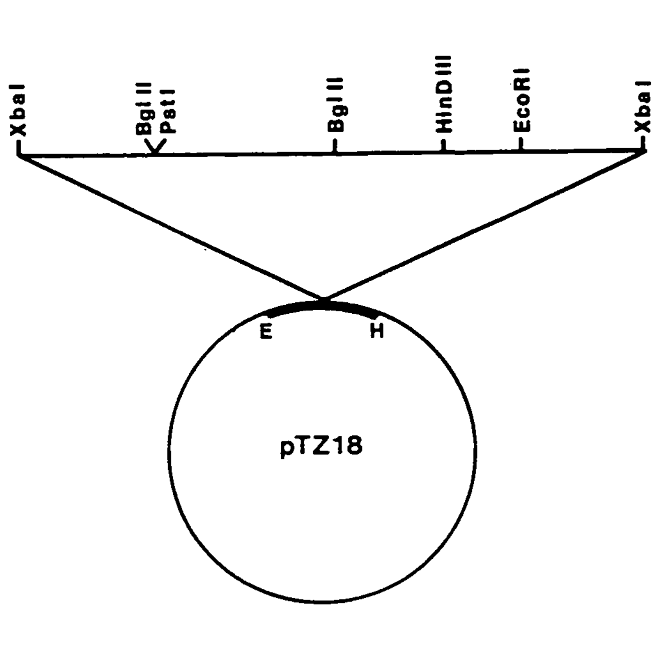
5.1.2: Analysis of pwis2-C1 and pwis2-C3

5.1.2.a: Delimitation of wis2 functional region by subcloning

A similar strategy of subcloning followed by transposon mutagenesis was followed in the case of *wis2*. HindIII subclones were made from pwis2-C1 (See Fig. 5.5). None of these plasmids showed any wis activity, as described above. In order to further delimit the *wis2* functional region, a construct was made which contained only the sequences to the right hand side of the central *Bam*HI site in the insert sequences of pwis2-C3. The strategy for the construction of this plasmid involved a transposon-containing plasmid clone of pwis2-C3. The plasmid pC3-Tn5-6 contained the transposon Tn5 integrated within vector sequences 0.5kb from the leftmost (vector) *Hin*dIII site in pwis2.C3 (Fig. 5.5). Due to the presence of a single *Bam*HI site within the transposon (Fig. 5.2), digestion of this plasmid with *Bam*HI, followed by religation, resulted in the loss of the *S.pombe* sequences to the left hand side of the central *Bam*HI site within the insert of pwis2-C3. The resulting plasmid, pC3-Tn5-6D, which contains only the insert sequences to the right hand side of the central *Bam*HI site, was shown to have wis activity. The combination of results from these experiments and the *Hin*dIII subclone analysis indicate that the functional sequence of pwis2 includes the *Hin*dIII site at the far right in Fig. 5.5. It is not possible to exclude the possibility that the *Hin*dIII site close to the *Bam*HI site within the pwis2-C3 is within the functional region, although this seemed unlikely, as it was within

Figure 5.4: Plasmid pX3.

pX3 consists of the 4.1kb *Xba*I fragment from pwis1-1 subcloned into pTZ18. Plasmid pX2 contains the same insert sequences, but in the opposite orientation in pTZ18.



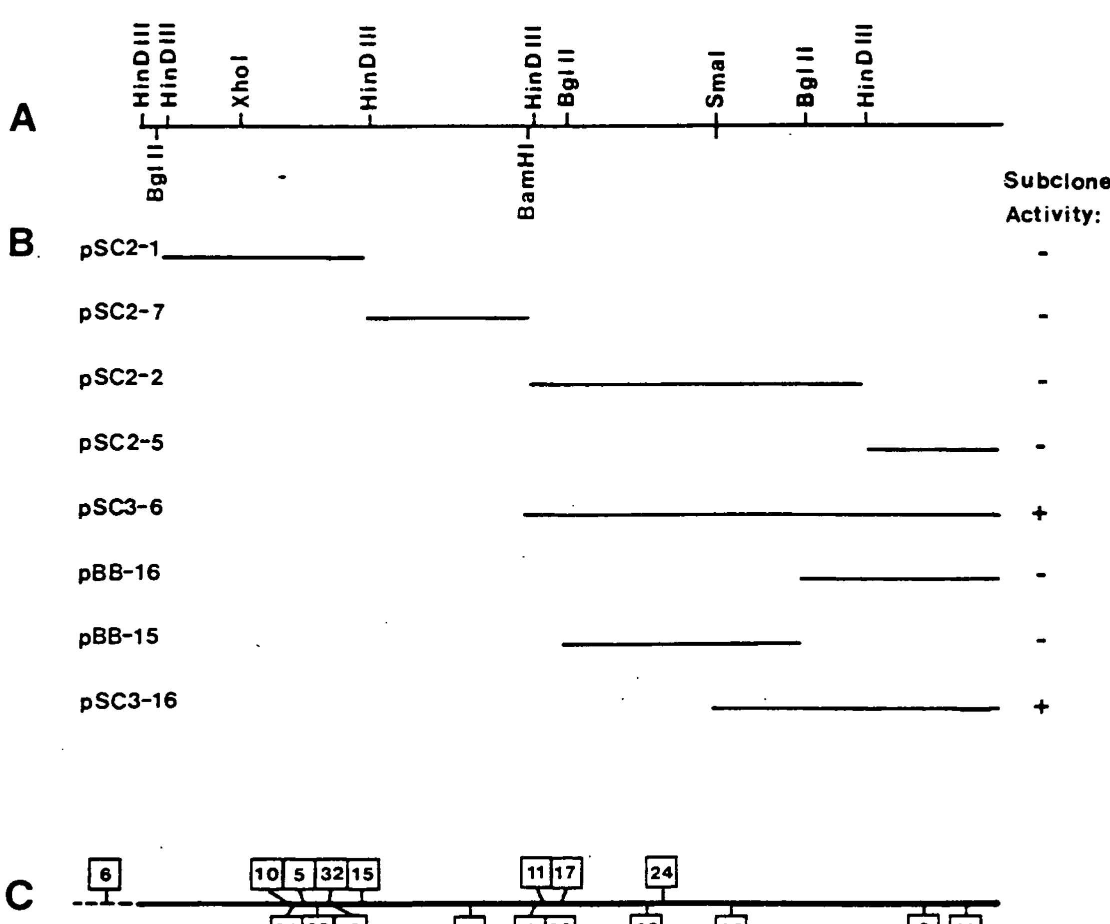
E H — Polylinker

Figure 5.5: The molecular analysis of wis2.

A: Restriction map of *S.pombe* insert in pwis2-C3.

B: Subclones of pwis2-C2 and pwis2-C3, and their *wis*2 activity.

C: Results of transposon mutagenesis. Numbers indicate individual transposon clones. Squares indicate the positions of transposons not affecting *wis*2 activity (no clones were identified with transposons which affected *wis2* activity).



0.1kb of the *Bam*HI site. This possibility was excluded by results from the transposon analysis described below.

In order to delimit the functional region of *wis2* further, subclones were constructed using the *Bgl*II sites contained within the insert sequences of pwis2-C3. The plasmid pBB-16 was derived by digestion of pwis2-c3 with BglII followed by religation at low DNA concentration. It contained mainly vector sequences, but included the very small fraction of *S.pombe* insert beyond the leftmost *Bgl*II site, and the 2kb of insert sequences to the right of the *Bgl*II site at the right hand side. pBB15 consisted of the 6.2kb *Bgl*II fragment of pwis2-C3 subcloned into pDB248 (Fig. 5.4). Neither of these constructs showed any activity in the assay described. The most likely explanation of this data is that the functional sequences of *wis2* are in the region of the right hand side *Bgl*II and *Hin*dIII sites, though from these experiments it is not possible to exclude the possibility that they include the central *Bgl*II site.

5.1.2.b: Transposon analysis of pwis2.C3

pwis2-C3 was subjected to transposon mutagenesis in the same way as that described for pwis1-1. Individual transposon containing plasmids were first subject to restriction analysis with *Hin*dIII, which was followed by analysis involving one or more of *Xho*I, *Bam*HI or *Pvu*II, which all have site within the transposon (Fig. 5.2), depending on the position of the transposon integration. Different restriction enzymes were employed in order to give restriction fragments of a small enough size for their length to be determined accurately by agarose gel electrophoresis.

Despite the analysis of 55 such transposon-containing plasmids, it was not possible to find one with an integration site within the region defined by clones 9 and 25 (Fig. 5.5). This "cold spot" was contrasted by two "hot spots" at the left hand side of the insert and in the centre. None of the integration events shown had any effect on wis function.

The results of the transposon mutagenesis experiments were in agreement with those derived from subcloning: the functional sequence of *wis2* includes the *Hin*dIII and *Bgl*II sites at the right hand side of the restriction map shown, and is within the integration sites of the transposons in clones 9 and 25. This delimits the functional sequence to 2.8kb. A subclone of this region was made using the *Sma*I site and the

*Sal*I site within the flanking vector sequences (Fig. 5.5). This fragment was subcloned into pTZ18 to give plasmid pC3-16.

5.1.3: Analysis of pwis 3-1

5.1.3.a: Restriction analysis of pwis3-1

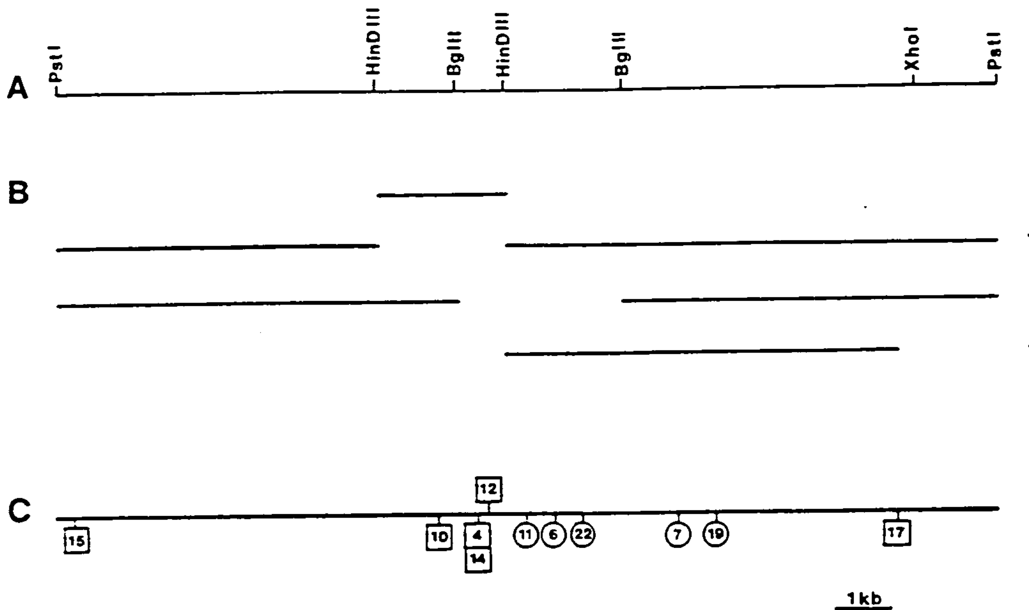
The plasmid pwis3-1, of approximately 17.5kb, was isolated from a library consisting of *S.pombe* genomic DNA partially digested with *Sau*3A contained within the vector pDB248. Restriction analysis showed that the vector, and possibly the *S.pombe* derived sequences, had undergone some form of re-arrangement. Because of this, restriction sites within the insert sequences difficult to map in relation to known sites within the vector. It was, however, possible to construct an outline map of the plasmid showing that it contained a single *Pst*I site, two *Hin*dIII sites and two *Bgl*II sites, and to determine their relative positions (Fig. 5.6). From this restriction analysis it was possible to deduce that the two *Bgl*II site lay within *S.pombe*-derived sequences, as the vector pDB248 contains no *Bgl*II sites. It was also possible to deduce that one of the *Hin*dIII sites was also derived from *S.pombe* sequences, as it lay between the two *Bgl*II sites (Fig. 5.6).

5.1.3.b: Delimitation of the pwis3-1 functional region by subcloning

The first experiments aimed at delimiting the functional sequences of *wis3* were to subclone the two fragments resulting from the *Hin*dIII digest of pwis3-1 into pDB262 (Fig. 5.6). The resulting plasmids, pSH3-11 (containing the 2.5kb fragment) and pSH3-16 (containing the 15.0kb fragment which consisted predominantly of vector sequences) were transformed into the strain *wee1.50 cdc25.22 win1.1 leu1.32* h^- to assess their wis function. pSH3-16 showed an effect similar to the parental plasmid pwis3-1 on this strain, while pSH3-11 had no effect. This suggests that the functional sequences of *wis3* were contained within pSH3-16.

A construct was made from pwis3-1 that lacked the 3.0kb fragment by total digestion with *Bgl*II followed by religation. This plasmid (pH3-B) showed no wis function when subjected to the assay as described above.

Figure 5.6: The molecular analysis of *wis3*



A: Restriction map of plasmid pwis3-1, linearised with *PstI*.

B: Subclones of pwis3-1 and their *wis3* activity.

C: Results of transposon mutagenesis. Numbers indicate individual transposon containing clones. Circles indicate the position of integration of transposons affecting *wis3* activity, and squares the positions of those not affecting *wis3* activity.

On the basis of the subcloning experiments with *Hin*dIII and *Bgl*II it could be concluded that the functional sequences contained no *Hin*dIII site, but one *Bgl*II site. It was also possible to deduce that this *Bgl*II site was not the one within the 2.5kb *Hin*dIII fragment, as loss of this fragment had no effect upon the wis function of pwis3-1.

5.1.3.c: Transposon analysis of pwis3-1

The next stage in the analysis consisted of transposon analysis as described for *wis1* and *wis2*. Individual transposon-containing plasmids were first subject to *Hin*dIII restriction analysis, followed by *Pst*I restriction analysis
to assign unequivocally the site of transposon integration (Fig. 5.5). The results of these experiments indicate that the transposons capable of affecting the wis function of pwis3.1 lie within a 3.6kb region flanking the right hand side *Bgl*II site, which is in agreement with the results of the subcloning experiments.

In order to facilitate further analysis, a DNA fragment containing the *wis3* functional sequences was subcloned. To do this, use was made of the *Hin*dIII sites contained within the transposon Tn5 in the transposon-containing clone no.17. A *Hin*dIII fragment of approximately 9kb was subcloned into the vector pDB262, resulting in the plasmid pW3. This plasmid was shown to have wis activity and was subjected to restriction analysis (Fig. 5.7).

5.1.4: Analysis of pwis4-1 and pwis5-1

Restriction analysis was performed upon pwis4-1 and pwis5-1, but no experiments to delimit their functional sequences were performed (Fig. 5.8).

5.1.5: Comparison of restriction data from wis functional regions with that from previously identified cell cycle genes

Once restriction maps of the *S.pombe* inserts contained within the pwis plasmids had been determined, it was possible to compare these with restriction maps of other cell cycle genes which had already been isolated and characterised. From this data it was possible to determine if the pwis plasmids contained sequences corresponding to any of these genes. None of the previously identified genes

Figure 5.7: Restriction map of the S.pombe insert sequences in plasmid pW3.

This insert was derived from the Tn5-containing clone of pwis3-1, H3-Tn5-17, and consists of a 9kb *Hin*DIII fragment containing both *S.pombe* and Tn5 sequences subcloned into pDB262.

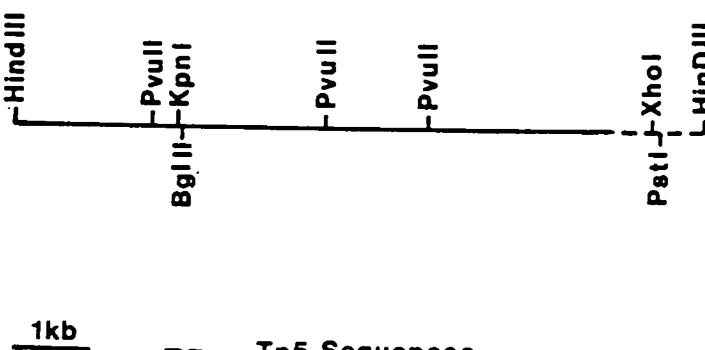
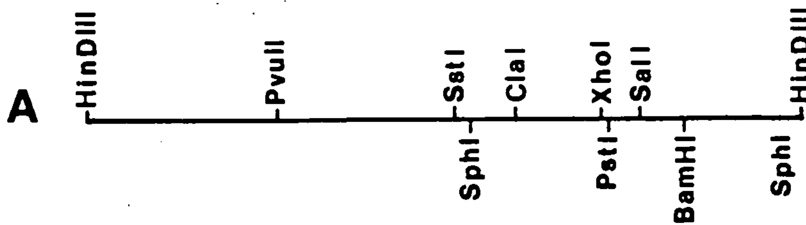
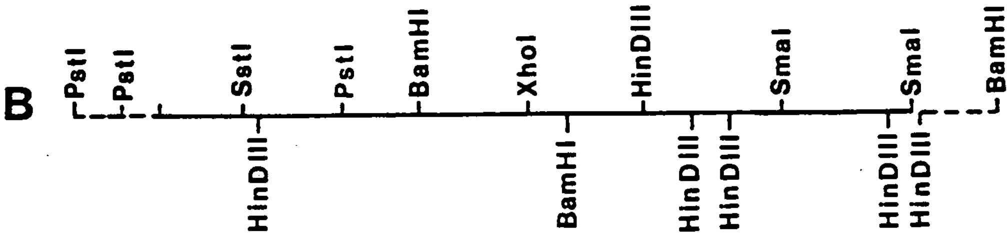


Figure 5.8: Restriction analysis of wis4 and wis5.

A: *S.pombe* genomic sequences contained within pN2 (pwis4-1). These insert sequences also contain at least a further 5 *Hin*DIII sites.

B: *S.pombe* genomic sequences contained within pSf (pwis5-1).



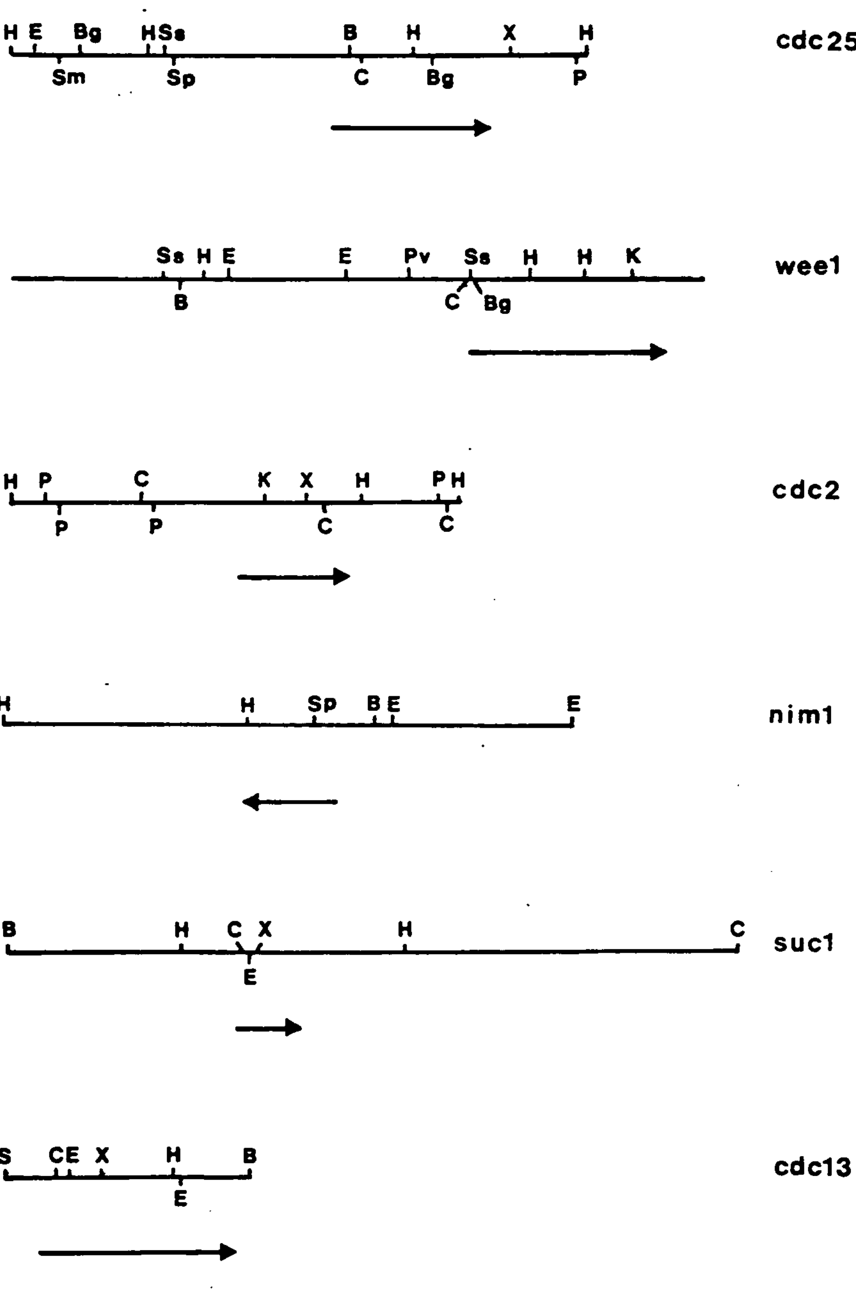


1kb ---- Vector Sequences

Figure 5.9: Restriction analysis of previously identified S.pombe cell cycle genes.

Arrows indicate positions and directions of open reading frames.

References: *cdc25*: Russell and Nurse (1986). *wee1*: Russell and Nurse (1987a). *cdc2*: Durkacz *et al* (1985). *nim1*: Russell and Nurse (1987b). *suc1*: Hayles *et al* (1986). *cdc13*: Hagan *et al* (1988); Booher and Beach, (1988).



examined showed any similarity to the pwis sequences. Those genes examined include *cdc2*, *cdc25*, *wee1*, *nim1*, *suc1* and *cdc13* (Fig. 5.9).

5.2: GENETIC ANALYSIS

5.2.1 Experimental strategy

In order to investigate possible interactions of the five *wis* genes with other cell cycle genes involved in the control over entry into mitosis, the effect of introducing the *wis* genes carried on multicopy plasmids into various cell cycle mutants was investigated.

5.2.2: Interactions of pwis plasmids with win1.1

Of primary interest was the effect on the *win1.1* mutation in an otherwise wild type background: only pwis1-1 (data no shown) and pwis4-1 (Table 5.1) were capable of suppressing the phenotype of increased cell length at division characteristic of this mutant. In order to determine if this were due to a specific suppression of *win1.1*, or a general effect upon cell length, plasmids carrying the *wis* genes were introduced into a *leu1.32* strain, and cell lengths at division determined (Table 5.2). These results suggest that *wis1* has a general effect upon cell length; in high copy number it will induce an early entry into mitosis. It is possibly this effect which results in the suppression of the *win1.1* single mutant phenotype. In contrast, *wis4* appears to be a specific suppressor of *win1.1*: when present in multicopy it has no significant effect upon wild type cells.

5.2.3: Interactions of pwis plasmids with cdc25

The pwis plasmids were isolated as suppressors of the temperature sensitive cdc phenotype of a strain carrying the mutation *cdc25.22*. These plasmids were transformed into a strain of genotype *cdc25.22 leu1.32 h$^-$* and the phenotype of the transformants examined at the restrictive temperature. The results of these experiments showed that the pwis plasmids showed no effect in suppressing the cdc phenotype of *cdc25.22* in an otherwise wild type background. This shows that the effect of these plasmids in multicopy is not directly to reverse the effect of loss of *cdc25* function.

Table 5.1. Effects of pwis4.1 upon cell length phenotype of win1$^+$ and win1.1 strains

Plasmid	Strain	Cell length/μm	SD
pwis4.1	*leu1.32 h$^-$*	13.56	0.84
pDB248	*leu1.32 h$^-$*	14.34	0.64
pwis4.1	*win1.1 leu1.32 h$^-$*	13.97	0.75
pDB248	*win1.1 leu1.32 h$^-$*	17.20	0.58

Cells were grown to mid log phase in EMM at 25°C.

Table 5.2. Cell lengths of transformants containing the pwis plasmids in the strain leu1.32 h$^-$. Cells were grown to mid log phase in EMM at 25°C.

Plasmid	Cell length/μm	SD
pDB248	12.99	0.85
pwis1.1	10.69	0.53
pwis2.C3	13.03	0.71
pwis3.1	12.83	0.50
pwis4.1	13.56	0.84
pwis5.1	14.01	0.69

5.2.4: Interactions of pwis plasmids with mcs3, mcs4 and mcs6

Several chromosomal mutations (*mcs3.12*, *mcs4.13* and *mcs6.13*) have been isolated which share with *win1.1* the capacity of reversing the suppression of *cdc25* by *wee1* (Molz *et al*, 1989). Thus, the phenotype resulting from the combination of one of these mutations with *wee1.50* and *cdc25.22* is cdc or semi-cdc. See Chapter 3 for a further description of the *mcs* mutations. The pwis plasmids were transformed into the following strains to determine if they were capable of suppressing their temperature sensitive phenotypes: *wee1.50 cdc25.22 mcs3.12 leu1.32 h^-*, *wee1.50 cdc25.22 mcs4.13 leu1.32 h^-*, *wee1.50 cdc25.22 mcs6.13 leu1.32 h^-*. For results, see Table 5.3.

In order to determine if the functional sequences contained within the pwis plasmids were allelic to the *mcs* mutations whose phenotypic effect they were capable of suppressing, a genetic analysis was performed. This involved constructing strains of the genotype *wee1.50 cdc25.22 [pwisN:LEU2] leu1.32 h^+*, where *[pwisN:LEU2]* denotes integrated copies of a pwis plasmid, which has previously been shown to have integrated by homologous recombination (Chapter 4). These crosses were performed in a *wee1.50 cdc25.22* genetic background as it was believed at the time that *mcs3.12*, *mcs4.13* and *mcs6.13* showed no observable phenotype in an otherwise wild type background. It later came to light that *mcs4.13* does show such a phenotype, which is one of a slight increase of length at division (Molz *et al*, 1989). The genetic locus of the *wis* sequence could be followed in these crosses by its close linkage to the leu^+ phenotype resulting from the associated plasmid sequences. The cdc phenotype was scored by microscopic examination of growing cells at the restrictive temperature. The crosses were examined by either tetrad analysis or free spore analysis, and the results shown in Table 5.4. These results show that none of the plasmids capable of suppressing *mcs* mutant alleles contain the equivalent *mcs^+* sequences.

The plasmids pwis1-1, pwis2-C3 and pwis3-1 were transformed into the strain *mcs4.13 leu1.32 h^-*. Of these, only pwis1-1 suppressed the phenotype of increased cell length at division shown by this strain. However, as in the case of *win1.1*, it is possible that this is a result of a general effect upon cell length show by *wis1* when present in multicopy.

Table 5.3. Effects of pwis plasmids upon phenotypes of strains carrying mcs mutant alleles in a wee1.50 cdc25.22 leu1.34 h⁻ genetic background.

Mutant allele	*mcs3.12*	*mcs4.13*	*mcs6.13*
Plasmid			
pwis1-1	-	+++	-
pwis2-1	++	++	++
pwis3-1	+	+	+
pwis4-1	-	-	-
pwis5-1	-	-	(+)

Levels of effect graded from +++ (strong suppression) to - (no effect) by microscopic examination of growing cells on EMM at 35°C.

