Genetic and molecular analysis of the *wis*2+ gene in fission yeast

Ronit Weisman

Thesis presented for the Degree of
Doctor of Philosophy
at the University of Edinburgh
1994

"One can't believe impossible things." [said Alice.]
"I daresay you haven't had much practice," said the Queen."... Why, sometimes I've believed as many as six impossible things before breakfast."
Through the looking glass by Lewis Carroll.

For my Family

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.

Ronit Weisman

July 1994

Acknowledgements

I am indebted to many people who made this research project possible.

Firstly, I would like to thank my supervisor Peter Fantes, for his continuous support, helpful discussions and advice, and for critical reading of the manuscript of this thesis.

I would also like to thank all members of the Fantes group; in particular, Chris McInerny, for his advice on experiments and valuable comments on the text; Stuart MacNeill for a wealth of ideas and technical tips; and Shaun Mackie for experimental assistance; my sincere thanks also to Pamela Harris, Paul Kersey and Sophie Stettler for their assistance and advice.

I would like to thank Emma Warbrick for introducing me to the *wis* genes, and for the care she took when providing the *wis2* plasmids.

Special thanks to Joan Davidson and Aileen Greig who never failed to keep up the supply of media, solutions and other essentials, all in all providing a superb technical support.

Thanks also to the photographers Frank Johnston and Graham Brown for their excellent work.

I would like to thank the Darwin Trust for financial support.

And not the least, thanks to my family for encouragement and support.

This work is dedicated to the memory of my father, with love, and appreciation for his good sense of judgement and for his ever enthusiastic curiosity.

Contents

Abbreviations

ATP	adenosine triphosphate
BSA	bovine serum albumin
bp	base pair
cAMP	cyclic adenosine monophosphate
cdc	cell division cycle
cDNA	complementary DNA
cM	centiMorgan
CsA	cyclosporin A
EMM	Edinburgh minimal medium
FKBP	FK506 binding proteins
IPTG	isopropylthio-β-D-galactoside
kb	kilobase
kDa	kiloDalton
ME	malt extract
mRNA	messenger RNA
MPF	maturation promoting factor
nt	nucleotide
OD	optical density
ORF	open reading frame
PCR	polymerase chain reaction
PPIase	peptidyl prolyl isomerase
rpm	revolutions per minute
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
ssDNA	single stranded DNA
ts	temperature sensitive
UV	ultraviolet light
UWGCG	University of Wisconsin GCG sequence analysis program
v/v	volume/volume
w/v	weight/volume
YE	yeast extract

Abstract

The *wis1*$^+$ - *wis5*$^+$ genes of the fission yeast *Schizosaccharomyces pombe* were isolated as multicopy suppressors of the cell cycle defect of the triple mutant *wee1-50 cdc25-22 win1-1*. The *wee1*$^+$ and *cdc25*$^+$ gene products are negative and positive regulators, respectively, of p34^{cdc2} protein kinase, the key regulator of entry into mitosis. The interaction of *win1-1* with mitotic control genes suggests that *win1*$^+$ plays a role in the control over entry into mitosis.

The work described in this thesis is primarily concerned with the genetic and molecular analysis of the *wis2*$^+$ gene. DNA sequence analysis of *wis2*$^+$ showed that it encodes a novel cyclophilin-like protein with the predicted molecular weight of 40 kDa. The predicted protein represents an unusual cyclophilin as the 18 kDa cyclophilin domain is followed by a C- terminal domain of 188 amino acid. Cyclophilins have been implicated in two separate cellular processes. One is protein folding and transport; the other is the blockage of signal transduction pathways when complexed with the immunosuppressive drug cyclosporin A.

The only genetic interaction detected that involves the *wis2*$^+$ gene is the suppression of the cell cycle defect of *wee1-50 cdc25-22 win1-1* by overexpression (referred to as wis2 activity). No effect associated with either overexpression or deletion of *wis2*$^+$ has been observed in either wild type strains or in a variety of cell cycle mutant strains.

Structure-function analysis has been carried out in order to determine the role of the two distinct regions of *wis2*$^+$ in wis2 activity. Several lines of evidence indicate that both the cyclophilin-like domain and the C-terminal region are required for wis2 activity. Mutations at highly conserved residues within the cyclophilin domain abolish wis2 activity, as does deletion of the C-terminus. The C-terminus appears to be responsible for the specificity of the molecule, since a hybrid protein consisting of a different *S.pombe* cyclophilin fused to the C-terminus of wis2 shows wis2 activity.

In addition, approaches towards cloning the *win1*$^+$ gene are described. The *win1*$^+$ gene has proved refractory to cloning by complementation screens

of genomic libraries. An alternative approach was undertaken in which *win1* was mapped genetically and a closely linked marker used as a hybridisation probe against an ordered cosmid library of the *S.pombe* genome. The cosmid clones were screened for sequences capable of suppressing the lethal phenotype of *wee1-50 cdc25-22 win1-1*. So far no such sequences have been isolated. It is possible that the set of cosmids spanning the *win1* region is incomplete; alternatively, the presence of multiple copies of $win1^+$ may be deleterious to *S.pombe* cells.

Chapter 1: Introduction

1.1 *Schizosaccharomyces pombe* and the eukaryotic cell cycle

i The eukaryotic cell cycle

The cell cycle is a period during which events required for successful cell reproduction are completed. The eukaryotic cycle has been traditionally divided into four distinct phases: G1, S, G2, and M. S phase represents DNA synthesis, the period during which the DNA of the cell is replicated. M phase represents mitosis, where the mitotic spindle is formed and the sister chromatids are separated. G1 and G2 were originally designated as gaps between these two phases, but are now known to contain two important control points of the cell cycle. Collectively, G1, S and G2 are called interphase, the period of the cell cycle distinct from division of the nucleus (mitosis) and cytoplasm (cytokinesis).

Cells can exit from the cell cycle during either the G1 or G2 phases. In multicellular eukaryotic organisms, most of the non-growing, non-proliferating cells in the intact organs are arrested at a phase known as G0. Such cells have left the cycle after division but before a certain point in G1, named the restriction point (Pardee 1974); cells past the restriction point are committed to traverse the remainder of the cycle independent of external cues. There is no specific name for the point in G2 where cells can exit the cell cycle.

In yeast cells, Start, a transition point in G1, is conceptually analogous to the restriction point in higher eukaryotes (Hartwell 1974). At Start, cells can become committed to traverse the remainder of the cell cycle. Alternatively cells can exit the mitotic cell cycle, switch into a sexual life cycle (see section 1.1 ii), or become arrested at a phase known as the stationary phase, in which viability can be maintained for long periods.

The cell cycle is a highly ordered and regulated biological process. One type of regulation concerns the control of cell size, which coordinates cell growth and cell division. Almost all cells must grow between each cell division (an exception is found in early stages in embryonic cells). Therefore, an unrestrained cell cycle that could run faster than cell growth, could lead to

a population of cells that would become progressively smaller with each division (reviewed in Murray and Kirschner 1991). Analysis of the coordination between cell cycles and cell growth in the unicellular eukaryotic cells of yeast has demonstrated that coordination is achieved by means of a threshold size control: cells must attain a critical size before they can pass through certain cell cycle stages. The existence of a threshold size control can be demonstrated by altering growth conditions to produce daughter cells of different sizes. In the budding yeast, *Saccharomyces cerevisiae* small daughter cells must grow for a longer time before they pass Start (Hartwell and Unger 1977). In the fission yeast, *Schizosaccharomyces pombe*, similar type of control is apparent, but the primary restraint by cell size control is manifested in G2 (Nurse 1975; Nurse and Fantes 1981, reviewed in Fantes 1989).

Another type of regulation of the eukaryotic cell cycle is the coordination of various events in the cycle. The two most obvious events are DNA replication and mitosis. Cells must avoid entering mitosis until their chromosomes have been replicated, since failure to do so can yield cells that lack a particular chromosome or that carry an extra chromosome. Similarly, DNA synthesis must be coupled to mitosis to avoid extra round(s) of replication as this may lead to polyploidy. Specific stages in the cycle where early events are monitored and their completion is coupled to the onset of later events have been referred to as 'checkpoints' (Hartwell and Weinert 1989).

Molecular mechanisms underlying the cell cycle regulatory process can be conceptually divided into two classes (reviewed in Nurse 1990; Murray 1992; Sherr 1993). One class constitutes the cell cycle machinery (sometimes referred to as the cell cycle clock) that brings about the transitions of the cell cycle phases in an ordered manner. The key components of the 'machine' are the cdks: cyclin-dependent kinases, the regulatory subunits cyclins and regulatory kinases and phosphatases. The other class is composed of several pathways and sensors that integrate intracellular or extracellular signals into the cell cycle machinery/clock. Much less is known about the components that make up this class, although proteins that are involved in pathways that monitor the completion of DNA replication, the nutritional status, or the presence of signal molecules from neighbouring cells are currently under investigation.

ii *S.pombe* physiology and genetics

The fission yeast *Schizosaccharomyces pombe* is a unicellular ascomycete fungus with rod shaped cells. This simple eukaryote has proved an ideal model organism for cell cycle studies mainly because of its mode of division (as will be discussed in section 1.1 iii), and its amenability to both classical and molecular genetic analysis (reviewed in Fantes 1989; MacNeill and Fantes 1993).

This section serves as a brief introduction for general features of the yeast *S.pombe*, in particular those features which have proved valuable for genetical and molecular studies.

S.pombe cells grow under relatively simple growth conditions. For laboratory use *S.pombe* cells are grown in either complex or minimal media at temperatures varying from 20°C to 36°C (Moreno et al. 1991). For most purposes 32°C is a convenient growth temperature; below 25°C cells grow only slowly and above 37°C cells do not grow. Depending on the medium and temperature conditions, the generation time of wild type cells is between 2- 5 hours (reviewed in MacNeill and Fantes 1993).

The nutritional conditions in combination with the presence of mating pheromones control changes in the life cycle of *S.pombe*. The life cycle consists of haploid and diploid mitotic cell cycles, conjugation, meiosis and sporulation. Haploid cells are of two mating types known as h^+ and h^-. Starvation induces haploid cells of opposite mating type to mate in pairs, forming diploid zygotes which are heterozygous at the mating type locus (h^+/h^-). The diploid formed by conjugation will undergo meiosis and sporulation if conditions of nutrient limitation continue. However, if the diploid is supplied with adequate nutrients, it will re-enter the mitotic cycle and divide vegetatively as a diploid (Egel and Egel-Mitani 1974; reviewed in Moreno et al. 1991).

The wild type *S.pombe* cells are homothallic (h^{90}), that is, they can switch their mating type between h^+ and h^- every other generation (reviewed in Egel 1989). The most commonly used heterothallic strains h^{-S} and h^{+N} (often referred to simply as h^- and h^+) contain deletions or rearrangements at the mating type locus which result in cells that cannot switch (h^{-S}), or that switch rarely (h^{+N}).

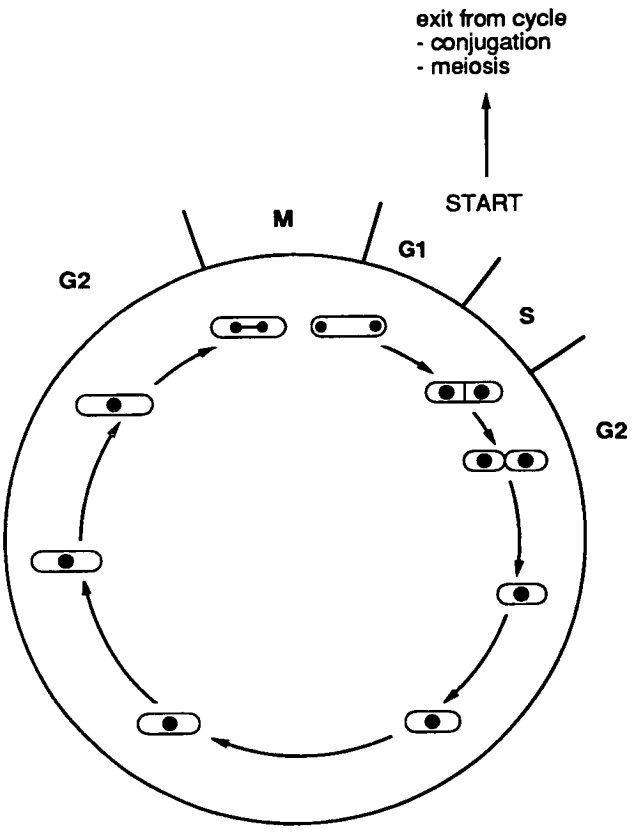
S.pombe cells contain a relatively small genome of about 14 Mbp, approximately 3 times as large as the *E.coli* genome, and 15 times smaller than the mouse genome. Three linkage groups have been genetically identified, consistent with the three chromosomes that can be visualised microscopically (Munz et al. 1989). Over 500 *S.pombe* genes have been identified, many of which have been placed on the genetic map of the organism. The genome of *S.pombe* has been covered by a yeast artificial chromosome (YAC) library (Maier et al. 1992), and more recently by high resolution of ordered bacteriophage P1 and cosmid libraries (Mizukami et al. 1993; Hoheisel et al. 1993). These maps are important for gene cloning and mapping procedures.

iii *S.pombe* cell cycle

The mode of growth of *S.pombe* cells by length extension allows the position of a cell in the division cycle to be estimated by a simple length measurement; this feature was one of the main reasons for which *S.pombe* was first chosen for cell cycle studies (Mitchison 1957 and see Figure 1.1). The mode of division of *S.pombe* into two equal daughter cells is more typical of higher eukaryotes than the budding mode of *S. cerevisiae* (Mitchison 1990). Notably, *S.pombe* and *S. cerevisiae* are two distantly related yeasts (Sipiczki 1989). Therefore, parallel cell cycle studies of these two model systems are particularly useful, indicating in some cases high conservation across evolution, or in other cases, demonstrating alternative strategies for similar goals.

The mitotic cell cycle of *S.pombe* is typically eukaryotic, with discrete G1, S, G2, and M phases (reviewed in Fantes, 1989). In rapidly growing cells, G2 is by far the longest phase, occupying about three quarters of the cycle (see Figure 1.1). During this phase most of cell growth takes place. Entry into mitosis is marked by chromosome condensation and by rapid microtubule rearrangement, as the cytoplasmic array of microtubles disappears and a short spindle is formed. Mitosis is followed by cell division when a septum is formed across the centre of the cell, and subsequent cell separation occurs when the septum is cleaved. G1 and S phases follow immediately after the completion of nuclear division, such that at the time of cell separation, the daughter cells are in S phase. The duration of each of the M, G1 and S phases at rapid growth rates is each approximately one tenth of the cell cycle.

Figure 1.1 A schematic representation of *S.pombe* cell cycle



The G1 and G2 phases in the cell cycle of *S.pombe* are two major control points, concerned with regulating the onset of S phase and mitosis, respectively, and ensure that these two major events are coupled together in the correct order. Under conditions of rapid growth, the G2 phase of *S.pombe* cells is rate limiting for the overall progress through the cell cycle, ensuring that mitosis and cell division are permitted only when cells have attained a critical size (Nurse and Fantes 1981). The G2-M transition also requires the completion of DNA replication, which is demonstrated by the finding that if DNA replication is blocked, cells do not enter mitosis (Enoch and Nurse 1990; Sheldrick and Carr 1993). Apart from maintaining dependencies characteristic of a normal cell cycle, a control point in G2 has been suggested to operate under adverse conditions, caused by DNA damage agents. This control is often referred to as radiation checkpoint control, and is demonstrated by a temporary arrest at G2 following treatment with ionising radiation (Al-Khodairy and Carr 1992; Rowley et al. 1992).

Controls that regulate growth and cell division exist in G1, although a threshold size control becomes apparent only under very slow growth conditions or in cell size mutants. Under normal conditions the cell size control in G1 is cryptic, presumably because cells are born at a cell size already larger than the required critical size (Nurse 1975). Entry into S phase also requires the completion of the previous mitosis, demonstrated by the fact that cells blocked in G2 cannot undergo S phase. The two requirements for entry into S phase are thought to operate through Start (Moreno and Nurse 1993).

vi *S.pombe* morphological and cytological changes

Parameters such as cell length and cytological changes provide useful markers for cell cycle stages. Cell length is the most common and convenient parameter to be assessed. Cells are born at a cell size of ~7 μm in length, and first grow only at the old end of the cell, which existed in the previous cell cycle. The new end starts to grow when cells reach ~9.5 μm, a stage which has been termed NETO for New End Take Off (Mitchison 1989). Cells grow until they reach the length of ~14 μm when growth stops and cells undergo mitosis and cytokinesis.

Changes in the cytoskeletal arrangements can be followed to a limited extent by light microscopy, but can be studied in detail by the application of fluorescence techniques and freeze substitution electron microscopy (reviewed in Robinow and Hyams 1989). Growth, nuclear positioning, mitosis and septation are all a function of the cytoskeleton. *S.pombe* contains two α tubulin, one β tubulin gene and one actin gene (Yanagida 1987). Actin filaments occur as two forms in *S.pombe*: dots and filaments (Marks and Hyams 1985). In actively growing cells during interphase, dots containing F-actin are concentrated at the growing tip(s). At the onset of mitosis, actin disappears from the cell ends to form a filamentous, equatorial belt that overlies the region that participates in the formation of the septum . Coincidentally, cytoplasmic microtubules depolymerise and a short spindle is formed between two spindle pole bodies associated with the nuclear envelope. Spindle elongation separates the daughter nuclei to the ends of cells, whence they subsequently relocate to the centre of the cell (Hagan and Hyams 1988)

Comparative cytological studies noted a significant difference concerning spindle formation in the fission and budding yeast. In fission yeast spindle formation marks the beginning of mitosis and the breakdown of the spindle occurs before the next interphase. These cytological events are different from that of the budding yeast, where the mitotic spindle is present for much of the cell cycle, being initiated during S phase (reviewed in Nurse 1985). Both fission and budding yeasts differ from higher eukaryotes in that the nuclear membrane remains intact during mitosis (reviewed in Nurse 1985).

v Universal cell cycle control mechanisms

An important discovery was the conserved nature of genes and biochemical mechanisms that regulate cell cycle progression. A particularly well studied example is the universal control over entry into mitosis. This section will focus on main features of this control.

Studies of the genetic control over the cell cycle were originated using yeast cells, while a parallel biochemical approach was taken using various invertebrate and vertebrate oocytes and eggs, particularly those of clams, sea urchins and *Xenopus*. Both genetic and biochemical approaches have now

been extended to a wider variety of organisms and cell systems (reviewed in: Nurse 1990; Norbury and Nurse 1992).

A breakthrough in understanding the mechanisms underlying the cell cycle came about when the serine/threonine protein kinase, cdc2, was found to be a key mitotic regulator, conserved from yeast to mammals. The *S.pombe* *cdc2*$^+$ gene was first isolated genetically, and was demonstrated to be absolutely required for entry into mitosis, as well as for regulation of its timing (Nurse et al. 1976; Nurse and Bisset 1981). Functional and structural homologues of cdc2 were subsequently found in all cell types examined. Most revealing was the demonstration that a cdc2 homologue is the active enzymatic component of MPF (Maturation Promoting Factor; Masui and Markert 1971), the biochemical entity responsible for induction of M phase and oocyte maturation in *Xenopus* (Dunphy et al. 1988; Gautier et al. 1988. The universal nature of cdc2 in controlling entry into mitosis was completely established when the human homologue of *cdc2* was cloned by complementation using a fission yeast *cdc2*$^+$ temperature sensitive strain, indicating an extensive functional overlap between the two diverse species (Lee and Nurse 1987).

The serine/threonine phosphorylation activity of cdc2 is thought to lead to the major mitotic events. cdc2 kinase activity is high in proliferating yeast and mammalian cells, but is much reduced in cells withdrawn from the cell cycle (Nurse 1990). It has been demonstrated in many cell types that the cdc2 kinase activity rises to a peak during M phase and falls to a low level during interphase (for examples see Draetta and Beach 1988; Moreno et al. 1989; Booher et al. 1989).

cdc2 is regulated both by physical interactions with other proteins and by post-translational modification. Prominent among cdc2 regulators are proteins called cyclins (reviewed in Norbury and Nurse 1991). Cyclins were first described as proteins that accumulate periodically during the rapid synchronous early division cycles of sea urchin embryos (Evans et al. 1983). Subsequently, it has become apperant that cyclins have an essential role in the initiation of mitosis. Cyclin B becomes complexed with cdc2 to form the heterodimer originally defined functionally as MPF (see above). The cdc2-cyclin B complex is now thought to be required for the initiation of M phase in all eukaryotes.

Besides the role of Cyclin B in the activation of cdc2 kinase activity, it is believed that cyclin B also influences cdc2 substrate specificity and subcellular localization (Booher et al. 1988; Alfa et al. 1990).

Another protein implicated in the regulation of cdc2 kinase activity is a product of a gene called *suc1*$^+$ in *S.pombe* (Hayles et at 1986; Hindley et al. 1987). Homologous genes have been identified also in *S.cerevisiae* and in vertebrates (reviewed in Nurse 1990). The function of the 13 kDa suc1 protein remains obscure, but genetic as well as biochemical studies demonstrate that the suc1 protein interacts physically with cdc2 (Brizuela et al. 1987) and that this interaction contributes to regulate cdc2 kinase activity (Draetta and Beach 1988).

Reversible phosphorylation of specific residues on cdc2 participates in regulation of the cdc2-cyclin activity. Substrates for these modifications are both cdc2 (Draetta et al. 1988; Gould and Nurse 1989) and cyclin subunits (Booher et al. 1989). Possible functions of phosphorylation on the cyclin subunit are less clear than those occurring on cdc2, and will not be discussed here (for review see Clarke and Karsenti 1991).

cdc2 is phosphorylated at several sites in a cell cycle dependent manner. These phosphorylation sites have been identified by a combination of genetic methods and phosphopeptide mapping. The most direct information about the role of phosphorylation of cdc2 in the regulation of the kinase activity stems from studies in *S.pombe* (Gould and Nurse 1989). In *S.pombe*, phosphorylation of Tyr15 negatively regulates cdc2 kinase activity: phosphorylation maintains the cdc2-cdc13 complex in an inactive form throughout S and G2, while removal of the inhibitory phosphate at the G2-M transition is absolutely required for activation of the cdc2 kinase activity. In vertebrates the phosphorylation of cdc2 kinase activity on Tyr15 is conserved. However, there is an additional inhibitory phosphorylation site on an adjacent residue, Thr14 (Krek and Nigg 1991; Norbury et al. 1991).

Another site which has been suggested to be phosphorylated in a cell cycle dependent manner is a conserved threonine residue of cdc2: Thr167 in *S.pombe* and Thr161 in human (Gould et al. 1991; Kerk and Nigg 1991; Nourbury et al. 1991). In contrast to the inhibitory function of Thr14/Tyr15 phosphorylation, Thr161/Thr167 phosphorylation is thought to be required for cdc2 kinase activity (Ducommun et al. 1991; Gould et al. 1991). Phosphorylation on Thr161 in *S.pombe* has been suggested to stabilise the binding of cdc2 to cyclin B (Gould et al 1991).

A recently completed X-ray crystal structure of the human cdk2 (closely related to the *S.pombe* and human cdc2 kinases) has mapped the Thr14 and Tyr15 residues at the ATP binding site. Thr160 (equivalent to Thr161/Thr167) has been mapped to a loop structure which appears to be acting as an auto-inhibitor of protein substrate binding (de Bondt et al. 1993).

While many features of cdc2 in the G2-M control are highly conserved, the role of this kinase in the G1-S transition is not. In fission and budding yeast, cdc2 (named cdc28 in budding yeast) plays a dominant role in regulation of both G1-S and G2-M transitions (Nurse et al. 1976; Nurse and Bissett 1981): cells carrying temperature sensitive mutations in the *cdc2*$^+$ gene become arrested in late G1 (at Start) or at the G2-M boundary on transfer to the restrictive temperature. In contrast, in higher eukaryotes additional diversity is apparent: the original cdc2 homologue, cdk1, has been suggested to operate in the G2-M transition alone (Th'ng et al. 1990; Hamaguchi et al. 1992), while related cyclin dependent kinases, such as cdk2 and possibly other cdks, act to regulate the G1-S transition (for review see Sherr 1993).

1.2 Cell cycle regulation in *S.pombe*

i Cell cycle mutants and cell cycle regulatory genes

Cell cycle studies in *S.pombe* have been largely based on the analysis of mutants. In the course of this analysis cell cycle mutants have been isolated that can fall into four broad categories: mutants which are blocked at a specific stage of the cycle; mutants that are defective in coordination of cell growth and cell division; mutants that are unable to maintain the dependency between cell cycle events; and mutants which show genetical interaction with already established cell cycle mutants.

In this section cell phenotypes which imply cell cycle defects and the gene products that can be altered to produce such phenotypes are discussed. An account of these gene products and their possible roles in cell cycle regulation under normal conditions will be given in the next section.

Cell cycle arrest in *S.pombe* typically leads to cell elongation, known as cdc phenotype (acronym for cell division cycle). Elongation occurs as little or no reduction in cellular growth rate takes place under condition of cell cycle

arrest (reviewed in: Fantes 1989; MacNeill and Fantes 1994). As cdc mutants confer a lethal phenotype, they have been isolated as conditional, usually, temperature sensitive mutants. Arrest may occur at different stages of the cell cycle and cdc genes can be classified according to their arrest point. These include genes required for entry into the cell cycle (Start); for initiation of DNA replication; for entry into mitosis and for septation (for examples see Fantes 1989). However, cell cycle arrest does not necessarily leads to cell elongation, as is the case for most mutants which block in mitosis itself (for example see Hirano et al. 1986).

Other types of mutations can alter the timing of entry into mitosis. Under conditions of rapid growth entry into mitosis is the rate limiting step for overall progression through the cycle (section 1.1 iii). Advancing the timing of entry into mitosis results in G2 being shortened and cells undergoing mitosis at a reduced size compared to wild type cells (Nurse 1975). The phenotype of abnormally small cells is referred to as wee (wee in Scottish means small). Controls can be altered to produce phenotypically small cells by altering the activity of the major mitotic regulator *cdc2*$^+$. Certain dominant hyperactive mutations in *cdc2*$^+$ result in a wee phenotype. Two such alleles are *cdc2-1w*, and *cdc2-3w* (Nurse and Theuriaux 1980; Fantes 1981). Cells of wee phenotype can also be produced by altering the activity of gene products that regulate cdc2 activity (see below section 1.2 ii).

Cell cycle mutations which break the normal dependencies of events in the cell cycle result in a variety of terminal lethal phenotypes. Cell cycle mutations that break the dependence of mitosis on the completion of the previous DNA replication have been identified under conditions which inhibit DNA synthesis, for example, in the presence of hydroxyurea (Enoch and Nurse 1990). Under such conditions wild type cells arrest reversibly in interphase, as highly elongated cells. In contrast, cell cycle mutants that allow entry into mitosis under condition of inhibition of DNA synthesis die in the presence of hydroxyurea, as they undergo cell division in the absence of DNA replication. Certain mutations of *cdc2*$^+$ or its regulators have been identified as defective in maintaining the normal dependency between DNA replication and mitosis (Enoch and Nurse 1990; Sheldrick and Carr 1993). In addition, mutations in number of genes which are otherwise non-essential for the regulation of mitosis have been speculated to be an integral part of a checkpoint mechanism that couple DNA synthesis and mitosis. The later

include certain *rad* (radiation-sensitive) and *hus* (hydroxyurea-sensitive) genes (Enoch and Nurse 1991; reviewed in Sheldrick and Carr 1993; MacNeill and Fantes 1994).

Cell cycle mutants in which the normal dependence of S-phase on the occurrence of the previous mitosis is perturbed can be identified by enlarged nuclei, indicating the content of twice or more the amount of DNA of a normal haploid cell. This phenotype reflects extra round(s) of replication without an intervening mitosis. Certain mutant alleles of *cdc2*$^+$ were first identified as being able to induce an extra round of replication in G2 cells, leading to diploidization (Broek et al. 1991). Overexpression of a recently isolated gene, *rum1*$^+$, has been found to cause a transient G1 delay followed by repeated rounds of DNA replication (Moreno and Nurse 1993). The investigation of the *rum1*$^+$ has only recently commenced, however, the findings suggest a role for *rum1*$^+$ in a pre-Start checkpoint control that restrains mitosis (Moreno and Nurse 1993).

Other genes have been identified by virtue of their interaction with known cell cycle regulatory genes. These include the genes *win1*$^+$; *wis1*$^+$-*wis5*$^+$ and *mcs1*$^+$-*mcs6*$^+$, all predicted to be involved in regulation of entry into mitosis. This prediction has been supported for several of the above genes by further studies (see below section 1.3).

ii Genetic network regulating entry into mitosis

Mutational analysis in combination with more recent biochemical studies have brought about a mechanistic model for the control over entry into mitosis in *S.pombe*. This section is concerned with a description of this model.

At the G2-M transition point cdc2 is complexed with cdc13, a G2 specific B-type cyclin (Booher and Beach 1987; Booher and Beach 1988). cdc13 is absolutely required for entry into mitosis and may have a role in directing cdc2 into the nucleus, or ensuring its retention within the nucleus (Booher et al. 1989; Alfa et al. 1990). Four other cyclin-like genes, *cig1*$^+$, *cig2*$^+$, *puc1*$^+$ and *mcs2*$^+$ have been isolated in *S.pombe* (Bueno et al 1991; Bueno and Russell

1993; Forsburg and Nurse 1991; Molz and Beach 1993) However, further studies are necessary to establish their cell cycle role.

The activity of the cdc2-cdc13 complex is regulated by the level of phosphorylation on Tyr15 residue of cdc2, determined by the balance of activity between specific inactivating protein tyrosine kinases, and specific activating protein tyrosine phosphatases (see Figure 1.2).

cdc25 protein is the major phosphatase thought to bring about activation of the cdc2-cdc13 complex by dephosphorylation of Tyr15 (Moreno et al. 1989; Millar et al. 1991). *cdc25*$^+$ is a dosage dependent inducer of mitosis: overexpression leads to entry into mitosis at reduced cell size, while temperature sensitive mutations result in a cdc phenotype and cells are arrested before mitosis (Fantes 1979; Russell and Nurse 1986). The role of *cdc25*$^+$ as a protein phosphatase was initially supported by limited sequence homology with a protein phosphatase isolated from vaccinia virus (Moreno and Nurse 1991). Subsequently, the biochemical role of cdc25 has been established by further studies, demonstrating that cdc25 has tyrosine phosphatase activity *in vitro* and is able to activate the cdc2 protein by Tyr15 dephosphorylation (Dunphy and Kumagai 1991; Millar et al. 1991).

Recently, the activity of another tyrosine phosphatase, pyp3, has been suggested to share overlapping function with cdc25. *pyp3*$^+$ encodes a protein tyrosine phosphatase which, based on sequence comparison, is only distantly related to cdc25. Overlapping function is supported by the finding that *pyp3* deletion exacerbates the phenotype of *cdc25* temperature sensitive mutations (Millar et al. 1992). In addition, pyp3 is capable of efficient dephosphorylation and activation of tyrosine-phosphorylated cdc2 *in vitro*. The extent to which pyp3 affects the regulation of entry into mitosis does not appear to be as dramatic as that of cdc25: a null mutation of the *pyp3*$^+$ gene does not result in a cdc phenotype but only in moderate elongation.

Two genes, *wee1*$^+$ and *mik1*$^+$, have been identified as negative regulators of cdc2-cdc13 activity by promoting phosphorylation of the Tyr15 residue of cdc2 (Russell and Nurse 1987a; Lundgren et al. 1991). The gene product of *wee1*$^+$ belongs to a rare class of protein kinase capable of phosphorylation of both tyrosine and serine residues, and there is strong evidence to suggest that it directly phosphorylates cdc2 on Tyr15 (Featherstone and Russell 1990; Parker et al 1991). The *mik1*$^+$ gene shares extensive sequence homology with *wee1*$^+$ (Lundgren et al. 1991). Loss of

either *wee1*$^+$ or *mik1*$^+$ activity is not lethal, though loss of both activities leads to rapid dephosphorylation of Tyr15 and to mitotic catastrophe, an apparently unregulated entry into mitosis, which is lethal (Lundgren et al 1991). These findings suggests that wee1 and mik1 share an overlapping function which is required for Tyr15 phosphorylation of cdc2. The *wee1*$^+$ gene is a dosage dependent inhibitor of mitosis: loss of *wee1*$^+$ function advances mitosis, while overexpression delays mitosis (Russell and Nurse 1987a). Loss of *mik1*$^+$ function has no effect upon cell length at division (Lundgren et al. 1991).

The activity of cdc25 and wee1 is subject to further regulation by phosphorylation. In *S.pombe*, the evidence for such regulation is better understood for the wee1 protein kinase (illustrated in Figure 1.2). The *nim1*$^+$ gene (allelic to *cdr1*$^+$; Young and Fantes 1987) encodes a protein kinase (Russell and Nurse 1987b), and there is strong evidence to suggest that it negatively regulates the wee1 protein by phosphorylation of one or more serine residues within the catalytic domain of wee1 (Coleman et al. 1993; Parker et al. 1993; Wu and Russell 1993).

More recent is the suggestion that the *pyp1*$^+$ and *pyp2*$^+$ genes promote wee1 kinase activity by tyrosine de-phosphorylation (Millar et al. 1992; Ottile 1992). *pyp1*$^+$ and *pyp2*$^+$ encode tyrosine phosphatases and genetical analysis suggest that they function to delay the timing of mitotic onset through the wee1 inhibitory pathway (Millar et al. 1992). Although genetical indications suggest that of pyp1 and pyp2 act directly on the wee1 protein, this suggestion awaits further support by biochemical evidence.

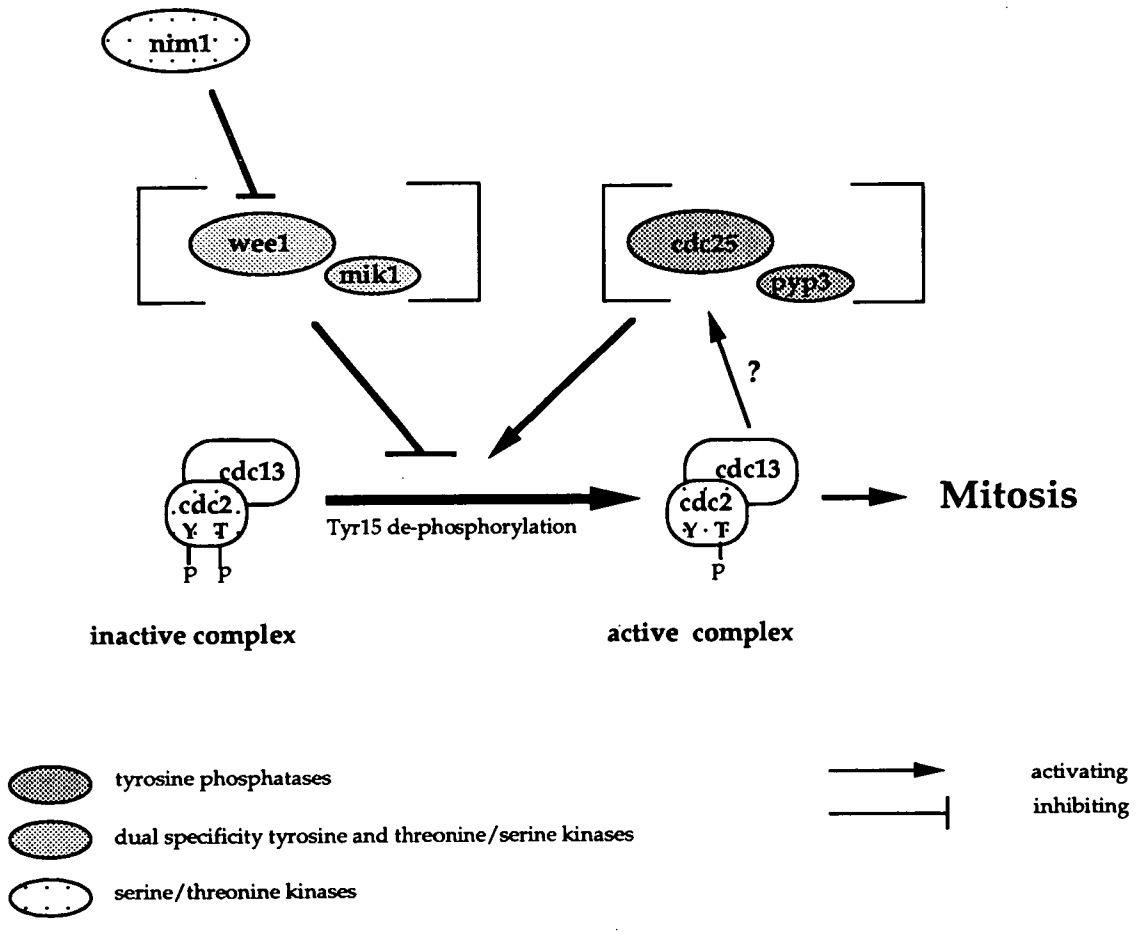
Regulation of the activity of cdc25 by phosphorylation is not well understood in *S.pombe*, although it is known to be a phosphoprotein. *S.pombe* cdc25 and its higher eukaryote homologues contain potential cdc2 phosphorylation sites, an observation that has raised the possibility that cdc2 has a role in activating cdc25 via a positive feedback loop (reviewed in Murray 1993). Phosphorylation of *Xenopus* and human cdc25 homologues was demonstrated to be associated with activation of cdc25 (Kumagai and Dunphy 1992). Anti cyclin B immunoprecipitates from mammalian cells were demonstrated to have kinase activity towards human cdc25 (Hoffmann et al. 1993). Furthermore, mutations in *Xenopus* cdc25 that eliminated conserved cdc2 phosphorylation sites prevented activation associated with *in vitro*

Figure 1.2 Current model of regulation over mitosis

The timing of activation of the cdc2-cdc13/cyclin mitotic kinase complex is determined by the relative activity of two antagonistic, inhibitory and activating, pathways (see text for details, section 1.2 ii).

Protein kinases and phosphatases are shaded according to their specificity for tyrosine or threonine/serine residues, based on sequence homology comparisons and biochemical assays.

Proteins in brackets indicate an overlap in function: proteins shown as a larger oval exhibit a more dramatic effect in mitotic regulation as demonstrated by mutational analysis (see text for details). Y indicates cdc2 Tyr15; T indicates cdc2 Thr167.



phosphorylation by cdc2 and failed to induce M phase in oocyte extracts (Izumi and Maller 1993).

cdc25 is also regulated at the level of its expression: the levels of both the *cdc25*$^+$ mRNA and cdc25 protein fluctuate modestly during the mitotic cell cycle of *S.pombe*, reaching a maximum at the G2-M transition point (Moreno et al. 1990; Ducommun et al. 1990).

Finally, phosphorylation of cdc2 on Thr167 residue of cdc2 is required for tight cyclin binding and activation of the cdc2 kinase activity (Gould et al. 1991). The protein kinase(s) and phosphatase(s) that are responsible for the level of Thr167 phosphorylation have not yet been identified yet in *S.pombe*, although a protein kinase called CAK (cdc activating kinase) has been isolated from both starfish and *Xenopus*, and been shown to phosphorylate Thr161, the equivalent site to Thr167, in higher eukaryotes (Fesquet et al. 1993; Poon et al. 1993; Solomon et al. 1993).

The phosphatase(s) that play a role in Thr167/161 dephosphorylation are yet to be established. *In vitro*, phosphatase type 2A (PP-2A) can dephosphorylate and inactivate cdc2 (Lee et al. 1991; Gould et al. 1991). However, it is not clear whether PP-2A acts in this way *in vivo*. In *S.pombe* cells, the ppa2 protein is responsible for the majority of PP-2A activity (Kinoshita et al. 1993). Deletion of *ppa2*$^+$ causes cells to divide at a reduced cell size (semi-wee phenotype), while overexpression causes delay in G2, reflected in an elongated phenotype (Kinoshita et al. 1993). This observation is consistent with ppa2 being a negative regulator of entry into mitosis. At present, the genetic analysis of interaction of *ppa2*$^+$ with other cell cycle genes is not conclusive, and cannot determine which of the mitotic control genes serves as a substrate for the activity of *ppa2*$^+$ gene product.

iii Cell cycle response to extracellular signals

Changes in the nutritional status exerts its effect on the cell cycle both at Start and at the G2-M transition point. The nutritional regulation at the G2-M transition exerts its effect on the critical size at which cells enter mitosis. This is clearly shown in experiments when cells are shifted between rich and poor media: transfer to poor media accelerates cells into mitosis, whereas transfer to rich media has the opposite effect (Fantes and Nurse 1977). The effect is most striking when exponentially growing cells are transferred to

nitrogen-free medium. Under these conditions, biomass accumulation is severely restricted, nevertheless, a large proportion of cells is stimulated into mitosis and division (Fantes 1977; Young and Fantes 1987).

Nutritional regulation is also apparent in cells undergoing transition into stationary phase. *S.pombe* cells that are grown to stationary phase under nitrogen-limiting conditions arrest in G1, whereas, cells grown under glucose limiting conditions arrest in G2 (Costello et al. 1986).

Several findings suggest that nutritional signals are monitored through the *wee1*$^+$ inhibitory pathway of cdc2. Mutations which abolish or reduce the activity of the wee1 kinase do not show any changes in the timing of mitosis after nutritional shifts (Fantes and Nurse 1978). Altered response to media shifts is also observed in *cdr1*$^-$ mutants, which arrest as highly elongated cells upon starvation (Young and Fantes 1987). Intriguingly, *cdr1*$^+$ was found to be allelic to *nim1*$^+$, the gene coding for a protein kinase which inhibits wee1 activity (Feilotter et al. 1991).

Of particular interest for the study of cell cycle response to extracellular signals is the possible role of MAP kinase(s) in such regulation (reviewed in Errede and Levin 1993). MAP kinases (or MAPK, for Mitogen Activated Protein Kinases) comprise a family of serine/threonine kinases. Like cdc2, the activity of MAP kinases is controlled by both tyrosine and threonine phosphorylation, although in this case both phosphorylations are required for activation (Clarke and Karsenti 1991). In *Xenopus*, the MAP kinase pathway is involved in oocyte maturation, while in mammalian cells activation of similar pathways can lead to proliferation or differentiation (reviewed in Errede and Levin 1993). In yeast similar pathways are involved in diverse aspects of cellular regulation. The best studied example is the mating factor signal transduction in *S.cerevisiae*. Other pathways in which MAP kinases have been implicated are protein kinase C mediated and high osmolarity glycerol (HOG) response pathways (reviewed in Ammerer 1994).

MAP kinase pathways are characterised by a phosphorylation cascade comprised of MAP kinase activators, named MAPKK (for MAPK kinases) and further upstream activating kinases named MAPKKK (for MAPKK kinases). Importantly, sequence analysis and studies of the hierarchy of MAP kinases and their activators showed a considerable conservation throughout evolution (Errede and Levin 1993).

A recent intriguing finding is the homology between the predicted protein product of *wis1*$^+$ and MAPKK (Warbrick and Fantes 1991). *wis1*$^+$ was

isolated as a multicopy suppresser of the cell cycle defect phenotype of the triple mutant strain *cdc25-22 wee1-50 win1-1* (the phenotype of this strain is discussed below, section 1.3). *wis1*$^+$ was demonstrated to be a dosage dependent activator of mitosis: overexpression results in reduction of cell size at division and deletion in moderate elongation (Warbrick and Fantes 1991). Strains deleted for *wis1*$^+$ are defective in their response to a variety of stress conditions, such as elevated growth temperature or medium of high osmotic strength (S. Stettler pers comm). In addition, the *wis1Δ* strains rapidly lose viability upon entry into stationary phase. Although the above observations may suggest that *wis1*$^+$ gene product play a role in mediating extracellular signals to the controls of the cell cycle via signal transduction pathways, the immediate targets of *wis1*$^+$ have not yet been identified, and the way *wis1*$^+$ may integrate signals into the cell cycle machinary awaits further elaboration.

1.3 *cdc25-22 wee1-50 win1-1* triple mutant

i The *win1-1* mutation

The *win1-1* chromosomal mutation was isolated in a screen that was designed for isolating genes that reverse the suppression of *cdc25-22* by *wee1-50* (Ogden and Fantes 1986). *cdc25*$^+$ is absolutely required for entry into mitosis but becomes dispensable in the absence of its antagonist *wee1*$^+$. The double mutant *cdc25-22 wee1-50* (both alleles are temperature sensitive mutations) is viable at the restrictive temperature, and cells are phenotypically wee (Fantes 1979). The *win1-1* mutation confers a conditional lethal phenotype in *cdc25-22 wee1-50* : on minimal medium and at the restrictive temperature, the majority of cells become extremely elongated, and are unable to form isolated colonies. In addition, a high proportion of the cells display the phenotype of branched or bent cells. Notably, the *win1-1* mutation exerts its effect only on minimal medium while growth on complex medium abolishes the effect. *win1-1* single mutant cells show moderate (30-50%) increase in length compared to wild type. This elongated phenotype is also strongly dependent on the medium composition, as described for the triple mutant *cdc25-22 wee1-50 win1-1*. The component of the growth media responsible for the sensitivity is not clear, although reduction of the elongated phenotype of *cdc25-22 wee1-50 win1-1* cells is observed when ammonium, the

usual nitrogen source in minimal medium, is replaced by amino acid mixtures (Warbrick 1990).

Temperature shift experiments indicated that *cdc25-22 wee1-50 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 (Warbrick 1990). Dominance relations suggested that *win1-1* is a recessive mutation (Ogden and Fantes 1986).

The *win1*$^+$ gene has not been cloned, but interactions of the *win1-1* mutation with other cell cycle regulatory genes suggest that the wild type allele has a role in the G2-M transition (Warbrick 1990). Notably, the *win1-1* mutation affects the terminal phenotype of *cdc13-117* mutant cells. *cdc13-117* mutants carry a temperature sensitive allele of the *cdc13*$^+$ gene, which encodes a cyclin type B protein. Such mutants show a 'mitotic' phenotype characterised by condensed chromosomes and high proportion of septated cells (Hagan et al. 1988). In contrast, *cdc13-117 win1-1* cells arrest with a terminal phenotype characteristic of G2, as majority of cells elongates without forming septa.

The wee mutations *wee1-50*, *cdc2-1w* and overexpression of *cdc25*$^+$ (see above section 1.2 i) are all capable of suppressing the increased cell length phenotype of *win1-1*. In contrast the combination of *win1-1* with *cdc2-3w* results in a phenotype more similar to that of *win1-1* (Warbrick 1990). These observations indicate that *win1-1* cells are still sensitive to both wee1 and cdc25 expression levels, and suggest an allele specific interaction between *win1-1* and *cdc2-3w*.

Like *win1-1*, the *mcs3-12*, *mcs4-13* and *mcs6-13* mutations are capable of reversing the suppression of *cdc25-22* by *wee1-50*. These chromosomal mutations were isolated by their ability to suppress the mitotic catastrophe phenotype of *wee1-50 cdc2-3w* (Molz et al. 1989). Although mutations of the *mcs* genes show a wide range of genetic interaction with other mitotic control genes the roles of the genes in cell cycle control await further characterisation at the molecular level.

The *win1-1* mutation is not capable of suppressing the mitotic catastrophe phenotype of *wee1-50 cdc2-3w*, and genetic analysis demonstrated that *win1-1* is not allelic to any of the above *mcs* mutations (Warbrick 1990). 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. The possibility that *mcs4*$^+$ and *win1*$^+$ may lie on the same pathway is supported by the similar phenotypes of the mutations and by the observation that the effect on cell length of these mutations is not additive.

ii Multicopy suppressors of *wee1-50 cdc25-22 win1-1*

In a large screen of *S.pombe* genomic libraries for plasmids able to complement the lethal defect of *cdc25-22 wee1-50 win1-1* five extragenic multicopy suppressors were isolated. Analysis of these plasmids has defined five new genes, designated *wis1*$^+$ to *wis5*$^+$ (for win suppression; Warbrick and Fantes 1992). Only *wis1*$^+$ and *wis4*$^+$ are capable of suppressing the *win1-1* phenotype in an otherwise wild type background.

wis1$^+$ has been further characterised (see above, section 1.2 iii). Two other genes, *wis2*$^+$ and *wis3*$^+$, show interaction with a range of mutations in a *wee1*^{ts} *cdc25*^{ts} genetic background, namely the suppression of the lethal phenotype caused by the combination of *wee1-50 cdc25-22* with *mcs3-12*, *mcs4-13* or *mcs6-13*. In contrast, the *wis1*$^+$ gene is capable of suppression the combination of *wee1-50 cdc25-22* with *mcs4-13* only. It has therefore been suggested that *wis2*$^+$ and *wis3*$^+$ may interact directly with elements central to the control on mitosis. The fourth, *wis4*$^+$, appears to be a specific suppressor of *win1-1*, as this gene is capable of reducing the cell size length of a single *win1-1* mutant strain but not the elongated phenotype of *mcs4-13* mutant strain. The *wis5*$^+$ exhibits genetic interactions with *cdc25-22 wee1-50 win1-1* only.

1.4 Cyclophilins

i Overview

Cyclophilins form a ubiquitous and highly conserved group of proteins. Members of the group have been found in all prokaryotic and eukaryotic cells investigated. In many cell types more than one cyclophilin gene is present and expressed (reviewed in Schreiber 1991; Sigal and Dumont 1992; Stamens el al. 1992; Galat 1993).

Cyclophilins display two properties. *In vitro* they catalyse the *cis-trans* isomerisation of peptidyl-prolyl bonds (PPIase activity). It has been proposed that *in vivo* cyclophilins accelerate rate limiting steps of folding/unfolding of cellular proteins, an action which they have been demonstrated to perform *in vitro*. Another proposal states that this enzymatic activity is not significant for protein biogenesis *in vivo*. Instead, the strong binding of cyclophilins to proline containing proteins implicates them in stabilising cellular proteins or assisting the correct non-covalent assembly of polypeptide containing structure *in vivo*, in a manner analogous to that of the non catalytic molecular chaperones.

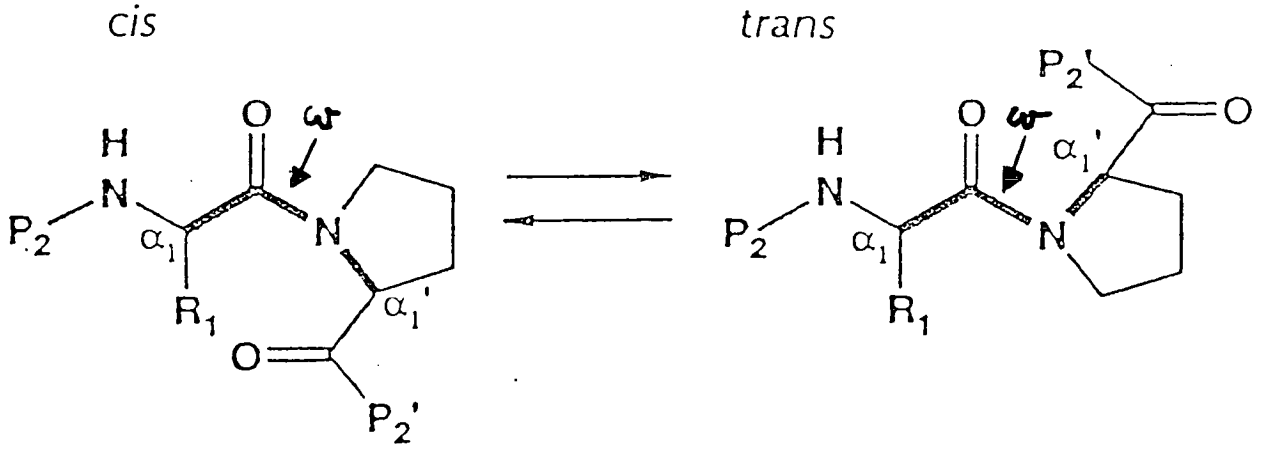
A second property was assigned to cyclophilins from an unrelated field of studies. It was found that cyclophilins serve as intracellular receptors for the immunosuppressive drug cyclosporin A (CsA). The drug-cyclophilin complex has been implicated in the disruption of Ca^{2+} mediated signal transduction pathways, initially in T-lymphocytes and subsequently in yeast cells.

ii Cyclophilins as PPIases: a role in protein biogenesis?

The enzymatic activity exhibited *in vitro* by cyclophilins is shown in Figure 1.3. The peptide bond has partial double bonded character. Rotation of this bond is therefore markedly restricted, but two configurations of the peptide bond are possible, one in which the C^{α} atoms are in *trans*, the other with them in *cis*. Proline containing oligopeptides and proteins in the unfolded and even in a few examples in the native state usually exhibit two slowly interconverting conformational states with respect to prolyl peptide bonds and the isomerisation of peptidyl-prolyl bonds have been

Figure 1.3 Reaction catalysed by peptidyl-prolyl *cis-trans* isomerase

The interconversion between the *trans* forms of peptidyl-prolyl bond is depicted. Rotation about the peptide (C'-N) bond is denoted by the torsion angle ω. (adapted from Hacker and Fischer 1993).



demonstrated to be a rate limiting step in the folding of proteins *in vitro* (Schmid et al. 1986).

The PPIase activity of cyclophilins can considerably accelerate *cis-trans* conversion in both directions. The catalysis of *cis* to *trans* interconversions is somewhat stronger. The PPIase activity of cyclophilins accelerates folding rates of oligopeptides by up to 300-fold *in vitro* compared with the uncatalysed reaction. Catalysis of *trans* to *cis* interconversions during the refolding of proteins is weaker but in some cases is still several orders of magnitude above the non-enzymatic reaction (reviewed in Hacker and Fischer 1993). The preferred substrate for cyclophilins *in vitro* is an oligopeptide in which the amino acid preceding the proline residue is alanine. The presence of glycine instead of alanine gives a poor substrate with a 10-fold lower catalytic constant (Hacker and Fischer 1993). PPIase activity for cyclophilins is routinely determined by using the tetrapeptide substrate *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide, an assay developed by Fischer et al. (1984).

The ability of cyclophilins to function as PPIase on peptides and proteins *in vitro* has led to the speculation that the biological function of cyclophilins *in vivo* is the acceleration of the rate of folding of cellular proteins. However, it is not clear whether the increase of the rate of protein folding *in vitro* is relevant within the cellular environment. An intriguing proposal is that the enzymatic activity of cyclophilins is a necessary consequence of the proline binding capability of these proteins, but is not functionally relevant *per se*. Binding of a proline-containing peptide or protein substrate may necessarily result in lowering of the activation barrier for proline isomerisation through stabilising a transition state conformation. It follows that the high affinity of cyclophilin for proline containing proteins may play a role in stabilising non-native conformations and facilitate protein trafficking in a fashion analogous to non-catalytic molecular chaperones (Schreiber and Crabtree 1992).

The cyclophilin whose biological role is best understood is the product of the *Drosophila* gene *ninaA* (Colley et al. 1991; Ondek et al. 1992; reviewed in Stamens et al. 1992). The *ninaA* gene was identified in a screen for mutants in *Drosophila* that display a visual defective phenotype. *ninaA* is expressed only in the retina and has been definitively implicated in the post-transcriptional regulation of the photopigment Rh1-rhodopsin. Rh1 is synthesised in the

endoplasmatic reticulum (ER) and transported via the secretory pathway to the rhabdomeres where it functions in visual transduction. Mutations in the *ninaA* gene lead to reduction in the level of rhodopsin. Notably, the small amount of rhodopsin present in the mutant flies fails to exit the ER and enter the Golgi stacks. The *ninaA* mutant phenotype is thus compatible with either proposal for the *in vivo* function of cyclophilins: the PPIase activity of the *ninaA* protein may be required for proper folding of rhodopsin; alternatively, it may act as a chaperone-like molecule escorting rhodopsin through the secretory pathway (Stamens et al. 1992).

