The WHI1 gene of Saccharomyces cerevisiae

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The *WHI1* gene of *Saccharomyces cerevisiae*.

Submitted by Nigel Francis Stenner

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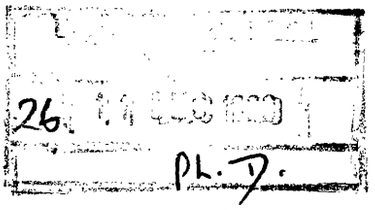

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ABSTRACT

The aim of this project was to study the gene *WHI1*,a gene known to be involved in cell size regulation. Three aspects were studied: 1 Interaction with other genes, 2 Mapping of the gene and 3 Cloning of the gene and structure of the protein product.

1)No interaction was found between *WHI1* and *DNA26* a *start* gene thought to be involved with DNA synthesis. Interactions were seen with *CDC28*, which encodes a protein kinase, *CDC63*, which is involved with protein synthesis and affects control at *start* and *CYR1*, the gene which encodes adenylate cyclase. These interactions give some clues to the action of *WHI1*. The *whi1* mutation did not affect the ability of the strain containing it to grow on non fermentable carbon sources.

2) It was thought necessary to map the gene accurately to aid cloning. Centromeric mapping data suggested the gene was present on chromosome I. Tetrad mapping of this arm did not prove successful. In order to locate the gene on a single chromosome, mapping using *rad52* induced chromosome loss was undertaken. As the *WHI1* gene acts in a dose dependent manner and it was not known how many genes were involved in the system, modelling of the method both physically and mathematically was carried out. The modelling also took into account the loss of unmarked chromosomes. Physical mapping showed that it was possible to distinguish the number of size control genes directly involved in the system. From the mathematical modelling it was possible to gain two independent estimates of death rate and predict the size distribution of cells based on different models. The number of cells exhibiting the *whi1* phenotype was greatly under-represented in the aneuploids leading to non assignment of the gene

to a single chromosome but analysis of the results did reveal some facts about the mapping method used: i) chromosome loss is not random, ii) partial chromosome loss is rare, iii) mitotic crossing over is rare and iv) there is a major source of death other than nullisomy. The frequency of cells of different sizes indicated that at least one other gene is involved in interaction with *whi1*.

3) Before cloning of the gene could proceed two other groups succeeded in cloning the *WHI1* gene and its mutant form. The null phenotype was found to be large cells and it was revealed that *whi1* was a hyperfunctional mutation. Initially no homology was found by other investigators between *WHI1* and any other protein sequence although subsequently limited homology was found with cyclin sequences. Examination of the sequence by Fast P showed homology with calcitonin gene related product. The homology was restricted to the pre-protein form around the area cleaved during maturation of the protein. Examination by eye showed that the sequence contained several structures not noted by other investigators at this time. These consisted of dibasic residues associated with threonine and serine residues, N-glycosylation sites and two small internal repeats. The C terminus, which is lost in both mutations isolated, is a PEST region. This region is where most of the dibasic residues were found. It is thought the dibasics may be targets for phosphorylation which would enhanced degradation of this region enabling function of the protein. Phosphorylation dependent activation of the protein also provides routes for control of this protein by the nutrient sensing system and pheromone system. These same structures were found in two other proteins, *SST2* and *SCG1*, both of which are involved in message transduction in the mating

pheromone system. Placing copies of the mutant gene into wild type haploid cells showed that *WHI1* interacted directly with at least one other protein.

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CONTENTS

CHAPTER 1: GENERAL INTRODUCTION

1.1 *SACCHAROMYCES CEREVISIAE.*

There is currently great interest in the mitotic cell division cycle of eukaryotes and the organisms in which it can be studied. One of these is the budding yeast *Saccharomyces cerevisiae*. Interest in this yeast was stimulated when it was found to be a true eukaryote which could be used as a model for higher systems and it has been extensively studied with respect to its biochemistry, physiology and genetic control. *S. cerevisiae* has many features which make it attractive, especially for the study of the complex controls and processes involved in the cell division cycle:

(1) Unlike many other eukaryotes *S. cerevisiae* has both vegetative haploid and diploid phases. This enables the isolation of recessive mutations in haploid cells and analysis of their effect in diploids (Mortimer & Schild, 1985).

(2) *S. cerevisiae* exhibits clear morphological events which are indicators of passage through the cell division sequence. The exact phase a cell is in can often be identified by microscopic examination (Hartwell, 1974).

(3) Mutational and recombinant studies of *S. cerevisiae* mean that the genetics and chromosome maps are well documented. This is useful when molecular DNA analysis is to be carried out on specific genes.

(4) The basic elements of cell structure, macromolecular synthesis, chromosome replication and segregation all show similarity to higher organisms. Once mechanisms are resolved in this organism results may indicate how the same processes are carried out in, for example, human cells. This may eventually lead to a better understanding of some diseases in man which are the result of loss of cellular

control (Botstein & Fink, 1988)

1.2 THE *SACCHAROMYCES CEREVISIAE* CELL CYCLE

In *S. cerevisiae* the mitotic cell cycle is an ordered sequence of events leading to cell division (Shilo *et al.*, 1976). It has been defined as the period between the birth of a cell and the time at which it undergoes division to form two daughter cells (Nurse and Fantes, 1981). A new cell cycle is signalled microscopically by the initiation of a bud, although DNA synthesis has already been initiated shortly before this (Rivin & Fangman, 1980; Brewer *et al.*, 1984). A series of reproducible events follows which ends in the segregation of genetic material and cell components into two more or less identical progeny cells. The cycle can be monitored by the passage of so called "landmark events", which may be morphological or biochemical (Pringle and Hartwell, 1981), and are summarised in Figure 1.1.

The cell cycle of yeast is usually described as having four phases; G1, S, G2 and M. Recently it has been proposed that G2 may be very short or may not exist in its own right (Nurse, 1985) as there are no clear landmarks which idicate the begining or end of this phase. Cells do not arrest but appear to pass directly from S to M phase.G1 is the phase of the cycle which is most variable; the length of time a cell spends in this phase being dependent on birth size and growth rate (Johnston, 1977; Lord & Wheals, 1984). G1 begins when nuclear division is complete and therefore contains both cytokinesis and cell separation (Pringle & Hartwell, 1981) as well as the major rate limiting step of the cell cycle (Shilo *et al.*, 1976; Johnston, 1977). G1 is followed by S phase in which the DNA is replicated. Its beginning is signalled by the appearance of the spindle pole body

FIGURE 1.1

Major Landmarks of the *S. cerevisiae* cell cycle.

Duration of events not to scale.

Abbreviations

SPBSF : spindle-pole-body-satellite formation

SPBD : spindle-pole-body duplication

CRF : chitin ring formation

MRF : microfilament ring formation

BE : bud emergence

iDS : initiation of chromosomal DNA synthesis

DS : chromosomal DNA synthesis

SPBS : spindle-pole-body separation

NM : nuclear migration

mND : medial stage of nuclear migration

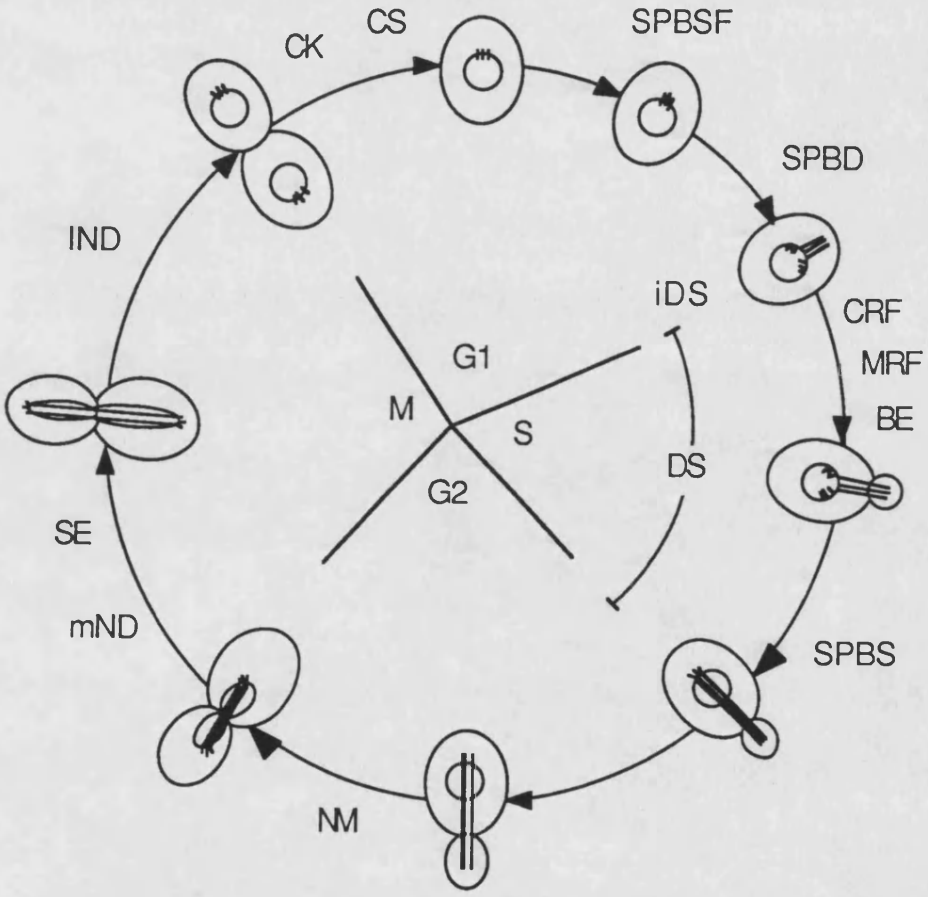
SE : spindle elongation

lND : late stage of nuclear division

CK : cytokinesis

CS : cell separation

(From Pringle and Hartwell, 1981)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G2
M

satellite which grows to duplicate the whole structure (Wheals, 1987). Bud emergence occurs after the appearance of the satellite and was thought to show the point of initiation of DNA synthesis (Hartwell, 1974; Slater *et al.*, 1977; Johnston *et al.*, 1979) although data also suggest that DNA synthesis may already be complete by this time (Slater, 1973; Hartwell, 1976). Although G1 is the most variable of the phases of the cell cycle the duration of S phase can be affected by the limitation of carbon and nitrogen sources and changes in temperature (Singer & Johnston, 1980; Rivin & Fangman, 1980). Separation of the spindle pole body and commitment to the formation of a spindle occurs at the end of S phase (Hartwell & Pringle, 1978). A short G2 may or may not occur next followed by the actual physical segregation of the nuclear material (mitosis). It has been postulated that mitosis is initiated in S phase (Nurse, 1985). During mitosis the nucleus migrates to the neck of the bud and nuclear division occurs. Unlike other eukaryotes the nuclear envelope does not break down during division. Once the DNA is partitioned a plasma membrane is formed separating the mother and daughter cytoplasms (cytokinesis) followed shortly by cell separation.

If the cell cycle is to be successful a cell must coordinate all of these processes with cellular growth. The cell division cycle does not appear to be a single pathway rather the processes involved are organised as a series of events in interlocking pathways. This is confirmed by such observations as bud emergence being independent of initiation of DNA synthesis, and functional sequence mapping of primary defect events of cell division cycle (CDC) mutants. The pathways appear to converge prior to, and diverge at the end of, G1.

This means that the entire cycle could be regulated by a single control event in G1. The exact mechanism of control is not yet known. Two models have been proposed which address the question of control. The first, deterministic growth controlled model, involves the traverse of a major rate limiting step being achieved when a critical amount of growth or mass is accumulated. This being said it is also known that the control is not dependent on the overall synthesis or accumulation of proteins in a cell (Singer *et al.*, 1978; Johnston & Singer, 1978). The second model is the transition probability model which was developed to explain the behaviour of growing mammalian cells. It states that the initiation of each cell cycle depends on traversing a single critical step but that this occurs at random and regulation of proliferation is achieved mainly through changing the probability of this transition rather than the rate at which some process is completed (Smith & Martin, 1973). Both of these initial models have been modified (Wheals, 1981; Tyson, 1986) to take into account the fact that, by use of mutants, which are usually conditional and arrest cells in the cell division cycle with a specific terminal phenotype (*cdc* mutations), two major cell cycle controls have been identified in yeasts. One acts in the G1 phase, resulting in the commitment to the mitotic cycle and the second acting at nuclear division leading to the initiation of mitosis (Nurse & Bissett, 1981; Piggott *et al.*, 1982). Both models contain a rate limiting step termed *start* (Hartwell, 1974) which is defined as the earliest gene controlled event in G1 and more importantly is the point, once passed, that commits cells to complete an initiated mitotic cell cycle (Nurse, 1985).

1.3 THE NATURE AND CONTROL OF *START*

Not only does regulation of the *S. cerevisiae* cell cycle at *start*

take the form of a rate limiting step but also as a point of decision for the cell as to which developmental pathway it will follow. The different pathways constitute the discontinuous processes of cell development (occurring only once or a few times in the cell cycle) which occur against the backdrop of normal continuous processes such as protein synthesis and cell metabolism. *Start* acts as a coordination point for the regulation of discontinuous and continuous processes and as such many inputs are known to affect this important period of the cell cycle. A summary of internal and external factors which affect the undifferentiated cell at *start* and alternative pathways to the mitotic cell cycle are shown in Figure 1.2.

Much has been written about the nature of *start*. It is functionally defined by a group of thirteen *cdc* mutations and in response to mating pheromone. On shift to their restrictive temperature, temperature sensitive *cdc start* mutants complete any unfinished cycles and arrest as undifferentiated cells prior to the *start* event.

The *CDC28* gene has been shown to be involved in the traverse of *start*, its function required in G1 before the initiation of DNA synthesis. It is an archetypal representative of the group of *cdc* mutations involved with *start*. Functional sequence mapping places it before the action of any other *cdc* mutations which function during G1 (Hartwell, 1973; Herefored & Hartwell, 1974). The function of the *CDC28* gene may explain and help reconcile the two models proposed for the passage of *start*. What is known about the function of the *CDC28* and other *CDC* mutations will be discussed later.

Shilo *et al.* (1976, 1977) presented data which they suggested showed

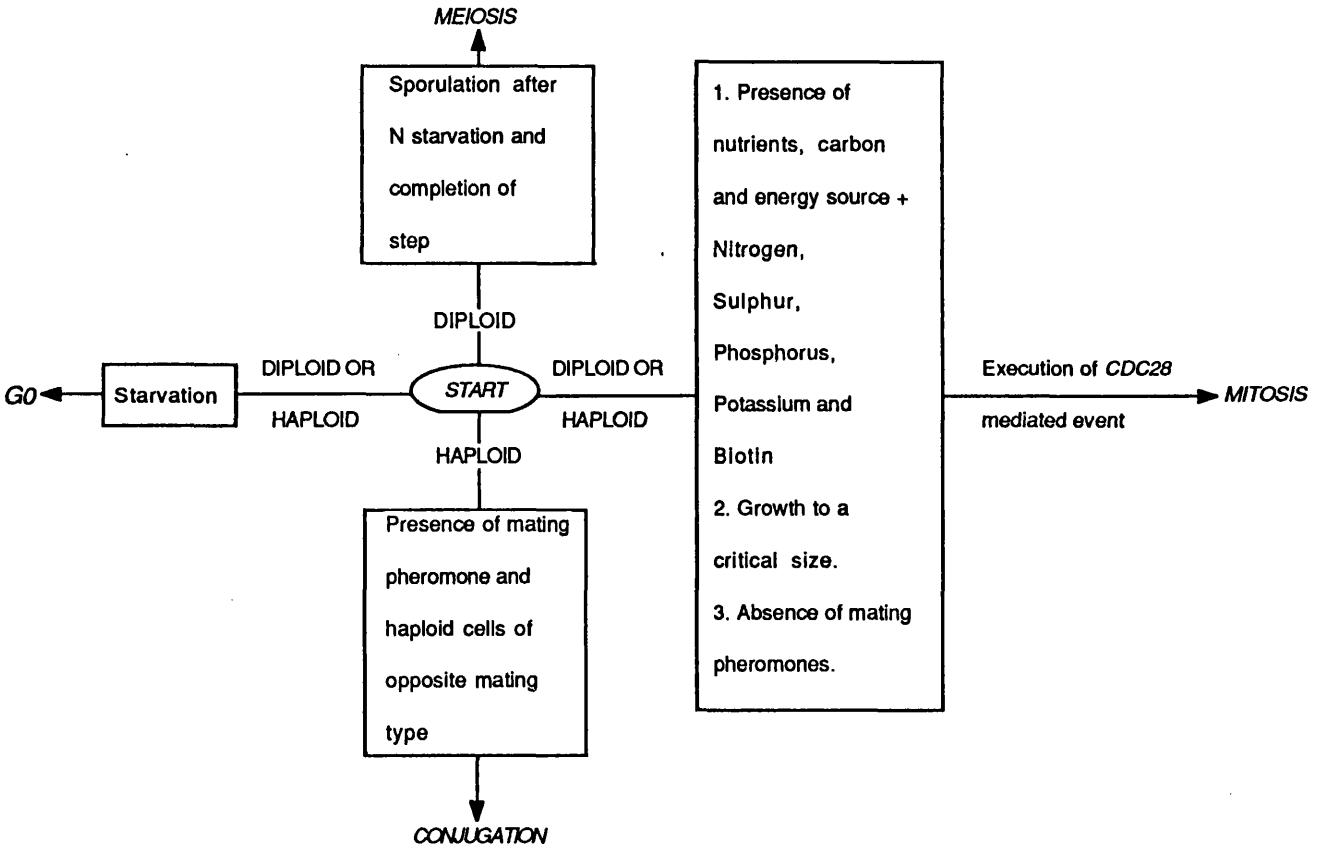


FIGURE 1.2 Possible Developmental Pathways From *Start*

Start; a decision point and the conditions required to complete it before cell cycle initiation. To read begin at *start* and follow any of the possible developmental pathways.

(adapted from Hartwell, 1974 and Wheals, 1987)

that cells emerged from a block at *start* with first order kinetics. These data suggested that the critical size criteria of Johnston (1977) could not apply to the traverse of *start*. On re-examination of the data however, (Nurse & Fantes 1977) it was shown that emergence did not show first order kinetics and that the critical size criteria could still apply.

Both models may apply to *start* if instead of being a point in the cell cycle it is a period. Entry into *start* may be deterministic, controlled by the critical size criteria, while exit is probablistic. Once traversing *start* cells have to complete all events which make up *start* before they become committed to the next mitotic cycle. Individual cells have a constant probability per unit time of doing this (Wheals, 1982). *Start* mutants may be involved in entry into *start*, passage through it or both.

Before cells traverse *start* they may be arrested by mating pheromone. This is a short polypeptide which is synthesised and released by haploid cells to arrest growth in cells of the opposite mating type so that mating can occur. MATa cells are arrested by alpha factor, the pheromone secreted by MAT*alpha* cells and *vice versa*. Arrest in G1 is essential for conjugation (Bucking-Thröm *et al.*, 1973). Experiments by Hartwell (1974) showed that *cdc28* and alpha factor cause arrest at approximately the same point, although *CDC28* does not complement the lesion in sterile alpha cells and alpha factor does not de-restrict *cdc28* at 36°C. (Nurse, 1985). Mutants have been isolated which do not arrest at *start* when exposed to pheromone of cells of the opposite mating type (MacKay & Manney, 1974a, 1974b; Manney & Woods, 1976; Hartwell, 1980; Whiteway *et al.*, 1989). The defect usually lies in the pheromone system

rather than in the *start* machinery. The pheromone system will be discussed in greater detail later.

Another condition which causes arrest prior to the *cdc28* block is nutrient depletion (Johnston *et al.*, 1977). Arrest due to lack of nutrients is not dependent on a single factor. Arrest can occur due to lack of carbon source, nitrogen, sulphur, phosphorus, potassium and biotin. One possible major nutrient control at *start* is thought to be through the adenosine 3′-5′ cyclic monophosphate (cAMP) pathway which relies on the sensing of glucose in the medium surrounding cells. Although this may be a major control pathway there are alternative pathways which rely on the sensing of other nutrients such as nitrogen (Cooper, 1982) and possibly the ability to synthesize certain organelles which may be dependent on a wider variety of nutrients. Glucose in the medium surrounding cells is sensed via *CDC25* which directly controls *RAS1* and *RAS2*. The *RAS* genes in turn control the activity of the *CYR1* gene. The protein product of this gene is in turn responsible for the production of cAMP. Low levels of cAMP due to the presence of a mutant *cyr1* gene cause arrest in G1. This is also true if the levels are lowered due to lack of glucose in spent medium. Cells containing this mutation arrest in G1 prior to the pheromone sensitive step (Matsumoto, Uno & Ishikawa, 1983). This shows the involvement of cAMP in control at *start*. How this control works is unclear although it may act through a phosphorylation cascade which involves *CDC28* (Mendenhall, Jones & Reed, 1987). Although there may be several control pathways they may operate through the same signal at *start*. Nutrient status is also connected with another parameter which is assessed at *start*, that of cell size.

1.4 CRITICAL SIZE AND MODELS FOR SIZE CONTROL

Fantes (1977), showed that dividing *Schizosaccharomyces pombe* cells dividing under fixed conditions display a constant cell size. This also applies to *Saccharomyces cerevisiae.* To maintain this consistency of cell size where growth is not linear, there must be a point in the cell cycle where the accumulation of mass and the mitotic cell cycle are coordinated. Coordination is demonstrated in cultures with slow growth rates by the phenomenon of asymmetrical division. This is when the daughter cell is smaller at birth than the parent cell although the overall size of cells in the population does not decrease. To maintain population size the daughters have consistently longer cycle times than the parents. The phase of growth which is extended is G1, the budded phase of parents and daughters remaining approximately the same. At faster growth rates the parent and daughter are equal in size at cell separation and consequently cycle times are much closer (Hartwell & Unger, 1977; Johnston *et al.*, 1977; Carter & Jagadish, 1978; Lord & Wheals, 1980; 1981) (see Figure 1.3). This indicates that cell size is being assessed somewhere in G1 and a critical size must be attained before the mitotic cycle can go ahead.

Investigation has shown that the mean cell size of a population is affected by growth rate. An increase in growth rate causes a rise in the mean cell size of the population and *vice versa.* This is only true down to specific growth rates at which point cell size becomes independent of growth rate and a minimum cell size at bud initiation is maintained (Johnston *et al.*, 1979; Tyson *et al.*, 1979). Data from these investigations also indicate that a critical cell size must be

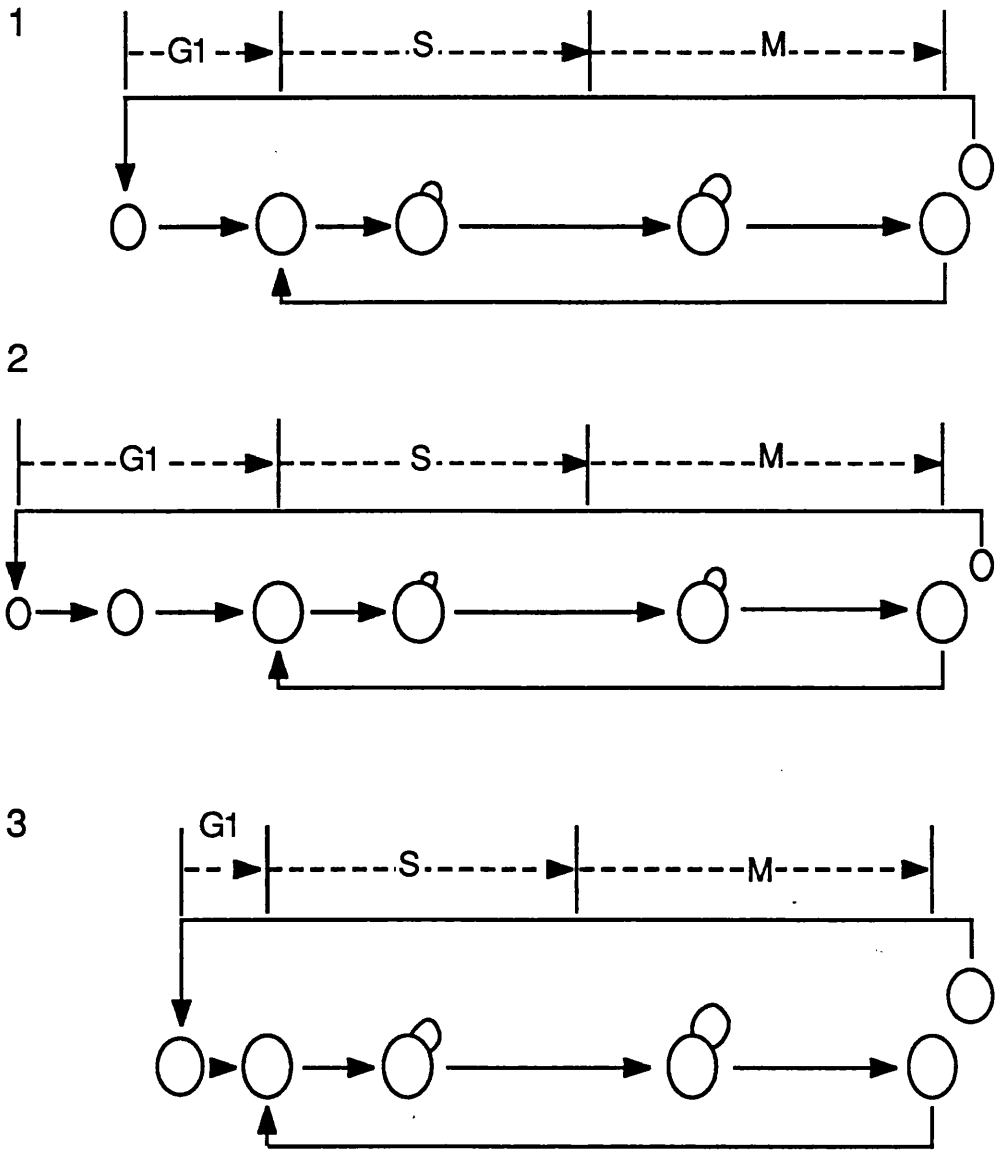


FIGURE 1.3 Affect of Growth Rate on Length of G1 Period for Daughter Cells

The rate at which cells grow affects the length of time the daughter cells spend in G1. If the S and M periods are fixed the amount of mass a daughter aquires is dependent on how fast cells are growing. With 1 as balanced growth, at a slower growth rate (2) the daughter is smaller at cell separation so must spend longer in G1. At faster growth rates (3) G1 is reduced as mother and daughter are closer in size at cell separation.

achieved before initiation of a cell cycle. Even under conditions of severely limited growth, attainment of a critical size is still a prerequisite for initiation of a new cycle. Cells grown on a medium containing no nitrogen soon exhibit nitrogen starvation and produce daughter cells which are abnormally small. Even so, young cells which initiate division had the size range consistent with that of cells growing exponentially in media containing a nitrogen source (Johnston, 1977).