Table 5.4. Results of crosses involving pwis integrant strains and those containing various mcs mutant alleles in a wee1.50 cdc25.22 leu1.32 genetic background.

[pwis1-1] wee1.50 cdc25.22 ura4.D18 leu1.32 h⁺ crossed to *wee1.50 cdc25.22 mcs4.13 leu1.32 h⁻.*

	mcs^+	$mcs^{+/-}$	mcs^-
leu^+	11	7	0
leu^-	6	0	8

[pwis2-C1] wee1.50 cdc25.22 leu1.32 h⁺ crossed to *wee1.50 cdc25.22 mcs3.12 leu1.32 h⁻.*

	mcs^+	$mcs^{+/-}$	mcs^-
leu^+	14	9	1
leu^-	13	0	9

[pwis2-C1] wee1.50 cdc25.22 leu1.32 h⁺ crossed to *wee1.50 cdc25.22 mcs4.13 leu1.32 h⁻.*

	mcs^+	$mcs^{+/-}$	mcs^-
leu^+	18	0	8
leu^-	7	0	3

[pwis2-C1] wee1.50 cdc25.22 leu1.32 h⁺ crossed to *wee1.50 cdc25.22 mcs6.13 leu1.32 h⁻.*

	mcs^+	$mcs^{+/-}$	mcs^-
leu^+	17	0	4
leu^-	16	0	11

Table 5.4. (continued)

[pwis3-1] wee1.50 cdc25.22 leu1.32 h^+ crossed to *wee1.50 cdc25.22 mcs3.12 leu1.32* h^-.

	mcs^+	$mcs^{+/-}$	mcs^-
leu^+	16	8	0
leu^-	5	1	16

[pwis3-1] wee1.50 cdc25.22 leu1.32 h^+ crossed to *wee1.50 cdc25.22 mcs4.13 leu1.32* h^-.

	mcs^+	$mcs^{+/-}$	mcs^-
leu^+	29	0	1
leu^-	14	0	4

[pwis3-1] wee1.50 cdc25.22 leu1.32 h^+ crossed to *wee1.50 cdc25.22 mcs6.13 leu1.32* h^-.

	mcs^+	$mcs^{+/-}$	mcs^-
leu^+	6	0	2
leu^-	0	0	2

5.2.5: Interactions of the pwis plasmids with other cell cycle mutations

No phenotypic effects were observed when the pwis plasmids were transformed into strains containing the following mutant alleles: *cdc2.33*, *cdc2.1w*, *cdc2.3w*, *wee1.50 cdc13.117*, *cdr1.34 (nim1)* and *cdr2.69*. This shows that the effect of these plasmids in multicopy is not acting to reverse the effect of loss of function of these genes.

5.3: DISCUSSION

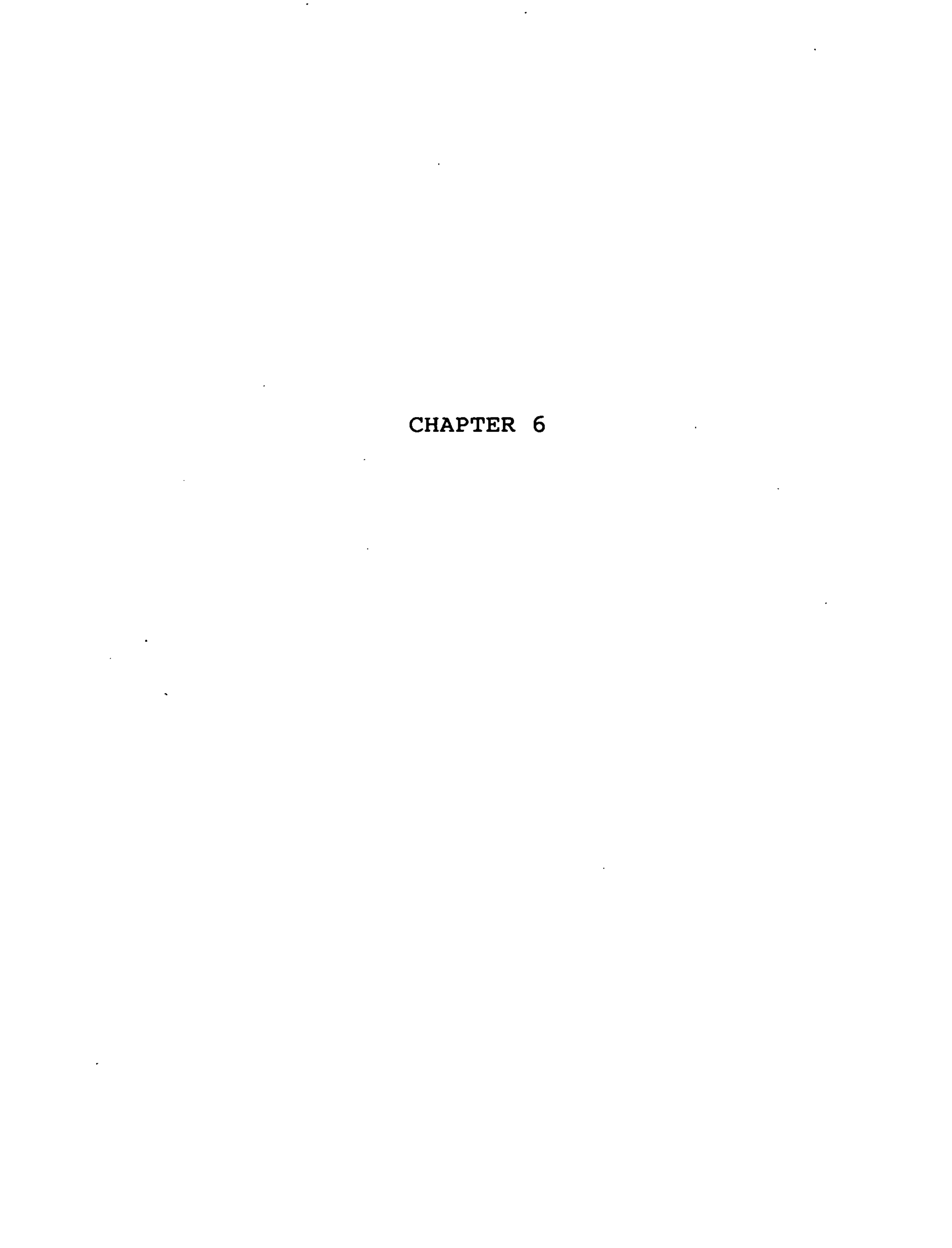
This chapter describes the analysis of the pwis plasmids by restriction mapping, subcloning, and by transposon mutagenesis. In the cases of *wis1*, *wis2* and *wis3*, functional sequences were identified within the original clone and subclones made. Restriction maps of the pwis genomic sequences were compared with those of previously identified cell cycle genes and no similarities found. The phenotypes of various cell cycle mutant strains containing the pwis plasmids in multicopy were examined. The most striking effect was shown by *wis1*, which was capable of reducing the cell length on division of an otherwise wild type strain when present in multicopy. This suggests a possible role for *wis1* as a dosage dependent inducer of mitosis.

None of the pwis plasmids showed any effect upon the phenotype of *cdc25.22* in an otherwise wild type background. This suggests that they are not acting to compensate directly for loss of *cdc25* activity. Only *wis1* and *wis4* were capable of suppressing the *win1.1* phenotype in an otherwise wild type background. Their effect upon the *wee1.50 cdc25.22 win1.1* phenotype may be due to an effect upon *win1* activity.

Several of the pwis plasmids were shown to affect the phenotype of strains with specific *mcs* mutations in a *wee1.50 cdc25.22* mutant background. Genetic mapping experiments showed that the *wis* genes were not allelic to the specific *mcs* mutations they were capable of suppressing. It is difficult to draw any conclusions from the interactions between pwis plasmids and *mcs* mutations, as so little is known about either. It is possibly worth noting that *wis2* and *wis3*, when present in multiple copies, are capable of affecting the phenotypes of *win1.1*, *mcs3.12*, *mcs4.13* and *mcs6.13* in a *wee1.50 cdc25.22* mutant background. In contrast, *wis1* only showed an interaction with *mcs4.13*, which shows a phenotype strikingly similar to that of

win1.1. The interactions between *win1.1* and the *mcs* mutations are described in Chapter 3.

CHAPTER 6



CHAPTER 6: GENETIC AND MOLECULAR ANALYSIS OF wis1

6.1: ANALYSIS OF wis1 TRANSCRIPTION

6.1.1: Identification of the wis1 transcript

RNA was prepared from both a wild type cells and an strain containing multiple integrated copies of pwis1-1. Following agarose gel electrophoresis, the samples were transferred to hybridisation membrane, and probed with *wis1* sequences in a Northern blot procedure. Two probes were used: one consisting of the 4.1kb *Xba*I fragment from pX2, and another consisting of a 1.5kb *Eco*RI fragment from deletion pX3-E2. The latter plasmid was constructed in the course of the sequencing of *wis1* (Chapter 7), and gives a 1.4kb DNA fragment from within the proposed *wis1* open reading frame upon digestion with *Eco*RI (Fig. 6.1). Both these probes showed hybridisation with a single mRNA species of approximately 2400 nucleotides (nt) which was present at a higher level in the pwis1-1 integrant strains (Fig. 6.2). The size of the hybridising band was calculated using the *S.pombe* 28S and 17S ribosomal RNAs as size markers (3400 and 1800 nt respectively).

6.1.2: Analysis of transcriptional direction

In order to determine the direction of transcription of *wis1*, Northern blots were carried out in a similar way to those described above. Single stranded DNA was prepared from plasmids containing *wis1* sequences cloned in opposite orientations in pTZ18 (Fig. 6.1), and used to prepare radioactively labelled single stranded DNA using the M13 reverse sequencing primer and Klenow enzyme. RNA samples from both wild type and a pwis1-1 integrant strain were probed separately with labelled DNA made from plasmids containing alternate orientations of *wis1*. Only one probe (derived from plasmid pC17) showed hybridisation with the RNA samples, and it gave a similar pattern of hybridisation to that of the double stranded probe derived from the *wis1* open reading frame, confirming that this was the *wis1* transcript. From the pattern of hybridisation of these two probes, it was possible to deduce that the direction of transcription was that shown in Figure 6.1.

Figure 6.1: Strategy for Northern blot analysis of wis1.

A: Restriction map of the 4.1kb XbaI fragment from pwis1-1, and the arrow above indicates the extent and transcriptional direction of the predicted *wis1* open reading frame.

B: Double stranded probes used for the detection of the *wis1* transcript, and the plasmids from which they were derived.

C: Plasmids from which single stranded probes were made which were used for the analysis of *wis1* transcript direction.

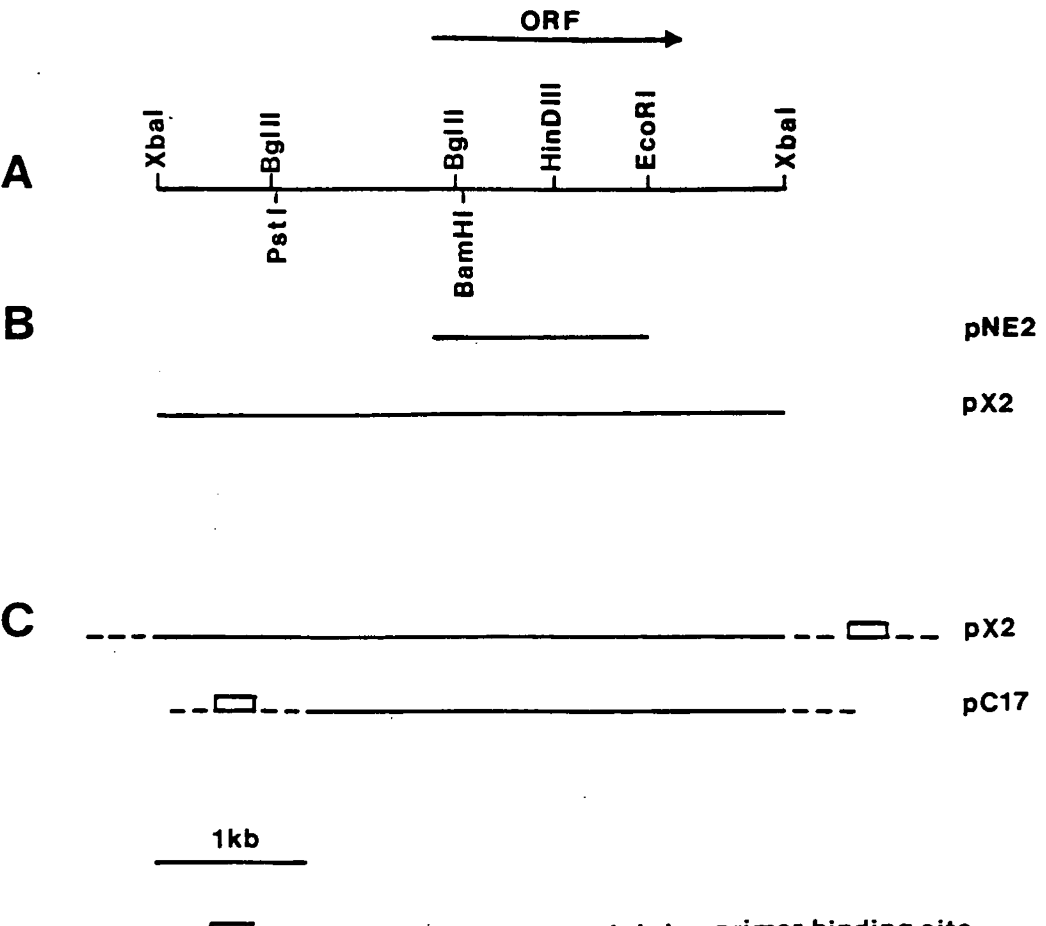


Figure 6.2: Northern blot analysis of wis1.

RNA was prepared from the strains indicated below, subject to denaturing agarose gel electrophoresis, Northern blotted and probed with the sequences indicated below:

Lane 1: wild type

Lane 2: D6X1-2

Lane 3: Int3

Lane 4: D4

Probed with the *Eco*RI fragment from plasmid pNE2

Lane 5: wild type

Lane 6: Int3

Probed with the *Xba*I fragment from plasmid pX2

(see Figure 6.1 for details of probes)

1 2 3 4 5 6 nt

←2400

←1500

6.2: EFFECTS OF INCREASED wis1 DOSAGE

6.2.1: Experimental strategy

As has already been described, multiple copies of *wis1*, either carried upon autonomously replicating plasmids or integrated into the *S.pombe* genome, resulted in a reduction of cell length at division of approximately 20%. In order to determine if this was a dosage dependent effect, strains were constructed containing one extra copy, and two extra copies of *wis1*, and the phenotypes of such strains compared with those containing multiple integrated copies.

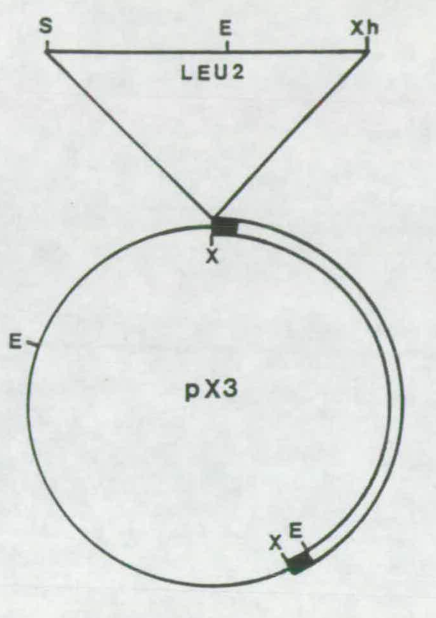
6.2.2: Construction of strains with increased wis1 copy number

To construct such strains, it was necessary to make a plasmid which contained the *wis1* functional sequence and an auxotrophic marker, but no autonomously replicating sequences, so that any transformant strains resulting from the treatment of cells this plasmid would contain a low number of integrated copies. Previously described integrant strains were obtained by the treatment of cells with linearised plasmid, which results in a large number of integrated copies. In order to construct such a plasmid, the 2.2kb *Sal*I - *Xho*I fragment from pDAM6 which contains the *LEU2* functional sequence was subcloned into the polylinker-derived *Sal*I site in pX3 to produce the plasmid pD6X1 (Fig. 6.3).

This plasmid was used to transform a strain of the genotype *leu1.32 h⁻*, and two stable transformants were selected for further analysis (D6X1-2 and D6X1-3). Total genomic DNA was prepared from these strains, digested separately with the restriction enzymes *Mlu*I and *Sst*I, and subjected to Southern blot analysis, probing with the 4.1kb *Xba*I fragment from pX3 (Fig. 6.4). Neither of these enzymes cuts within the *wis1* sequences in pD6X1, although one site for *Sst*I exists within the plasmid polylinker sequences. Wild type DNA shows one hybridising band when digested with either enzyme. In contrast, the integrant D6X1-2 shows two hybridising bands when digested with *Sst*I, which would be expected if it contained one integrated copy of pD6X1 (Fig. 6.5). The integrant D6X1-3 shows two bands similar to those in D6X1-2, (the larger band appears very faint in Figure 6.4) and an extra band of a size 9 kb equal to that of linearised pD6X1, suggesting that this strain contains more than one copy of integrated plasmid (Fig. 6.5). The *Mlu*I

Figure 6.3: Construction of the plasmid pD6X1.

pD6X1 consists of a *Sal*I- *Xho*I fragment containing the *S.cerevisiae LEU2* gene subcloned into pX3 (see text for details).



1kb

▭ Vector

▬ Polylinker

E-EcoRI S-SalI X-XbaI Xh-XhoI

Figure 6.4: Southern blot analysis of strains containing integrated copies of pD6X1.

S.pombe genomic DNA was digested with the restriction enzymes indicated below, subject to agarose gel electrophoresis, Southern blotted and probed with plasmid pD6X-1.

Lane 1: wild type	*Sst*I
Lane 2: D6X1-2	*Sst*I
Lane 3: D6X1-3	*Sst*I
Lane 4: wild type	*Mlu*I
Lane 5: D6X1-2	*Mlu*I
Lane 6: D6X1-3	*Mlu*I

6 5 4 3 2 1 kb

←25
←19
←13
←9

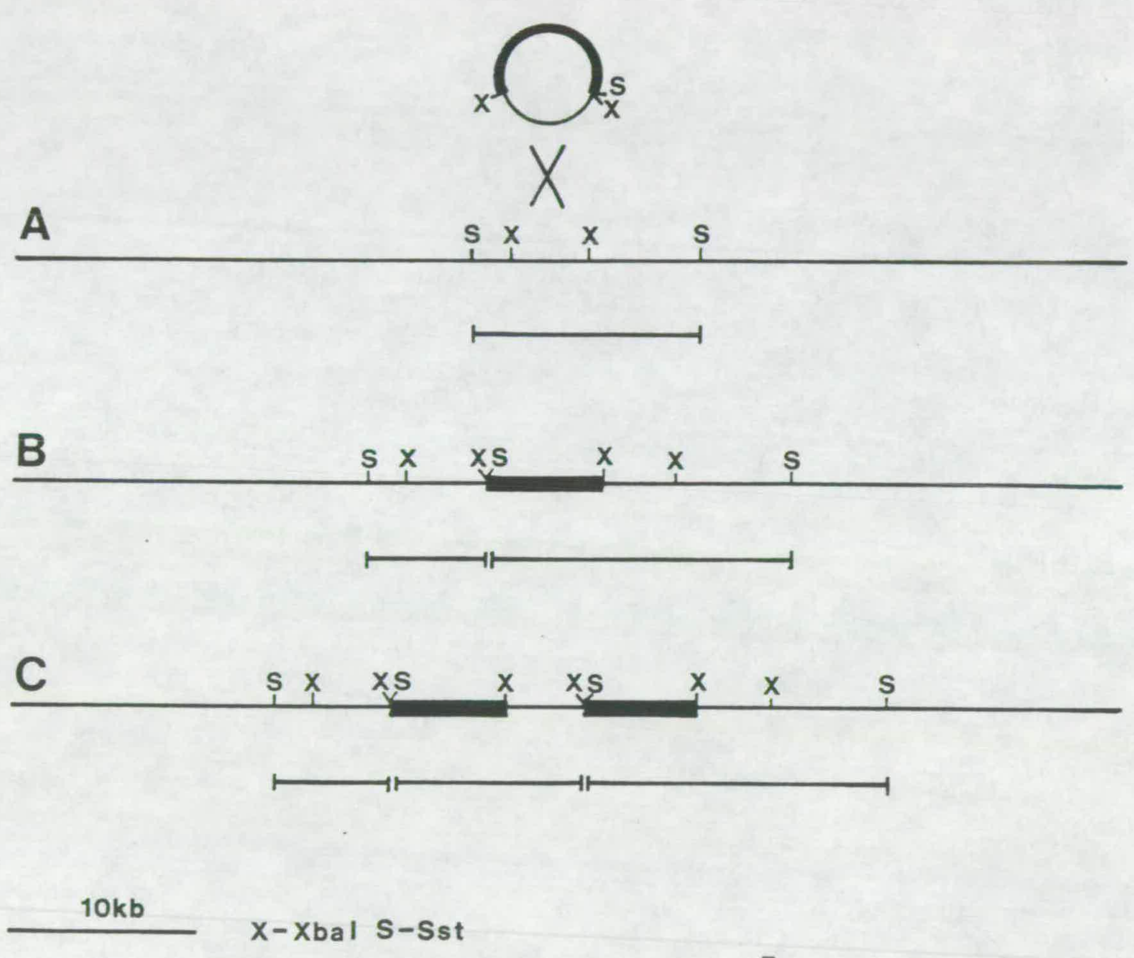
Figure 6.5: Schematic diagram showing the predicted results of the integration of one or two copies of pD6X1.

Bars indicate the sizes of *SstI* fragments which would be expected to hybridise to the 4.1kb *S.pombe* insert in pX3.

A: Integration of pD6X1 into the wild type genome.

B; One integrated copy of pD6X1.

C: Two integrated copies of pD6X1.



digested DNA from wild type, D6X1-2 and D6X1-3 show a single hybridising band in each case. The size of the hybridising fragment is increased in the integrants, being largest in D6X1-3. These results demonstrate that plasmid integration has taken place by homologous recombination in both integrant strains. Judging from the estimated sizes of the hybridising fragments in the *Mlu*I digested samples, 13kb for wild type, 19kb for D6X1-2 and 25kb for D6X1-3, it is possible to calculate that D6X1-2 contains one integrated copy, and D6X1-3 two integrated copies of pD6X1.

6.2.3: Analysis of the dosage dependent effects of wis1 upon the cell

In order to determine the effect of varying *wis1* copy numbers upon the cell, cell lengths at division were determined for the strains described above (Table 6.1). To confirm that increased *wis1* copy number results in a concomitant rise in *wis1* transcript level, Northern blot analysis was performed on RNA prepared from the following strains: wild type, D6X1-2 and Int3. Radioactively labelled DNA derived from the *wis1* 4.1kb *Xba*I fragment was used to probe the blot, and the results shown in Fig. 6.2. RNA prepared from D6X1-2 shows a similar or slightly increased level of *wis1* transcript to that in wild type RNA, but there is a significantly increased transcript level in the sample derived from Int3, which contains multiple integrated copies of pwis1-1.

The results from these experiments imply that the effect of *wis1* upon cell length at division is dosage dependent, with a progressive decrease in size correlating with increased *wis1* copy number (Fig. 6.6). This suggests that *wis1* is involved either directly, or indirectly, in a rate limiting step controlling entry into mitosis and cell division.

6.3: EFFECTS OF LOSS OF wis1 FUNCTION

6.3.1: Construction of strains resulting from the deletion of portions of the wis1 functional sequence

The next stage in the analysis was to determine the effect upon the cell of loss of *wis1* function. This was done by the technique of gene transplacement, described in *S.cerevisiae* by Rothstein, 1983). A portion of the functional region of the gene in question is replaced with a selectable marker, usually an auxotrophic one, in a plasmid construct. Restriction enzyme digests are then performed on this construct

Table 6.1: Cell length at division of strains containing one, two, three and multiple copies of wis1.

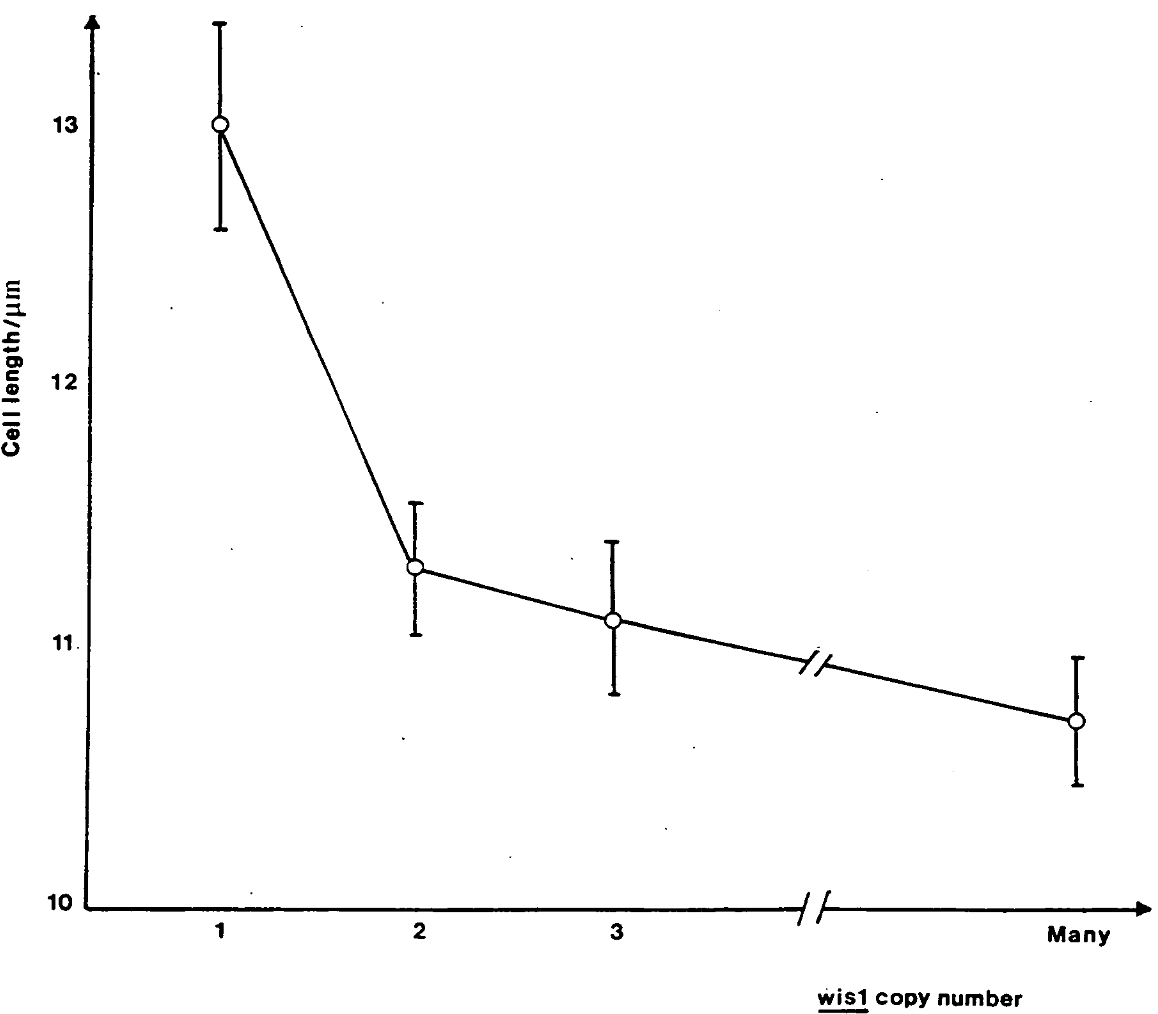
Strain	No. of copies	Cell length/μm	S.E.M
972	1	12.99	0.41
D6X1-2	2	11.34	0.27
D6X1-3	3	11.06	0.30
Int 3	multiple	10.75	0.25

S.E.M. = Standard error of mean

Cells were grown on EMM at 25°C.