Similar to *ninaA* , albeit far less well documented, is the activity suggested for human cyclophilins in their interaction with the Human-Immunodeficiency Virus Type 1 Gag protein. Human CyP-A and CyP-B proteins bind strongly to the viral Gag protein, and it has been speculated that they may be important for its proper folding or its targeting to the cell membrane (Luban et al. 1993).

iii Cyclophilin - cyclosporin A complex: disruption of signal transduction pathways

Cyclophilins display strong binding affinity to the immunosuppressive drug cyclosporin A (CsA). This feature was first observed by Handschumacher et al. (1984) who isolated a protein, which they named cyclophilin, by virtue of its high affinity to CsA. When a porcine enzyme peptidyl-prolyl *cis-trans* isomerase was sequenced it was realised that it was identical to the cyclophilin protein.

The drug CsA is a cyclic undecapeptide and a naturally occurring fungal secondary metabolite. CsA has revolutionised organ transplantation through its widespread use in the prevention of graft rejection. Because of this dramatic effect there has been much interest in deciphering the molecular mechanism of CsA immunosuppression (reviewed in: Schreiber and Crabtree 1992; Sigal and Dumont 1992; Galat 1993; Hacker and Fischer 1993). When bound to CsA, the PPIase activity is inhibited. However, it is now strongly believed that it is not the inhibition of the PPIase activity, but rather the strong binding affinity that is significant for mediating the immunosuppression effect (see below).

Another family of proteins, the FKBPs (an acronym for FK506 binding proteins) share with cyclophilins the ability to display PPIase activity. FKBPs are also known for the strong binding affinity to the immunosuppressant agents FK506 and rapamycin . Collectively, cyclophilins and FKBPs are known as immunophilins (reviewed in: Schreiber and Crabtree 1992; Kunz and Hall 1993; Galat 1993; Hacker and Fischer 1993).

Interestingly, cyclophilins and FKBPs show no structural similarities at the level of primary amino acid sequence or three dimensional structure (see Galat 1993; Hacker and Fischer 1993). The cognate immunosuppressive drugs of the immunophilins are also structurally unrelated. However, when cyclophilins and FKBPs are bound to CsA and FK506, respectively,they seem to interfere with the same step in the signal transduction pathway that lead to T lymphocyte cell proliferation.

The mechanisms by which the immunophilin-drug complex interfere with T-cell proliferation have been extensively investigated (see Figure 1.4, left panel). Briefly, T-cell activation includes the G0-G1 cell cycle transition that occurs in response to antigen binding to the T-cell receptor (TCR). The Ca^{2+}- dependent TCR signal transduction ultimately switches on transcription of several genes essential for T-cell proliferation, such as the gene encoding the lymphokine interleukin-2 (IL-2). T-cell activation involves not only IL-2 secretion but also expression of the lymphokine receptor IL-2R on the surface of the cell. After the binding of IL-2 to IL-2R, a lymphokine receptor (LKR) signal transduction pathway is activated. Transduction of this signal again proceeds through the cytoplasm to the nucleus, where a different set of genes from those activated by the TCR pathway, are transcribed. CsA and FK506 inhibit the TCR signal transduction pathway. Rapamycin, although structurally related to FK506, inhibits the LKR pathway (reviewed: Schreiber and Crabtree 1992; Sigal and Dumont 1992).

The PPIase activity of immunophilins is abolished upon binding to their cognate immunosuppressant. This finding first led to a model in which the PPIases are part of an intact TCR signal transduction pathway. At present the biological significance, if any, of the common features of PPIase activity is not clear, but it is strongly believed that it is not the inhibition of the enzymatic activity itself that is responsible for the immunosuppression. This has been realised by the findings that some drug analogues inhibit PPIase activity yet fail to suppress the immune response. This seemingly paradoxical

observation has recently been resolved. It is believed that the essential role of immunophilins is to form a complex with the drug and by doing this to present it to its biological target. The complex, but not the immunophilins or drugs alone, is capable of interaction with the biological target.

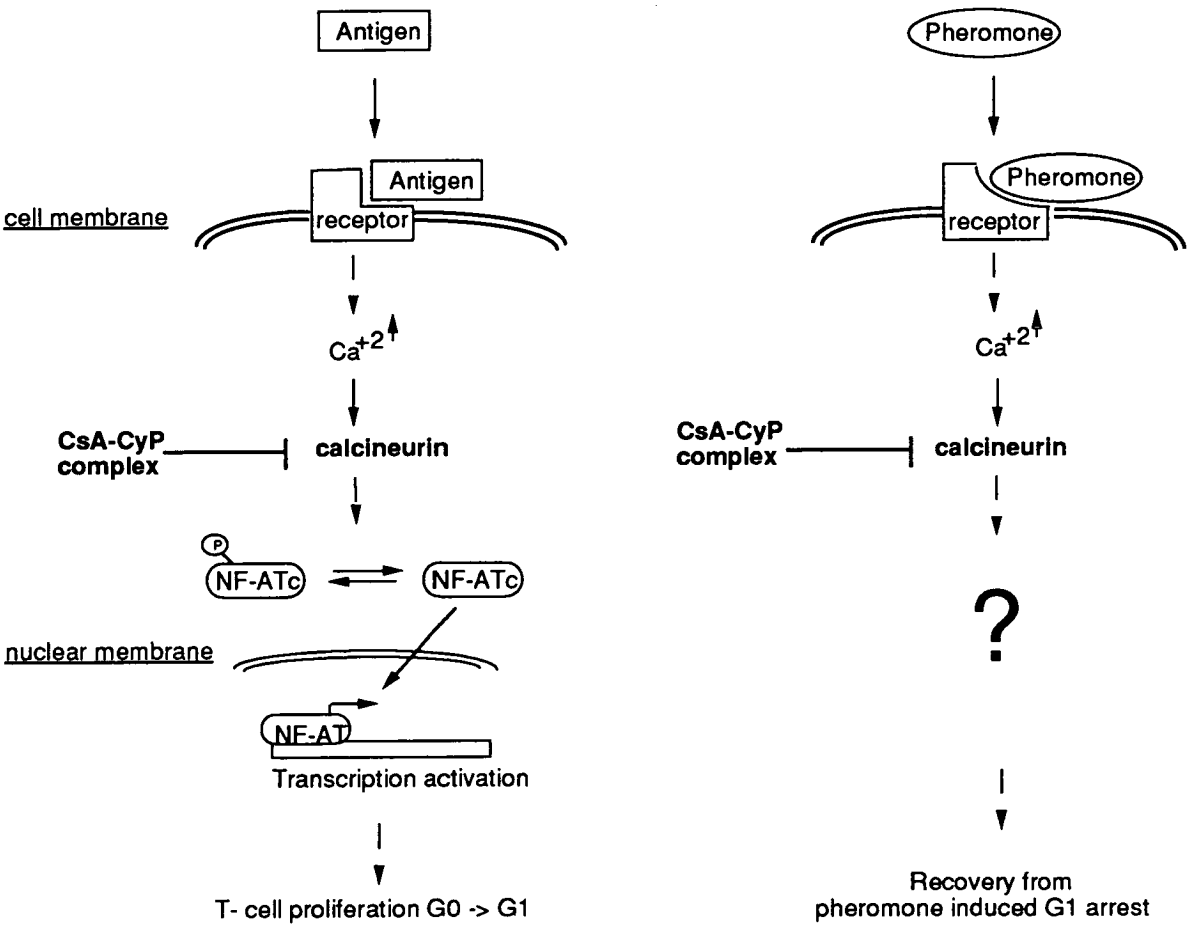
All available data strongly support the notion that the relevant target for the immunophilin-drug complex is the Ca^{2+}/calmodulin dependent phosphatase, calcineurin (Liu et al. 1991b; Cliptone and Crabtree 1992; O'keefe et al. 1992; Steiner et al. 1992; reviewed in: McKeon 1991; Schreiber 1992). The current model suggests that the immunophilin, when bound to the drugs acts in a gain-of-function manner to inhibit the calcineurin phosphatase activity. Dephosphorylation of transcription factors necessary for T-cell activation by calcineurin may be required for their nuclear translocation. The fact that the two immunophilin-drug complexes bind competitively to calcineurin suggests that they may bind to a common site. This is quite remarkable, given that there are no obvious structural similarities between the two immunophilins.

CsA, FK506 and rapamycin have effects on cells other than T lymphocytes. Relevant to the scope of this thesis is the effect seen in *S. cerevisiae*, in which effects of the drugs have been investigated. Rapamycin is extremely toxic for all laboratory *S. cerevisiae* strains tested (Kunz and Hall 1993). In contrast, CsA and FK506 are toxic for *S. cerevisiae* cells, albeit in a strain dependent way. In particular, it seems that most wild type strains of *S. cerevisiae* are naturally resistant to CsA, possibly due to permeability barriers in the cell membrane (Tropschug et al. 1989). At least in one case, it has been reported that the increased sensitivity to CsA segregates as a single recessive gene mutations when crossed to other yeast strains (Koser et al. 1991).

Five genes encoding cyclophilins and four genes encoding FKBPs have been identified in *S. cerevisiae* (reviewed by Kunz and Hall 1993). None of these genes is absolutely required for growth, moreover, simultaneous disruption of three of the cyclophilin genes (*CYP1*, *CYP2* and *CYP3*) and one of the FKBP genes (*RBP1*)resulted in viable cells indistinguishable from wild type cells. Notably, strains disrupted for certain cyclophilins or FKBPs become resistant to the corresponding drug. This observation gives further support to the mechanistic model in which it is not the PPIase activity that is responsible for the drug effect but rather that immunophilins are essential for targeting the drug to a third component. That only certain immunophilin disrupted alleles give rise to a resistance phenotype implies that some

Figure 1.4 Mechanisms of action and biological target of CsA-CyP complex

The illustration compares the modes of action of CsA-cyclophilin complex in human T lymphocytes and *S.cerevisiae* cells. In both cell types, the complex CsA-cyclophilin has been strongly suggested to inhibit the Ca^{2+}-dependent phosphatase, calcineurin. This inhibition results in disruption of a signal transduction pathways which normally lead to proliferation in T-cells and to recovery from α factor arrest in yeast (for details see text, section 1.4 iii).



members of this family are more readily complexed with the drug, or that only certain members can get access to the drug.

CsA and FK506 have an effect on sexual growth in *S. cerevisiae*. Both drugs block recovery from G1 arrest induced by mating pheromone. The inhibition of the mating response pathway occurs at drug concentrations much lower than those affecting vegetative growth (Foor et al 1992). The disruption of the mating transduction pathway in yeast resembles the disruption of the TCR signal transduction in T lymphocytes, at least in some aspects (see Figure 1.4). Notably, in both cases calcineurin is the target for the immunosuppressive drugs (Foor et al 1992). The high conservation, from yeast to mammals, of the interaction between the immunophilin-drug complexes and calcineurin may suggest that other elements of the Ca^{2+}/calcineurin-dependent signal transduction pathway may be also conserved.

Whether studies which employ the drugs CsA and FK506 are informative about the cellular function of cyclophilins and FKBPs in the absence of the drug is not clear. The implication of immunophilins in signal transduction pathways in the presence of the drug may reflect merely gain of function induced by the drug binding. Alternatively, if the drugs mimic the structure of an immunophilin cellular ligand, the effect seen with immunophilin-drug complex may reflect the true biological function of immunophilins under normal condition. A supportive observation for the second alternative is the finding that cyclophilins and FKBPs colocalize with calcium storage protein (Arber et al. 1992) and calcineurin (Steiner et al. 1992). However, no natural ligand of immunophilins has so far been identified.

The analysis of cyclophilin protein-protein interactions in the absence of the drug has only recently commenced. *In vitro* observations with yeast and rat liver protein extracts suggest that both cyclophilins and FKBPs bind the heat shock protein hsp90 (Nadeau et al. 1993). This association may indicate a role for immunophilins in protein folding as previously suggested (see section above), but it remains to be established whether the association of immunophilins with hsp90 does occur *in vivo*.

iv High resolution structure analysis

The three dimensional structure of human cyclophilin 18 kDa CyP-A protein has been solved by X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy, in the absence of ligand (Ke et al. 1992), when complexed with the proline containing tetrapeptide N-acetyl-Ala-Ala-Pro-Ala-amidomethylcoumarin and when complexed with the drug CsA (Kallen et al. 1991; Pflugl et al. 1993; Thériault et al. 1993).

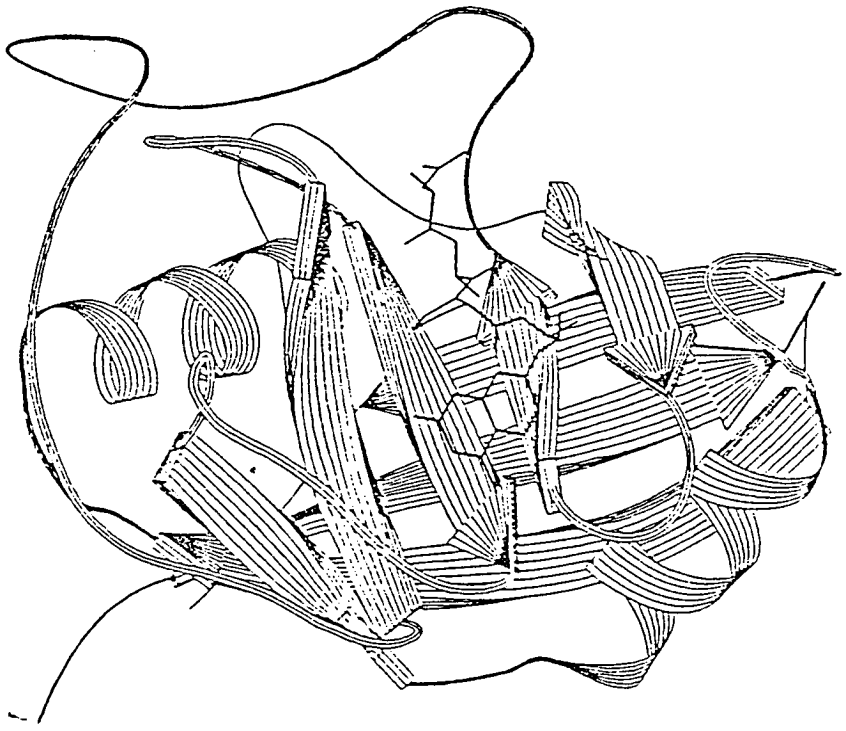
Human CyP-A is a roughly spherical molecule with a radius of about 17Å. The protein has an eight stranded antiparallel β barrel structure capped at each end by an α helix (Figure 1.5). The inner core of the molecule is tightly packed with hydrophobic residues. Other hydrophobic residues are located in the contact region of the two helices with the β barrel and in the ligand binding site.

Strikingly, CsA and the model tetrapeptide substrate bind to the same region of Hu-CyP-A (Thériault et al. 1993; Pflugl et al. 1993). The location of the binding site presents an unusual structural feature in that it lies on the protein surface. A binding pocket is formed between one face of the β barrel and a loop of 14 amino acids. It has been speculated that a portion of the bound CsA molecule mimics a twisted amide bond, similar to an intermediate state of the *cis* to *trans* peptidyl prolyl bond interconversion in the tetrapeptide N-acetyl-Ala-Ala-Pro-Ala-amidomethylcoumarin (Thériault et al. 1993).

Although CsA and the model tetrapeptide substrate are found to bind to the same site of the protein, decoupling of CsA binding from the PPIase activity has been demonstrated: while Trp 121 of Hu-Cyp-A is important for CsA binding it has little effect on prolyl isomerase activity (Liu et al. 1991b). Trp 121 is conserved in most eukaryotic cyclophilins reported to date. One of the few cyclophilin-like genes sequenced that does not have the a Trp at this position is the *E.coli* CypA (Liu et al. 1991a), in which it is replaced by Phe. The PPIase activity of CypA is comparable with that of Hu-Cyp-A, but is far less sensitive to inhibition by CsA. When the Phe *E.coli* CypA which aligns with the Trp121 region of Hu-Cyp-a was replaced by Trp, a 23 fold increase in CsA sensitivity was observed. Likewise when Trp121 of Hu-Cyp-A was mutated to Ala, the sensitivity of PPIase activity to CsA was decreased by 200 to 400 fold (Liu et al. 1991b; Bossard et al. 1991).

Figure 1.5 Overall folding of human cyclophilin

A ribbon plot illustrating the overall folding of human cyclophilin bound to the model tetrapeptide substrate, N-acetyl-Ala-Ala-Pro-Ala-amidomethylcoumarin (taken from Kallen et al 1991; Thériault et al. 1993). The tetrapeptide ligand binds in a long deep groove located on the protein surface as described in the text. Significantly, the overall fold of the human cyclophilin is the same when bound to CsA or when unbound.



Finally, a possible intermolecular interaction of cyclophilins which result in the formation of an oligomer has been recently suggested. Solving the crystal structure of human CyP-A complexed with CsA revealed a decameric cyclophilin- CsA complex (Pflugl et al 1993). The decameric complex is composed of two pentamers of 1:1 cyclophilin: CsA molecules. The pentamers associate in pairs, sitting on top of one another. The biological significance of the pentamer is as yet unclear but it seems that it is not merely an artefact of the crystallisation process.

v Cyclophilin subgroups

The most common type of cyclophilins are ~18 kDa soluble cytosolic proteins containing only the highly conserved 'core' domain responsible for the PPIase and CsA binding activities. They are the most ubiquitous and highly conserved class of cyclophilins, showing over 70% amino acid sequence similarity from organisms as divergent as human and yeast.

The second group of cyclophilins to be identified consists of cyclophilins which contain relatively short N- and C- terminal extensions (20-30 amino acids). These extensions were predicted to be involved in subcellular localization (reviewed by Stamnes et al. 1992), as they include sequences that encode signals for targeting to the secretory pathway (human CyP-B, *S.cerevisiae* Cyp2 and others) or to the mitochondria (*N.crassa* CyP-A), and hydrophobic sequences that anchor them into the membrane of the endoplasmatic reticulum (ER) and the transport vesicle (*D.melanogaster* NinaA *S.cerevisiae* Cyp3). Thus, cyclophilins specific for the mitochondria, the lumen of the ER and secreted forms of cyclophilins have been identified. Notably, no nuclear specific cyclophilin candidate is known (Stamnes et al 1992; Galat 1993). In multicellular organisms, some members of the cyclophilin family are tissue specific (reviewed by Galat 1993).

Recent studies which aimed at isolating more members of the cyclophilin family revealed that the core domain of cyclophilin may exist as a domain within a structurally complex protein (reviewed by Galat 1993). For example, the sequence of a 160 kDa mouse protein specific to killer cells has been shown to contain at its N terminus a cyclophilin segment. The protein has three charged domains which show sequence similarity to histone, and three other domains rich in serine, arginine and lysine (Anderson et al. 1993).

It is worth noting that in parallel to the rapid identification of new members of the cyclophilin family, the FKBP family is also expanding, showing similar structural variability.

Chapter 2: Sequence analysis of *wis2+*

2.1 Introduction: pwis2 genomic clone and wis2 activity

The plasmid pwis2-C3 was isolated from a *S.pombe* genomic library as a multicopy suppresser of the conditional lethal phenotype of the triple mutant strain *wee1-50 cdc25-22 win1-1* (Warbrick and Fantes 1992). The lethal phenotype is only exhibited at 35°C on minimal medium (Ogden and Fantes 1986). In this restrictive condition the majority of cells are highly elongated, and show aberrant morphology of branched and bent cells. In addition, cells are unable to form isolated colonies at low plating density ($<5x10^6$ cells per plate), although, bulk growth does occur at higher cell densities.

pwis2-C3 was isolated from a *Sau3A* gene library constructed in the yeast multicopy vector pDB248. The functional region of pwis2-C3 was delimited by combination of subclone analysis and transposon mutagenesis (Warbrick and Fantes 1992). The potential *wis2+* gene did not correspond to any mitotic control genes that had been previously described, as was shown by combination of restriction map comparison, complementation tests and genetic mapping (Warbrick and Fantes 1992).

Suppression of the triple mutant phenotype by pwis2-C3 is detectable by the ability of the transformed cells to form isolated colonies on minimal medium at 35°C. The suppressed phenotype is also distinct at the microscopic level (see Figure 2.1). The majority of cells divide at a reduced cell length compared with the unsuppressed phenotype, but the cells are still longer than that of wild type cells (~1.5 fold increase). Suppression is also exhibited in cell morphology, as cells are of normal shape compared with the morphological abnormalities of the unsuppressed phenotype.

The suppression activity described above will from now on be referred to as wis2 activity.

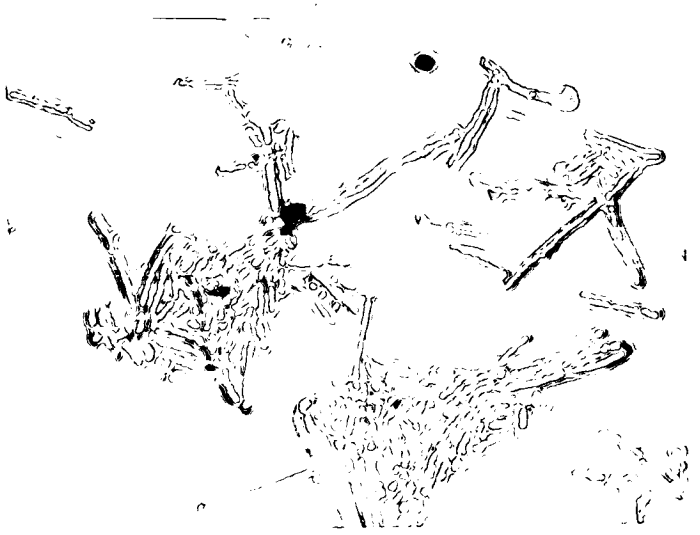
This chapter describes the determination of the nucleotide sequence of the *wis2+* gene. The strategy was to start sequencing within the predicted functional region. The sequence analysis was extended until a defined open reading frame (ORF) was apparent. Sequence analysis alone was not sufficient for unambiguous definition of an ORF. Therefore, the final

Figure 2.1 Suppression of *cdc25-22 wee1-50 win1-1* phenotype by pwis2

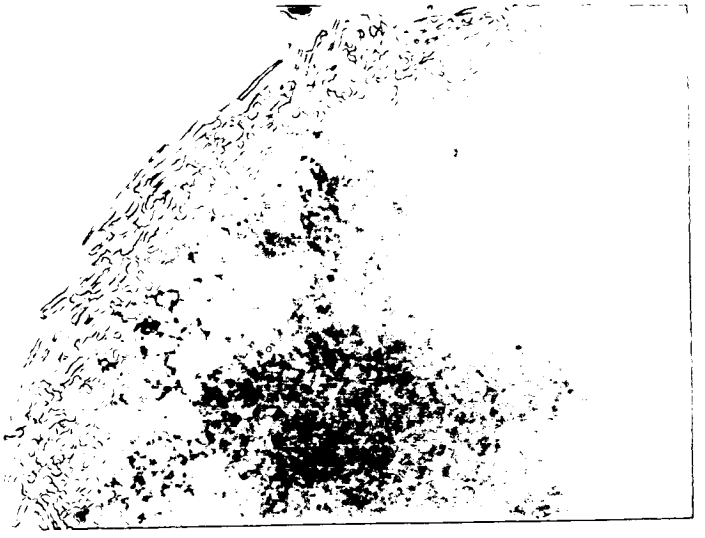
The unsuppressed phenotype of *cdc25-22 wee1-50 win1-1 leu1-2* transformed with pIRT2 alone (A) is compared with the suppressed phenotype resulting from overexpression of pIRT2/wis2-1 (B).

Cells are grown on solid minimal media at 35°C. The unsuppressed phenotype is characterised by elongated and branched cells, whereas the suppressed phenotype is characterised by the ability to form colonies (edge of a growing colony is shown), albeit cells are slightly longer than wild type cells.

A



B



determination of *wis2*$^+$ ORF was confirmed by analysis of *wis2*$^+$ cDNA sequences, as well as *in vivo* assays determining the ability of various subclones to confer wis2 activity.

The determination of the *wis2*$^+$ ORF is followed by comparative sequence analysis of the predicted polypeptide product.

2.2 Sequence analysis of *wis2*$^+$ genomic clone

i Experimental strategy

Previously, a 5 kb fragment of pwis2-C3 that was expected to contain the *wis2*$^+$ gene had been subcloned into pTZ18R (Warbrick and Fantes 1992). This subclone was initially named pTZ/SC3-16 but in order to simplify the terminology it will be referred to as pTZwis2-1. A restriction map of pTZwis2-1 is shown in Figure 2.2.

To confirm that the wis2-1 fragment contained the functional region of *wis2*$^+$ this fragment was cloned into the yeast autonomously replicating vector pIRT2. The resultant plasmid, pIwis2-1, showed the ability to confer wis2 activity.

To prepare a series of subclones suitable for sequencing, nested deletions were made of pTZwis2-1 following the method of Henikoff (1984) as described in Materials and Methods (section 7.6 i). The *Bgl*II site which lies within the region of wis2 activity was used to provide the susceptible end for Exonuclease III digestion. This strategy resulted in nested deletion clones that allowed sequencing to the right hand side of the *Bgl*II site, where *wis2*$^+$ was expected to lie.

Additionally, five subclones generated by restriction digestion were used for sequencing. Whenever a restriction site was used for creating subclones for DNA sequencing, the site was sequenced across using at least one other subclone.

Where no subclones or nested deletions were available, synthetic oligonucleotides were used as primers to initiate sequencing reactions.

The position and orientations of deletions, subclones and oligonucleotides used in the sequencing strategy are summarised in Figure 2.2B.

Figure 2.2: Restriction map of pTZwis2-1 and sequencing strategy

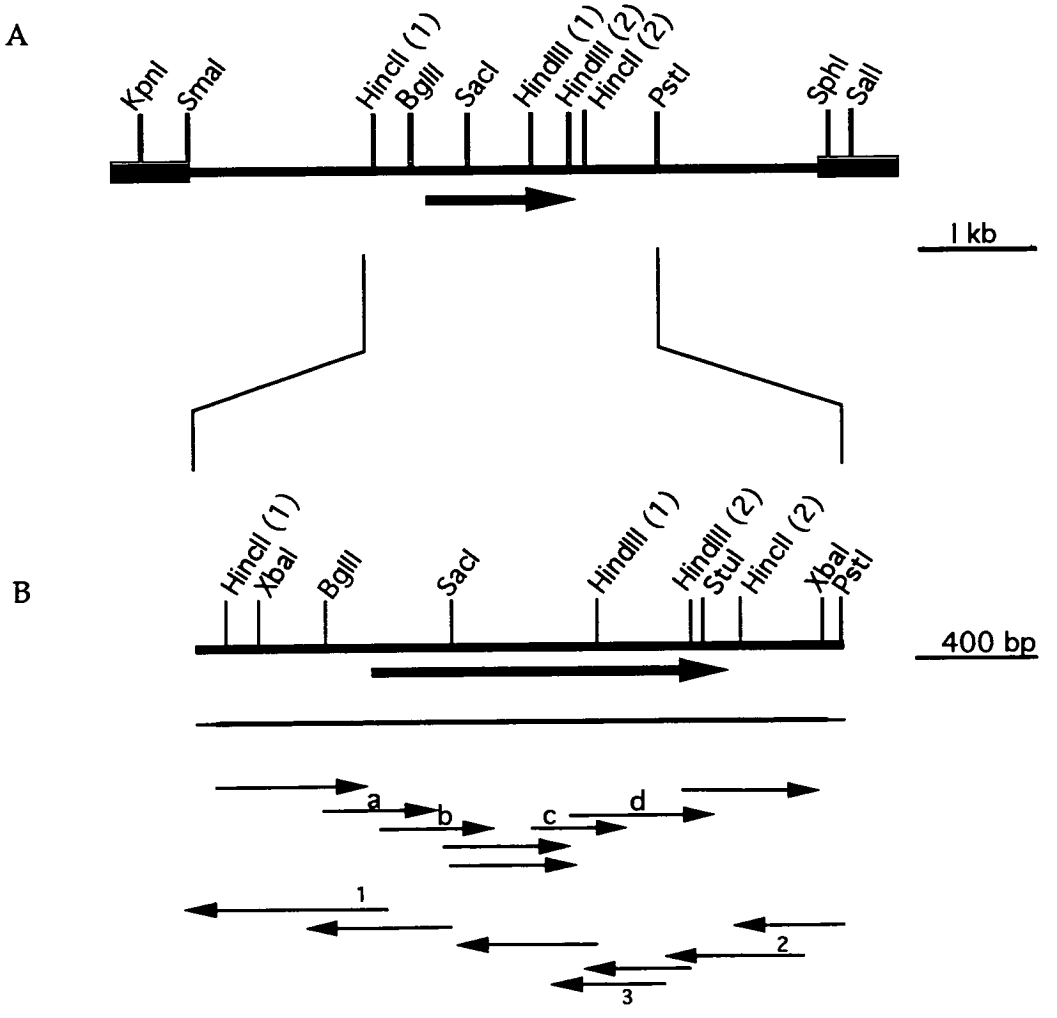
A. Outline map of restriction enzymes that cut pTZwis2-1.

Restriction sites within the pTZwis2-1 clone are indicated. Dark boxes indicate vector sequence. The predicted *wis2*$^{+}$ open reading frame is indicated by the thick arrow.

B. Sequencing strategy for pTZwis2-1.

A restriction map of a region expanded from (A) is shown. The region sequenced on both strands (line) and one strand (thin line) is indicated.

Thin arrows indicate orientation and approximate length of each subclone sequenced. Numbers indicate oligonucleotides (see Appendix A for details). Letters indicate ExoIII subclones. Remainder indicate direct subclones of the following fragments: 0.3 kb *Hin*dIII; 0.5 kb *Hin*dIII-*Pst*I; 1.6 kb *Hin*cII and 1.2 kb *Sst*I-*Pst*I.



Once suitable subclones were generated, they were used for the generation of single stranded or double stranded DNA (Materials and Methods, section 7.6 ii) Sequence was determined using the dideoxy chain termination method (Sanger et al. 1977; section 7.6 iii).

The sequences were assembled to form the whole continuous sequence, using Mac vector Sequence analysis software. DNA was sequenced on both strands for 1750 nt, from the left-hand *Hin*cII(1) site to the *Pst*I site. Additionally, DNA was sequenced on one strand for a further 80 nt upstream of the *Hin*cII(1) site and for 135 nt upstream of the *Pst*I site (Figure 2.2B).

The sequence analysis confirmed the presence and distribution of restriction enzyme recognition sites previously mapped by digest analysis. It also revealed the presence of other restriction sites which in some cases were useful for further subcloning (Figure 2.3).

ii Sequence and identification of ORF

Computer analysis of the sequence obtained revealed an ORF of 1068 nt starting 130 nt downstream from the *Bgl*II site. The putative start codon (ATG) most likely to initiate translation is indicated in Figure 2.4. No other ORFs above 200 nt long are present in either orientation within the sequenced region. The ORF terminates with a stop codon (TAA) 1068 nt downstream of the proposed start codon.

The length of the predicted ORF is consistent with the approximate size of 1500 nt of the *wis2*$^+$ transcript detected by Northern blot analysis (see section 3.1). Translation of the suggested ORF results in a putative polypeptide of 365 amino acids with a relative molecular weight of 40.1 kDa. The predicted molecular weight of the translated product is consistent with the detection of ~40 kDa protein by Western blot analysis (section 4.2 iii).

No sequence matching that of the 5' consensus for *S.pombe* introns (GTANG) was found within the proposed ORF. The presence of a 5' consensus splice site was identified upstream of the suggested initiating ATG codon, however, the presence of an intron at the *wis2*$^+$ 5' end was experimentally ruled out (see below, section 2.3).

Figure 2.3 Nucleotide sequence of the *wis2*$^{+}$ ORF and flanking sequences

Nucleotides are numbered relative to the predicted start codon. Sites cut by restriction enzymes referred to in the text are underlined, and predicted start and stop codons are in bold typeface.

```
-473  TACATTGCTGAAGACTCATCGAAATATGTTTAAAACTATTAGAAACACAATTTTGTGTTT  -414
                    HincII (1)
-413  TAAAATTTAAATACATTGTTGACTTCACTCAACAGCACTTATATTTCTTTATAGTCCGTG  -354
-353  TGTGCTAACTTAAATCAGGTATTTTATGAATTTTTAACGAAGAAGCAATCTAATTGAAAA  -294
-293  AAGTATAAATATTTTTTGGTCTAATAAAAAGTCAAATTTTCTTCTGCTAGCATTTTTTTA  -234
          XbaI
-233  ATACAGTATGTTTCTAGAAGTTTCGTAGAAAATCTCTTCGCAGTAGGTTGCAAATATAAA  -174
                                          BglII
-173  TCACCACCTTTTTAATAACTAAAACTAAATAGTTTGCACAGATCTGTATATTTCGTCTGC  -114
-113  GTAAAATCGCTATTCATTCTTCTTCTTAAAGCAACTTTGTGGTGTGAATACACTGAGGAG   -54
 -53  ACTGGCAGTGAAATATCATTACTTAAAAACGATCATTGTTTTTTTTTTGCACGAAATGAGT     6
   7  ACTTACGCCTATTTTAAAATTAGCATTGATGGTAAAATTCAACCAACAATCTACTTTGAA    66
  67  CTTTTCGACAACGTAGTTCCCAAAACTGTCAAAAACTTTGCTTCGTTGTGTAATGGGTTT   126
 127  GAGAAGGATGGCCGTTGTTTAACCTACAAAGGTTCAAGGTTCCATCGAGTAATTAAGAAC   186
 187  TTTATGCTTCAAGGTGGTGACTTTACTCGTGGTAATGGAACGGGTGGAGAAAGCATCTAC   246
                                  SstI
 247  GGCGAGAAGTTTGAGGACGAGAACTTTGAGCTCAAGCATGATAAGCCTTTCTTGCTTTCC   306
      BalI
 307  ATGGCCAATGCCGGCCCTAATACCAATGGATCTCAGTTCTTCATTACTACTGTTCCCACC   366
 367  CCTCATTTGGATGGAAAGCATGTTGTTTTTGGCAAGGTAATTCAAGGAAAATCTACTGTT   426
 427  CGTACCATTGAAAATTTGGAGACCAAAAATGACGATCCGGTTGTACCTGTTGTTATTGAA   486
 487  GAATGTGGAACTTGCACAAAGGATCAGATTGAGGCACCAAAGCCAGACGTTACTGGTGAT   546
 547  TCTCTGGAAGAATTTCCTGATGATTACGAGGGTGATAAGTCCGAGACTGCCATTTTCAAA   606
 607  ATTGCTAGCGACTTAAAGGGAATTGCTAACAAGCAATTTGCTCAGCAAAATTTGGATACA   666
                HindIII (1)
 667  GCCGTTGCCAAATGGCAAAAAGCTTTGCGCTACCTAATGGAATACCCTGTTCCCAACGAT   726
 727  GACTCTAAGGAATCCCCTGATTTCTGGAAAGAGTATAATGCTTTACGATACAGCATATAC   786
 787  GCAAACCTTGCCCTTGTAGCTTTGAAGCAGAACAAACCTCAGGAAGCTATTCGAAACGCC   846
 847  AACATTGTAATTGAGGCCAGTAACTCTACTGAGTTAGAGAAACAGAAAGCATACTATCGT   906
 907  TTGGGTTGTGCTCAAGGTCTCTTGAAGAATTTTGAAGAATCAGAGAAAGCACTTGCTAAG   966
                              HindIII (2)
 967  GCTGGTAACGATCCTGCAATTTCAAAGAAGCTTGCAGAAATTCGTCAAAAGAAAAAAGAT  1026
                StuI
1027  TATAAGAAGCGCCAACAAAAGGCCTATGCTAAGATGTTTCAGTAATTCCAAATTAATCGG  1086
1087  CGTCACGTGAATGCTGAGAAGTCAATTATGTTTTACGTTAATTTCTAGTTAGTCAATGAG  1146
                                    HincII (2)
1147  AGTTTTATCGCATTTACAAAATAACCATAGAAGGTCAACTACGTTGCGAGAAAGAGTGCA  1206
1207  AGATTTACTTATGGAGGATCTGGCTTCGTTTGTAAGACTGGTATGCCTACGTTTGCGTTT  1266
                                    XbaI
1267  CATACAGCAAAGTATCATCATGAATAGAAAGAACTCTAGAAACAAATGTGCATCAGTAAA  1326
1327  CTCAATAAGAATATCATATTTAAACATTTAATTCATTAAAATTAAATAAGACCGTCTCTT  1386
1387  ACCAAGTGTCGAAATTACATGAGTTTCCGCATTACCCTTCCTAAGGATGAGAGTGCTAAT  1446
                PstI
1447  GTGAACCCGCTAATACGAATCTGCAG  1472
```

Figure 2.4 ***wis2*$^{+}$ nucleotide and predicted amino acid sequence**

Nucleotide and amino acid sequence are numbered relative to the predicted start codon. A putative TATA box, in frame stop codons upstream of the predicted ORF and predicted start and stop codons are shown in bold.

```
-473  TACATTGCTGAAGACTCATCGAAATATGTTTAAAACTATTAGAAACACAATTTTGTGTTT  -414
                        HincII (1)
-413  TAAAATTTAAATACATTGTTGACTTCACTCAACAGCACTTATATTTCTTTATAGTCCGTG  -354
-353  TGTGCTAACTTAAATCAGGTATTTTATGAATTTTTAACGAAGAAGCAATCTAATTGAAAA  -294
-293  AAGTATAAATATTTTTTGGTCTAATAAAAAGTCAAATTTTCTTCTGCTAGCATTTTTTTA  -234
-233  ATACAGTATGTTTCTAGAAGTTTCGTAGAAAATCTCTTCGCAGTAGGTTGCAAATATAAA  -174
-173  TCACCACCTTTTTAATAACTAAAACTAAATAGTTTGCACAGATCTGTATATTTCGTCTGC  -114
-113  GTAAAATCGCTATTCATTCTTCTTCTTAAAGCAACTTTGTGGTGTGAATACACTGAGGAG  -54
 -53  ACTGGCAGTGAAATATCATTACTTAAAAACGATCATTGTTTTTTTTTGCACGAAATGAGT    6
                                                          M  S        2
   7  ACTTACGCCTATTTTAAAATTAGCATTGATGGTAAAATTCAACCAACAATCTACTTTGAA   66
   3  T  Y  A  Y  F  K  I  S  I  D  G  K  I  Q  P  T  I  Y  F  E    22
  67  CTTTTCGACAACGTAGTTCCCAAAACTGTCAAAAACTTTGCTTCGTTGTGTAATGGGTTT  126
  23  L  F  D  N  V  V  P  K  T  V  K  N  F  A  S  L  C  N  G  F    42
 127  GAGAAGGATGGCCGTTGTTTAACCTACAAAGGTTCAAGGTTCCATCGAGTAATTAAGAAC  186
  43  E  K  D  G  R  C  L  T  Y  K  G  S  R  F  H  R  V  I  K  N    62
 187  TTTATGCTTCAAGGTGGTGACTTTACTCGTGGTAATGGAACGGGTGGAGAAAGCATCTAC  246
  63  F  M  L  Q  G  G  D  F  T  R  G  N  G  T  G  G  E  S  I  Y    82
 247  GGCGAGAAGTTTGAGGACGAGAACTTTGAGCTCAAGCATGATAAGCCTTTCTTGCTTTCC  306
  83  G  E  K  F  E  D  E  N  F  E  L  K  H  D  K  P  F  L  L  S   102
 307  ATGGCCAATGCCGGCCCTAATACCAATGGATCTCAGTTCTTCATTACTACTGTTCCCACC  366
 103  M  A  N  A  G  P  N  T  N  G  S  Q  F  F  I  T  T  V  P  T   122
 367  CCTCATTTGGATGGAAAGCATGTTGTTTTTGGCAAGGTAATTCAAGGAAAATCTACTGTT  426
 123  P  H  L  D  G  K  H  V  V  F  G  K  V  I  Q  G  K  S  T  V   142
 427  CGTACCATTGAAAATTTGGAGACCAAAAATGACGATCCGGTTGTACCTGTTGTTATTGAA  486
 143  R  T  I  E  N  L  E  T  K  N  D  D  P  V  V  P  V  V  I  E   162
 487  GAATGTGGAACTTGCACAAAGGATCAGATTGAGGCACCAAAGCCAGACGTTACTGGTGAT  546
 163  E  C  G  T  C  T  K  D  Q  I  E  A  P  K  P  D  V  T  G  D   182
 547  TCTCTGGAAGAATTTCCTGATGATTACGAGGGTGATAAGTCCGAGACTGCCATTTTCAAA  606
 183  S  L  E  E  F  P  D  D  Y  E  G  D  K  S  E  T  A  I  F  K   202
 607  ATTGCTAGCGACTTAAAGGGAATTGCTAACAAGCAATTTGCTCAGCAAAATTTGGATACA  666
 203  I  A  S  D  L  K  G  I  A  N  K  Q  F  A  Q  Q  N  L  D  T   222
 667  GCCGTTGCCAAATGGCAAAAAGCTTTGCGCTACCTAATGGAATACCCTGTTCCCAACGAT  726
 223  A  V  A  K  W  Q  K  A  L  R  Y  L  M  E  Y  P  V  P  N  D   242
 727  GACTCTAAGGAATCCCCTGATTTCTGGAAAGAGTATAATGCTTTACGATACAGCATATAC  786
 243  D  S  K  E  S  P  D  F  W  K  E  Y  N  A  L  R  Y  S  I  Y   262
 787  GCAAACCTTGCCCTTGTAGCTTTGAAGCAGAACAAACCTCAGGAAGCTATTCGAAACGCC  846
 263  A  N  L  A  L  V  A  L  K  Q  N  K  P  Q  E  A  I  R  N  A   282
 847  AACATTGTAATTGAGGCCAGTAACTCTACTGAGTTAGAGAAACAGAAAGCATACTATCGT  906
 283  N  I  V  I  E  A  S  N  S  T  E  L  E  K  Q  K  A  Y  Y  R   302
 907  TTGGGTTGTGCTCAAGGTCTCTTGAAGAATTTTGAAGAATCAGAGAAAGCACTTGCTAAG  966
 303  L  G  C  A  Q  G  L  L  K  N  F  E  E  S  E  K  A  L  A  K   322
 967  GCTGGTAACGATCCTGCAATTTCAAAGAAGCTTGCAGAAATTCGTCAAAAGAAAAAAGAT 1026
 323  A  G  N  D  P  A  I  S  K  K  L  A  E  I  R  Q  K  K  K  D   342
1027  TATAAGAAGCGCCAACAAAAGGCCTATGCTAAGATGTTTCAGTAATTCCAAATTAATCGG 1086
 343  Y  K  K  R  Q  Q  K  A  Y  A  K  M  F  Q                     362
1087  CGTCACGTGAATGCTGAGAAGTCAATTATGTTTTACGTTAATTTCTAGTTAGTCAATGAG 1146
                                                  HincII (2)
1147  AGTTTTATCGCATTTACAAAATAACCATAGAAGGTCAACTACGTTGCGAGAAAGAGTGCA 1206
1207  AGATTTACTTATGGAGGATCTGGCTTCGTTTGTAAGACTGGTATGCCTACGTTTGCGTTT 1266
1267  CATACAGCAAAGTATCATCATGAATAGAAAGAACTCTAGAAACAAATGTGCATCAGTAAA 1326
1327  CTCAATAAGAATATCATATTTAAACATTTAATTCATTAAAATTAAATAAGACCGTCTCTT 1386
1387  ACCAAGTGTCGAAATTACATGAGTTTCCGCATTACCCTTCCTAAGGATGAGAGTGCTAAT 1446
1447  GTGAACCCGCTAATACGAATCTGCAG 1472
```

Analysis of the predicted 5' non coding region of *wis2*$^+$ shows a putative TATA box 180 nt upstream of the putative start codon. The TATA motif shows perfect match with the higher eukaryote TATA box sequence TATAAA. The location of the putative TATA box is in good agreement with that expected for *S.pombe* genes (within 250 nt upstream of the ORF, Russell 1989). In general, the consensus for *S.pombe* TATA motifs is not very strong, but TATA sequence motifs have been suggested for genes that are transcribed at moderate or high levels (Russell 1989).

Analysis of the predicted 3' non-coding region of *wis2*$^+$ reveals the presence of the sequence TAGTTAGTCAATTGAGAGTTT 60 nt downstream of the end of the *wis2*$^+$ ORF. This is similar to the sequence TAG...TAGT...TTT which has been proposed to serve as a transcription termination sequence in *S.cerevisiae* genes (Zaret and Sherman 1984). However, not all genes contain this sequence at their 3' end, and other termination motifs have been proposed (Humphrey et al. 1991). Mechanisms of mRNA 3' end formation in *S.pombe* closely resembles that of *S.cerevisiae* (Humphrey et al. 1991). In *S.pombe* polyadenylation signals like those of *S.cerevisiae* are AT rich but possess no single obvious sequence motif. The termination sequence TAG...TAGT...TTT is not always found in the 3' end of *S.pombe* genes, although derivations of this sequence can be identified in some cases.

iii wis2 activity and the predicted ORF

The 1.6 kb *Hin*cII fragment containing the predicted 1068 bp ORF (Figure 2.4) was cloned into the yeast vector pIRT2. The resultant plasmid, pIwis2-2, was able to suppress the triple mutant phenotype at the restrictive condition with comparable efficiency to pIwis2-1.

Note that the yeast vector pIRT2 does not contain *S.pombe* promoter sequences immediately upstream of the cloning site. Therefore, one possibility to account for the suppression activity of pIwis2-2 is to predict that the *Hin*cII fragment contains the *wis2*$^+$ endogenous promoter. This is plausible as the fragment contains 400 bp upstream of the putative initiating methionine. Another possibility is that *wis2*$^+$ transcription is the result of residual transcription activity derived from vector sequences. This is far less likely as Northern blot analysis showed that cells transformed with pIwis2-2

express mRNA that hybridises specifically to *wis2*$^+$ sequences and is of the same size as the wild type *wis2*$^+$ mRNA (see section 3.1).

2.3 Structure of the 5' end of *wis2*$^+$

i Analysis of 5' end sequences of *wis2*$^+$

Sequence analysis of the *wis2*$^+$ genomic clone could not determine satisfactorily the structure of the 5' end of the gene. Close examination, helped by computer analysis, revealed the presence of 5', branch and 3' intron splice sites at this region, which could indicate the presence of an intron.

The presence of introns is relatively common in *S.pombe* genes and consensus sequences for splice sites have been suggested (Russell 1989). Characteristically, *S.pombe* introns are short, ranging from 30-150 nt in total length (Russell 1989). Also, *S.pombe* introns, as in the case of *S.cerevisiae*, tend to be located towards the 5' end of genes (Fink 1987).

In the following discussion the consequence of a hypothetical splicing event are discussed in detail. Figure 2.6A shows the position and sequences of potential splice sites.

A potential 5' splice site is present at nucleotides (-209) - (-205) and encodes the relatively rare splice site of the sequence GTAGA. The consensus sequence for 5' splice sites is GTANG and the presence of an adenosine residue at the fifth position is found only in about 5% of spliced genes (Russell 1989). A potential branch site at nucleotides (-155) - (-151) is CTAAA (consensus CTPuAN) and a potential 3' splice site at nucleotides (-144) - (-142) is TAG (consensus NAG). Note that numbers mentioned above refer to the position of the nucleotides relative to ATG(1), the start codon predicted to initiate translation assuming that no splicing takes place.

If splicing occurred, an intron sequence of 67 nt would be removed and an ATG codon 17 nt upstream of the 5' splice site could function as the start codon. This ATG sequence is referred to as ATG(2). The sequence of the hypothetical intron includes two possible consecutive stop codons upstream ATG(1). Also, ATG(2) is in frame with ATG(1). It follows that if splicing occurred, the resulting ORF of the spliced gene would contain an additional 159 nt compared with the 1068 nt of the predicted unspliced gene.

The spliced gene potentially encodes 409 amino acids with a calculated molecular weight of ~46 kDa, about 6 kDa larger than the predicted unspliced gene product.

The presence or absence of an intron could not be resolved by the function analysis of the existing subclones (Figure 2.5):

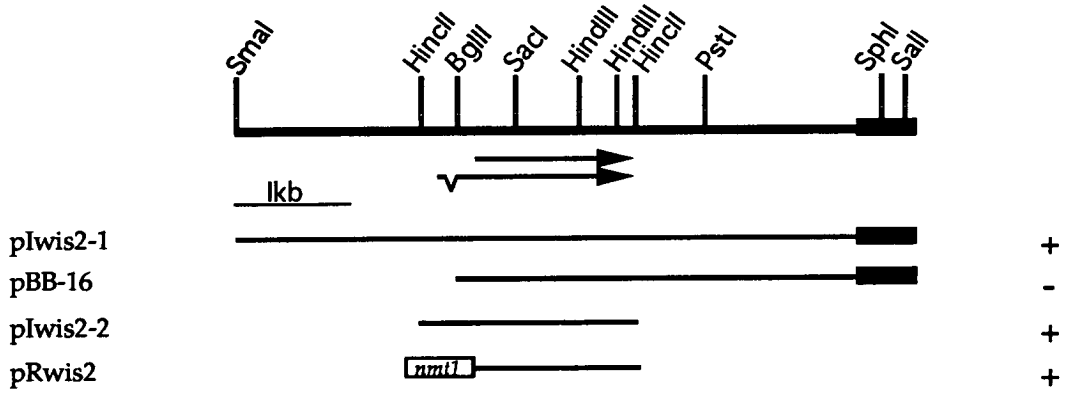
The subclone pBB-16 (*Bgl*II -*Sal*I subclone; Warbrick and Fantes 1992) was unable to confer wis2 activity. This inability could be either a consequence of a lack of promoter sequence or truncation of N-terminal amino acids, consistent with the gene being unspliced or spliced, respectively (Figure 2.5). The function of pIwis2-2 (*Hin*cII subclone) is consistent with both scenarios. pIwis2-2 insert contains 170 bp upstream ATG(2) and 400 bp upstream of ATG(1), thus the length of non-coding region upstream the putative initiating methionine is likely to be sufficient for gene expression either from ATG(1) or from ATG(2).

Figure 2.5 ***wis2*** **subclones and their wis2 activity**

The two potential ORF of *wis2*$^+$ as predicted from the sequence of genomic subclone. Top and bottom arrows represent the ORF in the absence or presence of a splicing event, respectively.

pBB-16 is a clone in pDB248 (Warbrick and Fantes 1992), pIwis2-1 and pIwis2-2 are clones in pIRT2, pRwis2 is a clone in pREP1 (described in sections 2.2i, 2.2iii and 2.3iv). Dark boxes indicate vector sequences.

+/- indicate wis2 activity. Note that activity of pRwis2 is tested in the derepressed conditions of the regulated *nmt1* promoter.



ii PCR analysis of *wis2*$^+$ cDNA clones

The strategy taken to resolve the structure of *wis2*$^+$ 5' end was to predominantly analyse *wis2*$^+$ cDNA sequences. cDNA libraries are constructed from terminally processed mRNAs, therefore, comparing the sequences of *wis2*$^+$ cDNA clones with the genomic clone should indicate whether or not *wis2*$^+$ is a spliced gene.

A *S.pombe* cDNA library made in the yeast vector pREP3X was a gift from C. Norbury. The presence or absence of coding sequences of *wis2*$^+$ cDNA was initially analysed by testing the ability to amplify *wis2*$^+$ fragments by the polymerase chain reaction (PCR) using the total cDNA library as a template.

Two synthetic oligonucleotide primers which can distinguish between the spliced and unspliced possibilities were designed (#1 and #2 in Figure 2.6A). Primer #1 includes ATG(2), the potential start codon if the suggested splicing event occurred. Primer #2 includes the two in frame stop codons upstream of ATG(1) and the potential initiating methionine predicted for the unspliced gene. It also contains the first two nucleotides of the splicing branch site.

Two additional synthetic oligonucleotides were employed. Primer #3 located 120 nt upstream of ATG(1) provided a positive control for the presence of *wis2*$^+$ 5' sequences in the cDNA library. Primer #4, 878 nt downstream of ATG(1) was used as the 3' end primer in all PCR reactions.

The conditions used for the PCR reactions are described in Section 7.8.

PCR reaction products were run on 0.7% agarose gels in the presence of ethidium bromide. The results of these PCR reactions are presented in figure 2.6 lanes 1-3.

A strong amplified signal was obtained when using primer #3, a weak signal when using primer #2 containing the in frame stop codons, and no signal at all when using primer #1 containing ATG(2). The observed products are of the expected sizes, 1030 bp and 1090 for primer #3 and #2 respectively.

The existence of *wis2*$^+$ cDNA sequences containing the in frame stop codons upstream of ATG(1) strongly suggests that no splicing event occurs at the 5' end. The weakness of the signal obtained with primer #2 may reflect the small population of cDNAs extending as far as - 174 nt upstream of ATG(1).

This explanation is plausible because 5' end sequences tend to be lost in the process of the construction of cDNA libraries (Sambrook et al. 1990).

The strategy taken to amplify the weak product of the PCR reaction with primer #2 involved a second set of PCR reactions, using as a template an enriched population of *wis2* cDNAs. The population enriched was that of *wis2* cDNAs of different length at their 5' end. To achieve this, a synthetic oligonucleotide was designed which anneals to vector sequences immediately upstream of the 5' end cloning site (pREP3X oligonucleotide, see Materials and Methods, section 7.8).

The product of the PCR reaction using the vector primer in combination with primer #4 resulted in a smeared band of about 1 kb length. This band was gel purified and was used as a template for the same reactions described for the total cDNA library.

The PCR reactions using the enriched *wis2* cDNAs as a template clearly showed that Primer #2, but not primer #1, was able to produce an amplified product (Figure 2.6 lanes 4-5). Note that both primers #2 and #1 are able to amplify the expected fragments when genomic DNA is used as a template (Figure 2.6 lanes 6-7).

The ability of primer #2, containing the in frame stop codons upstream ATG(1), to bind specifically to sequences in the cDNA library strongly suggests that these sequences are not part of an intron, as such sequences should not be present in the cDNA library. Therefore, these data strongly suggest that the downstream ATG(1) is the initiating codon for the *wis2*$^+$ ORF.

Figure 2.6 Analysis of *wis2*$^+$ 5'

A *wis2*$^+$ 5' sequence

wis2$^+$ sequences relevant for determining *wis2*$^+$ 5' end structure.

The two possible start codons for *wis2*$^+$ are indicated: the expected start codon for the non-spliced gene is in bold upper case characters at position 1; the alternative start codon for the spliced gene is in bold lower case characters at position -226.

Consistent with spliced *wis2*$^+$ is the removal of the in frame stop codons (*) by the intron splicing sequences (bold lower case) as described in section 2.3i.

A double underline indicates the furthermost 5' end nucleotide towards which the *wis2*$^+$ cDNA clone extended, as determined by sequence analysis.

The oligonucleotides used in PCR reactions are underlined and numbered.

B *wis2*$^+$ PCR products from a cDNA library

Total cDNA library, cDNAs enriched for *wis2* sequences (see section 2.3 iv) and total genomic DNA were used as templates in PCR reactions. In all reactions primer #4 was used as a 3' end primer in the amplification reaction. 5' end primers are indicated for each reaction.

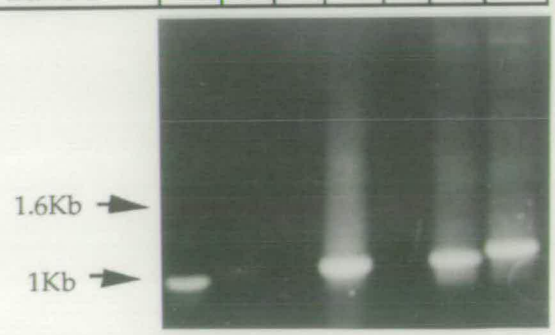
5, 10 or 30 µl out of total of 100 µl of the PCR reactions were analysed on a 0.7% agarose gel.