The attainment of a critical size is shown very clearly by the arrest of nutrient deprived cells. Such cultures demonstrate the two fundamental principles involved in coordinating continuous and discontinuous processes, these being:

i. Growth rather than progression through the events of DNA replication and mitosis is usually rate limiting for cell division.

ii. Attainment of a critical size must be achieved before passage through *start* can be carried out (Johnston, 1977).

On inoculation into fresh media arrested cells grow and attain the size necessary for passage through *start* , followed by division. Even though critical size theory is widely accepted as a method of cellular coordination, exactly how cells assess their size is unclear although several models have been put forward to explain the phenomenon.

Yeast demonstrates that cell size is a function of the number of nuclear equivalents of DNA present. This basically means that diploids are twice the size of haploids. This feature gives rise to the term "nuclear-cytoplasm ratio", a ratio which generally remains

the same regardless of natural, usually stable ploidy changes. All the models put forward for general size control must fit three criteria. These have been proposed on the observations of the behaviour of cells under various experimental conditions. Not all these criteria are applicable or testable in yeast but are general rules:

(i) Mitosis occurs at a controlled time in order to maintain the nuclear-cytoplasm ratio and is able to compensate for any fluctuations in this. Fluctuations may take the form of delaying mitosis, removal of cytoplasm from cells or the removal of nuclei.

(ii) The element of control is a diffusible cytoplasmic agent and its effect is proportional to the amount of cytoplasm present.

(iii) Nuclei in a common homogeneous cytoplasm undergo mitosis synchronously. Mixing nuclei from different intermitotic phases in a common cytoplasm will result in a hybrid, whose nuclei will undergo mitosis at the same time, this being intermediate between those of the unfused controls (Fantes *et al.*, 1975).

The models which fit these three criteria fall into two classes. Concentration models and structural models. The mathematics of how the models operate will not be discussed but the simple mechanics of each and any relevance to *S. cerevisiae* will be covered. The concentration models are of three types; simple concentration, linear exponential and unstable activator/inhibitor. Structural models all have the same concept but actual sub-units may change.

All the following models are from Fantes *et al.*,1975 and Wheals & Silverman, 1982. The simple concentration models involve a single

substance which changes in concentration at a rate which is proportional to the mass of the cytoplasm until a triggering concentration is reached. The change in concentration is discontinuous and is obviously proportional to the number of nuclei present. The substance may be an activator of mitosis, therefore concentration will rise with growth or an inhibitor in which case it will be diluted with growth or may be actively degraded, both of which mean concentration will fall with growth. If the substance is an activator, at or closely following mitosis each nucleus or genome equivalent removes a constant amount of effector or it may be diluted by the splitting of the cytoplasm between cells.

The linear exponential model differs from simple concentration models in that change in concentration of the effector substance is continuous. It in fact increases at a constant rate proportional to the number of nuclei or equivalents. The model involves another substance whose purpose is to titrate the amount of effector present or remove it from the system in some way. The titrator substance is produced exponentially in proportion to cell mass and mitosis is triggered when the effector concentration is equal to the concentration of titrator. The effector is proposed to be an inhibitor whose rate of synthesis doubles following mitosis.

As with the above model the unstable inhibitor model involves an effector which inhibits mitosis. The absolute amount of the effector is at all times proportional to the number of nuclei or equivalents. The amount made by the nucleus compared with the nucleus remains the same but because the cell grows the actual concentration in the cytoplasm drops. When a critical low value is reached the cell undergoes mitosis. As production of the effector is constant it must

have a half-life which is independent of the concentration. A similar model is the unstable activator model. In this model an effector is synthesised at a rate proportional to cell volume and is degraded at a rate proportional to its concentration as well as the number of genome equivalents present. As with the unstable inhibitor model mitosis occurs when a critical concentration is reached.

The structural model depends on the synthesis of sub-units of a mitotic structure. The production of the sub-units is at a rate which is proportional to cytoplasmic mass. Mitosis is triggered when a certain number of sub-units has accumulated and the mitotic structure may be completed. The structure is destroyed during mitosis, in effect removing all sub-units. One candidate for this structure is the spindle pole body (Dutcher & Hartwell, 1983).

As yet the exact mechanism in yeast by which cells control their size is not known. With mechanisms of this sort a good method for gaining some insight into the way they function is to obtain mutations in them. By isolating mutant alleles and sequencing them, some light may be shed on the mechanism. To this end mutants have been isolated which are deficient in cell size control.

1.5 THE *whi1* MUTATION

Cell size can be affected by many factors. Usually the effect of growth defects and some auxotrophies is to make cells larger. An auxotrophic mutation in the adenine pathway causes larger cells when compared with strains which are isogenic except for this mutation. Anything which will delay the completion of an event will lead to late division and therefore lead to larger cells. Mutations in regulatory genes can lead to acceleration of events, making cells

smaller. This type of mutation was sought. Small size mutants were isolated using a synchronous culture of mutagenized cells and the effect of alpha factor. It was postulated that if initially small cells were taken and allowed to grow, cells with defective size control would bud at a smaller size then wild type cells. It was found that if alpha factor was added when cells were approximately 38um^3 this would result in arrest and growth into excessively large cells, but cells defective in size control would have already budded and therefore would remain or be observed as small cells (Sudbery *et al.*, 1980). Using this method originally nine small size mutants were isolated, none from the same mutagenized culture to avoid the isolation of clones.

All the isolated mutants have been examined in detail and cell size when measured by a variety of methods was found to be approximately one half the parent strain (Sudbery *et al.*, 1980; Lord & Wheals, 1984). Parameters that have been measured include protein content, volume and dry mass. On crossing mutant strains with parent or other wild type strains the small size phenotype segregated 2:2 for all tetrads. This indicated that each of the mutants was due to a single nuclear gene mutation. When small sized phenotype progeny from these crosses were mated amongst each other and sporulated, although at least 20 tetrads for each intermutant cross where examined, no wild type recombinants were observed. This indicated that all mutations probably affected a single gene although two closely linked genes could not be ruled out.

The phenotypic characteristics of crosses within mutant strains and between mutants and wild type cells has led to speculation over the number of genes involved in this particular size control mechanism.

Heterozygous diploids produced by crossing the mutant and wild type cells resulted in cells with a volume which was intermediate between wild type haploid and diploid cells. This indicated that the mutant gene was semi-dominant to the wild type. Ratios of cell size of various genotypes indicated that the gene showed dosage effects; mutant haploids: wild type haploids: homozygous mutant diploids: heterozygous diploids: homozygous wild type diploids showing the size ratios of 1:2:2:3:4. The fact that homozygous mutant diploids were twice the size of the haploid suggested that there was still an element of size control present. This was confirmed by the observation of asymmetrical division, accompanied by the expected difference between parent and daughter cycles times in cells containing the *whi1* mutation. This also inferred that control was still exerted in the unbudded phase (Lord & Wheals, 1984). A feasible model for the explanation of size ratios and gene dosage effects was that there were two different genes operating in tandem to control cell size. The *whi1* mutation described a mutation in one of the genes which resulted in loss of function, the other remaining functional. This could be confirmed by isolation of mutants in the second gene. However this could not be detected by complementation using the *whi1* mutation because diploids still retained two functional genes and these would result in the size characteristic of the homozygous *whi1* diploid. Recombination between the mutant genes could result in the isolation of the second gene (Sudbery *et al.*, 1980) although this would be difficult, especially if they were close together. An alternative model to this is that the same gene is present in two copies per haploid complement rather than there being two different genes. The small size mutants containing one defective copy and one functional copy, resulting in the retention

of some control (Lord & Wheals, 1984).

A further model suggested that not only did the *whi1* mutation still exert control at the initiation of DNA synthesis, but also at or before nuclear division. This model was based on observations from media shift experiments. Cells containing the *whi1* mutation differed from wild type cells in three respects:

(i) On shifting from poor to rich media the percentage of cells budding in exponential growth increased rapidly in cultures containing the *whi1* mutation whereas it decreased in wild type cultures.

(ii) Cultures containing *whi1* budded at the size characteristic of poor media. This was not seen with wild type cells, which grew to the size characteristic of cells grown for several generations in rich media before budding.

(iii) In *whi1* cultures when the bud attained the size of the parent both bud and parent increased in size, only buds increased in size in wild type cultures (Wynne & Carter, described in Carter *et al.*, 1983).

Based on these observations, it was suggested that cells containing *whi1* must grow to a critical size before completing nuclear division. Further investigations of growth on various media, analysis of nuclear morphology in stationary phase, bud scar analysis and execution point analysis in coupling with a *cdc* mutation gave inconclusive evidence to this hypothesis. Many of the results from this investigation did indicate that control is still exerted in the unbudded phase in *whi1* cells (Rowley, 1986).

All of these models and work were based on a false assumption that

has come to light only recently with molecular manipulation of the *WHI1* gene. The *whi1* mutation does not describe a non functional gene but a hyperfuntional gene (Cross, 1988; Nash *et al.* , 1988). This conclusion is based on data from the production of cells null for the *WHI1* gene. In two cases the gene was destroyed by the insertion of another gene within the coding sequence. The null phenotype is larger cells. If a non functional gene results in larger than wild type cells then the small phenotype observed in *whi1* cells must be due to gain of or hyperfunction. This work will be discussed further in the introduction to Chapter 5. The work was carried out on independently isolated mutations, isolated in completely different ways. One of the isolation methods revealed another phenotype associated with the *whi1* small phenotype. Wild type cells of one mating type arrest in G1 when they come into contact with the mating pheromone of cells of the opposite mating type. *Whi1* cells no longer arrest. *Whi1* MATa cells are resistant to alpha factor. The mutants were originally isolated due to their dominant alpha factor resistance (Cross, 1988). This interaction between the mating pheromone system and size control has implications for control at *start*.

1.6 THE MATING PHEROMONE SYSTEM

Cell type in the yeast *S. cerevisiae* is controlled by the mating type locus which exists in two allelic forms; *MAT***a** and *MATalpha* (Herskowitz & Oshima, 1982). Haploid cells as well as containing one of these alleles in the active form have further copies of both alleles which exists as silent cassettes, *HMR* and *HML*. These genes control the expression of **a** and alpha specific genes. The alpha specific genes are turned on in alpha cells by the *alpha*1 protein

which is one of two proteins encoded by the *MATalpha* locus. In **a** cells the **a**-specific gene products are synthesised constitutively. This results in a need to turn these genes off in alpha cells. This is carried out by the second gene product from the *MATalpha* locus, *alpha2*. When cells fuse to form diploids, the a1 and *alpha2* gene products combine to turn off all the mating type specific genes and enable the genes required for meiosis and sporulation to be expressed

Cells only mate when unbudded in the G1 phase of the cell cycle. Both *MAT***a** and *MATalpha* cells produce pheromones which are secreted and arrest cells of the opposite mating type in G1 (Bucking-Thröm *et al.*, 1973). *MATalpha* cells produce alpha factor, a peptide of 13 amino acids. The **a** pheromone is also processed before secretion but is 11 amino acid residues in length. The mating pheromones bind to receptors on the surface of cells forming a complex. This complex is then thought to be internalised (Chvatchko *et al.*, 1986).

A series of mutations have been isolated which render the cells sterile (MacKay & Manney, 1974a). These *ste* mutations are of two classes; those which do not receive the signal from the pheromone and those which do receive the signal but are unable to transduce or respond to it. It is known that *STE2* and *STE3* code for the receptors found on the surface of **a** cells and alpha cells respectively (Jenness *et al.*, 1986; Hagen *et al.*, 1986; Nakayama *et al.*, 1985). Mutations in these genes are mating type specific and prevent cells from receiving the signal from any mating pheromone present (MacKay & Manney, 1974b). Other *ste* mutations interfere with normal pheromone response in different ways. Activation of the pheromone system leads to the transcription of specific genes. The expression

of genes encoding agglutinins (glycoproteins found on the surface of cells which aid agglutination of cells) and the mating pheromone are enhanced and the systems for arresting the cell in G1 and for recovery and return to normal growth are both activated. *STE* mutations can occur in these systems. *STE4*, *STE18* and *STE5* are thought to play roles in the transduction of the signal from the receptor to the nucleus (Whiteway *et al.*, 1989). It is thought that along with *SCG1* (also known as *GPA1*), *STE4* and *STE18* form a G-protein system analogous to that found in higher eukaryotes for the transduction of signals from cell surface receptors (Nakafuku *et al.*, 1987 & Whiteway *et al.*, 1989). Mutations in *STE12* are known to reduce the transcription from several genes required for mating (Fields & Herskowitz, 1985). Although not strictly *STE* genes, other mutations which interfere with the normal mating process occur in *BAR1* (formally *SST1*) and *SST2* genes. *BAR1* encodes a protease which specifically degrades alpha factor. This gene product along with the *SST2* gene product is thought to be part of the recovery system. Mutations in these two genes make cells supersensitive to alpha and a factor (Chan & Otte, 1982). Mutations in *BAR1* are mating type specific whereas those in *SST2* affect cells of both mating type. Another mutation which is mating type specific is in the *ARD1* gene. Mutations in this gene lead to the inability of **a** cells to respond to alpha factor. The cells are also blocked in their ability to enter stationary phase and to sporulate (Whiteway & Szostak, 1985). Although the gene is not essential, the mutant allele causes a defect in the expression of all **a**-specific functions and is thought to have been involved in primitive mating response (Whiteway & Szostak, 1985).

The *whi1* mutation has some features in common with *ard1* in that

cells do not arrest when exposed to alpha factor and it is also not essential. Whereas *ARD1* seems to show some common control between mating and nutrient response, *whi1* seems to link mating response and size control. *Whi1* cells do have receptors for alpha factor so the fault in response to alpha factor is probably in signal transduction. In shedding some light on the size control system in *S. cerevisiae* it is possible that more will become known about the way the arrest mechanism in mating response works as well.

1.7 AIMS OF THE PROJECT

The initial aim of the project was to map the *whi1* mutation to a chromosome, and then to close map it on that chromosome with a view to cloning it. Although it was hoped that some clues could be gained as to how the size control system works from sequencing the cloned gene, interactions with other genes involved in the cell cycle were also hoped to give indications and double mutants were made and investigated.

CHAPTER 2: MATERIALS AND METHODS

2.1 STRAINS

Various haploid strains of the budding yeast *Saccharomyces cerevisiae* were used . The yeast strains are laid out below with their genotype and where they were obtained from. Also shown are the details of *Escherichia coli* (*E. coli*) strains used in this project

Strain	Mating Type	Phenotype	Source
ISO19	alpha	*whi1 ade1 leu2 met2 ura3 his4 trp5 lys2 GAL1*	P.S.
X4003-5B	a	*WHI1 ade1 leu2 met2 ura3 his4 trp5 GAL1*	P. S.
S67-3a	a	*whi1 lys2*	P. S.
XS122-57D	a	*rad52-1 ura3*	P. J.
XS122-49C	alpha	*rad52-1 leu2*	P. J.
XS195-23B	a	*rad52-1 his7 leu2 trp1 ade5 arg4 ilv3 lys7*	P. J.
XS214-1B	a	*rad52-1 leu2 trp5 arg4 his6 ilv3 ura1 lys9 ade2 met2*	P. J.
XS209-11C	a	*rad52-1 leu2 trp1 his3 ura4 met10 ade4*	P. J.
XS206-9B	a	*rad52-1 leu2 his7 lys2 met6 ade2 arg1*	P. J.
XS194-23C	a	*rad52-1 ade1 trp1 ura3 his2 leu1 arg4 aro7*	P. J.
NF20	a	*whi1 rad52-1 lys2 leu2*	N. S.
NF23	alpha	*whi1 rad52-1 lys2 leu2*	N. S.
NF24	alpha	*rad52-1 met1 leu1 trp5*	N. S.
XS19-2A	a	*whi1 rad52-1 ura3 trp5 lys2 met2*	N. S.
RC631	a	*sst2-1 rme ade2 his6 met1 ura1 can1 cyh2*	R. C.

MIS1	alpha	*sst2-1 rme his6 met1 can1 cyh2*	N. S.
395	a	*cdc19-1 ade1 ade2 his7 ura1 lys2 tyr1 GAL1*	L. H.
2597	a	*mak16-1 ade1 ura3 trp1*	R. W.
B-614	a	*cyc3 his1 trp2 lys2*	F. S.
182-6-3	a	*cdc24-1 ade his ura1 trp arg4 tyr1 thr4 GAL1*	L. H.
JW4-5C	a	*cys1-3 CUP1 cys3*	YGSC
ISO8B	alpha	*mak16-1 whi1 ade1 ura3 trp1 lys2*	N. S.
ISO19a	a	*whi1 ade1 his4 met2 ura3 lys2*	N. S.
322	a	*cdc28-15 lys1 tyr1 cyh1*	J. D.
XS122-57D	a	*rad52-1 ura3*	P. J.
XS122-49C	alpha	*rad52-1 leu2*	P. J.
XS195-23B	a	*rad52-1 ade5 leu2 his7 trp1 lys7 arg4 ilv3*	P. J.
XS194-23C	a	*rad52-1 ade1 leu1 his2 ura3 trp1 arg4 aro7*	P. J.
XS206-9B	a	*rad52-1 ade2 ade4 leu2 his7 met6 lys2 arg1 ilv3*	P. J.
XS209-11C	a	*rad52-1 ade4 leu2 his3 met10 ura4 trp1*	P. J.
RC631	a	*sst2 rme ade2 his6 met1 ura1 can1 cyn2*	R. C.
RC757	alpha	*sst2 rme his6 met1*	R. C.

Key to sources

P.S. - Peter Sudbery

L.H. - Lee Hartwell

R.W. - Reed Wickner

F.S. - Fred Sherman

R.C. - R. K. Chan J.D. - Dick Dickinson

P.J. - P. Hanic-Joyce N.S. - Constructed by Author

YGSC - Yeast Genetic Stock Centre.

Yeast strains used in this investigation. All strains obtained from P. Hanic-Joyce were constructed for the original study of this method by Schild & Mortimer (1985).

STRAIN	GENOTYPE	SOURCE
LE392	F⁻, *hsdR514*(rk, mk), *supE44*, *supF58*, *lacY1* or delta(*lacIZY*)6, *galK2*, *galT22*, *metB1*, *trpR55*, lambda⁻	John Beeching
HB101	F⁻, *hsdS20*(rB, mB), *recA13*, *ara-14*, *proA2*, *lacY1*, *galK2*, *rpsL20*(Smr), *xyl-5*, *mtl-1*, *supE44*, lambda⁻	N.C.T.C.

E. coli strains used in this investigation.

2.2 MEDIA

YEPD

1% yeast extract, 2% mycological peptone, 2% glucose. 2% agar was added to solid medium.

Synthetic complete medium (S.C.)

0.67% yeast nitrogen base without amino acids, 4% glucose. 1.5% agar was added to solid medium. Nutrient supplements were added to selection and drop out media to give the following final concentrations:

Adenine	20mg/l
Uracil	20mg/l
Tryptophan	20mg/l
Histidine	20mg/l
Methionine	20mg/l
Tyrosine	30mg/l
Leucine	30mg/l
Isoleucine	30mg/l
Lysine	30mg/l
Phenylalanine	50mg/l
Aspartic Acid	100mg/l
Valine	100mg/l
Threonine	200mg/l

These were added to media before autoclaving with the exceptions of aspartic acid and threonine which were filter sterilised and added after autoclaving.

Presporulation Medium

10% glucose, 0.8% yeast extract, 0.3% mycological peptone, 2%

agar(lab M)

was added to solid media

Sporulation Medium

1% potassium acetate, 0.1% yeast extract, 0.05% glucose, 20mg/l lysine. 2% agar was added to solid medium.

Lactate Medium

0.67% yeast nitrogen base without amino acids(Difco), 2.5% agar. After autoclaving, 33mls/l of a filter sterilized 30% (v/v) lactic acid solution (pH 5.0) was added.

Chlorolactate Medium

1% yeast extract, 2% mycological peptone, 3% glycerol (v/v), 0.1% Beta-chlorolactic acid. 1% Agar was added to solid medium. The chlorolactate was dissolved (100mg per 1ml) in 0.1M K_2HPO_4, then filter sterilised and added after autoclaving.

LB (Luria-Bertani) Medium

1% bacteriological tryptone (Difco), 0.5% yeast extract, 1% sodium chloride. 2% agar was added to solid medium.

Antibiotics were added to a final concentration of:

Ampicillin	35-50ug/ml
Chloramphenicol	30ug/ml (bacterial selection)
	170ug/ml (plasmid amplification)
Tetracycline	12.5-15ug/ml

Magnesium ions were added to a final concentration of 10mM in media used to produce lambda lysates.

2.3 SOLUTIONS

Alkaline Sodium Dodecyl Sulphate

Equal volumes of sterile; 0.4M NaOH, 2.0% sodium dodecyl sulphate solution.

Dithiotreitol (DTT) Buffer

1.2M sorbitol, 25mM EDTA pH 8.0, 50mM dithiotreitol (DTT). The DTT was filter sterilised and added to the other components immediately prior to use.

Denhardts Solution

Stock solution (X5): 0.1% bovine serum albumen (BSA), 0.1% ficoll 400,000, 0.1% polyvinyl pyrollidone 40,000. All were mixed in sterile SSC (see later in solutions) and stored frozen until needed.

Frozen Storage Buffer (Competent Cells)

0.1M potassium chloride, 45mM $MnCl.4H_2O$, 10mM $CaCl_2.2H_2O$, 3mM $HACoCl_3$, 10mM K-MES. The final pH of the solution was 6.2. The salts were added to the filter sterilized K-MES solution, dissolved and refilter-sterilized.

Gel Loading Buffer

Stock solution (X6): Store at 4°C; 0.25% bromophenol blue (Sigma), 0.25% xylene cyanol (Sigma), 30% glycerol in H_2O.

Isotonic Sucrose

25% sucrose, 50mM Tris.HCl pH 8.0.

Ligation Buffer

Stock solution (x10): 2.5mM Adenosine triphosphate (ATP) (Sigma),

20mM DTT, 2mM EDTA pH 8.0, 10mM spermidine, 1mg/ml BSA. This was filter sterilized and stored at -20°C.

Ligation Salts

Stock solution (x5): 150mM NaCl, 150mM Tris.HCl pH 7.5, 38mM $MgCl_2$. This was autoclaved and stored at -20°C.

Lysis Buffer (Bacterial Extraction)

50mM Glucose, 10mM EDTA, 25mM Tris.HCl pH 8.0. This can be stored at room temperature. Lysozyme was added immediately before use at a concentration of 2mg/ml.

Lysis Buffer (Yeast Extraction)

50mM Tris.HCl pH 8.0, 0.02M EDTA, 1%(w/v) SDS. The pH is adjusted to 12.45 at 23°C with 1M NaOH.

Oligo Labelling Solution A

2M Tris.HCl pH 8.0 625ul, 5M $MgCl_2$ 25ul, dATP 5ul, dGTP 5ul, dTTP 5ul. Each of the triphosphates was dissolved in 3mM Tris.HCl, 0.2mM EDTA, pH 7.0 at a concentration of 0.1M.

Oligo Labelling Solution B

2M HEPES titrated to pH 6.6 with 1M NaOH and stored at 4°C

Oligo labelling Solution C

The hexadeoxyribonucleotides were evenly suspended in 3mM Tris.HCl, 0.2mM EDTA, pH 7.0 at 90 OD units/ml and stored at -20°C.

Solutions A, B and C were mixed in the ratio of 2:5:3 before use.

Oligo Labelling Stop Solution

20mM NaCl, 20mM Tris.HCl, 2mM EDTA, 0.25% SDS.

Pretreatment Buffer (Yeast Extraction)

0.2M Tris.HCl pH 8.0, 1.2M sorbitol, 0.1M EDTA, 0.1M beta-mercapto-ethanol pH 9.1.