Figure 6.6: The effect upon cell length at division of increased wis1 copy number.

Cells lengths at division were determined from the following strains: wild type, D6X1-2, D6X1-3 and Int3. Cells were grown in EMM at 25°C to mid log phase, and at least 20 cells measured from each strain. Bars indicate standard error of mean.



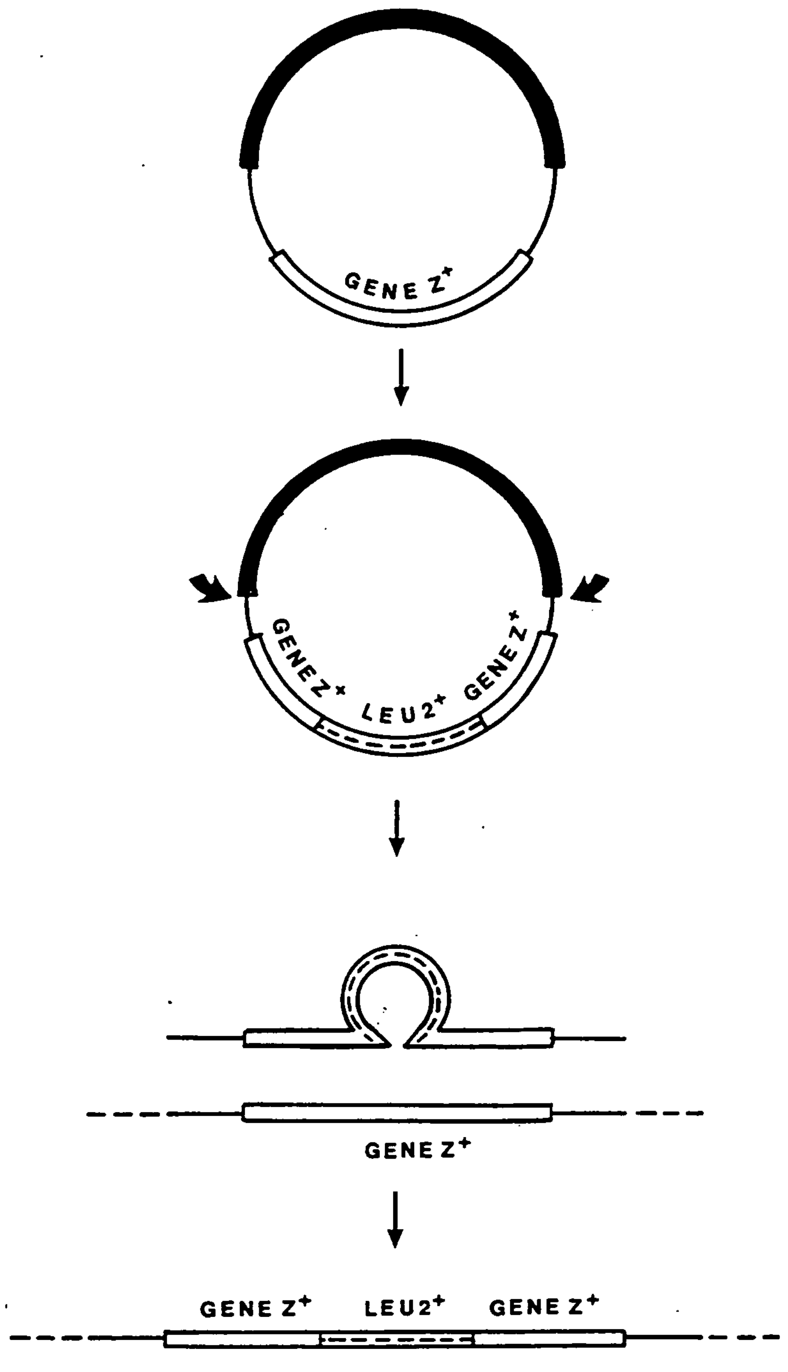
to give a fragment of DNA which consists of the selectable marker flanked with regions of *S.pombe* sequences from each side of the functional region. Cells are then treated with this fragment, using standard transformation techniques, and stable transformants isolated. Gene transplacement relies upon a double recombination event taking place (Fig. 6.7), so that the functional region of the gene in the *S.pombe* genome is replaced by the selectable marker sequences. If the gene in question were vital to cell growth and division, then loss of function would be lethal, and no transformants would be isolated. To avoid this, such experiments are normally performed with diploid strains, so that a heterozygous diploid will result which may then be induced to sporulate, and the haploid progeny analysed. Several methods for the maintenance of diploid strains in *S.pombe* exist (Russell, 1989), but the method chosen here was the utilisation of complementing alleles of *ade6* in haploids of h^+ and h^- mating type. This method which has the advantage of resulting in cells which will remain diploid under conditions of adenine selection, and which will sporulate spontaneously when placed under conditions of nitrogen starvation to give rise to haploid segregants which may then be analysed.

Two separate experiments were performed with the aim of producing a strain which lacked *wis1* function. The first involved the deletion of a small section (less that 100bp, see Chapter 7 for details) of functional sequence from *wis1*, and its replacement with a 3.0kb fragment containing the *LEU2* gene. Although the resulting construct contained a large segment of DNA within the functional sequence, it retained a detectable level of *wis1* function. The second experiment was then performed in which over 1kb of functional sequence was deleted and replaced with a *LEU2*-containing fragment. This gave rise to strains which were presumably completely lacking in *wis1* function, as over two thirds of the predicted *wis1* open reading frame had been lost (Chapter 7).

6.3.1.a: Deletion of <u>wis1</u> sequences with the construct pXPL-3

The first stage in this experiment was to create a version of pTZ19 which lacked the polylinker *Bam*HI site. To do this, pTZ19 was digested with *Bam*HI, treated with Klenow enzyme to produce 'blunt' ends, and subsequently religated. The resulting plasmid was named pTZ19B-5. The 3.5kb *Pst*I - *Xba*I fragment from pwis1-1 was then cloned into the corresponding polylinker sites of pTZ19B-5, to give the plasmid pXP-3 (Fig. 6.8). The 3.0kb *Bgl*II fragment containing the *LEU2* functional sequence from pDAM6 was then cloned into pXP3 using the closely spaced *Bgl*II and

Figure 6.7: One step gene transplacement in yeast.

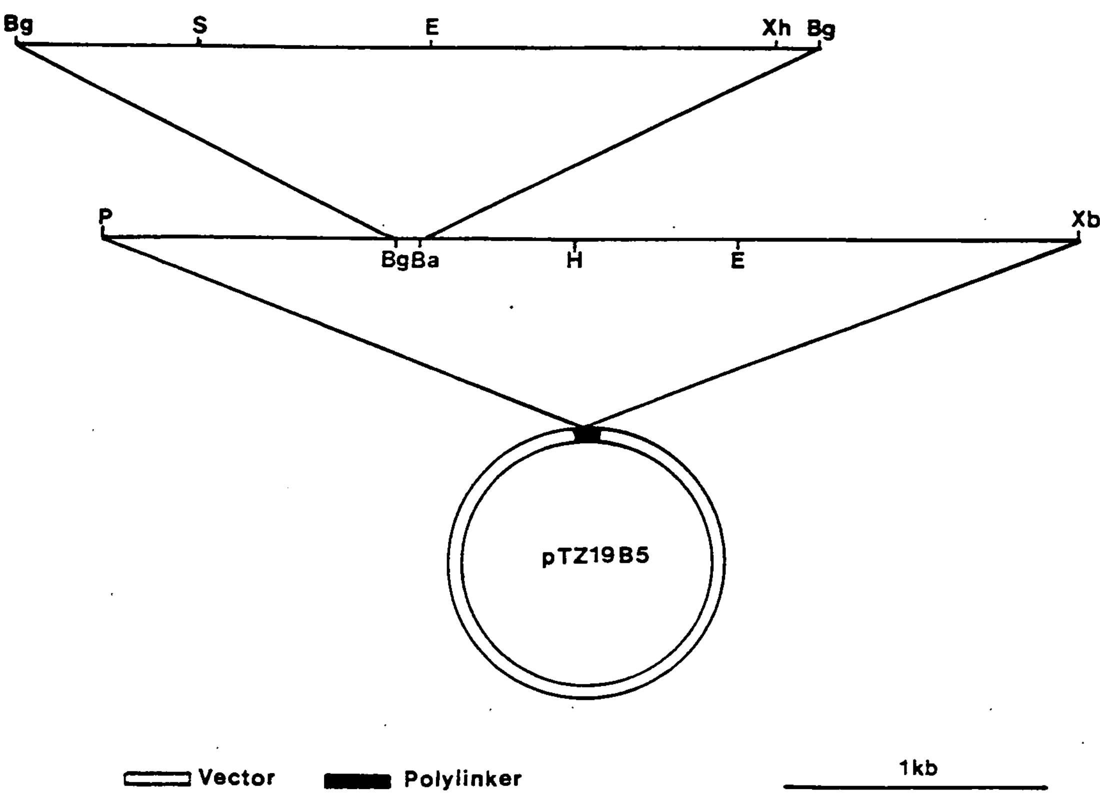


(Adapted from Rothstein, 1983)

The cloned fragment containing *GENE* Z^+ is digested with one or more restriction enzymes which cleave within the *GENE Z* sequence. A fragment containing a selectable yeast gene (*LEU2* in this example) is cloned in to the sites within *GENE Z*. The fragment containing the disrupted *gene Z* is liberated from the plasmid sequences, making certain that homology to the *GENE Z* region remains on both sides of the insert. Transformation of yeast cells with the linear fragment results in the substitution of the linear disrupted sequences for the chromosomal sequence.

Figure 6.8: Construction of pXPL-2.

The 3.5kb *Pst*I - *Xba*I from pwis1-1 was subcloned into pTZ19-B5 to give the plasmid pXP-3. The 3.0kb *Bgl*II fragment containing the *S.cerevisiae LEU2* gene was then cloned into the *S.pombe* sequences to give the plasmid pXPL-2 (see text for details).



*Bam*HI sites within the *wis1* sequences. This resulted in the plasmid pXPL-2 (Fig. 6.8). pXPL-2 was digested with *Pst*I and *Xba*I, and the 6.5kb fragment containing *LEU2* sequences flanked by *wis1* sequences was purified by electrophoresis, followed by isolation from low melting point agarose. Approximately 3μg of purified fragment was used to transform a diploid of the genotype *ade6.210/ade6.216 ura4.D18/ura4.D18 leu1.32/leu1.32* h^+/h^-.

Transformants were initially screened for the mitotic stability of the *LEU2* marker, and for their ability to sporulate. Two such stable transformants (D1 and D44) were selected for further analysis. These diploids were induced to sporulate by growth on ME medium, and tetrad analysis performed upon the resultant asci. Both strains gave a 2:2 segregation of the *LEU2* marker, with a phenotype of increased cell length at division cosegregating with *LEU2* (Table 6.2).

In order to confirm that the sequences containing the *LEU2* marker sequences had integrated at the *wis1* locus, *LEU2* segregants from D1 and D44 were crossed to strains of genotype [*wis1:ura4*$^+$] *ura4.D18 leu1.32*, containing the *ura4*$^+$ gene integrated adjacent to the *wis1* locus. Tetrad analysis of these crosses showed only parental ditypes in ten tetrads examined with respect to the *LEU2* and *ura4* markers. This evidence shows that the level of recombination between the *ura4*$^+$ and *LEU2*$^+$ tagged loci in these crosses is very low, indicating that the integration events giving rise to the leu$^+$ phenotypes of D1 and D44 both took place by homologous recombination.

Since these recombination events had not given rise to a lethal phenotype for the disruption, it remained a possibility that there was still *wis1* function present in these strains. To check the activity of the construct used to make these strains, the plasmid pXPL-2 was transformed into the strain *wee1.50 cdc25.22 win1.1 leu1.32* h^-. This plasmid could be relatively stably maintained, indicating that it contained ARS activity (see Chapter 7). The presence of this plasmid in multicopy means that any residual activity should be detectable by its ability to suppress the temperature sensitive phenotype of this strain. In this case, it was possible to detect a very low level of suppression, so it was assumed that D1 and D44 still retained a low level of *wis1* activity. A second experiment was therefore devised which involved deletion of a larger section of *wis1* functional sequence in order to completely abolish function.

Table 6.2: Cell length at division of strains containing disruptant alleles of wis1

Strain	Cell length/μm	SD
972	12.99	0.54
ED667	13.23	0.50
D1	21.24	1.36
D4	24.30	1.87
D4 (YE)	19.56	0.79

Cells were grown in supplemented EMM at 25°C unless otherwise indicated. ED667 has the genotype *leu1.32 ura4.D18 ade6.216*. SD = standard deviation.

6.3.1.b: Deletion of wis1 sequences with the construct pXPL-9

The plasmid pTZ19R-4, which consisted of the plasmid pTZ19 with the *Eco*RI site removed, was constructed in a similar way to that described for pTZ19B-5. The 3.5kb *Pst*I - *Xba*I from pwis1-1 was subcloned into this plasmid to produce the plasmid pXP-9 (Fig. 6.9). This plasmid was digested with *Eco*RI, treated with Klenow enzyme to produce blunt ends, and then treated with *Bgl*II. A DNA fragment was then subcloned into these sites which consisted of the *Bgl*II - *Xho*I *LEU2* fragment from pDAM6, which had been treated in a similar way to that described for pXP-9 to give a *Bgl*II - blunt end fragment. This gave rise to the plasmid pXPL-9 (Fig. 6.9).

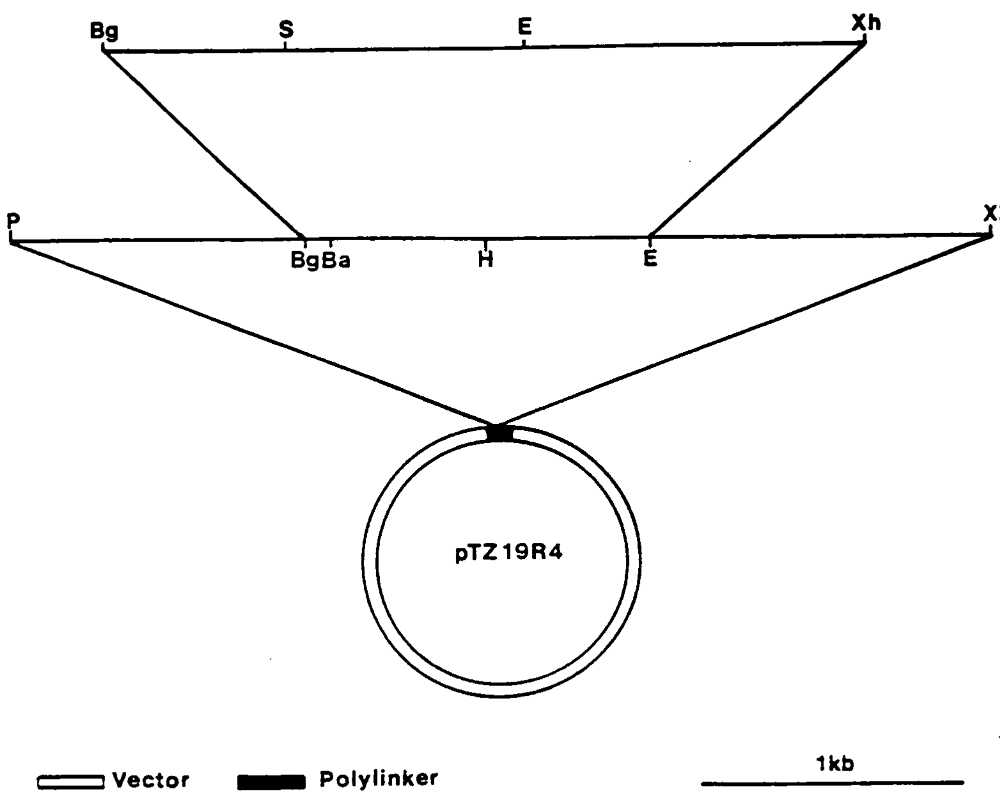
A similar procedure to that used in the case of pXPL-2 was used to treat diploid cells with over 10 g of purified *Pst*I - *Xba*I fragment from pXPL-9. Lesser amounts did not give rise to any stable transformants, possibly due to the reduced length of *S.pombe* flanking sequences in this fragment compared to that derived from pXPL-2. This difference may have led to reduced recombination frequencies.

Of 17 transformants examined, all were stably leu^+, one had lost the ability to sporulate, and, upon tetrad analysis, 11 showed a phenotype of increased cell length co-segregating with the *LEU2* marker. Five strains were selected and subjected to Southern blot analysis. A diagram showing the expected result from a one step gene transplacement event with this DNA construct is shown in Figure 6.10.

Genomic DNA was prepared from the five transformant strains described above, digested separately with *Hin*dIII and *Eco*RI, and this DNA subjected to Southern blot analysis. Such a blot probed with an equimolar mixture of the 4.4kb and 3.2kb *Hin*dIII fragments from pwis1-1 is shown in Figure 6.11. This probe shows hybridisation with the two equivalent fragments in wild type DNA digested with *Hin*dIII, but in the case of the disruptant strains, there is hybridisation with very high molecular weight DNA which has not been transferred efficiently to the filter. This result would be expected if multiple integration events had occurred involving the sequences between the flanking *Hin*dIII sites, which had also resulted in the loss of the central *Hin*dIII site. Since multiple gene transplacement events would simply result in repeated replacement of genomic sequences with incoming fragments, the

Figure 6.9: Construction of pXPL-9.

The 3.5kb *Pst*I - *Xba*I from pwis1-1 was subcloned into pTZ19-R4 to give the plasmid pXP-9. The 2.9kb *Bgl*II - *Xho*I fragment containing the *S.cerevisiae LEU2* gene was then cloned into the *S.pombe* sequences to give the plasmid pXPL-9 (see text for details).



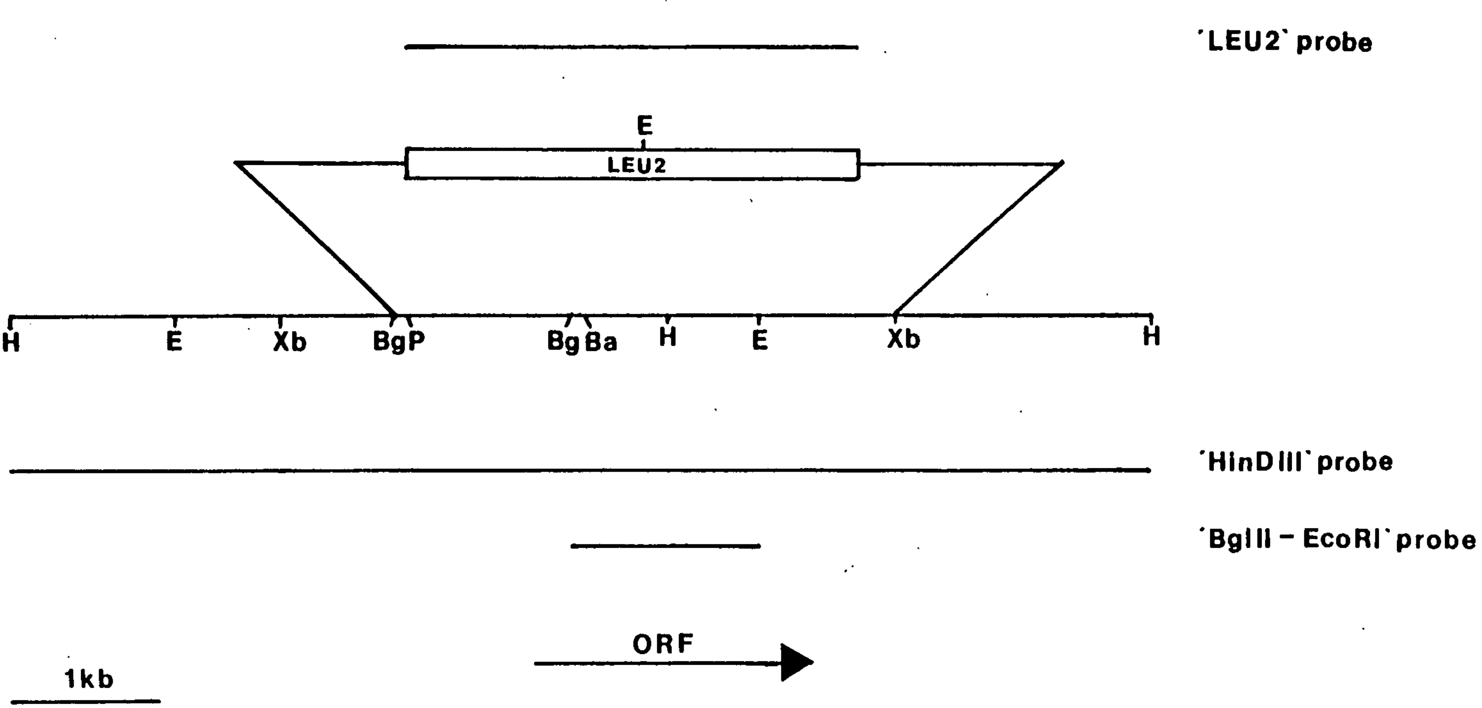


Figure 6.10: Schematic diagram indicating the expected result of gene transplacement with the pXPL-9 construct, and showing the probes used for the analysis of wis1 disruptant strains.

Arrow indicates the extent and direction of the predicted *wis1* open reading frame.

Figure 6.11: Southern blot analysis of wis1 disruptant strains

S.pombe genomic DNA was digested with the restriction enzymes indicated below, subject to agarose gel electrophoresis and Southern blotted.

Panel A - probed with the 4.4kb and 3.2 kb *Hin*DIII fragments from pwis1-1.*

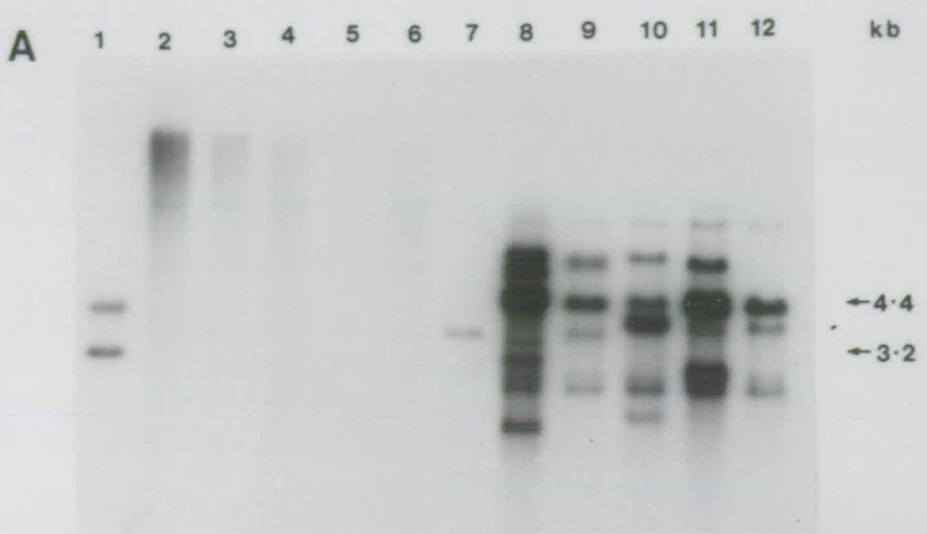
Panel B - probed with the *LEU2* containing *Bgl*II fragment from pDAM6.*

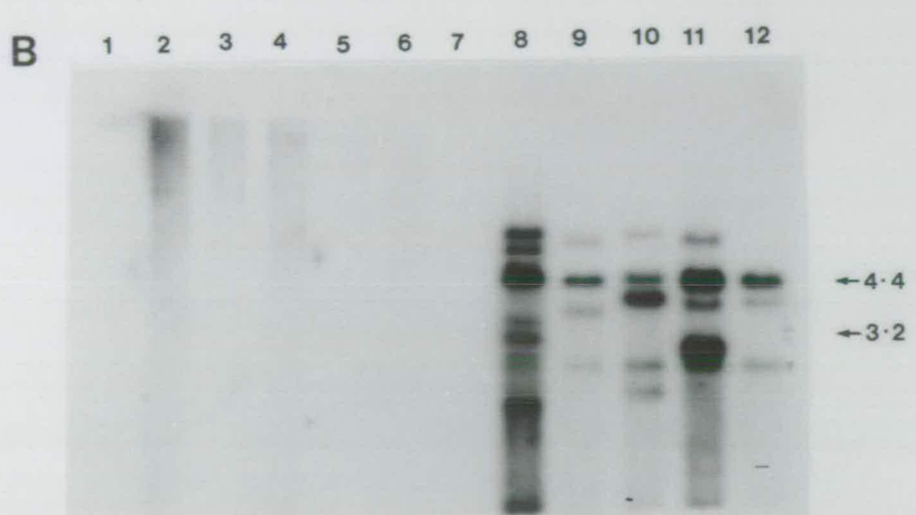
Lane 1: wild type *Hind*III
Lane 2: D2 *Hind*III
Lane 3: D4 *Hind*III
Lane 4: D5 *Hind*III
Lane 5: D7 *Hind*III
Lane 6: D10 *Hind*III
Lane 7: wild type *Eco*RI
Lane 8: D2 *Eco*RI
Lane 9: D4 *Eco*RI
Lane 10:D5 *Eco*RI
Lane 11:D7 *Eco*RI
Lane 12:D10 *Eco*RI

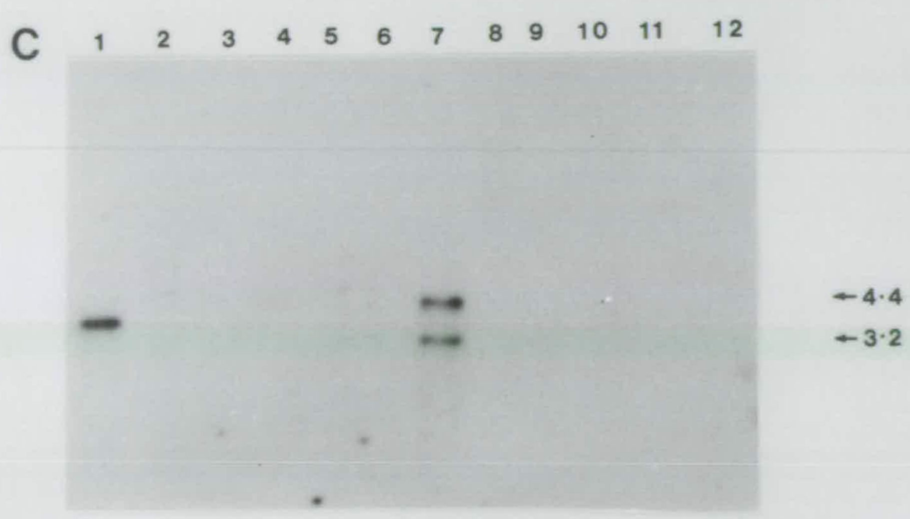
Panel C - probed with the BglII - EcoRI fragment from pwis1-1.*

Lane 1: wild type *Eco*RI
Lane 2: D2 *Eco*RI
Lane 3: D4 *Eco*RI
Lane 4: D5 *Eco*RI
Lane 5: D7 *Eco*RI
Lane 6: D10 *Eco*RI
Lane 7: wild type *Hind*III
Lane 8: D2 *Hind*III
Lane 9: D4 *Hind*III
Lane 10:D5 *Hind*III
Lane 11:D7 *Hind*III
Lane 12:D10 *Hind*III

* see Figure 6.10 for details of probes.