A

```
-473  TACATTGCTGAAGACTCATCGAAATATGTTTAAAACTATTAGAAACACAATTTTGTGTTT  -414
                          HindII
-413  TAAAATTTAAATACATTGTTGACTTCACTCAACAGCACTTATATTTCTTTATAGTCCGTG  -354

-353  TGTGCTAACTTAAATCAGGTATTTTATGAATTTTTAACGAAGAAGCAATCTAATTGAAAA  -294

-293  AAGTATAAATATTTTTTGGTCTAATAAAAAGTCAAATTTTCTTCTGCTAGCATTTTTTTA  -234
        #1 ->
-233  ATACAGTatgTTCTAGAAGTTTCgtagaAAATCTCTTCGCAGTAGGTTGCAAATATAAA  -174
        #2 ->      * *                    BglII
-173  TCACCACCTTTTTAATAActaaaACTAAATagTTTGCACAGATCTGTATATTTCGTCTGC  -114
           #3 ->
-113  GTAAAATCGCTATTCATTCTTCTTCTTAAAGCAACTTTGTGGTGTGAATACACTGAGGAG  -54

 -53  ACTGGCAGTGAAATATCATTACTTAAAAACGATCATTGTTTTTTTTTGCACGAAATGAGT   6
                                                        M S      2

   7  ACTTACGCCTATTTTAAAATTAGCATTGATGGTAAAATTCAACCAACAATCTACTTTGAA  66
   3  T Y A Y F K I S I D G K I Q P T I Y F E                       22

  67  CTTTTCGACAACGTAGTTCCCAAAACTGTCAAAAACTTTGCTTCGTTGTGTAATGGGTTT  126
  23  L F D N V V P K T V K N F A S L C N G F                       42
                                          <- #4
 127  GAGAAGGAT     /  /   TCTACTGAGTTAGAGAAACAGAAAGCATACTATCGT     906
  43  E K D         /  /   K Q N K P Q E A I R N A                  282

 907  TTGGGTTGTGCTCAAGGTCTCTTG.....
 283  N I V I E A S N .....
```

B

Template:	cDNA library			wis2 cDNA's		Genomic DNA	
5' primer:	3	2	1	2	1	2	1
µl loaded	10	30	30	10	30	5	5
Lane #	1	2	3	4	5	6	7



iii Sequence analysis of 5' end of *wis2*$^+$ cDNA

In order to further investigate the 5' end sequences of *wis2*$^+$, the gel purified fragments from the PCR product of the vector primed reaction were cloned into pBluescriptII KS+. Eight isolated clones were analysed by restriction digest at the 5' end. This analysis showed that 5 out of the 8 clones did not contain the *Bgl*II site at position (-135) - (-129) upstream of ATG(1). Three clones that contained the *Bgl*II site were subjected to sequence analysis. All three clones were found to extend to position -136 (indicated in Figure 2.6). Note, that the termination of *wis2*$^+$ cDNAs at position -136, or downstream of the *Bgl*II site, is consistent with the observation that PCR reactions with primer #2 result in a weak product as both observations are in agreement with respect to the proportion of *wis2*$^+$ cDNAs at their 5' end.

If the clones that terminate position (-136) are full length *wis2*$^+$ cDNAs, it follows that ATG(1) is the true initiating codon, as it is the first in frame ATG codon with the identified 1068 nt ORF. However, if the cDNA sequences analysed in this section are of truncated *wis2* cDNAs then the sequence analysis in itself could not distinguish between the possibilities of a spliced and unspliced gene, as position (-136) is downstream of the potential splice sites and the in frame stop codons.

iv *nmt-wis2*$^+$ construct

In order to determine if the ORF starting with ATG(1) was functional, this ORF was cloned under the control of the strong regulatable *nmt1* promoter in the pREP1 vector (Maundrell 1990; see section 7.2v). This cloning involved the modification by direct *in vitro* mutagenesis of three bases upstream of the ATG(1), so that an *Nde*I recognition site was introduced (Material and Methods section 7.7). Introduction of an *Nde*I site enabled the precise replacement of the *nmt1*$^+$ coding region with the predicted ORF of *wis2*$^+$ (see section 7.2 v).

The 1.2 kb *Nde*I - *Hin*cII fragment of *wis2*$^+$ was cloned into pREP1, and the resultant plasmid, pRwis2, was transformed into the *cdc25-22 wee1-50 win1-1* strain to test for its ability to confer wis2 activity.

Transformants were selected at 28°C on minimal medium supplemented with 4 μM thiamine (repressing conditions for *nmt1*-

dependent expression, Maundrell 1990). For full phenotypic expression under de-repressed conditions, transformants selected on thiamine plates were patched for overnight growth at 28°C on minimal plates lacking thiamine. Suppression of the triple mutant lethality was then tested in the absence of thiamine at the restrictive temperature of 35°C. It was found that pRwis2 was able to confer wis2 activity in the absence but not in the presence of thiamine.

The ORF starting at ATG(1) is therefore functional, at least in respect to the triple mutant suppression activity. This finding is consistent with the prediction that ATG(1) is the initiating methionine of *wis2*$^+$.

In conclusion, the set of experiments described above, in particular the PCR analysis of the 5' of *wis2*$^+$, support the prediction that *wis2*$^+$ ORF is initiated by ATG(1). Hereafter, the *wis2*$^+$ ORF will be referred to as the ORF initiated by ATG(1).

2.4 Predicted amino acid sequence of *wis2*$^+$

i Sequence comparisons

Translation of the predicted ORF predicts a polypeptide of 40.1 kDa containing 356 amino acids. The homology search algorithm FASTA (Lipman and Pearson 1985) was used to search the GenEMBL and SwissProt data bases for proteins showing similarity to the predicted *wis2*$^+$ product.

The search revealed that the first 168 amino acids located at the N-terminal of the predicted polypeptide show a high level of similarity to the cyclophilin domain of cyclophilin-related proteins: >60% identity to *S.pombe* or *S.cerevisiae* cyclophilins in an overlap region of ~160 amino acids; >50% identity to higher eukaryote cyclophilins in the same overlap region (Figure 2.7).

Cyclophilins share a common 18 kDa domain which exhibit *cis-trans* isomerisation of peptidyl-prolyl-peptide bonds (PPIase activity) *in vitro* and bind strongly to the immunosuppressant drug cyclosporin A (CsA). These features implicated cyclophilins in protein folding processes *in vivo*, and in signal transduction pathways when complexed to CsA (see section 1.4).

The 18 kDa cyclophilin domain is highly conserved throughout evolution, and cyclophilin related sequences have been isolated in a variety of prokaryotes and eukaryotes. The most abundant class of the family of

cyclophilins are 18 kDa proteins composed of the cyclophilin domain only. However, the cyclophilin domain may exist as a part of structurally complex proteins. For example, a 160 kDa human protein was isolated which contained a predicted cyclophilin domain at the N-terminal of the protein, three charged domains which show sequence similarity to histone and three other domains rich in Ser, Arg and Lys (Anderson et al. 1993).

wis2$^+$ is an unusual cyclophilin-like gene in that it contains a particularly long (188 amino acids) non cyclophilin-like domain at its C-terminus. This C-terminal region of *wis2*$^+$ encodes a highly charged (35% charged residues) and slightly basic amino acid sequence (estimated pI = 8.6). The last 85 C-terminal amino acids of the predicted *wis2*$^+$ gene product are particularly rich in the basic amino acids lysine and arginine. At the time this the work presented in this thesis was performed no homology was observed between the non-cyclophilin C-terminal region of *wis2*$^+$ and protein sequences in the data bases. However, towards the end of this work, the sequence of two human and one bovine cyclophilin-like genes, each of predicted molecular weight of 40 kDa, became available. These higher eukaryotic cyclophilins show high homology to *wis2*$^+$ (~ 44% identity) throughout the whole protein. The implications of this homology to the study of the *wis2*$^+$ gene will be considered in the Discussion chapter.

Figure 2.7: Comparison between *wis2*$^+$ predicted amino acid sequence and cyclophilins

The *wis2*$^+$ predicted polypeptide sequence is aligned with representatives of cyclophilin or cyclophilin related genes of different classes. Sequences were obtained from the SwissProt protein database. Alignment and consensus sequence were obtained using UWGCG software.

Sp (*S.pombe*)- Cyp1; Sc (*S.cerevisiae*)- Cyp1 and Hu (human)-CypA are of the class of 18 kDa cyclophilins.

Nc (*N.crassa*)- Cyph; Hu- CypB ; Sc-Cyp2 and Dm (*D. melanogaster*)- NinaA contain N-terminal or C-terminal extensions flanking the cyclophilin core domain which are thought to play a role in subcellular localisation (see section 1.4 v).

Ec (*E.coli*)- Cyph represents a eukaryotic cyclophilin related sequence, previously referred to as rotamase.

The consensus line at the bottom was derived from eukaryotic cyclophilins only: capital letters indicate identity, lower case letters indicate the most frequently occuring amino acid in the sequnces presented.

The C-terminal of *wis2*$^+$ is emphasised in bold letters.

```
Sp-wis2  ..........  ..........  ..........  ..........  ..MSTYAYFK
Sp-Cyp1  ..........  ..........  ..........  ..........  ...MSNCFFD
Sc-Cyp1  ..........  ..........  ..........  ..........  ...MSQVYFD
Hu-CypA  ..........  ..........  ..........  ..........  ..VNPTVFFD
Nc-Cyph  MFGPRHFSVL  KTTGSLVSST  FSSSLKPTAT  FSCARAFSQT  SSIMSKVFFD
Hu-CypB  .........M  KVLLAAALIA  GSVFFLLLPG  PSAADEKKKG  PKVTVKVYFD
Sc-Cyp2  .........M  KFSGLWCWLL  LFLSVNVIAS  DVGELIDQDD  EVITQKVFFD
Dm-Nina  ..........  ........MK  SLLNRIILCS  AFLAVASGLS  FTVTSRIYMD
Ec-Cyph  ..........  ..........  ..........  .....MFKST  LAAMAAVFAL
    con  ..........  ..........  ..........  ..........  ..vtskv.fd

Sp-wis2  ISIDGKI...  ..........  .QPTIYFELF  DNVVPKTVKN  FASLCNGFEK
Sp-Cyp1  VIANGQPL..  ..........  ..GRIVFKLF  DDVVPKTAAN  FRALCTG...
Sc-Cyp1  VEADGQPI..  ..........  ..GRVVFKLY  NDIVPKTAEN  FRALCTG...
Hu-CypA  IAVDGEPL..  ..........  ..GRVSFELF  ADKVPKTAEN  FRALSTG...
Nc-Cyph  LEWEGPVLGP  NNKPTSEIKA  QSGRINFTLY  DDVVPKTARN  FKELCTG...
Hu-CypB  LRIGDEDV..  ..........  ..GRVIFGLF  GKTVPKTVDN  FVALATG...
Sc-Cyp2  IEHGEEKV..  ..........  ..GRIVIGLY  GKVCPKTAKN  FYKLSTTTNS
Dm-Nina  VKHNKKPV..  ..........  ..GRITFGLF  GKLAPKTVAN  FRHIC...LR
Ec-Cyph  SALSPAAMAA  KGDPHVLLTT  SAGNIELELD  KQKAPVSVQN  FVDYVNSGF.
    con  .e.dgep...  ..........  ..grivfgLf  .dvvPKTa.N  Fralctg...

Sp-wis2  DGRCLTYKGS  RFHRVIKNFM  LQGGDFTRGN  GTGGESIYGE  KFEDEN..FE
Sp-Cyp1  .EKGYGYAGS  TFHRVIPQFM  LQGGDFTRGN  GTGGKSIYGE  KFPDEN..FA
Sc-Cyp1  .EKGFGYAGS  PFHRVIPDFM  LQGGDFTAGN  GTGGKSIYGG  KFPDEN..FK
Hu-CypA  .EKGFGYKGS  CFHRIIPGFM  CQGGDFTRHN  GTGGKSIYGE  KFEDEN..FI
Nc-Cyph  .QNGFGYKGS  SFHRIIPEFM  LQGGDFTRGN  GTGGKSIYGE  KFADEN..FA
Hu-CypB  .EKGFGYKNS  KFHRVIKDFM  IQGGDFTRGD  GTGGKSIYGE  RFPDEN..FK
Sc-Cyp2  KK...GFIGS  TFHRVIPNFM  VQGGDFTDGT  GVGGKSIYGD  TFPDEN..FT
Dm-Nina  GINGTSYVGS  RFHRVVDRFL  VQGGDIVNGD  GTGSISIYGD  YFPDEDKALA
Ec-Cyph  ......YNNT  TFHRVIPGFM  IQGGGFT..E  QMQQKKPNPP  IKNEADNGL.
    con  .ekgfgykgS  .FHRvip.Fm  lQGGDftrgn  GtGgkSIYGe  kFpDEn..fa

Sp-wis2  LKHDKPFLLS  MANAGPNTNG  SQFFITTV.P  TPHLDGKHVV  FGKVI..QGK
Sp-Cyp1  LKHNKPGLLS  MANAGPNTNG  SQFFITTV.V  TPWLDGKHVV  FGEVT..EGM
Sc-Cyp1  KHHDRPGLLS  MANAGPNTNG  SQFFITTV.P  CPWLDGKHVV  FGEVV..DGY
Hu-CypA  LKHTGPGILS  MANAGPNTNG  SQFFICTA.K  TEWLDGKHVV  FGKVK..EGM
Nc-Cyph  KKHVRPGLLS  MANAGPNTNG  SQFFVTTV.P  TSWLDGRHVV  FGEVADDESM
Hu-CypB  LKHYGPGWVS  MANAGKDTNG  SQFFITTV.K  TAWLDGKHVV  FGKVL..EGM
Sc-Cyp2  LKHDRKGRLS  MANRGKDTNG  SQFFITTTEE  ASWLDGKHVV  FGQVV..DGM
Dm-Nina  VEHNRPGYLG  MANRGPDTNG  CQFYVTTV.G  AKWLDGKHTV  FGKVL..EGM
Ec-Cyph  ..RNTRGTIA  MARTADKDSA  TSQFFINVAD  NAFLDHGQRD  FGYAVFGKVV
    con  lkHdrpglls  MANaGpnTNG  sQFfitTv.p  tpwLDGkHvV  FGkV...egm

Sp-wis2  STVRTIENLE  TKNDDP..VV  PVVIEECGTC  TKDQIEAPKP  DVTGDSLEEF
Sp-Cyp1  DVVKKVESLG  SNSGATR..A  RIVIDKCGTV  ..........  ..........
Sc-Cyp1  DIVKKVESLG  SPSGATK..A  RIVVAKSGEL  ..........  ..........
Hu-CypA  NIVEAMERFG  SRNGKTS..K  KITIADCGQL  E.........  ..........
Nc-Cyph  KVVKALEATG  SSSGAIRYSK  KPTIVDCGAL  ..........  ..........
Hu-CypB  EVVRKVESTK  TDS.RDKPLK  DVIIADCGKI  EVEKPFAIAK  E.........
Sc-Cyp2  DVVNYIQHV.  SRDANDKPLE  AVKIAKCGEW  TPELSS....  ..........
Dm-Nina  DTIYAIEDVK  TDTDDF.PVE  PVVISNCGEI  PTEQFEFYPD  DFNILGWIKA
Ec-Cyph  ..KGMDVADK  ISQVPTHDVG  PYQNVPSKPV  VILSAKVLP.  .........
    con  dvvk..eslg  s.sgatk..k  .vvia.cGel  ..........  ..........

Sp-wis2  PDDYEGDKSE  TAIFKIASDL  KGIANKQFAQ  QNLDTAVAKW  QKALRYLMEY
Sp-Cyp1  ..........  ..........  ..........  ..........  ..........
Sc-Cyp1  ..........  ..........  ..........  ..........  ..........
Hu-CypA  ..........  ..........  ..........  ..........  ..........
Nc-Cyph  ..........  ..........  ..........  ..........  ..........
Hu-CypB  ..........  ..........  ..........  ..........  ..........
Sc-Cyp2  ..........  ..........  ..........  ..........  ..........
Dm-Nina  AGLPVTSSFC  VLLIFHYFFR  QLNMYC....  ..........  ..........
    con  ..........  ..........  ..........  ..........  ..........

  Sp-wis2  PVPNDDSKES  PDFWKEYNAL  RYSIYANLAL  VALKQNKPQE  AIRNANIVIE
C-terminus ASNSTELEKQ  KAYYRLGCAQ  GLLKNFEESE  KALAKAGNDP  AISKKLAEIR
           QKKKDYKKRQ  QKAYAKMFQ
```

ii The cyclophilin-like region of *wis2*$^+$

The three dimensional structure of the cyclophilin-like region of *wis2*$^+$ gene product can be predicted by superimposing the predicted amino acid sequence of *wis2*$^+$ gene product on the sequence of the 18 kDa human cyclophilin protein, Hu-CypA.

The structure of Hu-CypA has been solved by X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy (Kallen et al. 1991; Thériault et al. 1993; and see section 1.4 iv). The tetrapeptide substrate for the enzymatic activity of Hu-CyPA binds in a long deep groove located on the protein surface between one face of the β barrel (β sheets 5 to 6) and the Thr116-Gly130 loop (Figure 2.8). It has been strongly suggested that this peptide binding site coincides with the CsA binding site.

Comparative analysis of the predicted amino acid sequence of *wis2*$^+$ with the amino acid sequence of Hu-CyPA reveals that all residues that are predicted to be in contact with CsA, except Trp121, are conserved in the predicted *wis2*$^+$ sequence. The sole Trp residue in Hu-CypA (position 121) is replaced by His in *wis2*$^+$ predicted protein (position 129).

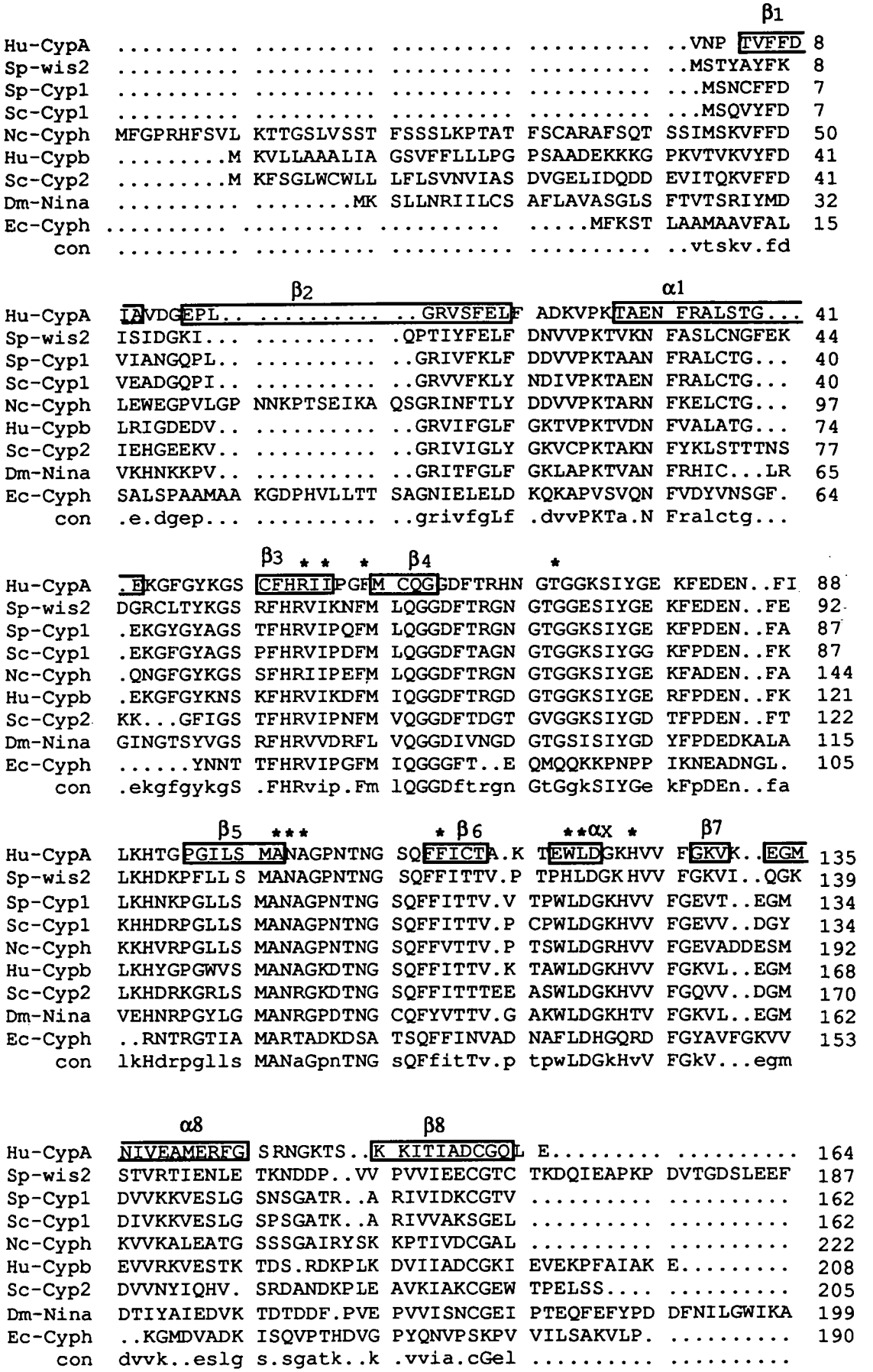
Notably, Trp 121 is highly conserved in most other cyclophilins and is the only residue known to date that is important for high affinity to CsA, but not necessary for prolyl isomerase activity (Liu et al. 1991a; and see section 1.4 iv).

Figure 2.8 Comparison analysis of cyclophilin conserved domain

Alignment of the cyclophilin region of various types of cyclophilin or cyclophilin related proteins. The amino acid sequences were obtained and aligned as described in figure legend 2.7.

The consecutively numbered secondary structures of Hu-CypA are as summarised by Kallen et al. (1991). α, α helix; β, β sheet; αχ, either a short α helix or a turn like structure. Amino acids comprising these secondary structures are boxed.

The binding of the drug CsA complexed with the Hu-CypA is stabilised by several hydrophobic interactions. The side chains of Hu-CyPA residues that interact with the hydrophobic surface of CsA are indicated by asterisks (Thériault et al. 1993). Several hydrogen bonds also stabilise the structure of the complex. The NMR studies indicate intermolecular hydrogen bonds between CsA and the following cyclophilin amino acid: Arg55, Asn102 and Trp121 (Thériault et al. 1993). These amino acids are also indicated by asterisks. Note particularly that in wis2 predicted protein the amino acid at position 121 is His, rather than the Trp found in other cyclophilins.


β1
Hu-CypA ....VNP TVFFD 8
Sp-wis2 ....MSTYAYFK 8
Sp-Cyp1 ....MSNCFFD 7
Sc-Cyp1 ....MSQVYFD 7
Nc-Cyph MFGPRHFSVL KTTGSLVSST FSSSLKPTAT FSCARAFSQT SSIMSKVFFD 50
Hu-Cypb .....M KVLLAAAALIA GSVFFLLLPG PSAADEKKKG PKVTVKVYFD 41
Sc-Cyp2 .....M KFSGLWCWLL LFLSVNVIAS DVGELIDQDD EVITQKVFFD 41
Dm-Nina ....MK SLLNRIILCS AFLAVASGLS FTVTSRIYMD 32
Ec-Cyph ....MFKST LAAMAAVFAL 15
con ....vtskv.fd
β2
α1
Hu-CypA IAVDGEPL.. ....GRVSFELF ADKVPKTAEN FRALSTG... 41
Sp-wis2 ISIDGKI... ..... .QPTIYFELF DNVVPKTVKN FASLCNGFEK 44
Sp-Cyp1 VIANGQPL.. ....GRIVFKLF DDVVPKTAAN FRALCTG... 40
Sc-Cyp1 VEADGQPI.. ....GRVVFKLY NDIVPKTAEN FRALCTG... 40
Nc-Cyph LEWEGPVLGP NNKPTSEIKA QSGRINFTLY DDVVPKTARN FKELCTG... 97
Hu-Cypb LRIGDEDV.. ....GRVIFGLF GKTVPKTVDN FVALATG... 74
Sc-Cyp2 IEHGEEKV.. ....GRIVIGLY GKVCPKTAKN FYKLSTTTNS 77
Dm-Nina VKHNKKPV.. ....GRITFGLF GKLAPKTVAN FRHIC...LR 65
Ec-Cyph SALSPAAMAA KGDPHVLLTT SAGNIELELD KQKAPVSVQN FVDYVNSGF. 64
con .e.dgep... ....grivfgLf .dvvPKTa.N Fralctg...
β3 * * * β4 *
Hu-CypA .EKGFGYKGS CFHRIIPGFM CQGGDFTRHN GTGGKSIYGE KFEDEN..FI 88
Sp-wis2 DGRCLTYKGS RFHRVIKNFM LQGGDFTRGN GTGGESIYGE KFEDEN..FE 92
Sp-Cyp1 .EKGYGYAGS TFHRVIPQFM LQGGDFTRGN GTGGKSIYGE KFPDEN..FA 87
Sc-Cyp1 .EKGFGYAGS PFHRVIPDFM LQGGDFTAGN GTGGKSIYGG KFPDEN..FK 87
Nc-Cyph .QNGFGYKGS SFHRIIPEFM LQGGDFTRGN GTGGKSIYGE KFADEN..FA 144
Hu-Cypb .EKGFGYKNS KFHRVIKDFM IQGGDFTRGD GTGGKSIYGE RFPDEN..FK 121
Sc-Cyp2 KK...GFIGS TFHRVIPNFM VQGGDFTDGT GVGGKSIYGD TFPDEN..FT 122
Dm-Nina GINGTSYVGS RFHRVVDRFL VQGGDIVNGD GTGSISIYGD YFPDEDKALA 115
Ec-Cyph ......YNNT TFHRVIPGFM IQGGGFT..E QMQQKKPNPP IKNEADNGL. 105
con .ekgfgykgS .FHRvip.Fm lQGGDftrgn GtGgkSIYGe kFpDEn..fa
β5 *** * β6 **αX * β7
Hu-CypA LKHTGPGILS MANAGPNTNG SQFFICTA.K TEWLDGKHVV FGKVK..EGM 135
Sp-wis2 LKHDKPFLLS MANAGPNTNG SQFFITTV.P TPHLDGKHVV FGKVI..QGK 139
Sp-Cyp1 LKHNKPGLLS MANAGPNTNG SQFFITTV.V TPWLDGKHVV FGEVT..EGM 134
Sc-Cyp1 KHHDRPGLLS MANAGPNTNG SQFFITTV.P CPWLDGKHVV FGEVV..DGY 134
Nc-Cyph KKHVRPGLLS MANAGPNTNG SQFFVTTV.P TSWLDGRHVV FGEVADDESM 192
Hu-Cypb LKHYGPGWVS MANAGKDTNG SQFFITTV.K TAWLDGKHVV FGKVL..EGM 168
Sc-Cyp2 LKHDRKGRLS MANRGKDTNG SQFFITTTEE ASWLDGKHVV FGQVV..DGM 170
Dm-Nina VEHNRPGYLG MANRGPDTNG CQFYVTTV.G AKWLDGKHTV FGKVL..EGM 162
Ec-Cyph ..RNTRGTIA MARTADKDSA TSQFFINVAD NAFLDHGQRD FGYAVFGKVV 153
con lkHdrpglls MANaGpnTNG sQFfitTv.p tpwLDGkHvV FGkV...egm
α8 β8
Hu-CypA NIVEAMERFG SRNGKTS..K KITIADCGQL E..... 164
Sp-wis2 STVRTIENLE TKNDDP..VV PVVIEECGTC TKDQIEAPKP DVTGDSLEEF 187
Sp-Cyp1 DVVKKVESLG SNSGATR..A RIVIDKCGTV 162
Sc-Cyp1 DIVKKVESLG SPSGATK..A RIVVAKSGEL 162
Nc-Cyph KVVKALEATG SSSGAIRYSK KPTIVDCGAL 222
Hu-Cypb EVVRKVESTK TDS.RDKPLK DVIIADCGKI EVEKPFAIAK E..... 208
Sc-Cyp2 DVVNYIQHV. SRDANDKPLE AVKIAKCGEW TPELSS.... 205
Dm-Nina DTIYAIEDVK TDTDDF.PVE PVVISNCGEI PTEQFEFYPD DFNILGWIKA 199
Ec-Cyph ..KGMDVADK ISQVPTHDVG PYQNVPSKPV VILSAKVLP. 190
con dvvk..eslg s.sgatk..k .vvia.cGel ....

2.5 Conclusion

This chapter describes the determination of the *wis2*$^+$ functional region by sequence and PCR analysis of genomic and cDNA clones.

The sequence of a fragment of DNA of 1960 nucleotides is presented and shown to contain an ORF of 1068 nucleotides. The predicted ORF is not interrupted by intron sequences.

The ORF predicts a polypeptide product of 356 amino acid and 40 kDa.

The N-terminus (168 amino acids) of *wis2*$^+$ predicted polypeptide shows a very high similarity with the cyclophilin family of proteins. It contains all the peptide motifs thought to be required for the *in vitro* enzymatic activity of cyclophilins. In contrast, *wis2*$^+$ does not contain the tryptophan residue implicated in binding of the immunosuppressant drug cyclosporin A.

The C-terminal 188 deduced amino acid sequence is unusual amongst the family of cyclophilins, it is highly charged and slightly basic.

Chapter 3: Genetics and physiology of *wis2+*

3.1 Overexpression of *wis2+* transcript

i Introduction

As described in chapter 2, multiple copies of *wis2+* expressed either from its own promoter (pIwis2-2) or the strong *nmt1* promoter (pRwis2), resulted in the rescue of the lethal phenotype of *cdc25-22 wee1-50 win1-1* (sections 2.1 i and 2.3 iv, respectively). In order to detect the overexpression of *wis2+* at the molecular level, transcript levels of *wis2+* were analysed by Northern blotting.

Confirmation of overexpression of *wis2+* transcript was followed by examination of the effects of overexpression in wild type and various cell cycle mutants. Such analysis may suggest possible biological roles for *wis2+*.

ii *wis2+* transcript

pIwis2-1, pIwis2-2 and pRwis2 were transformed into *cdc25-22 wee1-50 win1-1 leu1-32 h⁻*; as a control, the parental plasmid pIRT2 was included in the experiment. Total RNA was extracted from transformants grown on minimal medium at 28°C (see Materials and Methods, section 7.5 vii). In the case of pRwis2 transformants, total RNA was prepared separately from cells grown in the absence and in the presence of thiamine (de-repressed and repressed conditions, respectively).

The results of the Northern blot analysis are shown in Figure 3.1. The comparison of the level of *wis2+* mRNA in lanes 9 - 11 was assessed by Phosphor-imager analysis; variation in loadings were determined by the level of *adh1+* mRNA. RNA prepared from pIwis2-2 transformants shows about 12-fold increase in *wis2+* transcript level compared with the level observed in pRwis2 transformants grown in the presence of thiamine (lanes 9, 11). Yet a further increase, of about 40 fold, is detected in pRwis2 transformants grown in the absence of thiamine (lanes 10, 11).

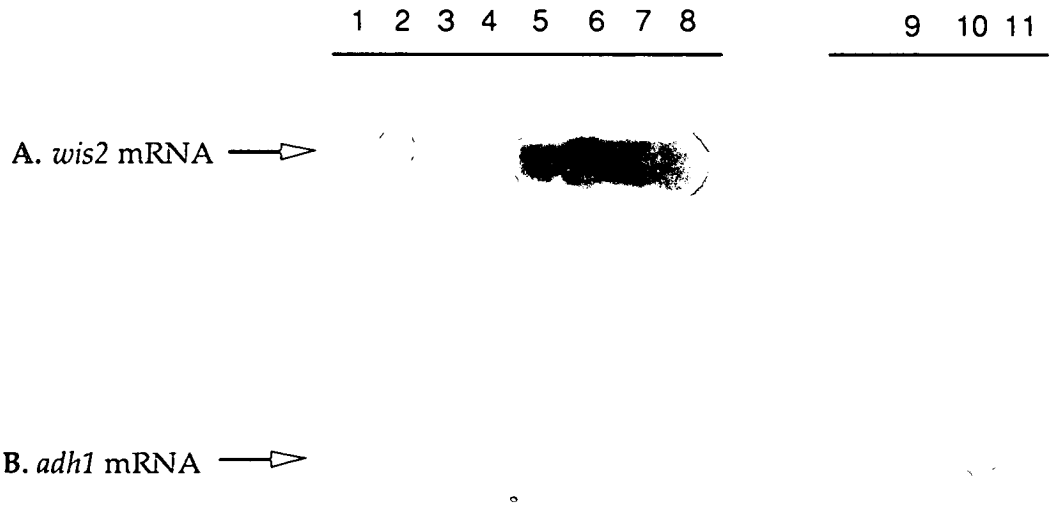
Figure 3.1 Northern blot analysis of wis2+ transcript

Total RNA was prepared from strains expressing various levels of *wis2*$^+$ (section 3.1 ii), run on agarose-formaldehyde gels and subjected to Northern blot analysis. Panel A shows hybridisation with the 800 bp *Bgl*II- *Hin*dIII fragment of pwis2-1, containing most of the *wis2*$^+$ ORF. Panel B shows hybridisation to a 1 kb *Eco*RI fragment internal to the *S.pombe adh1*$^+$ gene, which was used as a loading control.

Strains and growth conditions used for RNA extractions and quantity of total RNA used for the Northern blotting were as follows:

Lanes 1, 2: wild type, 5 and 10 µg, respectively.
Lanes 3, 4: *cdc25-22 wee1-50 win1-1*, 5 and 10 µg, respectively.

Lanes 5 - 11: *cdc25-22 wee1-50 win1-1* transformed as follows:
Lanes 5, 6: pIwis2-1, 5 and 10 µg, respectively.
Lanes 7, 8: pIwis2-2, 5 and 10 µg, respectively.
Lane 9: pIwis2-2
Lane 10: pRwis2, cells grown in the absence of thiamine.
Lane 11: pRwis2, cells grown in the presence of thiamine.



The suppression of the triple mutant phenotype is correlated with the overexpression of the *wis2*$^+$ transcript. pRwis2 is not capable of suppression of the triple mutant phenotype when the *nmt1* promoter is turned off, whereas pIwis2-2 or pRwis2 when the *nmt1* promoter is turned on are capable of suppression.

iii Overexpression of *wis2*$^+$ in various genetic backgrounds

pIwis2-2 and pRwis2 were used to transform various strains, in order to investigate possible effects of *wis2*$^+$ overexpression in different genetic backgrounds. These include *leu1-32 h*$^-$ ('wild type' control) as well as cells containing the following mutant alleles, which are defective in regulating the G2-M transition:

cdc2-33 (ED628), *cdc13-117* (ED684), *wee1-50* (ED835), *cdc25-22* (ED866), *win1-1* (ED623), *wee1-50 mik1Δ* (ED856), *wis1Δ* XX*wee1-50 cdc25-22* (ED626).

The full genotype of the transformed strains is given in section 7.5 i.

Possible effects were investigated on minimal media in a range of temperatures: 20, 25, 28, 32 and 35°C. This range includes permissive and restrictive temperatures for the cell cycle mutant alleles.

No phenotypic effect associated with the introduction of pwis2 plasmids was observed in any of the above genetic backgrounds, and no reduction of the restrictive temperature of the above mutant alleles was observed.

3.2 *wis2*+ null mutation

i Experimental strategy

A powerful means to study gene functions in *S.pombe* is to disrupt or to delete the genes at their chromosomal locus. In a disruption experiment, a selectable genetic marker is inserted into a single restriction site within the gene of interest; in a deletion experiment, a portion of, or preferably all the gene is replaced with a genetic marker. Techniques for deletion or disruption has been widely used to create null mutants of many yeast genes.

The method of one step gene replacement (Rothstein 1983) was employed to create a null mutation of *wis2*+. The method relies upon a double recombination event taking place at the chromosomal locus of the gene of interest: first, a selectable marker fragment is cloned such that it replaces part or most of the gene. Next, a linear fragment is released from the plasmid vector; the linear fragment should contain the selectable marker flanked by sufficient length of DNA sequence to permit homologous pairing with both sides of the chromosomal target sequence. Standard transformation techniques are used to transform the linear fragment and stable transformants for the genetic marker are isolated.

If the gene in question is vital to cell growth or division, then loss of function will be lethal and no transformants will be isolated. To avoid this, the replacement is carried out in a diploid strain. The replacement results in a heterozygous diploid which may then be induced to sporulate and the haploid progeny analysed.

ii Deletion of *wis2*+ at the chromosomal locus

The *wis2*+ ORF sequence had already been determined by the time the replacement experiment was performed, therefore, it was possible to construct a replacement experiment that would unambiguously create a null mutation of *wis2*+.

First, *wis2*+ sequences were replaced with the selectable marker *ura4*+. A 1.8 kb *ura4*+ fragment was used to replace a 1.2 kb fragment of pTZwis2-1 (Figure 3.2). The replaced *wis2* sequence consists of 135 bp of 5' non coding

sequences and 1056 bp of ORF sequences (all coding sequences excluding the last 12 bp at the 3' end). This replacement produced pTZwis2::ura4 .

The 4.2 Kb *Kpn*I- *Sph*I fragment of pTZwis2::ura4 was used for yeast transformation by electroporation (section 7.5 v). This fragment contains the *ura4*$^+$ sequences flanked by ~1.8 and ~2.4 kb of sequences adjacent to *wis2*$^+$. The extent of DNA sequence homology is well above the minimal length (500-bp) believed to be necessary for the event of homologous recombination (Rothstein 1983).

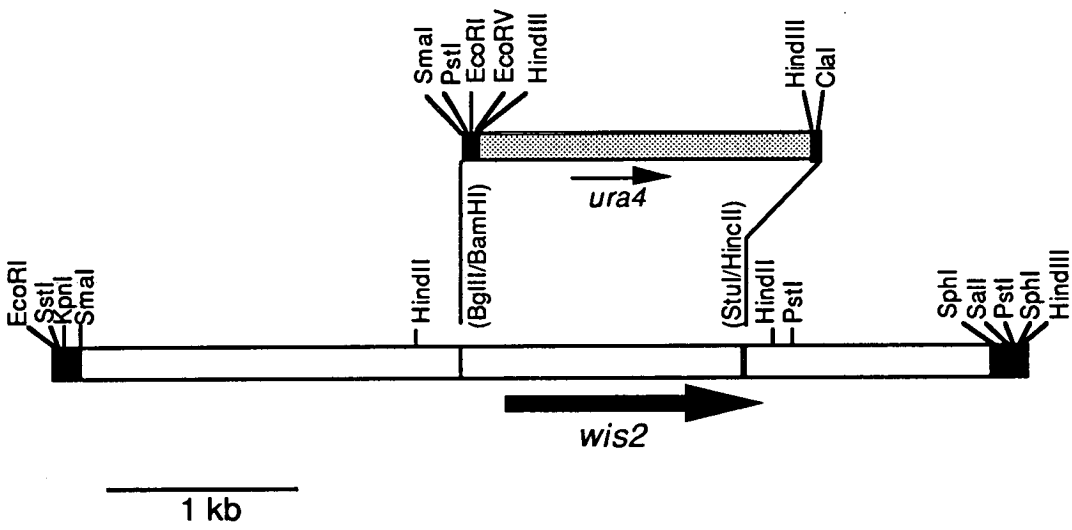
Approximately ~2 µg of gel purified *wis2* disrupted fragment was used to transform a diploid of the genotype *ade6-210/ade6-216 ura4-D18/ura4-D18 leu1-32/leu1-32 h*$^+$/*h*$^-$ (see section 7.5 iii for diploid construction and properties). Transformants were selected on minimal plates supplemented with leucine.

Six transformants (D1 - D6), which were shown to be stable for the *ura4*$^+$ marker and were able to sporulate, were subjected to random spore analysis. Haploids recovered showed 2:2 segregation of the *ura4*$^+$ marker, suggesting that the deletion of *wis2*$^+$ is not lethal. Before further characterisation of the *wis2Δ* strain took place, it was confirmed that the sequences containing the *ura4*$^+$ marker had integrated at the *wis2*$^+$ locus.

To achieve this, Southern blotting was performed with genomic DNA prepared separately from the D1- D6 strains. Preliminary analysis of *Eco*RI hybridisation fragments suggested that in all diploids strains analysed the same single integration event had occurred at *wis2*$^+$ chromosomal region (data not shown). Further Southern blot analysis confirmed the deletion of the *wis2*$^+$ gene by a single integration event. For this analysis genomic DNA was prepared from the D3 diploid strain and its progeny haploid cells: ura$^+$ and ura$^-$ (isolated by random spore analysis).

Figure 3.2 pwis2-1::ura4 construct

The *ura4*$^+$ gene used for the deletion of *wis2*$^+$ was obtained from pON160 (a gift from S. Aves). The *Hin*dIII fragment of pON160, containing the *ura4*$^+$ gene, was cloned into the *Hin*dIII site in pBCKS$^+$. Next, the the *ura4*$^+$ fragment was released from pBCKS$^+$ by double restriction digest with *Bam*HI and *Hin*cII, two of the polylinker restriction sites of pBCKS$^+$. This fragment was ligated with pTZwis2-1, double digested with *Bgl*II and *Stu*I restriction enzymes: *Bam*HI and *Bgl*II digests leave cohesive termini which are compatible; *Hin*cII and *Stu*I restriction digests leave blunt end termini.



Polylinker restriction sites

1.8 kb *ura4* fragment, thin arrow indicates ORF orientation

5 kb wis2-1 fragment, thick arrow indicates orientation and length of *wis2* ORF

The restriction endonucleases and probes used are summarised in Figure 3.3A and the results shown in Figure 3.3B. The patterns of hybridisation fragments obtained with the *wis2* radiolabelled fragments *Pst*I and *Hinc*II in the ura$^+$ haploid cells, blot #1 and #2, respectively, and the lack of any hybridisation signal in ura$^+$ haploid cells when the probe used is the *Bgl*II-*Hind*III fragment, are consistent with the deletion of the *wis2*$^+$ gene by a single integration event.

Finally, tetrad analysis of all six disrupted diploids (D1 -D6) confirmed segregation of 2:2 of ura$^+$ and ura$^-$ haploid cells (data not shown).

iii Phenotype of *wis2*$^+$ null mutation

Following the confirmation of *wis2*$^+$ gene deletion, the phenotype of haploid ura$^+$ cells derived from D3 was investigated. Cells were grown at a range of different temperatures (20, 25, 28, 32 and 35°C) on complex and supplemented minimal media, and were microscopically examined. No change associated with the deletion of *wis2*$^+$ was noticed, in particular, cells appeared to divide at the same cell size as wild type cells.

Cell size at time of division and generation time of *wis2Δ* strain:, *wis2::ura4*$^+$ *ade6-M216 ura4-D18 leu 1-32 h*$^+$, were compared with that of *ade6-M216 ura4-D18 leu1-32 h*$^+$ cells at 28°C. For estimation of cell size at division, cells containing septa were measured. Both strains divided at a cell size of 14.0 μm (standard deviation 0.4) and had a generation time of 2.5 hours.

Possible interactions of *wis2*$^+$ deleted allele with other cell cycle mutations were investigated.

The strain *wis2::ura4*$^+$ *ade6-M216 ura4-D18 leu1-32 h*$^+$ (ED912) was crossed with strains carrying the following mutations:

win1-1 (ED678), *cdc25-22* (ED866), *wee1-50* (ED909), *cdc2-33* (ED628) *cdc-13*(ED918).

All the above strains carry a deletion allele of *ura4*$^+$ in order to avoid possible meiotic or mitotic recombination between the *ura4*$^+$ locus of the cell cycle mutant strain and the *ura4*$^+$ sequences integrated in *wis2Δ* strain. Full genotypes of strains are listed in section 7.5i.

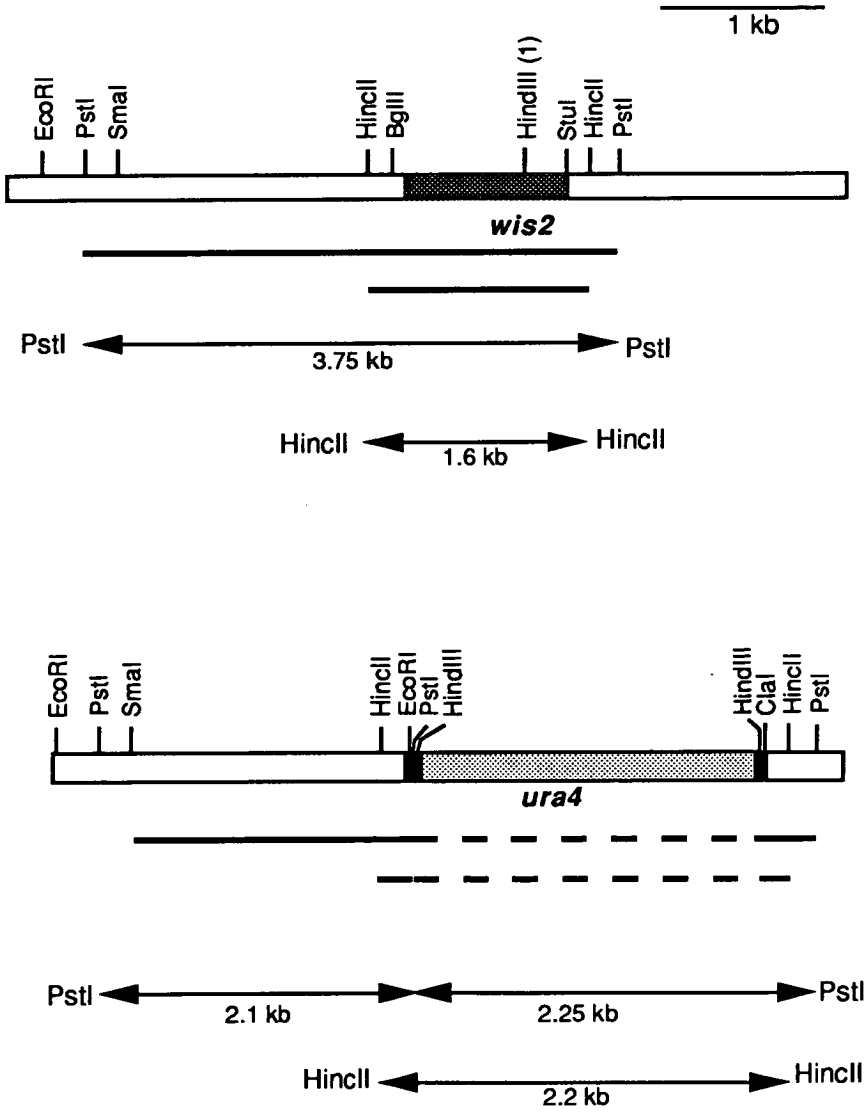
Double mutants were isolated by random spore analysis, using the ura$^+$ phenotype as an indicator for the presence of *wis2Δ* allele. Several such double mutant isolates of each cross were investigated for the ability to form

colonies and for cell size, under a variety of conditions as described above for the haploid ura$^+$ cells. Again, no defects associated with the deletion of *wis2*$^+$ were noticed.

Figure 3.3 Deletion of *wis2*$^+$ gene at the chromosomal locus

A Schematic diagram of the gene deletion of *wis2*$^+$

The diagram indicates the expected result of *wis2*$^+$ gene deletion with the *wis2-1::ura4* construct (figure 3.1). Restriction maps of the wild type chromosome (top) and the chromosome deleted of *wis2*$^+$ (bottom) are shown. The radioactively labelled *wis2* fragments, *Pst*I and *Hin*cII, used as probes in the Southern blot analysis are indicated as lines; dashed lines indicate non-hybridisation region due to the disruption by *ura4*$^+$ sequences. Arrows indicate length of fragments expected from *Pst*I and *Hin*dII restriction digests of chromosomal DNA derived from each allele.



B Southern blot analysis of *wis2*Δ strain.

S.pombe genomic DNA was extracted from the disuptant diploid D3, its progeny haploid ura4$^+$ and ura4$^-$ cells, and haploid wild type cells (972*h*$^-$).

The following genomic restriction digests and radiolabelled fragments of *wis2* were used for Southern blot analysis:

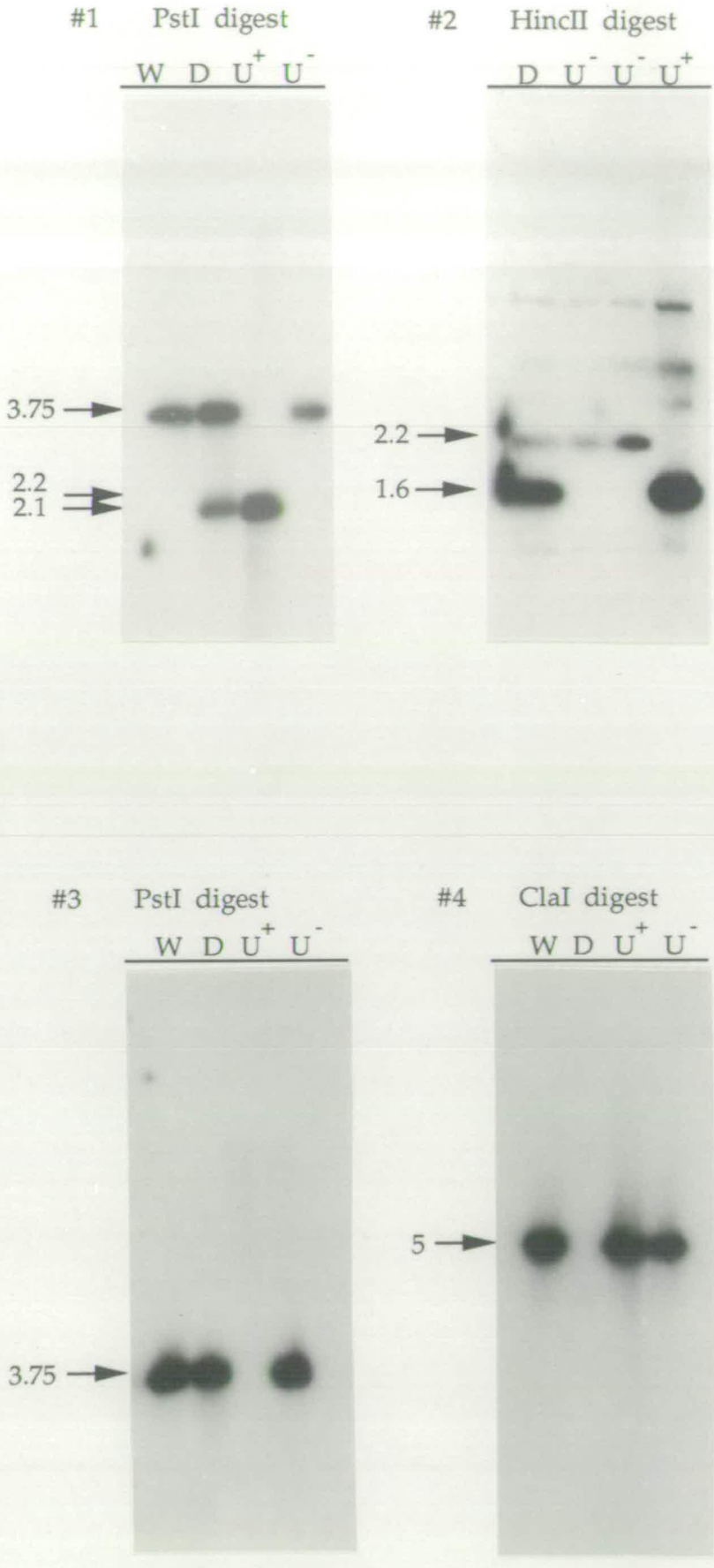
Blot #1: genomic DNA digested with *Pst*I and probed with *Sma*I-*Pst*I fragment.

Blot #2: genomic DNA digested with *Hin*cII and probed with *Hin*cII fragment.

Blot #3: genomic DNA digested with *Pst*I and probed with *Bgl*II-*Hin*dIII fragment. This fragment consists of sequences which are expected to be deleted in haploid ura$^+$ cells, therefore, no hybridsation signal should occur.

Blot #4: genomic DNA digested with *Cla*I and probed with *Bgl*II-*Hin*dIII fragment.

W Wild type haploid
D Disruptant diploid
U$^+$ Ura$^+$ haploid
U$^-$ Ura$^-$ haploid


#1 PstI digest
W D U+ U-
3.75
2.2
2.1
#2 HincII digest
D U- U- U+
2.2
1.6
#3 PstI digest
W D U+ U-
3.75
#4 ClaI digest
W D U+ U-
5

3.3 Effects of cyclosporin A drug on wild type and *wis2* mutants

The *wis2*$^+$ gene product potentially encodes a cyclophilin-like molecule (section 2.4). As cyclophilins are thought to be the intracellular mediators of the effect of the drug cyclosporin A (CsA), the effect of the drug was examined in wild type *S.pombe* cells (972 *h*$^-$) and in cells deleted for *wis2*$^+$ or overexpressing *wis2*$^+$. If CsA affects *S.pombe* cells, strains deleted for *wis2*$^+$ may be expected to abolish or reduce this effect, similair to the observation that deletion of the cyclophilin gene *CYP1* in *S.cerevisiae* results in resistance to the toxicity of CsA (Koser et al. 1991).

All *S.pombe* strains mentioned above produced colonies on complex or minimal plates containing CsA in concentrations varying from 50 to 400 µg/ml, growing at 28°C or 32°C. No growth defects judged by colony size were apparent when compared with colony formation in the absence of the drug. All strains could produce colonies even at the concentration of 400 µg/ml, which reaches the limit of the solubility of the drug in the growth medium.

Although the size of colonies was normal on CsA plates, microscopic examination of cells grown in liquid culture in the presence of CsA revealed that the majority of cells (more than 50%) were septated, either in wild type, *wis2Δ* or *wis2*$^+$ overexpressing strains. This is an unusual phenotype, as *S.pombe* cells grown on standard media normally reveal about 8 - 10% septated cells. In addition, on CsA plates, about 10% of cells showed aberrant septa formation, as cells contained 2-4 septa or (see Figure 3.4). The multiseptation resulted in forming cell compartments similar in size to that of wild type cells before cleavage of septa takes place. This phenotype is consistent with delay of septum cleavage.

Multiseptated phenotype was also observed in the following mutant strains:

leu1-32 (ED624), *ura4-D18 ade6-M210* (ED665), *win1-1* (ED631), *cdc25-22* (ED866), *wee1-50* (ED909), *cdc25-22 wee1-50* (ED626), *cdc25-22 wee1-50 win1-1* (ED565).