SCE Buffer (Yeast Extraction)

1.0M sorbitol, 0.1M sodium citrate, 60mM EDTA pH 5.8.

SM (Phage Storage Buffer)

0.1M NaCl, 10mM $MgSO_4.7H_2O$, 50mM Tris.HCl, 0.01% gelatin.

SSC

Stock solution (X20): 3.0M NaCl, 0.3M trisodium citrate.

TBE Electrophoresis Buffer

Stock solution (X10): 0.45M tris borate, 0.45M boric acid, 0.05M EDTA.

TE

10mM Tris.HCl, 1mM EDTA. The pH of this solution was dependent on the pH of the Tris.HCl.

TNE

Stock (X10):1.0M Tris.HCl, 500mM NaCl, 50mM EDTA. Again the pH of this solution was dependent on the pH of the Tris.HCl.

Triton-X100 Solution

0.2% Triton-X100, 50mM Tris.HCl pH 8.0, 62.5mM EDTA pH 8.0.

Universal Restriction Enzyme Digestion Buffer

Stock solution (X10): 0.33M tris acetate, 0.66M potassium acetate, 0.1M magnesium acetate, 0.04M spermidine, 5mM DTT. RNAse A (Sigma) (DNAse free) was added at 100ug/ml in stock solution. The buffer was stored at -20°C.

The above reagents were from Sigma, BDH or Lab M unless indicated.

All reagents for DNA manipulations should be either Analar (BDH Chemicals) or Ultra Pure (BRL).

2.4 BASIC YEAST METHODS

Mating and Selection of Diploids

Usually matings were carried out on YEPD at 25°C for approximately 12 hours by streaking heavily at 90° to each other. After incubation, cells were taken from the area where the two were actually touching. These were streaked onto selective media which would allow only diploids to grow. When no diploids resulted from this type of crossing, liquid mating was carried out. In this case, both strains were grown in liquid YEPD to exponential phase, half of each of these cultures were mixed and a further 100 mls of fresh YEPD added. This mixed culture was then grown with shaking at 25°C for 12 hours. Diploids were selected from this by plating out these cells onto selective media. If mating was still not achieved from this type of mating then alpha factor was added to the MATa cells in the liquid culture one hour before the cultures were mixed.

Selection of diploids was achieved using the complementation of auxotrophic markers (or forced crossing). The strains which were crossed usually contained a range of such markers with at least one marker difference. On Strains which must be mated but do not have unique auxotrophic markers can be mated in the same way. Diploids can be identified by the lack of production of mating pheromone (see mating type testing).

Tetrad Dissection and Analysis

Before the position of genes on chromosomes can be assessed by tetrad analysis, individual colonies must be produced from each of

the individual meiotic products of a mating. This was achieved by tetrad dissection and production of pure spore clones. The four spores of a meiotic event are naturally protected by the ascus wall. This was digested using beta-glucuronidase (Sigma H-3AF Crude Solution). One drop of beta-glucuronidase was placed in a sterile Eppendorf. A loopful of sporulating culture was then mixed with it to give an even suspension. The mixture was then incubated at 37°C for between 20 and 30 minutes depending on strain. Full digestion of the ascus wall was checked by eye using a microscope. Once this had been achieved dissection of the ascus was carried out. Only intact, four spored asci were dissected. These were usually found as a cruciform or intact pyramid although other shapes were seen they were usually the result of single spores coming together.

Once a number of intact tetrads have been broken open and their contents had been separated out the agar square on which they were spread was cut from the main plate and placed in a smaller sterile plate. This cut down loss caused by contaminating fungi. The spores were allowed to germinate at 25°C or 30°C, depending on their genetic make-up and grow into small colonies. If full tetrad analysis was to be carried out only asci where all four spores germinated were scored. The small spore clones were inoculated onto fresh plates and allowed to grow before being tested for mating type, chromosome markers and auxotrophies. Once a large enough number of asci had been scored the results were used to estimate map distances using maximum likelihood equations.

Random Spore Analysis (After Dawes, 1974)

This method frees spores and kills vegetative cells so that the

majority of colonies on a plate from a culture treated in this way are from individual spores. The general method is:

(1) Suspend a loop of sporulating culture in a single drop of beta-glucuronidase contained in a sterile Eppendorf.

(2) Incubate at 30°C for 25 minutes.

(3) Sonicate for 10 seconds to break open the asci.

(4) Place on ice and allow to cool (3 min).

(5) Add an equal amount of pre-cooled diethyl ether.

(6) Shake on ice for exactly one minute.

(7) Remove 100ul and add to 400ul of sterile liquid YEPD.

(8) Spread 100ul aliquots onto fresh YEPD plates.

(9) Incubate at 25°C or 30°C depending on genetic makeup.

(10) Pick off only small colonies for characterisation.

Auxotrophic mutants which give a colour change (*ade1*, *ade2*) can be use to give an indication of the number of haploids, but usually any of the smaller colonies taken will be haploid.

Characterisation of Markers

CELL SIZE

This was generally assessed by eye using a microscope with a X100 fold magnification. This was done by suspending a small amount of cells, from the edge of a growing colony, in a drop of half strength YEPD on a clean microscope slide. After placing on a coverslip the slide was examined with reference to parental strains. The cells can not only be distinguished by their size but also by their shape. The *whi1* strain used presented a uniform morphology throughout the population. All the crossing strains used showed a variety of shapes from round to cigar shaped within a sample from a single colony. Most showed the usual pear shaped cells. Having permanent fixed

slides of the parental strains was useful as an aid to size determination.

If size determination was difficult by this method, and as a random check of cultures that had been assessed, growth in liquid culture and electronic determination of size was carried out as follows: 20mls of liquid YEPD was pipetted into a sterile 100ml flask. A small amount of growing colony was added using a sterileloop or toothpick. This was grown overnight shaking at 25°C (to give a population of exponentially growing cells). A sample (0.8mls), was removed and sonicated for ten seconds. Cell size was then measured using an Electrozone Celloscope LT-III electronic sizer and counter which had been previously calibrated using latex particles (Sigma). The parental strains were used as controls.

TEMPERATURE SENSITIVE MARKERS

For all the strains which contained these mutations, the permissive temperature was 25°C and the restrictive was 36°C. Colonies which were to be tested were re-streaked out for single colonies on two YEPD plates. These two plates were then incubated, with control strains at either the permissive or restrictive temperature for two days and then scored.

AUXOTROPHIC MARKERS

Although replica plating, using a multipoint inoculator and the appropriate drop-out media, can be used to score this type of marker, streaking out for single colonies is more stringent and gives clearer results. All parents and progeny strains were therefore tested in this way. This method was also used for the *cys1-3* mutation using the appropriate medium. Cells containing the

cyc3-1 mutation were streaked out on either lactate or chlorolactate plates. Cells containing this mutation are resistant to chlorolactate and will also grow on lactate plates. Wild type cells do not grow on either.

MATING TYPE

Parent and progeny strains were assigned mating type using strains of *S. cerevisiae* containing the *sst2* mutation which causes sensitivity to mating pheromones. Strains containing it will not grow in the presence of either of the specific **a** or *alpha* pheromones (Chan & Otte, 1982). Testing is carried out by seeding overlay agar (YEPD but with only 0.7% Lab M Agar No. 2) with the appropriate a or alpha factor sensitive strain. Strains which are to be scored for mating type are then inoculated into this overlay and the plates incubated overnight at 25°C. Growth of the strains being tested results in the release of mating pheromone which diffuses into the overlay. If the cells are a mating type and the tester strain an a tester then a zone of inhibition results around the growing colony. If the strain is *alpha* then no zone is seen. All strains were tested in this way. Approximately 30 colonies can be tested per plate.

COULTER COUNTER USE

Samples were taken from liquid culture as described in cell size methods. An aliquot of the killed sonicated cell suspension was taken and added to 50mls of an ultra filtered (pore size 0.2um) 0.1% sodium azide, 0.9% sodium chloride solution. This was then read by the Electronic Particle Counter (Particle Data Inc.). The final value used for cell number calculations was a mean of at least three such readings. To get actual cell density (particles/ml) the final

mean reading was multiplied by 5 (since only 200ul are measured), the dilution factor in the counting vessel (1/50) and the initial dilution factor (250). Background readings were taken before each sample was read to ensure consistency of results. Calibration of the cell sizer (Nuclear Data Inc.) was carried out using commercially available latex spheres (Sigma Chemicals, 5.7um ± 1.1um and Polysciences Inc. Microspheres, 4.45um SD=0.35). 2ul of the latex spheres were added to 50mls of saline for calibration. After thoroughly mixing this suspension, samples were analysed. Before each sample was taken, the I (aperture current) and G (amplification) settings on the machine were changed. As a range of these values was required (total range of X64) ideal values for the initial settings were I=1/4 and G=12 although this was dependent on the spheres used as a standard. As the I and G values were changed the channel number on the screen where the peak appeared (which corresponds to the calculated volume) was noted. When all the volumes and their channel values were noted a calibration curve was drawn. I, G and volumes are shown in Table 2.1. To estimate the size of cells sampled from growing cultures, the diluted sample was run through the machine with I=1/4 and G=12, the channel number where the peak occurs was then noted. The channel values were all read off the calibration curve to give the size of cell samples in um^3.

2.5 CHROMOSOME MAPPING METHODS

Chromosome Mapping.

Chromosome mapping was carried out using tetrad analysis. Various markers were chosen which appear on the chromosome in question and strains containing these were crossed with a strain containing the *whi1* mutation. The assortment of the markers with the *whi1* mutation were analysed using a computer programme based on maximum likelihood

I	G	IG	VALUE	VOLUME(μm^3)
1/2	48	24	8 x 4/3 Y	95.50
1/4	48	12	4 x 4/3 Y	47.76
1/4	24	6	2 x 4/3 Y	23.88
1/4	12	3	4/3 Y	11.94
1/4	3	0.75	4/3 Y - 4	2.99
1/16	6	0.375	4/3 Y - 8	1.49

TABLE 2.1

Calibration Table For The Particle Sizer

Shown are a range of I and G settings and volumes used to obtain a calibration graph for the estimation of the size of yeast strains. With each calibration a different peak channel was obtained for each of the combinations shown, so there are no values included in the above table.

estimates of the distance between the various mutations (Snow, 1979). The markers used are shown in Figure 4.3 with their position on the left arm of chromosome I. After pair- wise crossings had been attempted, a triple point cross was carried out in an attempt to locate the gene more accurately.

Chromosome assignment using the *rad52-1* mutation.

As this method would work perfectly well with the *whi1* mutation either in coupling or repulsion, strain construction was relatively simple. As repulsion strains are much easier to construct than coupling strains, these were constructed. IS019 was initially mated with XS122-57D which contained the *rad52-1* mutation. Haploid strains were selected which exhibited not only the *whi1* phenotype but also the mutant *rad52-1* (see plate 2.1).

The method used was essentially as described by Schild and Mortimer (1985). Fresh diploids were inoculated into 1ml YEPD and incubated for 2hr at 30°C. These cultures were then irradiated for either 35 or 45min (20 or 25Krads). From these cultures 70ul aliquots were withdrawn and plated out onto dry YEPD plates. These were incubated for approximately 5 days after which colonies were picked off. Sterile toothpicks were used to pick off colonies of all sizes and to inoculate new YEPD plates at 30 colonies per plate. This plate was used as a master copy for replica-plating 3 days after inoculation. The colonies were replica-plated onto various drop out media dependant on the markers present in the parent strains. The master plate was also plated onto fresh YEPD (for size assessment) and control plates. Drop out plates were scored after 4 days incubation at 30°C, the size having already been scored by eye.

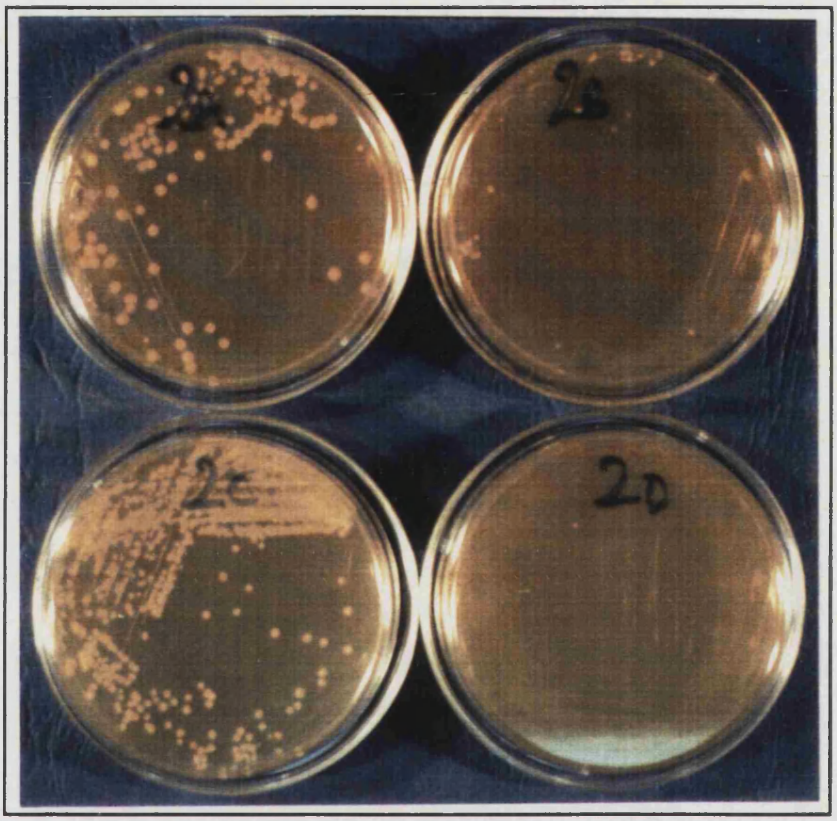


PLATE 2.1

Selection of Haploid Progeny Containing the *rad52* mutation

Four haploid progeny from a single ascus. The parents contained both the wild type gene and the *rad52* mutation. Progeny containing the mutant gene do not survive exposure to 50Krads of radiation (2B & 2D). This was used to construct double mutant strains (*whi1 rad52*).

Irradiation

A ^{60}Co fixed Gravator gamma radiation source was used. The dose rate had been determined with Fricke's dosimeter giving a value of 580 rads/min. A dose of 50Krads was used to score meiotic segregations for *RAD52* and *rad52*, with a dose of between 15-25Krads for chromosome loss induction. Diploids were inoculated into fresh YEPD (1ml) and incubated at 25°C for two hours before irradiation. Immediately after irradiation, cells were plated onto fresh YEPD or supplemented YEPD and incubated for 3-5 days before colonies were counted or sub-inoculated onto fresh media.

Determination of spontaneous chromosome loss in homozygous *rad52* diploids.

New diploids were used, as diploids stored for any length of time at 4°C may already have lost chromosomes, to inoculate 20mls of supplemented YEPD in a 250ml flask. This was incubated at 30°C shaking constantly. A sample was withdrawn immediately after inoculation and plated onto supplemented YEPD after appropriate dilution with fresh media. The culture was maintained by the inoculation of 0.1ml culture into 20mls fresh media every 24hr. Further samples were withdrawn, diluted and plated out at 24hrs, 72hrs and 170hr. The plates were incubated at 30°C for 3-5 days before 100 colonies for each sample time were tested for chromosome loss by the appearance of additional auxotrophies. This was shown by non growth on drop out plates. A control culture was also maintained and tested in the same way.

Determination of death rate caused by irradiation.

A new diploid was inoculated into 10mls YEPD and incubated for 2hrs

at 30°C shaking constantly. A sample was removed, and after dilution in fresh media, plated onto YEPD plates. Six samples of 1.5mls of the pre-grown culture were placed in sterile Eppendorfs and irradiated for 5, 10, 20, 30, 40 and 50 minutes. The samples were diluted appropriately and plated onto YEPD. All plates were incubated for 5 days at 30°C after which the number of colonies was counted. From these figures the number of cells/ml surviving after irradiation was calculated.

Determination of post-irradiation cell death due to non supplemented media.

This additional death rate experiment was carried out essentially as above but at a single time and using two types of media. Fresh diploids were inoculated into 100mls of YEPD and incubated for 3hr at 30°C shaking constantly. From this culture 2x2ml samples were withdrawn and the cells pelleted by centrifugation. The cells were resuspended in (i)1ml of YEPD and (ii)1ml of supplemented YEPD. From each of these control samples were withdrawn (4x100ul) and plated onto YEPD and supplemented YEPD respectively after dilution in appropriate media. Both resuspended samples were then irradiated for 25min after which four more samples were withdrawn (100ul) from each and plated, after dilution, onto either supplemented or unsupplemented YEPD. After incubation for five days at 30°C the number of red and total number of colonies were counted on each plate. The number of red colonies was used as an indication of the number of auxotrophic colonies present. The control plates were counted in the same way.

Determination of cell size of post radiation cells.

Cell size was determined by eye. The diploids (classified by eye)

produced for chromosome mapping were all homozygous for the *rad52-1* mutation. Heterozygosity at the *whi1* locus infers a size which is equivalent to 3 units (75-90um^3) (the *whi1* mutation being half that of a wild type gene conferring a size of 2 units). After irradiation as well as diploids, aneuploids should be observed with sizes of 2 (50-60um^3) and 1 (25-30um^3) units (equivalent to wild type and *whi1* size cells).

Fixed slides were made of all diploids from crosses as well as the *whi1* and wild type haploid parents. Samples for sizing were removed from the edge of overnight colonies with sterile toothpicks and suspended in 1/2 x YEPD. They were examined under x400 magnification and comparisons made with the fixed slides where appropriate.

2.6 MOLECULAR BIOLOGY METHODS

DNA Extraction Methods.

SMALL SCALE EXTRACTION OF PLASMID DNA. This was achieved using a modified alkaline/SDS method (Birnboim & Doly, 1979; Smith & Thomas, 1983) from saturated overnight cultures grown in the presence of the appropriate antibiotic to maintain the plasmid. Plasmid DNA extracted by this method was pure enough for general use, although it was sometimes cleaned using DE52 (see DNA cleaning).

LARGE SCALE EXTRACTION OF PLASMID DNA. A modified clear lysate method (modified from Godson & Vapnek, 1973) was used to obtain large very purified samples of plasmid DNA for general used. All volumes given are for 240mls of saturated culture grown overnight with appropriate antibiotic selection. Cells were harvested by spinning at 4050g for 10min in 250ml plastic centrifuge pots in a DuPont Sorvall RC-5C refrigerated centrifuge at 4°C. The cells were resuspended in 12mls of isotonic sucrose and split (2 x 6mls) into 50ml centrifuge tubes. To each tube, 3mls of lysozyme was added (7.5mg/ml lysozyme in isotonic sucrose) which was mixed well but not by vortexing. The tubes were then incubated for 15min. on ice. After incubation 3mls 0.25M EDTA p.H. 8.0 were added and the tubes incubated for a further 5min. 12mls of Triton-X100 solution was then added to each tube by blowing in quickly. The tubes were then mixed by gently rocking at room temperature for 5min. or until the suspension had become translucent. The debris was cleared from this suspension by spinning at 27,000g for 15mins in the RC-5C at 4°C. The supernatant was poured immediately into an equal volume of phenol which had been equilibrated with milliQ H_2O (Millipore). This was mixed thoroughly by shaking and the phases separated by spinning

at 3000g for 5min. The upper aqueous phase was removed and extracted four times with diethyl ether. Excess ether was removed by placing the solution in a water bath at 65°C for 10mins. The DNA was precipitated by adding an equal weight of -20°C isopropanol, mixing well and placing at -20°C for 20min. The DNA was pelleted by spinning at 7100rpm for 10min after which all the supernatant was removed. The pellet was dried under vacuum and resuspended in 2.5mls 1 x TNE. To further purify the DNA it was equilibium banded in a caesium chloride gradient. For each DNA species 4.5mls of DNA solution was placed in an ultracentrifuge tube. Exactly 4.62gms caesium chloride was added to each tube and shaken until the caesium chloride had dissolved. 0.5mls Ethidium Bromide was added to each tube (10mg /ml in 1 x TNE) and they were capped and labelled. The tubes were topped up with mineral oil and weighed to within 0.01gm of each other. Grub screws were finally placed in the tubes, which were then placed in a pre-cooled rotor. The DNA was banded by spinning at 48,000rpm for 48hr in a fixed rotor using a MSE Europa 75M ultracentrifuge. The DNA was drawn off using syringes. The top band (chromosomal DNA) was drawn off first and discarded. The plasmid band was drawn off and washed with room temperature isopropanol saturated with caesium chloride until no pink colour was left in the DNA sample. The caesium chloride was removed by overnight dialysis against 1 x TNE. The DNA was finally precipitated by the addition of an equal weight of -20°C isopropanol and placing the sample at -20°C for 20min. The precipitated DNA was spun down at high speed (6000g) and vacuum dried after the removal of all supernatant. The DNA samples were resuspended in 100ul 1/10 TNE. This DNA needed no further purification and was of high concentration.

EXTRACTION OF *Schizosaccharomyces pombe* CHROMOSOMAL DNA. Total DNA was extracted from *Sc. pombe* by the method described by Durkacz *et al.*, 1985. *Schizosaccharomyces pombe* was grown up overnight in 100mls YEPD at 25°C to mid exponential phase. The cells were harvested by spinning at 4050g for 5min in a DuPont Sorvall RC-5C and resuspended in 5ml 20mM citrate phosphate buffer, 40mM EDTA, 1.2M Sorbitol pH 5.6. 0.2ml Glusulase (Sigma) was added and cells incubated for 45min at 37°C. Cells were harvested by spinning at 3000g for 5min (DuPont Sorvall RC-5C) and resuspended in 15ml of 50mM Tris (pH 7.4), 5mM EDTA (pH 7.5) and 1.5ml 10% SDS was added to lyse the cells. 5ml of 5M potassium acetate was added and the lysate precipitated on ice for 30min. Debris was cleared from the lysate by spinning at 27,000g for 5min in the RC-5C at 4°C. The supernatant was decanted though gauze into a 50ml centrifuge tube and 20ml propan-2-ol added. The DNA was precipitated at -20°C for 20min. The precipitate was recovered by spinning at 15,000g for 10min in the RC-5C and the pellet dried. After resuspending in 50mM Tris, 5mM EDTA (pH 7.5) 50ug RNAse was added and an incubation was carried out 37°C for 2hr. This was then extracted with phenol, phenol/chloroform and finally chloroform. The DNA was finally precipitated by the addition of 3M sodium acetate (to 0.3M final concentration) and two volumes 95% ethanol. The pellet was washed in 70% ethanol before final resuspension in 200ul 10mM Tris, 1mM EDTA pH7.5.

Transformation Protocols

TRANSFORMATION OF *E. coli*. Cells were routinely transformed using a calcium chloride method. An overnight culture of *E. coli* was grown shaking at 37°C in 50ml LB. 1ml of this was added to 50ml fresh medium the following morning and allowed to grow for 2hr at 37°C.

Cells were harvested by spinning at 4000g for 5min. and the pellet resuspended in 20ml ice cold 100mM $CaCl_2$. This cell suspension was left on ice for 1hr. The cells were spun down again at the same speed and then resuspended in 5ml ice cold 100mM $CaCl_2$. After a further 1/2hr on ice the cells were considered competent. Competent cells were frequently pre-prepared and stored in tranformation storage buffer or 100mM $CaCl_2$ with glycerol added to a final concentration of 20%. These would be defrosted before using and protocol followed from the DNA addition step only.

Once the cells were prepared actual transformation was carried out by the addition of 1-10ul of DNA (dependent on whether it was pure prep. DNA or mini prep.) to 100ul cells. This was left on ice for 30 min. The cells were then heat shocked for 2min at 42°C. 1ml of LB (at 37°C) was added to the cells and then placed at 37°C for 11/2hr. The cells were spun down in a microfuge for 10sec and resuspended in 100ul of LB before being spread on selective media.

ONE STEP TRANSFORMATION OF *E.coli*. This was used when few transformants were required, although the author (see below) states that yield was 7 x 10^4per ug DNA. The method was carried as described by Golue, 1988. 5ul of competent cells were mixed on ice with 1ul plasmid DNA in an Eppendorf. The tube was immediately transferred to 44°C for 1min after which 100ul of LB was added. After mixing the suspension was plated onto selective media.

Lambda Techniques

PRODUCTION OF PLATING CELLS. After some initial investigations it was found that *E. coli* strain LE392 gave the best results with the lambda phage containing the *whi1* gene (lambda C1a). Plating cells were produced by the following method. From a fresh overnight Luria-

Bertani (LB) plate a single colony was picked into 10mls liquid LB + 2% maltose. This was grown overnight at 37°C without shaking. 100uls of this culture (after resuspension) were transferred to a fresh 10mls LB + 2% maltose which was incubated at 37°C for 6hr with shaking. The cells were pelleted and resuspended in 5mls of 10mM Mg^{++} (Cl_2 or SO_4). This suspension was shaken for 30min at 37°C. The cells were then used for plating. They were stored for up to four weeks at 4°C.