A
1 2 3 4 5 6 7 8 9 10 11 12
kb
←4·4
←3·2


B
1 2 3 4 5 6 7 8 9 10 11 12
←4·4
←3·2


C
1 2 3 4 5 6 7 8 9 10 11 12
←4·4
←3·2

net result would be in a small change in molecular weight: in this case a change from two bands of 4.4kb and 3.3kb to one of 8.3kb (Fig. 6.11). The results seen in Fig. 6.11 suggest that much more complicated recombination events have occurred, possibly involving concatenated fragments. These recombination events have resulted the loss of the central *Hin*dIII site, and the replacement of these sequences by a large fragment lacking in *Hin*dIII sites.

This probe shows hybridisation to three fragments in the case of *Eco*R1 digested wild type DNA. In the case of the corresponding disruptant samples, a very complex pattern of bands may be seen, again suggesting complex integration events have taken place. The smaller bands seen in this digest, compared to the *Hin*dIII treated DNA, very likely result from the presence of an *Eco*R1 site within the *LEU2* sequences.

The same blot probed with the *LEU2*-containing *Bgl*II fragment from pDAM6 is shown in Fig. 6.11. There is no hybridisation with wild type sequences, but the patterns of hybridising bands with the disruptant DNA samples are similar to that seen with the *Hin*dIII fragment probe. This confirms that the complex integration events have involved *LEU2* sequences. Given the complexity of the transplacement constructs, it was necessary to confirm that the *wis1* sequences replaced by the *LEU2* fragment in pXPL-9 were not present. A blot similar to that described above was probed with the *Bgl*II - *Eco*RI fragment from pX3, i.e. those sequences removed in the construction of the plasmid used for the disruptions. This fragment shows hybridisation with a fragment of approximately 4kb in wild type DNA digested with *Eco*RI, and with two bands of approximately 4kb and 3kb in wild type DNA digested with *Hin*dIII (Fig 6.11). There is no hybridisation, however, with any DNA samples derived from the disruptant strains, implying that this 1.2kb *Bgl*II - *Eco*RI fragment is absent from these strains. When probed with the 4.4 and 3.2kb *Hin*dIII fragments described above, hybridising bands are present in each track containing *S.pombe* DNA samples in this blot (data not shown).

In order to confirm that a strain with these sequences deleted showed no *wis1* activity, the plasmid pXPL-9 was transformed into the strain *wee1.50 cdc25.22 win1.1 leu1.32 h*$^-$ to assess its *wis1* functional activity. This plasmid showed no activity in suppressing the temperature sensitive phenotype of this strain. The disruptant strains described here were judged to have a complete lack of *wis1* activity, due to the loss of 1.2kb of functional sequence. The sequence analysis of

wis1 (Chapter 7) showed that the sequences deleted represented two thirds of the predicted *wis1* open reading frame. One strain (D4) was selected for further analysis. Analysis of the *wis1* transcript showed a truncated mRNA species present in D4 of approximately 1500 bases compared with the 2400 nt *wis1* mRNA in wild type cells. No transcript of a size corresponding to that of the wild type *wis1* mRNA could be detected in D4 (Fig. 6.2).

The disruptant strains (D1 and D44) described earlier showed some residual activity, though at a very reduced level compared to that in wild type. The fact that the plasmids pXPL-2 and pXPL-9 could be sustained as autonomously replicating plasmids suggests that they contain ARS function, which is very likely to be contained within the *S.pombe* sequences. For further details, see Chapter 7.

6.3.2: Characterisation of strains resulting from gene transplacement experiments

Strains with both forms of disruption construct described above showed phenotypes of an increased cell length at division. The increase was greater in the case of D4, and examination of this strain showed that the cell length increase was more marked on minimal medium compared to yeast extract medium (Table 6.2). D4 cells also showed a phenotype of very much reduced viability upon entry into stationary phase, which was first observed by microscopic examination of cells stored upon solid media for several days. In order to quantify this effect, known numbers of cells were plated onto solid media from exponentially growing cultures, and the platings repeated once these cultures had been allowed to enter stationary phase (Table 6.3).

Tetrad analysis was performed on a cross between two disruptant isolates which both lacked the *BglII* - *EcoRI* fragment missing in D4. The fact that this cross gave rise to relatively normal asci which, in the majority of cases, contained four viable spores suggests that *wis1* activity is not essential for conjugation, meiosis and sporulation.

In order to confirm that the disruptant phenotype was solely due to the loss of *wis1* function, wild type *wis1* sequences were introduced into the disruptant strains, and the phenotype of the transformant strains assessed. The 3.5kb XbaI - PstI fragment from pX3 was subcloned into the plasmid pIRTU to give the plasmid pXIU. This plasmid was transformed into the disruptant strain D4, and the phenotypes of one such transformant strain compared to one containing the plasmid pSP100 as a control

Table 6.3: Effect of the plasmid pXIU upon wis1 disruptant strains.

(a) Cell length at division

Plasmid	Cell length/μm	SD
pSP100	15.60	0.84
pXIU	9.35	1.22
None	24.30	1.87

(b) Viability upon entry into stationary phase

	Viability (%):	
Strain	Log phase	Stationary phase
ED667	97.2	68.3
D1	99.0	12.6
D4	51.0	3.2

Cells were grown to mid log phase in YEPD at 25°C and dilutions of each culture plated onto YE. Following 2 days' incubation, further samples were taken for plating. Viability was calculated from the number of colonies formed as a fraction of total cells plated.

(Table 6.3). The results from these experiments showed that the disruptant phenotype of increased cell length was solely due to the loss of *wis1* function.

Five independent disruptant strains were examined, all of which showed a phenotype of reduced viability upon entry into stationary phase which co-segregated with the *LEU2* marker integrated at the *wis1* locus. From this evidence, and from the examination of disruptant transformants containing the plasmid pXIU upon entry into stationary phase, it seemed very likely that both phenotypes of increased cell length, and low viability upon entry into stationary phase resulted from the loss of *wis1* function.

6.4: INTERACTIONS BETWEEN A DELETION ALLELE OF wis1 AND OTHER CELL CYCLE MUTATIONS

6.4.1: Interaction with wee1

In order to investigate the interaction of a *wis1* deletion allele with *wee1*, a double mutant strain was constructed and characterised. The cross between the strains *wee1.50 leu1.32 h⁻* and *wis1::LEU2 ade6.216 ura4.D18 leu1.32 h⁺* (D4) was subjected to tetrad analysis. Putative double mutant segregants were obtained from tetratype tetrads which showed a phenotype of cell length greater than wild type at 25°C, and shorter than wild type at 35°C. These putative double mutants were all leu$^+$, indicating that they were *wis1*$^-$.

One putative *wee1.50 wis1*$^-$ segregant was backcrossed to a strain of genotype *leu1.32 h*$^-$, to distinguish strains carrying the *wis1::LEU2* construct. The segregations observed in this cross indicated that the original segregant chosen had the genotype *wis1::LEU2 wee1.50 ura4.D18 ade6.216 leu1.32 h*$^+$. In order to investigate the phenotype of this *wee1.50 wis1*$^-$ strain more closely, the cell length at division of this strain was measured. Cells grown at the two temperatures 25°C and 35°C were used in order to assess the effect of the temperature sensitive *wee1.50* allele (Table 6.4). The *wee1.50 wis1*$^-$ strain showed a temperature sensitive wee phenotype indicating that *wee1.50* is epistatic to the *wis1*$^-$ deletion allele.

Table 6.4: Cell length at division of strains containing a wis1 deletion allele combined with a mutation which has a wee phenotype.

Strain	Cell length/μm	SD
972	12.99	0.87
wis1::LEU2	24.30	1.87
wis1::LEU2 win1.1	24.56	1.74
*wee1.50**	7.46	1.35
*wee1.50 wis1::LEU2**	8.00	0.93
cdc2.1w	8.65	0.64
cdc2.1w wis1::LEU2	13.60	0.67
cdc2.3w	9.05	0.51
cdc2.3w wis1::LEU2	13.35	0.71
adh-cdc25^{+}	8.44	0.77
adh-cdc25^{+} *wis1::LEU2*	11.20	0.45

Cells were grown in supplemented EMM at 25°C unless otherwise indicated. * indicates 35°C. At least 24 cells were measured in each sample.

4.2: Interactions with cdc2w alleles

A similar analysis to that described above was carried out to determine the effect of combining *cdc2.1w* and *cdc2.3w* with the *wis1* deletion allele. The cross between *cdc2.1w leu1.32 h⁻* and *wis1::LEU2 ura4.D18 ade6.216 leu1.32 h⁺* gave a particularly low spore viability. Putative double mutant strains were chosen from tetratype tetrads, which, in the case of both *cdc2w* alleles, were very slow growing and a dark red colour on Phloxin B containing media. Colonies from these strains consisted of slightly misshapen cells which were of a length between that of wild type and a *wis1⁻* strain. The genotypes of these strains were confirmed by backcrossing to a *leu1.32* strain, followed by tetrad analysis. Two strains of genotypes *wis1::LEU2 cdc2.1w ura4.D18 leu1.32 h⁻* and *wis1::LEU2 cdc2.3w ade6.216 leu1.32 h⁻* were chosen for further analysis, and cell lengths at division determined (Table 6.4). The *wis1::LEU2 cdc2.w* double mutant strain both showed cell lengths at division intermediate between those of the single mutants. These results indicate a form of interaction between the *cdc2w* alleles and *wis1* which is not strongly allele specific.

6.4.3: Interaction with over-expressed cdc25⁺

A cross between the strains [*adh-cdc25:ura4*] *ura4.D18 leu1.32 h⁻* and *wis1::LEU2 ura4.D18 ade6.216 leu1.32 h⁺* was subjected to tetrad analysis. It was possible to follow the *adh-cdc25* construct be the *ura4⁺* marker, and the *wis1* deletion by the *LEU2⁺* marker. Putative double mutant strains with a leu⁺ ura⁺ phenotype were selected. The *adh-cdc25* construct is present as an integrated plasmid which is lost at a relatively high rate. Following a period of growth on YE, it was possible to isolate *ura4⁻* segregants from the putative double mutants, and to confirm their *wis1⁻* genotype by examination of their cell length phenotype. The cell length at division was determined from a *wis1::LEU2* [*adh-cdc25:ura4*] *ura4.D18 leu1.32* strain (Table 6.4). These observations suggest that the division length of *wis1* deletion strains are sensitive to the *cdc25⁺* expression level.

6.4.4: Interaction with pat1

The strain *wis1::LEU2 ade6.216 ura4.D18 leu1.32 h⁺* was crossed to a strain of genotype *pat1.114 ade6.216 leu1.32 h⁻* (supplied by Maureen McLeod). Tetrad analysis showed no evidence of linkage between *wis1* and *pat1*. Putative double mutant strains were selected from tetratype tetrads. At 25°C the double mutants

showed a phenotype similar to that of the *wis1*⁻ strain. No sporulation at all was seen in the *wis1*⁻ *pat1.114* double mutant strains upon a shift to 35°C. The cells remained similar in length to *wis1*⁻ cells, but the cells were only capable of forming microcolonies consisting of up to 100 cells. These results indicate that loss of *wis1* function completely blocks the hypersporulation phenotype which normally results from loss of *pat1* activity, but does not completely relieve the growth defect shown by *pat1.114* strains at the restrictive temperature. This is similar to the interaction shown by *win1.1* with *pat1.114* (Chapter 3). Comparison of the restriction maps of sequences containing *wis1*, *cgs1* and *cgs2* established that *wis1* was not allelic to either *cgs1* or *cgs2*, mutations in which suppress both the growth and hypersporulation phenotypes of *pat1*^{ts} strains.

6.4.5: Interaction between win1.1 and wis1⁻

A cross was made between the strains *win1.1 ade6.216 ura4.D18 leu1.32 h*⁻ and *wis1::LEU2 ade6.216 ura4.D18 leu1.32 h*$^{+}$ which was subjected to tetrad analysis. Two putative *wis1*⁻ *win1.1* double mutant strains were selected from tetratype tetrads. These segregants showed a phenotype very similar to that of the parental *wis1*⁻ strain. Backcrossing to a *leu1.32* strain was used to confirm the phenotype of these putative double mutants and their cell length at division determined (Table 6.4). These results show that it is not possible to differentiate between *win1.1* and *win1.1 wis1*⁻ strains on the basis of cell length.

6.5: DISCUSSION

This chapter describes the analysis of the *wis1* transcript, including its levels in cells containing increased *wis1* copy number, and the effect upon transcription caused by deletion of large section of the *wis1* functional region. The effect upon the cell of altered *wis1* dosage is described, both in the form of increased copy number, and in the form of loss of *wis1* function, both partial and complete. Finally, the interactions between a *wis1* deletion allele and mutations which result in wee phenotype are described.

The fact that increased copy number results in a decrease in cell length, and that this effect is dependent on *wis1* dosage, suggests that *wis1* is involved in a rate limiting step controlling entry into mitosis and division. The observation that complete loss of *wis1* function is not lethal to the cell implies that *wis1* function is not vital for

cell growth and division. The genetic studies described here suggest that strains lacking in *wis1* function are still sensitive to levels of *wee1* and *cdc25* expression with respect to cell length, and that the combination of *cdc2w* alleles with a *wis1* deletion allele results in a substantial decrease in cell length. Loss of *wis1* function strongly suppresses the hypersporulation phenotype of *pat1.114*, which is similar to the effect shown by *win1.1*.

The phenotype of increased cell length of the disruptant strains again points to a role for *wis1* as a regulator of entry into mitosis and division, and the phenotype of low viability upon entry into stationary phase gives a further clue to the role of *wis1* in the cell. One possibility is a role in some form of nutritional sensing, so that *wis1*$^-$ cells are not receiving signals to stop growth and enter stationary phase upon starvation.

The epistasis of *wis1*$^-$ over *win1.1* suggests that *wis1* and *win1* may both lie in the same pathway.

CHAPTER 7

CHAPTER 7: SEQUENCE ANALYSIS OF wis1

7.1: INTRODUCTION

The determination of the DNA sequence of a gene isolated by molecular cloning techniques can give information concerning transcriptional regulation, transcript processing and the function and characteristics of the gene product. From the nucleotide sequence of an identified functional region of DNA, it is normally possible to identify an open reading frame, which may be interrupted by introns, and from this to deduce the amino acid sequence of the gene product. Identification of the open reading frame (ORF) and its flanking sequences facilitates further molecular manipulation of the isolated gene, such as deletion of the coding region by gene transplacement techniques, and gives an accurate and exhaustive restriction map of the sequences involved.

In some cases it is possible to identify a putative function for the gene product by the comparison of the predicted amino acid sequence with previously sequenced genes of known function. In this way, it may be possible to identify specific regions in the predicted gene product involved in protein function and regulation. The amino acid sequence may also yield information about structural features of the gene, such as the three-dimensional protein structure, and features such as hydrophobic regions which may be membrane associated.

In the sequence of regions flanking the coding region it may be possible to identify elements concerned with the regulation of transcription, and the processing of mRNA. The sequences include "upstream" elements such as the TATA box which has been shown to be involved in the initiation of transcription (Guarente, 1988), and downstream elements required for transcriptional termination and polyadenylation of mRNA (Zaret and Sherman, 1982; Proudfoot and Brownlee, 1976).

7.2: EXPERIMENTAL STRATEGY

The sequence of *wis1* was determined by a combination of the phagemid system devised by Vieira and Messing (1987) and the chain termination sequencing method of Sanger *et al*, (1977).

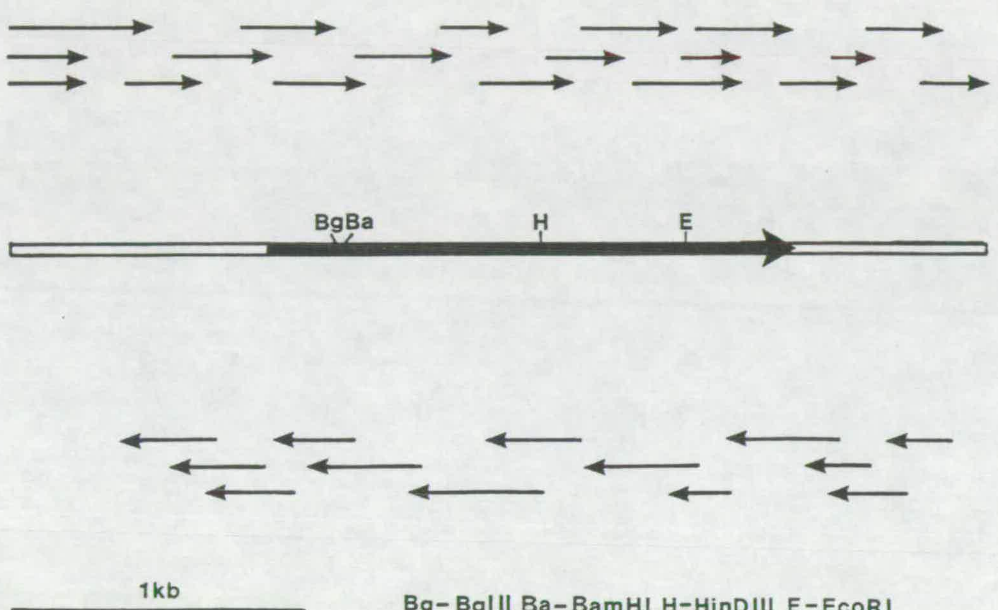
The plasmids pX2 and pX3 contain the 4.1kb *Xba*I fragment from pwis1-1 subcloned into the plasmid vector pTZ18R in opposite orientations (see Section 5.1.1). The pTZ18R and pTZ19R vectors contain both the pBR322 origin of replication for the generation of double stranded DNA within *E.coli*, and the M13 f1 origin, which may be used to generate single stranded DNA. In order to prepare single stranded DNA, cells carrying the phagemid are infected with the helper virus M13K07, which results in the production of bacteriophage particles containing single stranded copies of the phagemid genome (Section 2.2.2).

To prepare suitable subclones for the sequencing procedure, deletions were made from the ends of the inserts contained within the plasmids pX2, pX3, pBX2 and pBX3 proximal to the reverse sequencing primer hybridisation site in the phagemid. The plasmids pBX2 and pBX3 were generated from pX2 and pX3 by digestion with BamHI, followed by religation. The method used to generate deletions was that of Henikoff (1987) in which Exonuclease III is used to digest blunt or 5' protruding ends of DNA, followed by S1 nuclease to generate blunt ends for ligation. For the details of this procedure, see Section 2.6.1.

Once the sizes of the deletion constructs resulting from this procedure had been screened by restriction analysis, those of appropriate sizes were used for the preparation of single stranded DNA. These preparations were used in sequencing reactions, and the sequence determined by acrylamide gel electrophoresis, followed by the detection of radioactively labelled DNA by autoradiography. The DNA sequence described here was determined entirely by the use of deletion constructs, with the exception of one 200 - 300 bp section which was determined by the use of a synthetic 18bp oligonucleotide as a primer for the sequencing reactions (Fig. 7.1). The methods used are described in detail in Section 2.6.

Figure 7.1: Strategy for the sequencing of wis1.

Each arrow indicates the extent and direction of sequence determined from each deletion construct.



7.3: THE wis1 SEQUENCE

7.3.1: Results

The sequence of a section of DNA consisting of 3276bp was determined, and the results shown in Fig. 7.2. The entire sequence was determined on both strands, with the exception of the first 440nt, which were determined from three independently isolated clones on one strand only.

7.3.2: Restriction site analysis

The positions of *Bgl*II, *Hind*III and *Eco*RI restriction sites from the sequence were found to be in close agreement with those determined by previously described restriction analysis. The position of a *Pvu*II site known to be situated between the *Bam*HI and *Hin*dIII sites from subcloning experiments was confirmed. In addition, a second *Pvu*II site was detected approximately 390bp distant. A site for *Xba*I was detected at position 1310 (Fig. 7.2), which was surprising, as the DNA isolate used for sequencing had been isolated initially as a 4.1kb *Xba*I fragment. Examination of the sequences immediately adjacent to this *Xba*I site were shown to match the consensus for methylation by the *dam* methylase in *E.coli*. *XbaI* recognises the sequence 5'-TCTAGA-3', and the *XbaI* recognition sequence in this case was followed by 5'-TC-3'. The *dam* methylase will transfer a methyl group from S-adenosylmethionine to the N6 position of the adenine residue in the sequence 5'-GATC-3', and this will prevent recognition of the sequence by *XbaI* (Geier and Modrich, 1979). Since the *E.coli* strains used for plasmid propagation were all *dam*$^+$, it seems likely that this restriction site was not recognised in plasmid preparations due to methylation of the recognition sequence. Southern blot analysis with wild type *S.pombe* DNA (which is unmethylated) digested with *Xba*I and probed with sequences from this region revealed two hybridising fragments of approximately 2kb in place of the 4.1kb fragment seen in plasmid *Xba*I digests, which confirms this hypothesis (data not shown).

Figure 7.2: DNA sequence of the region containing wis1

```
   1  AACGTGTTGT CTGACTTTCG TTATCCTTTT ATCCTTCACC AACTCCATCC
  51  CATTCCACCT TCTTGTATAC CCAAACATAC CCCAGCCGGC TGGCGATACG
 101  TGGGATTCCT AATCTCTCAA GCATTCCCTT TCGACGTGTA TATCTTATTT
 151  ACATCGTACC GACTACACTT CCTATTTTAT CACCCTCCCT AAATTTTCCC
 201  ATTTCCTTTA AAGAGCAGAA TTTTTTTTGC TCTTTACTGT AAGAGAAAAG
 251  CGTTTCTGAA TTTTTTCTCT TCCTTCCACA CCCTTACGTG TTATCTTTTT
 301  AGAAAGATAA TTTCAAATTT CTTTCCTTTT TTATTTCGTG TTTGTGAATT
 351  ATTACTTTTT CTCCTTACCT TGCCTTCCTT ACTCGTCGGT CATACTCGGT
 401  TTAAAGGTAT TGACTTCTCG GACTTAAGAT TAACCACACT TTACTTTTTT
 451  TTCTTTAGTT CTCCTTGGGA AATTTATTAT CTAAGCCCCT GTTTCTTCCA
 501  GTTTCTGGCT TTTGCTGTTA AATTTAAACC CTTCCAAACC TCCTTTTTTT
 551  CCGTGGCATT TACCTACACA AAGCTACTCG TAGGTGATTG CCTTTAAACT
 601  TCCTTTTTTT TTCTTGGAAT ATCCTTCCTG CGGACTTTTT AGACCACCGC
 651  TTTTTTTTTC CTTCTTCGTC GGAGACGACT CGTAATTGAT TGTCTAATTT
 701  TAATTGTTCT TTTCACCCAA GATACCTTTT TTGTATTGCC ATCTCACTTT
 751  CGTTCATCTT CACTTTTGCT TCATTTATAT TACCGGAATT TAGTTTACCT
 801  AATTTTTTTT TCTTTTTTTT TAAGTTTGTG AAGCACATTT ATTTTATATG
                                                 PvuII
 851  TCTTCTCCAA ATAATCAACC CTTGTCTTGC TCATTGAGAC AGCTGTCTAT
 901  TTCTCCTACC GCACCTCCCG GTGATGTTGG TACTCCCGGC TCGCTCCTTT
 951  CTCTTTCGTC TTCAAGTTCT TCAAACACCG ATTCTTCTGG TTCTTCCTTG
1001  GGTTCCTTGT CTTTAAATTC TAACAGTAGT GGCAGTGACA ATGACTCAAA
1051  GGTTTCTTCT CCTAGTCGTG AAATACCTTC CGATCCCCCT CTTCCCCGTG
                                 BglII
1101  CCGTGCCTAC GGTCAGACTT GGCAGATcTA CGTCCAGTCG GAGTCGTAAC
                              BamHI
1151  TCTCTTAACC TTGACATGAA GGATCCTTCG GAAAAACCTA GACGTTCACT
                  PvuII
1201  TCCTACAGCA GCTGGTCAGA ACAATATTGG ATCTCCTCCT ACTCCACCGG
1251  GCCCATTTCC TGGAGGACTT TCAACTGATA TACAGGAGAA ATTGAAGGCC
                  XbaI
1301  TTCCATGCAT CTAGATCAAA ATCAATGCCG GAAGTAGTCA ACAAGATCAG
1351  TAGTCCAACT ACCCCTATTG TCGGTATGGG TCAACGAGGA AGTTATCCTT
1401  TGCCTAACTC TCAACTTGCT GGTCGATTAA GTAATTCGCC CGTAAAGTCT
1451  CCGAATATGC CAGAGTCCGG GCTTGCAAAA TCACTTGCTG CTGCTAGGAA
1501  TCCTTTACTC AACCGTCCAA CGTCCTTCAA TCGACAAACG AGAATCCGTC
1551  GTGCACCACC TGGAAAACTC GATTTATCCA ATTCCAATCC CACCAGCCCT
1601  GTCAGTCCGT CTAGCATGGC TTCTCGCCGT GGCCTAAACA TTCCTCCCAC
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Figure 7.2 contd.

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1651  CCTTAAACAG GCTGTTTCGG AAACCCCTTT TTCCACATTT TCGGATATTT
1701  TGGATGCAAA ATCAGGCACC TTAAATTTTA AAAACAAAGC CGTGTTAAAT
1751  TCAGAAGGTG TTAACTTTTC ATcTGGCTCT TCGTTTCGTA TTAATATGTC
                 HinDIII
1801  AGAGATTATT AAGCTTGAAG AACTTGGAAA AGGTAACTAT GGTGTTGTGT
1851  ATAAAGCATT GCATCAACCG ACTGGTGTCA CTATGGCCTT GAAGGAAATT
1901  AGGTTGTCCT TAGAAGAAGC AACATTTAAT CAAATTATAA TGGAATTGGA
1951  TATTTTACAT AAAGCAGTTA GTCCTTATAT CGTTGACTTT TATGGTGCCT
2001  TTTTTGTGGA AGGTTCTGTT TTTATTTGTA TGGAATATAT GGATGCTGGT
2051  AGCATGGACA AACTGTATGC TGGTGGTATC AAAGACGAAG GAGTTTTAGC
2101  TAGAACTGCT TATGCTGTAG TGCAAGGCCT CAAAACTTTG AAAGAGGAGC
2151  ATAATATCAT TCATCGTGAC GTTAAACCTA CTAATGTTTT GGTAAATTCT
2201  AATGGCCAGG TTAAGTTATG TGACTTTGGC GTGAGTGGGA ATCTTGTGGC
2251  TTCTATATCC AAAACGAACA TTGGATGTCA ATCTTACATG GCTCCTGAAA
      EcoRI
2301  GAATTCGTGT TGGTGGACCT ACCAATGGCG TCTTGACTTA CACCGTACAG
2351  GCTGATGTGT GGTCTCTAGG CCTTACCATT TTAGAAATGG CTTTAGGAGC
2401  TTATCCGTAT CCACCTGAAT CATATACTTC AATATTTGCA CAACTATCGG
2451  CGATTTGCGA TGGCGATCCA CCTTCTCTCC CCGATTCATT TTCTCCCGAA
2501  GCTCGTGATT TTGTAAACAA GTGTTTGAAT AAAAACCCGT CTTTGCGTCC
2551  CGATTATCAT GAGTTGGCTA ACCATCCATG GTTGTTAAAA TATCAAAATG
2601  CAGATGTGGA CATGGCTTCA TGGGCAAAAG GCGCTCTTAA AGAGAAAGGT
2651  GAAAAAAGAA GCTAAAGGTT CGCCTGCTTT CTAATTGCCT GCTCTGTTTT
2701  AAAGTACCCA TGCGCATTGG TGTTTGTCTT TAATTTCGAA TGCATGACTA
2751  TTACGTGATC CATAATTATG TTTCAGCAGA ACCGACGCTA TTTTGCATTT
2801  GTGCTTTTTC ATAAATTTAA TAATTTGGGT ATGATTCCGT ATAACGGTAG
2851  TtGATGTTTG CATTTTTGCT TTAAATTAAA ACGGGTATTT AATGTGTTAT
2901  TACATTTGTT TAAGGCATTT ACGTCCACCA TAAAAAACTT TTTTTTTATT
2951  TAACTAAGGG GCTTTAGAAG TGCAAACGAA AGTTTGCAAT TGTAAAAGTT
3001  ACTTGTACTG TATTAATTTA TTTCTTTTAA AACTTCGTTG ACTGGCTCCG
3051  TCGTTTTAGC TACTATGATC TCCTGTTTCC TACAATGGTG ATTACTTAGA
3101  GAGATTGAAT CCATCGTAAA ATGCAAGTGT TAATAGATAT TATATCGACC
3151  TAATAATAAT TACTTAAACT ATTAATATAT TACTCAAAGA GATTTGAGGT
3201  ATTCATTTAT TTAAAAAGTA CGAGACTTTT ATATATCAAA GAAACAAATT
3251  CTCCCAACAT AAGTCCAACA AGGTAA
```

Putative start and stop codons (green)

Restiction endonuclease sites (yellow)

Regions with ARS homology (pink)

7.3.3: ARS consensus sequences

The 3.5kb *Pst*I - *Xba*I fragment of pwis1-1 had been shown to contain ARS activity (described in section 6.3.1). The plasmid pXPL-9 which contained this *Pst*I - *Xba*I fragment with the *Bgl*II - *EcoR*I fragment replaced with 2.9kb containing the *S.cerevisiae LEU2* gene showed ARS activity. This suggests that ARS activity was present in the remaining *S.pombe* sequences, as the remainder of the plasmid was made up of pTZ19 and the *LEU2* region, neither of which show ARS activity.