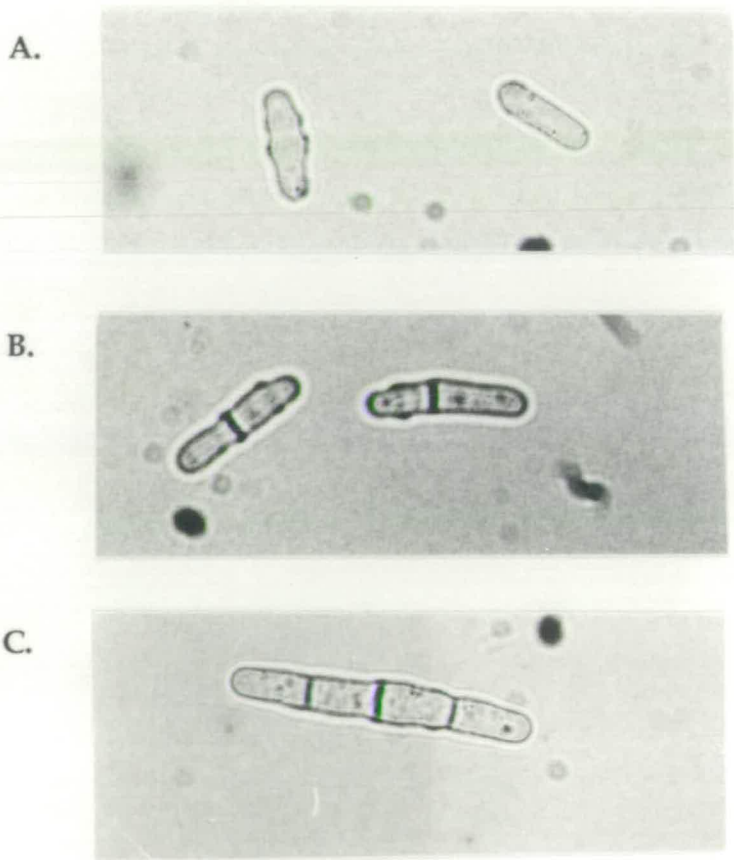
The nature of the multiseptated phenotype was not further investigated as it did not appear to be relevant for the study of the *wis2*$^+$ gene. As strains carrying mutations of the *wis2*$^+$ gene did not show any different response to treatment with CsA, it is unlikely that the effect of CsA

is mediated via the *wis2*$^+$ gene product. In this respect it is worth mentioning that studies in *S.cerevisiae* suggested that *CYP1*, the 18 kDa cyclophilin, and not *CYP2* or *CYP3* (19 and 20 kDa, respectively) mediate the cytotoxic effect of CsA in *S.cerevisiae* (Mclaughlin et al. 1992). By analogy, it is likely that the *S.pombe* cyp1 protein (predicted molecular weight of 18 kDa; de Martin and Philipson 1990), and not wis2, mediate the effect of CsA in *S.pombe*. Furthermore, the sequence analysis of *wis2*$^+$ has indicated that the cyclophilin region of *wis2*$^+$ lacks a conserved Trp (the equivalent to the human Trp121 residue), which is believed to be essential for high affinity of cyclophilins to CsA *in vitro* (section 2.4 ii). Therefore, it is not likely to complex readily with CsA *in vivo*.

Studies of CsA effects in *S.cerevisiae* cells brought about the suggestion that cyclophilins mediate the inhibition of recovery from α factor in cells treated with CsA (section 1.4). It is therefore possible that cyclophilins play a role in mating processes under normal condition as well as in the presence of CsA. To test this possibility, *wis2Δ* strains of opposite mating types were crossed on ME plates. However, no defect phenotype either in formation of asci or in sporulation was noted.

Figure 3.4 Effect of CsA on wild type cells

Wild type *S.pombe* cells (972 h^-) were grown for 16 hours in yeast extract liquid medium at 28°C either in the absence (A) or in the presence of CsA (B, C). Photographs were taken when the cultures were at growing at logarithmic phase. Characteristic to cells grown in the presence of CsA is the presence of a septum (B) or accumulation of multiple septa (C).



3.4 Conclusions

This chapter describes the analysis of *wis2*$^+$ transcripts, including the level in cells containing increased *wis2*$^+$ copy number.

In addition, a strain deleted for *wis2*$^+$ was constructed and possible effects of overexpression and of loss of function of *wis2*$^+$ were investigated in a variety of genetic backgrounds. This analysis includes the effects in wild type and cell cycle mutant strains defective in the control over mitosis. In particular, possible effects in genetic backgrounds of single mutation of *wee1*, *cdc25* or *win1*, the genes mutated in the triple mutant strain were examined. No effects associated with either overexpression or deletion of *wis2*$^+$ were observed.

Overexpression or deletion of *wis2*$^+$ did not show any further effects when cells were treated with CsA. Although *wis2*$^+$ predicted amino acid sequence contain a cyclophilin-like region, this region does not contain the conserved Trp 121 residue of cyclophilin, which is thought to be essential for CsA binding *in vitro*.

Chapter 4: Structure -function analysis of *wis2+*

4.1 Introduction

Sequence analysis predicts that the gene product of *wis2*$^+$ contains two distinct regions: the N-terminal half which is highly homologous to cyclophilins, while the C-terminal half does not show any homology to any other protein sequences in current databases (section 2.4). This is an unusual structure amongst the family of cyclophilins and therefore prompted a structure-function analysis, in particular, the involvement of the two regions of *wis2*$^+$ in the suppression activity of *cdc25-22 wee1-50 win1-1*. Previous functional analysis of subclones of the original pwis2-C3, in view of the sequence analysis of *wis2*$^+$, suggests that the C-terminal region of *wis2*$^+$ is required for wis2 activity: the subclone pSC2-2 (Warbrick 1990) lacks most of the C-terminal region (total of 129 amino acids), and is unable to confer wis2 activity.

The structure-function analysis described below consists of two lines of investigation. First, the effects of *wis2*$^+$ overexpression in *cdc25-22 wee1-50 win1-1* are compared with that of another *S.pombe* cyclophilin-like gene, *cyp1*$^+$ (de Martin and Philipson 1990; name of gene agreed by personal communication). *cyp1*$^+$ was isolated in a screen of *S.pombe* genomic library, employing degenerate oligonucleotides, containing sequences conserved between human and *S.cerevisiae* cyclophilins. The sequence of *cyp1*$^+$ predicts an ORF for an 18 kDa cyclophilin of the cytosolic subgroup. The homology between the predicted gene products of *cyp1*$^+$ and *wis2*$^+$ is 63% identity in an overlap region of 162 amino acids. This high homology suggests that the cyclophilin domain of *wis2*$^+$ and the *cyp1*$^+$ gene might be interchangeable for biological activities. A test for the ability of *cyp1*$^+$ to confer wis2 activity is performed and is followed by functional analysis of a hybrid cyp1-wis2 protein.

Secondly, direct analysis of the cyclophilin domain of wis2 is performed. The rationale of this strategy was to mutate nucleotides which encode highly conserved amino acids within the cyclophilin domain and test the mutated genes for the ability to confer wis2 activity.

The structure-function analysis includes the detection of protein levels of overexpressed wis2, cyp1 and mutated wis2 proteins by Western blot analysis.

4.2 Functional analysis of *cyp1*$^+$ and the *cyp1-wis2* hybrid

i Overexpression of *cyp1*$^+$ in *wee1-50 cdc25-22 win1-1*

A genomic clone of *cyp1*$^+$ in the pBKS$^+$ vector was a kind gift of R. de Martin (see Figure 4.1). For the ectopic expression of *cyp1*$^+$ in *cdc25-22 wee1-50 win1-1*, an *EcoRI* fragment containing the *cyp1*$^+$ gene was cloned into the *S.pombe* plasmid pIRT2. This *Eco*RI fragment contains 500 bp upstream of the predicted initiating ATG codon and 270 bp downstream of the 3' end of the predicted ORF of *cyp1*$^+$. The resultant plasmid, pIcyp1, was transformed into the triple mutant strain and the ability of *cyp1*$^+$ to suppress the lethal phenotype exhibited on minimal medium at 35°C was tested. No suppression was observed: no colony formation occurred at the restrictive conditions and cells displayed the typical abnormal lethal phenotype of highly elongated, bent and branched cells.

The overexpression of *cyp1*$^+$ gene in the triple mutant strain was confirmed by Northern blotting (Figure 4.2). Further analysis by Western blotting confirmed that the gene product of *cyp1*$^+$ is also expressed to high levels (see below, section 4.3 ii).

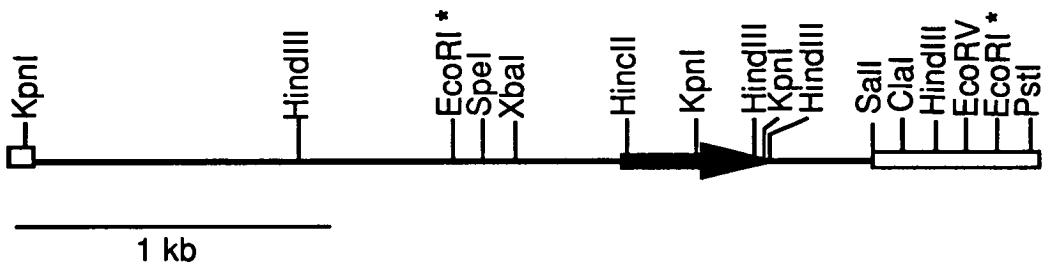
ii Overexpression of *cyp1-wis2 in wee1-50 cdc25-22 win1-1*

The inability of *cyp1*$^+$ to suppress the triple mutant phenotype is consistent with the subclone functional analysis which predicted that the C-terminal region of *wis2*$^+$ is essential for wis2 activity (see above, section i). Assuming that the cyclophilin domain of *wis2*$^+$ is also required for wis2 activity (see below section 4.3), the observation that pIcyp1-1 could not confer wis2 activity may be explained in at least two ways: the cyclophilin domain alone is insufficient for wis2 activity, or an intrinsic substrate specificity is contained within the cyclophilin domain. In order

Figure 4.1 ***cyp1*$^+$ genomic subclone**

The restriction map of pBKS$^+$ cyp1 is outlined; an arrow indicates the length and orientation of the putative *cyp1*$^+$ ORF.

The *Eco*RI restriction sites used for subsequent subcloning into the *S.pombe* plasmid pIRT2 are indicated by asterisks.

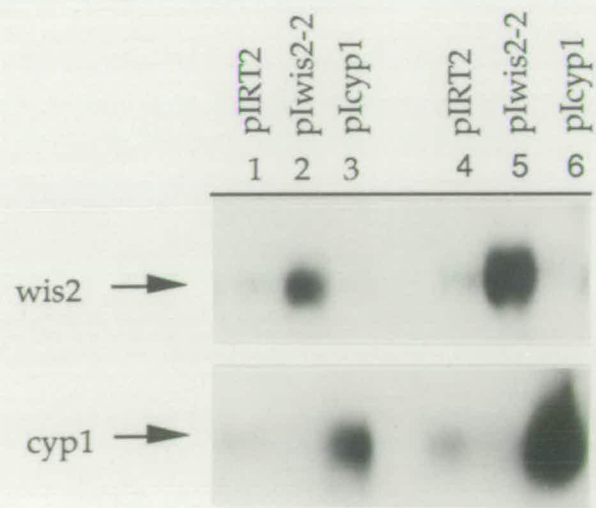


□ pBKS$^+$cyp1 vector sequences (not to scale)

Figure 4.2 Overexpression of *cyp1*$^+$ mRNA

Total RNA of *cdc25-22 wee1-50 win1-1 leu1-32 h-* strains, carrying either pIRT2 (control plasmid), pIwis2-2 or pIcyp1, was subjected to Northern blot analysis. The blot was probed sequentially with radiolabelled *Hin*cII and *Eco*RI fragments containing the predicted ORFs for *wis2*$^+$ and *cyp1*$^+$, respectively.

Lanes 1-3 and lanes 4-6 were loaded with 5 and 15 μg total RNA, respectively.



to test these possibilities, the C-terminal region of *wis2*$^+$ was fused to the 3' end of *cyp1*$^+$, and the ability of the hybrid protein to confer wis2 activity was examined.

The construction of the hybrid gene is shown in Figure 4.3. The border line between the cyclophilin domain and the C-terminal region of *wis2*$^+$ could be predicted as cyclophilin sequences are terminated with a highly conserved stretch of amino acids (see sequences presented in Figure 4.3 and the consensus sequence corresponding to wis2 amino acids at position 160-167 in chapter 2, Figure 2.7). The hybrid *cyp1-wis2* gene contains the predicted ORF of *cyp1*$^+$ (excluding the last codon encoding a valine, which is replaced by the *wis2*$^+$ codon for cysteine). The cyclophilin-like *cyp1*$^+$ sequence is followed by *wis2*$^+$ sequences, which potentially encode the C-terminal 188 amino acids.

cyp1-wis2 cloned into pIRT2 (pIcyp1-wis2) was transformed into the *cdc25-22 wee1-50 win1-1* strain. Remarkably, it was able to suppress the triple mutant phenotype with comparable efficiency to functional subclones of *wis2*$^+$ in pIRT2 plasmids (see Figure 4.4). This result is consistent with the prediction that the C-terminal domain *wis2*$^+$ is required for wis2 activity, furthermore, it suggests that the C-terminal domain can confer specificity on a cyclophilin domain of a different gene.

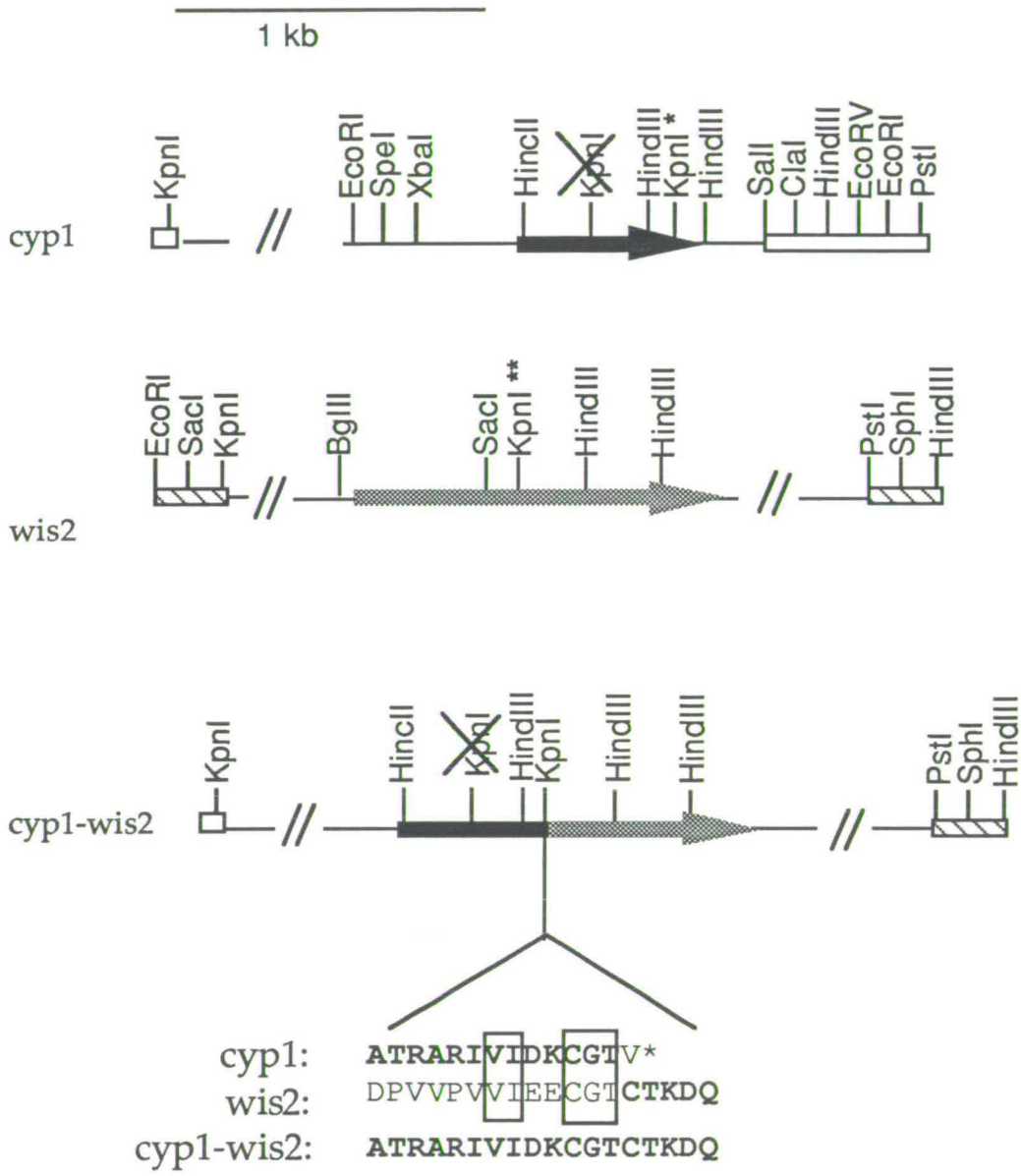
The experiments described so far can not role out the possibility that the C-terminal domain of *wis2*$^+$ alone is required for wis2 activity. Experiments that address this possibility will be described in section 4.3.

Figure 4.3 pIcyp1-wis2 construct

The strategy for the construction of a hybrid *cyp1-wis2* gene took advantage of a *Kpn*I site at the very 3' end of the *cyp1*$^+$ ORF (*Kpn*I*, indicated at the top bar, representing the *cyp1*$^+$ clone in pKS$^+$). In order to employ this *Kpn*I site for subsequent ligations, another *Kpn*I site, internal to the *cyp1*$^+$ gene, was removed. The removal of the *Kpn*I site resulted in a silent mutation at the predicted Gly60 residue of the cyp1 protein. In addition, a *Kpn*I site was introduced into *wis2*$^+$ at the border between the cyclophilin and the C-terminal region of *wis2*$^+$ (*Kpn*I**, indicated at the bar representing the *wis2*$^+$ clone in pIRT2). Introduction of this *Kpn*I site resulted in a silent mutation of the predicted Gly164 and Thr165 residues of wis2. All mutations were introduced by oligonucleotide directed *in vitro* mutagenesis (for details of oligonucleotide sequences see Materials and Methods, section 7.7).

Subsequently, the *Kpn*I fragment of the mutated *cyp1*$^+$ clone was cloned into the mutated *wis2* gene, such that it replaced the *Kpn*I fragment containing the cyclophilin region of *wis2*$^+$.

The predicted amino acid sequences of *cyp1*$^+$ and *wis2*$^+$ around the fusion point are shown. Highly conserved amino acid residues at the C-terminal of cyclophilins are indicated by boxes. The hybrid *cyp1-wis2* gene contains all the ORF of *cyp1*$^+$, excluding the last three base pairs, fused to the C-terminal domain of *wis2*$^+$. *cyp1-wis2* is predicted to be transcribed from the promoter of the *cyp1*$^+$ gene.

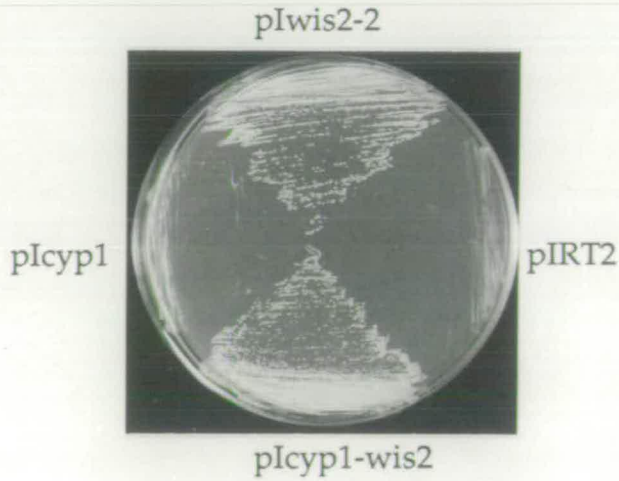


polylinker of pBKS^{+}

polylinker of pTZ18R

Figure 4.4 Test for suppression activity of *cyp1*$^+$ and *cyp1-wis2*

pIcyp1 and pIcyp1-wis2 were tested for the ability to suppress the triple mutant phenotype on minimal plates at 35°C.
cdc25-22 wee1-50 win1-1 leu1-32 h- transformed with either pIRT2, pIwis2-2, pIcyp1 or pIcyp1-wis2 were grown at the permissive temperature, then streaked onto fresh plates and incubated at the restrictive temperature for six days.



iii Detection of the wis2, cyp1 and cyp1-wis2 proteins

Detection of wis2, cyp1 and cyp1-wis2 proteins was performed by immunoblotting with polyclonal antibodies raised against human Cyp-A protein, a kind gift from C. Walsh (Nadeau et al. 1993). These antibodies were expected to recognise *S.pombe* cyclophilins since the homology between Cyp-A and the yeast cyclophilins wis2 and cyp1 is 58% and 78.9%, respectively, over the region spanning the cyclophilin domain.

Total *S.pombe* protein extracts were separated under denaturing conditions, blotted to nylon membranes, and immunodetected by using the ECL immunodetection system. Results are shown in Figure 4.5.

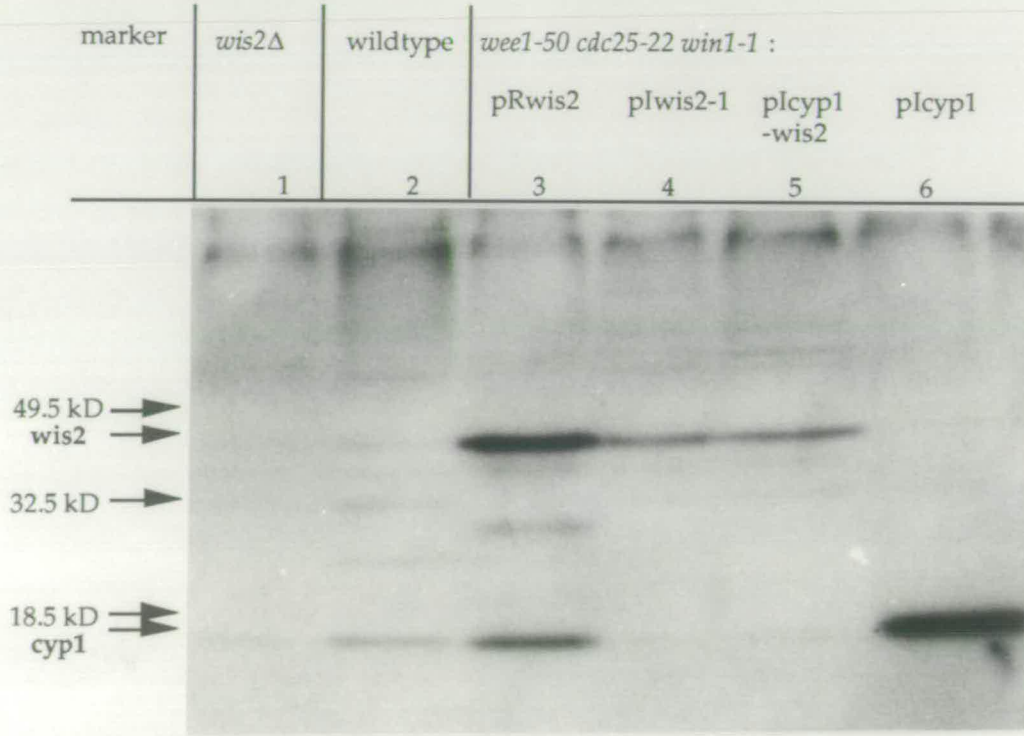
Cells transformed with pIwis2-1 and pIcyp1-wis2 exhibited increased expression of a ~40 kDa protein (lane 4,5) compared with wild type protein extract (lane 2) or compared with cells transformed with the control plasmid pIRT2 (Figure 4.5, lanes 1), consistent with the predicted molecular weight of wis2 and cyp1-wis2. Cells transformed with pIcyp1 showed increased expression of an ~18 kDa protein (lane 6), consistent with the predicted molecular weight of cyp1. The above observations indicate that the human CyP-A antibodies cross-reacted with the overexpressed *S.pombe* cyclophilin-like proteins. Note that the level of overexpression of the different kinds of cyclophilin-like proteins cannot be compared, as the anti-human-CyPA antibodies may have a different affinity to each of the *S.pombe* cyclophilins.

In cells carrying pRwis2 (*wis2*$^+$ is expressed from the strong *nmt1* promoter), a further increase of the 40 kDa protein is observed under derepressed conditions, compared with that of cells carrying pIwis2-2 (lanes 3 and 4). This observation is consistent with the Northern blot analysis (Figure 3.1), which showed a further increase in *wis2*$^+$ transcript level in cells transformed with pRwis2. In cells transformed with pRwis2 the overexpression of ~18, ~30 and ~38 kDa proteins was also observed. The presence of the ~30 and ~39 kDa proteins may have resulted from a proteolytic degradation of the overexpressed wis2 or from translation initiation of an internal methionine residue. The presence of elevated level of the 18 kDa species may reflect the presence of a particularly sensitive proteolytic site between the cyclophilin and the C-terminal region of wis2.

Figure 4.5 Detection of the proteins wis2, cyp1 and cyp1-wis2

Total protein extracts were prepared of the following strains: *wis2Δ*; wild type *972h*$^{-}$ and *cdc25-22 wee1-50 win1-1 leu1-32 h-* transformed with pRwis2, pIwis2-1, pIcyp1-wis2 or pIcyp1. Cells containing the pRwis2 plasmid were grown under derepression conditions for full activity of the *nmt1* promoter in pRwis2 (see section 7.5 viii for protein extraction method).

Protein extracts were blotted onto nylon membranes under denaturing conditions (Material and Methods, section 7.3 xiii). Each lane contains ~ 40 µg of total protein. Polyclonal antibodies raised against human CyP-A were used for detection at a dilution of 1: 200 from an unknown concentration of a polyclonal antiserum. The enhanced chemiluminescence detection (ECL) system was employed (section 7.3 xiv). Autoradiography films were exposed for 5-10 seconds.



The Western blot analysis included protein extracts of wild type cells and cells carrying a deletion of *wis2*$^+$ (Figure 4.5 lanes 1,2). The detection in these extracts is poor and is difficult to interpret. Three different protein species seem to be recognised by the antibodies: detection bands corresponding to approximate molecular weights of 18, 32 and 40 kDa are observed. The species of 18 kDa protein corresponds to the predicted molecular weight of cyp1 but as the 18 kDa band is somewhat stronger than the other bands in wild type extracts, it may also reflect the presence of other 18 kDa cyclophilins. This latter suggestion is plausible as the 18 kDa form of cyclophilin was found to be encoded by more than one gene in a variety of organisms (Galat 1993). The detection of the 32 and 40 kDa species is very weak and therefore can not be unambiguously discerned from non-specific binding.

In longer exposure of the blot shown in Figure 4.5 (data not shown), protein extracts of *wis2Δ* strain revealed the same detected species as observed in wild type cells, including the 40 kDa species which corresponds to the predicted molecular weight of wis2. If this detection reflects a specific interaction, the presence of another 40 kDa cyclophilin-like protein, in addition to wis2, must be assumed. This assumption is plausible as the cyclophilin family of proteins appears to reveal a great extent of redundancy.

4.3 Mutational analysis of wis2 cyclophilin-like region

i Overexpression of mutated *wis2* genes

In order to examine whether the cyclophilin domain of *wis2*$^+$ is required for wis2 activity, three different mutations were introduced. One mutation is a deletion of the sequence encoding eleven amino acids (*wis2Δ11*). This mutation results in the deletion of highly conserved amino acids, several of which are predicted to participate in the formation of the binding site of the cyclophilin enzyme (see Figure 4.6). Two other mutations are point mutations which convert His129 into either leucine or proline (*wis2L129* and *wis2P129*). His129 is a highly conserved residue which is found in all eukaryotic cyclophilins reported to date (see Figure 4.6). The equivalent His126 residue in human-CyPA has been predicted, based on the three dimensional structure of the crystallised cyclophilin and NMR studies, to participate in the actual

enzymatic activity of peptidyl-prolyl *cis trans* isomerisation (Kallen et al. 1991).

The above mutations were introduced into pTZwis2-1, employing the method of oligonucleotide directed *in vitro* mutagenesis (section 7.7). For the presence of the Δ11 and L129 mutations screens were carried out by restriction digest analysis: screening for the loss of a *Bal*I restriction site for *wis2Δ11* and for the introduction of an additional *Hind*III restriction site for *wis2L129* . The presence of the P129 mutation was screened for by sequence analysis. All isolated *wis2* mutated genes were sequenced across the mutated region, covering a region of at least 100 bp around the mutation.

The mutated *wis2* genes were cloned into pIRT2 and transformed into the *cdc25-22 wee1-50 win1-1* strain. None of the mutated genes were able to rescue the lethality of the triple mutant strain (Figure 4.7), and no alleviation of the aberrant phenotype of the strain at the restrictive temperature was noted.

Figure 4.6 Mutations within the cyclophilin region of *wis2*$^+$

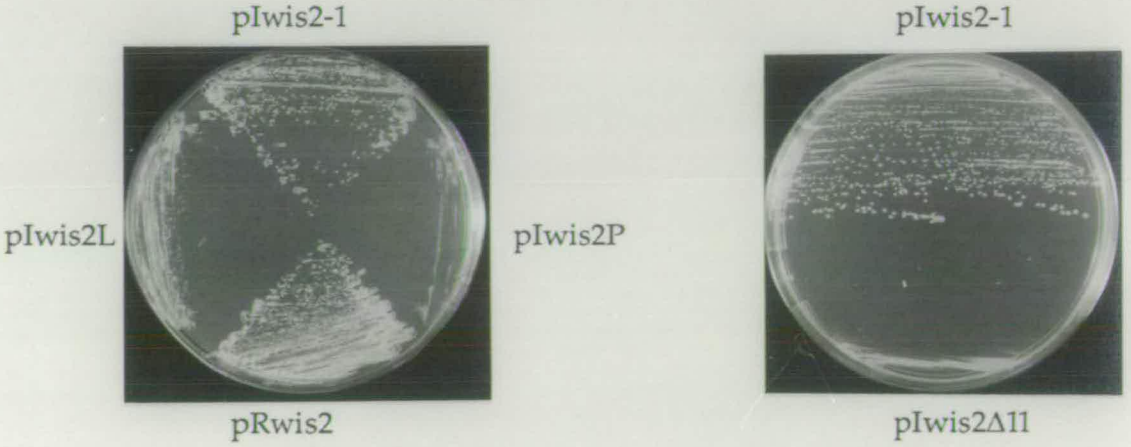
Alignment of the cyclophilin region of various types of cyclophilin or cyclophilin related proteins. The amino acid sequences were obtained and aligned as described in figure legend 2.8.

The predicted amino acids of the wis2 protein which were subjected to mutagenesis analysis are shaded: (1) a deletion of eleven amino acids: residues Ser102-Gly112; (2) point mutations at His129, converting it into either leucine or proline. The location of these mutations within the predicted secondary structure of the cyclophilin domain can be inferred by superimposing the amino acid sequence of wis2 onto that of the human CyP-A protein, the cyclophilin protein whose structure at the atomic level has been solved (Kallen et al. 1991; Thériault et al. 1993; see also figure legend 2.8).

```
                                                                                                β1
Hu-CypA  ..........  ..........  ..........  ..........  ..VNP TVFFD  8
Sp-wis2  ..........  ..........  ..........  ..........  ..MSTYAYFK  8
Sp-Cyp1  ..........  ..........  ..........  ..........  ...MSNCFFD  7
Sc-Cyp1  ..........  ..........  ..........  ..........  ...MSQVYFD  7
Nc-Cyph  MFGPRHFSVL  KTTGSLVSST  FSSSLKPTAT  FSCARAFSQT  SSIMSKVFFD  50
Hu-Cypb  .........M  KVLLAAALIA  GSVFFLLLPG  PSAADEKKKG  PKVTVKVYFD  41
Sc-Cyp2  .........M  KFSGLWCWLL  LFLSVNVIAS  DVGELIDQDD  EVITQKVFFD  41
Dm-Nina  ..........  ........MK  SLLNRIILCS  AFLAVASGLS  FTVTSRIYMD  32
Ec-Cyph  ..........  ..........  ..........  .....MFKST  LAAMAAVFAL  15
    con  ..........  ..........  ..........  ..........  ..vtskv.fd

                          β2                                                    α1
Hu-CypA  IAVDGEPL..  ..........  ..GRVSFELF  ADKVPKTAEN  FRALSTG...  41
Sp-wis2  ISIDGKI...  ..........  .QPTIYFELF  DNVVPKTVKN  FASLCNGFEK  44
Sp-Cyp1  VIANGQPL..  ..........  ..GRIVFKLF  DDVVPKTAAN  FRALCTG...  40
Sc-Cyp1  VEADGQPI..  ..........  ..GRVVFKLY  NDIVPKTAEN  FRALCTG...  40
Nc-Cyph  LEWEGPVLGP  NNKPTSEIKA  QSGRINFTLY  DDVVPKTARN  FKELCTG...  97
Hu-Cypb  LRIGDEDV..  ..........  ..GRVIFGLF  GKTVPKTVDN  FVALATG...  74
Sc-Cyp2  IEHGEEKV..  ..........  ..GRIVIGLY  GKVCPKTAKN  FYKLSTTTNS  77
Dm-Nina  VKHNKKPV..  ..........  ..GRITFGLF  GKLAPKTVAN  FRHIC...LR  65
Ec-Cyph  SALSPAAMAA  KGDPHVLLTT  SAGNIELELD  KQKAPVSVQN  FVDYVNSGF.  64
    con  .e.dgep...  ..........  ..grivfgLf  .dvvPKTa.N  Fralctg...

                      β3  * *  *   β4          *
Hu-CypA  .EKGFGYKGS  CFHRIIPGFM  CQGGDFTRHN  GTGGKSIYGE  KFEDEN..FI  88
Sp-wis2  DGRCLTYKGS  RFHRVIKNFM  LQGGDFTRGN  GTGGESIYGE  KFEDEN..FE  92
Sp-Cyp1  .EKGYGYAGS  TFHRVIPQFM  LQGGDFTRGN  GTGGKSIYGE  KFPDEN..FA  87
Sc-Cyp1  .EKGFGYAGS  PFHRVIPDFM  LQGGDFTAGN  GTGGKSIYGG  KFPDEN..FK  87
Nc-Cyph  .QNGFGYKGS  SFHRIIPEFM  LQGGDFTRGN  GTGGKSIYGE  KFADEN..FA  144
Hu-Cypb  .EKGFGYKNS  KFHRVIKDFM  IQGGDFTRGD  GTGGKSIYGE  RFPDEN..FK  121
Sc-Cyp2  KK...GFIGS  TFHRVIPNFM  VQGGDFTDGT  GVGGKSIYGD  TFPDEN..FT  122
Dm-Nina  GINGTSYVGS  RFHRVVDRFL  VQGGDIVNGD  GTGSISIYGD  YFPDEDKALA  115
Ec-Cyph  ......YNNT  TFHRVIPGFM  IQGGGFT..E  QMQQKKPNPP  IKNEADNGL.  105
    con  .ekgfgykgS  .FHRvip.Fm  lQGGDftrgn  GtGgkSIYGe  kFpDEn..fa

                  β5   ***          * β6        **αX  *     β7
Hu-CypA  LKHTGPGILS  MANAGPNTNG  SQFFICTA.K  TEWLDGKHVV  FGKVK..EGM  135
Sp-wis2  LKHDKPFLLS  MANAGPNTNG  SQFFITTV.P  TPHLDGKHVV  FGKVI..QGK  139
Sp-Cyp1  LKHNKPGLLS  MANAGPNTNG  SQFFITTV.V  TPWLDGKHVV  FGEVT..EGM  134
Sc-Cyp1  KHHDRPGLLS  MANAGPNTNG  SQFFITTV.P  CPWLDGKHVV  FGEVV..DGY  134
Nc-Cyph  KKHVRPGLLS  MANAGPNTNG  SQFFVTTV.P  TSWLDGRHVV  FGEVADDESM  192
Hu-Cypb  LKHYGPGWVS  MANAGKDTNG  SQFFITTV.K  TAWLDGKHVV  FGKVL..EGM  168
Sc-Cyp2  LKHDRKGRLS  MANRGKDTNG  SQFFITTTEE  ASWLDGKHVV  FGQVV..DGM  170
Dm-Nina  VEHNRPGYLG  MANRGPDTNG  CQFYVTTV.G  AKWLDGKHTV  FGKVL..EGM  162
Ec-Cyph  ..RNTRGTIA  MARTADKDSA  TSQFFINVAD  NAFLDHGQRD  FGYAVFGKVV  153
    con  lkHdrpglls  MANaGpnTNG  sQFfitTv.p  tpwLDGkHvV  FGkV...egm

             α8                        β8
Hu-CypA  NIVEAMERFG  SRNGKTS..K  KITIADCGQL  E.........  ..........  164
Sp-wis2  STVRTIENLE  TKNDDP..VV  PVVIEECGTC  TKDQIEAPKP  DVTGDSLEEF  187
Sp-Cyp1  DVVKKVESLG  SNSGATR..A  RIVIDKCGTV  ..........  ..........  162
Sc-Cyp1  DIVKKVESLG  SPSGATK..A  RIVVAKSGEL  ..........  ..........  162
Nc-Cyph  KVVKALEATG  SSSGAIRYSK  KPTIVDCGAL  ..........  ..........  222
Hu-Cypb  EVVRKVESTK  TDS.RDKPLK  DVIIADCGKI  EVEKPFAIAK  E.........  208
Sc-Cyp2  DVVNYIQHV.  SRDANDKPLE  AVKIAKCGEW  TPELSS....  ..........  205
Dm-Nina  DTIYAIEDVK  TDTDDF.PVE  PVVISNCGEI  PTEQFEFYPD  DFNILGWIKA  199
Ec-Cyph  ..KGMDVADK  ISQVPTHDVG  PYQNVPSKPV  VILSAKVLP.  ..........  190
    con  dvvk..eslg  s.sgatk..k  .vvia.cGel  ..........  ..........
```

Figure 4.7 Test for suppression activity of mutated *wis*2 genes

pIwis2L, pIwis2P, and pIwis2Δ11 were tested for the ability to suppress the triple mutant phenotype on minimal plates at 35°C. *cdc25-22 wee1-50 win1-1 leu1-32 h-* transformed with the above plasmid were grown at the permissive temperature, then streaked onto fresh plates and incubated at the restrictive temperature for six days. pIRT2 and pIwis2-1 plasmids were used as negative and positive controls, respectively.



ii Detection of wis2 mutated proteins

The level of expression of the mutated *wis2* genes was examined by Northern and Western blot analysis (Figures 4.8 and 4.9, respectively). The Phosphor-imager analysis of the Northern blot shown in Figure 4.8 indicated a slight decrease in the level of *wis2* mRNA detected in *cdc25-22 wee1-50 win1-1* transformed with the mutated genes compared with the level of *wis2* mRNA when the wild type gene is transformed: about 1.5 fold decrease in pIwis2L and pIwis2P transformants and 4 fold decrease in pIwis2Δ11 transformants. The reason for this decrease is not obvious, but as the decrease is not dramatic it may not indicate a significant difference between expression of the mutated and *wis2*$^+$ mRNAs.

In contrast to the Northern blot analysis, the Western blot analysis suggests a significant decrease in the protein level of wis2 in cells transformed with multicopy plasmid containing the mutated *wis2* gene, compared with cells transformed with the wild type gene (Figure 4.9). In cells transformed with pIwis2L or pIwis2Δ11, no increase of level of the 40 kDa signal is observed compared with the signal observed when cells transformed with the vector pIRT2 (lanes 1, 6, 7). Only in strains transformed with pIwis2P the signal at 40 kDa is slightly stronger than that detected in cells transformed with pIRT2, although it is still much weaker than the signal in cells transformed with pIwis2-1 (lanes 3, 5).

Several explanations might account for the poor detection of wis2 protein in strains expressing the mutated *wis2* genes. One possible explanation is that the mutations introduced reduce the stability of the proteins, therefore, the mutated proteins are rapidly degraded and can not accumulate to high levels, even when expressed from multicopy vectors. Another possible explanation is that the antibodies failed to recognise the mutated proteins, either because the mutation has introduced an overall change in the structure of the protein, or that the mutations specifically alter a highly antigenic region of the cyclophilin domain. This later possibility is less likely as the antibodies used are polyclonal antibodies.

The inability to detect overexpression of the wis2 proteins mutated at the cyclophilin domain makes it impossible to determine whether the mutated proteins are capable of wis2 activity. Such an activity is clearly dependent on overexpression of the wild type wis2 protein. In order to examine direct involvement of the cyclophilin domain in wis2 activity, in

Figure 4.8 Northern blot analysis of mutated *wis2* genes

Total RNA was extracted from cells of the *cdc25-22 wee1-50 win1-1 leu1-32 h*$^-$ strain transformed with the following plasmids: pIwis2-1 (lane 1), pRwis2 (lane 2, cells grown in the absence of thiamine for de-repression of the *nmt1* promoter in the pRwis2 plasmid), pRwis2 (lane 3, cells grown in the presence of thiamine for repression of the *nmt1* promoter), pIwis2L (lane 4), pIwis2P (lane 5), pIwis2Δ11 (lane 6). 10 μg of the RNA extracts were loaded in each lane, Northern blotting was performed and the blot probed sequentially with radiolabelled *Hin*cII fragment, containing the predicted ORFs for *wis2*$^+$, and radiolabelled *Eco*RI fragment internal to the *S.pombe adh1*$^+$ gene, which was used as a loading control.

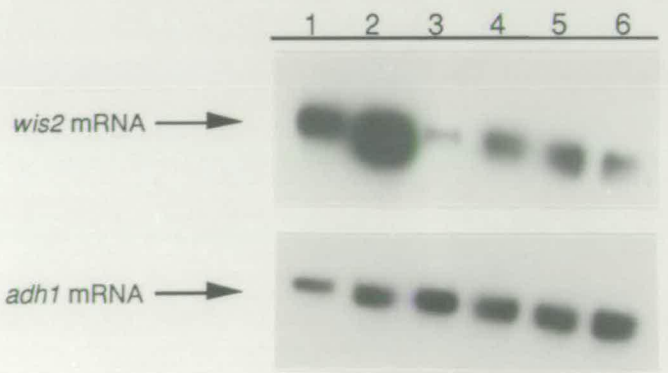
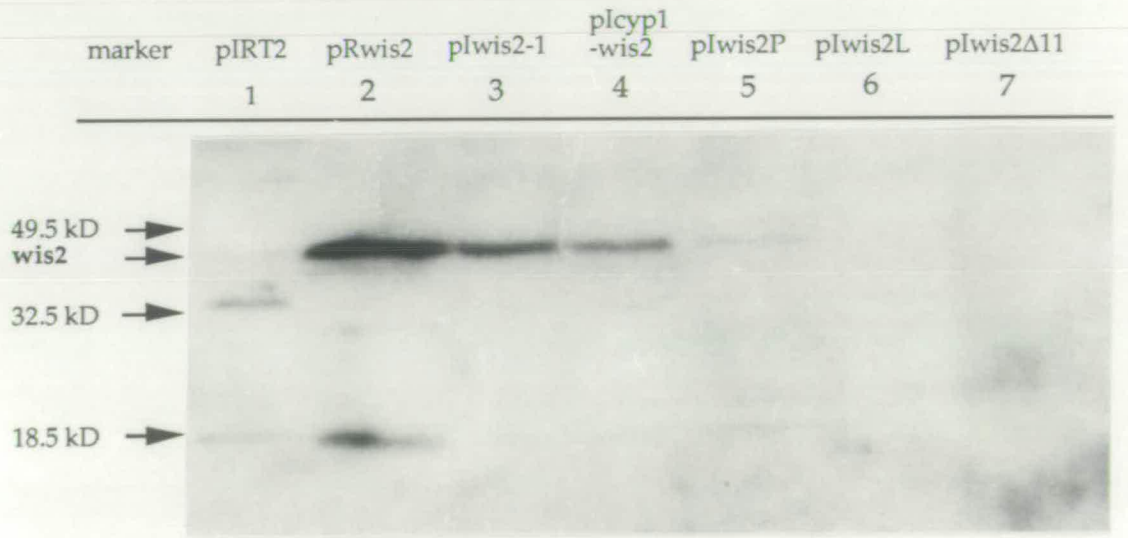


Figure 4.9 Western Blot analysis of *cdc25-22 wee1-50 win1-1* transformed with mutated *wis2* genes

Cells of the strain *cdc25-22 wee1-50 win1-1 leu1-32 h⁻* were transformed with the following plasmids: pIRT2 (control plasmid), pRwis2, pIwis2-1 , pIcyp1-wis2, pIwis2P, pIwis2L and pIwis2Δ11. Protein extracts were prepared from each transformant. Cells transformed with pRwis2 were grown in the absence of thiamine for full expression of *wis2*$^+$ from the *nmt1* promoter. Western blotting was performed as described in figure legend 4.4.



particular, the requirement for the putative enzymatic site of the cyclophilin, it must be ensured that the mutated wis2 proteins are expressed to a level that is known to be sufficient for the wild type protein to confer wis2 activity. Possible strategies for further analysis will be discussed in chapter 6.

4.4 Conclusions

This chapter described the detection of the wis2 protein when overexpressed in *S.pombe* cells. An increased signal of a ~40 kDa protein was detected by Western blot analysis, using polyclonal anti-human cyclophilin antibodies. These antibodies reacted only weakly with wild type protein extracts which could not be definitely discerned from non-specific reaction.

A structure-function analysis of the wis2 protein was carried out. The requirement for the cyclophilin domain was investigated by introducing a short deletion and point mutations into the cyclophilin domain of *wis2*$^+$. The mutated genes are no longer able to confer wis2 activity when expressed from a multicopy yeast plasmid, however, Western blot analysis suggested that the mutated proteins were not expressed to the same level as the wild type wis2 protein. Therefore, it is not clear whether the inability to confer wis2 activity stems from a specific defect of the cyclophilin active site, or whether it is a consequence of the instability of the mutated proteins.

The structure-function analysis of the predicted C-terminal region of wis2 was first assessed indirectly by expressing the *S.pombe* cyclophilin-like gene, *cyp1*$^+$, from a yeast multicopy plasmid. The *cyp1*$^+$ gene consists of a cyclophilin-like domain only and was unable to confer wis2 activity when overexpressed. Overexpression at the molecular level was confirmed by Northern and Western blot analysis. Significantly, a strong signal corresponding to the predicted molecular weight of the *cyp1*$^+$ gene product was observed by Western blot analysis, using anti-human Cyp-A antibodies. Although the wild type *cyp1*$^+$ was unable to confer wis2 activity, a hybrid gene which consists of the *cyp1* gene fused to the C-terminal region of *wis2*, exhibited wis2 activity with comparable efficiency to that of the *wis2*$^+$ gene. This result may suggest that the C-terminal domain of *wis2*$^+$ may confer specificity to the cyclophilin domain. Such a specificity may reflect targeting the cyclophilin domain to its substrate, either by allowing complexing with a

the target substrate, or by localising the protein to a special compartment of the cell.

Chapter 5: Towards cloning of *win1+*

5.1 Introduction

The genetic screen in which *wis2+* was isolated, along with *wis1+* and *wis3+* - *wis5+*, was originally attempted to clone the *win1+* gene (Warbrick 1990; see section 1.3 ii, 2.1 i). In this screen genomic libraries in yeast multicopy plasmid vectors were screened for complementation of the lethal phenotype of *cdc25-22 wee1-50 win1-1*. However, the *win1+* gene appeared refractory to cloning by such a method.

The failure to clone *win1+* could be due to a number of possible reasons, including *win1+* being deleterious to *S.pombe* when overexpressed, *win1+* being unclonable in *E.coli*, or *win1+* not being represented in the DNA libraries used. Another attempt to clone the *win1+* gene, which involves the screening of cosmids of ordered libraries by complementation tests, is described in this chapter. This alternative approach, although related to the previous method is improved in that it allows the examination of a specific chromosomal region where the *win1+* is expected to lie. Additionally, the method avoids the cloning of extragenic suppressors which could mask the presence of *win1+*.

The alternative approach for the cloning of *win1+* involved genetic mapping of *win1* relative to the *wis2*, *rad1*, and *tps19*. *tps19* sequences were then used to screen by DNA hybridisation ordered cosmid and bacteriophage P1 genomic libraries, made from the *S.pombe* strain *972h-* (Hoheisel et al. 1993). Cosmid clones that span the *tps19* region were obtained and screened either directly, by co-transformations of the cosmid clones into appropriate genetic backgrounds, or after an additional step of sub-cloning of the cosmid DNA into a *S.pombe* yeast vector.

5.2 Genetic mapping of *win1*

i Genetic mapping of *win1* , *tps19* and *wis2*

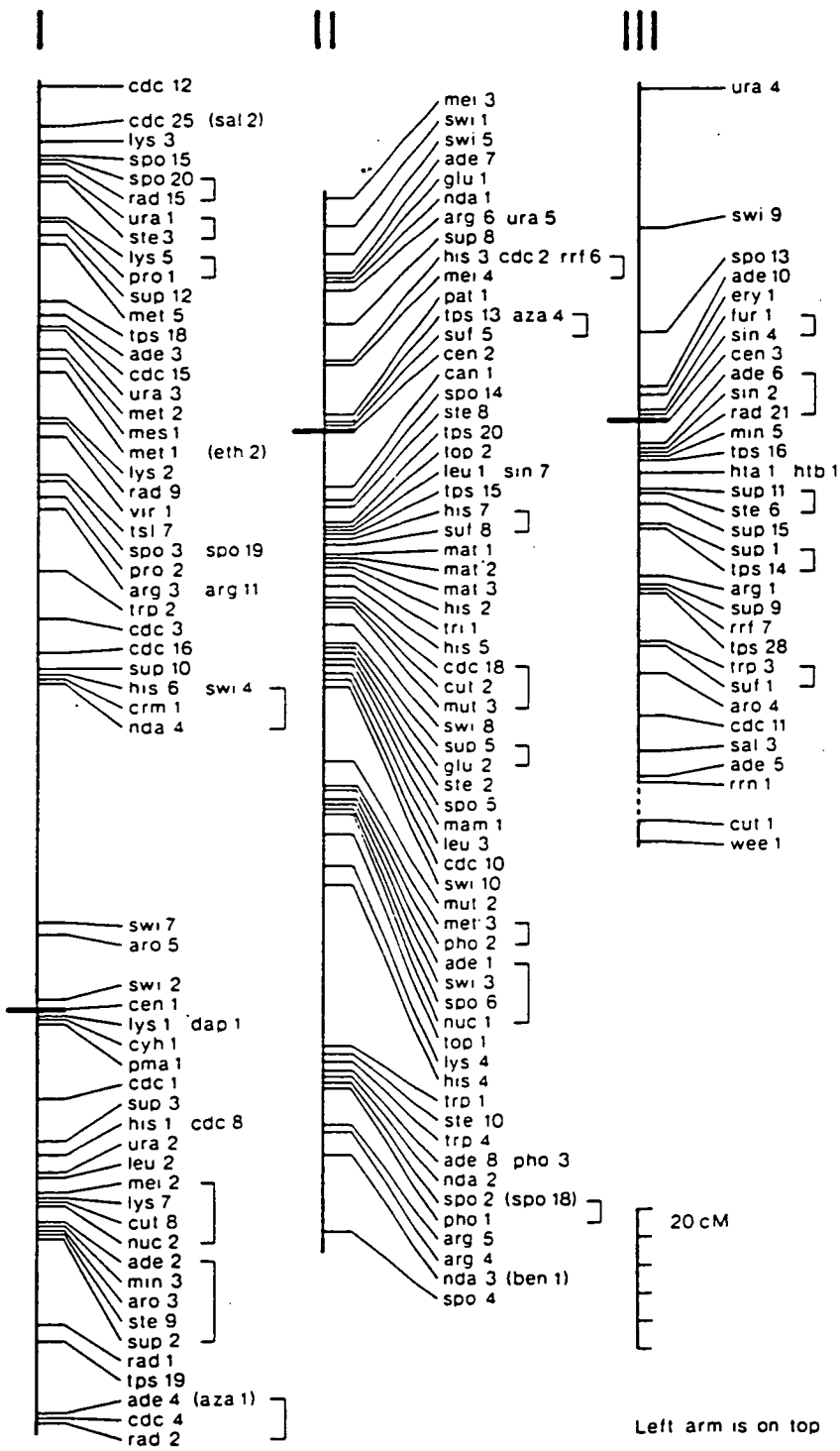
The *win1* locus was previously mapped by genetical procedures to the right arm of chromosome I (see Figure 5.1), employing the elongated phenotype to identify *win1-1* mutant segregants (Warbrick 1990). The *win1* locus was found to map close to two different genetic markers, *tps19* and *wis2* (Warbrick 1990). Genetic map distances were determined by scoring the recombination frequencies of meiotic progeny in random spore analysis. In such an analysis, one percent of recombinants is equivalent to one centiMorgan (cM), or to one map unit. The *tps19* locus was mapped 4 - 5 cM away from *win1*, using the temperature sensitive allele, *tps19-17*, as a genetic marker. The genetic mapping of *wis2* was carried out by integrating the *S.cerevisiae* genetic marker *LEU1* at the *wis2* locus. The segregation pattern of the *LEU1* marker was then followed in a *leu1-32* genetic background. This method mapped the *wis2* locus 12 cM away from the *win1* locus.

In order to further investigate the genetic distances and to determine the order of the loci described above, a three point cross test was employed. Particularly, it was important to re-examine the mapping data concerning *wis2*, as it was possible that the *LEU1* integrant used for the mapping of this locus was a result of a multicopy integration event at the *wis2* locus (E. Warbrick, pers comm). Such multicopy integration event could affect recombination events around the *wis2* locus. It is likely that multicopy integration of *LEU1* was selected for because of the inefficiency of a single copy of the heterologous *LEU1* gene to complement the *S.pombe leu1-32* mutation. Mapping of *wis2* was repeated, using as a tag the *S.pombe ura4*$^+$ selective marker. This marker is expected to complement efficiently the *ura4-D18* mutation in single copy.

Figure 5.1 Genetic map of *S.pombe*

From Munz et al. 1989.

The *tps19* and *rad1* loci on the right arm of chromosome I are highlighted.



For the construction of *ura4*$^+$ integrants, the plasmid pBC[wis2-5C; ura4$^+$] was constructed. To this aim, a 1.8 kb *ura4*$^+$ fragment was cloned into the *wis2* subclone pBCwis2-5C (Warbrick 1990; and see Figure 5.2). pBC[wis2-5C; ura4$^+$] was transformed by the protoplast method into *win1-1 tps19-17 leu1-32 ura4-D18 h*$^+$, and transformants were selected on minimal media supplemented with leucine but lacking uracil. Note that pBC[wis2-5C; ura4$^+$] is devoid of yeast autonomous replication sequences, therefore a high proportion of ura$^+$ transformants are expected to result from integration of the plasmid into the *S.pombe* genome. Stable ura$^+$ transformants were identified by plasmid stability tests (Materials and Methods, section 7.5 v). Colonies that were stable for the *ura4*$^+$ phenotype were further analysed by Southern blotting in order to determine if integration of the *ura4*$^+$ genetic marker had occurred at the *wis2* locus.

Chromosomal DNAs of wild type and two independent integrant strains (Int1 and Int2) were digested with the restriction enzyme *Mlu*I, which does not cut within pBC[*wis2*-5C; ura4$^+$]. The digest reactions were electrophoresed on agarose gel, and blotted into nylon membranes. The blots were then probed with the 3.7 kb *Pst*I fragment of pBC[*wis2*-5C; ura4$^+$]. The result is shown in Figure 5.3. Wild type restricted chromosomal DNA shows a hybridisation band of the size of ~ 7 kb, whereas integrant strains show a shift of the hybridising band to the molecular weight of ~ 14 kb. This shift is consistent with a single integration of a 7.3 kb pBC[*wis2*-5C; ura4$^+$] sequence at the *wis2* locus (see schematic diagram in Figure 5.3).

The two integrant strains, Int1 and Int2, were crossed with *ura4-D18 h*$^-$ and the cross subjected to random spore analysis. The phenotype of 100 colonies was scored in each cross. The ura$^+$ phenotype was scored by testing for growth on minimal medium at 28°C; the win1$^-$ elongated phenotype was scored by microscopic examination of cell length on minimal plates at 28°C, and the tps19$^-$ temperature sensitive phenotype was scored on YE plates at 35°C. The results are summarised in Table 5.1. The recombination frequencies observed place the *win1* locus 2 - 3 cM away from the *tps19* locus and 12 - 13 cM away from the *wis2* locus. These genetic distances are similar to the previous data described by Warbrick (1990).