PRODUCTION OF PRE-LYSATE STOCK AND ASSESSMENT OF PHAGE TITRE. 100ul of plating cells were added to 1ul of phage in a sterile Eppendorf. This was mixed thoroughly and 10-fold serial dilutions made, down to 10^{-6}. This was done using 90ul of plating cells with 10ul of the previous dilution in sterile Eppendorfs. The Eppendorfs were incubated for 15min at 37°C without shaking. The contents of the Eppendorfs were added to 3mls top LB agar (0.7% agar) which was kept molten at 45°C. The agar and cells were mixed by rolling. The top agar was then poured onto fresh thick LB plates and allowed to set. The plates were incubated upside down for 24hr at 37°C. The number of plaques per plate were counted and phage particles per ml calculated. The contents of two plaques were sucked up using an automatic pipette (20ul) and dispensed in 200uls of sterile SM in a sterile Eppendorf. Once sealed these were kept for 1-2 months at 4°C depending on phage. Another method of obtaining stock phage cultures used was to add 2mls SM to the incubated plates and place at 4°C for 15min. This prevented any agar from contaminating the phage. The SM was sucked off the plates and placed in sterile Eppendorfs and kept for 1-2 months.

SMALL SCALE LYSATES. 100ul of phage stock solution was added to

10mls LB + 10mM Mg^{++} in a 50ml flask. It was important that all glassware was acid washed as detergents appeared to adversely affect lysis. 100ul of plating cells was added to the flask and it was allowed to stand for 15min at 37°C. The flask was then incubated for 12-16hr shaking vigorously at 37°C. After incubation was complete chloroform was added to a final concentration of 0.1% and the flask was shaken for a further 10min. Lysates can be kept for up to 4 weeks with the addition of 10mM Mg^{++}. From lysates of this size small DNA samples were made.

LARGE SCALE LYSATES. These were made according to the method laid out by Maniatis *et al.*, 1982 and Kaslow, 1986. To a 500ml overnight lysate, chloroform was added to 2%. After 30min further shaking at 37°C, DNAse1 and RNAse were added to a final concentration of 1ug/ml. NaCl was then added to 1M the lysate incubated again for 30min at 37°C. Cell debris was removed by spinning at 6000g for 10min in a DuPont Sorvall RC-5C. The supernatant was recovered and polyethelene glycol (PEG) 8000 added to 10% weight/volume before placing at 4°C for 1hr. The phage particles were pelleted at 6000g for 20min at 4°C. The supernatant was removed and the pellet resuspended in 1ml TMN. DNAse and RNAse were added to 5ug/ml and 10ug/ml respectively and an incubation carried out at 37°C for 30min. The phage were lysed by the addition of SDS to 5%, EDTA to 20mM and Proteinase K to 100ug/ml. A further incubation was carried out for 30min at 68°C before extraction with an equal volume of phenol, phenol/chloroform and chloroform. The DNA was precipitated by the addition of 1/2 volume ammonium acetate and 2 total volumes of 95% ethanol. A microfuge was used to spin down the DNA at high speed for 15min. The pellets were dried completely before

resuspension in 1.6ml ddH_2O, 0.4ml 4M NaCl and 2ml 13% PEG 8000. This was placed on ice for 1hr before spinning at 10,000g in the RC-5C. All the supernatant was removed and the pellet washed with 70% ethanol before final drying. The DNA was resuspended in 1 x TE. The yeild was 1-2ug DNA per ml of lysate.

SMALL SCALE DNA PREPARATIONS. These were made from small scale lysis material. 800ul of a mini lysate was place in a sterile Eppendorf and DNase was added to 10ug/ml. This was incubated at room temperature for 15min. After incubation, 200ul 0.3M Tris.HCl (pH 9.0), 0.15M EDTA (pH 9.0) and 1.5% SDS was added. The contents were mixed by rocking and the Eppendorfs placed at 70°C for 15min. The Eppendorf was cooled to room temperature and 135ul 8M potassium acetate added. The contents were again mixed by rocking and the tubes incubated for 15min on ice. Debris was pelleted by spinning the Eppendorfs for 1min at high speed in a bench microfuge. 800uls of the supernatant was placed in a fresh sterile Eppendorf and 480ul -20°C isopropanol added. The contents were mixed well and left for 2min at room temperature. After pelleting by spinning for 1min at high speed in a microfuge, all the supernatant was carefully discarded. The pellet was washed with 70% ethanol and completely dried under vacuum. The pellet was resuspended in 50ul TE.

LARGE SCALE DNA PREPARATIONS. The method used was modified from Yamamoto *et al.*, 1970 and Maniatis *et al.*, 1982. Large lysed cultures (500mls) were cooled to room temperature and DNase and RNase added to a final concentration of 1ug/ml. After incubation for 30min at room temperature solid NaCl was added to a final concentration of 1M. This was dissolved by swirling and allowed to stand on ice for 1 hour. Debris was removed by centrifuging at

11,000g for 10min at 4°C. The supernatants were pooled and solid polyethylene glycol (PEG 6000) added to a final concentration of 10% w/v. The PEG was dissolved by slow stirring at room temperature on a magnetic stirrer. After the PEG had fully dissolved the phage mix was cooled on ice for at least one hour. The phage was pelleted by spinning at 11,000g for 10min at 4°C. All the supernatant was discarded and the tubes drained thoroughly. The pellet was resuspended in SM (8mls/500mls culture) using a wide mouthed pipette. An equal volume of chloroform was added to the phage suspension and vortexed for 30sec. The two phases were separated by centrifugation at 1600g for 15min at 4°C. The aqueous phase was recovered and the phage recovered by centrifugation at 25,000rpm for 2hr. The supernatant was discarded and the phage resuspended in 1.5mls of SM overnight with gentle shaking at 4°C. The following morning the resuspension was completed by gentle pipetting of the solution. Proteinase K was added to a final concentration of 50ug/ml and SDS to 0.5%. This was completely mixed and incubated at 65°C for 1hr. Protein was removed by the addition of an equal volume of phenol. This was mixed by inversion several times and the phases separated by centrifugation (1600g, 5min, room temperature). The aqueous phase was then extracted with phenol/chloroform (50:50) and chloroform in the same way. Finally the aqueous phase was dialysed overnight against 3000 volumes of TE. This method yielded pure samples of phage DNA (0.5-2.0ug DNA/ml lysate).

DNA Cleaning

DNA samples which could not be digested or which appeared "dirty" on agarose were cleaned using Whatman DE52 (Anion Exchanger) in a method described by P. Sudbery (*pers. comm.*). The DE52 was washed in

MilliQ water to remove fines. It was allowed to settle out and the water poured off. This was repeated until the water was virtually clear after washing. The cleaned DE52 was then loaded into a siliconized, glass wool plugged Pasteur pipette under water. Once evenly packed, the column was washed with 1 x TBE. The DNA sample was then added to the column and washed with MilliQ water. The DNA was eluted from the column with 1.1M NaCl. 200ul were added to the column and spun through; 500ul more was added and this was also spun through. This sample was collected as it was usually the one containing the clean DNA. The column was washed three more times with 500ul of 1.1M NaCl and the samples collected. The DNA was precipitated from the salt solution by the addition of 2 volumes of absolute ethanol and placing the samples at -70°C for 1hr. The DNA was collected in the usual way.

Gel Electrophoresis

Two types of gel electrophoresis were carried out; minigel and large gel. Both were carried out essentially as described in Maniatis *et al.*, 1982. In all cases the buffer used was 1 x TBE. Ethidium bromide was added to gels at a final concentration of 0.5ug/ml. The percentage of agarose added to the gel was dependent on the size of the restricted DNA fragments. Large gels were routinely run at 5V/cm of gel. Agarose used was Sigma No. A-6013.

Digestion of DNA with Restriction Endonucleases

Digestions were carried out again essentially as described in Maniatis *et al.*, 1982. Reactions were carried out routinely in volumes of 20ul. All digests were carried in a universal restriction buffer that was suitable for all enzymes. Preparative digests were

carried out in much larger volumes (100-300ul) and usually overnight. The temperatures at which digests were carried were dependent on enzyme but generally the temperature was 37°C. All enzymes used were obtained from Northumbria Biologicals Limited (NBL). DNA size standards were obtained by digestion of lambda DNA (NBL 0.2ug/ul) with the appropriate restriction enzymes.

Ligation of DNA Fragments

Ligation of fragments was always carried out with an excess of any insert (20 fold). The reaction was carried out in a total of 40ul in sterile Eppendorfs at 4°C. Ligation was achieved using T4 DNA ligase from NBL. Plasmids were prevented from self-ligating by dephosphorylating the 5′ ends. This was carried out according to the manufacturers protocol. Dephosphorylation was carried out using calf intestine alkaline phosphatase (BCL, Boehringer Mannheim). Inactivation of the phosphatase was achieved by incubation for 45min at 65°C in 10mM EGTA.

Isolation of DNA Fragments from Gels

DNA was recovered from gels by the freeze/thaw method (W. Lancashire *pers. comm.*). The gel containing the DNA fragment was cut from the large gel so as to avoid gel which did not contain DNA. This fragment was then chopped up and placed in a sterile, siliconised Eppendorf. This was placed in a dry ice/ethanol bath until the sample was completely frozen (approx. 2min.). The Eppendorf was then placed immediately in a 65°C water bath for 2min. This was repeated 4 times. The sample was then spun for 8min. high speed in a microfuge. The supernatant was placed in a fresh Eppendorf and extracted once with chloroform. The DNA was recovered by

precipitation and vacuum drying. If the DNA was to be ligated 100ug/ml bovine serum albumen was added to the ligation.

Southern Blotting

This was carried out according to the method described in Membrane Transfer and Detection Methods published by Amersham International plc (after Southern, 1975). DNA fragments were blotted onto Hybond-N nylon membrane and probed with radioactively and non-radioactively labelled probes.

Probe Production

Two types of probes were made from fragments containing the *whi1* gene, radioactive and non-radioactive. Radioactive probes were produced and hybridisations carried out according to the protocol in the Leicester University Cloning Course Manual 1986. The probes were oligo labelled using Klenow Fragment obtained from NBL. Non-radioactive probes were produced and used according to the manufactures protocol "DNA Labeling and Detection Kit-Nonradioactive". The kit used is produced by Molecular Biology Boehringer Mannheim and labels DNA fragments with digoxigenin-dUTP. Probes were removed and membranes decolourized according to the method of Gebeyehu *et al.*, 1987.

CHAPTER 3: GROWTH AND INTERACTION WITH *cdc* MUTATIONS

3.1 INTRODUCTION

Strains of *Saccharomyces cerevisiae* used generally for genetic analysis grow best by fermentation (Fraenkel, 1982). They can utilize a whole range of sugars in the production of biomass and secondary fermentation products (e.g. ethanol). Yeast cells may also grow by the oxidation of non-fermentable carbon sources such as lactate, acetate, pyruvate, glycerol and ethanol. When glucose is present in high enough concentrations cells will grow by fermentation; when the level of glucose drops, cells switch to oxidative growth utilising ethanol in the presence of oxygen.

Petite mutants of *S. cerevisiae* are deficient in the ability to grow oxidatively. They cannot grow on non-fermentable carbon sources and produce only small colonies on medium containing glucose. Although the colonies are small the cells are the same size as wild type. Cells containing the *whi1* mutation show an altered cell size. When grown on complex media containing glucose colonies are identical to wildtype, although there may be some initial difference in colony size, after 48hr growth the size is the same.

When mapping mutations, it is useful to have gross phenotypic differences. When carrying out tetrad analysis an easily distinguishable biochemical or growth phenotype leads to more accurate results. This type of phenotypic difference also makes cloning of genes by recovery of wild phenotype easier. In cells containing the *whi1* mutation the only phenotypic difference appeared to be the size of cells. Cells containing the *whi2* mutation, which leads to the cells being unable to enter stationary phase (Saul *et al.*, 1985) and exhibiting a small size phenotype on depletion of nutrients from media (Sudbery *et al.*, 1980), appear to grow faster

then wild type cells in media containing non-fermentable carbon sources (Harry Mountain *pers. comm.*). In order to assess if cells containing the *whi1* showed these sort of effects under differing conditions and in the hope of exploiting any differences in mapping, growth on different media was examined.

Although further examination of the phenotype of the mutation itself and the analysis of the cloned gene and its protein product may yield information on how *WHI1* functions in controlling cell size, it was hoped to gain yet more information by investigation of any interactions with cell division cycle (*cdc*) mutations.

By definition a cell division cycle mutation leads to a defect in a particular stage specific function of the cell cycle (Hartwell, 1974, 1978; Pringle, 1978). The actual lesion is known as the primary defect event and may be in the synthesis or function of a protein product. The tightness of a mutation will depend on whether it affects the action or synthesis of a molecule (Wheals, 1987). The mutations usually affect a function which is essential for division.

Cdc mutations are eventually lethal. This means that conditional mutants must be used in the study of the cell cycle. These are temperature, (usually heat) sensitive mutations although cold sensitive mutations are also used. The heat sensitives usually have a permissive temperature of 23°C and a non permissive temperature of 36°C. On switching a heterogenous culture of cells containing a temperature sensitive *cdc* mutation from the permissive to non permissive temperature the cells arrest, usually within one cell cycle, to become a population of cells which is morphologically homogeneous. The morphological point at which the cells arrest is

known as the terminal phenotype (Hartwell, 1974; Pringle, 1978). This phenotype does not represent the primary defect but the first event in the cell cycle which is affected by it and even though cells are arrested the continuous processes associated with growth and accumulation of mass often continue.

Cell cycle *cdc* mutations can be mapped in relation to each other. Using data from this type of mapping a functional sequence map can be formulated. A functional sequence map based almost entirely on *cdc* mutations is shown in Figure 3.1. This summarises the functional interrelatedness of cell cycle events and is arrived at by examination of the effects of single mutations, double mutations and examining reciprocal shift data. From the map two clusters of mutations can be seen before the dispersion of the cell cycle into several pathways. These represent some of the *start* mutants.

Most *start* mutations are *cdc* mutations which can be split into two main classes. Class I *start* mutations arrest in the same way as pheromone arrested cells. They continue to grow, have an unduplicated spindle pole body and are able to mate. Arrested class II *start* mutants rapidly stop growing, are unable to conjugate and have a spindle pole body satellite (Reed 1980). Class II mutants appear to be intimately involved in nutrient arrest recognition while some class I mutants may be involved in pheromone response (Wheals, 1987; Mendenhall *et al.*, 1987).

One *start* mutation which has been studied in great depth is *cdc28.* This gene has been cloned and by sequence analysis and antibody studies much is now known about some of its functions. After initial isolation further mutant alleles were isolated based on the fact

FIGURE 3.1 Functional Sequence Map of *cdc* Mutations of *S. cerevisiae.*

The numbers refer to the *cdc* mutation. Diagnostic landmark events are in boxes; dependent events are on consecutive arrows; independent events on parallel pathways; interdependent events on the same arrow. The positions of the landmark events across the figure are approximately to a correct temporal scale for the normal cell cycle.

Abbreviations are as for Fig. 1.1 with the exception of:

HU : hydroxyurea

MBC : methyl benzimidazole-2-ylcarbamate (benomyl)

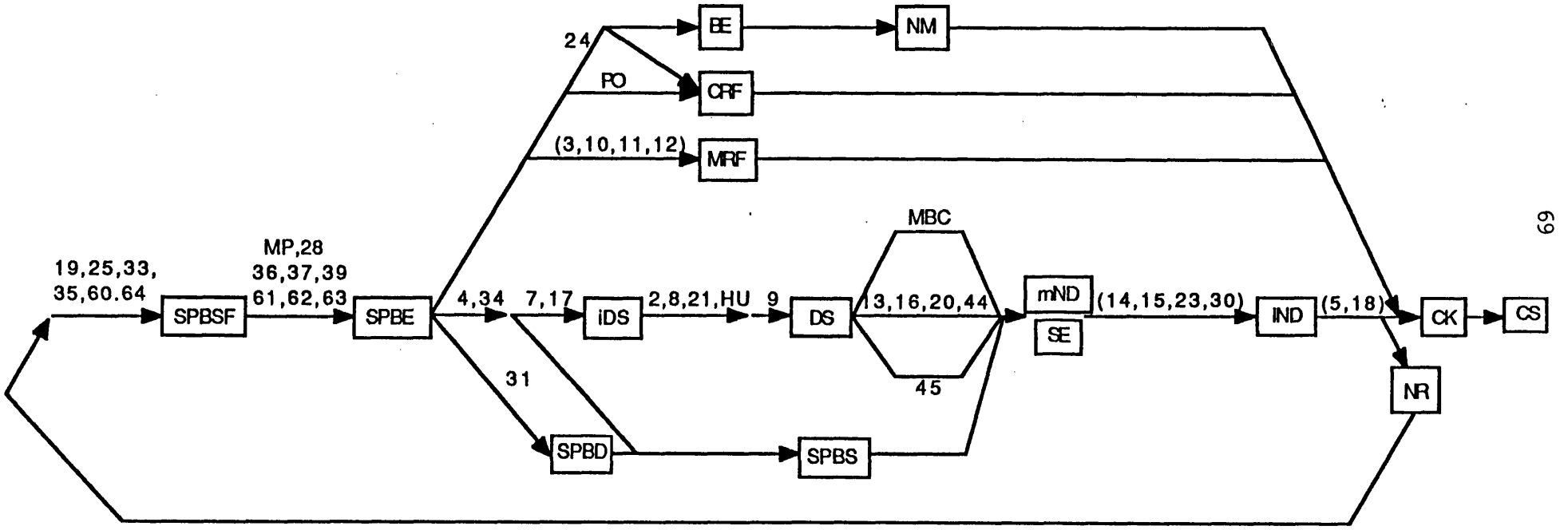
MP : mating pheromone

NR : nuclear reorganization

PO : polyoxin D

SPBE : spindle-pole-body-elongation

(From Pringle and Hartwell, 1981)


19,25,33,
35,60.64
SPBSF
MP,28
36,37,39
61,62,63
SPBE
4,34
7,17
iDS
2,8,21,HU
9
DS
MBC
13,16,20,44
45
mND
SE
(14,15,23,30)
IND
(5,18)
CK
CS
NR
24
PO
(3,10,11,12)
BE
CRF
MRF
NM
31
SPBD
SPBS

that class 1 *start* mutants are mating competent when arrested (Reed, 1980). The gene was cloned and characterised, revealing coding for a 34-36kd protein. Further investigation showed that it was transcribed at low levels, there being only 6-12 molecules of mRNA per haploid cell (Reed, Ferguson & Groppe, 1982). Analysis of the sequence showed that the gene had homology with protein kinases. Kinase activity of the gene product was demonstrated and the substrate shown to be a 40kd protein which coprecipitated with the *CDC28* gene product in immune complexes (Reed *et al,.* 1985). The reaction of the kinase and substrate was used to show when the *CDC28* protein kinase was active. Phosphorylation was shown to occur in G1. Cells which were nutrient limited or pheromone arrested were shown to have an inactive protein kinase. These data together indicated a role for the *CDC28* gene product in both nutrient response, via adenylate cyclase, and pheromone arrest (Lorincz & Reed, 1984; Reed *et al.*, 1985; Mendenhall *et al.*, 1987). A further true allele of *CDC28* has been isolated which acts at mitosis, causing arrest as budded cells. This demonstrates that *CDC28* may act at two points in the cell cycle (Piggott, Rai and Carter, 1982). Functional and structural homologues of this gene have now been found in other organisms. *CDC2*, the homologue from the fission yeast *Schizosaccharomyces pombe*, has been found to act at two points in the cell cycle of this yeast and to affect cell size by interacting with activators and inhibitors of mitosis (Russell & Nurse, 1987).

Other *start* mutations investigated here concerning their interactions with *WHI1* alleles are *cdc63-1*, *cyr1*, and *dna26-1*. Little is known at this point about the last in this list. Investigation is limited to its original isolation and work which has established that it

is a *start* mutation (Dumas *et al., 1982*). Its terminal phenotype is known (unbudded uninucleate cells) and it is thought to affect DNA synthesis. More is known about the other two mutations.

The mutation *cdc63-1* is known to be a classic temperature sensitive cell cycle arrest allele of the gene *PRT1.* Other available temperature sensitive alleles of this gene do not show class I arrest when switched to a non permissive temperature of 36°C but arrest randomly throughout the cell cycle. This is thought to be due to rapid inhibition of translation. It has been shown that mutations in *PRT1* not only affect cellular growth, by its effects on protein translation, but also specifically affect regulation of the cell cycle (Hanic-Joyce *et al.,* 1987a). These effects can be segregated with most mutant alleles by growth at different temperatures. Most *prt* mutant alleles will give first cycle arrest as unbudded cells when arrested at restrictive temperatures which are lower than 36°C. The arrest at *start* could be due to severe growth defects which prevent cells attaining critical size. This has been found not to be the case as *cdc63-1* continues to gain mass and retains the ability to mate. Effects on cell proliferation are different from those produced by a general inhibition of protein synthesis (Hanic-Joyce *et al.,* 1987b). The gene has been cloned and was found to be a gene of 2500 nucleotides in length. This gives coding capacity for a protein of Mr 88,000. Examination of the sequence shows it has no homology with any other protein known at this time, although it does have a region which may be involved in nucleotide binding (Hanic-Joyce *et al.,* 1986). As this gene has specific effects at *start,* interactions with *whi1* were investigated.

The class II *start* mutation *cyr1* is allelic with *cdc35*. Some of the effects of this mutation are mentioned in Chapter 1. This mutation causes a significant decrease in cellular cAMP levels. The mutation causes thermolability of its protein product, adenylate cyclase. Cells containing this mutation arrest as unbudded uninucleate G1 cells at the non-permissive temperatures in the absence of external cAMP (Boutelet *et al.*, 1985). The *CYR1* gene has been cloned and has a large open reading frame of 6078 bases corresponding to a protein of 2026 amino acids (Kataoka *et al.*, 1985). The catalytic domain is located in the 3'-terminal 1.2kb region of the open reading frame (Kataoka *et al.*, 1985). As this is a class II *start* mutation and the cAMP pathway is another facet of control at *start* not covered by the other mutations, interactions with the *WHI1* mutant alleles was also investigated.

Double mutants of all these mutant alleles with the *WHI1* alleles were produced and effects on growth at non-permissive temperatures were examined by looking at accumulation of mass, budding index and size. Accumulation of mass was examined by absorbance at 660nm. It was noted that absorbance can increase extensively, while increase in cell number is prevented by placing strains containing *cdc* mutations at their restrictive temperature (Hartwell, 1974). In some experiments this can lead to difficulty in interpreting results, especially when highly accurate measurement of cell concentration is required. This was not thought to be needed with this investigation. As only a single instrument was available for measuring both cell size and cell number, with cell size being thought the more important of these parameters, it was decided to use absorbance, coupled with the number of unbudded cells, to assess

division. Usually it is imperative to be able to convert absorbance data to a different, more useful, parameter (Pringle & Mor 1978). In this case the gross effect of temperature shift on the growth profiles was of major of interest. This it was thought could be seen clearly by the use of the the three parameters chosen.