No perfect matches to the 11nt ARS consensus identified by Maundrell *et al* (1988) were found in the sequence described here. However, three sequences with one mismatch were identified (Table 7.1) which all lie within 300nt in a region of DNA outside the proposed *wis1* open reading frame (Fig. 7.2). The section of DNA containing the three ARS matches were present in pXPL-9, a plasmid construct used for the deletion of *wis1* which showed ARS activity. pXPL-9 contains the 3.5kb *Xba*I- *Pst*I fragment from pwis1-1 subcloned into pTZ19-B, with the *Bgl*II - *Eco*RI fragment replaced with the *Bgl*II - *Xho*I *LEU2* fragment from pDAM6 (Section 6.3.1.b).

It is possible to draw several conclusions from these observations: an ARS consensus sequence may lie within the small region of the *Pst*I- *Xba*I subclone not sequenced. Alternatively, the consensus may lie outside the subclone, as Maundrell found that this consensus was found associated with ARS activity, rather that being essential for it. The third possibility is that the sequences shown in Table 7.1 are responsible for the ARS activity, despite their mismatches to the consensus.

7.4: IDENTIFICATION OF THE wis1 ORF

7.4.1: Identification of initiation and termination codons

The ATG codon shown is most likely to define the start of translation (Fig. 7.3a). It is preceded by a region of 847nt with no ATG codon in any reading frame, and is followed by a 1815nt open reading frame. Translation of eukaryotic genes has been shown to start at the first ATG codon in the majority of cases (Kozak, 1983). An optimum sequence for the site of initiation by eukaryotic ribosomes has been

Table 7.1: Sequences showing homology to the S.pombe ARS consensus sequence.

Consensus:	A/T	Pu	T	T	T	A	T	T	T	A	T/A
Posn. 2945:	T	t	T	T	T	A	T	T	T	A	A
Posn. 3206:	c	A	T	T	T	A	T	T	T	A	A
Posn. 3010:	A	A	T	T	T	A	T	T	T	c	T

Lower case letters indicate mismatches to the consensus sequence proposed by Maundrell *et al* (1988).

Positions refer to the numbering in Figure 7.2

determined (Kozak, 1986) which is a consensus of 5'-ACCATGG-3'. The sequences adjacent to the ATG proposed here as the initiation of translation (5'-TATATGT-3') show no homology to this consensus sequences. One possibility is that translation may occur at a second ATG codon if the first one encountered by the ribosome is not optimum for binding (Kozak, 1986). In this case the next ATG in frame is 318nt downstream, and also shows no match to the consensus sequence, making this possibility unlikely. A suggestion made by Kozak is that the start of a coding sequence with an ATG codon with low ribosome binding affinity may be to limit the synthesis of a protein which is potentially harmful to the cell in high dosage.

The open reading frame described here terminates in a TAA codon 1815 nucleotides downstream of the proposed initiation codon (Fig. 7.3d). The position of this open reading frame is within the *wis1* functional region defined by subcloning and transposon mutagenesis (see Section 5.1.1).

7.4.2: Introns

Many genes in *S.pombe* have been shown to contain introns (reviewed Russell, 1989). No sequences matching that of the 5' consensus for *S.pombe* introns ($^{G}/_{T}$GTANG$^{T}/_{A}$) were found within the *wis1* open reading frame, suggesting that no introns are present.

7.4.3: Codon usage

In *S.pombe*, as in *S.cerevisiae*, genes which are highly expressed use only a subset of the degenerate codons available. Using information about the codon usage in such highly expressed genes, it is possible to calculate a codon bias index (CBI) as a measure of the codon usage bias for a gene under investigation (Bennetzen and Hall, 1982; Russell and Hall, 1983). It may be possible to make a prediction concerning the level of expression of a gene from its CBI value, as CBI has been shown to correlate with transcript levels in several *S.pombe* genes examined (Russell, 1989).

Table 7.2 shows the CBI calculated for various *S.pombe* genes, including *wis1*. The CBI is calculated by the equation CBI=(P-R)/(T-R), where P is the number of times preferred codons are used, R the expected number of times the preferred codons

Table 7.2 : Codon bias index of the predicted wis1 open reading frame.

Gene	CBI value
adh	0.88
tpi	0.82
cyc	0.51
wee1	0.19
cdc25	0.16
cdc2	0.00
wis1	0.12

CBI was calculated as described in Section 7.4.3. Figures for CBI of *S.pombe* genes apart from *wis1* taken from Russell (1989)

would be used if codon usage were random, and T the total number of codons in the gene, not including Met, Asp and Trp codons. The preferred codons used to calculate this value were from a compilation of the *S.pombe*-genes *adh* (alcohol dehydrogenase) and *tpi* (triose phosphate isomerase) which are highly expressed (Russell, 1989). The CBI value for *wis1* is relatively low, similar to that of *wee1* and *cdc25*, which predicts a low level of expression.

7.4.4: Direction of transcription

The direction of transcription of the proposed open reading frame agrees with that predicted by Northern blot analysis (Section 6.1.2).

7.5: UPSTREAM ELEMENTS

The nature of RNA polymerase II promoters in *S.pombe* is not clear. The evidence available indicates that upstream regions contain upstream activator sites (UAS) which may include enhancer-like elements and regulatory regions, and TATA boxes, which specify the site of the initiation of transcription. Very little evidence is currently available concerning the nature of upstream regulatory elements in *S.pombe*. Deletions up to 1kb upstream of the transcriptional start site may impair promoter function (Russell, 1989). More specifically, sequences involved in the regulation of transcription of the mat1-Pi gene have been identified immediately upstream of the putative TATA box. This sequence is necessary and sufficient for the stimulation of transcription by nitrogen starvation signals of heterologous genes. This upstream activating sequence contains direct repeats of a nonamer 5'-CTTTGTTCC-3', which is also present in other mating type genes whose transcription is induced by nitrogen starvation (Aono and Shimoda, 1990). The nine histone genes of S.pombe contain a 17 nucleotide consensus sequence located upstream of the TATA box in each case, which is a likely candidate for the regulatory region involved in controlling the periodic transcription of these genes (Matsumoto and Yanagida, 1985)

The *S.pombe* TATA box, which has a consensus sequence TATAAA, is generally situated up to 250 nucleotides upstream of the initiation of translation. In those genes where the transcriptional start has been determined, this is generally 35 to 45

nucleotides downstream of the TATA box (Russell, 1989). There are several sequences with homology to a TATA motif within 250 nucleotides upstream of the proposed *wis1* translational start. At position -254 relative to the initiation codon, there is a sequence with one A/T mismatch to the TATAAA consensus, and at positions -71 and -148 there are sequences with two A/T mismatches (Fig. 7.3a). These observations raise the possibility of multiple sites for transcriptional initiation, though further experiments would be necessary to determine if this were the case.

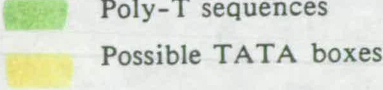
Struhl (1985) has shown that poly-A/poly-T homopolymer sequences are present in the upstream activating sequences in some *S.cerevisiae* genes. These act to stimulate transcription and are required for efficient expression. Similar homopolymer sequences have been observed in the upstream sequences of many *S.pombe* genes, and several such sequences are present in the *wis1* upstream region, notably $(T)_7$ at position -303, $(T)_8$ at position -402, and $(T)_9$ at positions -196 and -243. The striking sequence $(A)_2(T)_9C(T)_9(A)_2$ is present between -46 and -24 nucleotides upstream of the initiation codon (Fig. 7.3a). Its position downstream of identified TATA sequences suggests that it is unlikely to be a promoter element, but it may nevertheless play a role in transcriptional regulation.

7.6: DOWNSTREAM ELEMENTS

Most higher eukaryotic mRNAs have a polyadenylated 3' terminus that occurs approximately 20 nucleotides downstream from a sequence related to 5'-AATAAA-5'. Transcription may proceed beyond the polyadenylation site, with endonucleolytic cleavage and poly-A addition generating the mature 3' end (Birnstiel *et al*, 1985). Although nearly all higher eukaryotic genes contain the AATAAA signal downstream to the termination of translation, many genes in *S.cerevisiae* and *S.pombe* lack this sequence altogether. In *S.cerevisiae*, the consensus sequence 5'-TAG....TATGT....TTT-3' has been proposed as a terminator which signals transcriptional termination a short distance downstream (Zaret and Sherman, 1982). This motif is positioned up to 160 nucleotides downstream of the termination codon. There is no sequence corresponding to the higher eukaryotic AATAAA polyadenylation signal in the *wis1* downstream sequences. It is possible, however, to distinguish the motif TATGT....TTT similar to that described as a termination signal in *S.cerevisiae* (Fig. 7.3d).

Figure 7.3a: Upstream sequences and the -NH_4 terminus of the wisl product.

```
        GATTAACCACACTTTACTTTTTTTTCTTTAGTTCTCCTTGGGAAATTTATTATCTAAGCC
   -419 ---------+---------+---------+---------+---------+---------+  -360
        CTAATTGGTGTGAAATGAAAAAAAAGAAATCAAGAGGAACCCTTTAAATAATAGATTCGG

        CCTGTTTCTTCCAGTTTCTGGCTTTTGCTGTTAAATTTAAACCCTTCCAAACCTCCTTTT
   -359 ---------+---------+---------+---------+---------+---------+  -300
        GGACAAAGAAGGTCAAAGACCGAAAACGACAATTTAAATTTGGGAAGGTTTGGAGGAAAA

        TTTCCGTGGCATTTACCTACACAAAGCTACTCGTAGGTGATTGCCTTTAAACTTCCTTTT
   -299 ---------+---------+---------+---------+---------+---------+  -240
        AAAGGCACCGTAAATGGATGTGTTTCGATGAGCATCCACTAACGGAAATTTGAAGGAAAA

        TTTTTCTTGGAATATCCTTCCTGCGGACTTTTTAGACCACCGCTTTTTTTTTCCTTCTTC
   -239 ---------+---------+---------+---------+---------+---------+  -180
        AAAAAGAACCTTATAGGAAGGACGCCTGAAAAATCTGGTGGCGAAAAAAAAAGGAAGAAG

        GTCGGAGACGACTCGTAATTGATTGTCTAATTTTAATTGTTCTTTTCACCCAAGATACCT
   -179 ---------+---------+---------+---------+---------+---------+  -120
        CAGCCTCTGCTGAGCATTAACTAACAGATTAAAATTAACAAGAAAAGTGGGTTCTATGGA

        TTTTTGTATTGCCATCTCACTTTCGTTCATCTTCACTTTTGCTTCATTTATATTACCGGA
   -119 ---------+---------+---------+---------+---------+---------+  -60
        AAAAACATAACGGTAGAGTGAAAGCAAGTAGAAGTGAAAACGAAGTAAATATAATGGCCT

        ATTTAGTTTACCTAATTTTTTTTTCTTTTTTTTTAAGTTTGTGAAGCACATTTATTTTAT
    -59 ---------+---------+---------+---------+---------+---------+  0
        TAAATCAAATGGATTAAAAAAAAAGAAAAAAAAATTCAAACACTTCGTGTAAATAAAATA

        ATGTCTTCTCCAAATAATCAACCCTTGTCTTGCTCATTGAGACAGCTGTCTATTTCTCCT
      1 ---------+---------+---------+---------+---------+---------+  60
        TACAGAAGAGGTTTATTAGTTGGGAACAGAACGAGTAACTCTGTCGACAGATAAAGAGGA
        M  S  S  P  N  N  Q  P  L  S  C  S  L  R  Q  L  S  I  S  P

        ACCGCACCTCCCGGTGATGTTGGTACTCCCGGCTCGCTCCTTTCTCTTTCGTCTTCAAGT
     61 ---------+---------+---------+---------+---------+---------+  120
        TGGCGTGGAGGGCCACTACAACCATGAGGGCCGAGCGAGGAAAGAGAAAGCAGAAGTTCA
        T  A  P  P  G  D  V  G  T  P  G  S  L  L  S  L  S  S  S  S

        TCTTCAAACACCGATTCTTCTGGTTCTTCCTTGGGTTCCTTGTCTTTAAATTCTAACAGT
    121 ---------+---------+---------+---------+---------+---------+  180
        AGAAGTTTGTGGCTAAGAAGACCAAGAAGGAACCCAAGGAACAGAAATTTAAGATTGTCA
        S  S  N  T  D  S  S  G  S  S  L  G  S  L  S  L  N  S  N  S
```



Poly-T sequences

Possible TATA boxes

Figure 7.3b: <u>wis1</u> DNA sequence and predicted gene product

```
     AGTGGCAGTGACAATGACTCAAAGGTTTCTTCTCCTAGTCGTGAAATACCTTCCGATCCC
181  ---------+---------+---------+---------+---------+---------+  240
     TCACCGTCACTGTTACTGAGTTTCCAAAGAAGAGGATCAGCACTTTATGGAAGGCTAGGG
     S  G  S  D  N  D  S  K  V  S  S  P  S  R  E  I  P  S  D  P

     CCTCTTCCCCGTGCCGTGCCTACGGTCAGACTTGGCAGATcTACGTCCAGTCGGAGTCGT
241  ---------+---------+---------+---------+---------+---------+  300
     GGAGAAGGGGCACGGCACGGATGCCAGTCTGAACCGTCTAgATGCAGGTCAGCCTCAGCA
     P  L  P  R  A  V  P  T  V  R  L  G  R  S  T  S  S  R  S  R

     AACTCTCTTAACCTTGACATGAAGGATCCTTCGGAAAAACCTAGACGTTCACTTCCTACA
301  ---------+---------+---------+---------+---------+---------+  360
     TTGAGAGAATTGGAACTGTACTTCCTAGGAAGCCTTTTTGGATCTGCAAGTGAAGGATGT
     N  S  L  N  L  D  M  K  D  P  S  E  K  P  R  R  S  L  P  T

     GCAGCTGGTCAGAACAATATTGGATCTCCTCCTACTCCACCGGGCCCATTTCCTGGAGGA
361  ---------+---------+---------+---------+---------+---------+  420
     CGTCGACCAGTCTTGTTATAACCTAGAGGAGGATGAGGTGGCCCGGGTAAAGGACCTCCT
     A  A  G  Q  N  N  I  G  S  P  P  T  P  P  G  P  F  P  G  G

     CTTTCAACTGATATACAGGAGAAATTGAAGGCCTTCCATGCATCTAGATCAAAATCAATG
421  ---------+---------+---------+---------+---------+---------+  480
     GAAAGTTGACTATATGTCCTCTTTAACTTCCGGAAGGTACGTAGATCTAGTTTTAGTTAC
     L  S  T  D  I  Q  E  K  L  K  A  F  H  A  S  R  S  K  S  M

     CCGGAAGTAGTCAACAAGATCAGTAGTCCAACTACCCCTATTGTCGGTATGGGTCAACGA
481  ---------+---------+---------+---------+---------+---------+  540
     GGCCTTCATCAGTTGTTCTAGTCATCAGGTTGATGGGGATAACAGCCATACCCAGTTGCT
     P  E  V  V  N  K  I  S  S  P  T  T  P  I  V  G  M  G  Q  R

     GGAAGTTATCCTTTGCCTAACTCTCAACTTGCTGGTCGATTAAGTAATTCGCCCGTAAAG
541  ---------+---------+---------+---------+---------+---------+  600
     CCTTCAATAGGAAACGGATTGAGAGTTGAACGACCAGCTAATTCATTAAGCGGGCATTTC
     G  S  Y  P  L  P  N  S  Q  L  A  G  R  L  S  N  S  P  V  K

     TCTCCGAATATGCCAGAGTCCGGGCTTGCAAAATCACTTGCTGCTGCTAGGAATCCTTTA
601  ---------+---------+---------+---------+---------+---------+  660
     AGAGGCTTATACGGTCTCAGGCCCGAACGTTTTAGTGAACGACGACGATCCTTAGGAAAT
     S  P  N  M  P  E  S  G  L  A  K  S  L  A  A  A  R  N  P  L

     CTCAACCGTCCAACGTCCTTCAATCGACAAACGAGAATCCGTCGTGCACCACCTGGAAAA
661  ---------+---------+---------+---------+---------+---------+  720
     GAGTTGGCAGGTTGCAGGAAGTTAGCTGTTTGCTCTTAGGCAGCACGTGGTGGACCTTTT
     L  N  R  P  T  S  F  N  R  Q  T  R  I  R  R  A  P  P  G  K

     CTCGATTTATCCAATTCCAATCCCACCAGCCCTGTCAGTCCGTCTAGCATGGCTTCTCGC
721  ---------+---------+---------+---------+---------+---------+  780
     GAGCTAAATAGGTTAAGGTTAGGGTGGTCGGGACAGTCAGGCAGATCGTACCGAAGAGCG
     L  D  L  S  N  S  N  P  T  S  P  V  S  P  S  S  M  A  S  R

     CGTGGCCTAAACATTCCTCCCACCCTTAAACAGGCTGTTTCGGAAACCCCTTTTTCCACA
781  ---------+---------+---------+---------+---------+---------+  840
     GCACCGGATTTGTAAGGAGGGTGGGAATTTGTCCGACAAAGCCTTTGGGGAAAAAGGTGT
     R  G  L  N  I  P  P  T  L  K  Q  A  V  S  E  T  P  F  S  T

     TTTTCGGATATTTTGGATGCAAAATCAGGCACCTTAAATTTTAAAAACAAAGCCGTGTTA
841  ---------+---------+---------+---------+---------+---------+  900
     AAAAGCCTATAAAACCTACGTTTTAGTCCGTGGAATTTAAAATTTTTGTTTCGGCACAAT
     F  S  D  I  L  D  A  K  S  G  T  L  N  F  K  N  K  A  V  L

     AATTCAGAAGGTGTTAACTTTTCATcTGGCTCTTCGTTTCGTATTAATATGTCAGAGATT
901  ---------+---------+---------+---------+---------+---------+  960
     TTAAGTCTTCCACAATTGAAAAGTAgACCGAGAAGCAAAGCATAATTATACAGTCTCTAA
     N  S  E  G  V  N  F  S  S  G  S  S  F  R  I  N  M  S  E  I
```

Figure 7.3c: wis1 DNA sequence and predicted gene product

```
        ATTAAGCTTGAAGAACTTGGAAAAGGTAACTATGGTGTTGTGTATAAAGCATTGCATCAA
 961    ---------+---------+---------+---------+---------+---------+   1020
        TAATTCGAACTTCTTGAACCTTTTCCATTGATACCACAACACATATTTCGTAACGTAGTT
        I  K  L  E  E  L  G  K  G  N  Y  G  V  V  Y  K  A  L  H  Q

        CCGACTGGTGTCACTATGGCCTTGAAGGAAATTAGGTTGTCCTTAGAAGAAGCAACATTT
1021    ---------+---------+---------+---------+---------+---------+   1080
        GGCTGACCACAGTGATACCGGAACTTCCTTTAATCCAACAGGAATCTTCTTCGTTGTAAA
        P  T  G  V  T  M  A  L  K  E  I  R  L  S  L  E  E  A  T  F

        AATCAAATTATAATGGAATTGGATATTTTACATAAAGCAGTTAGTCCTTATATCGTTGAC
1081    ---------+---------+---------+---------+---------+---------+   1140
        TTAGTTTAATATTACCTTAACCTATAAAATGTATTTCGTCAATCAGGAATATAGCAACTG
        N  Q  I  I  M  E  L  D  I  L  H  K  A  V  S  P  Y  I  V  D

        TTTTATGGTGCCTTTTTTGTGGAAGGTTCTGTTTTTATTTGTATGGAATATATGGATGCT
1141    ---------+---------+---------+---------+---------+---------+   1200
        AAAATACCACGGAAAAAACACCTTCCAAGACAAAAATAAACATACCTTATATACCTACGA
        F  Y  G  A  F  F  V  E  G  S  V  F  I  C  M  E  Y  M  D  A

        GGTAGCATGGACAAACTGTATGCTGGTGGTATCAAAGACGAAGGAGTTTTAGCTAGAACT
1201    ---------+---------+---------+---------+---------+---------+   1260
        CCATCGTACCTGTTTGACATACGACCACCATAGTTTCTGCTTCCTCAAAATCGATCTTGA
        G  S  M  D  K  L  Y  A  G  G  I  K  D  E  G  V  L  A  R  T

        GCTTATGCTGTAGTGCAAGGCCTCAAAACTTTGAAAGAGGAGCATAATATCATTCATCGT
1261    ---------+---------+---------+---------+---------+---------+   1320
        CGAATACGACATCACGTTCCGGAGTTTTGAAACTTTCTCCTCGTATTATAGTAAGTAGCA
        A  Y  A  V  V  Q  G  L  K  T  L  K  E  E  H  N  I  I  H  R

        GACGTTAAACCTACTAATGTTTTGGTAAATTCTAATGGCCAGGTTAAGTTATGTGACTTT
1321    ---------+---------+---------+---------+---------+---------+   1380
        CTGCAATTTGGATGATTACAAAACCATTTAAGATTACCGGTCCAATTCAATACACTGAAA
        D  V  K  P  T  N  V  L  V  N  S  N  G  Q  V  K  L  C  D  F

        GGCGTGAGTGGGAATCTTGTGGCTTCTATATCCAAAACGAACATTGGATGTCAATCTTAC
1381    ---------+---------+---------+---------+---------+---------+   1440
        CCGCACTCACCCTTAGAACACCGAAGATATAGGTTTTGCTTGTAACCTACAGTTAGAATG
        G  V  S  G  N  L  V  A  S  I  S  K  T  N  I  G  C  Q  S  Y

        ATGGCTCCTGAAAGAATTCGTGTTGGTGGACCTACCAATGGCGTCTTGACTTACACCGTA
1441    ---------+---------+---------+---------+---------+---------+   1500
        TACCGAGGACTTTCTTAAGCACAACCACCTGGATGGTTACCGCAGAACTGAATGTGGCAT
        M  A  P  E  R  I  R  V  G  G  P  T  N  G  V  L  T  Y  T  V

        CAGGCTGATGTGTGGTCTCTAGGCCTTACCATTTTAGAAATGGCTTTAGGAGCTTATCCG
1501    ---------+---------+---------+---------+---------+---------+   1560
        GTCCGACTACACACCAGAGATCCGGAATGGTAAAATCTTTACCGAAATCCTCGAATAGGC
        Q  A  D  V  W  S  L  G  L  T  I  L  E  M  A  L  G  A  Y  P

        TATCCACCTGAATCATATACTTCAATATTTGCACAACTATCGGCGATTTGCGATGGCGAT
1561    ---------+---------+---------+---------+---------+---------+   1620
        ATAGGTGGACTTAGTATATGAAGTTATAAACGTGTTGATAGCCGCTAAACGCTACCGCTA
        Y  P  P  E  S  Y  T  S  I  F  A  Q  L  S  A  I  C  D  G  D

        CCACCTTCTCTCCCCGATTCATTTTCTCCCGAAGCTCGTGATTTTGTAAACAAGTGTTTG
1621    ---------+---------+---------+---------+---------+---------+   1680
        GGTGGAAGAGAGGGGCTAAGTAAAAGAGGGCTTCGAGCACTAAAACATTTGTTCACAAAC
        P  P  S  L  P  D  S  F  S  P  E  A  R  D  F  V  N  K  C  L
```