Figure 5.2 pBC[wis2-5-C; ura4$^+$]

The restriction map of the plasmid used for integration at the *wis2* chromosomal locus, pBCwis2-5C, is shown. The *ura4*$^+$ *Bam*HI fragment of the plasmid pON160 (a gift from S. Aves) was cloned into the *Bam*HI site of pBCwis2-5C, a *wis2* subclone constructed by Warbrick (1990).

The *Pst*I fragment used as hybridization probe for subsequent Southern blot analysis of putative pBC[wis2-5-C; ura4$^+$] integrants is indicated as a bar with an asterisk at its end (see section 5.2 i).

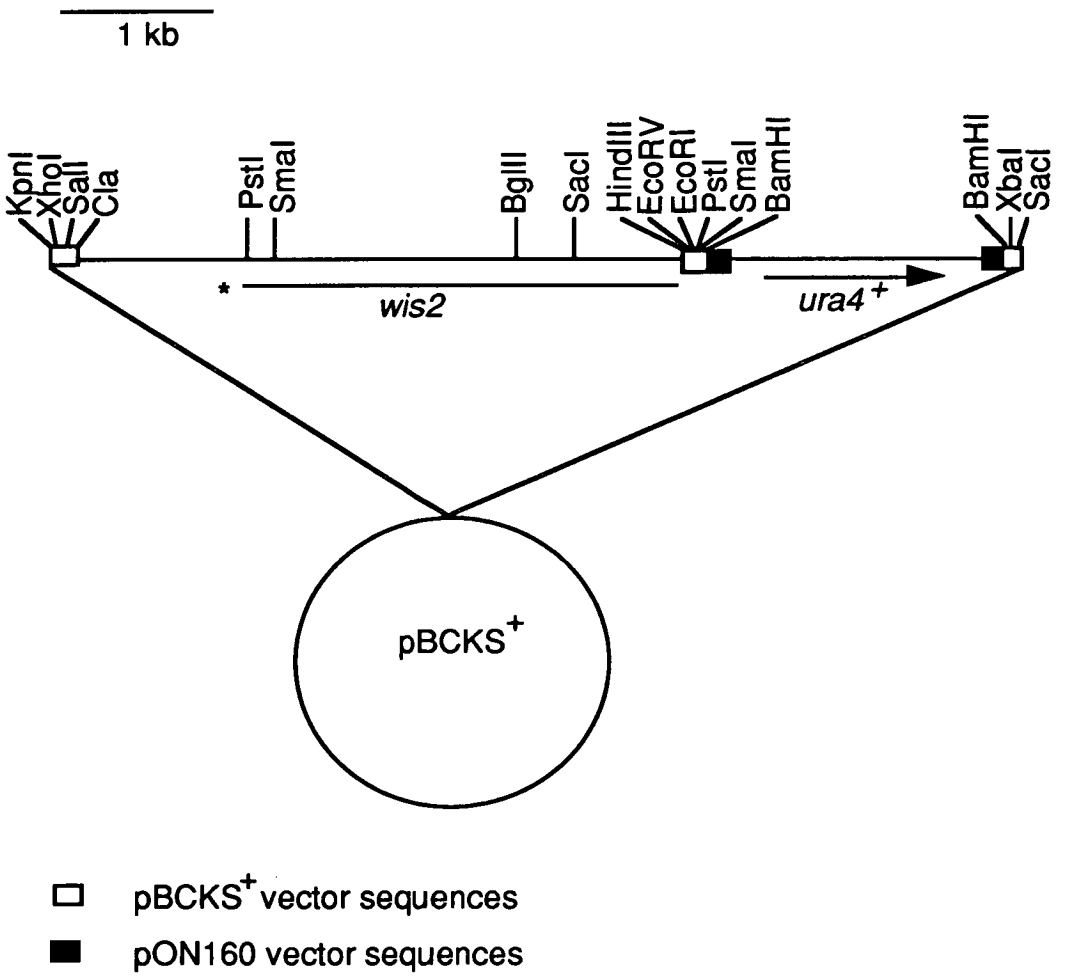


Figure 5.3 Southern Blot analysis of strains containing integrated copy of pBC[wis2-5-C; ura4$^+$] at the *wis2* locus

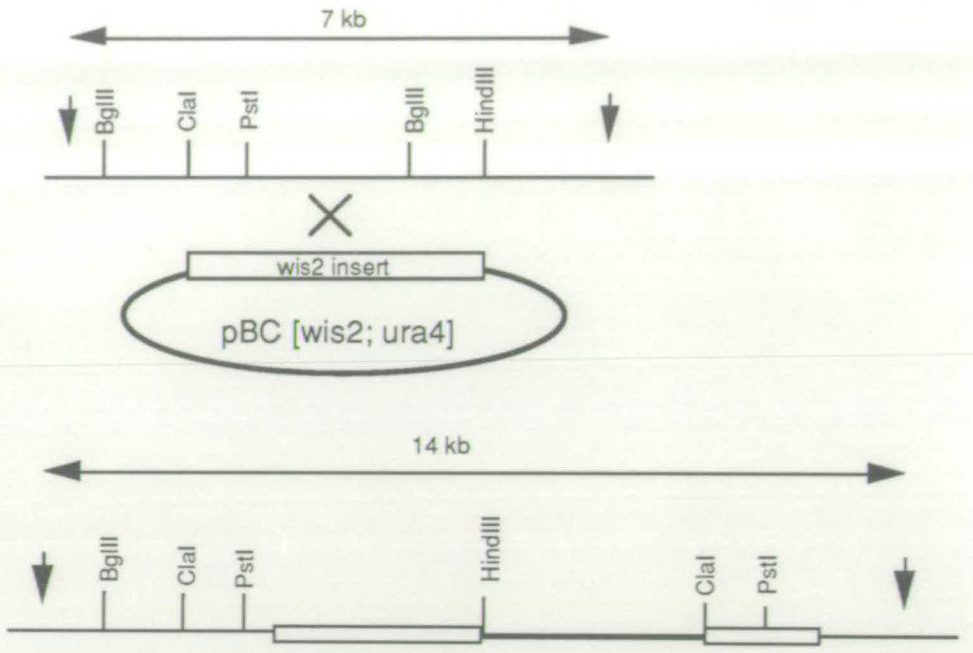
A Schematic diagram showing the predicted result of a single integration event of pBC[wis2-5-C; ura4$^+$] at the *wis2* locus.

Bars demonstrate the size of *Mlu*I fragments that showed hybridisation to the 3.7 kb *Pst*I *S.pombe* insert of pBC[wis2-5-C; ura4$^+$] (see Figure 5.2). Arrow heads indicate sites for *Mlu*I restriction endonuclease.

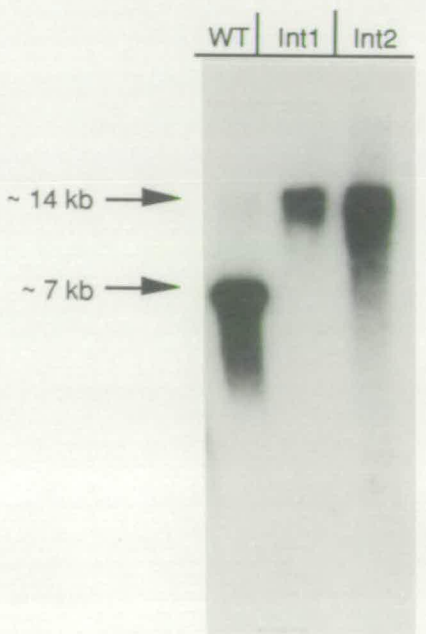
B *S.pombe* genomic DNA was digested with *Mlu*I, subjected to agarose gel electrophoresis, Southern blotted and probed with the 3.7 kb fragment of pBC[wis2-5-C; ura4$^+$].

Lane 1: wild type
Lane 2: Int1
Lane 3: Int2

A


7 kb
BglII
ClaI
PstI
BglII
HindIII
wis2 insert
pBC [wis2; ura4]
14 kb
BglII
ClaI
PstI
HindIII
ClaI
PstI

B


WT
Int1
Int2
~ 14 kb
~ 7 kb

The reduction in recombination frequencies between the *win1* and *tps19* (2 - 3 % compared with 4 - 5 % found by Warbrick 1990) may not reflect a true inconsistency, but may be the consequence of counter selection against the recombinant group of the genotype tps19^{-} ura^{-} win1^{+}. This group is under-represented as appears from comparison with its reciprocal group (see rows 5 and 6 in Table 5.1A).

In addition, the three point cross test suggests the order of the *win1*, *tps19* and *wis2* loci. The absence of one pair of reciprocal recombinant classes (Table 5.1A, rows 7 and 8) strongly suggests that these classes of recombinants represent double cross over events, thus locating the *win1* locus between the *tps19* and *wis2* loci on *S.pombe* chromosome I.

Table 5.1 Relative genetic mapping of *tps19*, *wis2* and *win1*

The table summarises the results of random spore analysis of the cross:

win1-1 tps19-17 ura4-D18 leu1-32 [pwis2-5C;ura4$^+$] X *ura4D18 h$^-$*

A The eight possible combinations of wild type and mutated alleles are shown in rows 1 - 8. (+) and (-) signs indicate the presence or the absence, respectively, of the wild type allele of the genetic marker indicated at the top of each column.

B A summary of the representation of the wild type and mutant alleles of each genetic marker.

A

					Int1	Int2
		tps19	ura4	win1		
1	Parental	+	-	+	42	39
2	Parental	-	+	-	42	45
3	Recombinant	+	+	+	7	10
4	Recombinant	-	-	-	6	4
5	Recombinant	-	-	+	0	0
6	Recombinant	+	+	-	3	2
7	Recombinant	+	-	-	0	0
8	Recombinant	-	+	+	0	0
Total segregants scored:					100	100

B

	Int1		Int2	
	wild type allele	mutant allele	wild type allele	mutant allele
win1	49	51	49	51
ura4	53	47	57	43
tps19	52	48	51	49

ii Genetic mapping of *win1*, *tps19* and *rad1*

Three point genetic crosses were also performed in order to determine the order of the *win1*, *tps19* and *rad1* loci and to estimate their relative genetic distances. The genetic mapping of *rad1* was of particular interest, as the *rad1* locus had already been mapped in the physical maps obtained from the *S.pombe* ordered cosmid and P1 libraries (Hoheisel et al. 1993). Comparison between the genetical and physical maps could be later used for the chromosomal walk from the *tps19* locus towards the *win1* locus (see below, sections 5.3 and 5.4).

In the genetic mapping experiment, the *rad1* locus was followed using the *rad1-1* allele as a genetic marker. The *rad1-1* mutation confers lethality on cells grown in the presence of hydroxyurea. Under such conditions, DNA replication is inhibited and wild type cells arrest as highly elongated cells, as the mitotic checkpoint control is activated (see Introduction, section 1.2 i). In contrast, *rad1-1* cells attempt to enter mitosis in the absence of DNA replication and so die.

As a preliminary experiment, the phenotype of the *win1-1* mutant strain in the presence of hydroxyurea was examined. Cells were plated on YE plates containing 11 mM hydroxyurea and incubated either at 28 or 35°C. At both temperatures, cells exhibited the extremely elongated phenotype typical of wild type cells grown in the presence of hydroxyurea. Therefore, it seems that *win1-1* mutants are not defective in the mitotic checkpoint control. Significantly, this experiment also indicates that *win1-1* and *rad1-1* phenotypes could be independently scored.

Random spore analysis of a cross between *rad1-1* h^- and *win1-1 leu1-32* h^+ identified putative *win1-1 rad1-1* double mutants. These mutants exhibited the win1$^-$ phenotype when grown on minimal plates and the rad1$^-$ phenotype when grown in the presence of hydroxyurea. The phenotypes of about 100 spores of two independent crosses were analysed (Table 5.2). This analysis suggests that the *rad1* locus is located about 16-cM away from the *win1* locus, a slightly higher estimate than the previous report, which estimated the distance between *rad1* and *tps19* to be 11.7 cM, based on tetrad analysis of a similar cross (Gygax and Thuriaux 1984).

Table 5.2 Relative genetic mapping of *win1* and *rad1*

The table summarises the results of random spore analysis of the cross

win1-1 leu1-32 h^{+} X *rad1-1h*$^{-}$

The four possible combinations of wild type and mutant alleles are shown in rows 1 - 4. (+) and (-) signs indicate the presence or the absence, respectively, of the wild type allele of the genetic marker indicated at the top of each column.

A

	win1	rad1	Cross I	Cross II
1	-	+	42 (44%)	46
2	+	-	38 (40%)	38
3	-	-	4 (4%)	6
4	+	+	12 (12%)	10
Total scored:			96	100

B

	Cross I		Cross II	
	wild type allele	mutated allele	wild type allele	mutated allele
win1	50	46	48	52
rad1	54	42	56	44

Finally *win1-1 tps19-17 leu1-32* h^+ was crossed with *rad1-1* h^- and random spore analysis was carried out. A total of 150 colonies were scored for the tps19, win1 and rad1 phenotypes. The results are shown in Table 5.3.

The three point cross suggests the order of *win1 tps19* and *rad1* loci. The absence of one pair of recombinant classes (Table 5.3A, rows 7 and 8) strongly suggest that these recombinants represent double cross over events, and thus locates the *win1* locus between the *rad1* and *tps19* loci. The *win1* locus has been located 12 - 15 cM away from the *rad1* locus and 2 cM away from the *tps19* locus.

In summary, the genetic mapping of *wis2*, *rad1* and *tps19* relative to *win1* demonstrated that the *tps19* locus is the nearest to the *win1* locus. Therefore, the genetic marker *tps19* is the most useful for the chromosomal walk from *tps19* towards *win1*. In *S.pombe*, one cM normally equals 7 - 10 kb (Gygax and Thuriaux 1984; Uzawa et al. 1990). The estimation of 2 - 3 cM (this thesis) and 4 - 5 cM (Warbrick 1990) between *tps19* and *win1* therefore predicts that the *win1* lies about 14 - 50 kb away from *tps19*.

Table 5.3 Three point genetic mapping of *tps19*, *wis2* and *win1*

Summary of the result of random spore analysis of the cross:

win1-1 tps19.17 leu1-32 h^{+} X *rad1-1h*$^{-}$

A The eight possible combinations of wild type and mutant alleles are shown in rows 1-8. (+) and (-) signs indicate the presence or the absence, respectively, of the wild type allele of the genetic marker indicated at the top of each column.

B A summary of the representation of the wild type and mutated alleles of each genetic marker.

A

Phenotype					Cross 1	Cross 2
		win1	tps19	rad1		
1	Parental	-	-	+	48	23
2	Parental	+	+	-	35	20
3	Recombinant	+	+	+	7	4
4	Recombinant	-	-	-	8	2
5	Recombinant	-	+	+	2	0
6	Recombinant	+	-	-	0	1
7	Recombinant	+	-	+	0	0
8	Recombinant	-	+	-	0	0
Total segregants scored:					100	50

B

	Cross I		Cross II	
	wild type allele	mutated allele	wild type allele	mutated allele
win1	42	58	25	25
tps19	44	56	24	26
rad1	57	43	27	23

5.3 Identification of cosmid and P1 clones of the *tps19* region

i Characterisation of *tps19*$^+$ clones

Since *tps19* is the locus that maps most closely to *win1*, it was decided to physically map the *tps19*$^+$ gene into the ordered cosmid and P1 libraries. Such a mapping procedure should identify cosmids clones close to the chromosomal region of *win1*.

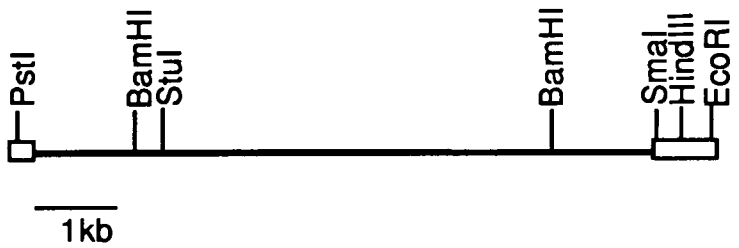
Previously, putative *tps19*$^+$ clones had been isolated by transforming a *tps19-17 leu1-32* strain with a *S.pombe* genomic library and selecting for temperature resistant transformants (M.V. Zarate and E. Warbrick, pers comm). Three different groups of plasmids were identified by restriction digest analysis: two plasmids were 6.6 kb, two plasmids were 11.5-kb, and one plasmid was 18.5 kb (E. Warbrick, pers comm). As these plasmids are expected to derive from a genomic library constructed in pWH5, a plasmid vector of 10.6 kb, it appeared that at least the 6.6 kb plasmids had undergone major rearrangements.

The putative *tps19*$^+$ plasmids were subjected to further restriction digest analysis. Only the 18.5 kb plasmid showed restriction fragment patterns expected from a pWH5 plasmid containing an insert, suggesting that the 11.5 kb plasmids, as well as the 6.6 kb plasmids, had undergone major rearrangements. Further analysis was carried out on the 18.5 kb plasmid, which is referred to as pWtps19-2 (for a restriction map see Figure 5.4).

pWtps19-2 was re-transformed into *win1-1 tps19-17 leu1-32 h*$^+$ and tps19$^+$ transformants were analysed for the co-segregation of the temperature resistance and leu$^+$ phenotypes (Material and Methods, section 7.5 v). Co-segregation of the leu$^+$ and the temperature resistance phenotypes confirmed that pWtps19-2 was responsible for the complementation of the *tps19-17* mutation. Subsequently, Southern blot analysis was carried in order to determine whether the insert of pWHtps19-2 was collinear within the *S.pombe* genome. To this aim, wild type DNA was digested with *Pst*I, a restriction enzyme which does not cut pWHtps19-2. The digest reaction was electrophoresed and Southern blotted (Material and Methods, section 7.3 ix).

Figure 5.4 Restriction map of pWtps19-2 insert

Outline map of restriction enzymes that cut the insert fragment of pWtps19-2.



□ pWH5 vector sequences (not to scale)

Only one band showed hybridisation to labelled plasmid sequences (data not shown), strongly suggesting that the insert sequence is collinear with the *S.pombe* genome.

ii Hybridisation screens of ordered *S.pombe* libraries

A 5 kb *Bam*HI insert DNA of pWHtps-2 was used for hybridisation of ordered P1 and cosmid libraries gridded on high density filters (a gift of J. Hoheisel and E. Maier). Putative tps19 positive signals were identified as strong hybridisation signals (Figure 5.5). A competitor radiolabelled DNA, derived from the cosmid LoristX, was included in the hybridisation experiment (a gift from S. Cross; Little and Cross 1985). The radiolabelled LoristX DNA was added so that the ratio between the LoristX probe and the *tps19* probe is 1:500, with respect to the radioactivity (see section 7.3 v). LoristX shares extensive sequence homology with the cosmid Lawrist4, used for the construction of the *S.pombe* cosmid library, and limited sequence homology with the bacteriophage P1 vector (section 7.2 v). The LoristX probe cross-hybridised to the whole array of the cosmid and P1 clones, giving rise to weak hybridisation signals, which facilitated the determination of the coordinates of the strong, putative positive signals.

The conditions used for the hybridisation of the cosmid and P1 filters are described in Materials and Methods section 7.9.

A total of 15 and 9 strong hybridisation signals were obtained on the cosmid and P1 filters, respectively, and the coordinates of 10 and 8 signals of the cosmid and P1 filters, respectively, could be unambiguously assessed (Figure 5.5). These coordinates were analysed by J. Hoheisel and E. Maier (pers comm), and the positions of the corresponding clones within the *S.pombe* physical map were determined. 8 out of 8 of the putative positive P1 clones, and 8 out of 10 of the putative positive cosmid clones were found to map to the right arm of chromosome I, about 100 kb distal to the *rad1* locus. The physical mapping of *tps19* is thus in agreement with the genetical mapping described above (section 5.2 ii; see Figure 5.6).

Figure 5.5 Hybridisation screen of *S.pombe* ordered libraries

A 5 kb *Bam*HI fragment of the *S.pombe* insert of pWtps19-2 and the cosmid LoristX were labelled by random hexamer priming. A mixture of the two probes, in which the ratio of the radioactive amount of the *tps19* probe and the LoristX probe was 500: 1, was used to hybridise high density filters of *S.pombe* ordered libraries in cosmids (A) or in bacteriophage P1 (B).

The cosmid and P1 libraries are composed of 3456 clones which represent about 17 and 23 fold of the *S.pombe* genome, respecticely. The clones are arranged on high density filters in 96 boxes (12 x 8), each box contains a 6 x 6 array of cosmid or P1 alones. The coordinates of the clones therefore mean column (A - H), row (1-12) and location within the box (1-36).

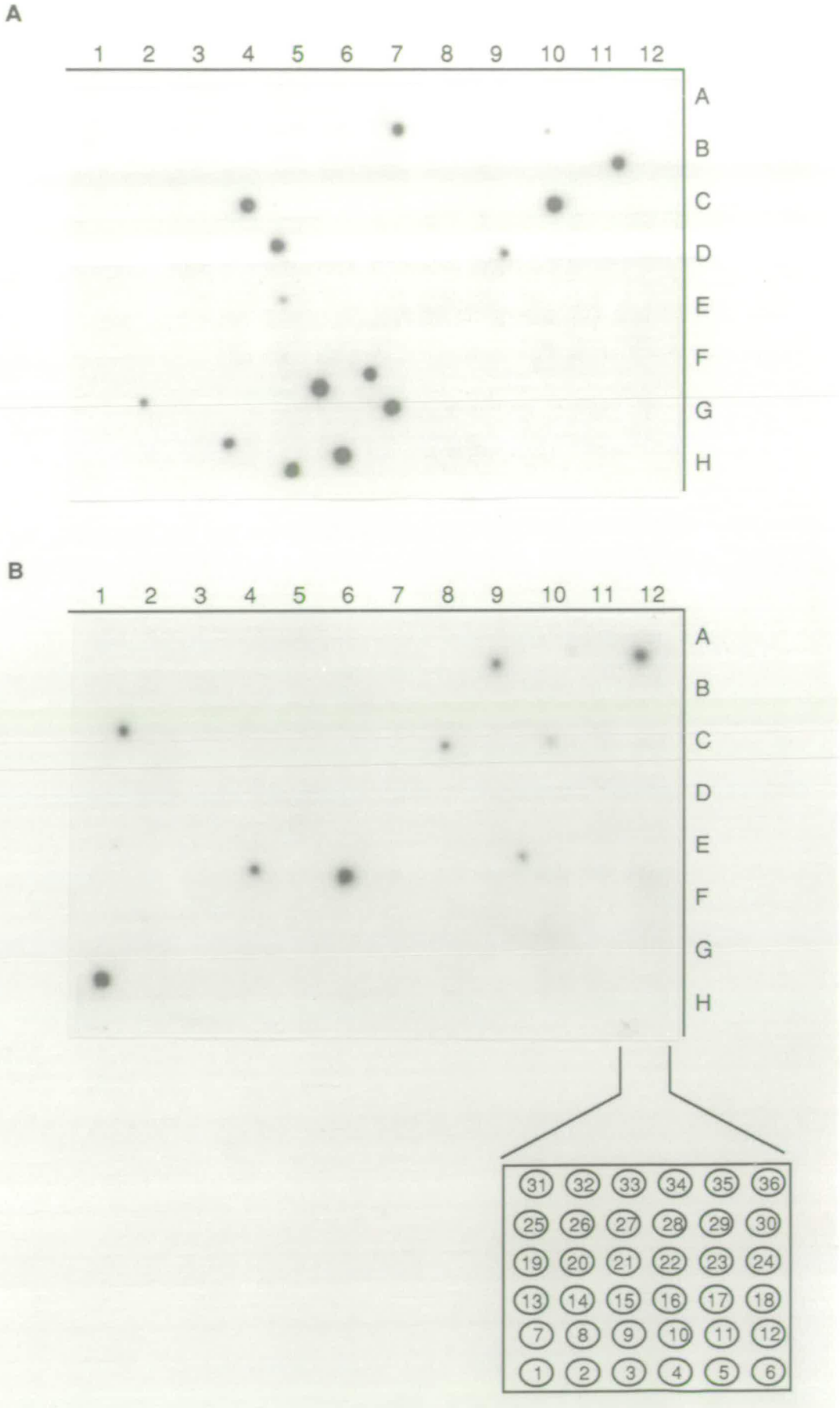

A
1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H
B
1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H
31 32 33 34 35 36
25 26 27 28 29 30
19 20 21 22 23 24
13 14 15 16 17 18
7 8 9 10 11 12
1 2 3 4 5 6

Figure 5.6 Physical map of the chromosomal region of *tps19*$^+$.

A. Genetic map of the *win1* chromosomal region (see text in section 5.2).

B. The order of YAC (Yeast Artificial clones), P1 and cosmid clones spanning the region of *tps19*$^+$ on the right arm of chromosome I (taken from Hoheisel et al. 1993). The letters y (YAC) c (cosmid) and p (P1) identify the clone type. Note that only a subset of cosmid clones that map into this region are shown.

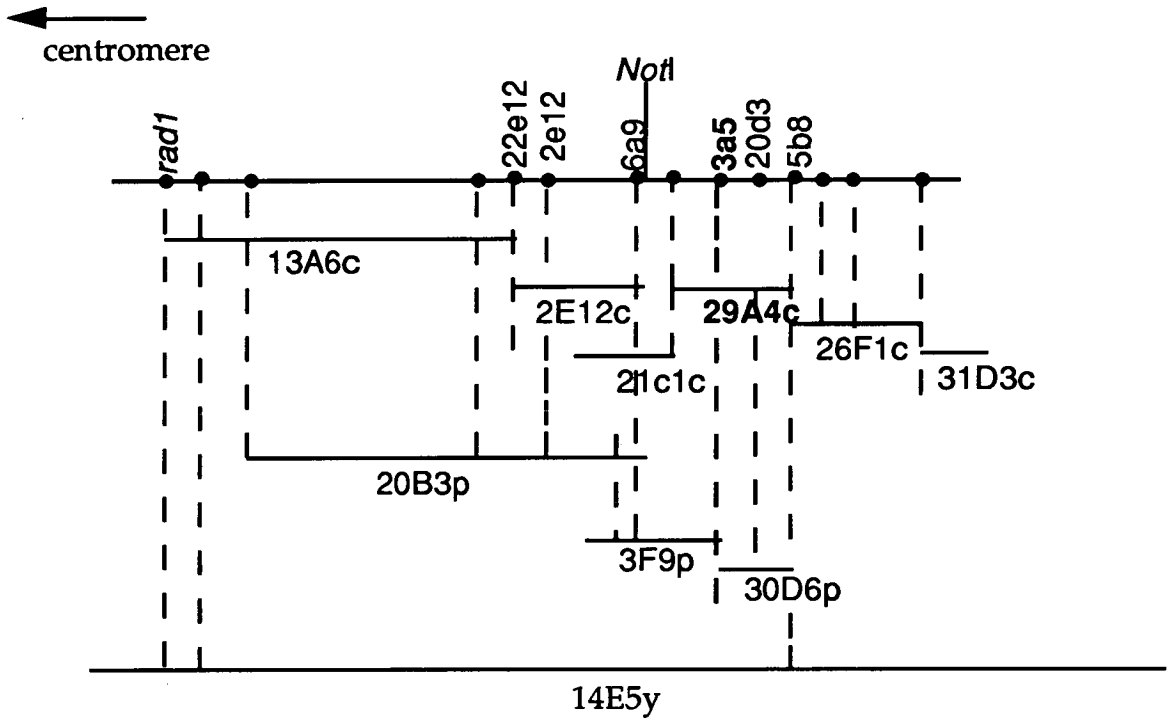
Black dots on the top bar represent probes which were used for ordering the cosmids and P1 clones of the presented contig. In some cases, the probes are themselves cosmid clones. If so, the name of the probe is given on top of the bar. Broken lines show which probe was hybridised to which library. In addition, the position of a *Not*I restriction site and the *rad1* gene are indicated. Note that clones that have only one probe in common do not necessarily overlap, since the probes are themselves clones.

Names of clones shown in bold indicate clones that hybridised with the *tps19* -2 probe.

A.


rad1
win1
tps19
12-16cM
2-5cM

B.


centromere
rad1
22e12
2e12
NotI
6a9
3a5
20d3
5b8
13A6c
2E12c
29A4c
26F1c
31D3c
211c1c
20B3p
3F9p
30D6p
14E5y

~ 1 cM/ 7-10 kb

2 out of 10 of the strong hybridisation signals of the cosmid filters mapped elsewhere on the genome, and are therefore false-positive signals. These signals could result due to cross-hybridisation with clones carrying similar inserts sequence. Alternatively, DNA contamination might have occurred during the construction of the cosmid filters.

Based on the hybridisation result of the *tps19* probe, a total of 37 cosmids (#1-28 and #30-38) that flank the *tps19* locus were obtained from the ordered *S.pombe* gene bank (see Table 5.4). These cosmids form a contig, a term that indicates that the ordered cosmids are expected to cover a certain chromosomal region without intervening gaps. The *tps19* contig spans a region starting from the *rad1* locus (cosmid 13A6), through the predicted *tps19* locus, and ~ 70 kb to the right of this position (cosmid 31D3).

Table 5.4 A contig of the tps19 region in cosmids

The table presents cosmids in the *tps19* region (obtained from E. Maier, pers comm). The first two columns indicate the name of the cosmids as referred to in this thesis (numbers) and in the cosmid database (Hoheisel et al 1993). The third column represents the hybridisation results with a set of ordered probes used in the construction of the cosmid contig (Hoheisel et al. 1993). X indicates a positive hybridisation whereas (-) indicated negative hybridisation. X signs in bold indicate positive hybridisation with the *tps19* probe.

The last two columns indicate the results of complementation screens for the *tps19*$^+$ and *win1*$^+$ genes. N.D stands for not determined.

Name of cosmids		Hybridisation results	tps19$^+$	win1$^+$
1	13A6	xxxxx----------	N.D	-
2	10F2	-xxxxxx----------	N.D	N.D
3	19C6	--xxxxx----------	N.D	N.D
4	29F10	--xxxxx----------	N.D	-
5	16A5	---xxxx----------	N.D	-
6	6D12	-x--x----------	N.D	-
7	5G2	----xxx----------	N.D	N.D
8	2E12	----xxx----------	N.D	-
9	17C11	----xxx----------	N.D	-
10	7B9	----xxx----------	N.D	N.D
11	23E7	-----xx----------	N.D	N.D
12	6A9	-----xx----------	N.D	N.D
14	12F2	-----xx----------	N.D	-
15	12B6	-------x----------	-	-
16	21B2	-------xxx----------	+	-
17	8H10	-------xx----------	-	N.D
18	25E9	-------xxx----------	+	-
19	3A5	-------xxx----------	+	N.D
20	25F10	-------xxx----------	+	N.D
21	15B7	-------xxx----------	N.D	-
22	15A6	-------xxx----------	+	-
23	8H7	-------xxxx----------	+	-
24	29A4	-------xxxx----------	+	N.D
25	26F4	-------xxxx----------	+	-
26	24G12	--------xxx----------	+	-
27	28D8	----------x----------	-	-
28	20D3	----------x----------	-	-
30	8G11	----------xx----------	-	-
34	13D1	----------xx-x-x----------	N.D	N.D
35	14B8	----------xx-x----------	N.D	-
36	22A12	----------x----------	N.D	-
37	18C12	----------x----------	N.D	N.D
38	31D3	----------xx----------	N.D	-

iii Restriction digest analysis of cosmid clones

As a first step in analysing the contig of the *tps19* region, the cosmids of the contig were subjected to restriction digest analysis. Two kinds of information can be gained from such analysis. Firstly, the total size of the inserts of the cosmids can be estimated and compared with the expected average size of 37.5 kb (Hoheisel et al. 1993). Secondly, the extent of overlap between adjacent clones can be roughly estimated by comparing the pattern of restricted fragments produced by digest reactions.

Cosmid DNA was extracted (section 7.4 iii) and restriction digest reactions were performed by double *Hin*dIII and *Stu*I restriction digests. This double digest released most of the cosmid vector sequences (a restriction fragment of 4.8 kb). The digest reactions were electrophoresed on 0.7 % agarose gels, along with the 1 kb DNA ladder marker. An example is shown in Figure 5.7 A.

The size of the inserts of the cosmids was estimated by adding up the sizes of the restricted fragments of each cosmid. For most clones this gave a rough estimation of 30 - 34 kb, a slightly lower figure than expected for the average insert size of 37.5 kb. This discrepancy is likely to stem from the inherent inaccuracy of the method employed, as small fragments (< 300 bp), cannot be detected on agarose gels.

More significant was the examination of possible overlap between the cosmid clones. Restriction fragments derived from different cosmid clones, which appear to have identical size by the gel electrophoresis analysis, are likely to indicate shared sequences between the different clones. In Figure 5.7 A the cosmid clones #16, #22 and #23 have in common at least four restriction fragments of the approximate sizes of 7, 6.5, 3.8 and 2.2 kb. The cosmid clones #14, #9 and #8 have in common at least two restriction fragments of the approximate sizes of 2.8 and 2.1 kb. In contrast, the cosmid clone #15 gives a completely different restriction digest pattern.

The diagram in Figure 5.7 B summarises the results of restriction digest analysis of 17 cosmids. Significantly, cosmid #15 appears to be completely different from its presumptive flanking cosmids (see Table 5.4), suggesting the possibility of a gap in the ordered cosmid library between the cosmid group A (composed of cosmids that hybridise to the *tps19* probe) and the cosmid group B (composed of cosmids that lie between *tps19* and *rad1*). The possibility of limited overlap between cosmid #15 and its adjacent cosmids

cannot be ruled out, and further analysis by Southern blotting is required (as will be discussed later, chapter 6). Significantly, the possible gap in the *tps19* contig in cosmids is located between the *tps19* and *rad1* loci, where *win1* gene is expected to lie (see Table 5.4).

The possibility that cosmid #15 is not related to the *tps19* contig was later supported by re-examination of hybridisation data of the cosmid library (J. Hoheisel, pers comm), but as yet there has not been a definite conclusion regarding the position of cosmid #15 in the *S.pombe* physical map.

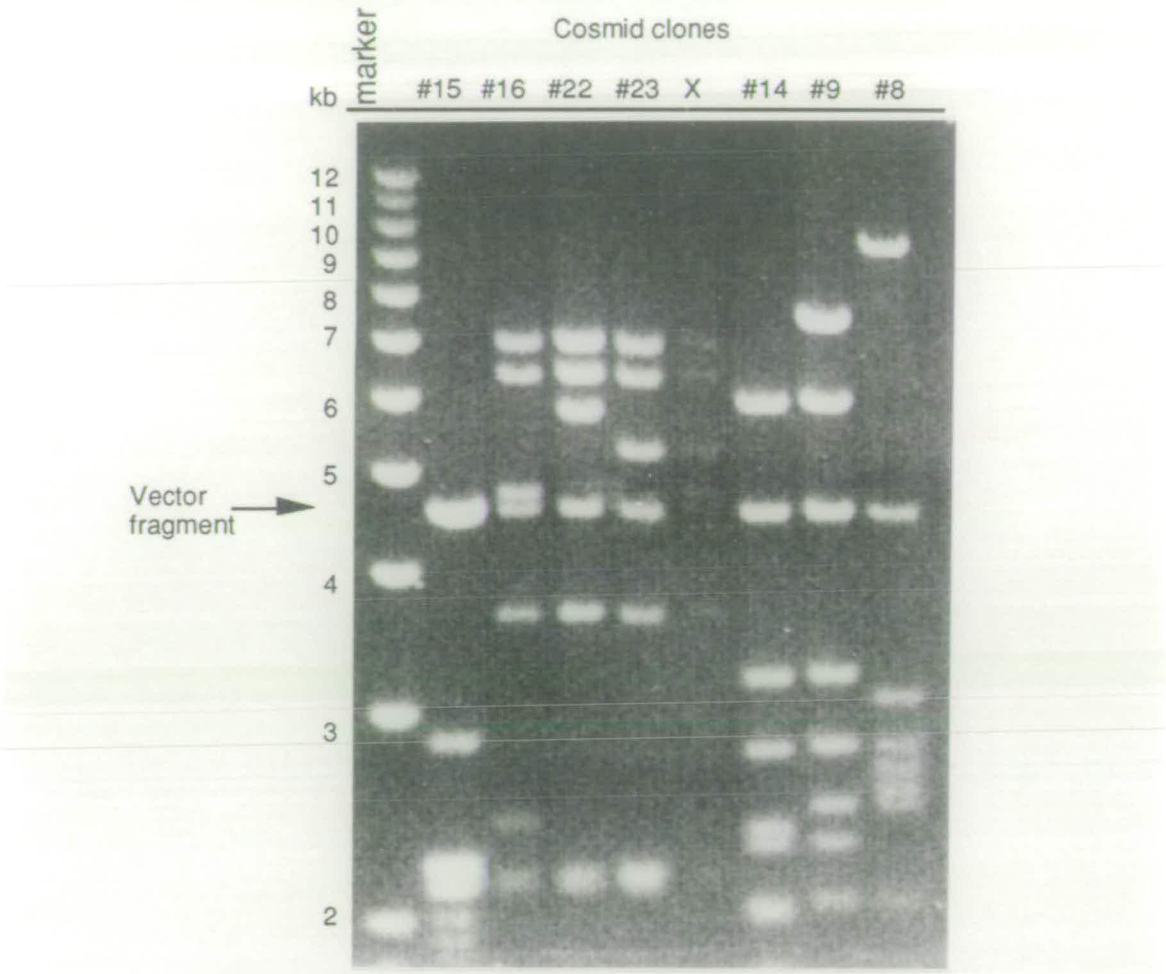
Bearing in mind the possibility of a gap in the *tps19* contig, cosmid clones within the contig were screened by complementation tests for the *win1*$^+$ gene, as described below.

Figure 5.7 Restriction digest analysis of *S.pombe* cosmid clones

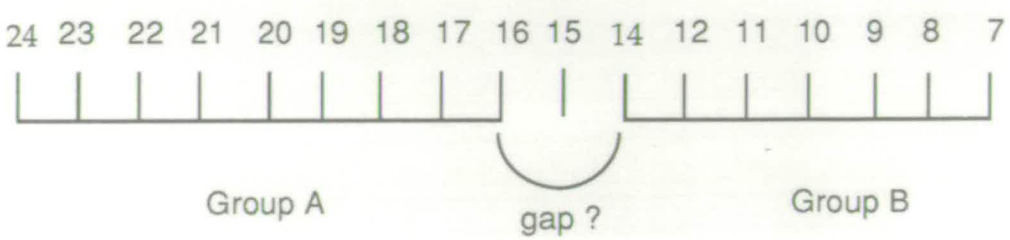
A Cosmid DNA of seven different clones was subjected to restriction digest analysis. 6 µl out of 50 µl (~ 0.6 µg) of Magic minipreparation of cosmid DNA (section 7.4 iii) were double digested with the restriction endonucleases *Hin*dIII and *Stu*I, in total volume of 10 µl and the digest reactions were electrophoresed on a 0.7% agarose gel. The photograph shown was taken after about 1.5 hours of electrophoresis at 70 V. Electrophoresis was then continued for 1-2 hours, in order to further separate the restricted bands. Only then was the number and length of the restricted bands of each cosmid assessed, as described in the text (section 5.3 iii).

B Schematic diagram summarising the analysis of potential shared restricted fragments between different cosmid clones. The order of cosmid clones is based on the data in Table 5.4.

A.


marker
Cosmid clones
kb
#15
#16
#22
#23
X
#14
#9
#8
12
11
10
9
8
7
6
5
4
3
2
Vector
fragment

B.


24 23 22 21 20 19 18 17 16 15 14 12 11 10 9 8 7
Group A
gap ?
Group B

5.4 Complementation tests for cloning the *win1*$^+$ gene

i Screens of cosmids in *win1-1 tps19-17*

Complementation screens of the *tps19* contig in cosmids were first carried out by co-transformations of individual cosmids, along with a yeast multicopy vector, into strains with appropriate genetic backgrounds. Co-transformation rather than transformation was performed because the cosmid vectors do not contain any *S.pombe* selective marker or autonomous replication sequence. Cosmid clones were first tested for their ability to complement the temperature sensitive phenotype of the *tps19-17* mutation in the strain *win1-1 tps19-17 leu1-32*. This test could indicate whether co-transformation of cosmids is an adequate procedure for cloning by complementation, as there are strong expectations that copies of the *tps19*$^+$ gene are present in at least most of the cosmids that hybridise to the *tps19* probe.

The yeast plasmid that was used for co-transformations was constructed so that it contained sequence homology with the cosmid vector sequences. Sequence homology between the co-transformed vectors was likely to increase the probability of intracellular homologous recombination, thus increasing the co-transformation efficiency. To this aim, a 2.8 kb *Sph*I fragment of LoristX was cloned into the yeast multicopy vector pIRT2. The resultant plasmid was named pIlorist. Co-transformations of cosmids were carried out using the protoplast method (section 7.5 v). This procedure was chosen as it gave higher and more reproducible results than the electroporation method.

Transformants were initially selected for the leu1$^+$ marker on minimal sorbitol plates at 28°C. The leu1$^+$ transformants were then replicated onto YE plates and incubated at 35°C, in order to test for complementation of the *tps19-17* mutation. Direct selection for *tps19*$^+$ transformants was not performed because the *tps19-17* mutation was found to be suppressed on 1.2 M sorbitol plates, the media used for regeneration of the transformed protoplasts.

Initially, two cosmids, #16 and #22, were tested for complementation of *win1-1 tps19-17 leu1-32*. These cosmids were both identified as positive

clones in the hybridisation screen with the *tps19* probe. Co-transformation of these cosmids with pIlorist gave rise to leu1$^+$ tps19$^+$ transformants. Several of these transformants showed mitotic co-segregation of the leu1$^+$ and tps19$^+$ genetic markers, confirming that the cosmids were responsible for the complementation of the *tps19-17* mutation.

The best transformation efficiency, about 100 leu1$^+$ tps19$^+$ transformants per μg cosmid DNA, was obtained in experiments in which the ratio of pIlorist to cosmid was 1: 25 (0.2 μg pIlorist DNA and 5 μg cosmid DNA). This ratio and the corresponding amounts of DNA were maintained in all subsequent transformations.

A total of 15 cosmids were tested for their ability to complement the temperature sensitive phenotype of *win1-1 tps19-17 leu1-32* (as shown in Table 5.4). The result of the co-transformation experiments can be summarised as follows: all cosmids that gave rise to leu1$^+$ tps19$^+$ transformants were clones that also gave positive hybridisation signals with the *tps19* probe; none of the cosmids tested that did not hybridise to the *tps19* probe gave rise to leu1$^+$ tps19$^+$ transformants.

No positive selection for *win1*$^+$ can be applied in *win1-1 tps19-17 leu1-32*, as *win1-1* does not confer a lethal phenotype in this genetic background. However, *win1*$^+$ sequences may be expected to suppress the elongated phenotype associated with the *win1-1* mutation. Therefore leu1$^+$ tps19$^+$ transformants, which indicate a successful transformation of cosmids, were examined microscopically for cell size complementation of the *win1-1* mutation. Five to ten colonies of each leu1$^+$ tps19$^+$ transformant from each cosmid transformation were examined. All transformants exhibited the characteristic elongated phenotype of the *win1-1* mutation, thus failing to identify cosmids that contain the *win1*$^+$ gene.

The failure to identify suppression of the *win1-1* mutation in leu1$^+$ tps19$^+$ transformants may suggest that the physical distance between *tps19*$^+$ and *win1*$^+$ is greater than the distance that would allow these two genes to lie within the same cosmid insert. The genetic mapping data, suggesting a distance of 2 - 5 cM between *tps19* and *win1* (section 5.1) cannot definitely support or rule out such a suggestion. If each cM equals about 7 - 10 kb then the expected distance between *tps19* and *win1* is 14- 50 kb. As the average insert size of a cosmid is 37.5 kb, the *tps19*$^+$ and *win1*$^+$ genes may, or may not, be contained within the same cosmid.

ii Screens of cosmids in *cdc25-22 wee1-50 win1-1*

In order to extend the range of cosmids screened for *win1*$^+$, a second set of complementation screens was carried out in *cdc25-22 wee1-50 win1-1* background. As the *win1-1* mutation confers a lethal phenotype in combination with the *cdc25-22* and *wee1-50* alleles, this second screen allowed for positive selection for *win1*$^+$.

A total of 21 cosmids were examined (see Table 5.4). Co-transformations were carried out as described in section 5.4 i.

Colonies were first selected for leu1$^+$ by plating the transformed protoplasts on minimal sorbitol plates at 28°C. Selection for cells able to grow at 35°C was performed following the procedure described by Warbrick (1990). Cells were scraped off the sorbitol containing plates, resuspended in a small volume of minimal medium and immediately re-plated onto minimal plates at a density of approximately 10^4 cells per plate and incubated at 35°C. In each experiment, a number of cells equal to more than ten times the original number of transformants was plated, to ensure the representation of each original transformant. Direct replication of the minimal sorbitol plates to minimal plates, followed by incubation at 35°C, was not possible as the strain *cdc25-22 wee1-50 win1-1* shows significant bulk growth on minimal plates at 35°C (section 2.1).

For each cosmid, at least 500 leu1$^+$ transformants were screened for growth at 35°C. Although occasionally a few colonies were formed at the restrictive conditions, the change in phenotype of the cells was determined not to be due to cosmid-borne sequences by two criteria. Firstly, some of these cells did not show the ability to grow at 35°C in further experiments, suggesting that the conditional lethal phenotype of the triple mutant is somewhat leaky, even at a low density plating. Secondly, transformants that were confirmed for growth at 35°C did not show co-segregation of the win1$^+$ and leu1$^+$ phenotypes. Instead, the leu1$^+$ marker was found to be mitotically unstable whereas the win1$^+$ phenotype was stable, suggesting that a spontaneous chromosomal mutation is responsible for the suppression.

As a positive control, pTZwis2-1, a *wis2* subclone in a non-yeast-vector (section 2.2 i), was included in the co-transformation experiments. Re-plating the leu1$^+$ transformants arising from co-transformation with pTZwis2-1 into

minimal plates at 35°C resulted in ~ 200 colonies per plate, indicating that the co-transformation procedure described above can identify suppressors of the triple mutant phenotype.

iii Screens of cosmid mini-libraries

Without direct means to assess the success of a transformation event into *cdc25-22 wee1-50 win1-1*, it was unclear whether *win1*$^+$ was not isolated due to problems inherent in the cosmid co-transformation procedure, such as high mitotic instability or frequent rearrangements of the transformed cosmids. Therefore the cosmid inserts were subcloned into *S.pombe* replicative vector prior to transformation into the *cdc25-22 wee1-50 win1-1* background.

To this aim, two different mini-libraries of cosmids #1 - #38 were constructed: a mini-library of *Bam*HI digest in pIRT2 and a mini-library of partial *Hin*dIII digest in pWH5. The construction of these mini-libraries is described in Materials and Methods, section 7.10.

The two mini-libraries were checked for the proportion of plasmids containing inserts. Eight individual *E. coli* colonies were picked from each mini-library transformation, and the plasmid DNA was extracted and analysed by restriction digest analysis. This analysis showed that only a low number of clones of the *Bam*HI mini-library (1 out of 8) contained insert DNA, but a high proportion of the clones of the *Hin*dIII mini-library (7 out of 8) showed the presence of inserts (data not shown).

The transformants of each mini-library were pooled together by washing colonies off the transformation plates. Each pool, of about 10^3 colonies, was used as an inoculum for a medium scale preparation of plasmid DNA. These plasmid preparations were used for transformation into *win1-1 tps19-17* and *cdc25-22 wee1-50 win1-1* backgrounds.

About 1.5 µg of each mini-library was used for transformation. Transformants were first selected for the leu1$^+$ selective marker and then tested for the tps19$^+$ or the win1$^+$ phenotype.

Transformations of the mini-libraries into *win1-1 tps19-17* showed that *tps19*$^+$ clones constitute 2.5 or 5.8 % of the pIRT2/*Bam*HI mini-library and 2.7 or 3 % of the pWH5/*Hin*dIII mini-library (percentage are calculated separately for two independent transformation experiments).

Following the confirmation of the presence of *tps19*$^+$ in the mini-libraries, two independent transformations were performed for each mini-

library into *cdc25-22 wee1-50 win1-1*, but no plasmids capable of suppression of the conditional lethal phenotype of the strain were isolated.

5.5 Conclusions

This section describes an alternative approach for the cloning of *win1*$^+$ in which ordered cosmid clones of the *win1* chromosomal region were screened by complementation tests for their ability to rescue the lethal phenotype of *win1-1* in a *wee1-50 cdc25-22* genetic background, or alternatively, for their ability to reduce the elongated phenotype of *win1-1* in a *win1-1 tps19-17* background. This approach involved several steps.

First, genetic mapping data were obtained for the *win1* locus and nearby genetic markers. The *win1* locus was located between the *wis2* and *tps19* loci, 12 - 13 cM away from *wis2* and 2 - 5 cM away from the *tps19* locus. A separate three point cross located the *win1* locus between the *rad1* and *tps19* loci, 12 - 16 cM away from *rad1*. Of the available genetic markers, the *tps19* locus is thus the most closely linked genetic marker to *win1* and is therefore most suitable for chromosomal walk procedures towards the *win1* locus.

Putative *tps19*$^+$ clones have been previously cloned from a yeast genomic library by complementation of the temperature sensitive phenotype of *tps19-17* mutants (M.V Zarate and E Warbrick Pers Comm). Analysis of theses clones demonstrated that one clone carried the *tps19*$^+$ gene. An insert DNA fragment of this clone was used for hybridisation of *S.pombe* ordered cosmid and bacteriophage P1 libraries (Hoheisel et al. 1993). This hybridisation experiment has physically mapped *tps19* to chromosome I, 100 kb away the *rad1* locus (Hoheisel et al. 1993). The physical mapping of *tps19* is in agreement with the genetical mapping data.

A total of 37 clones that flank the *tps19* positive cosmid clones were obtained. The cosmids were screened by complementation for the presence of *win1*$^+$ and *tps19*$^+$, either by co-transformations of the cosmids into appropriate genetic backgrounds, or after additional steps of constructing mini-libraries of the cosmid DNA. These screens have failed to identify *win1*$^+$ clones. In contrast, *tps19*$^+$ cosmid and plasmid clones were identified both by complementation screens of cosmids and the mini-libraries, suggesting that both procedures are appropriate for isolation of genes.

The failure to clone *win1*$^+$ by the methods described in this chapter may be due to the same reasons that it was not cloned in the past by Warbrick (1990). *win1*$^+$ may be deleterious to *S.pombe* when overexpressed, alternatively, *win1*$^+$ may be unclonable in *E.coli*. Difficulties in cloning *win1*$^+$ in *E.coli* may arise from the nature of the *win1*$^+$ gene product, or, alternatively, from the DNA structure of *win1*$^+$ or its surrounding sequences. Indeed, restriction digest analysis of cosmids of the *tps19* contig suggests a possible gap in the cosmid library between *tps19* and *rad1*. The presence of such a gap is consistent with the suggestion that the *win1*$^+$ could not be cloned in *E.coli*. Further discussion of the possibilities for the failure to clone *win1*$^+$ and alternative methods to overcome these difficulties are considered in the Discussion chapter 6.

Chapter 6: Discussion

6.1 Summary of the molecular and genetical analysis of *wis2*$^+$

The molecular and genetical analysis of the *wis2*$^+$ gene can be summarised in the following points:

1. Overexpression of the *wis2*$^+$ gene suppresses the cell cycle defect of the triple mutant strain *cdc25-22 wee1-50 win1-1* on minimal medium at 35°C, this suppression is defined as wis2 activity.

2. Sequence analysis of *wis2*$^+$ reveals an ORF of 1068 nucleotides. Analysis of *wis2*$^+$ cDNAs support the position of the predicted initiating ATG codon, as this is the first in frame ATG codon downstream of two consecutive stop codons.

3. The ORF predicts a polypeptide product of 356 amino acid and 40.1 kDa. The N-terminus of the *wis2*$^+$ predicted polypeptide (186 amino acids) shows a very high similarity with the cyclophilin family of proteins. The rest of the protein, comprising 188 C-terminal amino acids, is highly charged and slightly basic.

4. The length of the predicted ORF is consistent with the detection of a ~1500 nt transcript by Northern blot analysis; and the predicted molecular weight of the translated product of *wis2*$^+$ is consistent with the detection of a ~40 kDa protein by Western blot analysis.

5. Deletion of the *wis2*$^+$ gene results in a viable phenotype and cells are indistinguishable from wild type cells. No effect associated with either overexpression or deletion of *wis2*$^+$ has been observed in a variety of strains carrying mutations in genes that regulate entry into mitosis.

6. A short deletion or any of two point mutations in the cyclophilin domain of *wis2*$^+$ result in mutated genes which are no longer able to confer wis2 activity when expressed from a multicopy yeast plasmid. However, Western blot analysis suggests that the mutated proteins are not expressed in

S.pombe cells to the same level as the wild type wis2 protein. Therefore, it is not clear whether the inability to confer wis2 activity stems from a specific defect of the cyclophilin active site, or whether it is a consequence of the instability of the mutated proteins.

8. The C-terminal half of *wis2*$^+$ is absolutely required for wis2 activity. Furthermore, the C-terminus appears to confer specificity on the cyclophilin domain, since a hybrid protein consisting of a different *S.pombe* cyclophilin fused to the C-terminus of wis2 shows wis2 activity.

9. The cyclophilin domain of *wis2*$^+$ is unique amongst the eukaryotic cyclophilins in that it does not contain the highly conserved Trp121 residue. Trp121 is essential for binding the drug cyclosporin A (CsA) *in vitro*. Consistently, overexpression or deletion of *wis2*$^+$ does not affects the sensitivity of cells to CsA.

6.2 Genetic analysis considerations

i Overview

The *wis2*$^+$ gene was isolated in a genetic screen as a multicopy suppressor of the triple mutant *cdc25-22 wee1-50 win1-1*. As the triple mutant strain carries mutations in genes involved in mitotic control, the suppression activity of *wis2*$^+$ suggests a role of *wis2*$^+$ in regulating entry into mitosis.

This prediction was tested by investigating the phenotypes associated with a null mutation of *wis2*$^+$, or with overexpression of *wis2*$^+$ (chapter 3). The *wis2*$^+$ gene was found to be non essential for either growth or division: deletion of essentially all coding sequences of *wis2*$^+$ in a wild type background resulted in a viable strain indistinguishable from wild type strains (section 3.2). The possibility that *wis2*$^+$ is non essential because it shares an overlapping function with other genes is discussed below (section 6.2 v). No effect associated with *wis2*$^+$ deleted allele has been observed in a variety of cell cycle mutant strains. In particular, no additional effects have been observed when a deleted allele for *wis2*$^+$ was combined with either *wee1-50*, *cdc25-22* or *win1-1*, the alleles combined to produce the triple mutant phenotype.

Overexpression of *wis2*$^+$ from a yeast multicopy plasmid, either from its own promoter or from the strong *nmt1* promoter, did not produce any effects in wild type or cell cycle mutant strains other than *cdc25-22 wee1-50 win1-1* (section 3.1).

How then can one account for the suppression activity of *wis2*$^+$ in *cdc25-22 wee1-50 win1-1* cells? The combination of three mutated alleles results in a complicated genetic background which makes the interpretation of wis2 activity difficult. In order to understand the phenotype of the triple mutant strain one can look at the effect associated with each of the mutated genes and then try to understand the effect produced when the mutated alleles are combined to a novel phenotype.