3.2 RESULTS

Calculations

To compare cultures of wild type and mutant cells growth rates were calculated using the following formulae:

$$(a) \quad TD = \frac{t}{\ln(xt/x0)} . \ln 2 \text{ hrs}$$

where TD is the doubling time

t is the amount of time over which growth is examined

x_0 is the cell density at time zero

x_t is the cell density after time t

$$(b) \quad \mu = \frac{(xt)\ln - (x0)\ln}{t} \text{ hrs}^{-1}$$

where U is the specific growth rate

Other parameters as above

N.B. $$TD = \frac{\ln 2}{\mu}$$

Growth and Cell Size

Growth measurements were carried out to check that both *whi1* and *WHI1* parent strains had similar growth characteristics and that they showed significant differences in cell size (Figures 3.2. A and B. Doubling times were comparable (*WHI1* = 2.70hr, *whi1* = 2.40hr) as were the specific growth rates growth rates (*WHI1* = 0.256hr^{-1}, *whi1* = 0.288hr^{-1}). Both have similar growth characteristics and show difference in cell volume during exponential growth. The wild type strain showed approximately twice the cell volume of the *whi1* mutant strain in mid exponential growth. The major difference between the two strains, besides actual size, is the change in size over the period of growth. The *WHI1* cells actually decreased in size (a fall

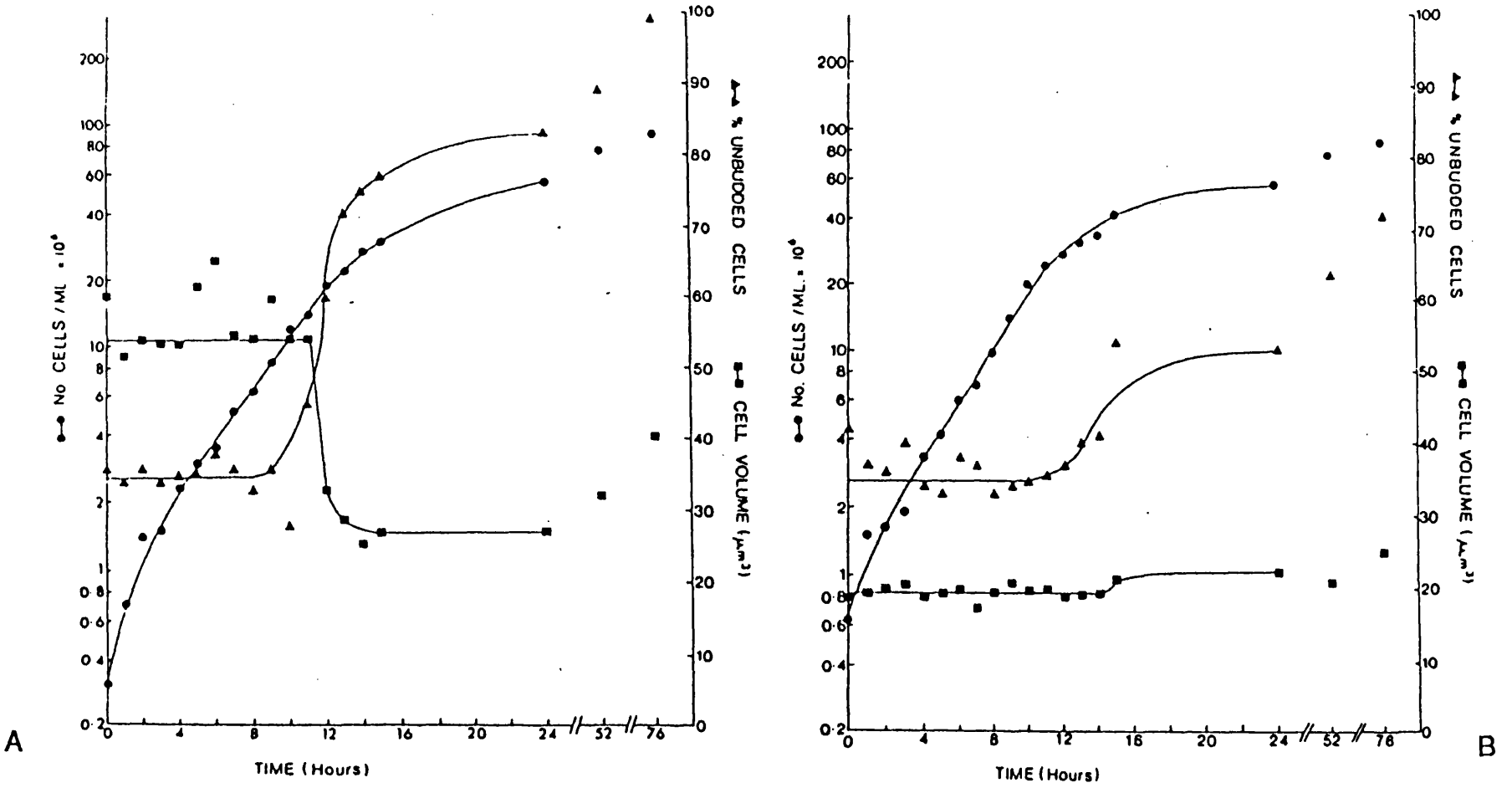


FIGURE 3.2 Growth Profiles of Wild Type and Mutant Strains: Glucose Media

A = Wild Type

B = *whi1*

of approximately 50%) as they approached stationary phase, whereas the *whi1* cells increased slightly (approximately 17%) during the same period. A further difference was noted between the budding index profiles of the two strains when grown on media containing glucose. The budding index of cells containing the mutant size control gene began to rise later in the exponential growth phase than in the wild type. It also rose more slowly and gave a value after 24hr of growth lower than that of the wild type cells. Although the cell number had not doubled after the 24hr period the difference in budding index showed a difference of 30%. This difference was still approximately 30% when the medium was exhausted and all growth had ceased.

Growth on glycerol and ethanol media

Growth of the wild type, *WHI1* strain, and the mutant *whi1* strain, was carried out on these non-fermentable carbon sources to give direct comparisons with growth of the strains on glucose media and to explore any differences between the strains that may have become apparent. It was hoped that there may have been some easily scorable difference associated with the *whi1* phenotype which could have been exploited in the mapping of the mutant gene. Figures 3.3 A and B show the growth of the two strains on glycerol media. The doubling times (and growth rates) were comparable, *WHI1* = 4.21hr ($0.164hr^{-1}$) and *whi1* = 4.52hr ($0.153hr^{-1}$). As expected the cells grew more slowly on this medium and due to this, over the fourteen hour period the cultures were analysed hourly no real phases were apparent in the growth profile. Cells would enter stationary phase over this time period in media containing glucose. Cell number still showed significant increase after twenty four and forty eight hours and budding index was high enough to indicate that cells were still

growing. The initial difference in cell volume was not as great as in the glucose grown cells but both cultures showed a drop in cell size after about eight hours of growth, wild type cells dropping quickly and mutant cells falling more gradually. Budding index in the wild type culture appeared to rise slowly until the change in cell size when it reached a plateau, only rising as the medium became exhausted. The unbudded index in the *whi1* culture appeared to rise slowly as the culture grew, reaching around 60% after fourteen hours growth. In both cultures the final number of unbudded cells was around 80%.

The growth profiles of cultures grown on media with ethanol as the only major carbon source (Figures 3.4 A and B) show two distinct phases when compared with the glycerol growth profiles. This was apparent in both the mutant and wild type cultures. The doubling times for the wild type culture were 4.13hr initially falling to 4.70hr and for the mutant culture 4.18hr and 4.96hr. Although the growth rate of both cultures appear to slow down, growth continued for at least ten hours (note the OD values at 24hr). Again the difference in cell volume between cultures at the outset was smaller than had been observed in glucose media although the *whi1* cells appeared larger. Cell volume remained stable until around the shift in growth rate when it fell gradually in both cultures. The change in growth rate and cell volume was matched by a change in budding index. The numbers of unbudded cells remained static until the change in growth rate, after which the numbers began to rise. After the rise, the number of unbudded cells seemed to reach a plateau. The final number of unbudded cells in both cases was just below 80%.

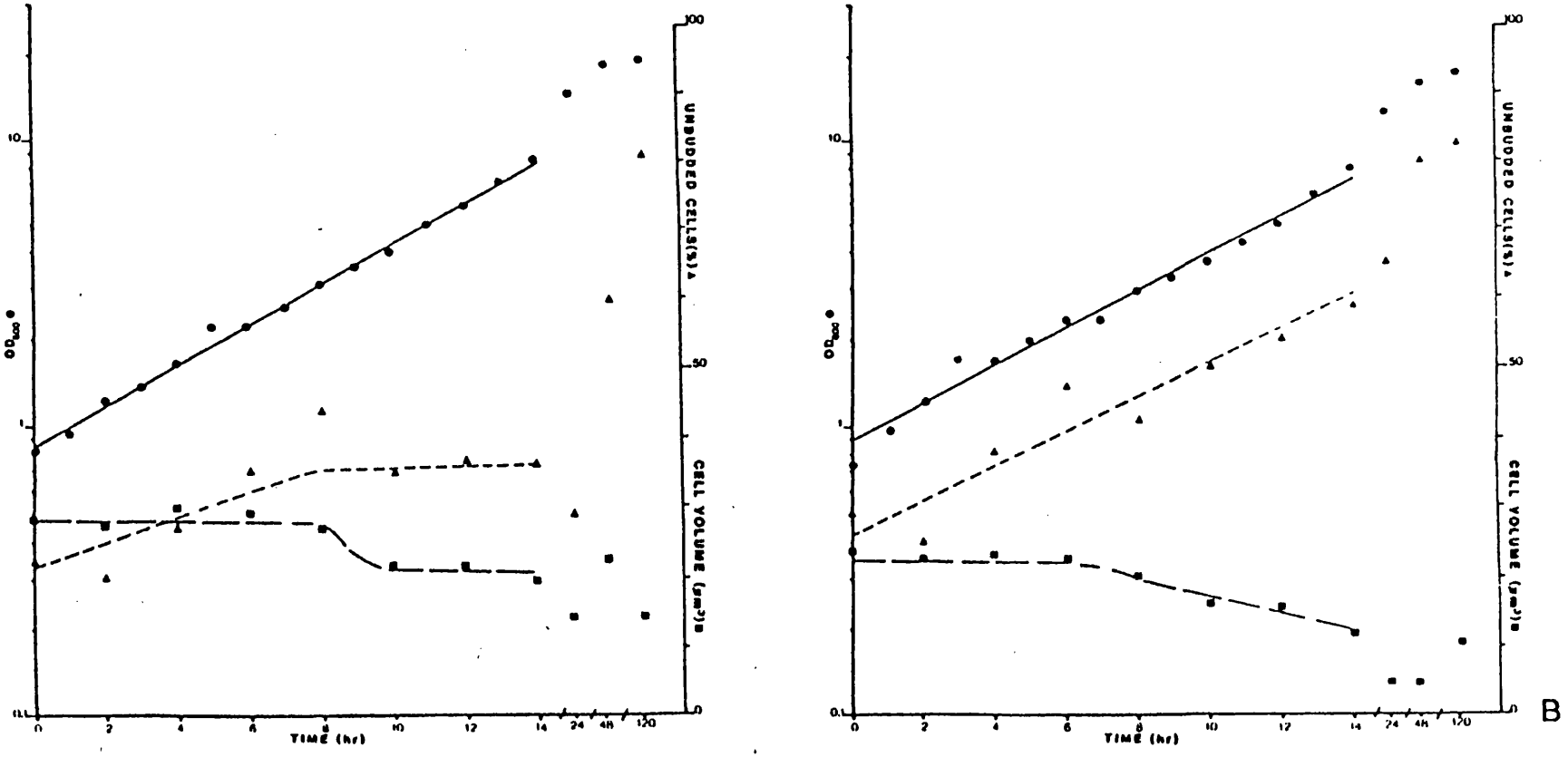


FIGURE 3.3 Growth Profiles of Wild Type and Mutant Strains: Glycerol Media

A = Wild Type

B = *whi1*

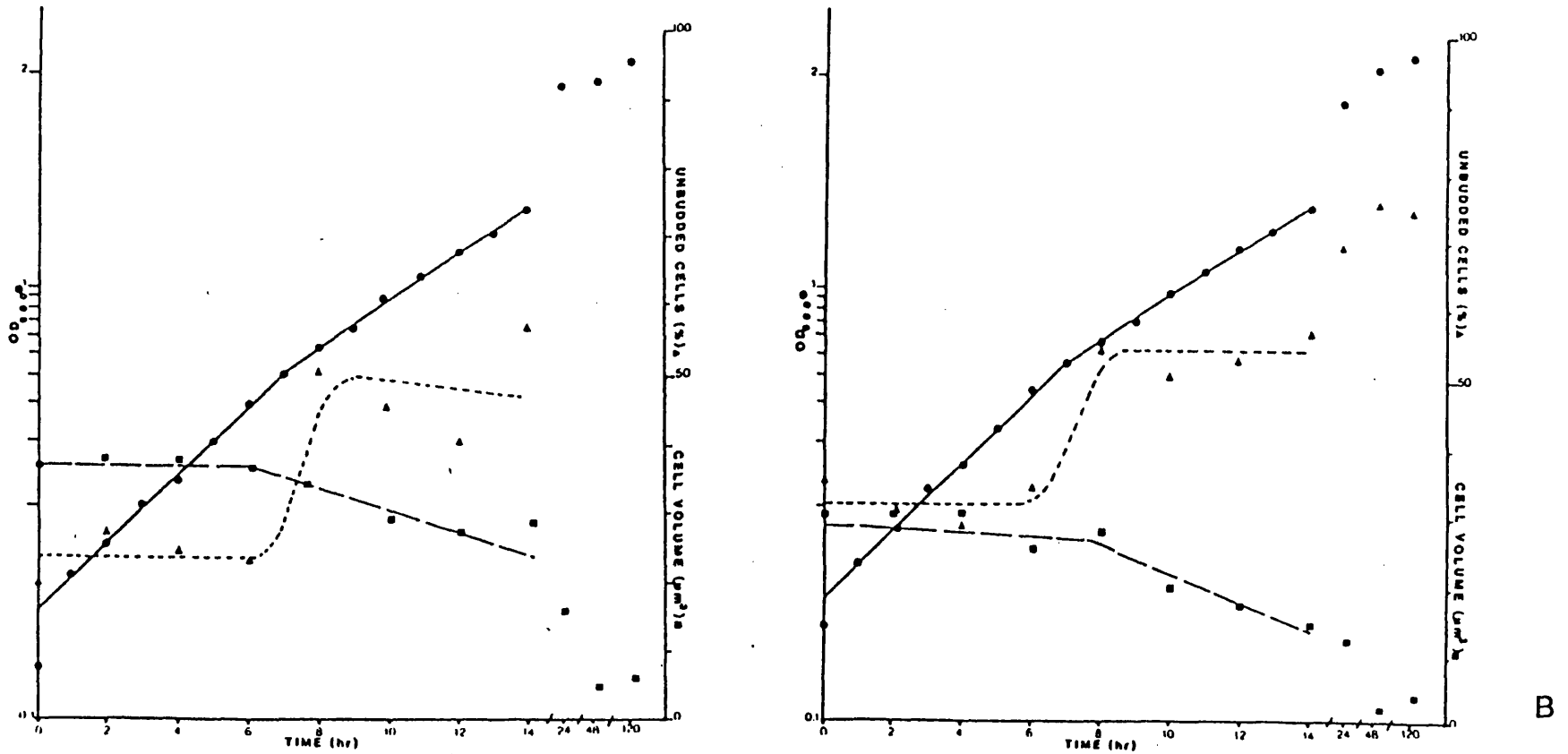


FIGURE 3.4 Growth Profiles of Wild Type and Mutant Strains: Ethanol Media

A = Wild Type

B = *whi1*

Strain construction

Before investigation of any interactions between the *whi1* mutation and *cdc* mutations could be investigated strains had to be constructed which contained both mutations. At a late stage in the project strains also became available which contained 4 x *whi1* and no functional copies of *WHI1* (null phenotype cells or *WHI1*^{0}). These were also crossed with strains containing various *cdc* mutations. Mutations crossed into the above strains were *cdc28*, *cdc35*(*cyr1*), *cdc63* and *dna26*. The strains containing these mutations were crossed and sporulated in the normal way. Sporulating cultures were examined at regular intervals to assess how long cells took to sporulate and the actual amounts of sporulation. When (or if) significant amounts of sporulation had occurred, 20 full tetrads were picked for each cross (where possible) and the amount of germination and growth noted. Table 3.1 shows the sporulation profiles of the crosses carried out and tables 3.2 and 3.3 the germination of actual spores and the resultant phenotypes.

Interaction of *dna26* and alleles of *whi1*

All of the allelic variants of *whi1* were successfully crossed with a strain containing *dna26* and double mutants recovered. The variants of *whi1* which were used were *whi1*, *WHI1*, 4x*whi1* and *WHI1*^{0}. These four variants were crossed with all the following cell division cycle mutants. Once the double mutants had been isolated temperature shift experiments were carried out in liquid media to assess any interaction between the *WHI1* allele (or alleles) and the cell division cycle mutation present. As well as examining the effect of the *WHI1* allele on growth at restrictive temperature, the size of cells was also examined to assess any effect the division mutation may have had on cell volume throughout exponential growth. To obtain

CELL CYCLE MUTATION	WHI1 PHENO.	3 DAYS			7 DAYS			14 DAYS		
		TOTAL	2'S	4'S	TOTAL	2'S	4'S	TOTAL	2'S	4'S
dna26	d	2	6	94	43	5.5	94.5	67	3	97
	D	0	0	0	15	2	98	60	2	98
	N	16	2	98	40	2.5	97.5	72	2	98
	4D	0	0	0	12	3	97	51	2	98
cdc28	d	3	1	99	21	1.5	98.5	51	2	98
	D	0	0	0	3	17	83	13	16	84
	N	7	12	88	14	12	88	29	12	88
	4D	0	0	0	3.5	12	88	11	11	89
cdc63	d	3.5	2	98	31	2	98	60	2	98
	D	0	0	0	19	3	97	51	3	97
	N	8	2	98	41	1.7	98.3	63	2	98.1
	4D	0	0	0	14	3	97	56	2.5	97.5
cdc35	d	3	2.5	97.5	18	3	97	41	3	97
	D	0	0	0	17	2	98	41	2	98
	N	3	3.5	96.5	21	3	97	49	3.5	96.5
	4D	0	0	0	10	3	97	33	3	97

TABLE 3.1 Sporulation Profiles of Interaction Crosses.

Sporulating cultures were examined after 3, 7 and 14 days. The total amount of sporulation was estimated (as a percentage of all cells), the number of two spored asci (2'S) and the number of four spored asci (4'S). All figures are expressed as percentages.

d = Wild type D = *whi1*

N = *WHI1*^{o} (null) 4D = *4xwhi1*

TOTAL = Total sporulation (including 2, 3 and 4 spored asci) at the time of examination.

CELL CYCLE MUTATION	WHI PHENO.	NO. OF COLONIES FROM SINGLE ASCUS					MICRO COLONIES	NUMBER NON GERM. SPORES
		0	1	2	3	4		
dna26	d	0	0	2	2	6	1	5
	D	0	0	3	0	7	1	5
	N	0	0	1	4	5	0	6
	4D	0	0	0	2	8	1	1
cdc28	d	0	1	3	3	3	6	6
	D	1	2	5	2	0	7	15
	N	1	2	2	3	2	4	13
	4D	4	2	4	0	0	8	22
cdc63	d	0	2	3	3	2	1	14
	D	1	2	6	1	0	0	23
	N	1	2	4	3	0	0	20
	4D	2	4	3	1	0	2	25
cdc35	d	0	2	2	3	3	6	7
	D	7	0	2	0	1	11	21
	N	0	1	3	4	2	3	10
	4D	3	3	1	3	0	2	24

TABLE 3.2 Germination Profiles of Interaction Crosses

The number of germinating spore from any individual asci are shown (at 25^0 C). Spores which only resulted in micro-colonies are also shown. Phenotypes are d = *WHI1*, D=*whi1* N = the null pheno type and 4D = 4 x *whi1*. Note the number of fatalites is lower in the dna26 crosses than all others.

CELL CYCLE MUTATION	*WHI1* PHENO.	GENOTYPE OF SPORE				TOTAL GERM. SPORES
		PARENT 1 *WHI1*	PARENT 2 CELL CYCLE	DOUBLE WILD TYPE	DOUBLE MUTANT	
dna26	d	9	10	7	8	34
	D	10	10	7	7	34
	N	9	10	7	8	34
	4D	11	8	10	9	38
cdc28	d	6	8	8	6	28
	D	7	7	3	1	18
	N	7	7	6	3	23
	4D	4	4	2	0	10
cdc63	d	7	8	6	4	25
	D	6	5	3	3	17
	N	6	5	5	3	19
	4D	5	5	1	2	13
cdc35	d	8	8	5	6	27
	D	3	3	2	0	8
	N	9	6	7	5	27
	4D	6	4	3	1	14

TABLE 3.3 Genotypes of Germinated Spores

Shown are the genotypes of all spores which germinated. Germinations were carried out at 25°C on YEPD. Parent 1 always contained the *WHI1* allele, parent 2 the cell cycle mutation. Double mutants or double wild type contained the recombined genotypes not seen in the parents. The *WHI1* phenotypes are as seen in Table3.2.

a clear picture of any interactions, cultures were examined every half hour after the time of shift for the following two hours. Figure 3.5 shows a growth curve of a stain containing the *whi1* mutation alone. Cells containing *WHI1*, 4x*whi1* and *WHI1*^{0} alone had similar growth curves, although the size profile was not the same in all cases. This should be referred to as a control for all growth curves in this section as far as the *whi1* mutation is concerned. Strains with the phenotype *cdcX WHI1* should be regarded as controls as far as the cell division mutations are concerned.

The growth profiles for *dna26* are shown in Figures 3.6 A and B . The control (Fig. 3.6 A)shows that after shift to 37°C proliferation rapidly ceases. The slope continues to rise as the absorbance increases due to continued cell growth. The sharp increase in the number of unbudded cells demonstrates that cell division has decreased. As well as a rise in the percent of unbudded cells the cell size increased. Both these parameters began to fall as the nutrients in the media became exhausted. The halt in proliferation with the continuation of cell growth is typical of true cell cycle mutations.

Figure 3.6 B shows the growth profile of the strain *dna26* 4x*whi1*. This is the only profile from this series of crosses to be shown as, apart from differences in size, all were essentially the same. Cell division ceased after the shift to the restrictive temperature (R.T.), the number of unbudded cells rose and the size of cells increased. In all cases the double mutant strains behaved as though *dna26* single mutants.

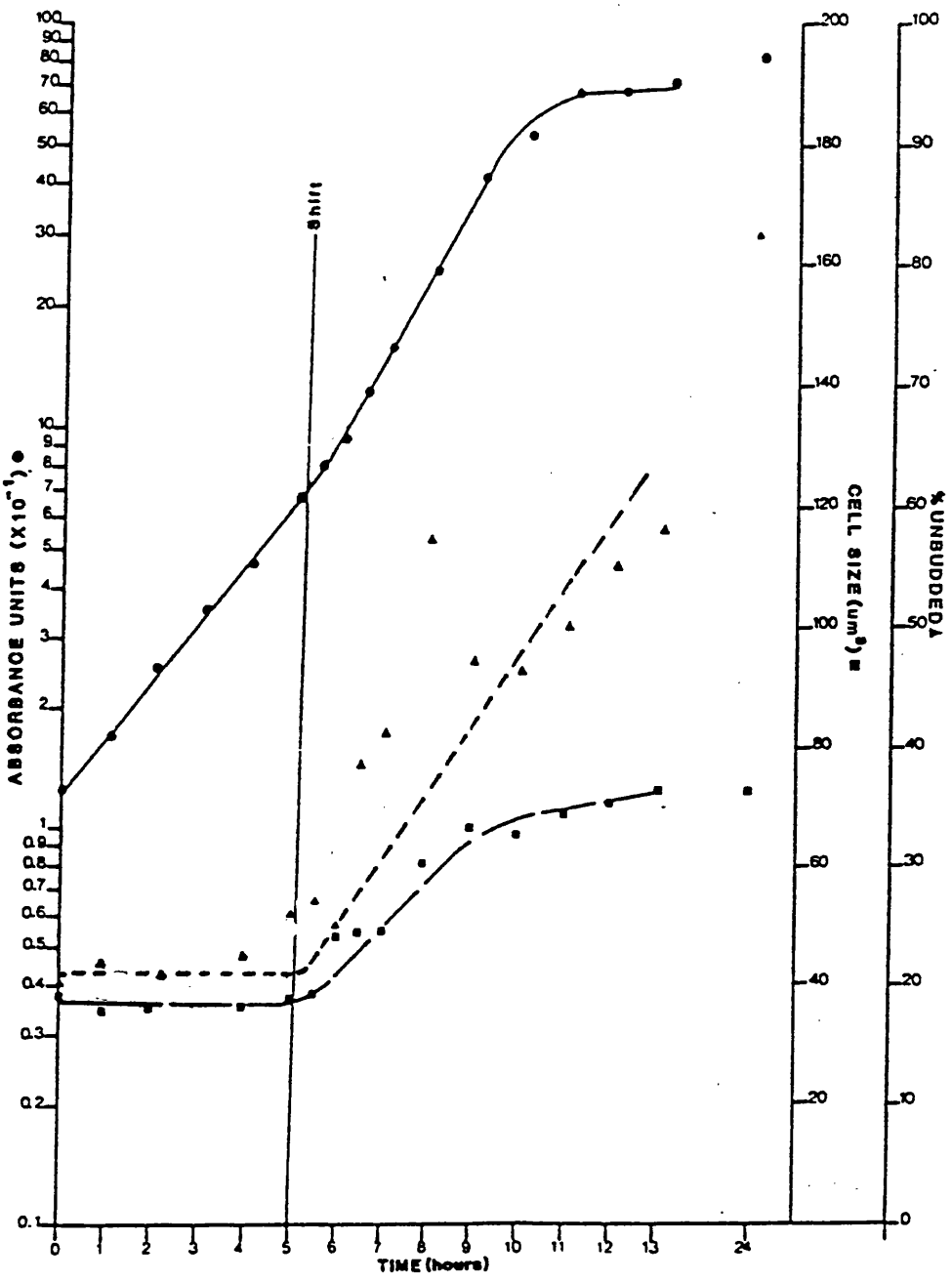


FIGURE 3.5 Growth Profile of *whi1* control strain

Strain was grown at 25°C until mid stationary phase then shifted to 36°C. This growth profile is the *whi1* control for all following growth curves.