Figure 7.3d: Downstream sequences and -COOH terminus of the predicted wis1 gene product

```
      AATAAAAACCCGTCTTTGCGTCCCGATTATCATGAGTTGGCTAACCATCCATGGTTGTTA
1681  ---------+---------+---------+---------+---------+---------+  1740
      TTATTTTTGGGCAGAAACGCAGGGCTAATAGTACTCAACCGATTGGTAGGTACCAACAAT
      N  K  N  P  S  L  R  P  D  Y  H  E  L  A  N  H  P  W  L  L

      AAATATCAAAATGCAGATGTGGACATGGCTTCATGGGCAAAAGGCGCTCTTAAAGAGAAA
1741  ---------+---------+---------+---------+---------+---------+  1800
      TTTATAGTTTTACGTCTACACCTGTACCGAAGTACCCGTTTTCCGCGAGAATTTCTCTTT
      K  Y  Q  N  A  D  V  D  M  A  S  W  A  K  G  A  L  K  E  K

      GGTGAAAAAAGAAGCTAAAGGTTCGCCTGCTTTCTAATTGCCTGCTCTGTTTTAAAGTAC
1801  ---------+---------+---------+---------+---------+---------+  1860
      CCACTTTTTTCTTCGATTTCCAAGCGGACGAAAGATTAACGGACGAGACAAAATTTCATG
      G  E  K  R  S  *

      CCATGCGCATTGGTGTTTGTCTTTAATTTCGAATGCATGACTATTACGTGATCCATAATT
1861  ---------+---------+---------+---------+---------+---------+  1920
      GGTACGCGTAACCACAAACAGAAATTAAAGCTTACGTACTGATAATGCACTAGGTATTAA

      ATGTTTCAGCAGAACCGACGCTATTTTGCATTTGTGCTTTTTCATAAATTTAATAATTTG
1921  ---------+---------+---------+---------+---------+---------+  1980
      TACAAAGTCGTCTTGGCTGCGATAAAACGTAAACACGAAAAAGTATTTAAATTATTAAAC

      GGTATGATTCCGTATAACGGTAGTtGATGTTTGCATTTTTGCTTTAAATTAAAACGGGTA
1981  ---------+---------+---------+---------+---------+---------+  2040
      CCATACTAAGGCATATTGCCATCAaCTACAAACGTAAAAACGAAATTTAATTTTGCCCAT

      TTTAATGTGTTATTACATTTGTTTAAGGCATTTACGTCCACCATAAAAAACTTTTTTTTT
2041  ---------+---------+---------+---------+---------+---------+  2100
      AAATTACACAATAATGTAAACAAATTCCGTAAATGCAGGTGGTATTTTTTGAAAAAAAAA

      ATTTAACTAAGGGGCTTTAGAAGTGCAAACGAAAGTTTGCAATTGTAAAAGTTACTTGTA
2101  ---------+---------+---------+---------+---------+---------+  2160
      TAAATTGATTCCCCGAAATCTTCACGTTTGCTTTCAAACGTTAACATTTTCAATGAACAT

      CTGTATTAATTTATTTCTTTTAAAACTTCGTTGACTGGCTCCGTCGTTTTAGCTACTATG
2161  ---------+---------+---------+---------+---------+---------+  2220
      GACATAATTAAATAAAGAAAATTTTGAAGCAACTGACCGAGGCAGCAAAATCGATGATAC

      ATCTCCTGTTTCCTACAATGGTGATTACTTAGAGAGATTGAATCCATCGTAAAATGCAAG
2221  ---------+---------+---------+---------+---------+---------+  2280
      TAGAGGACAAAGGATGTTACCACTAATGAATCTCTCTAACTTAGGTAGCATTTTACGTTC

      TGTTAATAGATATTATATCGACCTAATAATAATTACTTAAACTATTAATATATTACTCAA
      ACAATTATCTATAATATAGCTGGATTATTATTAATGAATTTGATAATTATATAATGAGTT

      AGAGATTTGAGGTATTCATTTATTTAAAAAGTACGAGACTTTTATATATCAAAGAAACAA
2341  ---------+---------+---------+---------+---------+---------+  2400
      TCTCTAAACTCCATAAGTAAATAAATTTTTCATGCTCTGAAAATATATAGTTTCTTTGTT

      ATTCTCCCAACATAAGTCCAACAAGGTAA
2401  ---------+---------+---------  2429
      TAAGAGGGTTGTATTCAGGTTGTTCCATT
```

(highlighted) Putative 3' processing sequences

Of higher eukaryotic mRNAs, many show an over-representation of the trinucleotide TGT, found sometimes repeated and in conjunction with poly-T stretches, in region known as a G/T cluster. This is found generally 30 nucleotides downstream of the AATAAA motif, and less than 20 nucleotides downstream of the polyadenylation site, and may be involved in mRNA processing (Birnstiel *et al*, 1985). *wis1* downstream sequences contain a motif similar to a G/T cluster situated approximately 50 nucleotides downstream of the termination codon, which consists of the sequences 3'-TTGGTGTTTGT-3' (Fig. 7.3d). One possibility for the processing of the 3' terminus of the *wis1* mRNA is termination of transcription downstream of the TATGT sequence, followed by endonucleolytic cleavage and polyadenylation at a site upstream of the proposed G/T cluster. Very little is known about mRNA terminal processing in *S.pombe*, and further experiments would be required to determine the *wis1* polyadenylation site.

7.7: THE wis1 GENE PRODUCT

The *wis1* ORF described above predicts a protein of 605 amino acids with a relative molecular mass of approximately 60kD which appears to be relatively hydrophilic (Figs. 7.3a, b, c, d). The amino acid composition of the *wis1* gene product is shown in Table 7.3, along with that of an "average" protein. The most notable features of this comparison are the high levels of serine and proline, and the relatively low levels of histidine, tryptophan, and glutamine in the predicted *wis1* product.

7.8: HOMOLOGIES BETWEEN wis1 AND PREVIOUSLY IDENTIFIED GENES

7.8.1: Results

The homology search algorithm FASTA (Lipman and Pearson, 1985) was used to search both the NBRF and EMBL protein sequences data bases for proteins showing similarity to the predicted *wis1* gene product. The results of these data base searches strongly indicate that the *wis1* product is related to the protein kinase family of polypeptides, as the 50 highest scoring matches were all kinase, or kinase related, proteins. The 20 best matches from a search of the EMBL data base are shown in

Table 7.3: Amino acid composition of the predicted wis1 gene product.

Amino acid	No. of residues	% composition	
		wis1	Average
A Ala	39	6.4	7.7
C Cys	6	1.0	1.8
D Asp	26	4.3	5.2
E Glu	26	4.3	6.3
F Phe	17	2.8	4.0
G Gly	45	7.4	7.2
H His	7	1.2	2.3
I Ile	27	4.5	5.4
K Lys	33	5.5	5.9
L Leu	58	9.6	9.1
M Met	15	2.5	2.3
N Asn	39	6.5	4.4
P Pro	54	8.9	5.1
Q Gln	16	2.6	4.1
R Arg	29	4.8	5.2
S Ser	87	14.4	7.1
T Thr	29	4.8	5.8
V Val	34	5.6	6.5
W Trp	3	0.5	1.3
Y Tyr	15	2.5	3.2
Total	605		

The figures for the amino acid composition of an average protein were obtained by taking the average composition of 15409 entries in the EMBL protein data base.

Table 7.4: Proteins with homology to the predicted wis1 gene product identified from data base searches.

	Gene	Organism
1	*PBS2* Polymyxin B resistance protein	*S.cerevisiae*
2	*byr1* Protein kinase	*S.pombe*
3	*STE7* Regulatory protein	*S.cerevisiae*
4	ninA long protein	*Drosophila*
5	*cdc2* homologue	Human
6	*cdc2* homologue	Mouse
7	*cdc2* homologue	Chick
8	*SCH9* cAMP dependent protein kinase homologue	*S.cerevisiae*
9	Protein kinase C	*Drosophila*
10	*CDC28*	*S.cerevisiae*
11	NimA G2 specific protein kinase	*Aspergillus*
12	*cdc2*	*S.pombe*
13	*KIN28*	*S.cerevisiae*
14	LSK proto-oncogene tyrosine kinase	Mouse
15	*TPK2* cAMP dependent protein kinase	*S.cerevisiae*
16	*TPK1* cAMP dependent protein kinase	*S.cerevisiae*
17	*YPK1* protein kinase	*S.cerevisiae*
18	*TPK3* cAMP dependent protein kinase	*S:cerevisiae*
19	Protein kinase C (type epsilon)	Mouse
20	c-tkl tyrosine kinase	Chick

Matches 4 - 20 show an identity of approximately 25% to the predicted *wis1* gene product in an overlap of approximately 200 to 270 amino acids.

PBS2, *byr1* and *STE7* showed a higher homology (see Figs. 7.5, 7.6, 7.7 and text).

Table 7.4, and the optimum alignment between the predicted *wis1* gene product and the three best matches are shown in Figures 7.5, 7.6 and 7.7.

In Figure 7.4, the *wis1* amino acid sequence is compared to the predicted amino acid sequences of the *PBS2* and *STE7* genes from *S.cerevisiae*, and the *byr1* gene from *S.pombe*, which show the closest homologies to the predicted *wis1* product. All protein kinases are closely related over a stretch of about 260 amino acids which constitutes the kinase catalytic domain. Of particular note are the sequence elements outlined in Figure 7.4 which were identified by Hanks *et al*, (1988) as highly conserved protein kinase domains. The sequence Gly-Xaa-Gly-Xaa-Xaa-Gly is thought to be involved in ribose ring interactions with ATP. A second conserved sequence, Ala-Xaa-Lys, is found another 15-20 amino acids towards the COOH terminus in all protein kinases. There is evidence that the conserved lysine residue is involved in a phosphate group interaction at the catalytic centre. A second group of conserved sequences [His-Arg-Asp-Leu-$(Xaa)_{17}$-Asp-Phe-Gly-$(Xaa)_{20}$-Ala-Pro-Glu-$(Xaa)_{16}$-Asp-Xaa-Trp-Xaa-Gly] thought to be involved in phosphoreceptor activity is also present (Fig. 7.4). The sequences following the His-Arg-Asp-Leu motif indicate the serine/threonine or tyrosine specificity of the kinase. In the case of *wis1*, this sequence is Lys-Pro-Thr-Asn, which is closely related to the Lys-Pro-Glu-Asn consensus for serine/threonine protein kinases.

Protein kinases related to that encoded by the *cdc2* gene in *S.pombe* all contain a tyrosine residue at position 5 within the Gly-Xaa-Gly-Xaa-Xaa-Gly conserved region, and in *cdc2*, phosphorylation of this tyrosine residue has been shown to be important for the regulation of *cdc2* kinase activity (Gould and Nurse, 1989). Both the *wis1* and *PBS2* predicted gene products contain a tyrosine residue at this site, and of other putative protein kinases identified in *S.pombe*, only the *ran1* protein kinase contains a tyrosine residue at this position (McLeod and Beach, 1986). These observations raise the possibility that the activity of the *wis1* gene product may also be regulated by the phosphorylation of the equivalent tyrosine residue.

Nearly all of the protein sequences identified in the data base searches described above share a high degree of homology in a region of approximately 260 amino acids, which represents the protein kinase functional region. The *PBS2* and *STE7* gene products share a strong homology with *wis1* over a more extensive region (Figs. 7.5 and

Figure 7.4: Comparison between the protein sequences of PBS2, STE7, byr1 and wis1.

```
      350                                                   399
Byr1  RPAWISD... ........LD NSSLEVVRHL GEGNGGAV.. SLVKHRNIFM
Ste7  ......DTLS GTSNGNY.IQ LQDLVQLGKI GAGNSGTVVK ALHVPDSKIV
Pbs2  KLSLSSKGID FSNGSSSRIT LDELEFLDEL GHGNYGNVSK VLHKPTNVIM
Wis1  KAVLNSEGVN FSSGSSFRIN MSEIIKLEEL GKGNYGVVYK ALHQPTGVTM
      k..l.sdg.. fssgss..i. lsele.l.el G.GNyG.V.k aLhkptnvim

      400                                                   449
Byr1  ARKTVYVGSD SKLQ.KQILR ELGVLHHCRS PY..IVGFYG AFQ...YKNN
Ste7  AKKTIPVEQN NSTIINQLVR ELSIV.KNVK PHENIITFYG AYYNQHINNE
Pbs2  ATKEVRLELD EAKF.RQILM ELEVLHKCNS PY..IVDFYG AFF...IEGA
Wis1  ALKEIRLSLE EATF.NQIIM ELDILHKAVS PY..IVDFYG AFF...VEGS
      A.K..r.eld eatf.nQil. EL..lhkcvs Py..IvdFYG Aff...ie..

      450                                                   499
Byr1  ISLCMEYMDC GSLDA.I..L ...R.EGG.P ..I....PLD ILGKIINSMV
Ste7  IIILMEYSDC GSLDKILSVY KRFVQRGT.V SSKKTWFNEL TISKIAYGVL
Pbs2  VYMCMEYMDG GSLDK.I..Y D..E..SSEI GGI....DEP QLAFIANAVI
Wis1  VFICMEYMDA GSMDK.L..Y ...A..GG.I ..K....DEG VLARTAYAVV
      ..icMEYmDc GSlDk....y ......gg.i .......de. .lakia.avv

      500                                                   549
Byr1  KGLIYLYNVL HIIHRDLKPS NVVVNSR.GE IKLCDFGVSG ELVNSVAQTF
Ste7  NGLDHLYRQY KIIHRDIKPS NVLINSK.GQ IKLCDFGVSK KLINSIADTF
Pbs2  HGLKELKEQH NIIHRDVKPT NILCSANQGT VKLCDFGVSG NLVASLAKTN
Wis1  QGLKTLKEEH NIIHRDVKPT NVLVNSN.GQ VKLCDFGVSG NLVASISKTN
      .GLk.L.eqh nIIHRDvKP. Nvlvnsn.Gq .KLCDFGVSg nLv.SiakT.

      550                                                   599
Byr1  VGTSTYMSPE RIRGG..... ..KYTVKSDI WSLGISIIEL ATQELPWSFS
Ste7  VGTSTYMSPE RIQ.G...N. V..YSIKGDV WSLGLMIIEL VTGEFPLG..
Pbs2  IGCQSYMAPE RIKSLNPDRA ..TYTVQSDI WSLGLSILEM ALGRYPYP..
Wis1  IGCQSYMAPE RIRVGGPTNG VLTYTVQADV WSLGLTILEM ALGAYPYP..
      .G...YM.PE RIr.g...n. ..tYtv.sD. WSLGlsI.E. a.geyPyp..

      600                                                   649
Byr1  NIDDSIG..I LDLLHCIVQE EPPRLP.SS. FPEDLRLFVD ACLHKDPTLR
Ste7  .GHNDTPDGI LDLLQRIVNE PSPRLPKDRI YSKEMTDFVN RCCIKNERER
Pbs2  .PETYDN..I FSQLSAIVDG PPPRLPSDK. FSSDAQDFVS LCLQKIPERR
Wis1  .PESYTS..I FAQLSAICDG DPPSLP.DS. FSPEARDFVN KCLNKNPSLR
      .pe.yt...I .d.LsaIvd. ppPrLP.ds. fs..ardFVn .Cl.Knp.lR

      650                                                   699
Byr1  ASPQQLCAMP YFQQALMINV D.LAS.WA.S NFRSS
Ste7  SSIHELLHHD LIMKYVSPSK DDKFRHWCRK IKSKIKEDKR IKREALDRAK
Pbs2  PTYAALTEHP WLVKYRNQDV H.MSE.YI.T ERLERRNKIL RERGENGLSK
Wis1  PDYHELANHP WLLKYQNADV D.MAS.WA.K GALKEKGEKR S
      psyheL..hp wl.ky.n.dv d.mas.wa.k ..lk.k..kr ..R......K
```

The protein sequences were aligned with the "Bestfit" program from the UWGCG package. The lowest line indicates a consensus sequence where capital letters indicate identity between all sequences.

[highlight] Highly conserved protein kinase domains.

7.6), possibly suggesting similar functions for these proteins. The *PBS2* and *STE7* gene products have previously been assigned to a particular subfamily of protein kinases, though it is not clear if *byr1* also belongs to this subfamily as its sequence was not available at the time of the analysis (Hanks *et al*, 1988). A further data base homology search was undertaken with the 311 amino acid amino-terminal region of the predicted *wis1* gene product. This was done to determine if the *wis1* product showed homologies to previously identified proteins in a region outside that containing highly conserved protein kinase domains. Monomeric protein kinases commonly include discrete regulatory domains, which may contain pseudosubstrate sequences involved in negative regulation (Hunter, 1987). However, the only significant homology detected in this search was between *wis1* and the equivalent region in the *PBS2* gene product.

7.8.2: Homology between wis1 and PBS2

The gene product showing the highest homology to the predicted *wis1* sequence is that of the *S.cerevisiae* gene *PBS2*, with a 49% identity (82% homology) in a 460 amino acid overlap, and 45% identity (58% similarity) overall (Fig. 7.5). The *PBS2* gene was identified by it ability to confer resistance to the antibiotic polymyxin B when present in multicopy (Boguslawski and Polazzi, 1987). Analysis of the regions of *PBS2* and *wis1* not showing significant homology revealed several highly hydrophilic regions, with a high serine content. Boguslawski and Polazzi (1987) describe a hydrophobic pocket containing proline and leucine residues starting at position 94 in the *PBS2* gene product. Examination of the amino acid sequence of the *wis1* gene product shows that the equivalent protein regions also show strongly hydrophilic areas, with a similar hydrophobic pocket consisting of the sequence PPLPRAVP situated between two hydrophilic regions particularly high in serine residues (Fig. 7.5). The significance of these observations is not clear, but they are a further pointer to a possible similarity in function between *PBS2* and *wis1*. One striking difference between the two proteins is that the *PBS2* polypeptide terminates in a short, strongly hydrophobic string of amino acids, reminiscent of the *S.cerevisiae* *RAS* proteins, while no equivalent hydrophobic region is present in the *wis1* gene product.

Figure 7.5: Comparison between the PBS2 and wis1 gene products.

(*wis1* sequence on top line.)

```
                 .         .         .         .         .
  9 LSCSLRQLSISPTAPPGDV.......GTPGSLLSLSSSSSSNTDSSGSSL 51
    :. .: .||: .....:.:       :. || :. .||.||: :. ...|
  1 MEDKFANLSLHEKTGKSSIQLNEQTGSDNGSAVKRTSSTSSHYNNINADL 50
                 .         .         .         .         .
 52 G.............SLSLNSNSSGSDNDSKVSSPSREIPSDPPLPRAVP 87
                  | |:.|| |: |.:|. |..  :    :.|||.
 51 HARVKAFQEQRALKRSASVGSNQSEQDKGSSQSPKHIQQIVNKPLPP... 97
                 .         .         .         .         .
 88 TVRLGRSTSSRSRNSLNLDMKDPSEKP..RRSLPTAAGQN....NIGSPP 131
       |. ..||: ...:. :: ::|.|.   :. |.....||     ||. ..
 98 ...LPVAGSSKVSQRMSSQVVQASSKSTLKNVLDNQETQNITDVNINIDT 144
                 .         .         .         .         .
132 TPPGPFPGGLSTDIQEKLKAFHASRSKSMPEVVNKISSP.......TTP 173
    |. .: . |:.|::...  .   .|.. | .. .. :..        .|.
145 TKITATTIGVNTGLPATDITPSVSNTASATHKAQLLNPNRRAPRRPLSTQ 194
                 .         .         .         .         .
174 IVGMGQRGSYPLPNSQLAGRLSNSPVKSPNMPESGLAKSLA..AARNPLL 221
     ..... :... |.    ..: | |: :...:|.:|:. .::  .|..|
195 HPTRPNVAPHKAPAIINTPKQSLSARRAVKLPPGGMSLKMPTKTAQQPQQ 244
                 .         .         .         .         .
222 NRPTSFNRQTRIRRAPPGKLDLSNSNPTSPV......SPSSMASRRGLNI 265
      |.. |:.     . .  :: ..|||.| :        |.||  .. .  .:
245 FAPSPSNKKHIETLSNSKVVEGKRSNPGSLINGVQSTSTSSSTEGPHDTV 294
                 .         .         .         .         .
266 PPTLKQAVSETP...........FSTFSDILDAKSGTLNFKNKAVLNSEG 304
    ..| : : |:..            |..||..:| |||.||| .|  |.|.|
295 GTTPRTGNSNNSSNSGSSGGGGLFANFSKYVDIKSGSLNFAGKLSLSSKG 344
                 .         .         .         .         .
305 VNFSSGSSFRINMSEIIKLEELGKGNYGVVYKALHQPTGVTMALKEIRLS 354
    ::||.||| ||.:.|:  |:|||.|||| | |.||.||.|.|| ||:||.
345 IDFSNGSSSRITLDELEFLDELGHGNYGNVSKVLHKPTNVIMATKEVRLE 394
                 .         .         .         .         .
355 LEEATFNQIIMELDILHKAVSPYIVDFYGAFFVEGSVFICMEYMDAGSMD 404
    |:||.|.||:|||::|||. ||||||||||||:||.|::||||||:||:|
395 LDEAKFRQILMELEVLHKCNSPYIVDFYGAFFIEGAVYMCMEYMDGGSLD 444
                 .         .         .         .         .
405 KLYAG....GIKDEGVLARTAYAVVQGLKTLKEEHNIIHRDVKPTNVLVN 450
    |:|.:    |  ||. || .| ||::|||.|||:||||||||||||:|..
445 KIYDESSEIGGIDEPQLAFIANAVIHGLKELKEQHNIIHRDVKPTNILCS 494
                 .         .         .         .         .
451 SN.GQVKLCDFGVSGNLVASISKTNIGCQSYMAPERIRVGGPTNGVLTYT 499
    .| | ||||||||||||||||:.||||||||||||||||:   .|..:  |||
495 ANQGTVKLCDFGVSGNLVASLAKTNIGCQSYMAPERIKSLNPDRA..TYT 542
                 .         .         .         .         .
500 VQADVWSLGLTILEMALGAYPYPPESYTSIFAQLSAICDGDPPSLP.DSF 548
    ||.|:||||||.||||||| ||||||.|..||.|||||.||.||.|| |.|
543 VQSDIWSLGLSILEMALGRYPYPPETYDNIFSQLSAIVDGPPPRLPSDKF 592
                 .         .         .         .         .
549 SPEARDFVNKCLNKNPSLRPDYHELANHPWLLKYQNADVDMASWAKG... 595
    |.:|.|||. ||.| |. ||.| .|.:||||:||.|.||.|..: .:
593 SSDAQDFVSLCLQKIPERRPTYAALTEHPWLVKYRNQDVHMSEYITERLE 642
                 .
596 ....ALKEKGEK 603
        |:||:||.
643 RRNKILRERGEN 654
```

Figure 7.6: Comparison between the STE7 and wis1 gene products.

(*wis1* sequence on top line.)

```
                   .         .         .         .         .
 90 RLGRSTSSRSRNSLNLDMKDPSEKPRRSLPTAAGQNNIGSPPTPPGPFPG 139
    .:.|.|  | ||  .|::.          |....|.|.   ..|.. . .:
  1 MFQRKTLQR.RNLKGLNLN.........LHPDVGNNGQLQEKTETHQGQS 40
                   .         .         .         .         .
140 GLSTDIQEKLKAFHASRSKSMPEVVNKISSPTTPIVGMGQRGSYPLPNSQ 189
     :...: ..:.|::....  :. .:.|      . .: ::|: .... ||.
 41 RIEGHVMSNINAIQNNSNLFLRRGIKK....KLTLDAFGDDQAISKPNTV 86
                   .         .         .         .         .
190 LAGRLSNSPVKSPNMPESGLAKSLAAARNPLLNRPTSFNRQTRIRRAPPG 239
    : ..    |.||     :. |:|..| ...... |. |. ::       | ..
 87 VIQQPQNEPV....LVLSSLSQSPCVSSSSSLSTPCIID.......AYSN 125
                   .         .         .         .         .
240 KLDLSNSNPTSPVSPSSMASRRGLNIPPTLKQAVSETPFSTFSDILDAKS 289
    .::|| |...|  .||.:.: ..:..|.. .:.:|    :..:.: |.: .
126 NFGLSPSSTNS..TPSTIQGLSNIATPVENEHSIS...LPPLEESLSPAA 170
                   .         .         .         .         .
290 GTLNFKNKAVLNSEGVNFSSGSSFRINMSEIIKLEELGKGNYGVVYKALH 339
    :.|.            :.:. .|.:.: |.: :::.|:.:| || |.| ||||
171 ADLK.........DTLSGTSNGNY.IQLQDLVQLGKIGAGNSGTVVKALH 210
                   .         .         .         .         .
340 QPTGVTMALKEIRLSLEEAT.FNQIIMELDILHKAVSPY..IVDFYGAFF 386
     |.: .:| |.|.:. ::.| :||::.||.|: |.|.|.   |:.||||::
211 VPDSKIVAKKTIPVEQNNSTIINQLVRELSIV.KNVKPHENIITFYGAYY 259
                   .         .         .         .         .
387 ...VEGSVFICMEYMDAGSMDKLYA........GGIK......DEGVLAR 419
       ::..::| ||| |.||:||:..        |.:.        :| .:.:
260 NQHINNEIIILMEYSDCGSLDKILSVYKRFVQRGTVSSKKTWFNELTISK 309
                   .         .         .         .         .
420 TAYAVVQGLKTLKEEHNIIHRDVKPTNVLVNSNGQVKLCDFGVSGNLVAS 469
    .||:|:.||. |   :..|||||:||.|||:||.||:||||||||| .|:.|
310 IAYGVLNGLDHLYRQYKIIHRDIKPSNVLINSKGQIKLCDFGVSKKLINS 359
                   .         .         .         .         .
470 ISKTNIGCQSYMAPERIRVGGPTNGVLTYTVQADVWSLGLTILEMALGAY 519
    |..| :|. .||.||||...         .|.:.:||||||| |:|:. |.:
360 IADTFVGTSTYMSPERIQGN.......VYSIKGDVWSLGLMIIELVTGEF 402
                   .         .         .         .         .
520 PYPPESYT..SIFAQLSAICDGDPPSLPDS..FSPEARDFVNKCLNKNPS 565
    |..... |  :|:. |  |.::..|.||..  :|.|   ||||:|  ||..
403 PLGGHNDTPDGILDLLQRIVNEPSPRLPKDRIYSKEMTDFVNRCCIKNER 452
                   .         .         .         .         .
566 LRPDYHELANHPWLLKY.....................QNADVDMASWAK 594
     |...||| :|.:::||                      ::  :....:.:
453 ERSSIHELLHHDLIMKYVSPSKDDKFRHWCRKIKSKIKEDKRIKREALDR 502
                   .
595 GALKEKGEKRS 605
    : |..|...||
503 AKLEKKQSERS 513
```

Figure 7.7: Comparison between the byr1 and wis1 gene products.
(*wis1* sequence on top line.)

```
             .         .         .         .         .
234 RRAPPGKLDLSNSNPTSPVSPSSMASRRGLNIPPTLKQAVSETPFSTFSD 283
    ||  |  | |   ||...|..|.           . |:.: :.. |  |:
  4 RRRNPKGLVL...NPNASVKSSD..........NDHKEELINNQKSFESN 40
             .         .         .         .         .
284 ILDAKSGTLNFKNKAVLNSEGVNFSSGSSFRINMSEIIKLEELGKGNYGV 333
    : . .... ::..::.: |:           :: |.:  : .||.|| |.
 41 VEAFMEQCAHMNRRPAWISD...........LDNSSLEVVRHLGEGNGGA 79
             .         .         .         .         .
334 VYKALHQPTGVTMALKEIRLSLEEATFNQIIMELDILHKAVSPYIVDFYG 383
    |  .| .. .: || |.: :: :.   .||:.||.:||.. |||||:|||
 80 V..SLVKHRNIFMARKTVYVGSDSKLQKQILRELGVLHHCRSPYIVGFYG 127
             .         .         .         .         .
384 AFFVEGSVFICMEYMDAGSMDKLY.AGGIKDEGVLARTAYAVVQGLKTLK 432
    ||  ...: :||||||.||:| :. .||  . ::|::.  .:|.||  |
128 AFQYKNNISLCMEYMDCGSLDAILREGGPIPLDILGKIINSMVKGLIYLY 177
             .         .         .         .         .
433 EEHNIIHRDVKPTNVLVNSNGQVKLCDFGVSGNLVASISKTNIGCQSYMA 482
    :  :|||||:||.||:|||.|::|||||||||:||.|:..| :|. .||.
178 NVLHIIHRDLKPSNVVVNSRGEIKLCDFGVSGELVNSVAQTFVGTSTYMS 227
             .         .         .         .         .
483 PERIRVGGPTNGVLTYTVQADVWSLGLTILEMALGAYPYP...PESYTSI 529
    |||||.|       .|||..|:||||:.|:|:| ...|:.    :. .:|
228 PERIRGG.......KYTVKSDIWSLGISIIELATQELPWSFSNIDDSIGI 270
             .         .         .         .         .
530 FAQLSAICDGDPPSLPDSFSPEARDFVNKCLNKNPSLRPDYHELANHPWL 579
    :. | .|.:::||.||.||..: | ||: ||:|:|.||:. ::|.. |::
271 LDLLHCIVQEEPPRLPSSFPEDLRLFVDACLHKDPTLRASPQQLCAMPYF 320
             .
580 LKYQNADVDMASWAKG 595
     .    :||:||||..
321 QQALMINVDLASWASN 336
```

The *wis1* gene was tested for its ability to confer polymyxin B resistance on *S.pombe*. Cultures of cells containing either pwis1-1, or pDB248 as a control, were grown to mid-log phase in selective medium, and plated at two different dilutions onto EMM-glut plates containing various concentrations of polymyxin B. Transformants containing either pDB248 or pwis1-1 showed a similar sensitivity to Polymyxin B: cells were resistant to a concentration of 0.2mg/ml, but sensitive to 0.3mg/ml polymyxin B. These observations indicate that *wis1* when present in multicopy was not capable of conferring polymyin B resistance on *S.pombe*.