The following sections discuss possible molecular events which may be responsible for the phenotype of the double mutant *cdc25-22 wee1-50* and the triple mutant *cdc25-22 wee1-50 win1-1*.

ii The *wee1-50 cdc25-22* double mutant strain

The *cdc25*$^+$ gene is a dosage dependent activator of mitosis and is absolutely essential for entry into mitosis in an otherwise wild type background (Fantes 1979; Russell and Nurse 1986). The *cdc25*$^+$ gene product is a protein tyrosine phosphatase which acts as the major activator of the cdc2-cdc13 complex by promoting tyrosine de-phosphorylation on the Tyr15 residue of cdc2 (Moreno et al. 1989; Millar et al. 1991). *cdc25-22* temperature sensitive mutants arrest as highly elongated cells before mitosis. This lethal phenotype is suppressed by the temperature sensitive loss of function mutation *wee1-50* (section 1.3 i). The *wee1*$^+$ gene is a dosage dependent inhibitor of mitosis and its gene product has been established as the major tyrosine kinase responsible for inactivation of cdc2 by tyrosine phosphorylation (Russell and Nurse 1987a; Featherstone and Russell 1990; Parker et al 1991). Therefore, it may not be surprising that cdc25 becomes dispensable in the absence of its antagonist.

The *wee1-50* mutation is also capable of suppressing a *cdc25* null mutation (Russell and Nurse 1986), demonstrating that the viability of the double mutant *cdc25-22 wee1-50* at the restrictive temperature is not simply due to residual activity of the *cdc25-22* gene product. However, it is

likely that cdc25 residual activity accounts for the phenotypic difference between *cdc25-22 wee1-50* and *cdc25Δ wee1-50* double mutants at the restrictive temperature: *cdc25-22 wee1-50* cells divide at a smaller cell size than wild type cells, whereas *cdc25Δ wee1-50* are elongated (Fantes 1979; Russell and Nurse 1986).

Several lines of evidence, including the dosage dependent manner in which the *cdc25*$^+$ and *wee1*$^+$ exert their effects, suggest that in *S.pombe* the timing of mitosis is normally regulated by the phosphorylation state of cdc2 Tyr15 (Moreno et al. 1990; Russell and Nurse 1986; Russell and Nurse 1987). What mechanism could account for the regulation of the entry into mitosis in the absence, or greatly reduced activity of cdc25 and wee1? One possibility is that in *cdc25-22 wee1-50* cells, the regulation of cdc2 by Tyr15 phosphorylation is determined by the activities of other tyrosine phosphatases and tyrosine kinases. Support for this possibility is found by the isolation and characterisation of *pyp3*$^+$ and *mik1*$^+$, whose gene products have been suggested to share overlapping function with cdc25 and wee1, respectively (Millar et al. 1992; Lundgren et al. 1991). Another possibility is that in the absence of cdc25 and wee1, other(s) components of the cell cycle machinery become rate limiting for entry into mitosis. One possibility is that the rate of accumulation of B type cyclins can regulate entry into mitosis. The proposal that the onset of mitosis is regulated by cyclin B accumulation has been a popular model for eukaryotic cell cycles (Nurse 1990), but has never been proven. In *S.pombe*, it seems that the cyclin type B encoded by *cdc13*$^+$ is not rate limiting for entry into mitosis, in particular, overexpression of *cdc13*$^+$ does not advance mitosis. It is however possible that accumulation of B-type cyclin becomes rate limiting when cdc2 Tyr15 de-phosphorylation does not occur or is unregulated.

At the moment, analysis of genetic interactions of a variety of genes in *cdc25-22 wee1-50* background does not exclusively support one of the above alternatives as is discussed in the section below.

iii Genetic interactions in *cdc25-25 wee1-50* background

The *win1-1* chromosomal mutation was isolated by its virtue of reversing the suppression of *cdc25-22* by *wee1-50* (Ogden and Fantes 1986). The range of genetic interactions of the *win1-1* mutation with alleles of the *cdc25*, *wee1*, and *cdc2* genes suggests that *win1-1* is involved in mitotic control. However, there is no simple explanation for the effect of *win1-1* in the *cdc25-22 wee1-50* background (Warbrick 1990; see section 1.3 i).

The genetic interactions of *win1-1* with alleles of the *wee1* and *cdc25* genes do not exclusively imply a role for *win1*$^+$ either in the inhibitory wee1 pathway or the activating cdc25 pathway: both *wee1-50* and the overexpression of *cdc25*$^+$ are epistatic to *win1-1*. Indeed, the observation that the *win1-1* mutation exacerbates the terminal phenotype of a temperature sensitive allele of the mitotic cyclin *cdc13* (see section 1.3-i), may suggest that *win1-1* reverses the suppression of *cdc25-22* by *wee1-50* by altering some aspects concerned with cyclin accumulation or its ability to form an active complex with cdc2.

Intriguingly, the *win1-1* mutation exhibits allele specific interactions with *cdc2-w* activated alleles (Warbrick 1990): *cdc2-1w* is capable of suppressing the elongated phenotype of *win1-1*, whereas the combination of *cdc2-3w* with *win1-1* results in a cell length more similar to that of *win1-1*. Combination of *cdc2-3w* , but not *cdc2-1w*, with *wee1-50* results in mitotic catastrophe phenotype, suggesting that only the *cdc2-3w* allele is sensitive to wee1 inhibition. In contrast, *cdc2-3w* is a better suppressor of the *cdc25-22* mutation, suggesting that the *cdc2-3w* allele requires to a lesser extent the cdc25 activity (reviewed in Fantes 1989). One possible interpretation of the interactions of *win1-1* with the *cdc2-w* alleles is that *win1-1* acts through the wee1 pathway, as the elongated effect of *win1-1* is exerted only in *cdc2-3w* cells which are still sensitive to wee1 activity. However, the *cdc2-w* alleles are likely to differ from each other in other aspects, notably, these allele show specific interaction with a *cdc13* mutant allele: *cdc2-3w* is lethal in combination with the *cdc13-c1* allele, whereas no genetic interaction has been observed between *cdc2-1w* and *cdc13-c1* (Booher and Beach 1987). Therefore, the possibility that *win1-1* exerts its effects through some aspect of the complexing between cdc2 and cdc13 cannot be ruled out.

A most striking characteristic of the *win1-1* mutation is the nutritional sensitive nature of its interaction in a *wee1-50 cdc25-22* background. Cells of the genotype *cdc25-22 wee1-50 win1-1* show a predominantly cdc phenotype on minimal media at 35°C, while cells grown on rich medium, such as yeast extract, are much shorter and are capable of growth and division (Ogden and Fantes 1986). The elongated phenotype of the single *win1-1* mutation in an otherwise wild type background is also highly dependent on the nutritional composition of the media, as is observed for the interaction with the *cdc25-22* and *wee1-50* alleles. The component of the growth media responsible for the sensitivity is not clear, although reduction in the elongated phenotype of *cdc25-22 wee1-50 win1-1* cells is observed when ammonium, the usual nitrogen source in minimal medium, is replaced by amino acid mixtures (Warbrick 1990). This observation may suggest that the lethality of the triple mutant phenotype stems from a defect of coupling nutritional cues with the mitotic control machinary. However, this proposition requires further investigation of signal transduction pathway(s) in which *win1*$^+$ may play a role.

Other mutations which share with *win1-1* the ability to reverse the suppression of *cdc25-22* by *wee1-50* are the three *mcs* mutations, *mcs3-12*, *mcs4-13* and *mcs6-13* (Molz et al. 1989). These alleles were originally identified as mutations in genes which are capable of suppressing the lethal mitotic catastrophe phenotype of *cdc2-3w wee1-50*. Mutations of the *mcs* genes show a wide range of genetic interaction with other mitotic control genes (see Table 6.1), but the roles of the corresponding wild type genes await their cloning and characterisation at the molecular level.

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. The possibility that *mcs4*$^+$ and *win1*$^+$ may lie on the same pathway is supported by the similar phenotypes of the correspondent mutations and by the observation that the effect on cell length of these mutations is not additive (Warbrick 1990). Again, the analysis of genetic interactions alone is not conclusive. In particular, the genetic interaction of *mcs6-13*, and not *mcs4-13*, is more similar to the genetic interactions of *win1-1* with the *cdc2-w* and *cdc13-117* alleles (see Table 6.1).

Table 6.1 Phenotype of the *win1-1* and *mcs* chromosomal mutations in wild type and mutant strains.

	phenotype in wild type background	Interaction with mitotic control genes
mcs3-12[1]	Wild type phenotype.	No indications of specific genetic interactions.
mcs4-13[1]	Cells divide at a length ~1.5 times larger than wild type on minimal media but at a wild type cell length on yeast extract media.	Non conditional lethal in *cdc25-22* mutant background
mcs6-13[1]	Wild type phenotype.	Allele specific interaction with *cdc2-w* alleles: *mcs6-13 cdc2-3w* at 35°C forms slowly growing colonies consisting of highly elongated cells, whereas *mcs6-13 cdc2-1w* are wee. Exacerbate the phenotype of *cdc13-117* allele.
win1-1[2]	Cells divide at a length ~1.5 times larger than wild type on minimal media but at a wild type cell length on yeast extract media.	Allele specific interaction with *cdc2-w* alleles: *win1-1 cdc2-3w* cells are ~1.5 times larger than wild type, whereas *win1-1 cdc2-1w* cells are wee. Exacerbates the phenotype of *cdc13-117* allele.

1 Molz et al. 1989

2 Warbrick 1990

More recent is the genetic interaction observed between a deletion allele of *pyp3*$^+$ and the double mutant *wee1-50 cdc25-22* (Millar et al. 1992). *pyp3*$^+$ encodes a protein tyrosine phosphatase which is capable of efficient dephosphorylation and activation of tyrosine-phosphorylated cdc2 *in vitro* and acts as a mitotic inducer *in vivo* (Millar et al. 1992). Loss of pyp3 function in an otherwise wild-type background results in a small increase in cell size at division but has stronger effects in genetic backgrounds in which cdc25 activity is lost. In particular, *pyp3Δ* is capable of reversing the suppression of *cdc25-22* by *wee1-50* in a similar way to the reverse of suppression by *win1-1*, although this genetic interaction is not medium dependent. Genetic studies have shown that *win1*$^+$ and *pyp3*$^+$are different genes (P. Fantes pers comm). The ability of *pyp3Δ* to reverse the suppression of *cdc25-22* by *wee1-50* supports the prediction that in the *cdc25-22 wee1-50* background activation of cdc2 by Tyr15 de-phosphorylation is carried out by tyrosine phosphatase that share overlapping function with cdc25. However, this finding does not exclude the possibility that other mechanisms, independent of the cdc2 Tyr15 phosphorylation state, are responsible for the reversion of suppression of *cdc25-22* by *wee1-50* .

iv Suppression activity of the *wis1*$^+$ - *wis5*$^+$ genes

Suppression of *cdc25-22 wee1-50 win1-1* by *wis1*$^+$ - *wis5*$^+$ may be carried out by several mechanisms. As the *cdc25-22* allele is not functionally equivalent to a *cdc25* null allele at 35°C (see above), one possibility is that the *wis1*$^+$ - *wis5*$^+$ genes enhance the residual activity of *cdc25-22* gene product. Overexpression of the *wis1*$^+$ - *wis5*$^+$ genes in a *cdc25-22* background did not suppress the lethal phenotype of this strain at 35°C, thus failing to support interaction between *cdc25-22* and the *wis1*$^+$ - *wis5*$^+$ gene products (Warbrick and Fantes 1992). Further examination has been carried out in this thesis by examining the phenotypes of *wis2*$^+$ overexpression and *wis2* deletion in *cdc25-22* background at a variety of temperatures (section 3.2 and 3.3). Neither change in wis2 level affected the restrictive temperature of *cdc25-22*, again, failing to support interaction between *cdc25-22* and *wis2*$^+$.

The genetic interactions of the *wis1*$^+$ - *wis5*$^+$ genes with *win1-1* and the *mcs* mutations (Warbrick 1990) are summarised in Table 6.2. The variety of genetic interactions suggests that different mechanisms underlie the suppression activity of the various *wis* genes in the *cdc25-22 wee1-50 win1-1* background. For example, only *wis1*$^+$ and *wis4*$^+$ are capable of suppressing the *win1-1* phenotype in an otherwise wild type background. The genetic interactions of *wis1*$^+$ are consistent with its involvement in a pathway that includes the *win1*$^+$ and *mcs4*$^+$ genes. In contrast, the *wis4*$^+$ is capable of reducing the cell size length of a single *win1-1* mutant strain, but not the elongated phenotype of *mcs4-13* mutant strain. *wis4*$^+$ therefore appears to be a specific suppressor of *win1-1*.

A particular feature of the *wis2*$^+$ and *wis3*$^+$ genes is their ability to suppress either the *win1-1*, *mcs3-12*, *mcs4-13* or *mcs6-13* mutations in a *wee1-50 cdc25-22* background. The common factor in these triple mutant strains is the presence of the *cdc25-22* and *wee1-50* mutations. It is therefore possible that *wis2*$^+$ and *wis3*$^+$ interact directly with elements central to the control on mitosis.

One possibility for further investigation of this suggestion is to extend the analysis of the genetical interaction of *wis2*$^+$. For example, the phenotype of mutant cells of the genotype *wis2Δ wee1-50 cdc25-22* may prove informative: if wis2 suppression activity results from interaction of the *wis2*$^+$ gene product with a component that becomes rate limiting in the *wee1-50 cdc25-22* background, the interaction with *wis2Δ* allele may be lethal. In addition, it could be interesting to investigate possible interaction between the *cdc2-w* alleles and the *wis2Δ* allele or *wis2*$^+$ overexpression, as *cdc2-w* alleles display a range of genetic interaction with *cdc13*, *wee1* and *cdc25* alleles.

Table 6.2 Genetic interactions of the *wis1*$^+$ - *wis5*$^+$ genes

The table summarises the phenotypic effect of overexpression of the *wis1*$^+$ - *wis5*$^+$ genes from yeast multicopy plasmids (taken from Warbrick 1990).

(+) and (-) refer to the suppression activity of the *wis* genes

	wild type background		*cdc25-22 wee1-50* background			
	win1-1	*mcs4-13*	*win1-1*	*mcs3-12*	*mcs4-12*	*mcs6-13*
***wis1*$^+$**	+	+	+	-	+	-
***wis2*$^+$**	-	-	+	+	+	+
***wis3*$^+$**	-	-	+	+	+	+
***wis4*$^+$**	+	-	+	-	-	-
***wis5*$^+$**	-	-	+	-	-	(+)

An alternative approach for identifying interactions between wis2 and other cellular proteins is to employ the method of the yeast two hybrid system (Fields and Song 1989). This method scores for *in vivo* interactions between two proteins expressed in *S.cerevisiae* cells. The proteins in question are fused to the DNA-binding domain and to the activation domain of the *S.cerevisiae* transcriptional factor GAL4, respectively. An interaction between these proteins brings together the two GAL4 domains, allowing the formation of a functional GAL4 complex, which activates the expression of a *GAL1-lacZ* reporter gene. To identify proteins that interact specifically with wis2, a library of plasmids that express GAL4 activation domain- cDNA library fusion proteins could be co-transformed into a *GAL1-lacZ* integrant strain along with a plasmid containing wis2 fused to the GAL4 DNA binding domain (for examples see Yang et al. 1992; Luban et al. 1993).

The two hybrid system could be of particular use for investigating possible roles of the two distinct regions of wis2. The structure-function analysis of wis2 has suggested that the C-terminal of the protein is responsible for the specificity of the molecule (chapter 4). The observation that the 18 kDa cyp1 protein did not exhibit wis2 activity suggests that the cyclophilin domain alone is capable of interactions with different protein(s) than when attached to the C-terminal domain of wis2. If so, similar gene(s) may be isolated by testing for protein interactions against GAL4 fusion proteins of cyp1 or of the cyclophilin domain of wis2. In contrast, different gene(s) are predicted to be isolated when protein interactions are tested against a GAL4 fusion protein of the C-terminal domain of wis2.

6.3 Sequence homology considerations

i PPIase activity of cyclophilins

Sequence analysis of *wis2*$^+$ revealed that the N-terminal half of the predicted protein shows strong homology with the cyclophilin family of proteins. Cyclophilins, along with FKBPs (FK506 binding proteins) belong to the superfamily of immunophilins (see section 1.4 iii). Cyclophilins and FKBPs are structurally unrelated, but both exhibit peptidyl *cis-trans* isomerase (PPIase) activity *in vitro* (Kunz and Hall 1993). In addition, both cyclophilin and FKBPs bind to, and mediate, the effects of the immunosuppressive drugs CsA and FK506, respectively (discussed below, section 6.3 iii).

In vitro, the PPIase activity of immunophilins can accelerate the rate of protein folding (Schmid et al. 1986). The *in vivo* function of the immunophilins is less clear, although several lines of investigation suggest that immunophilins are involved in protein folding, protein transport and assembly of protein complexes (reviewed in Galat 1993; see section 1.4).

The study of cyclophilins in the yeast *S.cerevisiae* has so far failed to discover a cellular function. None of the five *S.cerevisiae* cyclophilin or cyclophilin-like genes so far identified is essential. Moreover, simultaneous disruption of three of the cyclophilin genes (*CYP1*, *CYP2* and *CYP3*) and one of the functionally related FKBP genes (*RBP1*) resulted in viable cells indistinguishable from wild type cells (McLaughlin et al 1993). These results suggest that cyclophilins and FKBP gene functions are not essential under normal growth conditions. Alternatively, cyclophilins and FKBPs form a highly redundant family of proteins.

In *S.pombe*, only one other cyclophilin-like gene, apart from *wis2*$^+$, has been isolated (*cyp1*$^+$; de Martin and Philipson 1990) and no FKBP-like genes have been identified. The phenotype of a strain deleted for *cyp1*$^+$ has not been determined. The possibility that *wis2*$^+$ and *cyp1*$^+$ share an essential function could be tested by constructing a *cyp1Δ* deletion strain and examining the phenotype of a *wis2Δ cyp1Δ* double mutant. However, the genetical analysis of cyclophilins in *S.cerevisiae* suggests that more members of the family of cyclophilins exist in *S.pombe* and that the double mutant may not exhibit a defective phenotype.

An interesting feature of the cyclophilin family of proteins is the high degree of sequence conservation across the cyclophilin domain (CyP-18 domain), compared with the diversity observed for regions flanking it. In this respect, it has been suggested that members of this family will range from general house-keeping proteins to others with extreme subcellular compartment or substrate specificity (Stamnes et al 1991). Consistent with this suggestion is the finding that in several cyclophilins N- or C-terminal extensions play a role in subcellular localisation of the cyclophilin molecule (for examples see Koser et al. 1991; McLaughlin et al. 1992). Most striking is the example of the *Drosophila melanogaster ninaA* cyclophilin, which shows extreme tissue and substrate specificity (Ondek et al. 1992). ninaA is a photoreceptor-specific integral membrane cyclophilin, with the cyclophilin domain located in the lumen of the ER and in intracellular transport vesicles. Genetical and biochemical studies suggest that the cyclophilin domain of ninaA is involved in aspects of folding or transport of the blue-sensitive photopigment rhodopsin Rh1, whereas the C-terminal tail of ninaA may play a role in the specific interaction of ninaA with Rh1. Interestingly, the structure function analysis of wis2 suggests a similar relationship between the cyclophilin and the C-terminal region of the molecule (chapter 4 and see below, section 6.4).

ii Possible roles for a new 40 kDa cyclophilin subgroup

Sequence analysis of *wis2*$^+$ revealed that it encodes an unusual cyclophilin of 40 kDa. The 18 kDa cyclophilin-like domain lies at the N-terminal of the protein and is followed by a non-conserved region encoding for 188 amino acids. In addition, the sole Trp residue in the cyclophilin-like domain, important for CsA binding (Liu et al. 1991a; and see section 1.4 iv) and highly conserved in most other cyclophilins (section 2.4 ii), is replaced by His in wis2. At the time the analysis of *wis2*$^+$ was carried out the sequence of wis2 was unique amongst cyclophilins present in the sequence data bases. However, more recently, human and bovine cyclophilin-like proteins have been isolated which share with wis2 not only the non CyP-18 like domain, but also the substitution of the conserved Trp with His (Kieffer et al. 1993).

The human and bovine 40 kDa cyclophilin (referred to as CyP-40; Kieffer et al. 1993) share extensive homology with each other. The complete ORF of *wis2*$^+$ is 44% identical to that of the human or bovine 40 kDa cyclophilins. The identity between the non-CyP-18 domain of *wis2*$^+$ and that of either of the CyP-40 proteins is 31% (see Figure 6.1A). The homology between *wis2*$^+$ and the higher eukaryote 40 kDa cyclophilins indicate that the CyP-40 subgroup of cyclophilins is highly conserved across evolution. This is also one of the few examples where a significant homology exists between the non CyP-18 domain of cyclophilin related proteins isolated from highly diverse organisms.

The analysis of the higher eukaryote CyP-40 genes may suggest new aspects for wis2 activity. Kieffer et al. noted that the non CyP-18-like domain of CyP-40 is 31% identical to a certain region of the FKBP-59 proteins, a group of proteins related to the cytoplasmic binding proteins for the drug FK506. The homology between the non CyP-18 domain of wis2 and FKBP-59 is also limited to the same region of FKBP-59 and is slightly lower than that observed between CyP-40 and FKBP-59 (27% identity; Figure 6.1 B).

The amino acid sequence of FKBP-59 predicts a 59 kDa protein of three globular domains followed by a short C-terminal tail. The first two domains are structurally related to the cytoplasmic binding protein for FK506 (FKBP-12); the third domain is distantly related to the first two (Callebaut et al. 1992). It is this third domain and the C terminal tail which are structurally related to the non CyP-18 domain of CyP-40 or wis2 (see Figure 6.2 and 6.3).

A putative calmodulin binding site is present in the C-terminal tail of FKBP-59 (Lebeau et al. 1992) and appears to be conserved in the predicted sequences of wis2 and CyP-40.

Significantly, the homology shared between the PPIases CyP-40 and FKBP-59 is the first instance of substantial homology between cyclophilin and FKBP proteins and draws yet another parallel between these two families of proteins.

Figure 6.1 **Comparison between wis2 C-terminal region and human CyP-40**

The alogarithm FASTA (Lipman and Pearson 1985) was used to align the predicted amino acid sequences of wis2 with amino acid sequences of human CyP-40 (A) or rabbit-FKBP59 (B).
The percentage of identity in each of the overlap regions is indicated at the top.
Dashed lines indicate identity and dots indicate similarity.

A 31.3% identity in 183 amino acid overlap

```
                                    176       186       196       206
wis2                          CTKDQIEAPKPDVTGDSLEEFPDDYEGD-KSETAIFKIASD
                                      | :|||  :||:| : | |: : |: |::|
Hu-40  EVKGEKPAKLCVIAECGELKEGDDGGIFPKDGSGDSHPDFPEDADIDLKDVDKILLITED
         170       180       190       200       210       220

              216       226       236       246       256       266
wis2   LKGIANKQFAQQNLDTAVAKWQKALRYLMEYPVPNDDSKESPDFWKEYNALRYSIYANLA
       ||:|:|: |  || : |: |::::|||:     ::::  |::| : : :::  |   |::
Hu-40  LKNIGNTFFKSQNWEMAIKKYAEVLRYV----DSSKAVIETAD-RAKLQPIALSCVLNIG
         230       240       250           260       270       280

              276       286       296       306       316
wis2   LVALKQNKPQEAIRNANIVIEASNSTELEKQKAYYRLGCAQGLLKNFEESEKALAK----
          || :: |:|| :   ::| : |    : || || : :   ||:::::  :| |
Hu-40  ACKLKMSNWQGAIDSCLEALELDPS----NTKALYRRAQGWQGLKEYDQALADLKKAQGI
              290       300           310       320       330

        326       336       346       356
wis2   AGNDPAISKKLAEIRQKKKDYKKRQQKAYAKMFQ
       | :| ||  :| :::|| |: |:::: :|||||
Hu-40  APEDKAIQAELLKVKQKIKAQKDKEKAVYAKMFAX
         340       350       360       370
```

B 27.4% identity in 106 amino acid overlap

```
          230       240       250       260       270       280
wis2   AKWQKALRYLMEYPVPNDDSKESPDFWKEYNALRYSIYANLALVALKQNKPQEAIRNANI
                                    :||| : : |||:  || :   :|: : |
Rb-59  KYKQALLQYKKIVSWLEYESSFSSEEVQKAQALRLASHLNLAMCHLKLQAFSAAVESCNK
          290       300       310       320       330       340

          290       300       310           320       330       340
Wis2   VIEASNSTELEKQKAYYRLGCAQGLLKNFE----ESEKALAKAGNDPAISKKLAEIRQKK
       ::| ::::|    |: :| | |:  :::|:    : :|:|:   :: | : :||  :|:
Rb-59  ALELDSNNE----KGLFRRGEAHLAVNDFDLARADFQKVLQLYPSNKAAKAQLAVCQQRI
          350           360       370       380       390       400

              350
Wis2   KDYKKRQQKAYAKMFQ
       ::   |::| ||:||:
Rb-59  RKQIAREKKLYANMFERLAEEENKAKAEVAAGDHPMDTEMKDERNDVAGSQSQVETEA
              410       420       430       440       450
```

The study of FKBP-59 proteins has been more extensive than that of CyP-40. FKBP-59 proteins have been isolated from higher eukaryotic cells including various cell types of human, rabbit and calf. These proteins have been shown to exhibit PPIase activity as well as binding to the drug FK506 and rapamycin. Sequence analysis of human FKBP-59 revealed that it is identical to the previously identified hsp56 heat shock protein (Yem et al. 1991), hence, the FKBP59 protein family has been termed hsp binding immunophilins (HBIs; Callebaut et al. 1992).

A cellular function has been suggested for FKBP-59 in the regulation of steroid receptor complexes. FKBP-59 is a component of non-active steroid complexes composed of steroid receptors and of the heat shock proteins hsp90 and hsp70 (Yem et al. 1991; Lebeau et al. 1991; Schreiber et al. 1992). Binding of the steroid results in dissociation of the heat shock proteins (hsp's), transformation of the steroid receptor to an active DNA binding state and translocation of the complex into the nucleus (Pratt 1987). It has been speculated that FKBP-59 may help the assembly/disassembly mechanisms involved in steroid receptor transport (Callebaut et al. 1992). Alternatively, FKBP-59 may have a role in regulating the transport of steroid receptors from the cytoplasm into the nucleus (Nadeau et al. 1993).

It possible that the CyP-40 proteins modulate the formation or dissociation of protein complexes, in a similar way suggested for the FKBP-59 proteins. The substrate for CyP-40 proteins is uknown. In view of the genetic analysis of wis2 it is intriguing to speculate that wis2 participate in the assembly or transport of the major cell cycle regulatory complex cdc2-cdc13 (see also above section 6.1 iv). However, at the moment this is a highly speculative suggestion, which awaits experimental investigations.

Figure 6.2 Comparison between wis2, CyP-40 and FKBP-59

Alignment of the C-terminal of wis2, human (Hu-) CyP-40, bovine (Bv-) CyP-40, human FKBP-59 and rabbit (Rb-) FKBP-59.

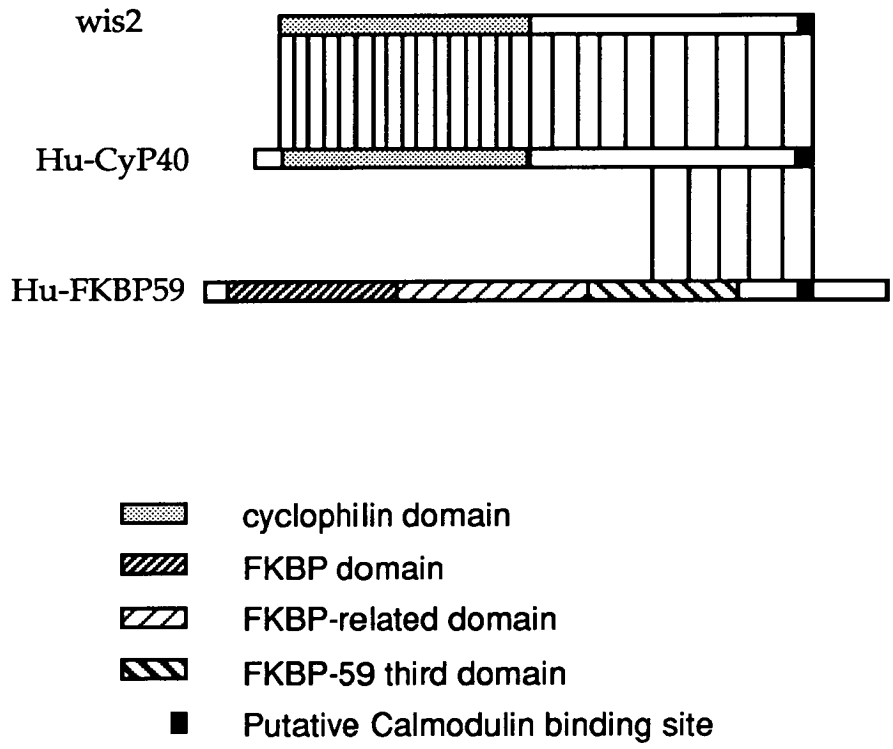
The homology between the non cyclophilin domain of the 40 kDa cyclophilins and FKBP-59 proteins is limited to the third domain and the C-terminal domain of FKBP-59 (see text for details). It is this region of homology which is shown below.

On the bottom line, upper case letters indicate identical (5/5) amino acids. Lower case letters indicate identity between at least three amino acid sequences. A putative calmodulin binding site is boxed (Lebeau et al. 1992).

```
     wis2  ..........  .........  YNALRYSIYA NLALVALKQN KPQEAIRNAN 283
 Bv-Cyp40  ..........  .........  LQPVALSCVL NIGACKLKMS DWQGAVDSCL
 Hu-Cyp40  ..........  .........  LQPIALSCVL NIGACKLKMS NWQGAIDSCL
Hu-FKBP59  ..........  .........  .QALRLASHL NLAMCHLKLQ AFSAAIESCN
Rb-FKBP59  ..........  .........  .QALRLASHL NLAMCHLKLQ AFSAAVESCN
           ..........  .........  .qalrls..l Nla.c.LK.. a.q.Ai.scn

     wis2  IVIEASNSTE LEKQKAYYRL GCAQGLLKNF EES....EKA LAKAGNDPAI 329
 Bv-Cyp40  EALEIDPSNT ....KALYRR AQGWQGLKEY DQALADLKKA QEIAPEDKAI
 Hu-Cyp40  EALELDPSNT ....KALYRR AQGWQGLKEY DQALADLKKA QGIAPEDKAI
Hu-FKBP59  KALELDSNNE ....KGLFRR GEAHLAVNDF ELARADFQKV LQLYPNNKAA
Rb-FKBP59  KALELDSNNE ....KGLFRR GEAHLAVNDF DLARADFQKV LQLYPSNKAA
           .alEld.sne ....KalyRr g.a...lk.f d.a.ad..Ka lq.ap.dkAi

     wis2  SKKLAEIRQK KKDYKKRQQK AYAKMFQ... .......... .......... 356
 Bv-Cyp40  QAELLKVKQK IKAQKDKEKA AYAKMFA*.. .......... ..........
 Hu-Cyp40  QAELLKVKQK IKAQKDKEKA VYAKMFA*.. .......... ..........
Hu-FKBP59  KTQLAVCQQR IRRQLAREKK LYANMFERLA EEENKAKAEA SSGDHPTDTE
Rb-FKBP59  KAQLAVCQQR IRKQIAREKK LYANMFERLA EEENKAKAEV AAGDHPMDTE
           .a.La...Qk ikaqk.rekk .YAkMF.... .......... ..........

     wis2  .......... ..........
 Bv-Cyp40  .......... ..........
 Hu-Cyp40  .......... ..........
Hu-FKBP59  MKEEQKSNTA GSQSQVETEA
Rb-FKBP59  MKDE.RNDVA GSQSQVETEA
           .......... ..........
```

Figure 6.3 Schematic comparison of CyP-40 and FKBP-59 proteins

The wis2, human CyP-40 and human-FKBP-59 proteins are represented as boxes. Lines joining boxes indicate similarity; density of lines is proportional to degree of similarity.



Another point to be considered is the association of FKBP-59 with heat shock proteins in non-active steroid receptors. It is of interest that this association bridges two classes of proteins implicated in protein folding and assembly. Physical associations between the immunophilins and hsp90 proteins have been supported by their interaction *in vitro* (Nadeau et al. 1993). In four parallel experiments partner proteins that bind the hsp90 proteins from human, rat, *S. cerevisiae* and *E.coli* were identified. This was achieved by immobilising hsp90 from the four organisms and passing over protein extracts of the cognate organisms. Human and rat hsp90 columns retained hsp70, FKBP59 and 18 kDa cyclophilin . The proteins identified in the yeast hsp90 columns were the 130 kDa transcriptional activation factor HSF (Heat Shock Factor), the 18 kDa Cyp1 and a putative 53 or 54 kDa protein, which is likely to be the yeast homologue of FKBP-59, based on its interaction with anti-human FKBP-59 antibodies. Proteins identified by *E.coli* hsp90 columns further support a conserved pattern of interaction between hsp90 and the immunophilins as these columns identified the *E.coli* 18 kDa cyclophilin-like protein.

The major role attributed to hsp90 has been the recognition of unfolded proteins, the prevention of aggregation and the formation of specific complexes with partner proteins (Weich et al. 1992; reviewed in Gething and Sambrook 1992). It is possible that hsp90, FKBP59 and cyclophilins may all interact to refold individual proteins or protein complexes (Nadeau et al. 1993).

One particular interesting question yet to be answered is whether CyP-40 proteins themselves are heat shock induced. The implication of cyclophilins and in particular of FKBP-59 in heat shock response led to a search for heat shock elements (HSEs) in the promoter region of *wis2*$^+$. HSEs serve as a binding site for the heat shock transcriptional activation factor HSF (Heat Shock Factor) and they are conserved from yeast to mammals. HSEs consist of inverted repeats of the sequence nGAAn (Perisic et al. 1989). In *S. cerevisiae*, most heat shock responding genes have been shown to contain an HSE element in their promoter sequences and the sequence CNNGAANNTTCNNG has been suggested to function as the HSF binding site (Pelham 1985).

Intriguingly, *wis2*$^+$ contains a putative HSE sequence CTAGAAGTTTCGTAG, 218 bp upstream of the putative initiating ATG codon (and 30 bp upstream of the putative TATA box). This sequence is

almost identical to the consensus sequence shown above except for the 3' terminal G. This 3' terminal G is also not conserved in a functional HSE found upstream the ORF of the *S.pombe* hsp70 homologue *Ssp1*$^+$ (Kasai and Isono 1991), whose transcript is induced by heat shock (Powell and Watts 1990).

The latter findings suggest the involvement of *wis2*$^+$ in heat shock response is intriguing in view of wis2 activity as a suppressor of the cell cycle defect phenotype of *wee1-50 cdc25-22 win1-1*. Although the presence of the HSE element in the *wis2*$^+$ promoter region may imply that one function of wis2 is in response to stress conditions, there is much evidence to suggest that a variety of hsp's and the HSF factor itself are involved in regulating cellular events under normal growth conditions. For example, both the *S.pombe* and the *S.cerevisiae* HSF homologues are essential for growth under normal conditions (Gallo et al. 1993; Sorger et al. 1988), even though the activity of HSF as a transcriptional activator is largely restricted to stress conditions. Several hypotheses for the function of HSF could explain these observations. One possibility is that the yeast HSFs are required for basal level of expression of certain hsp under normal cellular growth (Gallo et al. 1993; Sorger et al. 1988).

Another possibility is that HSF becomes active at certain phases of the cell cycle and thus is required for normal growth despite being in an inactive form the majority of the time (Gallo et al 1993). Some support for this suggestion is that *S.cerevisiae* cells containing a certain mutation in the HSF gene display a cell cycle defect phenotype consistent with a delay in progression through the G2 phase of the cell cycle (Smith and Yaffe 1991). In addition, the lethality of *S.pombe* cells deleted for the HSF gene can be rescued by episomal expression of either the *S.pombe* or the *D.melanogaster* HSF, but this rescue results in a high percentage of cells displaying a moderate elongated phenotype characteristic of certain cell cycle mutants (Gallo et al 1993).

Future experiments could address the possible involvement of *wis2*$^+$ in heat shock response. For example, the level of expression of *wis2*$^+$ transcript or wis2 protein in response to heat shock or other stress conditions could be examined. In addition, cells deleted for *wis2*$^+$ could be tested for their ability to survive heat shock, prolonged incubation at high temperature, or other stress conditions. In order to examine the suggestion that the HSF may have some cell cycle regulatory character it will be of

interest to examine whether the level of *wis2*$^+$ transcript varies during the cell cycle.

ii The interaction of cyclophilins with CsA

A different line of investigation has shown that cyclophilins are the cellular receptor for the immunosuppressive drug CsA. The drug-cyclophilin complex has been implicated in the disruption of Ca^{2+} mediated signal transduction pathways, initially in T-lymphocytes activation in the immune response and subsequently in the α factor signalling pathway in *S.cerevisiae* cells (Friedman and Weissman 1991; Foor et al. 1992). It has been suggested that in both cell systems the effect of CsA is mediated by a dominant activity of cyclophilin-CsA complex, rather than simply by the inhibition of the cyclophilin PPIase activity by CsA. Therefore, it is unclear whether the studies which employ the drug CsA are informative about the normal cellular function of cyclophilins.

In certain genetic backgrounds, CsA has a cytotoxic effect on *S.cerevisiae* cells (see section 1.4 iii). The mechanism by which the cytotoxic effect is mediated is largely unknown, although the presence of the 18 kDa cytosolic cyclophilin Cyp1 is necessary for the exertion of the CsA effect, suggesting that the cytotoxic effect is mediated by Cyp1-CsA complex.

The effect of CsA on the vegetative growth of *S.pombe* has been investigated in this thesis (section 3.3). CsA did not affect the ability of *S.pombe* cells to form colonies in the presence of the drug, although cells exhibited the phenotype of accumulated septa, suggesting abnormal septum formation or delay in cleavage of the septa. Neither deletion of *wis2*$^+$ nor overexpression affected the phenotype of *S.pombe* cells treated with CsA, suggesting that *wis2*$^+$ does not participate in mediating the effect of CsA.

Several points may be considered in this respect. First, it is likely that not all members of the family of cyclophilins participates in mediating the effect of CsA. Studies in *S.cerevisiae* demonstrated that only Cyp1 participates in mediating CsA cytotoxicity, even though all the three cyclophilins, Cyp1, Cyp2 and Cyp3 are capable of binding CsA *in vitro.*, (McLaughlin et al. 1992). Cyp2 and Cyp3 are of 19 kDa each, and differ from Cyp1 in that they contain extensions at the N-terminus of the

cyclophilin domain. These extensions are thought to target Cyp2 and Cyp3 to the ER and mitochondria, respectively (Koser et al. 1991; McLaughlin. et al. 1992). It is therefore possible that differences in subcellular localisation of Cyp1, Cyp2 and Cyp3 affects their interactions with other putative downstream targets in the cell. The studies of cyclophilins in *S.cerevisiae* may imply that the major receptor for CsA in *S.pombe* is the 18 kDa cyclophilin cyp1, and not wis2. If so, it is expected that deletion of *cyp1*$^+$ will abolish, or alleviate, the multiseptation phenotype associated with treatment of *S.pombe* cells with CsA. Such an experiments can be conducted once a strain deleted for *cyp1*$^+$ is constructed.

A second point of consideration derives from the sequence analysis of *wis2*$^+$. *wis2*$^+$ does not contain a tryptophan residue (Trp121), which is required for high affinity to CsA *in vitro* (Lie et al. 1991a). Trp121 is conserved amongst most known eukaryotic cyclophilins, but not in most of the bacterial cyclophilin-like genes. It has been shown that the *E.coli* cyclophilin (CypA) exhibits PPIase activity comparable to eukaryotic cyclophilins, but its binding to CsA is poor (Lie et al. 1991a).

It is worth noting that the higher eukaryotic CyP-40 proteins, which like wis2 do not contain the conserved Trp residue, were isolated by virtue of their binding to CsA affinity columns. This finding indicates that replacement of the conserved Trp with histidine residue does not abolish the ability of the cyclophilin to bind CsA *in vitro* , however Kieffer et al. (1992) noted that the affinity of bovine CyP-40 to CsA is 15 fold lower compared with that of bovine CyP-18. In addition, the cellular abundance of the CyP-40 protein is much lower than that of CyP-18. Together these findings suggest that *in vivo* the major target for CsA binding is CyP-18 and not CyP-40.

6.4 Structure-function analysis of wis2

The unusual structure of *wis2*$^+$ of a cyclophilin domain and a C-terminal region of nonconserved nature has prompted an *in vivo* structure-function analysis (chapter 4). The aim of this analysis was to determine the involvement of the two regions of *wis2*$^+$ in the suppression activity of wis2 in the triple mutant phenotype *cdc25-22 wee1-50 win1-1*.

The requirement for the cyclophilin domain was investigated by introducing a short deletion and two point mutations into the cyclophilin domain of *wis2*$^+$(section 4.3). The two point mutations replaced the His129 residue with either leucine or proline. The equivalent His126 residue of the human Cyp-A protein has been mapped to the active site of the cyclophilin domain on the basis of crystallography and NMR analysis (see section 1.4 iv). His126 has also been suggested to actively participate in the PPIase enzymatic mechanism (Kallen et al 1991).

wis2P129 and *wis2L129* were no longer able to confer wis2 activity when expressed from a multicopy yeast plasmid. However, Western blot analysis of whole *S.pombe* protein extracts did not show an increase in the level of the mutated wis2 proteins upon overexpression. Therefore, it is not clear whether the inability to confer wis2 activity stems from a specific defect of the cyclophilin active site, or whether it is a consequence of gross misfolding of the mutated proteins, making them sensitive to proteolysis *in vivo*.

Interestingly, a mutagenesis screen of mutant alleles of the *D.melanogaster* cyclophilin gene *ninaA* revealed that all mutations that abolished ninaA activity and which were located near or at the cyclophilin active site, also resulted in significant decrease of protein levels (less than 25% compared to the wild type protein level, Ondek et al. 1992).

The mutagenesis analysis of *ninaA* and *wis2*$^+$ may suggest that the amino acid residues which map to or near the active site of cyclophilins are also required for the correct conformation of cyclophilins, or that the overall structure of the cyclophilin domain is particularly sensitive to non-conserved amino acid substitutions. However, in both cases the mutagenesis analysis introduced non-conserved mutations, therefore, in future experiments it will be interesting to examine the effect of conserved substitutions within wis2, as such substitutions are expected to introduce less overall changes in the protein structure.

The structure-function analysis of wis2 is consistent with the suggestion that regions spanning the cyclophilin domain are responsible for the specificity of the molecule. Overexpression of the 18 kDa cyp1 protein in a *cdc25-22 wee1-50 win1-1* strain showed that cyp1 is not capable of suppressing the lethal phenotype of this strain. In contrast, a hybrid protein that contained cyp1 fused to the C-terminal domain of wis2 is an efficient suppressor of *cdc25-22 wee1-50 win1-1*.

The possibility that the C-terminal alone can confer wis2 activity has not been directly ruled out in this thesis and should be addressed in future experiments. In order to examine the ability of the C-terminal region of *wis2*$^+$ to confer wis2 activity it is necessary to express the C-terminal on its own in a *cdc25-22 wee1-50 win1-1* background. The level of the C-terminal domain should then be monitored either by specific antibodies against the C-terminal domain, or alternatively by tagging the C-terminal domain with a short peptide sequence against which commercial antibodies are available.

6.5 Strategies for cloning the *win1*$^+$ gene

An attempt to clone the *win1*$^+$ gene was previously described by Warbrick (1990). In this attempt four different partial digest genomic libraries in yeast multicopy vectors were screened for complementation of the lethal phenotype of *cdc25-22 wee1-50 win1-1*. This attempt was not successful, therefore, a second attempt to clone the *win1*$^+$ was carried out. In an alternative approach for cloning of *win1*$^+$(chapter 5), ordered cosmid genomic libraries were screened by DNA hybridisation, using as a probe a genetic marker, *tps19*, which is closely linked to *win1*. Cosmid clones spanning the *tps19* region were obtained, and were screened for complementation in *cdc25-22 wee1-50 win1-1* and *tps19-17 win1-1* backgrounds. This approach allowed an extensive screen of relatively small region of chromosome I, where the *win1*$^+$ gene is expected to lie. However, the *win1*$^+$ was not cloned.

Several possibilities can account for the failure to clone *win1*$^+$. One possibility is that *win1*$^+$ is unclonable in *E.coli* cells, either because the gene product is toxic or, alternatively, because the structure of the *win1*$^+$ gene or its flanking DNA induce rearrangements in plasmid or cosmid clones. Indeed, the restriction endonuclease mapping of cosmids of the *tps19* contig suggests a possible gap which lies between *tps19* and *rad1*, where the *win1*$^+$ gene is expected to lie (section 5.3 iii). The presence of such a gap could be explained if *win1*$^+$ could not be propagated in *E.coli*.

A different possibility for the failure to clone *win1*$^+$ is that *win1*$^+$ has deleterious effects on *cdc25-22 wee1-50 win1-1* cells when overexpressed. It has been previously noted that overexpression of *cdc25*$^+$

is deleterious in *cdc25-22 wee1-50 win1-1* cells (Warbrick 1990), and by analogy, similar reasoning might account to the failure to clone *win1*$^+$. It is also possible that the gene product of *win1*$^+$ is deleterious when overexpressed in any genetic background as has been described for several *S.pombe* genes (for example, see the deleterious effect of S.pombe gene *Nda3*$^+$ encoding for β tubulin; Hiraoka et al. 1984).

Further analysis of the *tps19* contig in cosmids is required in order to determine what renders the *win1*$^+$ gene unclonable. As a first step, the possibility of gap in the *tps19* contig should be investigated. In order to identify unambiguously overlap between adjacent cosmid clones, restriction mapping of the *tps19* contig can be carried out by Southern hybridisation analysis, with terminal insert sequences of cosmid clones as probes. In addition, cosmid insert fragments (1 - 2 kb long) could be genetically mapped relative to the *win1* and *tps19* loci. This can be done by cloning these fragments into integrating *S.pombe* plasmids as described for the genetic mapping of *wis2* (section 5.2 i). Comparing the genetical and physical mapping data of insert fragments of cosmids should further delimit the chromosomal region where *win1*$^+$ is expected to lie, and thus indicate whether or not *win1*$^+$ sequences are expected to be present in the *tps19* contig.

Several alternative strategies exist for the isolation of the *win1*$^+$ gene. One possibility would be to avoid problems due to high level of *win1*$^+$ expression by constructing a mini-library of the *tps19* contig in cosmids in integrating yeast plasmids. Such mini-library could then be screened in *cdc25-22 wee1-50 win1-1* and *tps19-17 win1-1* backgrounds. This strategy is most suitable for avoiding possible deleterious effects of *win1*$^+$ in *S.pombe* cells, but it may also circumvent problems due to *win1*$^+$ being unclonable in *E.coli*. Screening by integration can identify plasmids which carry partial (truncated) sequences of *win1*$^+$, as integration of such plasmids could reconstitute a complete wild type copy of *win1*$^+$, depending on the site of homologous recombination.

A second possibility would be to screen by complementation insert sequences of the ordered bacteriophage P1 clones that span the *tps19* region. Four such P1 clones have been identified by hybridisation of the *S.pombe* P1 libraries with the *tps19* probe (section 5.3 ii). In contrast to the cosmid clones of the *S.pombe* library, which propagate at high copy number in *E.coli* cells

(Cross and Little 1984), P1 clones propagate at a low copy number (Sternberg 1992). If the *win1*$^+$ gene product is toxic to *E.coli*, the reduced level of its expression may alleviate its toxic effect. Screening P1 clones for the presence of *win1*$^+$ will involve the construction of mini-libraries of the P1 DNA in plasmids that have low copy number in *E.coli* (such plasmids are described in Rose and Broach 1991). The P1 mini-libraries could then be screened in appropriate genetic backgrounds as described for the cosmid mini-libraries.

Chapter 7: Materials and Methods

7.1 Commonly used reagents and buffers

Most of the methods used in this work were based on those described in Sambrook *et al.*. (1989).

All chemicals used were of analytical grade, and were bought from Sigma, British Drug Houses (BDH), Gibco-BRL, Fisons, or Pharmacia.

H_2O refers to distilled autoclaved water.

Eppendorf tube refers to 1.5 ml centrifuge tube. High speed and low speed centrifugation in Eppendorf tubes refers to a Microcentaur centrifuge at 13,000 rpm and 6,500 rpm, respectively.

Tris-HCl

Tris base (tris[hydroxymethyl]aminomethane) was dissolved in H_2O, and the pH adjusted to the required value by addition of HCl. H_2O was added to give a 1 M stock solution.

EDTA

A stock solution of 0.5 M EDTA (ethylenediaminetetramino acid, di-sodium salt) was made by dissolving solid EDTA in H_2O, adjusting the pH to 8.0 with NaOH, and adding water to the required volume.

TE

A buffer solution, suitable as a routine DNA solvent, consisting of 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA.

Phenol

Phenol was pre-equilibrated with 1 M Tris-HCl (pH 7.5), followed by TE buffer. To retard oxidation of the phenol solution 0.1% (v/v) hydroxyquinoline and 0.2% (v/v) β–mercaptoethanol were added. For work with RNA, phenol pre-equilibrated with H_2O (Rathburn 3024), was used. Phenol solutions were stored at 4°C in the dark.

Chloroform

Chloroform (BDH 10077) refers to a 24:1 (v/v) mixture of chloroform and isoamyl alcohol (Sigma I-1381).

Sodium acetate

Sodium acetate was dissolved in H_2O, the pH adjusted to 5.2 with HOAc, and H_2O added to a final concentration of 3 M.

Ethidium bromide

Ethidium bromide (Sigma E-875) was dissolved as a stock solution of 10 mg ml^{-1} in H_2O, and stored at 4°C in the dark.

Loading buffer

5 x loading buffer for gel electrophoresis of nucleic acids was prepared and stored at room temperature:

0.25% (w/v) bromophenol Blue
15% (w/v) ficoll in H_2O.

TBE

TBE was routinely made up as a 10 x stock solution, and stored at room temperature:

	stock	1 l
0.89 M Tris base		108 g
0.89 M boric acid		55 g
20 mM EDTA	0.5 M	40 ml.

TAE

TAE was made as a stock solution of 50 x, and stored at room temperature:

	stock	1 l
2 M Tris base		242 g
HOAc		57.1 ml
50 mM EDTA	0.5 M	100 ml

DEPC H_2O

RNAase free water, suitable for dissolving RNA, was made by adding 0.1% (v/v) diethyl pyrocarbonate to H_2O, mixing for 5 minutes at room temperature, and autoclaving.

7.2 Nucleic acid manipulations

i Dissolving and storage

All DNA and RNA was dissolved in a solution of either TE or H_2O, depending on the subsequent use of the nucleic acid. Nucleic acids were routinely stored as solutions at -20°C.

ii Extraction with phenol/chloroform

Proteins were removed from solutions containing DNA and RNA, by extraction with an equal volume of a 1:1 mixture of phenol and chloroform. Traces of phenol were removed by a further extraction with chloroform.

Extractions were carried out by adding a volume of phenol/chloroform equal to that of the nucleic acid solution. The two solutions were mixed thoroughly by vortexing to form an emulsion, and separated by centrifugation, routinely 4 minutes high speed, at room temperature. The aqueous phase was then transferred to a fresh tube carefully, avoiding protein at the interface of the two phases, and the nucleic acids recovered by precipitation.

iii Precipitation of nucleic acids

DNA was precipitated by the addition of a $^1/_{10}$ volume of 3 M sodium acetate pH 5.2, followed by 2 volumes of -20°C absolute ethanol. RNA was precipitated by the addition of $^1/_{10}$ volume 3 M sodium acetate pH 5.2, followed by 2.5 volumes of -20°C absolute ethanol. In each case the solution was mixed thoroughly by vortexing, frozen at -20°C for 30 minutes, and the nucleic acids pelleted by centrifugation, at high speed at 4°C. Small amount of DNA were precipitated as above, with the addition of 20 µg of glycogen (Boehringer Mannheim 12463420), before freezing at -20°C.

To remove trace amounts of salt remaining from the precipitation, the nucleic acid pellet was washed in 100 µl of either 70% (v/v) -20°C ethanol (for RNA, or large fragments of DNA >500 nucleotides), or 90%

(v/v) -20°C ethanol (for small fragments of DNA <500 nucleotides), and pelleted by centrifugation. The nucleic acid pellet was air dried, and dissolved in an appropriate volume of H_2O or TE buffer.

iv Quantification and quality control

DNA and RNA quality and amount was assayed by using two methods: spectrophotometry, and ethidium bromide staining after gel electrophoresis.

The first method involves taking an absorbance reading at wavelengths 260 and 280 nm. The reading at 260 nm allows calculation of the concentration of nucleic acid in the sample. An A_{260} of 1 corresponds to approximately 50 µg/ml for double stranded DNA, 40 µg/ml for single stranded DNA and RNA, and 20 µg/ml for single stranded oligonucleotides. The ratio between the readings at 260 nm and 280 nm provides an estimate of purity of the nucleic acid. Pure preparations of DNA and RNA have ratios of 1.8 and 2.0, respectively. Any ratios less than these values indicate protein and/or phenol contamination of the sample.

The second method utilises ethidium bromide, which specifically and proportionately binds to DNA, and fluoresces under UV light. The nucleic acid sample is run in an appropriate gel containing ethidium bromide at a concentration of 0.5 µg/ml, in parallel with a nucleic acid sample of known amount (usually standard molecular weight markers), and visualised on a trans-illuminator. A rough estimate of DNA amount can be made from the relative staining intensities of the bands.

v Plasmid vectors

Propagation of plasmids in *E.coli*

The pUC based plasmids were used for the manipulation of foreign genes in *E. coli* (Vieira and Messing 1982). They contain the pBR322 derived ampicillin resistance gene, origin of DNA replication and a polylinker inserted into a portion of the *lacZ* gene of *E. coli*. Non-recombinant plasmids are able to synthesize this enzyme which breaks down X-Gal (Section 7.4 ii), to release a blue pigmented derivative. In recombinant plasmids the *lacZ* gene is interrupted by foreign DNA,

resulting in colourless colonies.

The vectors pTZ18/19 (Pharmacia) are similar to the pUC plasmids, but contain in addition the f1 origin of replication. Consequently, if the host cell is super-infected with the helper phage M13K07, replication will be initiated at the f1 origin, resulting in the excretion of phage coats containing single stranded DNA of the plasmid.

pBluescript SK/KS (Stratagene) plasmids have an alternative more extensive polylinker than the pTZ plasmids, and the ampicillin resistance gene has been replaced by the chloramphenicol resistance gene, for alternative selection.

Yeast autonomously replicating vectors

The plasmids described below are capable of autonomous replication both in *E.coli* and *S.pombe*. The presence of *S.pombe ars1*$^+$ sequences or the *S.cerevisiae* 2µm origin lead to high frequency transformation (review in Moreno et al.. 1991). Both plasmid vectors based on *ars1*$^+$ sequences and the 2µm origin are mitotically unstable, the copy number of the latter is generally lower.

As a selective marker for propagation in yeast cells, plasmid vectors contain either the *S.cerevisiae LEU2* gene or the *S.pombe ura4*$^+$ gene, capable of complementing mutations of *S.pombe* in the *leu1* or the *ura4* gene, respectively.