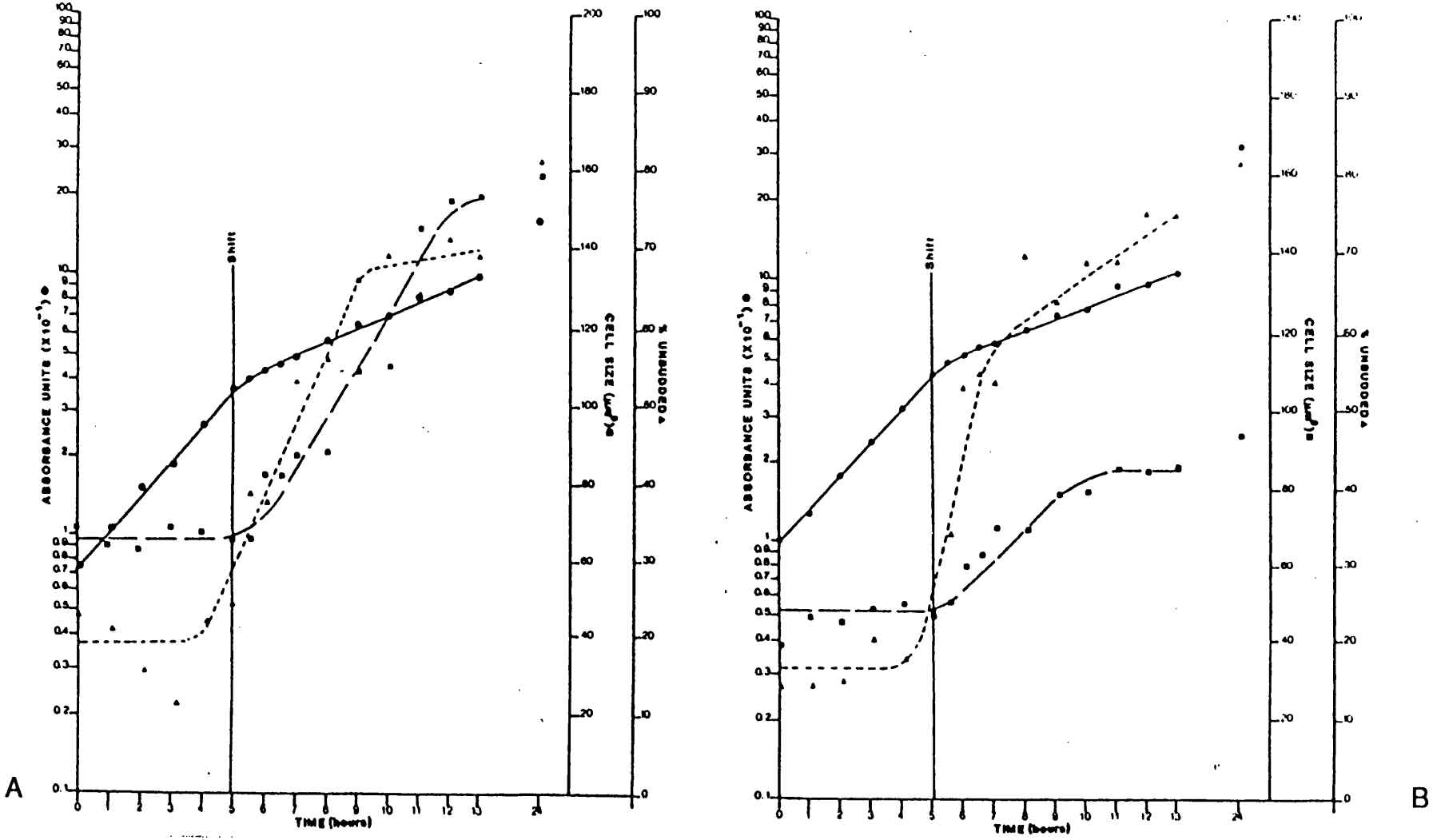


FIGURE 3.6 Interactions in Double Mutants: Growth Curves

A = *dna26* control

B = *dna26 whi1*

Interaction between *cdc28* and alleles of *whi1*

The same alleles of *whi1* were crossed into a strain containing a mutant *cdc28* gene. Again progeny were selected which contained both mutations. No double mutant was isolated which contained *cdc28* and 4x *whi1*. These did not appear viable (see Table 3.3). There was also great difficulty in obtaining *cdc28 whi1* double mutants. The control strain (*cdc28 WHI1*) is shown in Figure 3.7 A. On shift to the restrictive temperature the number of unbudded cells rapidly rises as proliferation ceases. The rise in optical density slows and the cell size begins to climb. Although as with *dna26* the growth curve does not plateau altogether, parameters taken together indicate the proliferation has stopped. This is not seen in the growth profile of the *cdc28 whi1* double mutant (figure 3.7 B). Although the number of unbudded cells and cell size both rise quickly the optical density plot seems to indicate that the culture arrested briefly before continuing to proliferate but with a slightly faster doubling time and specific growth rate (initially 2.3hr ($0.301hr^{-1}$) falling to 2.0hr ($0.33hr^{-1}$)). The rise in the number of unbudded cells also seems to last for a shorter time (one hour as opposed to two) and this is reflected in a lower percentage of unbudded cells after the rapid rise. The effect of the shift to the restrictive temperature on cell size seems to be the same in both cases.

Although there seems to be some interaction between *whi1* and *cdc28* there does not seem to be the same interaction between *cdc28* and *WHI1*^{0} (Figure 3.8 B). Proliferation in this strain seems to cease as quickly as in the *cdc28* control strain. The number of unbudded cells rises very quickly as does the size and although proliferation stops cell growth continues.

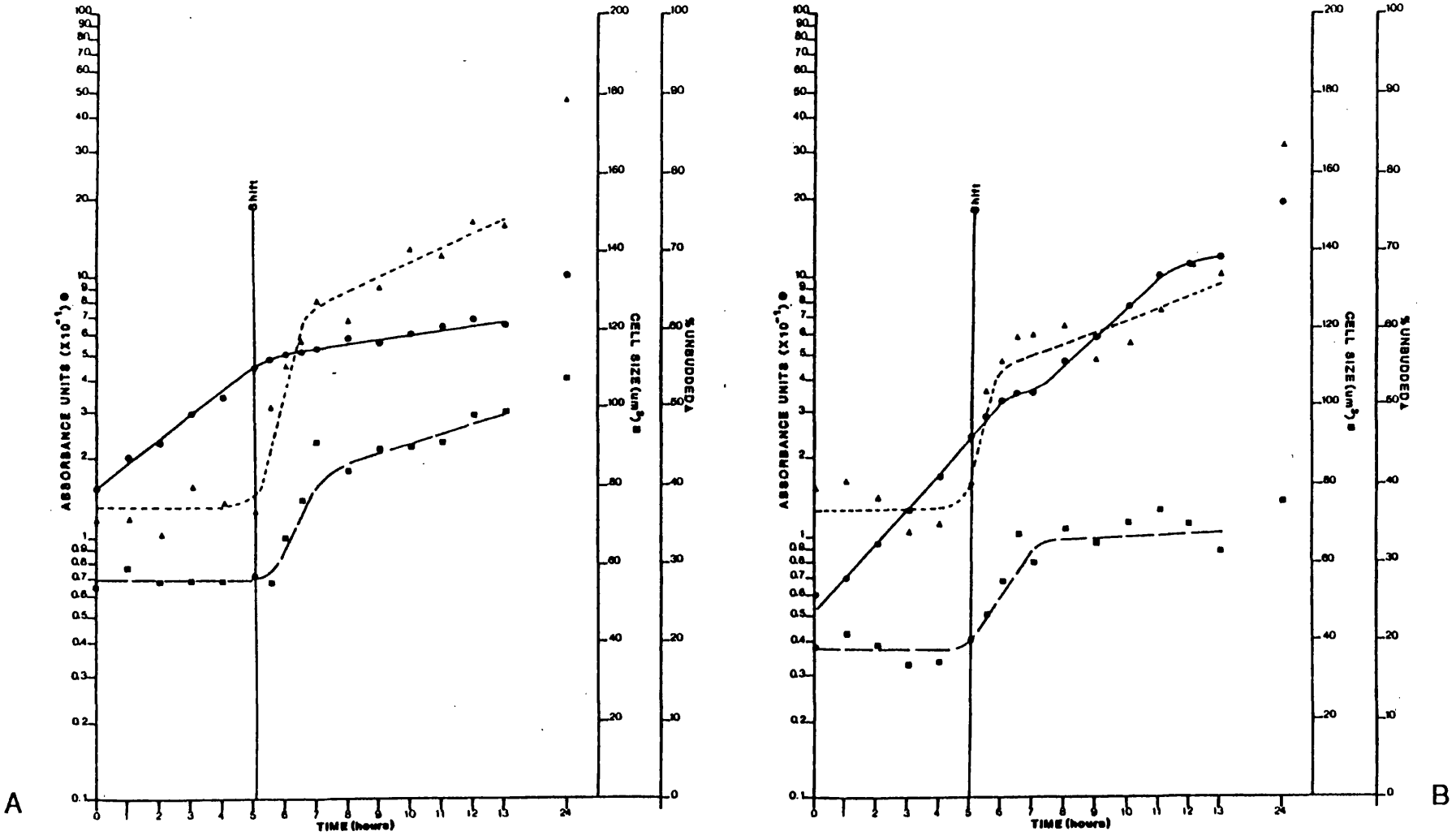


FIGURE3.7 Interactions in Double Mutants: Growth Curves

A = *cdc28* control

B = *cdc28 whi1*

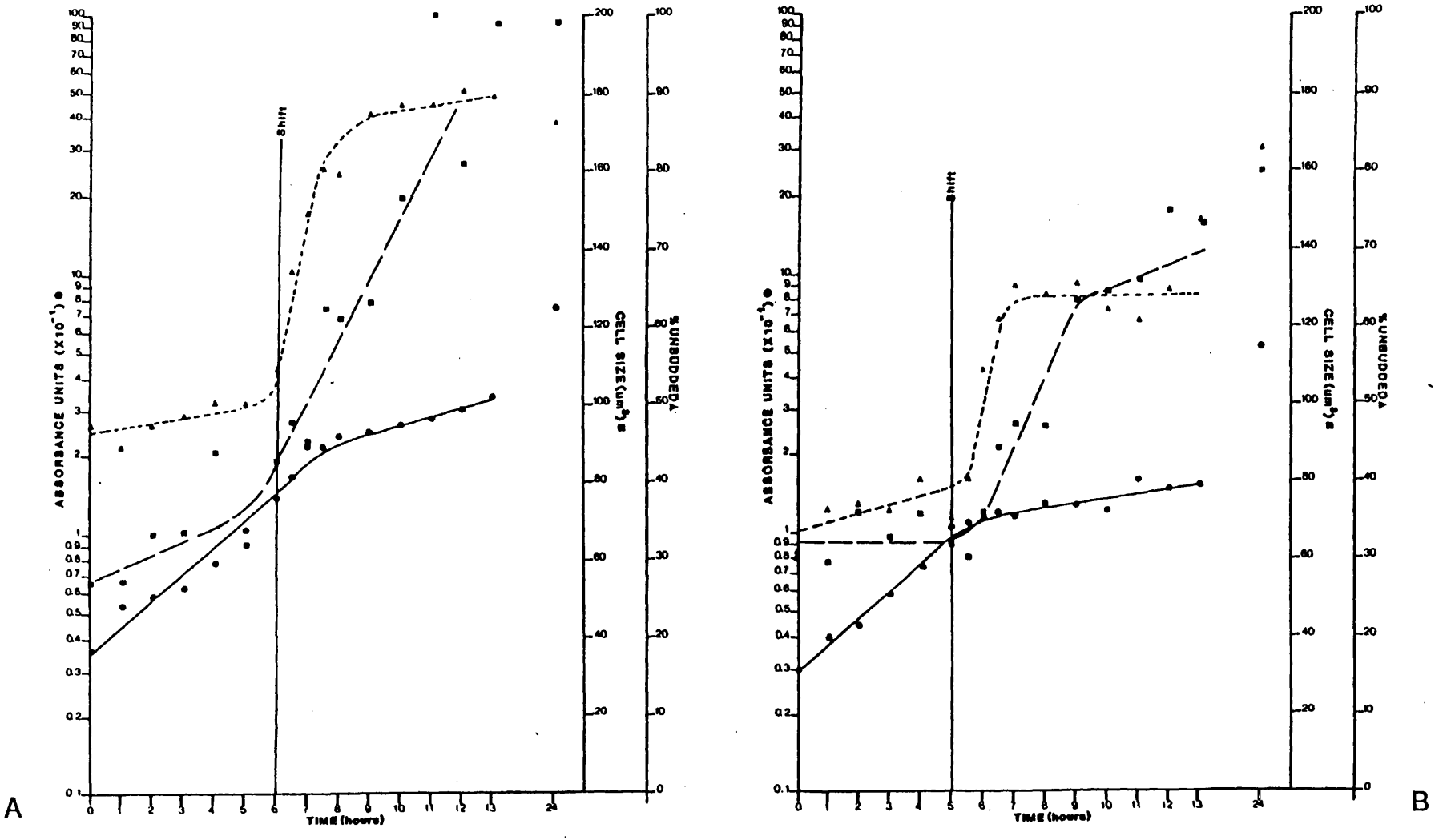


FIGURE3.8 Interactions in Double Mutants: Growth Curves

A = *cdc63 WHI1N*^{0}

B = *cdc28 WHI1N*^{0}

Interaction between *cdc63* and alleles of *whi1*

All combinations of *cdc63* and alleles of *whi1* could be isolated in progeny strains. As 4x *whi1* exhibited the same growth profile as *whi1* in a single copy, its profile is not shown. The profiles of the *cdc63* control and the *whi1* double mutant are shown in Figure 3.9 A and B respectively. On shift to the restrictive temperature the number of unbudded cells rises dramatically in the control strain. This is matched be a rapid rise in the size of the cells. The change in the rate of increase of optical density does not occur as sharply as in the other parameters. Some residual proliferation is indicated by the absorbance while the sharp rise in the number of unbudded cells would indicate that proliferation ceases abruptly. The rapid rise in cell size coupled with the rise in number of unbudded cells shows that this mutant allele of *cdc63-1* is showing classic cell cycle arrest. The double mutant shows no residual proliferation although the post shift rate of increase of absorbance is higher than that seen in the control strain. Both cell size and the number of unbudded cells rise sharply after the shift to the restrictive temperature.

The *cdc63 WHI1*^{0} (Figure 3.8 A) also has an optical density which continues to rise at a rate which is possibly a little too fast for a culture of non proliferating, growing cells. It does however exhibit a dramatic rise in the number of unbudded cells and a major increase in cell size which seem to indicate that the cells have stopped proliferating.

Interaction between alleles of *whi1* and *cyr1* (*cdc35*)

As with *cdc28*, isolating double mutants for all of the *whi1* alleles was a problem as there seemed to be some interaction which affected

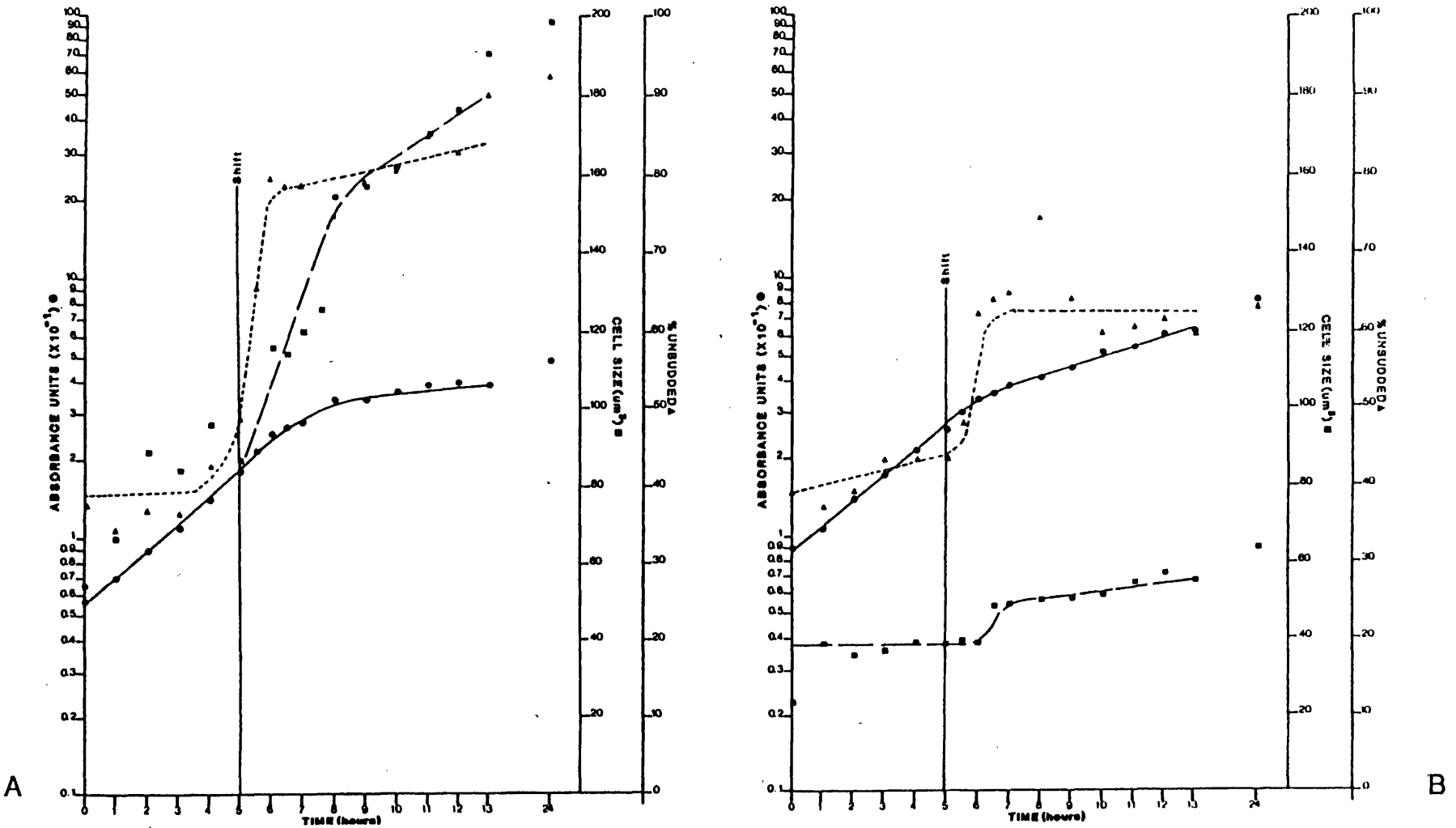


FIGURE3.9: Interactions in Double Mutants: Growth Curves

A = *cdc63* control

B = *cdc63 whi1*

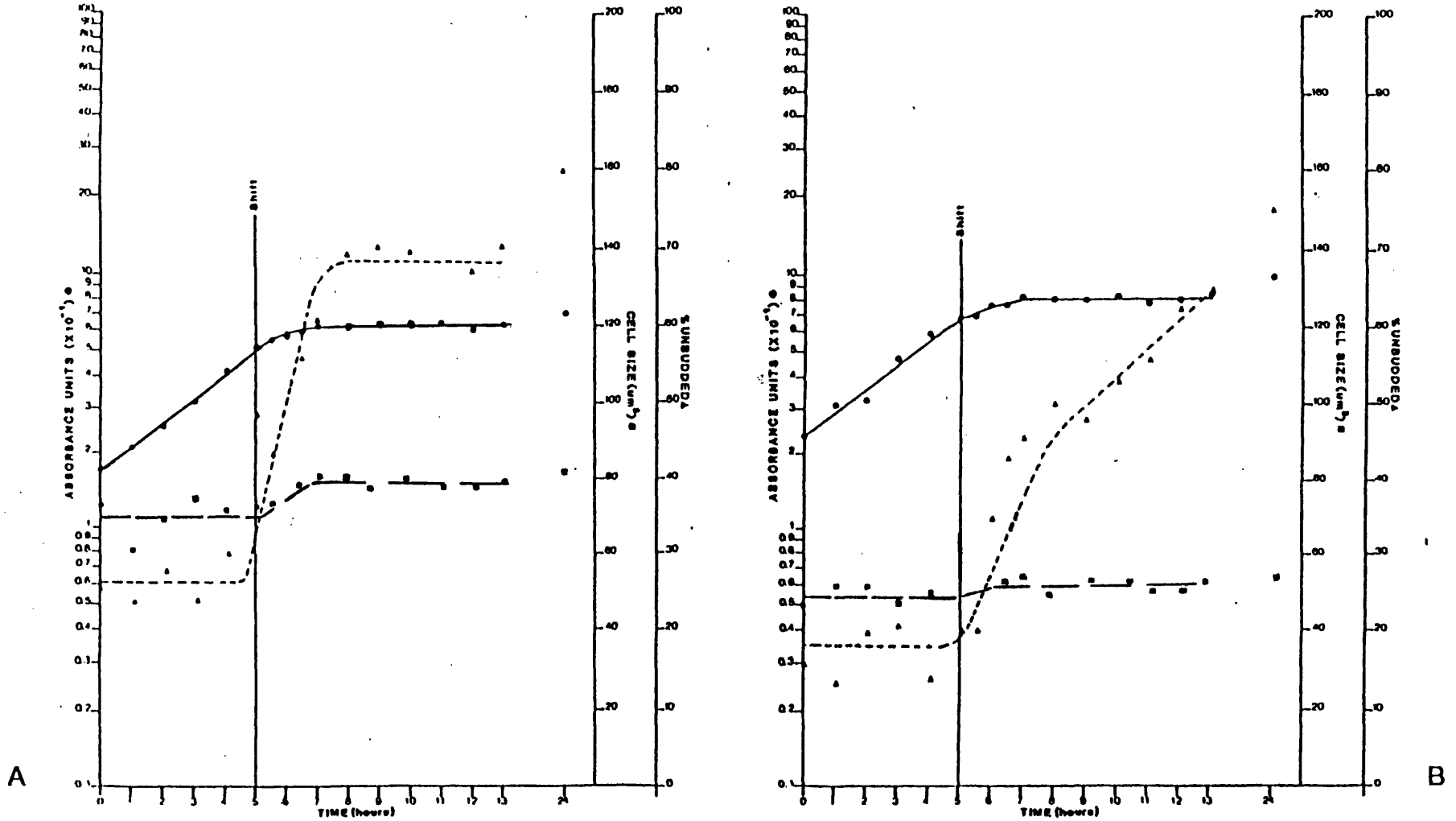


FIGURE3.10 Interactions in Double Mutants: Growth Curves

A = *cyr1* control

B = *cyr1* *whi1*

viability in progeny of crosses. As no double mutant actually containing *whi1* alone was isolated, the 4 x *whi1* was examined in growth experiments. The growth profiles of the *cyr1* control and the small double mutant are shown in Figure 3.10 A and B. Unlike the other cell division cycle mutants, not only did *cyr1* affect proliferation but it also affected growth itself. On shift to the restrictive temperature the optical density of the control culture stopped increasing altogether and levelled out. The number of unbudded cells rose rapidly before reaching a plateau just after the culture ceased all growth. This pattern was mirrored by cell size which also increased rapidly then levelled off. On examination of the growth profile for the *cyr1 4xwhi1* culture you will note the similarity with that of the control. The only major difference is that the number of unbudded cells does not rise as sharply and does not appear to level off. Cell size does undergo an increase before settling down to a new value. The figure for *cyr1 WHI1^0* is not shown as it showed the same characteristics as the *cyr1* control, it did not exhibit the difference in the rise in the number of unbudded cells.

3.3 DISCUSSION

Growth Rate and Cell Size

Growth of the wild type and mutant strains on glucose media was carried out to show that there were no major differences between their growth characteristics except that due to the *whi1* mutation. Doubling times, growth rates and final cell number of both strains were approximately the same. The fraction of unbudded cells and cell size differed during exponential growth. The final values for the numbers of unbudded cells differed by 30%. Cells containing the *whi1* mutation arrested not only in G1, the unbudded portion of the cell cycle, but also in other phases as budded cells. One explanation for this is a cryptic cell size control at mitosis. In wild type cells, growing at moderate or fast growth rates, size control at *start* would be enough to maintain population size. When growing at slower growth rates wild type cells would become small and a second, cryptic cell size control may become important. This size control may not be modulated, as is the one at *start,* and may be situated at mitosis. The changes in cell size seen in the cultures as they reach stationary phase for the first time may be due to this hypothetical size control. The small rise in cell size may be due to the growth rate falling below that necessary to reveal the cryptic cell size given the size the cells already exhibited. The size of the wild type culture may be maintained by the control at *start.*

Although this size control is hypothetical, its presence may not be without precedence. The major size control in *Schizosaccharomyces pombe* is located at mitosis rather than at the initiation of the cell cycle. Although located at different places in the repective cell cycles these major cell size controls do have certain

components in common, namely *CDC28s.c.* and *CDC2s.p.* which are hmologues. These major size controls may be of the same type, modulating size with growth through some cAMP dependent pathway. The minor size control in *Saccharomyces cerevisiae* may not be a modulated control but may define the lower limit or minimum cell size for slow growing cells. Maintenance of population size may be incidental as the cells are kept at a size from which they may recover if or when growth conditions improve.

Growth on glycerol and ethanol media

Although these cultures had been pre-adapted for approximately 100hr in the above media, balanced growth did not seem to have been achieved in all cases. This became clear only as hourly sampling was carried out. Although no clear phases of growth were noted, absorbance did increase in an exponential manner. It was hoped that there were some clear differences between the wild type and mutant strain that could be exploited when mapping the mutation. This was not found. The slightly higher growth rate achieved by the mutant strain may have been due to its increased surface area to volume ratio. As far as is known glycerol enters the cell passively. If this is the case cells with a larger surface area in relation to the amount of cytoplasm should be able to grow quicker if the system for used of glycerol is not fully saturated at low levels. The difference was very small and may just be due to inherent differences between strains.

Real differences occurred between the number of unbudded cells and cell volume. The number of unbudded wild type cells levelled off at 37%. It remained at this value until the hourly readings were stopped (14hr). In the mutant strain however the final value was

around 60% after fourteen hours. This increase in unbudded number did not correspond with a slowing of proliferation, therefore cells were spending more time in the unbudded phase of the cell cycle. This appears to be a paradox. Both strains will have a reduced size due to slower growth on glycerol. The reduction in mutant cells would be relatively less than the wild type if there were constraints on the size down to which cell size could modulated at *start* (or if a minimum cell size existed). This smaller amount of change in cell size meant that cells containing the mutant must increase their biomass by less before traversing *start*. If true then cells containing the mutant should spend less time in the unbudded phase then the wild type cells. The increasing number of unbudded cells showed that there was still major control over entry into mitosis at *start*. It may have been that the carbon source itself was important in the cells ability to measure its cell size and this effects the mutant *whi1* more than the wild type. This was not exploitable in the mapping of the mutant gene.