In mammalian cells, polymyxin B has been shown to be a specific inhibitor of protein kinase C (Nel *et al*, 1985). It seems highly unlikely that *PBS2* and *wis1* are the yeast equivalents of protein kinase C, as they do not show any homology to cloned protein kinase C sequences apart from those in the kinase functional region. In particular, *PBS2* and *wis1* lack the characteristic sequences present in this family of protein kinases necessary for interaction with Ca^{2+}/calmodulin, diacylglycerol and phospholipid which include cysteine-rich sequences similar to the "zinc finger" motifs found in metallo-proteins (Nishizuka, 1988). It is interesting to note, however, that a region of the protein kinase C epsilon subgroup also shows homology with a region of *wis1* outside that conserved between all protein kinases. This is a region highly conserved within the epsilon subgroup, but not within the protein kinase C family as a whole (Nishizuka, 1988).. The role of these sequences is not presently clear, but it seems likely that they play some role in regulatory functions unique to the epsilon subgroup. The significance of the homologies between these sequences and the sequence of *wis1* is not clear.

7.8.3: Homology between wis1 and STE7

The *S.cerevisiae* gene *STE7* encodes a putative protein kinase which appears to be involved in the pathway leading to the transcriptional induction of a-specific and Á-specific genes by mating factors (Teague *et al*, 1986; Herskowitz, 1989). The mating-factor receptors themselves, products of the *STE2* and *STE3* genes communicate with a G protein complex consisting of alpha, beta and gamma subunits. The *STE7* and *STE11* gene products, which are both putative protein kinases, are believed to act downstream of the G protein complex, although their precise roles and substrates are not clear. The product of the *STE12* gene has been

demonstrated to be a phosphoprotein capable of binding a short region of DNA known as the "pheromone response element" which is involved in the induction of transcription by mating factors. An appealing hypothesis is that the activity of the *STE12* gene product is determined by its phosphorylation state, which is in turn determined by the activity of protein kinases involved upstream in this signalling pathway (Herskowitz, 1989).

7.8.4: Homology between wis1 and byr1

The predicted *wis1* and *byr1* gene products show a high degree of homology, although the homologous region in each case is limited to that defined as the protein kinase catalytic region (Fig. 7.7). There is a strong homology, however, in sequences between the highly conserved kinase domains, and this sequence similarity is not specific to *S.pombe* protein kinases. *byr1* was identified as a gene which may mediate the meiosis and sporulation function of *ras1*, and has been shown to allelic to *ste1*: mutant strains are completely defective in conjugation and sporulation (Nadin-Davis and Nasim, 1988; 1990). In *S.pombe*, *ras1* has been shown to be important for sexual differentiation, though *ras* function does not seem to be mediated by changes in cAMP levels, as has been observed in *S.cerevisiae* (Fukui *et al*, 1986a). Loss of *ras1* function leads to sterility, and *ras1* has been shown to be allelic to *ste5* (Nadin-Davis and Nasim, 1990). The *ste* gene family in *S.pombe* are believed to function in a signal transduction pathway co-ordinating the elevated expression of certain mating type specific genes with low levels of extracellular nutrients such as nitrogen (Nadin-Davis and Nasim, 1990).

7.9: DISCUSSION

This chapter describes the determination of the sequence of the *wis1* functional region, and its analysis. The sequence of a section of DNA consisting of 3276bp was determined, which contained a 1815nt open reading frame. The position of this ORF was in agreement with previous experiments defining the *wis1* functional region. It was possible to confirm the positions of previously identified restriction sites from the sequence, with the exception of one *Xba*I site, which is presumed not to have been recognised in previous experiments due to *dam* methylation of plasmid clones. Three sequences with a close agreement to the consensus for ARS activity in

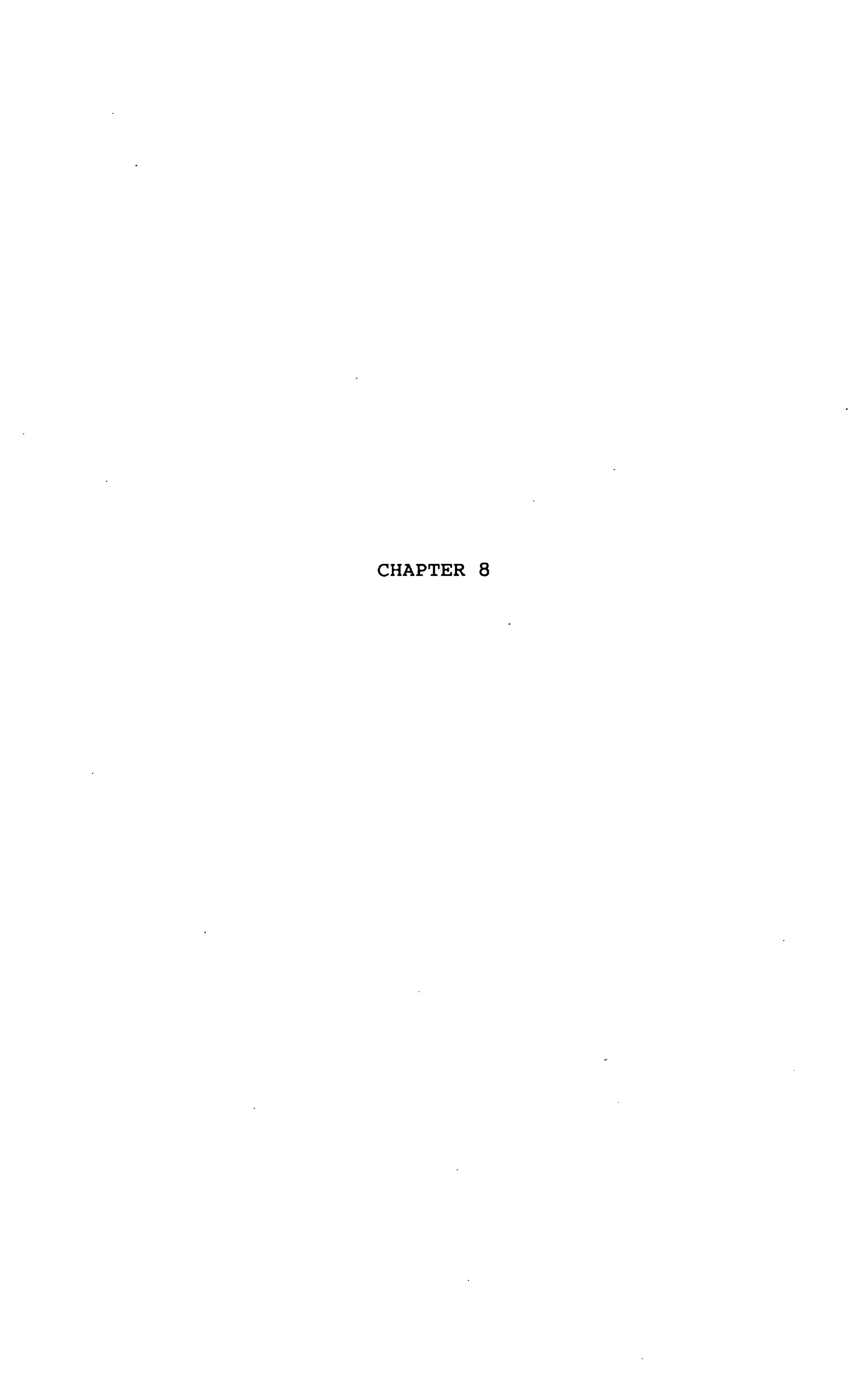
S.pombe were identified in a 280bp region outside the ORF, which correlates with ARS activity shown by plasmids containing this sequence.

The open reading frame identified here contains no consensus sequences for intron splicing in *S.pombe*, and predicts a *wis1* gene product of 605 amino acids. The -COOH terminal half of the predicted *wis1* protein shows a strong homology to serine/threonine protein kinases, and contains all the conserved domains necessary for protein kinase function, described by Hanks *et al* (1988). The -NH_4 terminal half shows no significant homologies to non-kinase proteins.

The predicted wis1 gene product shares the closest homology with the *PBS2* and *STE7* genes in *S.cerevisiae*, and the *byr1* gene in *S.pombe*. The *PBS2* and *STE7* genes have previously been identified as related, and assigned to a subfamily of protein kinases (Hanks *et al* 1988). It is not clear if the *byr1* gene product may be assigned to this family. The strongest homology is between *PBS2* and *wis1*: when present in multicopy, the *PBS2* gene confers on *S.cerevisiae* cells resistance to the antibiotic polymyxin B (Boguslawski and Polazzi, 1987), though *wis1* is not capable of conferring such resistance in *S.pombe*. The biological role of *PBS2* is not clear, though one model is that the *PBS2* kinase is involved in the phosphorylation of a membrane component (possibly encoded by the *PBS1* gene) which is affected by polymyxin B. The effect of polymyxin B upon the phosphorylation of the receptor may be overcome by the overexpression of the *PBS2* protein kinase.

The *STE7* and *byr1* genes also appear to involved in signal transduction pathways. *STE7* is believed to be involved in the pathway leading to the transcriptional induction of mating type specific genes in response mating factor signalling (Teague *et al*, 1986; Herskowitz, 1989), and *byr1* in the pathway co-ordinating the elevated expression of certain mating type specific genes with low levels of extracellular nutrients such as nitrogen (Nadin-Davis and Nasim, 1988; 1990).

Based on the evidence described here it is possible to postulate a role for the *wis1* gene product in a signalling pathway regulated by phosphorylation. A likely function for such a pathway would be the mediation of the effects of extracellular nutrients upon the cell cycle. Such possibilities are discussed in Chapter 8.



CHAPTER 8

CHAPTER 8: DISCUSSION

8.1: OVERVIEW

The study of elements involved in the control of the eukaryotic cell cycle has recently entered a highly productive phase. In the last few years, it has been possible to bring together the powerful genetic techniques available in the study of the yeast cell cycle with biochemical studies undertaken in other organisms. Evidence is mounting that conserved mechanisms for the control of the cell cycle exist in organisms which are highly evolutionarily divergent.

This study concerns the analysis of elements involved in the control over entry into mitosis in the fission yeast *Schizosaccharomyces pombe.* The initial aim of the project was to characterise the role of the *win1* gene in this control system. *win1* was initially defined by a mutant allele which showed a strong interaction with *wee1* and *cdc25* (Ogden and Fantes, 1986), genes which had previously been shown to play an important role in the control over entry into mitosis, probably acting through the *cdc2* protein kinase (Russell and Nurse, 1986; Russell and Nurse, 1987a). The aims of this project included the isolation and characterisation of *win1*, and the investigation of the genetic interactions between *win1* and other cell cycle genes. The strategy for cloning *win1* was dependent upon the isolation of sequences capable of suppressing the cdc phenotype arising from the combination of *win1.1* with *wee1.50* and *cdc25.22* at the restrictive temperature. Following the extensive screening of gene libraries, it proved impossible to isolate *win1* using this approach, although 5 new genes presumed to be involved in the mitotic control were isolated as multicopy suppressors of this phenotype. These were named *wis* (win suppressing) 1 to 5.

The work described here mainly concerns the molecular analysis of the *wis* genes, a more detailed molecular and genetic analysis of *wis1* (including the determination of its DNA sequence), and the study of interactions between *win1.1* and previously identified genes involved in the mitotic control in *S.pombe.*

8.2: SUMMARY OF RESULTS

8.2.1: Genetics and physiology of winl

The most striking characteristic of the *win1.1* mutation is the nutritionally sensitive nature of its interaction with *wee1* and *cdc25*. Cells of the genotype *wee1.50 cdc25.22 win1.1* show a predominantly cdc phenotype on EMM at 35°C, while cells grown on rich medium, such as YE, are much shorter and are capable of growth and division (Ogden and Fantes, 1986). An investigation of the effects of growth medium on *wee1.50 cdc25.22 win1.1* strains suggested that nitrogen source was an important factor, and that media rich in amino acids suppressed the cdc phenotype of *wee1.50 cdc25.22 win1.1* strains. Temperature shift experiments indicated that *wee1.50 cdc25.22 win1.1* cells did not show a first cell cycle arrest upon shift to the restrictive temperature, implying that it is some form of cumulative effect which gives rise to the cdc phenotype shown by such cells when grown on minimal medium at 35°C.

The fact that *win1.1* shows little or no interaction when combined with many other cell cycle mutants had already been demonstrated by Ogden and Fantes, (1986). Work described here shows that *win1.1* interacts with *cdc13*: the terminal phenotype of a *win1.1 cdc13.117* double mutant upon a shift to the restrictive temperature was that of an arrest in G2, in contrast to that of a *cdc13.117* strain, which shows a cdc phenotype, but with many characteristics of mitotic cells (Nasmyth and Nurse, 1981).

wee1 is epistatic to *win1.1* (Ogden and Fantes, 1986), and experiments described here show that over-expression of *cdc25* also results in the suppression of the *win1.1* phenotype of increased cell length at division, implying that *win1.1* cells are still sensitive to levels of *cdc25* expression. The *win1.1* mutation is not capable of suppressing the two forms of mitotic catastrophe phenotype which result from the combination of *wee1.50* with either *cdc2.3w* or with high levels of *cdc25*$^+$ expression. Interestingly, the combination of *win1.1* with two *cdc2w* alleles resulted in different phenotypes: *cdc2.1w* was capable of suppressing the *win1.1* phenotype, though the combination of *cdc2.3w* with *win1.1* resulted in a cell length phenotype more similar to that of *win1.1*.

Six *mcs* genes were identified by Molz *et al* (1989) by mutations which were capable of suppressing the lethal mitotic catastrophe phenotype arising from the combination

of *cdc2.3w* with loss of *wee1* function. Three *mcs* mutations, *mcs3.12*, *mcs4.12* and *mcs6.13* share with *win1.1* the property of reversing the suppression of *cdc25.22* by *wee1.50*. These three *mcs* mutations were shown not to be allelic to *win1.1*, and the phenotypes of the quadruple mutants *mcsX wee1.50 cdc25.22 win1.1* examined. The results of these experiments are difficult to interpret, as the resulting strains contain four cell cycle mutations. However, one possible interpretation of the results is that *win1* and *mcs4* lie in the same pathway, judging not only from the evidence of their strikingly similar phenotypes, but from their mutual epistasis when combined in a *wee1.50 cdc25.22* genetic background.

The combination of *win1.1* with *pat1.114* was found to suppress the hypersporulation phenotype normally shown by *pat1*^{ts} strains at the restrictive temperature (Iino and Yamamoto, 1985; Nurse, 1985), although cells of the double mutant strain do not continue to grow and divide. *win1.1* is not allelic to *cgs1* or *cgs2*, mutant alleles of which are also capable of suppressing the hypersporulation phenotype of *pat1.114* (McLeod and Beach, 1989). *win1.1* strains show an increased sensitivity to caffeine, which is an inhibitor of cAMP phosphodiesterase, suggesting that such strains have an altered response to intracellular cAMP levels. The implications of these results are discussed in detail in Section 8.5.

The *win1* locus was mapped to a position within 4 - 5 cM of *tps19*, which is situated on the short arm of chromosome I. *win1* was first mapped to chromosome I by a mitotic haploidisation procedure, and subsequently allocated to a region of Chromosome I by a series of crosses undertaken in a *swi5.39* genetic background, which results in a reduction in meiotic recombination frequency (Gutz and Schmidt, 1985). Finally, *win1* was mapped relative to *tps19* using conventional techniques.

8.2.2: The isolation and analysis of plasmids capable of suppressing a win1.1-conferred phenotype

Five independent sequences were isolated which were capable of suppressing the temperature sensitive phenotype of strains of the genotype *wee1.50 cdc25.22 win1.1*. None of these contained the authentic *win1* gene, as demonstrated by integration and genetic mapping, although one showed loose genetic linkage with the *win1* locus. These were named *wis* (win suppressing) 1 to 5. A molecular analysis was carried out on these plasmids, including the determination of restriction maps of the *S.pombe* sequences contained within them to confirm that none represented previously isolated

cell cycle genes. In the cases of *wis1*, *wis2* and *wis3*, functional sequences were identified within the original clone by a combination of subcloning and transposon mutagenesis, and functional subclones isolated.

The phenotypes of various cell cycle mutant strains containing the pwis plasmids in multicopy were also examined. None of the plasmids showed any effect upon the *cdc25.22* phenotype in an otherwise wild type background, suggesting that their effect upon a *wee1.50 cdc25.22 win1.1* strain was not simply to compensate for loss of *cdc25* activity. Only *wis1* and *wis4* were capable of suppressing the *win1.1* phenotype in an otherwise wild type background.

Several of the pwis plasmids were shown to affect the phenotype of strains with specific *mcs* mutations in a *wee1.50 cdc25.22* mutant background. Genetic mapping experiments showed that the *wis* genes were not allelic to the specific *mcs* mutations they were capable of suppressing. *wis2* and *wis3*, when present in multiple copies are capable of affecting the phenotypes of *win1.1*, *mcs3.12*, *mcs4.13* and *mcs6.13* in a *wee1.50 cdc25.22* mutant background. In contrast, only *wis1* showed an interaction with *mcs4.13*, which shares many phenotypic similarities with *win1.1*. The most striking result from the study of the effects of the pwis plasmids was that *wis1* was found to be capable of reducing the cell length on division of an otherwise wild type strain when present in multicopy. This suggested a possible role for *wis1* as a dosage dependent inducer of mitosis, and *wis1* was selected for further analysis.

8.2.3: The analysis of wis1

In the study of *wis1*, emphasis was laid upon the examination of changed *wis1* dosage upon the cell. This included a study of the dosage dependent effects of increasing copy number, and the effect of deleting a large section of the *wis1* functional region.

An increase in the number of copies of the *wis1* functional region present in the cell was found to decrease cell length at division in a dosage dependent manner. This suggests that *wis1* is involved in a rate limiting step controlling entry into mitosis and division. A single transcript of approximately 2400 nucleotides was identified, and was found to be present in elevated levels in strains containing increased *wis1* copy number. A large section of the *wis1* functional region was deleted by one step gene transplacement, and was found to result in highly elongated cells which showed a dramatic drop in viability upon entry into stationary phase.

A genetic study was carried out to determine the effect of combining a *wis1* deletion allele with mutations or genetic constructs which result in a wee phenotype. These experiments suggested that strains lacking in *wis1* function were still sensitive to levels of *wee1* and *cdc25* expression, as a *wis1* deletion allele in combination with either over-expressed *cdc25*$^{+}$, or a *wee1.50* mutation at the restrictive temperature, resulted in a wee phenotype. The combination of a *wis1* deletion allele with *cdc2.1w* or *cdc2.3w* mutations resulted in a substantial decrease in cell length, although the double mutant strains were significantly longer than either the parental *cdc2w* strains. Loss of *wis1* function was found to strongly suppress the hypersporulation phenotype of *pat1.114*, which is similar to the effect shown by *win1.1*, though, as in the case of *win1.1*, *wis1*$^{-}$ *pat1.114* double mutants were not capable of vegetative growth at the restrictive temperature.

The sequence of a 2.5kb region of DNA containing the *wis1* functional region was determined on both strands using exonuclease III deletion constructs (Henikoff, 1884), and the chain termination sequencing method of Sanger *et al* (1977). This contained a 1815nt open reading frame encoding a predicted *wis1* gene product of 605 amino acids. Protein sequence data base homology searches revealed that the *wis1* gene product showed a strong homology to the serine/threonine protein kinase family.

8.2.4: Unanswered questions

This summary of results leaves many questions unanswered. The following are amongst the most important:

(i) What is the role of the *win1* gene product in the control of the cell cycle? Since it proved impossible to clone *win1* using the method described here, models for the role of *win1* must be based on purely genetic evidence involving the *win1.1* mutant allele.

(ii) Why did it prove impossible to isolate the *win1* gene by complementation of the cdc phenotype shown by *wee1.50 cdc25.22 win1.1* strains on minimal medium at 35°C, and what alternative strategies exist for the cloning of *win1*?

(iii) Why are the five *wis* genes capable of suppressing the cdc phenotype of *wee1.50 cdc25.22 win1.1* strains when present in multicopy, and, with special emphasis on *wis1*, what is their normal role in the cell?

8.3: PLASMIDS CAPABLE OF SUPPRESSING A win1.1-CONFERRED PHENOTYPE

8.3.1: Why were win1 and cdc25 not isolated?

Several plasmids from various gene libraries were identified by their ability to suppress the cdc phenotype of a *wee1.50 cdc25.22 win1.1* strain on EMM. These plasmids defined five functional regions which were named *wis1 - 5*. It was predicted that such a gene library screen would identify both the *win1* and *cdc25* genes, although none of the plasmids isolated carried either of these genes. It has previously been shown that strong over-expression of *cdc25⁺* in combination with loss of *wee1* function is lethal (Russell and Nurse, 1986). This suggests that a possible reason why *cdc25⁺* transformants of the *wee1.50 cdc25.22 win1.1* strain were not identified is that the combination of multicopy *cdc25⁺* with a wee1.50 mutation was either lethal or detrimental to cell growth. The transformation of *wee1.50 cdc25.22 win1.1* with a plasmid carrying *cdc25⁺* revealed that such transformants were capable of growth at 35°C, but were very slow growing, and so would probably not have been identified in this screen.

The result obtained with *cdc25* suggested a possible reason why the *win1* gene was not isolated. The exhaustive screening of gene libraries without isolating *win1* suggests either that the sequence is not present in any of the libraries used, which seems unlikely, or that high levels of *win1* expression are detrimental to growth in a *wee1.50 cdc25.22 win1.1* genetic background, as is the case for *cdc25*.

8.3.2: Alternative strategies for the cloning of win1

Several alternative strategies exist for the isolation of the *win1* gene: one possibility would be to avoid problems due to high levels of *win1* expression by using a gene library constructed in a low copy number plasmid to transform a *wee1.50 cdc25.22 win1.1* strain, and to select for growth at 35°C on EMM. Unfortunately, although such plasmids are available for use in *S.cerevisiae*, they are not yet available in *S.pombe*.

A second possibility would be to use the *win1.1* phenotype of slightly elongated cells to screen gene libraries for plasmids containing the *win1⁺* gene. No positive selection

would be possible using this strategy, which would involve the detailed microscopic examination of many thousands of transformants.

A third possibility is the use of *win1* genetic mapping data to clone *win1*. This would involve the cloning of the closely linked *tps19* gene by complementation of the temperature sensitive phenotype of *tps19.14*, which would be followed chromosome walking, initially using *tps19* sequences as a probe, to isolate *win1*.

8.3.3: How do the five wis genes suppress the cdc win1.1-conferred phenotype used for gene library screening?

The question remains of why the five *wis* gene are capable of suppressing the cdc phenotype of a *wee1.50 cdc25.22 win1.1* strain when present in multicopy. There has been shown to be negligible *wee1* activity in *wee1.50* strains at the restrictive temperature (Russell and Nurse, 1987a), suggesting that the *wis* gene products do not interact with *wee1*. In contrast, the *cdc25.22* allele is not functionally equivalent to a *cdc25* null allele at 35°C (Russell and Nurse, 1986), so it is possible that high levels of *wis* expression are raising residual levels of *cdc25* activity. If this were the case, then it would be expected that the pwis plasmids would show suppression of *cdc25.22* in an otherwise wild type background, which was demonstrated not to be the case.

Little is known concerning levels of *win1* activity in *win1.1* strains, so a third possibility is that the effect of the pwis plasmids was to raise the level of *win1* activity. Only pwis1-1 and pwis4-1 are capable of suppressing the *win1.1* phenotype of increased cell length at division in an otherwise wild type background, suggesting that they may be acting directly to reverse the effect of decreased *win1* function, although it is impossible to determine from the available data if they interact directly with the *win1* gene product. *wis1* appears to have a more general effect upon cell length, as it is capable of reducing the cell division length of wild type cells when present in multicopy.

The mutations *mcs3.12*, *mcs4.13* and *mcs6.13* share with *win1.1* the property of reversing the suppression of *cdc25.22* by *wee1.50* (Molz *et al*, 1989). The plasmids pwis2.c1 and pwis3.1 are capable of suppressing the cdc phenotypes which arise from the combination of either of these four mutations with *wee1.50* and *cdc25.22*. Of the three *mcs* mutations, only *mcs4.13* shows a mutant phenotype in an otherwise wild type background, and this is not affected by the presence of either pwis2.c1 or

pwis3.1. *wis2* and *wis3* are not allelic to any of these three *mcs* genes. These results suggest that *wis2* and *wis3* show some form of general activity which will reverse the action of any mutation which results in a cdc phenotype when combined with *wee1.50* and *cdc25.22*. It is possible that *wis2* and *wis3* share some enzymatic function which may be extended to non-specific substrates when these genes are highly expressed. The interactions between *mcs4.13*, *win1.1* and *wis1* will be discussed in Section 8.4.5.

There is strong evidence that *wee1* and *cdc25* interact to control the activity of the *cdc2* gene product (Russell and Nurse 1986; 1987a). It is possible that the action of the *wis* genes, when present in multicopy, is to bypass the control of *cdc2* by *wee1* and *cdc25*, and since their action is to suppress a block in division, they would be predicted to activate the *cdc2* gene product. None of the pwis plasmids are capable of suppressing the cdc phenotype of a *cdc2.33* strain at the restrictive temperature, suggesting that this simple model is not an explanation, though this evidence does not exclude some form of allele specific interaction between *cdc2* and the *wis* gene products. No phenotypic effects were observed when the pwis plasmids were transformed into strains containing mutant alleles of *wee1.50*, *cdc13.117*, *cdr1.34* and *cdr2.69*, suggesting that the effect of these plasmids in multicopy is not to reverse the loss of function of these genes. *cdr1* has been recently shown to be allelic to *nim1*, which is believed to regulate *wee1* function (Russell and Nurse, 1987b).