For selection in *E.coli* cells, plasmids contain antibiotic resistance genes.

pDB248

pDB248 was derived from the *E.coli* plasmid pBR322 and the *S.cerevisiae* plasmid pJDB248 (Durkacz et al.. 1985). pDB248 is 2µm based and contains the *S.cerevisiae* LEU2 gene and the antibiotic resistance genes for both ampicillin and tetracycline .

pWH5

pWH5 was constructed by a modification of the yeast vector pDB262 (Wright et al.. 1986). The plasmid is 2µm based and contains the *S.cerevisiae LEU2* gene and the antibiotic resistance genes for ampicillin and tetracycline. The tetracycline gene is under the control of the bacteriophage λ *Pr* promoter. pWH5 contains the λ *cI* suppressor gene, so that only inactivation of the suppressor gene product allows expression of

the tetracycline gene. Such inactivation can be induced by cloning fragments into either the *Hin*dIII or *Bcl*I sites of λ *cI* . This cloning procedure is especially useful for gene library construction, since selection for tetracycline resistance ensures that all *E. coli* transformants contain recombinant plasmids.

pIRT2

pIRT2 was constructed from the vector pUC118 by inserting an *Eco*RI fragment containing *ars1*$^+$ into the *Eco*RI in the polylinker, and a *Hin*dIII fragment containing the *LEU2* gene into the *Hin*dIII polylinker site. This plasmid allows the use of polylinker sites for the cloning of DNA fragments (Booher and Beach 1986; see Figure 7A).

pREP1

The pREP1 plasmid allows expression of DNA fragments from the strong and repressible *nmt1*$^+$ promoter of *S.pombe* (Maundrell 1990). pREP1 was constructed by cloning the two *cis* regulatory elements of *nmt1*$^+$ into the polylinker of pUC119: the *nmt1* promoter extending upstream of the initiator ATG codon, and the transcription stop element extending downstream from the stop codon of *nmt1*$^+$. In addition, the *LEU2* gene of *S.cerevisiae* and the *ars1*$^+$ sequence of *S.pombe* were cloned into the *Hin*dIII and *Eco*RI sites, respectively, of the pUC119 polylinker.

For the cloning of DNA fragments into pREP1, immediately downstream of the *nmt1* promoter, four restriction sites are available: *Nde*I *Sal*I, *Bam*HI and *Sma*I (see Figure 7B). The *Nde*I cloning site was introduced by altering the sequences of the initiating ATG codon of *nmt1*$^+$. *Nde*I contains an ATG sequence and thus is particularly useful for further subcloning into pREP1 as it allows the precise replacement of the coding region of *nmt1*$^+$ by the coding region of a heterologous gene.

The *nmt1* promoter is repressed in media supplemented with thiamine. For the repression of genes cloned under the regulation of the *nmt1* promoter, 3 - 5 μm/ml of thiamine are added to the growth media. Kinetic studies demonstrated that addition of thiamine to exponentially growing cells result in a complete disappearance of the *nmt1*$^+$ mRNA within 3 hours. Removal of thiamine from the media produced the first detectable message after 10 hours and maximal steady state levels after 16 hours (Maundrell 1990).

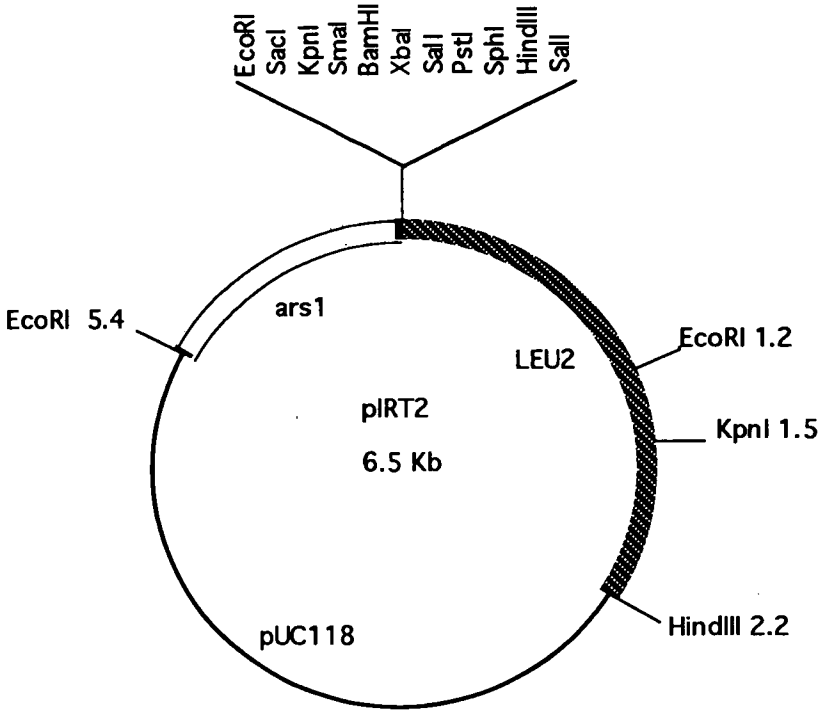


Figure 7A pIRT2

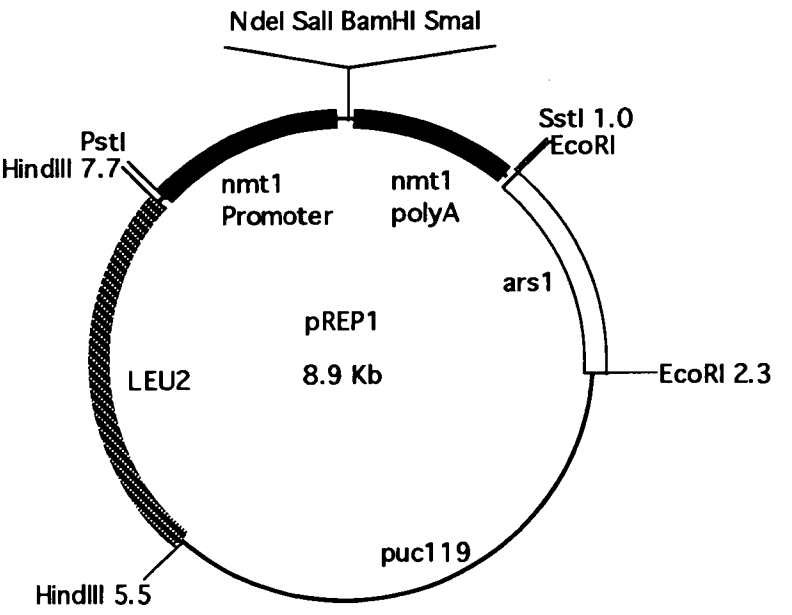


Figure 7B pREP1

pREP3X

pREP3X is a derivative of pREP1, constructed by C. Norbury (personal communication). pREP3X differs from pREP1 only in the restriction sites available for cloning of DNA fragments downstream of the *nmt1* promoter. These restriction sites are: *XhoI*, *Sal*I *Bam*HI and *Sma*I.

vi Cosmids and plasmid P1 vectors

Ordered genomic libraries of *S.pombe*, constructed in Lawrist4 cosmid and bacteriophage P1 vectors (Hoheisel et al.. 1993), were used for physical mapping of *tps19*$^+$ (chapter 5). Cosmid and P1 vectors which had been mapped to the same region as *tps19*$^+$ were then used in various strategies in an attempt to clone *win1*$^+$.

Lawrist4 cosmid

Lawrist4 is a cointegrate of the cosmid LoristX and pUCcos1. Lorist X is an *E.coli* cosmid based on λ *ori* , the origin of replication of phage λ (Gibson et al. 1987). pUCcos1 is a plasmid based on the pUC series in which the polylinker containing *Pvu*II fragment was replaced by the λ *cos* region (Hinshelwood and Stoker 1992).

Sequences of Lawrist4 derived from LoristX include the phage λ *cro*, *cII*, *O* and *P* genes and the *Pr* promoter and the kanamycin resistance gene from the transposon Tn5. pUCcos1 has been inserted at the single *cos* site of Lawrist4 to produce a chimeric vector containing two *cos* sites. The introduction of the second *cos* site facilitates cloning (the *BamH*I cloning site was used for the construction of the cosmid *S.pombe* library; Hoheisel et al.. 1993). After packaging the pUC sequences are lost and the vector is identical to LoristX.

Bacteriophage P1

The bacteriophage P1 cloning system can package and propagate DNA inserts that are up to 95 kb long (reviewed in Sternberg 1992). Clones are maintained in *E.coli* by low copy replicon in the P1 cloning vector, under selection of kanamycin (the kanamycin resistance gene is derived from the transposon Tn903). Clones can be amplified by inducing a second replicon in the vector with isopropyl β–D-thiogalactopyranoside, and can be readily isolated as supercoiled circles by standard molecular techniques.

The pAd10sacBII bacteriophage system was developed by Pierce et al. (1991), and provides a positive selection system for cloning foreign DNA. The positive selection is based on the properties of the *SacB* gene: expression of this gene kills *E.coli* cells that are grown in the presence of sucrose. In pAd10sacBII *SacB* expression is regulated by a synthetic *E.coli* promoter that contains a P1 C1 repressor binding site. A unique *Bam*HI cloning site is located between the promoter and the *SacB* structural gene. Cloning DNA fragments into the *Bam*HI site interrupts *SacB* expression and permits growth of plasmid containing cells in the presence of sucrose.

7.3 Molecular analysis of nucleic acids and proteins

i Restriction enzyme digestions

Restriction endonucleases were used, as recommended by the manufacturers, to cut DNA. Usually, 1-5 µg of DNA was incubated with 10 units of restriction enzyme and appropriate buffer, in a final volume of 10 µl, at 37°C for 1-1.5 hours. Digestions were stopped by the addition of $^1/_{25}$ volume of 0.5 M EDTA, and freezing at -20°C.

ii Modification enzymes

Ligations

Ligation of DNA, either of cohesive or blunt end termini, was performed using T_4 DNA ligase (Boehringer Mannheim, 481220) in T_4 ligase buffer supplied by the manufacturer. Routinely, between 0.5-2 µg of DNA was ligated with 0.5-1 µl of ligase (0.5-1 or 1 units), in a volume of 10 µl. Ligation reactions were incubated for 16 hours at 14°C for cohesive termini, and at 25°C for blunt ended termini.

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 was treated with the Klenow fragment of DNA polymerase I (Boehringer Mannheim, 1008412).

0.5 - 2 µg DNA was incubated at 25°C in a volume of 25 µl, with 0.5-1 unit of Klenow fragment, 2.5 µl buffer (500 mM Tris-HCl pH7.5; 100 mM

$MgCl_2$; 1mM DTT, 500 µg/µl BSA) and 2 mM dNTPs. The reactions was incubated for 30-60 minutes and stopped by adding 1 µl of 0.5 M EDTA.

Alkaline phosphatase

The removal of 5' phosphate groups of linearized plasmid DNA was performed to avoid re-circularization of plasmid vectors in subsequent ligation reactions. The reaction was carried out using Calf intestinal alkaline phosphatase (Boeringer Mannhim, 713 023). Alkaline phosphatase was deactivated by incubation at 68°C for 15 minutes, followed by phenol/chloroform extraction.

iii Gel purification of DNA fragments

To isolate restricted DNA fragments of the length of 0.3 -10 kb, restriction digest reactions were separated on agarose gels (section 7.3 vi), and a gel slice containing the appropriate fragment was excised. The DNA fragment was purified using the GeneClean kit (Bio 101) which is based on the method of Volgelstein and Gillespie (1979). Purified fragments were resuspended in water or TE buffer.

iv Radio-labelling of DNA fragments

The method used was that of random-primed labelling, based on that described by Feinberg and Vogelstein (1983).

The labelling reaction consisted of:

DNA	10-50 ng (dissolved in H_2O or TE)
BSA 10 mg/ml	2 µl
OLB[1]	10 µl
[α-^{32}P]dCTP	5 µl (Amersham 10 µCi/µl)
H_2O	to 50 µl total
Klenow (2 U/µl)	1 µl (Boehringer Mannheim 100840)

The reaction was incubated for 4-6 hours or overnight at room temperature and was stopped by addition of 200 µl stop buffer (20 mM NaCl/20 mM Tris. Cl/2 mM EDTA).

1 OLB:

OLB was made from the following components:

O: to 1 ml of 1.25 M Tris. Cl (pH 8.0) and 0.125 M $MgCl_2$, 18 µl of β-mercaptoethanol added, and 5 µl each of 100 mM dATP, dTTP, and dGTP (Pharmacia 27-2035-01).

L: 2 M hepes (Sigma H-3375), titrated to pH 6.6 with 4 M NaOH.

B: Hexa-deoxyribonucleotides at 90 OD units/ml (Pharmacia 27-2166-01).

Solution O, L, and B were mixed at a ratio of 100: 250: 150, to make OLB. OLB stored as 20 µl aliquots at -20°C.

v Purification of labelled DNA

Radiolabelled DNA was separated from un-incorporated radio-nucleotide by the use of a G-50 matrix size exclusion column.

1. G-50 (Pharmacia 17-0042-01) beads were prepared by adding two volumes of TE and autoclaving.
2. The plunger of a 1 ml disposable syringe (Plastipak) was removed, and a small wad of siliconized glass wool[1] pushed into the bottom.
3. G-50 was added until the syringe was full: the column was spun for 2 minutes at 2000 rpm, more G-50 was added and the column spun again until the G-50 had shrunk to about 800 µl.
4. Labelled DNA was added in a volume of about 100 µl, with 1 µl of glycogen carrier, and spun again for 2 minutes at 2000 rpm.
5. The eluted DNA was sodium acetate precipitated, washed with 90% ethanol and resuspended in water.
6. Measurements of the amount of radioactivity of the DNA eluted from the G-50 column was carried out using scintillation counter (Beckman LS1701).

1 Siliconized glass wool:

Glass wool was soaked in 5% solution of trimethyl-chlorosilane and this allowed to evaporate in a fume hood. The wool was then washed thoroughly in water and baked dry at 180°C for 2 hours.

vi Agarose gel electrophoresis

For analysis of DNA fragments of the size of 0.3-10 kb, 0.6 -1% (w/v) agarose gels in the horizontal gel system were used.

Agarose (Sigma A-6877) was dissolved in 1 x TAE buffer by brief boiling. DNA samples were mixed with a $^1/_5$ volume of loading buffer, before loading into gel wells. Routinely 0.2 µg of molecular weight markers (1 kb ladder, Gibco-BRL 5615SA) was loaded. Gels were run with an applied voltage of 4-8 V/cm for 1-3 hours. DNA was stained with ethidium bromide at a final concentration of 0.5 µg/ml, visualized with a UV trans-illuminator, and photographs taken with a Mitsubishi Video Copy Processor Model P65B.

vii Acrylamide gel electrophoresis

For the analysis of DNA fragments of the size of 50 - 200 nucleotides, 6% acrylamide gels in the vertical Bio-Rad Gel system were used.

1. A stock solution was prepared by dissolving acrylamide: bisacrylamide at a ratio of 33: 1 (125 g : 3 g) in 206 ml of H_2O.
2. 5 ml of acrylamide stock was added to 50 ml of 1 x TBE buffer and mixed.
3. 200 µl of 10% (w/v) ammonium persulphate and 25 µl of TEMED (N,N,N',N'-Tetramethylethylenediamine) were added, and the solution poured immediately.
4. DNA samples were mixed with a $^1/_5$ volume of loading buffer and loaded onto the gel. Gels were run at 10 V/cm for 2-3 hours.
5. DNA was visualised and photographed as for agarose gels.

viii Formaldehyde gel electrophoresis

RNA was separated using formaldehyde gels (method described in Golberg 1980).

1. 1.0 g agarose (Sigma A-6877) was dissolved in 63 ml H_2O and 16 ml 5 x MNE[1] buffer by boiling. The agarose solution was cooled to 60°C, 17 ml of 38% (w/v) solution of formaldehyde were added, and the solution was poured immediately into a horizontal gel apparatus
2. To each sample containing 5 - 10 µg of fission yeast RNA, 15 µl of RNA buffer[2] and 1 µl of 0.5 mg/ml ethidium bromide were added. These samples were incubated at 60°C for 5 minutes, and loaded onto the gel.
3. Gels were run in 1 x MNE buffer with an applied voltage of 10 V/cm for 3 hours.

1 5 x MNE buffer:

	500 ml
0.12 M MOPS (Sigma M-1254)	13.08 g
25 mM NaOAc	1.03 g
5 mM EDTA	0.19 g.

Adjusted to pH 7 with NaOH, and stored at 4°C in the dark.

2 RNA buffer:

formaldehyde	600 µl
formamide (BDH 28241)	200 µl
MNE Buffer	240 µl
H_2O	160 µl.

ix Filter hybridization of DNA

A modification of the method of Southern (1975) was used to detect specific DNA fragments from plasmids or from digested *S.pombe* chromosomal DNA. DNA digest reactions were seperated on agarose gels (section 7.3 vii) and GeneScreen Plus nylon membranes (NEN Research, NEF-976) were used to immobilise DNA of the size of 0.2-12 kb.

Transfer of DNA fragments was performed using the capillary method, according to the manufacturer's instructions. Hybridization was carried following the preferred protocol; this included performing the hybridization at 65°C and allowing it to proceed for at least 6 hours. The membrane was washed according to manufacturer's instructions and briefly air dried before being sealed in plastic bags and autoradiographed.

x Filter hybridization of RNA

Transfer of *S.pombe* RNA samples to nylon membranes was performed using GeneScreen plus membrane (NEN Research, NEF-976), using the capillary method, similar to that described for Southern blotting.

Transfer was performed from formaldehyde gels (section 7.3 viii). Once transfer was completed the membrane was baked in a vacuum oven at 80°-100°C for 2-3 hours. Hybridization was carried following the preferred protocol described in the manufacturer's instructions. This included hybridization at 42°C, which was allowed to proceed for 12-24 hours. The membrane was washed according to the manufacturer's instructions and briefly air dried, before being sealed in plastic bags and autoradiographed.

When further hybridizations with other probes were performed, RNA blots were stripped of the previous radiolabelled probe. The removal was achieved by following the preferred protocol described in the manufacturer's instructions. Alternatively, when radiolabelled probes were difficult to remove, the blots were placed in a boiling solution of 10 mM Tris-HCl pH 7.5-8, 1mM EDTA and 1% SDS, and kept in the boiling solution for 20-30 minutes.

xi Autoradiography and Phosphor-image analysis

GeneScreen plus membranes sealed in plastic bags were placed in contact with Fuji Blue X-ray film in cassettes. For the detection of ^{32}P, cassettes were placed at -70°C and intensifying screens used. For the detection of ^{35}S, cassettes were placed at room temperature without intensifying screens. Exposure times varied from 1 hour to 5 days. Films were developed using an X-ograph Compact X2.

Phosphor-image analysis was performed for detection and quntification of signals produced by ^{32}P. GeneScreen plus membranes sealed in plastic bags were placed against phosphor screens (Molecular Dynamic) for exposure time of 30 minutes. The phosphor screens were than scanned by a PhosphoImager (Molecular Dynamic). ImageQuant Software was used for analysing the data, and the measurments expressed in numerical values.

xii SDS-polyacrylamide gel electrophoresis

Discontinuous SDS-polyacrylamide gels were used for separation of total *S.pombe* protein extracts, under denaturing conditions. Gels were poured using the Mini-PROTEAN II dual slab cell for miniature polyacrylamide gels (BioRad 165-2940).

Discontinuous polyacrylamide consists of a resolving (lower) gel and a stacking (upper) gel. The stacking gel acts to concentrate large sample volumes, resulting in a better gel resolution. Molecules are then completely separated in the resolving gel.

Reagents and gel preparation described below are based on the method described by Laemmli (1970) and on the instruction manual of the BioRad Mini-PROTEAN II system.

1. The gel apparatus was assembled according the manufacturer's instruction. Resolving gel monomer solution[1] was prepared and poured. The monomer solution was immediately overlaid with a water-saturated isobutanol.
2. The resolving gel was allowed to polymerize for 45 minutes to 1 hour. The water-saturated isobutanol solution was rinsed off with distilled water.
3. The stacking gel monomer solution[2] was prepared, poured immediately and combs fitted. The stacking gel was allowed to polymerize for 45 minutes to 1 hour; the combs were removed and the wells were rinsed with distilled water.
4. The glass sandwich gel was fitted into the buffer tank, and the tank filled with Tris-Glycine buffer[3].

5. Protein samples prepared in Loading buffer[4] were loaded into the gel. As a molecular marker, prestained SDS-PAGE low range molecular weight marker (Bio-Rad 161-0305) was used.
6. Gels were run at 100-150 volts (constant voltage setting), for approximately 45 minutes.

1 Resolving gel (10%):

30% Acrylamide [37.5:1] Bisacrylamide solution (NBL 077234)	2 ml
Tris-HCl pH 8.8	2.25 ml
H_2O	1.7 ml
SDS 20% (w/v)	30 µl
Amonium persulfate 10% (w/v) (Freshly made stock solution)	30 µl
TEMED (N,N,N',N'-Tetramethylethylenediamine)	5 µl

2 Stacking gel (4%):

30% Acrylamide [37.5:1] Bisacrylamide solution (NBL 077234)	0.55 ml
Tris-HCl pH 6.8	0.5 ml
H_2O	2.9 ml
SDS 20 % (w/v)	20 µl
Amonium persulfate 10% (w/v) (Freshly made stock solution)	20 µl
TEMED (N,N,N',N'-Tetramethylethylenediamine)	5 µl

3 Tris Glycine buffer:

25 mM Tris
250 mM glycine pH 8.3
0.1% SDS

4 X2 Loading buffer:

100 mM Tris-HCl pH6.8
4% SDS
0.2% bromophenol blue
20% glycerol
700 mM β-mercaptoethanol (added from 14.3 M solution immediately before loading)

In order to visualize polypeptides separated by SDS-polyacrylamide gels, gels were soaked in 0.1% PAGE-Blue G-90/4% perchloric acid on a rocking platform for 5 minutes. Staining solution was removed, and excess dye was then allowed to diffuse from the gel by soaking it in distilled water and leaving it to shake gently for 0.5-3 hours.

xiii Filter hybridization of proteins

The Mini Trans blot cell (BioRad) was used for blotting proteins from SDS-polyacrylamide gels by electric current. Electrophoretic transfer conditions were used according to the manufacturer's instructions; including the transfer buffer of 25 mM Tris/250 mM Glycine/20% methanol (v/v). The membrane of choice for the blotting was 0.2 micron PVDF (polyvinylidene difluoride) membrane (Bio-Rad, 162-0184).

Transfer was allowed to proceed for 1-1.5 hours at 100-150 volt. If transfer was longer than 1 hour, the cooling unit of the Mini Trans blot cell was changed every 30-45 minute, in order to keep the buffer temperature below 30°C. In all transfers the current was monitored and was not allowed to exceeds the value of 300 mA.

xiv Detection of immobilized proteins

Two different protein detection systems were used: ProtoBlot Western blot AP (alkaline phosphatase) system (Promega); and ECL (enhanced chemiluminescence) system (Amersham).

Prior to the application of either of the above detection systems, the membranes carrying the immobilized proteins were treated as follows:

1. Non-specific binding sites were blocked by immersing the membrane in 4-5% dried milk in phosphate buffered saline: PBS[1]. This step was allowed to proceed overnight at 4°C, with agitation.
2. The membrane, routinely cut to the size of 48 mm x 82 mm, was removed from the blocking solution and placed, protein side up, in a small dish containing 3-5 ml of PBSP-T[2].
3. Incubation with the primary antibody was allowed to proceed with very gentle agitation for 30 minutes. This and the following incubation steps were performed in room temperature.
4. The membrane was removed from the primary antibody solution and washed three times in a large volume (20-50 ml) of PBS-T for 5-10 minutes each time.
5. The membrane was placed into a fresh small dish containing 5 ml of PBS-T and the secondary antibody was added. For Promega AP system, the anti-rabbit secondary antibody was diluted 1:7500; for Amersham ECL system anti-rabbit the secondary antibody was diluted 1:1500 or 1:2500.
6. Incubation with the secondary antibody solution was allowed to proceed with very gentle agitation for 30 minutes.
7. The membrane was washed three times in a large volume of PBS-T for 5-10 minutes each time.
8. The membrane was removed from the secondary antibody solution, excess of liquid was removed by blotting for few seconds onto 3MM paper.
9. The membrane was placed into a fresh small dish.

When the Promega AP system was used, colour developing solution (5 ml) supplied by the manufacturer was added. Incubation allowed to proceed with very gentle agitation for about 10 - 30 minutes, which is the normal length of time needed for colour development.

When the Amersham ECL system was used, the membrane was first allowed to equilibrate in 2.5 ml of detection reagent, solution #2, supplied by the manufacturer. Then an equal quantity of solution #1 was added, and incubation was proceeded with gentle agitation for exactly 1 minute. Excess of detection reagents was removed by blotting for few seconds onto 3MM. The drained membrane was placed in a plastic bag, the plastic bag sealed and placed in a film cassette. Hyperfilm-ECL autoradiography film was used. Exposure time varied from few seconds to 15 seconds.

1 PBS pH 7.5:

di-sodium hydrogen orthophosphate anhydrous (80 mM)	11.5 g
sodium dihydrogen orthophosphate (20 mM)	2.96 g
sodium chloride (100 mM)	5.84 g

Dissolved in 1000 ml distilled water.

2 PBS-T:

Tween-20 was diluted in PBS buffer to a concentration of 0.1% v/v.

7.4 *Escherichia coli* manipulations

i *E.coli* strains

E.coli strains for the propagation of plasmids:

HB101 *supE44 hsdS*20($r_B m_B$)*recA*13 *ara*-14 *proA*2 *lacY*1 *galK*2 *rpsL*20 *xyl*-5 *mtl*-1

JA226 *ecBC leuB6 trpE5 hsdR*$^-$ *hsdM*$^+$ *lacY600.*

E.coli strains for the propagation of plasmids and the production of single stranded DNA

JM101 *supE thi* Δ(*lac-proAB*)
F'[*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]

JM109 recA1 *supE44 endA*1 *hsdR*17 *gyrA*96 *relA*1 *thi* Δ(*lac-proAB*)
F'[*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15)

DH5α *supE44* Δ*lac*U169 (ϕ80 *lacZ*ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1

XL1-Blue *supE44* Δ *hsdR*17 *recA*1 *endA*1 *gyrA*46 *thi relA*1 *lac*$^-$
F'[*proAB*$^+$*lacI*^{q}*acZ*ΔM15Tn10(tet^r)]

E.coli strains for oligonucleotide-directed *in vitro* mutagenesis procedure:

CJ236 *dut1 ung1 thi-1 relA*1/pCJ105(cam^r F')

ii Media and growth conditions

L-Broth (LB)

E. coli strains were routinely grown in rich medium LB

Bacto-tryptone	10 g/l
NaCl	10 g/l
Yeast extract	5 g/l
glucose	1 g/l

glucose was added from a sterile stock after autoclaving. For solid plate medium 15 g/l Bactoagar was added before autoclaving.

M9 minimal medium

750 ml of H_2O containing 12 g of Bactoagar was autoclaved and cooled to 55°C in a water bath. Each of the following were autoclaved separately and then added together:

200 ml of 5 x M 9 salts[1]
2 ml of 1 M $MgSO_4$
100 µl of 1 M $CaCl_2$
20 ml of 20% glucose.

1 5 x M 9 salts:

$Na_2HPO_4.7H_2O$	64 g
KH_2PO_4	15 g
NaCl	2.5 g
NH_4Cl	5.0 g.

The solution was dissolved to final volume of 1 litre in H_2O, the pH adjusted to 7.4 with HCl, split into 200 ml aliquots, and autoclaved.

Thiamine was added as supplement at a final concentration of 1 mM thiamine-HCl.

2XYT

Bactopeptone 16 g/l
yeast extract 10 g/l
NaCl 5 g/l .

Selective antibiotics

Ampicillin

A 1000 x stock solution of 100 mg/ml of the sodium salt was dissolved in 50% ethanol.

Chloramphenicol

A 1000 x stock solution of 34 mg/ml chloramphenicol was dissolved in 100% ethanol.

Kanamycin

A 1000 x stock of 70 mg/ml kanamycin was dissolved in H_2O.

Tetracycline

A 500 x stock of 5 mg/ml of tetracycline was dissolved in 50% ethanol.

Antibiotic stock solutions were stored at -20°C.

Colour selection

Where blue/white colour selection was available in cloning vectors, X-Gal (5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside) to a final concentration of 40 μg ml^{-1}, from a freshly made solution of 20 mg ml^{-1} made in dimethyl formamide, and IPTG (isopropylthio-β-D-galactoside) to a final concentration of 100 μM, from a stock of 100 mM in H_2O, were added to media.

Growth conditions

All bacteria were grown at 37°C, either on solid medium or in liquid medium in a shaking incubator.

Storage

Bacteria were stored on LB agar plates for periods up to 4 weeks. Long term storage was in LB medium containing 20% (v/v) glycerol frozen at -70°C. Bacteria were revived from long term storage by removing a small stab from the frozen culture, and streaking out onto LB medium with appropriate antibiotic.

iii Plasmid and cosmid preparation

Small scale plasmid and cosmids preparations

Minipreparations of plasmid DNA were performed for screening of large numbers of transformant colonies, and were obtained by the boiling method (Sambrook *et al*, 1989). When more pure preparations were required, Magic DNA Clean up system (Promega A7390) was used. This system incorporates a column purification step and gives DNA pure enough for subsequent ligation reactions and for yeast transformations. Both methods produced 2-5 μg of plasmid or cosmid DNA per 1-3 ml of *E.coli* culture, incubated overnight.

The following procedure describes the boiling method:

1. Single colonies were inoculated into 5 ml of LB with selective antibiotic, and grown overnight shaking at 37°C.
2. 1.5 ml of each cultures was transferred to Eppendorf tubes and cells pelleted by centrifugation at high speed for 2-4 minutes

3. Supernatants were removed, and each pellet re-suspended in 200 µl of STET[1]; suspensions were transferred to Eppendorf tubes and incubated on ice for about 5 minutes.
4. A small hole was pierced in the lid of each Eppendorf tube to stop them opening when boiled at the next stage.
5. Eppendorf tubes were plunged into boiling water for 45 seconds, cooled on ice for 30 seconds, and centrifuged at high speed for 10 minutes.
5. Gelatinous pellets were removed with a tooth-pick from each tube.
6. All remaining volumes were made up to 200 µl with STET, and 200 µl of isopropanol added, mixed, and incubated at -20°C for 20 minutes.
7. Solutions were centrifuged at high speed for 10 minutes, supernatants discarded, and pellets were vacuum dried, washed in 70% ethanol and resuspended in 50 µl of TE. Plasmid preparations were frozen at -20°C for storage.

1 STET:

		stock	1 l
sucrose	8%		80 g
Triton X-100	5%		50 ml
EDTA	5 mM	0.5 M	10 ml
Tris-HCl	50 mM	1 M	50 ml.

Medium scale plasmid preparation

This method, based on that of Birnboim and Doly (1979), produced ~ 1 µg/µl amounts of plasmid DNA, which was used for all common molecular biology techniques.

1. Single colonies were inoculated into 100 ml of LB with appropriate antibiotic, and grown overnight shaking at 37°C.
2. Cultures were aliquoted into two 50 ml screw cap tubes (Falcon 7050) and cells pelleted by centrifugation at 10,000 rpm for 5 minutes.
3. Pellets were carefully resuspended in a final volume of 3 ml of

TESL[1] by gentle pipetting, and incubated on ice water 20 minutes.

4. 6 ml of NS[2] was added, mixed carefully by inversion, and incubated on ice for 10 minutes.
5. Cells were pelleted by centrifugation at 15,000 rpm for 15 minutes, in a swing-out rotor, and the supernatant carefully removed; this was transferred to another 50 ml screw cap tube, 50 µl of RNAase[3] added and incubated for 20 minutes at 37°C.
6. Supernatants were phenol/chloroform extracted twice (Section 7.2 ii).
7. Two volumes of ice cold ethanol were added to precipitate DNA, and DNA pelleted by centrifugation at 10,000 rpm for 10 minutes.
8. Supernatants were discarded, and pellets dissolved in 1.6 ml of H_20; 400 µl of 4 M NaCl added and mixed; 2 ml of 13% (w/v) PEG 6000 added, mixed, and the tubes incubated on ice water for 60 minutes.
9. DNA was pelleted by centrifugation at 10,000 rpm for 10 minutes, supernatants were discarded, washed pellets with 70% ethanol, pellets dried and dissolved in 100 µl of TE.

1 TES:

	stock	100 ml
25 mM Tris. Cl (pH 8.0)	1 M	2.5 ml
10 mM EDTA	0.5 M	2 ml
15% sucrose		15 g

2 mg/ml lysosyme (added before use).

2 NS:

	stock	100 ml
0.2 M NaOH		800 mg
1% SDS	20% (w/v)	5 ml.

3 RNAse (Bovine pancreatic, Sigma R-5503)
Dissolved at a concentration of 10 mg/ml in Tris. Cl (pH 7.5) and 15 mM NaCl. Incubated at 100°C for 15 minutes , and then allowed to cool slowly to room temperature. Stock was stored at -20°C.

iv Small scale bacteriophage P1 vector preparation

The method used is that of Pierce and Sternberg (1992). It includes a step for activating the lytic replicon in the P1 vector, and is otherwise based on the alkaline lysis procedure. This method produced about 1-2 μg of P1 plasmid DNA. It can be scaled up to 250 ml with the expected increase in DNA recovery.

1. Single colonies were picked from agar plates and inoculated into 10 ml of LB medium containing 25 μg/ml kanamycin. The culture was shaken at 37°C until it appeared slightly turbid (OD_{590} 0.1), then IPTG was added to a final concentration of 1mM, and growth continued for an additional 5-12 hours.
2. The cells were centrifuged for 15 minutes at 3000 rpm and the pellet was resuspended in 150 μl of solution A[1] containing 2 mg/ml lysosyme. The cell suspension was incubated at room temperature for 10 minutes, and transferred into Eppendorf tubes.
3. To the cell suspension, 300 μl of a freshly prepared solution B[2] were added. The tubes were gently inverted several times and incubated on ice for 10 minutes.
5. 235 μl of potassium acetate (solution C[3]) were added and mixed in by inverting the tube several times. Incubation then continued for further 10 minutes.
6. The lysate was centrifuged at high speed at room temperature for 10 minutes and the supernatant was transferred into a fresh Eppendorf tube.
7. The supernatant was phenol/chloroform extracted and ethanol precipitated (section 7.2 iii). The ethanol pellet was washed with 70% ethanol and resuspended in 40 μl TE buffer.
8. Routinely, 12 μl out of the total 40 μl preparation was used in restriction digest reactions. When the digest reaction was completed, loading buffer was added and the digest reaction was incubated at 70°C for 10 minutes, before loading onto the gel.

1 Solution A:
50 mM glucose
25 mM Tris-HCl
10 mM EDTA pH 8.0

2 Solution B:

0.2 N NaOH

1% SDS

3 Solution C:

5 M potasium acetate	60 ml
glacial acetic acid	11.5 ml
H_2O	28.5 ml

iv Competent cells and transformation

This method is based on that of Hanahan (1983), and produced transformation frequencies of up to 10^8 colonies per µg of plasmid DNA.

Competent cells

1. Pre-cultures were grown in LB overnight shaking at 37°C.
2. 200 ml of pre-culture was inoculated into 20 ml φ broth[1] and grown at 37°C shaking to an OD_{550} of 0.25: this usually took about 2 hours.
3. 5 ml of cells were inoculated into 20 ml ψ broth, and grown to an OD_{550} of 0.48.
4. Cultures were cooled briefly on ice, and cells pelleted by centrifugation for 5 minutes at 9000 rpm at 4°C.
5. Pellets were resuspended in 33 ml of ice cold TfBI[2], and incubated on ice for 15 minutes.
6. Cells were pelleted again by centrifugation for 5 minutes at 9000 rpm at 4°C.
7. Pellets were resuspended in 4 ml of ice cold TfBII[3] and incubated on ice for 20 minutes.
8. 200 µl of cells aliquoted into ice-cold Eppendorf tubes, snap frozen on solid CO_2, and stored at -70°C.

1 ψ broth was made of 10 ml of salts added to 490 ml tryptone/yeast extract. 10 g of tryptone and 2.5 g of yeast

extract were dissolved in 490 ml of H_20, and autoclaved.

Salts:

	100 ml
1M $MgSO_4$. 7 H_2O	24.6 g
0.5 M NaCl	2.9 g
1.25 M KCl	9.3 g

and filter sterilized.

2 TfBI:

	500 ml
NaOAc	1.43 g
$CaCl_2$. 6 H_2O	1.1 g
glycerol	75 g

and pH to 5.9 with HOAc.

Added solid:

RbCl	6.0 g
$MnCl_2$	4.95 g

and autoclaved in 50 ml aliquots.

3 TfBII:

	100 ml
MOPS	209 mg
$RbCl_2$	120 mg
$CaCl_2$. 6 H_2O	1.64 g
glycerol	15 g

pH to 6.8 with KOH and autoclaved in 10 ml aliquots.

Transformation

1. Cells were thawed on ice for 10-15 minutes.
2. DNA was added in a minimum volume - less than 10 µl.
3. Cells were incubated on ice for 30 minutes.
4. Cells were heat-shocked in a 42°C water bath for 60 seconds.
5. 400 µl of LB was added and incubated at 37°C for 60 minutes.
6. 250 µl of cells were spread onto appropriate plates.

v Production of single-stranded DNA

Single-stranded DNA (ssDNA) was produced using helper phage M13KO7, following a modified method of Vieira and Messing (1987). This method yielded about 5 μg of ssDNA template, suitable for dideoxy sequencing.

Cultures and infections

1. Single transformant colonies in *E. coli* strains were streaked onto appropriate selective plates to enrich for the F9 episome (minimal plates for JM101 strains; tetracycline containing plates for XL1-Blue strains).
2. Single colonies were inoculated into 5 ml LB with appropriate antibiotic(s), and incubated overnight at 37°C.
3. 400 ml of culture were inoculated into 10 ml of 2XYT in a 50 ml screw cap tube (Falcon 7020), and incubated shaking at 37°C.
4. Cultures were monitored until OD_{660} was between 0.5-0.8 (this usually took between 2 and 2.5 hours).
5. Cultures were infected with M13KO7 (Bio-Rad 1703578) at a multiplicity of infection of 10. Infected cultures were shaken at 37°C for further 60 minutes.
6. 200 μl of each culture were added to 10 ml of 2XYT containing kanamycin at a final concentration of 70 μg/μl. The cultures were then grown overnight at 37°C.

Preparation of ssDNA

7. Cultures were poured into 30 ml Corex tubes and centrifuged for 5 minutes at 9,000 rpm; supernatants were decanted into fresh 30 ml Corex tubes, and the spin repeated.
8. 14 ml of each supernatant were removed into fresh 30 ml Corex tubes and 2.1 ml of 20% PEG 6000/2.5 M NaCl added. Phage particles were allowed to precipitate on ice for 30 minutes.
9. Phage particles were pelleted by centrifugation at 12,000 rpm for 10 minutes.
10. Supernatant was discarded, and the pellet was re-centrifuged briefly to remove residual liquid.

11. Pellets were resuspended in 500 µl of: 0.3M sodium acetate pH 6.0/1mM EDTA, and removed to Ependorff tubes
12. Each solution was phenol/chloroform extracted 2-3 times, the final aqueous phase removed to a fresh tube and 1 ml of ice cold ethanol added. DNA precipitation was followed as described in section 7.2 iii, and pellets were resuspended in 10 µl of TE.
13. 0.5-1 µl of the resuspended pellets were run on an agarose gel in parallel with ssDNA from the Pharmacia T7 sequencing kit (27-1682-01) to assay the amount of the ssDNA.

7.5 Fission yeast manipulations

i Strains

The wild type strain and mutant strains of the fission yeast *Schizosaccharomyces pombe* Linder, were all derived from the haploid heterothallic 972h^- and 975 h^+ isolates described by Leupold (1950).

In the following list the genotypes and catalogue numbers of strains used in this work are indicated. Asterisks indicate strains constructed in this work; X indicate undetermined allele.

Wild-type strains

ED878 975 h^+

ED812 972 h^-

Cell cycle mutants

ED566 *win1-1 wee1-50 cdc25-22 leu1-32* h^-

ED623 *win1-1 leu1-32* h^+

ED626 *wee1-50 cdc25-22 leu1-32* h^-

ED628 *cdc2-33 leu1-32 ura4-D18* h^-

ED631 *win1-1* h^-

ED677 *win1-1 wee1-50 cdc25-22 leu1-32* h^+

ED678 *win1-1 leu1-32 ura4-D18 ade6-M216* h^-

ED684 *cdc13-117 leu1-32* h^-

ED716 *cdc13-117* h^-

ED803 *win1-1 tps19-17 leu1-32* h^+

ED804 *win1-1 rad1-1 leu1-32 hX*

ED814 *cdc25-22 leu1-32* h^-

ED835 *wee1-50 leu1-32* h^-

ED856 *wee1-50 mik1::ura4* $^+$*leu1-32 ura4-D18 ade6-M210* h^+

ED866 *cdc25-22 leu1-32 uraD18* h^-

ED888 *pyp3::ura4*$^+$ *leu1-32 ura4-D18* h^-

ED890 *wis1::LEU2 leu1-32 ura4-D18 ade6-M216* h^-

ED909 *wee1-50 leu1-32 ura4-D18* h^-

ED918 *cdc13-117 ura4-294 leu1-32* h^-

ED947 *wis1::his1*$^+$ *his1-102 ade6-M216 ura4-D18* h^+

* *win1-1 tps19-17 leu1-32 ura4-D18* h^+

* *win1-1 tps19-17 leu1-32 h⁻[pwis2-5C, ura4⁺] h⁺*
* *win1-1 rad1-1 hX*

Others
ED624 *leu1-32 h⁻*
ED665 *leu1-32 ura4-D18 ade6-M210 h⁻*
ED666 *leu1-32 ura4-D18 ade6-M210 h⁺*
ED741 *tps19-17 h⁻*
ED804 *rad1-1 h⁻*
ED862 *leu1-32 ura4-294 h⁺*
* *ura4-D18 h⁻*

wis2 disruptant strain
*ED912 *wis2::ura4⁺ leu1-32 ura4-D18 ade6-M216 h⁺*
* *wis2::ura4⁺ leu1-32 ura4-D18 ade6-M216 h⁻*
* *cdc25-22 wis2::ura4⁺ leu1-32 ura4-D18 hX*
* *wee1-50 wis2::ura4⁺ leu1-32 ura4-D18 hX*
* *cdc2-33 wis2::ura4⁺ leu1-32 ura4-D18 hX*
* *win1-1 wis2::ura4⁺ leu1-32 ura4-D18 ade6-M216 hX*
* *cdc23-117 wis2::ura4⁺ leu1-32 ura4-D18 hX*
* *wis2::ura4⁺ leu1-32 ura4-D18 ade6-M216 hX*
* *wis2::ura4⁺ leu1-32 ura4-D18 ade6-M216 hX*

ii Media and growth conditions

YE

Strains of fission yeast were routinely grown on solid YE complex medium supplemented with adenine and uracil.

YE contains per litre:

glucose	30 g
yeast extract	5 g
adenine	75 mg
uracil	75 mg
Difco-Bacto agar	20 g

For complex liquid medium YE was used minus Difco-Bacto agar.

EMM

EMM (Moreno *et al*., 1991) was used as minimal medium, and is a modification of EMM2 (Mitchison 1970; Nurse, 1975), and contains the following per litre:

glucose	20 g
KH phthalate	3 g
Na_2HPO_4	1.8 g
NH_4Cl	5 g
$NaSO_4$	100 mg
$CaCl_2$	15 mg
$MgCl_2$	1 g
vitamins	1 ml
minerals	100 µl.

Vitamins: 5 g inositol, 5g nicotinic acid, 0.5 g calcium pantothenate and 5 g biotin, all dissolved in 500 ml H_2O.

Minerals: H_3BO_3 1 g, $MnSO_4$. 4 H_2O, 1.04 g, $ZnSO_4$. 7 H_20 800 mg, $FeCl_3$. 6 H_2O 400 mg, H_2MoO_4 288 mg, $CuSO_4$. 5 H_2O 80 mg, Citric acid 2 g, and KI 20 mg, all dissolved in 200 ml H_2O.

Amino acids, leucine, adenine or uracil, were added when appropriate at 75 mg/liter.

EMM/sorbitol

1.2 M sorbitol was included in EMM solid media for the regeneration of osmotically sensitive spheroplasts.

ME

For the induction of conjugation and consequent sporulation, nitrogen limiting medium ME, consisting of 30 g/l malt extract and 20 g/l agar, was used.

Phloxin B

To check ploidy of cells, phloxin B was added, from a stock solution of 10 mg/ml, to a final concentration of 20 µg/ml in solid media. Diploid cells die faster than haploid cells and accumulate the dye more quickly, and therefore can be differentiated by relative colour staining.

Storage

Short term storage of fission yeast (up to 4 weeks) was on solid medium at 4°C. Long term storage was in medium containing 15% (v/v) glycerol at –70°C.

iii Genetical analysis

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 (mating types h^+ and h^-), on the surface of an ME plate. The mating mix was incubated at 25°C for 2-3 days to allow zygotes to form. The progeny of the crosses were examined either by random spore analysis or by tetrad analysis.

Random spore analysis

A loopful of mating mix was suspended in 1 ml of sterile distilled water containing 20 µl of a stock solution of the snail gut enzyme (Suc d'Helix pomatia, Industrie Biologique, France) and incubated overnight at 35°C. The stock solution is a 1:10 dilution of the preparation supplied, which is stored at 4°C. The spore concentration was estimated using a haemocytometer and an appropriate dilution, to give about 200 spores per plate, spread onto solid YE media.

Tetrad Analysis

Single asci were isolated from a streak of the mating mix on a YE 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 to breakdown. The spores were then separated on the surface of the plate with the micromanipulator and allowed to form colonies.

Analysis of phenotypes

Phenotypes of cells were tested by replica plating or by streaking, from a master plate onto EMM plus or minus growth supplements for auxotrophs; and onto YE at the restrictive and permissive temperatures, for temperature-sensitive strains.

Diploid construction

For sporulation competent diploid strain, h^+/h^- strains were constructed using complementing alleles of *ade6*. The *ade6-M210* and *ade6-M216* both confer adenine requirement for growth, but heterozygous diploids *ade6-M210 ade6-M216* are prototrophic. The advantage of this system is that very little recombination occurs between these two loci, so that very few prototrophic haploids arise.

Strains carrying the *ade* mutated alleles were crossed in the normal way, left to conjugate overnight and than streaked onto selective media for adenine prototrophs. Diploid colonies were recognised by their colour on phloxin-containing plates, and their ability to sporulate checked microscopically.

iv Cell physiology

Growth of liquid cultures

Exponentially growing cells were obtained by inoculating a single colony into a 10 ml YE pre-culture and incubating until the cells had entered the stationary phase of growth (1-2 days). An aliquot of the pre-culture (usually to give $^1/_{100}$ dilution) was inoculated into 200 ml of EMM in a 500 ml conical flask , and incubated shaking for 14-18 hours at 28°C or 32°C, giving a density of 3-8 x 10^6 cells/ml.

Estimation of cell number

Cell number was estimated using a Coulter counter (Industrial D). A 100 µl sample of cell culture was mixed in 10 ml of ISOTON solution and sonicated for 5 seconds at setting 2 on a Lucas Soniprobe Sonicator. Usually, 2 counts of 500 µl volume were taken, and combined to give a cell count per ml.

The optical density (OD) of a culture was used to measure the approximate concentration of cells. The OD reading is approximate as the reading of cells of different strains is affected by cell length and cell composition. For wild type strains an OD595 = 0.1 is roughly equivalent to 2 x 10^6 cells/ml.

Cyclosporin A treatment

Cyclosporin A (CsA) is a fungal natural product, used as immunosuprressant drug (section 1.4). It is a cyclic undecapeptide with low solubility in water. CsA was a gift of Sandoz Pharma. For stock solution 1 mg of CsA was dissolved in absolute ethanol/0.1% tween 20; filter sterelised and kept in a glass container at 4°C. CsA was added to a final concentration of 50-400 µg/ml in liquid or agar containing media.

v Transformation methods and stability test

Protoplast transformation

The method used for fission yeast transformation was essentially that of Beach & Nurse (1981), and gave up to 1 - 2 $x10^4$ transformant colonies per µg of plasmid DNA.

1. Cells were grown in 200 ml of EMM with appropriate supplements to a density of approximately 0.5 - 1 $x10^7$ cells /ml
2. The culture was transferred to a 250 ml Beckman bottles and pelleted by centrifugation at 5000 rpm for 10 minutes.
3. The supernatant was discarded and cells resuspended in 10 ml of CPE[1] and transferred to a 50 ml screw cap tube (Falcon 2070) .
4. Cells were pelleted, and the supernatant discarded.
5. The pellet was resuspended in 5 ml of CPS[2] and incubated at 37°C.
6. After 20 minutes, 5 µl of cells were removed to check spheroplast formation under the microscope. When sample contained approximately 50-75% spherical protoplasts, the next stage was started. If the cells had not spheroplasted the incubation was continued and the cells checked every ten minutes.
7. 25 ml of TS[3] was added. Protoplasts were mixed gently and centrifuged at 2000 rpm for 4 minutes.
8. The protoplasts were washed two more times in TS[3].
9. Cells were finally resuspended at a density of 2.5 x 10^8 cells/ml in TCS[4] (TS solution containing 10 mM $CaCl_2$).
11. 100 µl aliquots of cell suspension were transferred to Eppendorf tubes. Transforming DNA (1- 5 µg) in a volume of not more than 10 µl was added, mixed gently, and protoplasts incubated at room temperature for 15 minutes.

12. 1 ml of TCP[5] was added and cells incubated at room temperature for further 15 minutes.
13. Cells were pelleted by centrifugation at low speed in a microcentrifuge (6500 rpm), and supernatants discarded.
14. Cells were resuspended in 500 µl plating medium, PM[6].
15. Cells were incubated for 60 minutes at permissive temperature, and 250 µl spread gently onto each of two EMM/1.2 M sorbitol plates.
16. Colonies appeared in four to six days at 28°C.

1 CPE:

20 mM	citrate-phosphate buffer pH 5.6
40 mM	EDTA
0.1% (v/v)	β-mercaptoethanol (added before use).

2 CPS:

50 mM	citrate-phosphate buffer pH 5.6
1.2 M	sorbitol
0.1% (v/v)	β-mercaptoethanol (added before use)
2 mg/ml	Novozyme 234 (Novo Industries, Bagsvaerd, Denmark 69-289-01) (added before use).

3 TS:

10 mM	Tris. Cl pH 7.6
2 mg/ml	1.2 M sorbitol

4 TCS:

10 mM	Tris. Cl pH 7.6
10 mM	$CaCl_2$
1.2 M	sorbitol.

5 TCP:

10 mM	Tris. Cl pH 7.6
10 mM	$CaCl_2$
20%	PEG-4000.

6 PM:

1.2M	sorbitol
10 mM	Tris- HCl pH 7.6
0.5 mg/ml	YE
0.5 mg/ml	Amino acid supplements, as required.

Transformation by electroporation

Transformation by electroporation was performed according to Prentice (1991). It achieves high transformation efficiency (10^4 to 10^5 colonies per μg DNA) and compared with the protoplast transformation method it requires less time, and no special media.

1. Cells were grown in 200 ml of EMM with appropriate supplements to a density of approximately 0.5 - 1 x10^7 cells/ml.
2. Cells were transferred to a 250 ml Beckman bottle and pelleted by centrifugation at 5000 rpm for 10 minutes.
3. Cells were washed three times in 10 ml ice cold autoclaved 1.2 M sorbitol.
4. Cell were resuspended in 1.2 M sorbitol to a concentration of 1×10^9 cells/ml.
5. Aliquots of 0.2 ml of the cell suspension were mixed with 0.1-2 μg of DNA and immediately transferred to ice cold cuvettes.
6. Cells were pulsed at 2.25 kV (11.25 kV/cm), 200 Ω and 25 μF. Immediately after the pulse 0.5 ml of ice cold 1.2 M sorbitol was added to the cuvette.
7. Electroporated cells were diluted with 1 ml of 1.2 M sorbitol, and 0.5 ml were spread on very dry selective media.
8. Colonies appeared in four to six days at 28°C.

Stability and co-segregation test

The procedure described below is useful for distinguishing between a an autonomously replicating plasmid and an integration event. It is also used to determine co-segregation of genetic markers.

The rationale of the procedure is that if a plasmid replicates autonomously it will be lost in the absence of selection. On the other hand if the plasmid has integrated, or there has been a reversion or gene

conversion event, the phenotype will be maintained after relaxing the selection.

1. A transformant colony was streaked out to single colonies on YE plate, YE(1). Colonies were grown under no selection for about 3 days.
2. YE(1) was replica plated to selective media, e.g. to YE plates at restrictive temperature or to minimal plates at permissive temperature. Alternatively, 20-50 single colonies of YE(1) were picked and allowed to grow overnight on a second YE plate, YE(2), with no selection; then YE(2) was replicated to the appropriate selective media.
3. The instability of a selective marker or the co-instability of selective markers were scored.

vi Preparation of *S.pombe* chromosomal DNA

S.pombe chromosomal DNA was prepared as described in Durkacz et al. (1985). This method yields DNA of sufficient quantity and quality for variety of purposes such as Southern blotting, plasmid recovery or PCR amplification. This method can be scaled up if required.

1. 10 ml cultures of media were inoculated with a single yeast colony, and incubated until the culture reached stationary phase (1-2 days).
2. Cells were harvested for 5 minutes at 2500 rpm in 15 ml centrifuge tubes (Falcon 2095).
3. Supernatants were discarded and cells resuspended in 1.5 ml CPS[1] containing 2.5 mg/ml zymolyase 20 T (Seikaguku Kogyo 120491). The suspensions were transferred to Eppendorf tubes.
4. Cells were incubated at 37°C for 60-80 minutes.
5. Cells were harvested by centrifugation at high speed for 2 minutes.
6. Supernatants were discarded and cells resuspended in 300 µl of 5 x TE. 35 µl of 10% SDS (w/v) was added and the mixture incubated at 65°C for 5 minutes.
7. 100 µl of 5 M potassium acetate (pH 5.6) was added and the mixture incubated on ice for 30 minutes.

8. Centrifuged at 4°C at high speed for 15 minutes: the supernatant was removed, and 1 ml ice-cold 95% ethanol was added and mixed.
9. Centrifuged at high speed at 4°C for 10 minutes.
10. Supernatant was discarded and pellet resuspended in 400 µl of 5 x TE containing 100 µg/ml RNAase (bovine pancreatic, Sigma R-5000); then incubated for 2-4 hours at 37°C.
11. Suspension was extracted once each with an equal volume of phenol, phenol/chloroform, and chloroform.
12. The final aqueous phase was transferred to another Eppendorf tube, sodium acetate precipitated, and washed with 70% ethanol. Pellets were dried, dissolved in 100 µl of TE

1 CPS:

50 mM	citrate-phosphate buffer pH 5.6
1.2 M	sorbitol
0.1% (v/v)	β-mercaptoethanol (added before use)
2 mg/ml	Novozyme 234 (Novo Industries, Bagsvaerd, Denmark 69-289-01).

vii Preparation of *S.pombe* total RNA

This method, based on that of Kaufer et al.. (1985), yields *S.pombe* total RNA of good quality, and was used for Northern blotting .

Gloves were worn during all RNA procedures to prevent RNAase contamination.

1. Cells were grown in 200 ml of EMM with any appropriate supplements to a density of approximately 0.5 - 1 $x10^7$ cells /ml, and harvested by centrifugation at 5000 rpm for 10 minutes in four 50 ml screw cap tubes (Falcon 7020).
2. Each cell pellet was resuspended in 1 ml of sterile saline[1] and transferred to an Eppendorf tube; cells were pelleted by centrifugation at high speed, and the supernatant discarded (cells could be stored at -70°C at this stage).
3. Cells were resuspended in 75 µl of STE[2].