An unexpected feature of the growth profiles was the reduction in size after seven hours. This was not coupled to any reduction in proliferation rate and could not be explained by diauxic growth as the carbon source would not have become exhausted. It might have been a response to a lower concentration of glycerol. As the level in the media fell, proliferation may have been maintained at the cost of a reduction in cell size. Cell growth appeared to halt at the same time as proliferation. This was to be expected as no secondary source of carbon was available.

A small shift in growth rate was apparent in the cultures which were grown on media with ethanol as the carbon source. The shift in

growth rate, cell size and the number of unbudded cells occurred at approximately the same time as the changes occurred in the cultures grown on glycerol. The growth rates of the two cultures were comparable both before and after the apparent shift in growth rate. With the wild type and mutant cultures grown on ethanol there were no differences at all. The change in cell size seemed to occur later in the mutant culture but this may have been an artifact of the sampling. This experiment was carried out to identify any differences between wild type and mutant cultures which could be exploited in mapping but none were observed. Why the shift occurred is unknown. The changes were similar to those undergone by a culture which experiences a shiftdown in media or a drop in temperature. Diauxic growth was not possible. No other evidence of these shifts is available and while interesting, they were common to both cultures and not a feature which could be exploited in mapping of this gene.

Strain construction

The number of germinating spores which occurred during production of double mutants revealed where interactions would occur during growth experiments. The number of four spored asci was also an early indicator. No difficulties were encountered during the construction of strains containing the *dna26* mutation and no interactions were noted. The mutations appeared to be totally independent of each other. In contrast there were few four spored asci from the cross containing *whi1* mutations and the *cdc28* mutation. Those that did result refused to germinate in most cases. This interaction at spore level was matched when the double mutants were grown at the restrictive temperature of the cell cycle mutations.

Calvert & Dawes (1984) had noted that cells would only sporulated once they had again attained a certain size. The nature of this size control is not understood but seems to be linked to control mediated by *WHI1*. Whether the actual number of functional *WHI1* genes and their products affects sporulation directly or whether the control is exerted indirectly through size is not known. Examination of the sporulation data seem to indicate that the *WHI1* gene product is an inhibitor of meiosis. In strains that contain the non-functional *WHI1* gene the heterozygous diploids are larger and in most cases sporulate much quicker than the other heterozygous diploids. This may be purely because they are larger. The heterozygous diploid strains which contain the mutant *whi1* gene or 4 x *whi1* are the same size, yet those which contain multiple copies of the mutant gene take in one case, up to a week longer to produce greater than 20% spores on sporulation media (either four or two spored asci). It would seem that although the cell size mechanism concerned with cell cycle becomes saturated at two doses of the mutant gene, the size control concerned with meiosis does not. This indicates that *WHI1* as well as being acted on directly by two inputs (pheromone pathway and nutritional pathway) may itself affect two morphological processes. A single polypeptide possibly having an inducing as well as an inhibitory function.

Interaction of *WHI1* mutants with cell cycle mutations

Although size is reduced by approximately 50% in cells containing *whi1*, modulation of size with growth rate appears intact. In the *whi1* growth curve, control for all interaction curves which follow, (Figure 3.5) both size and growth rate increase within 30 min. Size almost doubles over the next four hours before reaching a value at

which it plateaus out. Increase after this time is due to cell cycle arrest with continued growth. The increase in the number of unbudded cells will be due initially to cells being arrested in G1 for longer until they attain the new higher value set for critical size at the faster growth rate. This is seen in shift up experiments with rich and poor media. The number continued to climb due to cells arresting as the media became exhausted.

In the majority of interaction growth curves results were clear on the question of whether the cultures stopped proliferating on shift to the restrictive temperatures or not. *Cdc63, dna26* and *cyr1* double mutants all appear to behave in the same way as the control strains. At this level absorbance was relatively successful. Had more time been available then the actual cell numbers would have been used as a parameter with greater accuracy. This may have given a more accurate picture of what occurred at time of shift and allowed the calculation of execution points. While being a poor parameter for general use, absorbance, did give clear indications of interactions in most cases.

There appears to be no interaction between the cell cycle mutation *dna26* and any of the *WHI1* alleles. The exact nature of the *dna26* mutation is not known although it is thought to be involved in the initiation of DNA synthesis. It is known that cell division is independent of DNA synthesis. Size control may be of paramount importance in regulating cell division but not involved at all with DNA synthesis. These data indicate that *DNA26* is not directly connected to the control of size at *start*.

Although *DNA26* seemed to be completely independent of *WHI1*, this was

not seen to be the case with *CDC28*. Isolating double mutants proved very difficult. In fact no double mutant was isolated for *cdc28*, 4 x *whi1* as none of the spores seemed viable. There were also problems with obtaining *cdc28 whi1* single clones. This inviability of the spores was not due to the genetic background. The control strain *cdc28 WHI1* was isolated from crosses with identical strains, as was the double mutant containing the null *WHI1* gene. The control *cdc28* strain showed normal characteristics. On shift to the restrictive temperature proliferation ceased rapidly. This was also true of cells containing no functional *WHI1* gene (*cdc28*, *WHI1*^{0}) which arrested normally. This was not seen in the double mutant containing the hyperfunctional *whi1* mutation.

After shift to the restrictive temperature the size of the cells increased and the number of unbudded cells rose indicating that the cells were arresting. This apparent arrest was only transient in cells containing both mutations. Growth definitely appeared to be interrupted before it continued at approximately the same rate as before. In a previous investigation of *whi1 cdc28* interaction there was no slowing of growth. It continued as if no *cdc28* mutation was present (Ashcroft, 1987). The nature of this interaction is unknown and may be until the exact nature of the action of *WHI1* is understood but it is clear that *whi1* is a suppressor of the mutation in the *CDC28* gene.

Fortunately more is known about the nature of the product of *CDC28*. It is a protein kinase which interacts directly with another protein during G1 to enable the traverse of *start*. The *CDC28* protein kinase appears to phosphorylate the protein just before mitosis begins. When cells are unable to continue proliferation, due to pheromone

arrest or the action of another cell division cycle mutation, the ability of the *CDC28* gene product to phosphorylate the other protein falls. A model has been proposed (Mendenhall *et al.* , 1987) which suggest that the protein which the *CDC28* gene product phosphorylates prevents DNA synthesis in the unphosphorylated state. Phosphorylation by the *CDC28* protein removes the block and synthesis occurs. The action of *WHI1* gene product as an inducer of mitosis may be dependent on this phosphorylation event. It is clear, now that the *WHI1* gene has been cloned and sequenced, that it resembles cyclins (Nash *et al.*, 1988). It is possible that the *WHI1* gene product is one substrate for the *CDC28* protein kinase specifically at *start.* Under normal conditions the *WHI1* protein may require phosphorylation by the *CDC28* protein to enable traverse of *start.* At restrictive temperature the mutant *cdc28* is unable to carry this function out and *WHI1* is unable to induce mitosis. It is known that the mutant *whi1* protein is a truncated form (Nash *et al.*, 1988 & Cross, 1988). The lost portion may contain the phosphorylation sites which enable activation (see Chapter 5). In its truncated form the *whi1* protein may therefore be independent of the phosphorylation event and function even when no functional *CDC28* protein is present to activate it. The mutant protein is known to be hyperfunctional in the truncated form (Nash *et al.*, 1988 & Cross,1988) so it is not unreasonable to assume that the lost C-terminus is a controlling element.

There is no interaction seen between the null *WHI1* allele and the mutant *cdc28* allele. If the model above were in any way correct, no growth would be expected at all in cells with a non functional *WHI1* gene. This is known not the case (Nash *et al.* , 1988, Cross, 1988). This problem would be overcome if there were more than one copy of

this gene in the genome or other cyclin like proteins which could carry out the function of *WHI1*. Two other genes have been found which suppress the mutant *cdc28* and resemble cyclins (Wittenberg *et al.* ,1989). It is possible that these function in the place of *WHI1* when this function is lost.

Another model is one where there are two components which interact. An inhibitor and activator of mitosis. The function of *CDC28* may be to bind the inhibitor allowing the activator to push cells into mitosis. When the truncated activator is present the *CDC28* function may not be required. In spores initial growth may be dependent on the correct function of both components and therefore they may be much more sensitive to disruptions.

Whatever the actual mechanics of the control of the cell cycle at *start*, it is clear that there is an interaction between the *CDC28* protein kinase and the cyclin like *WHI1* protein. Examples of cyclins interacting with *CDC28* like protein kinases to control events in the cell cycle are already documented (Draetta *et al.*, 1989, Pines & Hunter, 1989, Booher *et al.* , 1989 and North, 1989). Although the event controlled is mitosis in these cases, it is possible that the major event controlled by *CDC28* in the *Saccharomyces cerevisiae* cell cycle is at *start* rather than mitosis and that the *WHI1* protein plays an intimate role in this control.

The interaction seen here is not seen with all alleles of mutant *CDC28*. Exact location of all alleles which do interact with *whi1* may reveal clustering. This would indicate different functional domains of the *CDC28* protein.

The reactions between the *WHI1* alleles, *dna26* and *cdc28* are clear. This also appears to be the case with *cdc63.* The control *cdc63* strain growth profile showed the anomaly of what appeared to be residual proliferation. This is the one case where counting cell number would have indicated exactly when cell proliferation ceased. Proliferation did appear to cease after 11/2 hours. Why this delay occurred or if it is a real phenomenon is unclear. It did not occur in the double mutants to the extent seen in the control. Both the other parameters behaved as expected if all the cultures had arrested after the shift in temperature. There is no clear indication of a major interaction between *cdc63* and the mutant *WHI1* alleles. although repeats of the growth curves, counting cell number, would clarify what exactly happened at shift. It may be that this particular strain is leaky for the *cdc63* mutation showing itself as delayed arrest.

Although isolation of double mutants containing *cyr1* was not successful in all cases and interaction was indicated, none was seen in any of the growth profiles. As with all class II *start* mutants, on shift to the restrictive temperature, all growth ceased. This was clear in all cultures. There was a small increase in the size of cells in all cultures, which although not expected, was not as large as in all the class I cultures. The shift up in modulation in cell size usually seen with an increase in growth rate may be responsible, although the *cyr1* mutation should stop growth too quickly to allow this to occur. All cultures exhibited the expected rise in number of unbudded cells of cultures which were undergoing arrest.

Although no mitotic interaction was noted with *cyr1* there did appear to be an interaction which prevented germination of some genotypes.

The lack of sporulation and germination could be explained by something inherent in the strains which produced lethal progeny. This is not thought to be the case as parentals and double wild type strains were recovered in all crosses. It is possible that there is an interaction which only affects germination or which germination is particularly sensitive to. Neither of the mutations on its own is enough to prevent germination but both mutations together prevent some process required for the initial mitotic cycle or so weaken spores that they cannot undergo division. As the spores were germinated at non restrictive temperatures why the *cyr1* mutation should be expressed is not clear. It is know that the product of the *cyr1* mutation is a heat labile cAMP. These results indicate that mutant protein also has an affect at germination, although this is cryptic unless in the presence of a hyperfunctional *whi1* protein. The mutant *whi1* protein may so stress spores at this important stage in development that the defect in the cAMP is expressed and germination fails. Investigation of germination in a larger number of spores should be investigated to assess the reality of this phenomenon and the extent of the lethality.

Although there was no interaction noted between this particular mutation in the cAMP pathway it does not rule out input from this pathway into size control at *start*. The rise and fall in levels of cAMP in the cell according to nutrient status of the surrounding media would be an ideal component in a size modulation mechanism. Further investigation of interaction between mutations in this pathway and *whi1* should be investigated.

The data covered in this section gives some clues as to which areas

of *start* should be further investigated and the gene products which should be looked at in greater detail. Perhaps investigations using plasmid borne *WHI1* alleles in cells carrying *cdc* mutations would yield other interesting results. The actual structure of the *WHI1* gene itself and its protein product may give further clues to the interactions involved in this system and which genes are involved in cell size.

CHAPTER 4: MAPPING OF *whi1*

4.1 INTRODUCTION

Often much can be learned about the nature of a gene product by the cloning and detailed analysis of the coding sequence although the sequence of a cloned gene may be completely different to anything seen before and may contain no sites which have a recognised function and thus nothing may be learned by examination alone. Another approach which may yield some information about the function of the gene is to use the clone to examine effects on the phenotype of cells by over expression or disruption. To this end, cloning of a gene is desirable.

It is usually straightforward to clone a gene where a mutation in that gene gives a clear and easily scorable phenotype. Genomic DNA fragments are inserted into cells and those exhibiting wild phenotype selected. Where the mutation inhibits growth on a particular medium or inhibits growth at a particular temperature selecting the transformed cells containing the wild type gene is simple. This is not the case with cell size. The *whi1* mutation leads to reduced size and as far as was known when this work commenced no other phenotypic change. If the loss in size is due to loss of function in the *WHI1* gene then, after transforming cells with genomic DNA, those which were now showing wild type size would contain the fully functional *WHI1* gene. All transformants, of course, would have to be screened by eye to see which were exhibiting the wild type size. Although this was not an impossible task, it was a daunting one. However it was envisaged that a number of other effects could produce normal size in cells: (i) suppression of the mutant phenotype by other genes involved in size control, (ii) cloning of *cdc* mutations which may have the effect of delaying

DNA synthesis and therefore producing larger cells or (iii) cloning genes which affected the growth rate of cells, again producing cells with a larger size. If direct selection were to be used, any of these problems could arise. It was therefore thought to be impracticable and another strategy was devised.

The technique of co-cloning allows much more accurate and reliable results. It involves the cloning of two genes rather than one. This of course means that a strain must be produced which has two mutations rather than just the single one of interest. As two phenotypes are selected at the same time the chance of selecting suppressors or other individual genes affecting one of the phenotypes is much reduced. Although two genes are neighbours it is unlikely that suppressors for mutations in both those genes are also neighbours. It is also highly unlikely that if the neighbouring genes affect different processes that a single gene will be cloned which will produce a fully wild type strain. Co-cloning thus effectively reduces the number of false positives. It can also make screening for a difficult phenotype easier. When working with a phenotype such as size which is difficult to select for, it is hoped that mutations in neighbouring genes are much easier to score. If a mutation in either neighbour produces a temperature, than recovery of growth at the restrictive temperature (a very easy phenotype to score) will reduce the number to be scored for regain of wild type size. Therefore by creating a strain which has mutations in both the gene and its neighbour, scoring transformants is much quicker. For co-cloning to be successful the location of the gene must be known and its neighbours identified. This is dependent on accurate mapping to a single chromosome and to a small area on that chromosome. Unfortunately the location of *WHI1* is not known. As co-cloning is to

be carried out the gene must initially be mapped to a chromosome and then linked to genes on that chromosome.

There are now several methods which enable the assignment of genes to particular chromosomes in the budding yeast *S. cerevisiae.* All the methods have their advantages and disadvantages according to the gene in question. All the methods devised overcome the major obstacles of the high level of meiotic recombination and the relatively large number of chromosomes. The two basic methods are trisomic analysis (Mortimer & Hawthorne, 1973; Wickner, 1979; Hilger, Prevot & Mortimer, 1982) and chromosome loss methods. Chromosome loss can be induced by chemical methods, as in the use of methyl benzimidazole-2-yl-carbamate (Wood, 1982), or by the use of genetic mutations. The mutations used in mapping include *cdc6* and *cdc14* (Kawasaki, 1979), *ch11* (Liras *et al.* , 1978) and *rad52* (Schild & Mortimer, 1985; Hanic-Joyce, 1985). Other genetic mutations, not based on chromosomes loss, have also been used with some success. The method chosen in this study was the chromosome loss method mediated by the *rad52* mutation.

The *rad52* mutation confers distinct properties on diploids which are homozygous for the mutation. It causes spontaneous chromosome loss or non-disjunction at high frequency in cells undergoing mitosis. This is enhanced by ionising radiation (Mortimer *et al.*, 1981). This sensitivity to radiation (X-rays and UV to a lesser extent; Game & Mortimer, 1974) may be caused by defects in the DNA and ultimately the chromosome repair mechanism (Parry *et al.*, 1979). A defective DNA repair system may also be responsible for the blocked meiotic and mitotic recombination associated with diploids homozygous for the *rad52* mutation, (Prakash *et al.*, 1980; Saeki

et al., 1980; Game *et al.*, 1980). This allows the use of the mutation for mapping genes whose chromosomal location is unknown. Although sensitive to radiation, *rad52* homozygous diploids do not show increased mutation rates when exposedto gamma radiation (McKee & Lawrence, 1979). Spontaneous mutability is raised 10-20 fold by the mutant gene (Prakash *et al.* , 1980) and sporulation is impaired. Few spores are produced by homozygous diploids and those that appear are inviable. This again seems to be a defect in the DNA repair system. Several of these characteristics are relied upon when mapping genes using the *rad52* mutation.

The method of mapping using the *rad52* mutation with its characteristic spontaneous and enhanced chromosome loss is also possible because *S. cerevisiae* can survive as an aneuploid. If at least one of a pair of homologues is present in an aneuploid and that lone homologue carries no lethal mutations the cell will survive and of course all genes on the single homologue will be expressed. Mapping can be carried out in two ways. The gene to be mapped may be located on the same homologue as an auxotrophic chromosome marker or on the homologue which is unmarked. When on the same homologue (or in coupling) the genes will always be expressed together; when on opposite homologues (or in repulsion) they will never be expressed together. The chromosomes are marked with recessive auxotrophic mutations. Diploid strains are heterozygous for all markers as well as the unmapped gene but homozygous for the *rad52* mutation. After irradiation chromosomes are lost, revealing markers (if the dominant wild type allele is lost), with no or insignificant mitotic recombination. The unmapped gene will (1) always be revealed with only one of the chromosome markers, if in

coupling or (2) never found with only one of the chromosome, if in repulsion. As the chromosome location of all the markers is known the unmapped gene may then be assigned to a chromosome (Schild & Mortimer, 1985).

The location of the *WHI1* gene which affects the cell size of *S. cerevisiae* was not known with any certainty and the *rad52* mapping method was to be employed in the hope of locating it on a chromosome. A strain with a mutation in the size control gene was crossed with a strain containing the *rad52* mutation. Progeny from this cross, which contained both mutations, were then crossed with strains containing the chromosome markers as well as a *rad52* mutation. These strains were then used to try and map the *whi1* mutation. Due to the fact that at this stage the number of genes involved in the *WHI1* size control mechanism was unknown mapping using the *rad52* method presented some unique problems. Without knowing the number of genes affecting this phenotype, accurate mapping of the *WHI1* gene could not be carried out. In order to resolve the question of the number of genes involved in cell size control, models were devised which would distinguish between one gene or two.

It is known that mitosis occurs at a controlled time in order to maintain the nuclear-cytoplasm ratio (Fantes *et al.*, 1975). In cells expressing *whi1* phenotype, that control operates at a time resulting in cells with half the wild type size. Although the control seems to reside in one (possibly two) genes the question of the effect of further sets of chromosomes in cells with such a small size should also be examined. Does an almost complete set of chromosomes have any effect on a haploid complement of size control

genes or does the control reside entirely with these genes? If some insight into the actual number of chromosomes present in irradiated diploid cells displaying chromosome loss could be achieved this question might be answered. To this end and because the genes in question displayed dosage effects, chromosome loss was modelled mathematically. The full mathematical model is shown in Appendix I.

It was based on a limited number of assumptions, some of which had been previously verified. The question of the probability of any chromosome being lost due to damage by radiation was approached first. This value could be obtained by two methods. Firstly it could be estimated by looking at the number of cells which died and survived after exposure to a known dose of radiation. The probability of chromosome being lost is calculated by knowing the original number of cells, the number of survivors and the number of homologues each cell contains. As yeast cells can survive aneuploidy, it was assumed that cells only perished if they lost both of any pairs of homologues. Another method of calculating this value, p, was to look at the the distribution of revealed markers in cells after exposure to radiation. From this the mean number of chromosomes lost can be estimated and from this value the probability of any single chromosome being lost can be calculated.

Once a value of p has been calculated for each of the crosses, it was possible to use this value to calculate the probable number of chromosomes lost in any cell showing a given number of extra markers. This is done by use of a binomial expression. The major parameters used in the calculation of this expression were; p, the probability of a chromosome being lost; n, the number of genetically marked chromosomes; k, the mean number of chromosomes lost and m,

the number of unmarked chromosomes. Of these values m and n are known, as stated above, p can be calculated by two methods and knowing these allowed the calculation of k. Expansion of the binomial using a specific value of p allowed the number of chromosomes present in a cell exhibiting a known number of extra markers to be estimated. The size of these same cells can be assessed in a number of ways and these values compared. In this way the relationship between the number of genomes and cell size can be explored. Using this method it is possible to see if the actual number of size control genes alone was important, or their interaction with the number of chromosomes, in controlling cell size. The main thrust of this investigation was the mapping of the *WHI1* gene, other questions which were still unresolved at this time might be answered by the use of this method.

Once the chromosome on which a gene is located is identified accurate mapping using tetrad analysis can be carried out. In this investigation some tetrad analysis was carried out initially as *WHI1* was thought to be located on chromosome I. This was indicated by centromeric mapping data carried out by Finnuala Wynne (pers. comm.).

This type of analysis can be used to show linkage between two genes and between a gene and the centromere of the chromosome it is on. Using a large numbers of spores and mathematical analysis of results, accurate mapping with respect to known markers can be carried out. If gene conversion is taken into account and there is no segregation of suppressors a 2:2 segregation is seen for heterozygous nuclear genes. Tetrad analysis is usually carried out with diploids which are heterozygous at more than one locus. Each

locus is considered independently but when both segregate independently only three types of asci may result; parental ditypes (PD), non parental ditypes (NPD) and tetratypes (TT). Parental ditypes contain spores which show the same segregation of markers as the parental strains, non parental ditypes are spores which contain gene combinations not seen in the parents and tetratypes contain spores with all four combinations of the two heterozygous markers. Map distances can be calculated depending on the relative frequencies of these types of asci using mathematical equations derived by Perkins (1949). Although this is useful in most cases it only accounts for single and double cross-overs. As map distances become greater the probability of getting triple cross-overs increases and the method underestimates distance. More useful are equations using maximum likelihood estimation (Snow, 1979). These equations use the same data but calculate distances with greater accuracy using computer programmes. This sort of accuracy is required if the data from mapping are to be used in the cloning of a gene. Using the methods described above mapping the *WHI1* gene was attempted. It was hoped that once the gene had been mapped it could be cloned.

4.2 RESULTS

Mapping of chromosomes by the *rad52-1* method

Before actual mapping of the gene began models were devised to resolve the question of the number of genes involved and preliminary experiments were carried out to determine i) the spontaneous loss from diploids in a *whi1* background, ii) death rate for given times using the available gamma radiation source and iii) a death rate experiment to assess the effect of supplementing complex media. The death rate experiment also enabled the value of p, the probability of a chromosome being lost, to be calculated.

Based on a limited number of assumptions, some of which have been previously verified, mathematical statements have been formulated which allow the calculation of the probable number of chromosomes present in a population of cells showing random chromosome loss and expressing recessive auxotrophic markers (see Appendix 2). The model is based on an expansion of the binomial theorem as applied to the presence or absence of marked or unmarked chromosomes distributed over 16 pairs with the proviso that loss of both homologues is lethal. The model enables the investigation of properties of the system with respect to several major parameters; p, the probability of loss of any given chromosome; n, the number of genetically marked chromosomes; k, the mean number of chromosomes lost; and m, the number of unmarked chromosomes.

Modelling of chromosome loss and size

Both models were proposed for genes in repulsion. All mapping auxotrophic mutations were on different homologues to the gene being mapped. The models shown only apply if wild type cells can be

distinguished from mutant *whi1* cells. More involved models were proposed which took into account difficulties in distinguishing the size of cells. These predicted that in fact if two genes were present and wild type and mutant cells appeared the same size, the location of the wild type gene or the second size control gene would be indicated with no way of knowing which. Also there would be no way of telling whether there were one or two genes involved in the system. Fortunately it was found that the sizes of wild type and mutant cells could be distinguished from each other after irradiation and only the simple models were required. These are shown in Figures 4.1A and 4.1B. They show that genes do not need to be in coupling for accurate mapping as with other genes mapped using this system. They also show that the one or two gene issue would be resolved by the number of markers associated with the wild type cells (size 2) and diploid cells (size 3). If all size 2 and size 3 cells are found with all the wild type alleles of the auxotrophic markers then two genes control cell size directly. If all but one are found with these size cells then only *WHI1* controls size. In both models the location of the *WHI1* gene will be marked by a single auxotrophic marker never occurring in size 1 cells.

Spontaneous chromosome loss

Previous investigations with *rad52-1* homozygous diploids (Mortimer, Contopolou & Schild, 1981; Schild & Mortimer, 1985) have reported spontaneous loss of chromosomes demonstrated by the appearance of recessive auxotrophic markers. The rate of loss reported was between 10-38% for strains used. As diploids stored for any length of time at 4°C may already have lost chromosomes, only diploids produced from recent matings were used. Batch cultures of cells containing

FIGURE 4.1A One Gene Model For Chromosome Loss

The figure shows the effect loss of chromosomes containing the wild type or *whi1* mutation would have on size and the link with auxotrophic markers. The mutant *whi1* allele is assumed to be non functional.