8.4: The roles of win1 and wis1 in the control of mitosis

As described above, the *win1.1* mutant allele was isolated by Ogden and Fantes (1986) on the basis of its interaction with *wee1* and *cdc25* mutant alleles, and shows a phenotype of a slight increase in length in an otherwise wild type background. Their results, examining dominance relationships for *win1.1* in a *wee1.6 cdc25.22* genetic background, indicate that *win1.1* is a recessive mutation, suggesting that it encodes a protein product with substantially reduced function. Unfortunately, it is presently impossible to deduce to what extent the activity of the *win1.1* gene product is affected, making the interpretation of genetic data difficult. In contrast, it could be concluded that the *wis1* deletion allele resulted in a complete loss of *wis1* function, as two thirds of the predicted *wis1* open reading frame was deleted in the construct used for genetic studies. The observation that complete loss of *wis1* function is not lethal to the cell implies that *wis1* function is not vital for cell growth and division.

8.4.1: The interaction of win1 and wis1 with wee1 and cdc25

The over-expression of *cdc25* has a similar effect upon *win1* and *wis1* mutant cells as does the loss of *wee1* function. This result suggests that both strains are sensitive to *cdc25* expression.

wee1 is epistatic to both *win1* and *wis1*$^-$ mutant alleles with respect to cell length, demonstrating that both mutant strains are sensitive to *wee1* activity. This may be because the effect on the cell length due to the loss of *wee1* function overrides the effect of reduced *win1* and *wis1* function, or that the effects of *win1* and *wis1* are mediated by the *wee1* gene product. If this were the case then it would be predicted that the *wis1* gene product would inhibit *wee1* activity, as *wee1* acts as an inhibitor over entry into mitosis. The observations that increased *wis1* expression results in a reduced cell length, and that an increased level of *wis1* expression has no effect upon the cell length of *wee1*$^-$ strains fit with this model.

wee1 and *cdc25* have been shown to act independently in a dosage dependent manner to control entry into mitosis. A control element which inhibits *wee1* function might be expected to suppress *cdc25*^{ts} alleles to some extent. Such a role has been proposed for the *nim1* gene, which was isolated as a multicopy suppressor of *cdc25.22*. In contrast to *nim1*, *wis1* does not suppress *cdc25* when present in multicopy, suggesting that a similar argument can not be applied in this case. Increased levels of *wis1* expression result in a decrease in cell length in wild type cells and also in strains carrying either *win1.1 or mcs4.13*, mutations which lead to an increased cell length at division. Interestingly, increased *wis1* expression has very little effect on the cell length phenotype of either *cdr1.34* or *cdr2.69* strains, suggesting that these genes may play a role in mediating *wis1* function.

8.4.2: The interaction of win1.1 with cdc13.117

The interaction of *win1.1* with *cdc13.117* lends further support to the proposal that *win1* is involved in the control over entry into mitosis. The *cdc13* gene product is a cyclin homologue (Goebl and Byers, 1988; Solomon *et al*, 1988; Hagan *et al*, 1988) which shows a physical interaction with the *cdc2* protein kinase (p34), and may be involved in the nuclear localisation of p34 at mitosis (Booher *et al*, 1989). A deletion of the *cdc13* gene results in a block in G_2 prior to entry into mitosis, which suggests that the "mitotic" phenotype associated with the *cdc13.117* mutation is due to residual

cdc13 activity (Hagan *et al*, 1988; Booher and Beach, 1988). At the restrictive temperature, *cdc13.117 win1.1* cells show a phenotype similar to that resulting from a complete loss of *cdc13* function, suggesting that *win1.1* affects *cdc13* activity. It is not possible to determine if *win1* is required for *cdc13* action, as it is not known to what extent *win1.1* affects *win1* activity. If such a requirement did exist, then complete loss of *win1* function would be expected to result in a block in the cell cycle prior to mitosis.

Several possibilities exist to explain the nutritionally sensitive phenotype of the *wee1.50 cdc25.22 win1.1* strain. One possibility is that such cells are in a finely balanced state, possibly due to a very low residual *win1* activity, and that a small change in the cells' biochemistry, such as that induced by growth rates on various media, may be enough to shift a balance between a cdc phenotype, and the ability to form colonies. A second, more attractive possibility is that *win1* is directly involved in the nutritional sensing machinery of the cell. Such a signal has been proposed a a modulator of the cell size control over entry into mitosis (Fantes and Nurse, 1977) and it seems likely that *wee1* and *cdc25* are involved in this control (Fantes and Nurse, 1978; Nurse and Thuriaux, 1984). In double *wee1 win1.1* mutants, the *wee1* mutation is epistatic to *win1.1*, independent of the *wee1* allele. This suggests that *win1* may act through *wee1*, which has been shown to have a dosage dependent effect on cell size at division (Russell and Nurse 1987a), and this model is supported by the interaction of *win1.1* with *cdc2w* alleles.

8.4.3: The interaction of win1 with cdc2

Several alleles of *cdc2* have been identified which show a phenotype of small cells, resulting from an early entry into mitosis and division (Nurse and Thuriaux, 1980); Fantes, 1981). These wee alleles fall into two classes (Thuriaux *et al*, 1978; Fantes, 1981; Russell and Nurse, 1987a): one, typified by *cdc2.1w*, is specifically insensitive to *wee1* expression, though responding essentially normally to *cdc25* levels . A second class, typified by *cdc2.3w*, is sensitive to levels of *wee1* expression, but show little response to changes in *cdc25* levels. The cell length phenotype of *win1.1* is strongly suppressed by *cdc2.1w*, though the combination of *win1.1* with *cdc2.3w* has little effect upon the *win1.1* phenotype. One possible explanation for these results is that *win1* negatively regulates *wee1* levels, so that loss of *win1* function gives a raised level of *wee1* function, which results in longer cells. This model would explain the specific interactions between *cdc2w* alleles and *win1.1*, though would predict that

wee1⁻ cells would be completely insensitive to *win1* dosage. This is evidently not the case, since *wee1.50 cdc25.22 win1.1* cells show very different phenotypic characteristics from *wee1.50 cdc25.22 win1⁺* cells.

8.4.4: Possible roles for wis1

The cell length phenotype of *wis1⁻* strains is strongly suppressed either by loss of *wee1* function, or by over-expression of *cdc25*, and is also affected by either of the *cdc2w* alleles tested. These observations suggest that *wis1* function either regulates both *wee1* and *cdc25* activity, or acts independently of *wee1* and *cdc25* on *cdc2*, assuming that *wis1* is involved in this control mechanism. It seems most likely that *wis1* acts upstream of *wee1* and *cdc25* in some form of signal transduction pathway involved in the sensing of nutritional conditions. The phenotype of *wis1* deletion strains also lends support to the theory that *wis1* is involved in the sensing of nutritional conditions, as such strains appear either to be unable to recognise conditions of starvation, or to respond to them by entering stationary phase. It is not yet known if loss of *wis1* function affects the starvation induced transcription of genes such as *mei2*, *mei3* and the mating type genes. If *wis1* activity were required for the function of these genes, then it might be expected that *wis1⁻* strains would be sterile, which is not the case.

8.4.5: win1, wis1 and mcs4 may lie on the same control pathway.

The *mcs4.13* mutation shares many phenotypic characteristics with *win1.1*: both result in slightly elongated cells, and both reverse the suppression of *cdc25⁻* by *wee1⁻* in a medium dependent manner. A cross between strains of the genotype *wee1.50 cdc25.22 win1.1* and *wee1.50 cdc25.22 mcs4.13* gives rise to a quadruple mutant strain *wee1.50 cdc25.22 win1.1 mcs4.13* which is indistinguishable from its parents. In addition a *win1.1 mcs4.13* strain is only slightly longer than the single mutant parent strains. An important difference between the two mutations is that *mcs4.13* is capable of suppressing the mitotic catastrophe phenotype, whereas *win1.1* is not. These observations suggest that *win1* and *mcs4* may share related, though not identical, roles in the control of the cell cycle. The observation that *win1.1 wis1⁻* double mutant strains appear to be phenotypically identical to *wis1⁻* single mutants suggests that *wis1* and *win1* may lie on the same pathway.

A high level of expression of *wis1* can suppress both the single mutant phenotype of *mcs4.13*, and the *cdc⁻* phenotype which results from the combination of *mcs4.13* with *wee1.50* and *cdc25.22* at 35°C on EMM. Plasmid borne *wis1* sequences are not capable of suppressing the cdc phenotype resulting from the combination of either *mcs3.12* or *mcs6.13* with *wee1.50* and *cdc25.22*. This suggests that *wis1* does not have some form of general activity which will reverse the action of any mutation which results in a cdc phenotype when combined with *wee1.50* and *cdc25.22*.

8.4.6: wis1 encodes a putative protein kinase

Further clues to the role of *wis1* in the cell may be gleaned from the comparison of the predicted *wis1* sequence with those of proteins of known function.
The three proteins showing the highest homology to the predicted *wis1* gene product (*PBS2* and *STE7* from *Saccharomyces cerevisiae*, and *byr1* from *Schizosaccharomyces pombe*) are all thought to be serine/threonine protein kinases involved in signalling mechanisms. The *PBS2* gene was identified by its ability to confer resistance to the antibiotic polymyxin B when present in multicopy in *S.cerevisiae*, and has not been extensively characterised. Boguslawski and Polazzi (1987) suggest that phosphorylation of the *PBS1* gene product by the *PBS2* protein kinase may block its mediation of the polymyxin B signal.

The role of the *S.cerevisiae STE7* protein has been more extensively characterised, and is believed to act in a signalling pathway mediating the effects of mating factors on the induction of gene expression. The *STE7* and *STE11* gene products are both phosphoproteins with protein kinase activity which are believed to act downstream of the G protein complex in this pathway (Teague *et al*, 1986; Errede *et al*, 1990; reviewed by Herskowitz, 1989).

The *S.pombe byr1* gene was first identified as a multicopy suppressor of the sporulation defect observed in *ras1⁻* strains (Nadin-Davis and Nasim, 1988). *S.pombe* contains a single *ras* gene, which is not essential for vegetative growth, but is important in sexual differentiation (Fukui and Kaziro, 1985; Nadin-Davis *et al*, 1986a and 1986b; Fukui *et al*, 1986). *ras1⁻* strains are incapable of conjugation, but are capable of sporulation. A possible role for *ras1* lies in the nutritional sensing apparatus of the cell, which prepares both h^- and h^+ strains to receive specific mating signals. Loss of *byr1* function results in sterility, whilst over-expression overcomes loss of *ras1* function, suggesting it plays a role in the *ras* signalling

pathway (Nadin-Davis and Nasim, 1988; 1990). Neither *byr1* or *ras1* mutations suppress the hypersporulation phenotype of *pat1*^{ts} mutations, suggesting that they function upstream of *pat1* (Nadin-Davis and Nasim, 1990).

8.5: THE ROLES OF win1 AND wis1 IN THE CONTROL OVER ENTRY INTO CONJUGATION AND MEIOSIS

The observation that both *win1.1* and *wis1*$^{-}$ alleles are capable of suppressing the hypersporulation phenotype of *pat1.114* strains suggests that these genes may play a role in the control over entry into meiosis and/or sporulation.

6.1: Genetic elements involved in the control over entry into meiosis

Loss of *pat1* function results in a complex phenotype: the mitotic cell cycle ceases, and cells undergo meiosis irrespective of *mat1* configuration and nutritional conditions. At the semi-permissive temperature, conjugation occurs irrespective of nutritional conditions, although this will only occur between cells of opposite mating type (Iino and Yamamoto, 1985a; Nurse, 1985). Partial inactivation of the *pat1* gene product induces transcription of the *mat1* genes (Nielson and Egel, 1990) thus mimicking conjugation of wild type cells under conditions of nitrogen starvation. *pat1*^{ts} mutants undergo premeiotic DNA synthesis and meiotic recombination when shifted to the restrictive temperature, suggesting that inactivation of *pat1*$^{+}$ gene product activates an early step in the normal meiotic pathway (Beach *et al*, 1985).

The *mei3* gene is only expressed when the two conditions of mating type heterozygosity and nitrogen starvation are met. Artificially high levels of expression of *mei3* result in uncontrolled sporulation (McLeod *et al*, 1987). Loss of *mei3* activity does not affect the *pat1* phenotype (Iino and Yamamoto, 1985a; Beach *et al*, 1985), and meiosis is derepressed when both genes are simultaneously over-expressed (McLeod and Beach, 1988). These results suggest that the decision between meiosis and mitosis is decided by a balance of *mei3* and *pat1* activities, and direct interactions between the *mei3* and *pat1* gene products have been demonstrated. The *pat1* gene product shows protein kinase activity, which is inhibited by physical association with the *mei3* gene product (McLeod and Beach, 1988).

Changes in several parameters can suppress the expression of the *pat1* phenotype:

(i) Loss of *mei2* activity.

Transcription of *mei2* is stimulated by nitrogen starvation, and is not dependent on mating type (Shimoda *et al*, 1987; Watanabe *et al*, 1988). It is possible is that the *mei2* gene product acts as *a* substrate for the *pat1* protein kinase.

(ii) Loss of steX activity.

Mutations in the *steX* gene (probably allelic to *aff1*) result in sterility, and block the induction of *mei2* by nitrogen starvation (Watanabe *et al*, 1988).

(iii) High levels of *pac1* activity.

The *pac1* gene (pat compensating) when present in multicopy blocks the induction of *mei2* by nitrogen starvation, and also inhibits mating and sporulation. *pac1* activity is essential for vegetative growth, and the *pac1* gene product shows a strong homology with ribonuclease III from *E.coli*, and also shows a ribonuclease III-like activity in cleaving dsRNA *in vitro* (Sugimoto *et al*, 1990). A possible role for *pac1* lies in the post-transcriptional regulation of expression of genes concerned with meiosis and sporulation.

(iv) Mutations in *cgs1* or *cgs2*.

Cells with mutations in either of the *cgs* genes are sterile and meiotically defective. *cgs1*⁻ cells rapidly loose viability as they become limited for nutrients, and become aberrantly elongated in response to nutrient depletion. Sequence analysis of the *cgs1* gene reveals that the predicted gene product is highly homologous to the regulatory subunit of cAMP dependent protein kinase from *S.cerevisiae* and other eukaryotes (McLeod and Beach, 1989).

8.5.2: A model for the control of conjugation and meiosis

Conjugation is normally regulated by the mating types of the cells concerned, and is triggered by nitrogen starvation. *pat1* obviously plays some role in the control of sporulation, as $pat1^{ts}$ cells no longer require starvation as a prerequisite for conjugation at the semipermissive temperature (Iino and Yamamoto, 1985a; Nurse, 1985). The influence of various *ste* genes on *pat1*-driven conjugation and/or meiosis has been analysed by Sipiczki (1988). None of the previously known mutants (*ste1-9*) interfered with *pat1*-driven sporulation, but "untimely" conjugation was possible in mutants of *ste1* (*byr1*), *ste3* and *ste8*. Cells with mutations in *ste5/ras1* and *ste6* are able to undergo meiosis when diploid, suggesting that these genes play a role in conjugation specifically. Further mutants, such as *steX* and the *cgs* mutants, have been identified as sterile suppressors of the temperature sensitive growth arrest of $pat1^{ts}$ strains.

Meiosis is also dependent on mating type configuration and nitrogen starvation. A model has been proposed for the regulation of meiosis (Fig. 8.1) in which the combination of starvation, and the expression of both mating type loci results in the expression of *mei3*. Starvation also signals a stimulation of *mei2* transcription (Watanabe *et al*, 1988). In this model, *mei3* functions to inactivate the *pat1* protein kinase, which would otherwise act to reduce *mei2* activity, probably by post-transcriptional regulation. An attractive possibility is that *pat1* regulates *mei2* activity by phosphorylation. *mei2* activity then leads to meiosis.

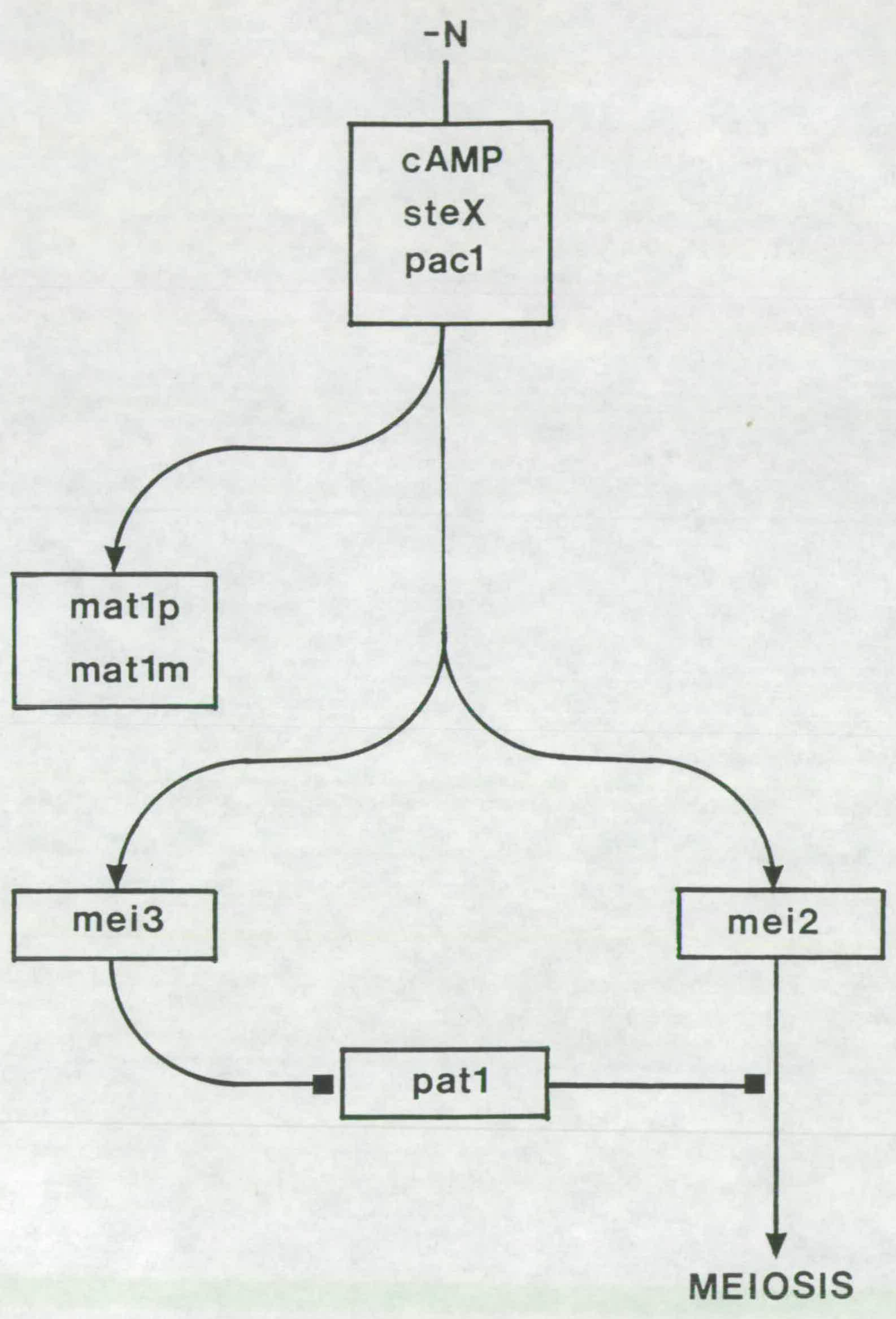
8.5.3: The cAMP connection.

Artificially high levels of cAMP are capable of suppressing the phenotype of *pat1*^{ts} strains, an effect which is potentiated by caffeine, a known inhibitor of cAMP phosphodiesterase. cAMP shows a range of effects upon the sexual life cycle of *S.pombe*: it has been demonstrated to reduce the conjugation efficiency of haploids, and the sporulation efficiency of diploids, at levels which do not affect the growth rate of vegetative cells, suggesting that the effect is specific to sporulation and meiosis (Watanabe *et al* 1988). The treatment of cells with a combination of cAMP and caffeine not only prevents the induction of the *mat1* transcripts Pm and Mm, but also inhibits the inducible transcription of *mei2*, suggesting that cAMP generally counteracts the induction of sexual life cycle genes by starvation (Watanabe *et al*, 1988). It has been demonstrated that cAMP can no longer suppress the *pat1* phenotype when *mei2* is constitutively supplied, suggesting that the inhibition of *mei2* transcription by cAMP is the primary reason for its suppression of *pat1* (Watanabe *et al*, 1988).

It has recently been reported that the adenylate cyclase gene of *S.pombe* (*acy1*) has been isolated (Maeda *et al*, 1989). Surprisingly, increased dosage of *acy1* does not suppress *pat1*, possibly suggesting *acy1* activity is regulated post-transcriptionally. In contrast, transformation of *pat1*^{ts} strains with the adenylate cyclase gene of S.cerevisiae (*CYR1*) results in a high
level of intracellular cAMP and in the suppression of the *pat1* phenotype (Beach *et al*, 1985). When a *pat1*^{ts} strain containing the *CYR1* gene is grown to stationary phase, cells become highly elongated compared to wild type cells, due to a continuation of cell growth following the cessation of cell division.

Figure 8.1: A model for the control of meiosis in S.pombe.

(adapted from Watanabe *et al*, 1988)



Positive, transcriptional regulation

Negative, post-transcriptional regulation

The *cgs1* gene in *S.pombe* is highly homologous to the regulatory subunit of cAMP dependent protein kinase. Mutations in *cgs1* result in a cell length phenotype similar to that resulting from the transformation of $pat1^{ts}$ strains with *CYR1*. *cgs1* mutants are sterile, and show a reduced viability upon entry into stationary phase, though it is not clear if *S.pombe CYR1* transformants share these attributes.

In *S.cerevisiae*, cAMP is though to be the positive signal for growth elicited in response to a sufficient nutrient supply. This signal pathway acts through one of the two *ras* homologues which have been identified in *S.cerevisiae*. The *RAS* gene products are GTP-binding proteins which are believed to transduce information concerning environmental conditions into the activity of membrane bound adenylate cyclase. *RAS1* and *RAS2* appear to have overlapping functions, as only one is essential for cell growth and adenylate cyclase activity. $ras1^{-}$/*ras2ts* double mutants arrest as single unbudded cells, a phenotype similar to that of nutritionally arrested cells, and to that of *CDC19*, *CDC25*, *CDC33* and *CDC35* mutants. *CDC35* (*CYR1*) is the structural gene for adenylate cyclase, and *CDC25* is believed to interact in a regulatory manner with the long variable regions of the *RAS* gene products.

In contrast, the single *ras* gene identified in *S.pombe* is not required for vegetative growth, but plays an essential role in mating. $ras1^{+}$, $ras1^{-}$ and activated $ras1^{VAL-17}$ strains all contain similar cAMP levels, and comparable adenylate cyclase activities, suggesting that adenylate cyclase modulation is not the basis of *ras1* function in *S.pombe*. The region of the *S.cerevisiae RAS2* polypeptide required for adenylate cyclase modulation has been mapped, and found to lie in sequences that are either lost or not conserved in the *S.pombe* gene. It is not clear if cAMP is involved in growth control in *S.pombe*, though it has been found that the transcript levels of the adenylate cyclase gene in *S.pombe* are not affected by starvation, suggesting there is no strong involvement.

Strains lacking in *wis1* activity show a phenotype similar in many ways to that which would be expected from an elevated intracellular cAMP concentration. Such cells become elongated upon nutritional limitation, and appear to be unable to respond to starvation by entering stationary phase. However, if this were the simple explanation of the $wis1^{-}$ phenotype, then $wis1^{-}$ strains would also be expected to be sterile. Surprisingly, such strains appear to be capable of responding to starvation by undergoing conjugation followed by normal meiosis and sporulation.

A very attractive explanation for the suppression of the *pat1* phenotype by *wis1*⁻ is that the effect is due to raised intracellular levels of cAMP. However, the conditions described above which raise intracellular levels of cAMP suppress not only the hypersporulation of *pat1*^{ts} strains, but also suppress the block in vegetative growth which results from loss of *pat1* function. In contrast, the combination of *wis1*⁻ with *pat1*^{ts} results in the complete suppression of the hypersporulation defect at the restrictive temperature, but does not allow vegetative growth. A similar effect arises from the combination of *win1.1* with *pat1*^{ts}. This result may suggest that loss of *pat1* function causes hypersporulation and the cessation of vegetative growth through separate signalling pathways, one of which involves *wis1* and *win1.1*. It is not yet known if the derepression of conjugation by *pat1*^{ts} at the semi-permissive temperature is affected by *wis1*⁻ or *win1.1*, or if diploids homozygous for *pat1*^{ts} and *wis1*⁻ or *win1.1* undergo meiosis at the restrictive temperature. cAMP levels in *wis1*⁻ and *win1.1* strains have not yet been investigated, although *win1.1* strains are sensitive to caffeine.

8.6: SUMMARY

The *wis1* gene was isolated by virtue of its interaction with the mitotic genes *cdc25*, *wee1* and *win1*, and appears to play an important role in the regulation of entry into mitosis, as it acts as a dosage dependent inducer of entry into mitosis and cell division. *wis1* activity is not essential for vegetative growth and division, and does not prevent conjugation, meiosis and division. *wis1*⁻ cells show an elongated cell morphology, suggesting that their entry into mitosis and division is delayed relative to wild type cells, though this has not been directly demonstrated. A second notable characteristic of *wis1*⁻ cells is that they do not respond normally to nutrient starvation, becoming aberrantly elongated, and undergoing a rapid reduction in viability; a phenotype similar to that caused by elevated intracellular cAMP levels. This evidence suggests that *wis1* may play a role in the regulation of mitosis by nutritional signals, possibly in a mechanism involving cAMP. *wis1* encodes a putative protein kinase, which shows homology to several protein kinases involved in signal transduction pathways.

win1.1 was isolated on the basis of its interaction with *cdc25* and *wee1*, which are both involved in the control over entry into mitosis (Russell and Nurse 1986; 1987a). The interaction between *win1.1* and *cdc13.117* confirms the role of *win1* in the regulation of mitosis. The interaction of *win1.1* with *cdc2w* alleles suggests that *win1*

may act through *wee1*. Further genetic evidence suggests that *win1*, *wis1* and *mcs4* may act through the same pathway.

The artificial induction of sporulation by loss of *pat1* function is suppressed both by *win1.1*, and by loss of *wis1* function, though *wis1*$^-$ cells are capable of undergoing normal meiosis and sporulation. *pat1*^{ts} strains cease vegetative grow that the restrictive temperature, a phenotype which is not suppressed by *win1.1* or *wis1*$^-$. The question of why *win1* and *wis1* affects *pat1*-induced, but not normal meiosis and sporulation, must remain unanswered for the present.

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VLADIMIR: That passed the time.

ESTRAGON: It would have passed in any case.

VLADIMIR: Yes, but not so rapidly.

(Pause)

ESTRAGON: What do we do now?

VLADIMIR: I don't know.

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