4. Acid washed beads (425-600 micron, Sigma G-9268; treated with concentrated hydrochloric acid) were added to just beneath meniscus, and the cells briefly vortexed.
5. 600 µl of NTES[3] was added, and the mixture briefly vortexed again,
6. 500 µl of hot phenol (65°C) was added, and the mixture immediately vortexed.
7. The mixture was incubated at 65°C for 5 minutes with frequent vortexing.
8. The mixture was centrifuged for 1 minute at high speed, and the aqueous phase and protein interface were removed to a second 500 µl aliquot of hot phenol.
9. Incubated at 65°C for 2 minutes with frequent vortexing.
10. Centrifuged for 1 minute at high speed, and again the aqueous phase and interface were removed to a third 400 µl aliquot of hot phenol.
11. Incubated at 65°C for 2 minutes with frequent vortexing.
12. Aqueous phase only was removed into 400 µl of phenol/chloroform at room temperature, vortexed, and spun at high speed for 1 minute.
13. Aqueous phase was re-extracted with 300 µl of chloroform, vortexed, and spun for one minute at high speed.
14. Aqueous phase was sodium acetate precipitated (section 7.2 iii).
15. Supernatant was discarded and the RNA pellet washed with 70% ethanol in DEPC H_2O, and briefly dried.
16. RNA pellet was resuspended in 55 µl of ice cold DEPC H_2O and was stored at -20°C.

17. 5 µl of each sample was used to estimate amount and quality by spectrophotometry (Section 7.2. iv).

1 Sterile saline:

0.9% (W/V)	NaCl, autoclaved.

2 STE:

0.32 M	sucrose
20 mM	Tris. Cl (pH 7.5)

10 mM	EDTA (pH 8.0)
0.5 mg/ml	heparin (Sigma H-7005) added solid just prior to use.

3 NTES:

100	mM NaCl
5 mM	EDTA
50 mM	Tris. Cl (pH 7.5)
1% (w/v)	SDS
0.5 mg/ml	heparin added solid just prior to use.

viii Preparation of *S.pombe* denatured protein extract

This method is described by Moreno et al.. (1991). It yields *S.pombe* total protein extract of sufficient quantity and quality for Western blotting.

1. Cells were grown in 200 ml of YE or EMM with appropriate supplements to a density of approximately 0.5 - 1 $x10^7$ cells/ml.
2. Cells were harvested by centrifugation in four 50 ml screw cap tubes (Falcon 2070) at 2000 rpm for 10 minutes.
3. Supernatant was discarded and pellet resuspended in 10 ml of ice cold Stop buffer[1]. The suspension was transferred to heat resistant 12 ml Greiner tubes and spun down by centrifugation at 2000 rpm for 5 minutes. The pellet was spun again briefly and residual liquid was removed.
4. The pellet was resuspended in 50 µl of RIPA[2] and mixed thoroughly.
5. 3 grams of acid washed glass beads (425-600 micron, Sigma G-9268) were added. Cells were broken by vigorous vortexing: alternating 1 minute of vortexing with 1 minute of incubation on ice, for four times. Cell breakage was monitored microscopically.
6. 100 µl of 1% SDS were added, and tubes plunged into boiling water for 3 minutes. A small hole was pierced in the lid of each tube to stop them opening when boiled.
7. 1 ml of RIPA was added, and tubes vortexed briefly.
8. Liquid was drawn up using a Pasteur pipette and transferred to Eppendorf tubes.

9. The extracts was centrifuged at high speed at 4°C and the supernatant was transferred to a fresh test tube.
10. 5-10 µl of the protein extract were used for estimation of protein concentration[3]. Equivalent volume of X2 loading buffer was added to half of the protein extract (section 7.3 xii) and the tubes plunged into boiling water for 4 minutes. Protein extracts, with or without loading buffer were stored frozen (-20°C).

1 Stop buffer:
150 mM NaCl
50 mM NaF
10 mM EDTA
1 mM NaN_3 pH 8

2 RIPA
10 mM sodium phosphate, pH 7
1 % Triton X-100
0.1 % SDS
2 mM sodium vandate
4 µg/ml leupeptin
1 mM PMSF

3 Protein concentration was estimated using the Pierce BCA Protein Assay kit (23225).

7.6 DNA sequencing

Determination of the DNA sequence of the *S.pombe wis2*$^+$ gene involved the preparation of suitable DNA templates and the performance of sequencing reactions. DNA subclones that allow sequencing reactions with limited number of synthetic oligonucleotides were designed by the combination of nested deletion subclones and direct subcloning. In addition, three synthetic oligonucleotides which hybridise to *wis2*$^+$ sequences were designed.

Sequence reactions were performed using the method based on the chain termination sequencing method of Sanger *et al.*. (1977).

i Double-stranded nested deletions

A series of unidirectional nested deletions of *wis2*$^+$ subclone were made, using the Pharmacia Deletion kit, designed on the basis of the method described by Henikoff (1984).

Nested deletions were performed using pwis2-1 subclone in pTZ18R vector. pTZ18R contains a DNA sequence adjacent to the cloning site which allows sequencing reactions with the commercially available Reverse primer. To create a linearized molecule with resistant and sensitive ends to Exo III, 5 μg of pTZ/wis2-1 were cut by the *Bgl*II and *Kpn*I restriction endonucleases (section 2.2; Figure 2.2). The resistant end digestion (*Kpn*I) was completed first, and monitored by gel electrophoresis to ensure completion of digest. The sensitive end digestion (*Bgl*II) was then performed. Both enzymes were inactivated by freezing at -20°C.

In order to obtain 300 nucleotide spaced deletions, ExoIII digestion was performed at 30°C in 75 mM NaCl and samples were removed at 3 minute intervals.

Analysis of samples from each time point was carried out by gel electrophoresis, using half of each sample. The remaining half of each timed sample was used for re-ligation. The extent of deletion in each nuclease-treated sample was judged by comparing its mobility with that of two control samples: recombinant DNA linearized but not subjected to the deletion reaction, and linearized vector containing no insert. Timed samples that contained deletions of interest were used to transform the *E.coli* strain JM101.

Small scale plasmid DNA preparations were prepared from 10

transformant colonies from each time point, and the size of resultant inserts estimated. Initially, an *Eco*RI digestion was performed to ensure presence of the reverse sequencing primer site, adjacent to the polylinker. Those clones containing the primer site were then double digested with *Eco*RI and another restriction enzyme to release the DNA insert fragment which had been deleted by Exo III. Restriction enzymes cutting at sites progressively further into the clones were used as they produced small DNA fragments, which could be accurately sized.

ii DNA templates for sequencing reactions

Single-stranded DNA templates of good purity for sequencing reactions were obtained by the method described in section 7.4 vi.

In addition, double-stranded DNA templates were directly used for sequencing reactions. Prior to DNA sequencing the double-stranded DNA was treated by the alkaline denaturing method as follows:

1. The concentration of RNA free plasmid DNA preparation was adjusted so that 32 µl contained 1.5-2 µg.
2. 8 µl 2 M NaOH were added, and the tube vortexed gently, then centrifuged briefly and incubated at room temperature for 10 minutes.
3. 7 µl of 3M sodium acetate (pH 4.8) and 4 µl of distilled water were added.
4. 120 µl absolute ethanol was added, mixed, and place on dry ice. The precipitated DNA was collected by centrifuging and the pellet washed and dried as described in section 7.2 iii.

iii Sequence reactions

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

The chain termination method involves the synthesis of a DNA strand by DNA polymerase *in vitro* using a single stranded DNA template. The synthesis reaction is terminated by the incorporation of a nucleotide analogues, 2', 3' -dideoxynucleoside 5' -tri phosphates (ddNTPs), that will

not support continued DNA elongation. Four separate reactions with different ddNTPs give complete sequence information. A radioactivity labelled nucleotide is also included in the synthesis, so that the labelled chains of various length can be visualised by autobiography after separation by high resolution electrophoresis.

For each sequencing reaction 1.5-3 μg of single-stranded or double-stranded DNA were used. Priming of the sequencing reactions was performed using a variety of synthetic oligonucleotides. These include the commercially available synthetic oligonucleotides, which anneal to commonly used plasmid cloning vectors: the Universal primer (supplied with the kit) and the Reverse primer (Amersham, NH512). Specific oligonucleotides, designed on the basis of sequencing data of *wis2-1*, were obtained from the Oswel DNA service, University of Edinburgh. 1-2 pmol and 5-10 pmol of the appropriate primer were used for ssDNA and double-stranded DNA sequencing, respectively.

The sequences of these oligonucleotides are listed below, indicating the position of the furthermost 5' end oligonucleotide relative to the putative ATG initiator codon, subsequently assigned to *wis2*$^+$. All oligonucleotides comprise of sequences of the predicted non coding strand of *wis2*$^+$.

S1	5'-CACTGCCAGTCTCCTCAG-3'	*wis2*	-45
S2	5'-TGAAACGCAAACGTAGGC-3'	*wis2*	+1267
S3	5'-GCTTTCTGTTTCTCTAACT-3'	*wis2*	+895

iv Denaturing gel electrophoresis

Electrophoresis was carried out using the BRL Model S2 sequencing gel electrophoresis system. Routinely, between 300-400 nucleotides of each sequencing reaction could be clearly determined.

1. Glass plates were cleaned with H_2O and ethanol, treated with trimethylchlorosilane, separated by spacers, and sealed with tape.

2. To 100 ml of sequencing mix[1], 1 ml of freshly prepared 10% (w/v) ammonium persulphate and 25 µl of TEMED (N,N,N',N'-tetramethylethylenediamine) were added, the solution mixed, and immediately poured.
3. Loading wells were formed using 'shark's-tooth' combs.
4. Gel were pre-run at a constant 43 Watts for 15-30 minutes in 1 x TBE buffer, the wells flushed out with 1 x TBE buffer, and samples loaded after incubation at 75-80°C for 3 minutes.
5. Gels were run at 43 Watts for 3-12 hours.
6. Glass plates were separated so that the gel remained attached to one plate.
7. Gels were transferred to a sheet of Whatman filter paper (3 MM), covered with SaranWrap, and dried on a vacuum gel dryer.
8. Gels were processed for autoradiography (Section 7.3 xi).

1 Sequencing mix:

40% acrylamide (19:1 acrylamide: bisacrylamide)	75 ml
10 x TBE	50 ml
urea	250 g
H_2O added for final volume of 500 ml.	

All reagents used were Gibco-BRL Ultra-Pure.

7.7 Oligonucleotide-directed *in vitro* mutagenesis

Oligonucleotide-directed *in vitro* mutagenesis of *S.pombe wis2*$^+$ and *cyp1*$^+$ genes was performed using the MutaGene Phagemid Kit (BioRad 170-3576). The kit is based on the method described by Kunkel (Kunkel 1985). The method provides a strong selection against the non-mutagenised strand of a double stranded DNA. When DNA is synthesised by a *dut ung* double mutant bacterium (CJ236 section 7.4 i) the nascent DNA carries a number of uracil residues in thymine positions as a result of the *dut* mutation which inactivates the enzyme dUTPase and results in high intracellular level of dUTP. The *ung* mutation inactivates uracil N glycosylase which allows the incorporated uracil to remain in the DNA. This uracil containing strand is then used as a template for the synthesis *in vitro* of a complementary strand, primed by an oligonucleotide containing the desired mutation. When the resulted double stranded DNA is transformed into a cell with proficient uracil N-glycolase, the uracil containing strand is inactivated with high efficiency, leaving the non-uracil-containing strand to replicate.

Oligonucleotide-directed *in vitro* mutagenesis was performed following the instructions of the manufacturer. Normally, mutagenesis frequencies obtained with the MutaGene Phagemid Kit were greater than 60%.

Synthetic oligonucleotides were purchased from Oswel DNA service, University of Edinburgh. Oligonucleotides designed for introduction of point mutations (alteration of 1-3 adjacent nucleotides) were of 24-33 nt long, allowing the pairing of about 12 bp with the wild type strand on both sides of the point mutation. The oligonucleotide used for deletion of 33 bp of *wis2*$^+$ sequence was 41 nt long and was HPLC purified.

The oligonucleotides used for *in vitro* mutagenesis are listed below: the introduced mutagenising nucleotides are underlined, an asterisk indicates the point of a gap of 33 bp in the deleting oligonucleotide.

		Gene	Purpose	Section
M1	5'-GGATGGAAAGC(C/T)TGTTGTTTTTGG C-3'	*wis2*	H129 -> L/P	4.3 i
M2	5'-CATGATAAGCCTTTCTTGCTT*TCTCA GTTCTTCATTACTAC-3'	*wis2*	Δ11	4.3 i
M3	5'-CATTGTTTTTTTTTGCACCATATGACT TAC-3'	*wis2*	Introduce *Nde*I	2.3 iii
M5	5'-GAAGAATGTGGTACCTGCACAAAGG ATC-3'	*wis2*	Introduce *Kpn*I	4.2 ii
M6	5'-CTTTCCACCGGTTCCGTTACCGCG-3'	*cyp1*	Remove *Kpn*I	4.2 ii

In addition, oligonucleotides for sequencing reactions were used to confirm, or screen for the introduction of the desired mutation. Routinely about 400 nucleotides covering the region mutagenised were sequenced. These oligonucleotides are listed below, indicating the position of the furthermost 5' end oligonucleotide relative to the putative ATG initiator codon of *wis2*$^+$ or *cyp1*$^+$.

		Gene	Position
MS1	5'-GAGAAGTTTGAGGACGAG-3'	*wis2*	+248
MS2	5'-GTGCTCTCTGCACCGGCC-3'	*cyp1*	+102
MS3	5'-CTGCGTAAAATCGCTATTC-3'	*wis2*	-115

7.8 Polymerase chain reactions

The polymerase chain reaction (PCR) was used for the amplification of *wis2* fragments (section 2.3 i - ii).

Amplification was performed over 30 cycles using 0.1 μg of either *S.pombe* genomic or plasmid DNA, using the GenAmp DNA reagent kit (Perkin Elmer Cetus N801-0055). The following cycles were performed:

94°C for 2 minutes	(denature)
55°C or 48°CC for 2 minutes	(annealing)
72°C for 4 minutes	(elongation)

and finishing with:

72°C for 5 minutes
25°C for 30 minutes.

Each reaction mixture, made up to the manufacturer's instructions, consisted of the following:

Template DNA	10 μl of 0.1 μg
primer 1	5 μl of 20 μM
primer 2	5 μl of 20 μM
Reaction Buffer[1]	10 μl
dNTP mix[2]	16 μl
H_2O	52.5 μl
Taq DNA polymerase	0.5 μl

To prevent evaporation each reaction mix was overlaid with 100 μl of light mineral oil (PCR Grade, Sigma M-3516).

1 Reaction buffer:
10 mM Tris-HCl (pH 8.3)
50 mM KCl
1.5 mM $MgCl_2$
0.01% gelatin.

2 dNTP mix:
Working solution of dNTP's was made up to give 1.25 mM of each dATP, dCTP, dGTP, and dTTP (Pharmacia 27-2035-01).

The sequences of synthetic oligonucleotides used for PCR are listed below, indicating the G+C and A+T content and the position of the furthermost 5' end oligonucleotide relative to the putative ATG initiator of *wis2*$^+$ or the ATG initiator of *nmt1*$^+$ (see also Figure 2.6A).

		Gene	Position	G+C	A+T
P1	5'-ACAGTATGTTTCTAGAAG-3'	*wis2*	-233	6	12
P2	5'-CACCACCTTTTTAATAACT-3'	*wis2*	-174	6	12
P3	5'-CTGCGTAAAATCGCTATTC-3'	*wis2*	-119	8	10
P4	3'-AGTTAGAGAAACAGAAAGC-5	*wis2*	+915	7	12
PREP3X	5'-GGAATCCTGGCATATCATCAATTG-3'	*nmt1*	-89	10	14

7.9 Screens of cosmid and P1 libraries by DNA hybridisation

The DNA hybridisation protocol for the *S.pombe* ordered cosmid and bacteriophage P1 genomic libraries (section 5.3 ii) was as described by Hoheisel et al.. (1993). Hybridisation were carried out in 0.5 M Na-phosphate pH 7.2/ 7% SDS/ 1 mM EDTA, at 65°C for 12-16 hours. The filters were briefly rinsed in 40 mM Na-phosphate pH 7.2/ 0.1% SDS, twice at room temperature and once at 65°C for 30 minutes.

Subsequently the filters were briefly dried, sealed in plastic bags and films exposed overnight at -70°C using intensifying screen.

7.10 Construction of cosmids mini libraries

The method for constructing cosmid mini-libraries is based on the method described by Rose and Broach 1991. Two different mini-libraries of cosmids #1 - #38 were constructed: a mini-library of complete *Bam*HI digest in pIRT2 and a mini-library of partial *Hin*dIII digest in pWH5.

pIRT2 and pWH5 vector DNA was prepared by medium scale plasmid preparation (section 7.4 iii). For each plasmid vector, 4 μg of DNA was digested with 10 units of the appropriate enzyme for 1 hour. Subsequent to extractions with phenol and chloroform, and a precipitation with ethanol, the vector DNA was dephosphorylated using calf intestinal phosphatase.

The insert DNA was prepared from a pool of cosmid DNA. For the *Bam*HI mini-library, a total of 2 μg cosmid DNA was cut with 10 units of enzyme at 37°C for 3 hours. A sample of the *Bam*HI digest was fractionated by size on agarose gel. This analysis showed that the majority of fragments were of the size of 12 - 15 kb. The cosmid *Bam*HI digest was phenol:chloroform extracted, ethanol precipitated and ligated with linearized pIRT2 DNA (total size of 6.5 kb). For the ligation, 0.36 μg cosmid DNA was ligated with 0.72 μg pIRT2 DNA in total of 40 μl ligation reaction (a molar ratio of 1 to 2 insert:vector DNA; total DNA concentration of 27 μg/ml).

For the *Hin*dIII mini-library, a total of 3 μg cosmid DNA was cut with 1 unit of enzyme at 37°C for 1 hour. Under such conditions, the cosmid DNA was only partially digested as shown by size fractionation of the above digest reaction along with a sample of a similar *Hin*dIII digest which was left for a longer incubation time. The sample of the partial digest reaction showed that the majority of restricted fragments were of the size of ~6 kb, whereas longer incubation resulted in majority of fragments of ~4 kb in size. For the ligation, 0.72 μg of partially restricted cosmid DNA was ligated with 0.5 μg linearized pWH5 (10.6 kb) in total of 40 μl ligation reaction (an approximate molar ratio of 1.5 to 1 insert:vector DNA; total DNA concentration of 30 μg/ml).

For both mini-libraries, ligation was performed using T_4 DNA

ligase (Boehringer Mannheim) in T_4 ligase buffer supplied by the manufacturer. Prior to adding the enzyme, the ligation mixture was heated to 37°C for 5 minutes to separate cohesive ends. The ligation mixture was then incubated overnight at 15°C.

The ligation products were transformed into the *E.coli* strain DH5α. Transformants of the pIRT2/*Bam*HI mini-library were selected on 50 μg/ml ampicillin plates, and transformants of the pWH5/*Hin*dIII mini-library were selected on plates containing both 50 μg/ml ampicillin and 12.5 μg/ml tetracycline. Selection for tetracycline resistance increases the possibility that the *E. coli* transformants contain recombinant plasmids, as cloning fragments into the *Hin*dIII site of pWH5 causes the inactivation of the λ *cI* repressor gene, which otherwise suppresses the expression of the pWH5 tetracycline gene (see section 7.2 v).

References

Al-Khodairy F and Carr AM (1992)
DNA repair mutants defining G2 checkpoint pathways in *Schizosaccharomyces pombe*. EMBO J 11:1343-1350.

Alfa CE, Ducommun B, Beach D and Hyams JS (1990)
Distinct nuclear and spindle pole body populations of cyclin-cdc2 in fission yeast. Nature 347:680-685.

Ammerer G (1994)
Sex, Stress and integrity: the importance of MAP kinases in yeast. Curr Opin Cell Biol 4:90-95.

Anderson SK, Gallinger S, Roder J, Frey J, Young HA and Ortaldo JR (1993)
A cyclophilin-related protein involved in the function of natural killer cells. Proc Natl Acad Sci USA 90:542-546.

Arber SA (1992)
s- cyclophilin is retained intracellularly via unique COOH terminal sequence and colocalizes with the calcium storage protein calreticulin. J Cell Biol 116:113-125.

Beach DH and Nurse P (1981)
High frequency transformation of the fission yeast *Schizosaccharomyces pombe*. Nature 290:140-142.

Birnboim HC and Doly J (1979)
A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res 7:1513-1523.

Booher R and Beach D (1986)
Site specific mutagenesis of cdc2, a cell cycle control gene of the fission yeast *Schizosaccharomyces pombe*. Mol Cell Biol 6:3523-3530.

Booher R and Beach D (1987)
Interaction between *cdc13*$^+$ and *cdc2*$^+$ in the control of mitosis in fission yeast: dissociation of the G1 and G2 roles of the *cdc2*$^+$ protein kinase. EMBO J 6:3441-3447.

Booher R and Beach D (1988)
Involvement of *cdc13*$^+$ in mitotic control in *Schizosaccharomyces pombe*: possible interaction of the gene product with microtubules. EMBO J 67:2321-2327.

Booher RN, Alfa JS, Hyams JS and Beach DH (1989)
The fission yeast cdc2/cdc13/suc1 protein kinase: regulation of catalytic activity and nuclear localization. Cell 58:485-497.

Bossard MJ, Koser PL, Brandt M, Bergsma DJ and Levy MA(1991)
A single Trp121 to Ala121 mutation in human cyclophilin alters cyclosporin A affinity and peptidyl prolyl isomerase activity. Biochem Biophys Res Commun 176:1142-1148.

Brizuela L, Draetta G and Beach D (1987)
$p13^{suc1}$ acts in the fission yeast cell division cycle as a component of the $p34^{cdc2}$ protein kinase. EMBO J 6:3507-3514.

Broek D, Bartlett R, Crawford K and Nurse P (1991)
Involvement of $p34^{cdc2}$ in establishing the dependency of S phase on mitosis. Nature 349:388-393.

Bueno A and Russell P (1993)
Two fission yeast B-type cyclins cig2 and cdc13 have different functions in mitosis. Mol Cell Biol 13:2286-2297.

Bueno A, Richardson H, Reed SI and Russell P (1991)
A fission yeast B-type cyclin functioning early in the cell cycle. Cell 66:149-159.

Callebaut I, Renoir J, Lebeau M, Massol N, Burny A, Baulieu E-E and Mornon J (1992)
An immunophilin structure that binds Mr 90,000 heat shock protein: main structure of the mammalian p59 protein. Proc Natl Acad Sci USA 89:6270-6274.

Clipstone NA and Crabtree GR (1992)
Identification of calcineurin as a key signalling enzyme in T-lymphocyte activation. Nature 357:695-697.

Coleman TR, Tang Z and Dunphy WG (1993)
Negative regulation of the wee1 protein kinase by direct action of the nim1/cdr1 mitotic inducer. Cell 72:919-1005.

Colley NJ, Baker EK, Stamnes MA and Zuker CS (1991)
The cyclophilin ninaA is required in the secretory pathway. Cell 67:255-263.

Costello G, Rodgers L and Beach D (1986)
Fission yeast enters the stationary G0 state from either mitotic G1 or G2. Curr Genet 11:119-125.

Cyret MS and Thorner J (1992)
Regulatory subunit of yeast Ca^{2+}/calmodulin-dependent phosphoprotein phosphatases is required for adaptation to pheromone. Mol Cell Biol 12:3460-3469.

de Bondt P, Rosenblatt J, Jancarik J, Jones HD, Morgan DO and Kim S-H (1993)
Crystal structure of cyclin-dependent kinase 2. Nature 363:595-602.

de Martin R and Philipson L (1990)
The gene for cyclophilin peptidyl -prolyl *cis trans* isomerase from *Schizosaccharomyces pombe*. Nucleic Acids Res 18:4917.

Draetta G and Beach D (1988)
Activation of cdc2 protein kinase during mitosis in human cells: cell cycle dependent phosphorylation and subunit rearrangement. Cell 54:17-26.

Draetta G, Piwnicia-Worms H, Morrison D, Druker B, Roberts T and Beach D (1988)
Human cdc2 protein kinase is a major cell cycle regulated tyrosine kinase substrate. Nature 336:738-744.

Ducommun B, Brambilla P, Felix M-A, Franza BR, Karsenti E, Draetta G (1991)
cdc2 phosphorylation is required for its interaction with cyclin. EMBO J 10:3311-3319.

Ducommun B, Draetta G, Young P and Beach D (1990)
Fission yeast cdc25 is a cell-cycle regulated protein. Bioc Biophys Res Comm 167:301-307.

Dunphy WG and Kumagai A (1991)
The cdc25 protein controls tyrosine dephosphorylation of the cdc2 protein in a cell free system. Cell 64:903-914.

Dunphy WG, Brizuela L, Beach D and Newport J (1988)
The *Xenopus* cdc2 protein is a component of MPF, a cytoplasmic regulator of mitosis. Cell 54:423-431.

Durkacz B, Beach D, Hayles J and Nurse P (1985)
The fission yeast cell cycle control gene cdc2 - structure of the cdc2 region. Mol Gen Genet 201:543-545.

Egel R (1989)
Mating type genes, meiosis and sporulation. In: Molecular Biology of the Fission Yeast (Nasim A Young P and Johnston BF Eds) Academic Press New York. pp 31-68

Egel R and Egel-Mitani M (1974)
Premeitotic DNA synthesis in fission yeast. Exp. Cell Res 88:127-134

Enoch T and Nurse P (1990)
Mutation of fission yeast cell cycle control genes abolishes the dependence of mitosis on DNA replication. Cell 60:665-673.

Errede B and Levin DE (1993)
A conserved kinase cascade for MAP kinase activation in yeast. Curr Opin Cell Biol 5:254-260

Evans T, Rosenthal ET, Youngblom J, Distel D and Hunt T (1983)
Cyclins: a protein specified by maternal mRNA in sea urchin eggs that is destroyed at each cleavage division. Cell 33:389-396

Fantes PA (1979)
Epistatic gene interactions in the control of division in fission yeast. Nature 279:428-430.

Fantes PA (1981)
Isolation of cell size mutants of a fission yeast by a new selective method: characterisation of mutants and implication for division control mechanisms. J Bacteriol 146:746-754.

Fantes PA (1984)
Cell cycle control in *Schizosaccharomyces pombe*: In: The microbial cell cycle (Nurse P and Steiblova E Eds) CRC Press Boca Raton, Florida pp 109-125.

Fantes PA (1989)
Cell cycle controls. In: Molecular Biology of the Fission Yeast (Nasim A Young P and Johnston BF Eds) Academic Press New York. pp 128-195.

Fantes PA and Nurse P (1977)
Control of cell size at division in fission yeast by a growth modulated size control over nuclear division. Exp Cell Res 107:377-386.

Fantes PA and Nurse P (1978)
Control of the timing of cell division in fission yeast: cell size mutants reveal a second control pathway. Exp Cell Res 115:317-329.

Featherstone C and Russell P (1991)
Fission yeast p107^{wee1} mitotic inhibitor is a tyrosine/serine kinase. Nature 349:808-811.

Feinberg AP and Volgelstein B (1983)
A technique for radiolabelling restriction endonuclease fragments to high specific activity. Anal Biochem 1:6-13.

Fesquet D, Labbé J-C, Derancourt J, Capony J-C, Galas S, Girard F, Lorca T, Shuttleworth J, Doreé M and Cavadore J-C (1993)
The *MO15* gene encodes the catalytic subunit of a protein kinase that activates cdc2 and other cyclin-dependent kinases (CDKs) through phosphorylation of Thr161 and its homologues. EMBO J 12:3111-3121.

Fields S and Song O (1989)
A novel genetic system to detect protein-protein interactions. Nature 340:245-246.

Fink GR (1987)
Pseudogenes in yeast? Cell 49:5-6.

Fischer G, Bang H and Mech C (1984)
Detection of enzyme catalysis for *cis -trans* isomerization of peptide bonds using proline containing peptides as substrates. Biomed Biochem Acta 43:1101-1078.

Foor F, Parent SA, Morin N, Dahl AM, Ramadan N, Chrebet G, Bostian KA and Nielsen JB (1992)
Calcineurin mediates inhibition by FK506 and cyclosporin of recovery from α -factor arrest in yeast. Nature 360:682-684.

Forsburg SL and Nurse P (1991)
Identification of a G1-type cyclin *puc1*$^{+}$ in the fission yeast *Schizosaccharomyces pombe*. Nature 35:245-248.

Friedman J and Weissman I (1991)
Two cytoplasmic candidates for immunophilin action are revealed by affinity for a new cyclophilin: one in the presence and one in the absence of CsA. Cell 66:799-806.

Galat A (1993)
Peptidyl -prolyl *cis trans* isomerases: immunophilins. Eur J Biochem 216:689-707.

Gallo JG, Prentice H and Kingston RE (1993)
Heat shock factor is required for growth at normal temperature in the fission yeast *Schizosaccharomyces pombe*. Mol Cell Biol 13:749-761.

Gautier J, Norbury C, Lohka M, Nurse P and Maller J (1988)
Purified maturation promoting factor contains the product of a *Xenopus* homologue of the fission yeast cell cycle control gene *cdc2*$^{+}$. Cell 54:433-439.

Gething MJ and Sambrook J (1992)
Protein folding in the cell. Nature 355:33-45.

Gibson TJ, Coulson AR, Sulston JE and Little PFR (1987)
Lorist2, a cosmid with transcriptional terminators insulating vector genes from interference by promoters within the insert: effect on DNA yield and cloned insert frequency. Gene 53:275-281.

Goldberg DA (1980)
Isolation and partial characterisation of the *Drosophila* alcohol dehydrogenase gene. Proc Natl Acad Sci USA 77:5794-5802.

Gould KL and Nurse P (1989)
Tyrosine phosphorylation of the fission yeast cdc2 protein kinase regulates entry into mitosis. Nature 342: 39-45.

Gould KL, Moreno S, Owen DJ, Sazer S and Nurse P (1991)
Phosphorylation at Thr167 is required for *Schizosaccharomyces pombe* p34^{cdc2} function. EMBO J 10:3297-3309.

Gould KL, Moreno S, Tonks NK, and Nurse P (1990)
Complementation of the mitotic activator p80^{cdc25} by a human protein-tyrosine phosphatase. Science 250:1573-1576.

Grimm C, Kohli J, Murray J and Maundrell K (1988)
Genetic engineering of *Schizosaccharomyces pombe*: a system for gene disruption and replacement using the *ura4* gene as a selectable marker. Mol Gen Genet 215:81-86.

Gutz H, Heslot H, Leupold U and Loprieno N (1974)
Schizosaccharomyces pombe. In: Handbook of Genetics, Volume 1 (RC King Ed.) Plenum Publishing Corp, New York.

Gygax A and Thuriaux P (1984)
A revised chromosome of the fission yeast *Schizosaccharomyces pombe.* Curr Genet 8:85-92.

Hacker J and Fischer G (1993)
Immunophilins: structure -function relationship and possible role in microbial pathogenecity. Mol Microbiology 10:445-456.

Hagan I, Hayles J and Nurse P (1988)
Cloning and sequencing of the cyclin related *cdc13*$^{+}$ gene and a cytological study of its role in fission yeast mitosis. J Cell Sci 91:587-595.

Hagan IM and Hyams JS (1988)
The use of cell division cycle mutants to investigate the control of microtubule division in the fission yeast *Schizosaccharomyces pombe.* J Cell Sci 89:343-357.

Hagan IM, Riddle PN and Hymas JS (1990)
Intramitotic controls in the fission yeast *Schizosaccharomyces pombe*: the effect of cell size on the spindle length and the timing of mitotic events. J Cell Biol 110:1627-1621.

Hamaguchi JR, Tobey RA, Pines J, Crissman HA, Hunter T and Bradbury EM (1992)
Requirement for p34^{cdc2} kinase is restricted to mitosis in the mammalian cdc2 mutant FT210. J Cell Biol 117:1041-1053.

Hanahan D (1983)
Studies on transformation of *Esherichia coli* with plasmids. J Mol Biol 166:557-580.

Handschummacher RE, Harding MW, Rice J, Drugge RJ and Speicher DW (1984)
Cyclophilins: a specific cytosolic binding protein for cyclosporin A. Science 226:544-546.

Hartwell LH and Weinert T (1989)
Checkpoints: controls that ensure the order of cell cycle events. Science 246:629-634.

Hartwell LH (1974)
Saccharomyces cell cycle. Bact Rev 38:164-198.

Hartwell LH and Unger MW (1977)
Unequal division in *Saccharomyces cerevisiae* and its implication for the control of cell division. J Cell Biol 75:422-435.

Hartwell LH, Culotti J, Pringlr JR and Reid BJ (1974)
Genetic control of the cell division cycle in yeast: a model. Science 183:46-51.

Hayles J, Beach D, Durkacz B and Nurse P (1986)
The fission yeast cell cycle control gene *cdc2* and a sequence *suc1* that suppresses *cdc2* mutant function. Mol Gen Genet 202:291-293.

Heitman H, Movva RN, Heistand PC and Hall MN (1991)
FK506-binding protein proline rotamase is a target for the immunosuppressive agent FK506 in *Saccharomyces cerevisiae*. Proc Natl Acad Sci 88:1948-1952.

Henikoff S (1984)
Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. Gene 28:351-359.

Hindley J, Phear G, Stein M and Beach B (1987)
suc1$^+$ encodes a predicted 13-kilodalton protein that is essential for cell viability and is directly involved in the division cycle of *Schizosaccharomyces pombe*. Mol Cell Biol 7:504-511.

Hinshelwood S and Stoker NG (1992)
An *Escherichia coli-Mycobacterium* shuttle cosmid vector, pMSC1. Gene 110:115-118.

Hirano T, Funahashi S, Uemura T and Yangida M (1986)
Isolation and characterisation of cut mutants that block nuclear division but not cytokinesis. EMBO J 5:2973-2979.

Hiraoka Y, Toda T and Yanagida M (1984)
The NDA3 gene of fission yeast encodes β tubulin: a cold sensitive *nda3* mutation reversibly blocks spindle formation and chromosome movement in mitosis. Cell 39:349-358.

Hoffmann I, Clarke P, Marcote MJ, Karsenti E and Draetta G (1993)
Phosphorylation and activation of human cdc25-C by cdc2-cyclin B and its involvement in the self amplification of MPF at mitosis. EMBO J 12:53-63.

Hoheisel JD, Maier E, Mott R, McCarthy L, Grigoriev AV, Schalkwyk C, Nizetic D, Francis F and Lehrach H (1993)
High resolution cosmids and P1 maps spanning the 14 Mb genome of the fission yeast *S.pombe*. Cell 73:109-120.

Humphrey T, Sadhale P, Platt T and Proudfoot N (1991)
Homologous mRNA 3' end formation in fission and budding yeast. EMBO J 10:3503-3511.

Izumi T and Maller JL (1994)
Elimination of cdc2 phosphorylation sites in the cdc25 phosphatase block intiation of M phase. Mol Biol Cell 4:1337-1350.

Kallen J, Spittzfaden C, Mauro G, Zurini M, Wider G Widmer H, Wuthrich K and Walkinshaw MD (1991)
Structure of human cyclophilin and its binding site for CsA determined by X-ray crystallography and NMR spectroscopy. Nature 353:276-279.

Kasai H and Isono K (1991)
Dual modes of transcriptional and translational initiation of SSP1, the gene for a mitochondrial HSP70, responding to heat shock in *Schizosaccharomyces pombe*. Nucleic Acids Res 19:53331-5337.

Kaufer N, Simanis V and Nurse P (1985)
Fission yeast *Schizosaccharomyces pombe* correctly excises a mammalian RNA transcript intervening sequence. Nature 318:78-80.

Kieffer JK, Seng TW, Wei L, Osterman DG and Handschumacher RE (1993)
Cyclophilin-40, a protein with homology to the P59 component of the steroid receptor complex. J Biol Chem 268:12303-12310.

Kieffer JK, Theresia T and Handschumacher RE (1992)
Isolation and characterisation of a 40-kDa cyclophilin-related protein. J Biol Chem 267:5503-5507.

Kinoshita N, Yamano H, Niwa H, Yoshida T and Yanagida M (1993)
Negative regulation of mitosis by the fission yeast protein phosphatase ppa2. Genes Dev 7:1059-1065.

Kohli J, Hottinger H, Munz P, Strauss A and Thuriaux P (1977)
Genetic mapping in *Schizosaccharomyces pombe* by mitotic and meiotic analysis, and induced haploidization. Genetics 87:423-471.

Koser PL, Bergsma DJ, Cafferkey R, Eng W-K, McLaughlin NM, Ferrara A, Silverman C, Kasyan K, Bossard MJ, Johnson RK, Porter TG, Levy MA and Livi GP (1991)
The *CYP2* gene of *Saccharomyces cerevisiae* encodes a cyclosporin A peptidyl prolyl *cis trans* isomerase with N-terminal signal sequence. Gene 108:74-80.

Krek W and Nigg EA (1991)
Mutation of $p34^{cdc2}$ phosphorylation site induce premature mitotic events in Hela cells: evidence for a double block to $p34^{cdc2}$ kinase activation in vertebrates. EMBO J 10:3331-3341.

Kunkel TA (1985)
Rapid and efficient site specific mutagenesis without phenotypic selection. Proc Natl Acad Sci USA 82:488-492.

Kunz J and Hall MN (1993)
Cyclosporin A, FK506 and rapamycin: more than just immunosuppression. Trends Biochem 18:334-338.

Laemmli UK (1970)
Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680.

Lebeau, Massol N, Herrick J, Faber LE, Renoir JM, Radanyi C and Baulieu E-E (1992)
P59 and hsp90- binding protein. J Biol Chem 267:4281-4284.

Lee MG and Nurse P (1987)
Complementation used to clone a human homologue of the cell cycle control gene cdc2. Nature 327:31-35.

Lee TH, Solomon MJ, Mumby MJ and Kirschner MW (1991)
INH, a negative regulator of MPF, is a form of protein phosphatase 2A. Cell 64:415-423.

Leupold U (1950)
Die vererbung von homothallie und heterothallie bie *Schizosaccharomyces pombe.* CR Trav Lab Carlesberg Ser Physiol 24:381-480.

Lipman DJ and Pearson WR (1985)
Rapid and sensitive protein similarity searches. Science 227:1435-1441.

Little PFR and Cross SH (1985)
A cosmid vector that facilitates restriction enzyme mapping. Proc Natl Acad Sci USA 82:3159-3163.

Liu J, Albers AL, Chen C, Schreiber L and Walsh T (1990)
Cloning expression and purification of human cyclophilin in *Escherichia coli* and assessment of the catalytic role of cysteines by site-directed mutagenesis. Proc Natl Acad Sci USA 87:2304-2308.

Liu J, Chen C and Walsh CT (1991a)
Human and *Escherichia coli* cyclophilins: Sensitivity to inhibition by the immunosuppressant CsA with specific tryptophan residue. Biochemistry 30:2306-2310.

Liu J, Farmer JD, Lane WS, Friedman I and Schreiber L (1991b)
Calcineurin is a common target of cyclophilin - cyclosporin A and FKBP-FK506 complexes. Cell 66:807-815.

Luban J, Bossolt KL, Franke EK, Kalpana GV and Goff P (1993)
Human Immunodeficiency Virus Type 1 Gag protein binds to cyclophilins A and B. Cell 73:1067-1078.

Lundgren K, Walworth N, Booher R, Dembski M, Kirschner M and Beach D (1991)
mik1 and wee1 cooperate in the inhibitory tyrosine phosphorylation of cdc2. Cell 64:1111-1122.

MacNeill SA and Fantes PA (1993)
Methods for analysis of the fission yeast cell cycle In: The cell cycle: A practical approach (Fantes PA and Brooks R Eds) IRL Press, Oxford UK.

MacNeill SA and Fantes PA (1994)
Controlling entry into mitosis in fission yeast. In: Frontiers in Molecular Biology: the cell cycle (Glover D and Hutchison C Eds) IRL Press Oxford UK.

Maier E, Hoheisel JD, McCarthy L, Mott R, Grigoriev AV, Monaco AP, Larin-Z and Lehrach H (1992)
Complete coverage of the *Schizosaccharomyces pombe* genome in yeast artificial chromosomes. Nature Genet 1:273-277.

Marks J and Hyams J (1985)
Localization of F-actin through the cell division cycle of *Schizosaccharomyces pombe*. Eur J Cell Biol 3:27-32.

Masui Y and Markert CL (1971)
Cytoplasmic control of nuclear behaviour during meiotic maturation of frog oocytes. J Exp Zool 177:129-146.

Maundrell K (1990).
nmt1 of fission yeast. J Biol Chem 265:10857-10864

McKeon F (1991)
When worlds collide: immunosuppressants meet protein phosphatases. Cell 66:823-826.

Mclaughlin MM, Bossard MJ, Koser PL, Cafferkey R, Moris RA, Miles LM, Stricker J, Bergsma DJ, Levy MA and Livi GP (1992)
The yeast cyclophilin multigene family: purification and characterisation of new isoform. Gene 111:85-92.

Millar JBA, Lenaers G and Russell P (1992)
pyp3 PTPase acts as a mitotic inducer in fission yeast. EMBO J 11:4933-4941.

Millar JBA, McGowan CH, Lenaers G, Jones R and Russell P (1991)
$p80^{cdc25}$ mitotic inducer is the tyrosine phosphatase that activates cdc2 kinase in fission yeast. EMBO J 10:4301-4309.

Millar JBA, Russell P, Dixon JE and Guan KL (1992)
Negative regulation of mitosis by two functionally overlapping PTPases in fission yeast. EMBO J 11:4943-4952.

Mitchison JM (1957)
The growth of single cells In *Schizosaccharomyces pombe*. Exp Cell Res 13:244-262.

Mitchison JM (1970)
Physiological and cytological methods for *Schizosaccharomyces pombe*. Methods Cell Physiol. 4:131-165.

Mitchison JM (1989)
Cell cycle and periodicities In: Molecular Biology of the Fission Yeast (Nasim A Young P and Johnston BF Eds) Academic Press New York. pp 128-195.

Mitchison JM (1990)
The fission yeast *Schizosaccharomyces pombe*. Bioassays 12:189-191.

Mizukami T, Chang WI, Garkavatsev I, Kaplan N, Lombardi D, Matsumoto T, Niwa O, Kounosu A, Yanagida M, Marr TG and Beach D (1993)
A 13 kb resolution cosmid map of 14 Mb fission yeast genome by non-random sequence-tagged site mapping. Cell 73:121-132.

Molz L and Beach D (1993)
Characterisation of the fission yeast mcs2 cyclin and its associated protein kinase activity. EMBO J 12:1723.

Molz L, Booher R, Young P and Beach D (1989)
cdc2 and the regulation of mitosis : six interacting *mcs* genes. Genetics 122:773-782.

Moreno S and Nurse P (1991)
Clues to action of cdc25 protein. Nature 351:194.

Moreno S and Nurse P (1993)
Regulation of progression through G1 phase of the cell cycle by the *rum1*$^+$ gene. Nature 367:236-242.

Moreno S, Hayles J and Nurse P (1989)
Regulation of p34^{cdc2} protein kinase during mitosis. Cell 58:361-372

Moreno S, Klar A and Nurse P (1991)
Molecular genetic analysis of *Schizosaccharomyces pombe*. Methods Enzymol 194:795-823.

Moreno S, Nurse P and Russell P (1990)
Regulation of mitosis by cyclic accumulation of p80^{cdc25} mitotic inducer in fission yeast. Nature 344:549-552.

Munz P, Wolf K, Kohli J and Leoupold U (1989)
Genetics overview In: Molecular Biology of the Fission Yeast (Nasim A Young P and Johnston BF Eds) Academic Press New York. pp 1-25.

Murray AW (1992)
Creative blocks: cell cycle checkpoints and feedback controls. Nature 359:599-604.

Murray AW and Kirschner MW (1991)
What controls the cell cycle. Scientific American. 264:34-41.

Nadeau K, Das A and Walsh CT (1993)
Hsp90 chaperonins possess ATPase activity and bind heat shock transcription factors and peptidyl-prolyl isomerases. J Biol Chem 268:1479-1487.

Norbury C and Nurse P (1991)
Cyclins and cell cycle control. Curr Biol 1:23-24.

Norbury C and Nurse P (1992)
Animal cell cycles and their control. Annu Rev Biochem 61:441-470.

Norbury C, Blow JJ and Nurse P (1991)
Regulatory phosphorylation of the $p34^{cdc2}$ protein kinase in vertebrates. EMBO J 10:3321-3329.

Nurse P (1975)
Genetic control of cell size at cell division in yeast. Nature 256:547-551.

Nurse P (1985)
Cell cycle controls in yeast. Trends Genet 1:51-55.

Nurse P (1990)
Universal control mechanism regulating onset of M-phase. Nature 344:503-508.

Nurse P and Bissett Y (1981)
Gene required in G_1 for commitment to cell cycle and in G_2 for control of mitosis in fission yeast. Nature 292:558-560.

Nurse P and Fantes P (1981)
Cell cycle controls in fission yeast: a genetic analysis. In: The cell cycle (P. C. L. John, ed.), pp. 85-98. Cambridge University Press.

Nurse P and Thuriaux P (1977)
Controls over the timing of DNA replication during the cell cycle of fission yeast. Exp Cell Res 107:365-375.

Nurse P and Thuriaux P (1980)
Regulatory genes controlling mitosis in the fission yeast *Schizosaccharomyces pombe*. Genetics 96:627-637.

Nurse P, Thuriaux P and Nasmyth KA (1976)
Genetic control of the cell division cycle in the fission yeast *Schizosaccharomyces pombe*. Mol Gen Genet 146:167-178.

O'keffe SJ, Tamura J, Kincaid RL, Tocci MJ and O'neill EA (1992)
FK506- and CsA- sensitive activation of interleukin-2 promoter by calcineurin. Nature 357:692-694.

Ogden JE and Fantes PA (1986)
Isolation of a novel type of mutation in the mitotic control of *Schizosaccharomyces pombe* whose phenotypic expression is dependent on the genetic background and nutritional environment. Curr Genet 10:509-514.

Ondek B , Hardy BW, Baker EK, Stamnes MA, Shieh B and Zuker CS (1992)
Genetic dissection of cyclophilin function. J Biol Chem 267:16460-16466.

Ottilie S, Chernoff J, Hnnig G, Hoffman CS and Erikson RL (1992)
The fission yeast gene *pyp1*$^+$ and *pyp2*$^+$ encode protein tyrosine phosphatases that negatively regulate mitosis. Mol Cell Biol 12:5571-5580.

Pardee AB (1974)
A restriction point for control of normal animal cell proliferation. Proc Natl Acad Sci USA 71:1286-1290.

Parker LL, Walter SA, Young PG and Piwnica-Worms H (1993)
Phosphorylation and inactivation of the mitotic inhibitor wee1 by the nim1/cdr1 kinase. Nature 363:736-741.

Pelham H (1985)
Activation of heat shock genes in eukaryotes. Trends Genet 1:31-35.

Perisic O, Xiao H and Lis JT (1989)
Stable binding of *Drosophila* heat shock factor to head-to-tail repeats of a conserved 5 bp recognition unit. Cell 59:797-806.

Pfügl G, Kallen J, Schirmer T, Jansonius JN, Zurini MG and Walkinshaw MD (1993)
X-ray structure of a decameric cyclophilin /cyclosporin crystal complex. Nature 361:91- 94.

Pierce JC and Sternberg NL (1992)
Using bacteriophage-P1 system to clone high molecular weight genomic DNA. Method Enzymol 216:549-574.

Pierce JC, Brian S and Sternberg NL (1992)
A positive selection for cloning high molecular weight DNA by the bacteriophage P1 system: improved cloning efficacy. Proc Acad Sci USA 89:2056-2060.

Poon RYC, Yamashita K, Adamczewski JP, Hunt T and Shuttleworth J (1993)
The cdc2-related protein $p40^{MO15}$ is the catalytic subunit of a protein kinase that can activate $p33^{cdk2}$ and $p34^{cdc2}$. EMBO J 12:3123-3132.

Pratt WB (1987)
Transformation of the glucocortcoid and progesterone receptors to the DNA binding state. J Cell Biochem 35:51-68.

Prentice HL (1991)
High efficiency transformation of *Schizosaccharomyces pombe* by electroporation. Nucleic Acids Res 20:621.

Robinow CF and Hyams JS (1989)
General cytology of fission yeast cell cycle controls. In: Molecular Biology of the Fission Yeast (Nasim A Young P and Johnston BF Eds) Academic Press New York. pp 273-324.

Rose MD and Broach JR (1991)
Cloning genes by complementation in yeast. In: Guide to yeast genetics and molecular biology (Guthrie C and Fink GR Eds) Academic Press New York. pp 230-239.

Rothstein RJ (1983)
One step gene disruption in yeast. In: Methods in Enzymology (R Wu, L Grossman and K Moldave eds), Academic Press 101:202-211.

Rowley R Subramani S and Young PG (1992)
Checkpoint controls in *Schizosaccharomyces pombe*. EMBO J 11:1335-1342.

Ruderman JV (1993)
MAP kinase and activation of quiescent cells. Curr Opin Cell Biol 5:207-213.

Russell P (1989)
Gene cloning and expression In: Molecular Biology of the Fission Yeast (Nasim A, Young P and Johnston BF Eds). Academic Press New York. pp 224-267.

Russell P and Nurse P (1986)
cdc25$^+$ functions as an inducer in the mitotic control of fission yeast. Cell 45:145-153.

Russell P and Nurse P (1987a)
Negative regulation of mitosis by *wee1*$^+$ a gene encoding a protein kinase homologue. Cell 49:559-567

Russell P and Nurse P (1987b)
The mitotic inducer *nim1*$^+$ function in a regulatory network of protein kinase homologies controlling the intiation of mitosis. Cell 49:569-576.

Sambrook J, Fritch EF and Maniatis T (1989)
Molecular cloning: a laboratory handbook. Cold Spring Harbour Press, Cold Spring Harbour New York.

Sanger F, Nicklen S and Coulson AR (1977)
DNA sequencing with chain terminating inhibitors. Proc Natl Acad Sci USA 74:5463-5467.

Schmid FX, Grafl R, Wrba A and Beintema JJ (1986)
Role of proline peptide bond isomerization in unfolding and refolding of ribonuclease. Proc Natl Acad Sci USA 83:872-876.

Schreiber SL (1991)
Chemistry and Biology of the immunophilins and their immunosuppressive ligands. Science 251:283-287.

Schreiber SL (1992)
Immunophilin sensitive protein phosphatases action in cell signalling pathways. Cell 70:365-368.

Schreiber SL and Crabtree GR (1992)
The mechanism of action of cyclosporin A and FK506. Immunol today 13:136-142.

Sheldrick KS and Carr AM
Feedback controls and G2 checkpoints: fission yeast as a model system. BioEssays 15:775-782.

Sherr CJ (1993)
Mammalian G1 cyclins. Cell 73:1059-1065.

Sigal NH and Dumont FJ (1992)
Cyclosporin A, FK-506, and rapamycin: pharmacologic probes of lymphocytes and signal transduction. Annu Rev Immunol 10:519-560.

Sipiczki M (1989)
Taxonomy and phylogenesis. In: Molecular Biology of the Fission Yeast (Nasim A Young P and Johnston BF Eds) Academic Press New York. pp 431-448.

Smith BJ and Yaffe MP (1991)
A mutation in the yeast heat shock factor gene causes temperature sensitive defects in both mitochondrial protein import and the cell cycle. Mol Cell Biol 11:2647-2655.

Solomon MJ, Harper JW and Shuttleworth J (1993)
CAK the $p34^{cdc2}$ activating kinase contains a protein identical or closely related to $p40^{MO15}$. EMBO J 12:3133-3142.

Sorger PK and Pelham HRB (1988)
Yeast heat shock factor is an essential DNA binding protein that exhibits temperature dependent phosphorylation. Cell 54:855-864.

Southern EM (1975)
Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98:503-517.

Stamens MA, Rutherford SL and Zuker CS (1992)
Cyclophilins: a new family of proteins involved in intracellular folding. Trends Cell Biol 2:272-276.

Stein RL (1993)
Exploring the catalytic activity of immunophilins. Curr Biol 1:234-236.

Steiner JP, Dawson TM, Fotuhi M, Glatt CE, Snowman AM, Cohen N and Snyder S (1992)
High brain densities of the immunophilin FKBP colocalized with calcineurin. Nature 358:584-587.

Sternberg NL (1992)
Cloning high molecular weight DNA fragments by the bacteriophage P1 system. Trends Genet 8:11-16.

Tai P, Albers MW, Chang H, Faber LE and Schriber SL (1992)
Association of a 59 kDa immunophilin with glococorticoid receptor complex. Science 256:1315-1318.

Th'ng JPH, Wright PS, Hamaguchi J, Lee MG, Norbury CJ, Nurse P and Bradbury EM (1990)
The FT210 cell line is a mouse G2 phase mutant with a temperature sensitive *CDC2* product. Cell 63:313-324.

Thériault Y, Logan TM, Meadows R, Yu L, Olejniczak ET, Holzman TF, Simmer RL and Fesik SW (1991)
Solution of the cyclosporin-A/cyclophilin complex by NMR. Nature 361:88-91.

Thuriaux P Nurse P Carter B (1978)
Mutants altered in the control co-ordinating cell division with cell growth in the fission yeast *Schizosaccharomyces pombe.* Mol Gen Genet 161:215-220.

Tropschug M, Barthelmess IB and Newpert W (1989)
Sensitivity to cyclosporin A is mediated by cyclophilin in *Neurospora crassa* and *Saccharomyces cerevisiae.* Nature 342:953-955.

Uzawa A, Samajima I, Hirano T. Tanaka K and Yanagida M (1990)
The fission yeast *cut1*$^+$ gene regulates spindle pole body duplication and has homology to the budding yeast *ESP1* gene. Cell 62:923-925.

Vieira K and Messing J (1982)
The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene 19:259-268.

Volgelstein B and Gillespie D (1979)
Preparative and analytical purification of DNA from agarose. Proc Natl Acad Sci USA 76:615-619.

Warbrick E (1990)
New elements of the mitotic control in *Schizosaccharomyces pombe*. Ph.D. thesis. University of Edinburgh

Warbrick E and Fantes PA (1991)
The *wis1*$^+$ protein kinase is a dosage dependent regulator of mitosis in *Schizosaccharomyces pombe*. EMBO J 302:4291-4299.

Warbrick E and Fantes PA (1992)
Five novel elements involved in the regulation of mitosis in fission yeast. Mol Gen Genet 232:440-446.

Weinert TA and Hartwell LH (1988)
The *RAD9* gene controls the cell cycle response to DNA damage in *Saccharomyces cerevisiae*. Science 241:317-322.

Wiech H, Buchnner J, Zimmerman R and Jakob U (1992)
Hsp90 chaperones protein folding *in vitro*. Nature 358:13115-13118.

Wright A, Maundrell K, Heyer WD, Beach D and Nurse P (1986)
Vectors for the construction of gene banks and the integration of clones genes in *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*. Plasmid 15:156-158.

Wu L and Russell P (1993)
nim1 kinase promotes mitosis by inactivating wee1 tyrosine kinase. Nature 363:738.

Yanagida M 1987
Yeast tubulin genes. Microbiol Sci 4:115-11.

Yang X, Hubbard J and Carlson M (1992)
A protein kinase substrate identified by the two hybrid system. Science 257:680-682.

Yem AW, Tomasselli AG, Heinrikson RL, Zurcher H, Ruff VA, Johnson RA and Deibel MR (1992).
The Hsp56 component of steroid receptor complexes binds to immobilised FK506 and shows homology to FKBP-12 and FKBP-13. J Biol Chem 267:2868-2871.

Young PG and Fantes PA (1987)
Schizosaccharomyces pombe mutants affected in their division response to starvation. J Cell Sci 88:295-304.

Zaret KS and Sherman F (1982)
DNA sequence requirement for efficient transcription termination in yeast. Cell 28:563-573.