1 The wild type *WHI1* is lost giving the cell a size of 1. These cells will always be *ADE* $^{+}$ as the mutant gene and wild type auxotrophic markers are on the same chromosome. It may be auxotrophic or wild type for any of the other markers.

2 The mutant *whi1* gene is lost giving the cell a size of 2. These cells will always be *ade* $^{-}$ as the mutant auxotrophic marker is on the same chromosome as the wild type size control gene. Again it may be auxtrophic or wild type for any of the other markers.

3 Neither size control genes are lost, but as the wild type gene is dominant over the auxotrophic mutation it will always be *ADE* $^{+}$ and size 3

This all means that the location of the size control gene will be shown by the fact that size 1 cells will never be found with a single auxotrophic mutation. This will locate the gene to a single chromosome.

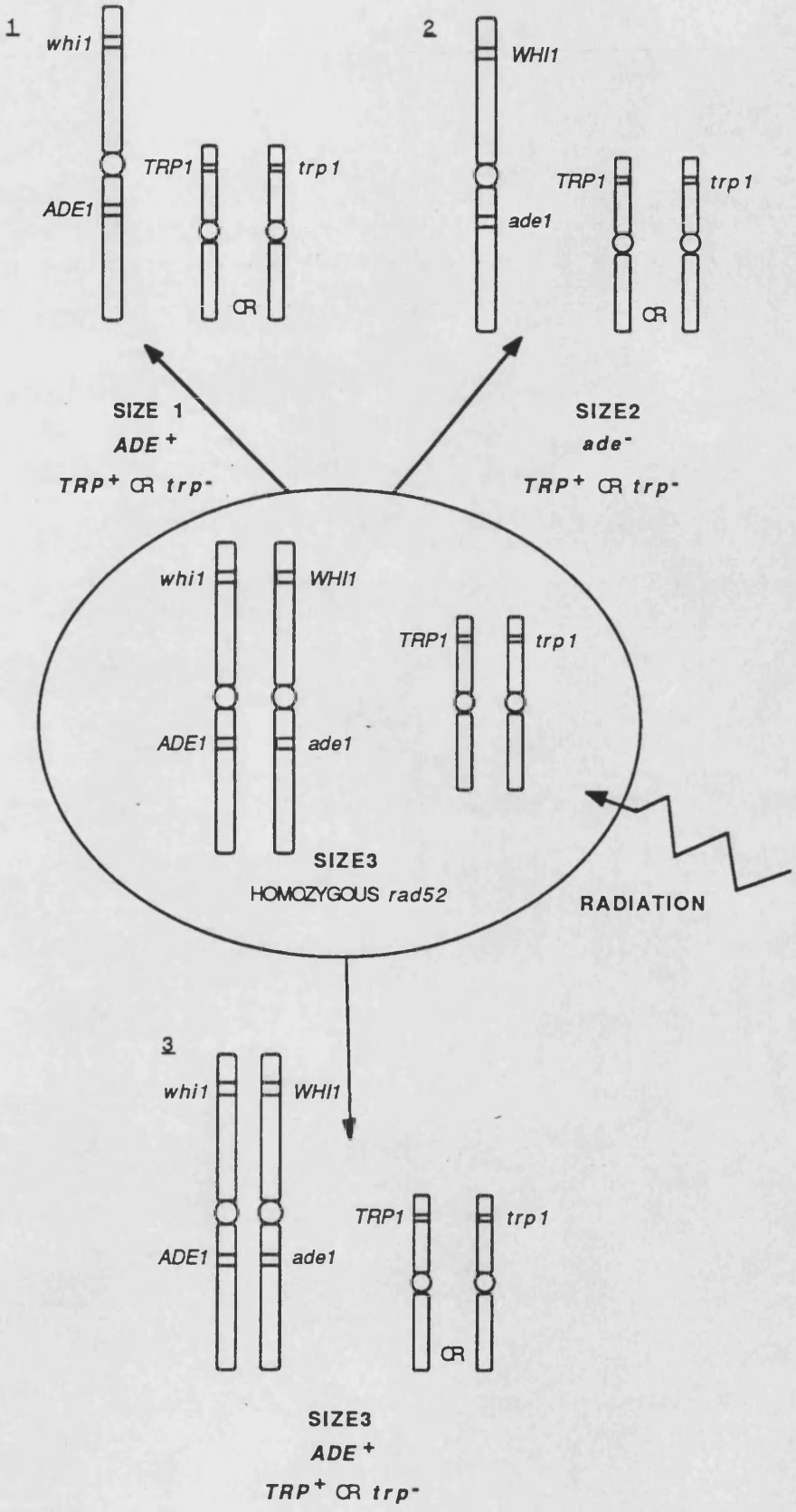

1
whi1
ADE1
TRP1
trp1
CR
2
WHI1
ade1
TRP1
trp1
CR
SIZE 1
ADE+
TRP+ CR trp-
SIZE2
ade-
TRP+ CR trp-
whi1
WHI1
ADE1
ade1
TRP1
trp1
SIZE3
HOMOZYGOUS rad52
RADIATION
3
whi1
WHI1
ADE1
ade1
TRP1
trp1
CR
SIZE3
ADE+
TRP+ CR trp-

FIGURE 4.1B Two Gene Model For Chromosome Loss

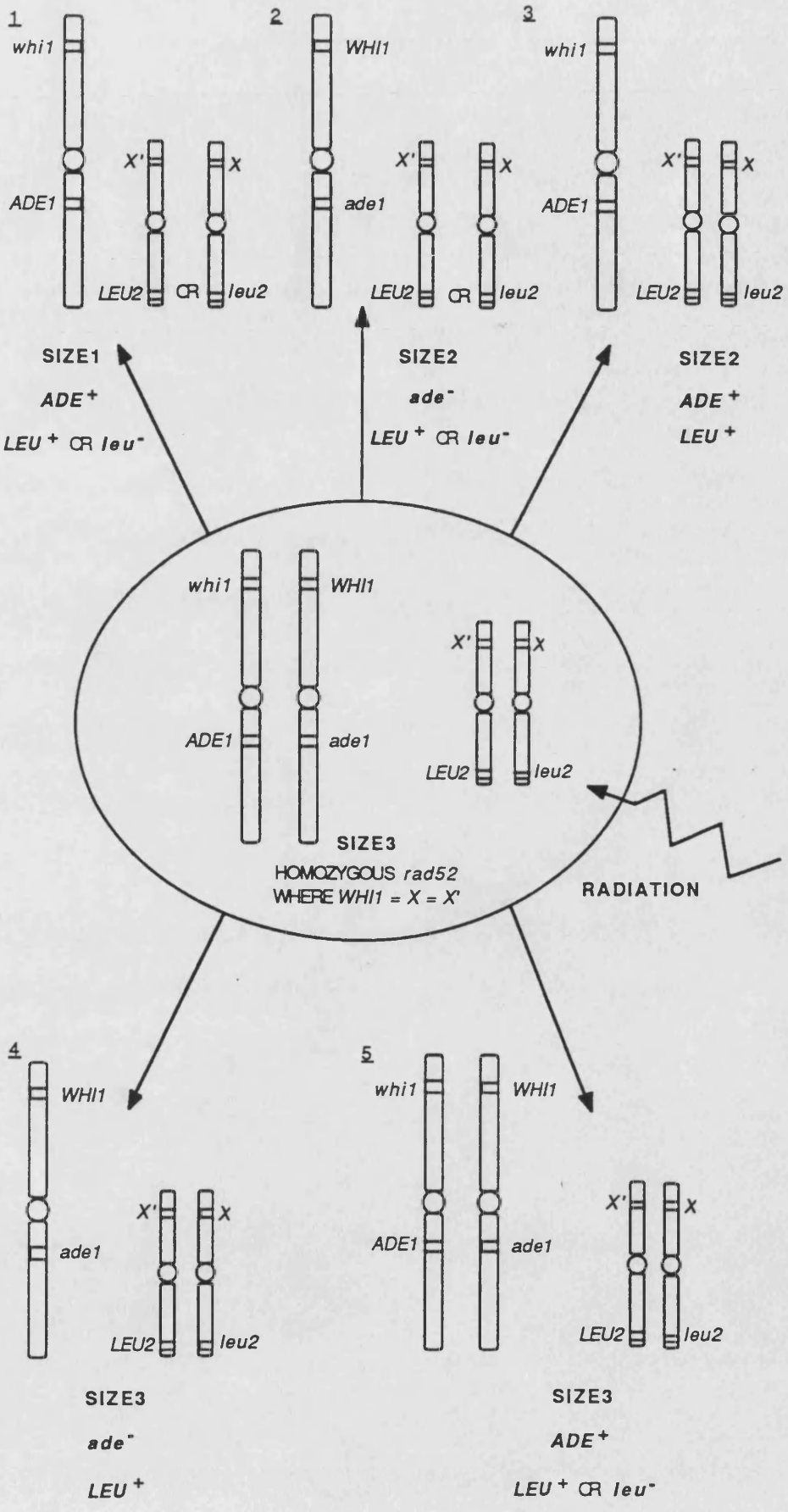
Again this figure shows the effect loss of chromosomes containing the wild type or *whi1* mutation would have on size and the link with auxotrophic markers but assumes that there are two genes controlling size. The second gene (marked *X* and *X'*) are both fully functional and are equivalent to the wildtype *WHI1* gene. The mutant gene again is assumed to be non functional.

1 The wild type *WHI1* is lost giving the cell a size of 1. These cells will always be *ADE*$^+$ as the mutant gene and wild type auxotrophic markers are on the same chromosome. It may be auxotrophic or wild type for any of the other markers.

2 & 3. Two genotypes giving a phenotypic size of 2. Unlike the one gene model these can be found with any wild type marker or auxotrophic marker. One auxotrophic mutation is not found to always be associated with this size class.

4 & 5. Two genotypes giving the phenotypic size of 3. Again unlike the one gene model, this size class is not always associated with any single wild type chromosome marker. Both wild type and mutant phenotypes may be found for all markers in this size class.

To find out which marker gives the chromosome location of the *WHI1* gene look at all size 1 class of cells and identify which marker never occurs in the mutant form. This will mark the location of the gene. Note also by examining whether this marker occurs in both forms or not in the size 2 and and size 3 classes will indicate the number of genes involved in the system.


1
whi1
ADE1
X'
X
LEU2
OR
leu2
2
WHI1
ade1
3
SIZE1
ADE+
LEU+ OR leu-
SIZE2
ade-
LEU+ OR leu-
SIZE2
ADE+
LEU+
SIZE3
HOMOZYGOUS rad52
WHERE WHI1 = X = X'
RADIATION
4
5
SIZE3
ade-
LEU+
SIZE3
ADE+
LEU+ OR leu-

the *whi1* mutation were diluted daily 200 fold into fresh YPD, sampled at intervals and 100 of the resulting colonies grown on YPD were tested for chromosome loss by the appearance of additional auxotrophies on synthetic omission plates. Spontaneous chromosome loss would confirm that the strain used was homozygous *rad52-1*. A control strain was grown under the same conditions with a heterozygous *rad52* genotype. The results are shown in table 4.1. Initially loss was seen in only 7% of the colonies but rose to 23% after 170hr of semi-continuous growth. A sample of the 170hr cultures was plated out for comparison with a new diploid. The comparison is shown in plate 4.1.

Death rate experiment

To estimate p, the probability of a chromosome being lost, and to give an indication of the dose needed to result in less than 1% survival, one of the homozygous *rad52* diploids was irradiated for varying times and the survival rate estimated. The control strain used was one which was heterozygous for the *rad52* mutation. With a dose of 20.3krads, strains homozygous for *rad52* were 2 orders of magnitude more sensitive to killing by irradiation than strains heterozygous for the *rad52* mutation.

After the dose for chromosome loss had been decided a further death rate investigation was carried out at that dose to estimate p. The results from all these investigations are shown in tables 4.2. and 4.3. Although the value of p varied from strain to strain, the death rate experiment was carried out on only one strain, this being chosen at random to give a general idea of the magnitude of the value.

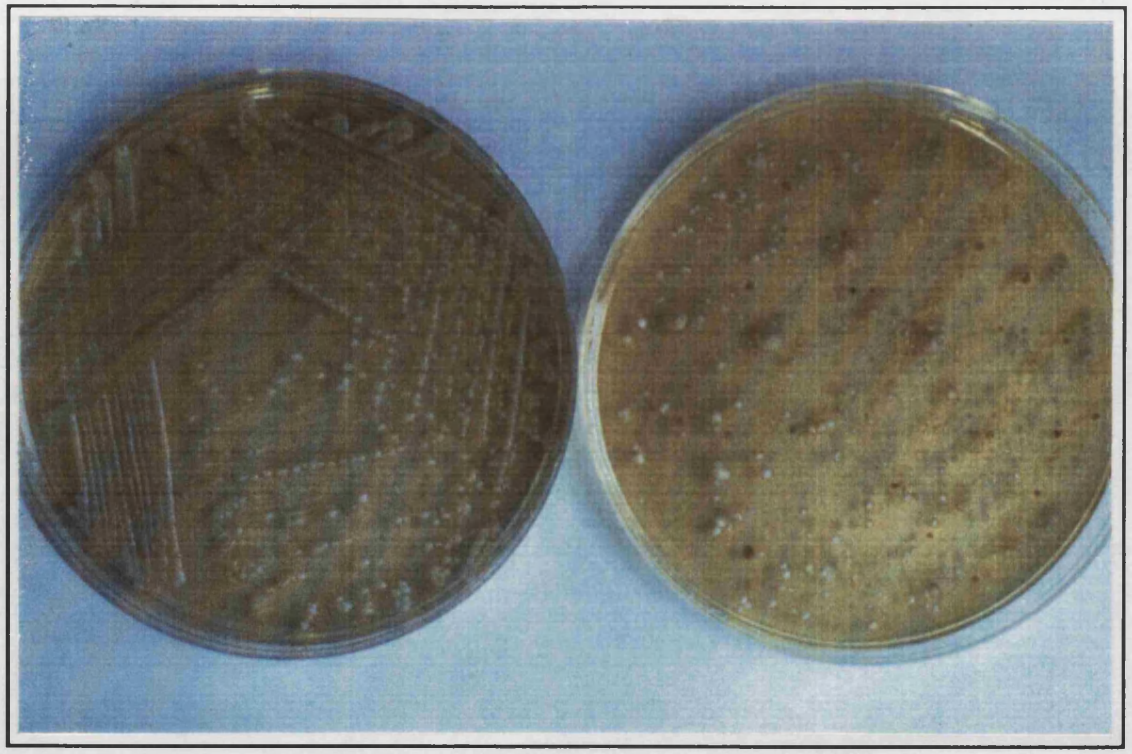


PLATE 4.1 Spontaneous Chromosome Loss From *rad52* Homozygous Diploids

Comparision of a new diploid (a) with one which has been grown semi-continuously for 170hr before plating out for single colonies (b). Red colonies have grown from cells which have aquired an additional adenine auxotrophy due to spontaneous chromosome loss during mitosis due to the presence of the *rad52* mutation.

TABLE 4.1 Spontaneous Loss of Chromosomes From Homozygous *rad52* strains

Both strains were grown semi-continuously for 170hr in liquid YEPD. Samples were withdrawn (100µl) at the times shown and plated out to give single colonies on YEPD.

After 5 days growth, 100 colonies from each was regrown on YEPD before being analysed for the appearence of addition auxotrophic markers. The control strain was heterozygous for the *rad52* mutation. The number of strains showing 1 or 2 additional markers is shown.

Time after inoculation (hr)	% Showing additional auxotrophies	Number of additional markers		
		0	1	2
Homozygous Diploid				
0	7	93	7	0
24	9	91	8	1
72	19	81	16	3
170	23	77	20	3
Control				
0	0	100	0	0
24	0	100	0	0
72	0	100	0	0
170	0	100	0	0

TABLE 4.2 Rate of Cell Death in Cells Homozygous For the *rad52* Mutation.

Cells homozygous for the *rad52* mutation were exposed to radiation for increasing lengths of time before being plated out for single colonies on YEPD. Cell number was accurately measured before exposure. After exposure cells were diluted x 1000 before plating. Replicates were plated out to give greater accuracy in results.

The control strain was heterozygous for the *rad52* mutation. While some mortality was noted in the control strain, it in no way matched the rate of death in the homozygous diploid strain.

		TIME OF IRRADIATION						
		0	5	10	20	30	40	50
CONTROL MATERIAL	PLATE COUNTS	25 31 27 39 30.5	34 26 30 33 30.25	31 26 28 22 26.75	24 24 30 22 25	22 22 24 18 21.5	19 22 18 23 20.5	17 18 13 17 16.25
	ACTUAL CELLS PER ML	3.05×10^4	3.03×10^4	2.68×10^4	2.50×10^4	2.15×10^4	2.05×10^4	1.63×10^4
	% OF ORIGINAL VALUE	100	99.2	87.7	81.9	70.5	67.2	53.3
TEST MATERIAL	PLATE COUNTS	29 22 30 29 27.5	108 109 99 106 105.5	208 234 231 215 224.3	101 82 93 87 90.75	43 32 35 45 38.75	7 8 5 6 6.5	20 13 22 30 21.3
	ACTUAL CELLS PER ML	2.74×10^4	1.06×10^4	3.36×10^3	5.67×10^2	5.80×10^1	9.75	3.00
	% OF ORIGINAL VALUE	100	38.5	12.2	2.06	0.21	0.03	0.01

Table 4.3 Death Rate Experiment to Obtain Values of p.

Cells were grown up in YEPD until in mid to late exponential phase. Cell number was obtained by direct counting. Cells were then irradiated for 45 min and plated out to give single colonies (100µl per plate). The number of cells surviving were counted and calculations of p the probability of a chromosome being lost, calculated using expression [A7] (see Appendix I) for each cross.

	Cross Number			
	1	2	3	4
Pre-Irradiation Counts	112 120 116	52 63 56	163 142 152	126 139 105
Mean	116	57.5	152	122
Cells per 100 µl	1.16×10^5	5.75×10^5	1.52×10^5	1.22×10^5
Post-Irradiation Counts	324 337 321	233 199 205	269 274 292	74 80 76
Mean	327.5	212.3	278.3	76.7
Cells per 100µl	327	212	278	76
% Survivors	0.282	0.369	0.183	0.628
Equivalent p value	0.55	0.54	0.57	0.52

The irradiation times of 35 and 45min were used to carry out the supplementary death rate experiment on all the diploids used to carry out the mapping. The values range from 0.06-0.18% survival which corresponds to p values ranging from 0.52-0.57. As the p values are of such a small range a mean value was taken for the calculation of probable chromosome loss numbers in irradiated cells.

Determination of survival on supplemented media

This additional experiment was carried out after the mapping had been completed. The values for the probability of a chromosome being lost had been calculated from the death rate experiment and also from the distributions of the number of colonies showing the range of additional auxotrophies. There was some discrepancy between these values which may have been due to cells failing to grow after irradiation even though they actually survived the effects of the gamma radiation. Supplementing the regularly used YEPD with all the amino acid requirements which may become essential after weakening by irradiation was proposed as a way of increasing the survival of weakened cells and showing the true number of non-lethal radiation damage events. If more cells survive due to the supplements then the proportion of cells showing auxotrophies should increased as well as the total number of cells. To this end the number of red colonies (those showing adenine auxotrophy) was counted and taken as representing the proportion of auxotrophies. The results are shown in table 4.4.

With survival rates of approximately 1.5% there were 25% more survivors on the supplemented plates compared to the unsupplemented plates. The percentage of red adenines doubled to 7.7% of the survivors. The difference between the supplemented and

TABLE 4.4 Growth on Supplemented and Unsupplemented Media

A sample of cells was spilt and divided into four aliquots. These were all irradiated for the same time. One group were plated on YEPD the other on YEPD supplemented with amino acids. The total number of colonies which resulted were counted as well as just reds. These were taken as a measure of cells which showed additional auxotrophies.

Plate No.	UNSUPPLEMENTED MEDIA				SUPPLEMENTED MEDIA			
	Pre-Rad Controls	Total Cols.	Reds	% Reds	Pre-Rad Controls	Total Cols.	Reds	% Reds
1	30080	584	20	3.4	31680	626	52	8.3
2	37000	438	17	3.8	37290	650	73	11.2
3	36470	491	14	2.9	34360	616	34	5.5
4	35940	340	13	3.8	33000	416	18	4.3
MEANS	34870	463	16	3.45	34080	577	44	7.67

unsupplemented total numbers as well as the percentage of red adenine auxotrophic colonies was highly significant although not large enough to explain the difference in the values of p, the probability of chromosome loss.

Actual chromosome mapping

Between 700 and 1000 irradiated cells were examined for each of the mapping crosses 1-4. All the chromosomes were included by these crosses except numbers 11 and 12. These were covered by two additional crosses in which only the specific chromosome markers for 11 and 12, with one other as a control, were examined. The results are laid out in tables 4.5-4.9. Two radiation doses were used, the higher dose giving a higher mean number of chromosomes lost in all crosses. All the values for the probability of a chromosome being lost, as calculated from the distribution of additional auxotrophic markers revealed, were less than one half those calculated from the death rate experiment for the same radiation dose. Unfortunately the data fitted none of the models proposed in that the specified phenotypes were not found associated with only one auxotrophy as expected therefore as far as they were concerned no clear conclusion could be drawn (see figure 4.1A and 4.1B).

The distribution of markers revealed by chromosome loss after irradiation fitted expected distributions in all but one case (cross 2) with a 26Krad dose where there was a significant difference. Table 4.10. shows the data from co-ordinate expression calculations. In a few cases there was small significant difference between the expected and observed values. The differences were however not as large as would be expected if true repulsion of markers were being observed.

TABLES 4.5-4.9

Mapping *whi1* Using the *rad52* Crossing Strains

All the following five tables have the same format. Each shows the names of the strains involved in the cross. The genotypes of these strains can be found in Table 2.1. The cross numbers are arbitary and only refer to the order the crosses were carried out in. The size of cells in all crosses was assessed by eye. Samples were taken and checked by electronic particle sizer. Where two mutations were present for the same amino acid requirement no attempt was made to distinguish between them.

DIP (3) = Size of 3, corresponding to the heterozygous diploid.

WT (2) = Size of 2, corresponding to the wild type haploid.

WHI (3) = Size of 1, corresponding to the mutant *whi1* haploid.

/ = No cells corresponding to this size class were found.

Marker Revealed	Chromo. No.	Size			Totals
		DIP (3)	WT (2)	WHI (1)	
None	/	147	78	1	226
ade5	7	51	42	1	94
arg4	8	25	26	1	52
his7	2	38	49	1	88
ilv3	10	49	36	2	87
leu2	3	26	24	1	51
trp1	4	75	54	3	132
ura3	5	57	44	/	101
lys2 or *lys7*	2 or 13	52	58	3	113
Totals		520	411	13	944

TABLE 4.5 Results From Cross 1

Cross between:

XS195 x NF30

Marker Revealed	Chromo. No.	Size			Totals
		DIP (3)	WT (2)	WHI (1)	
None	/	133	109	3	245
ade1	1	50	45	2	97
arg4	8	19	35	3	57
aro7	16	17	30	1	48
his2	6	8	20	/	28
leu1	7	25	42	2	69
lys2	2	36	49	2	87
trp1	4	44	58	3	105
Totals		332	388	16	736

TABLE 4.6 Results From Cross 2

Cross between:

XS194 x NF30

Marker Revealed	Chromo. No.	Size			Totals
		DIP (3)	WT (2)	WHI (1)	
None	/	188	134	1	323
ade2	15	11	13	1	25
ade4	13	42	73	8	123
arg1	15	23	24	2	49
his7	2	33	52	6	91
ilv3	10	25	59	4	89
leu2	3	28	28	4	60
met6	5	9	13	/	22
ura3	5	47	65	3	115
Totals		406	461	29	897

TABLE 4.7 Results From Cross 3

Cross between:

XS206-9B x NF30

Marker Revealed	Chromo. No.	Size			Totals
		DIP (3)	WT (2)	WHI (1)	
None	/	88	66	/	154
ade2	15	5	16	1	22
arg4	8	14	42	2	57
his6	9	29	33	5	67
ilv3	10	40	72	5	127
leu2	3	19	30	3	52
met4	14	15	23	/	38
trp5	7	46	63	4	113
lys2 or *lys9*	2 or 4	64	102	3	171
ura1 or *ura3*	11 or 5	43	80	5	128
Totals		374	527	28	929

TABLE 4.8 Results From Cross 4

Cross between:

XS214-1B x NF30

Marker Revealed	Chromo. No.	SIZE			Totals
		DIP(3)	WT(2)	WHI(1)	
Cross 5					
met1	11	12	27	1	40
lys2	2	21	21	2	44
	From a total of 360 colonies				84
Cross 6					
trp1	4	19	31	3	53
ura4	12	28	18	2	48
	From a total of 360 colonies				101

TABLE 4.9 Results From Crosses 5 and 6

Cross 5 was between:

NF24 x NF20

Cross 6 was between:

XS209 x NF23

TABLE 4.10

Co-ordinate Expression Data

Given the probability of any marker (including either *WHI1* or *whi1*) being expressed in a cell after irradiation, it is possible to calculate the probability of two occurring together. This will be affected on were the markers are. If they are on the same homologue they will never be expressed at the same time. The table shows the predicted number of size two cells (those expressing *WHI1*) cosegregating with any given. Also shown is the actual number occuring. The significance of difference is based on a 2 x 2 contingency table with one degree of freedom. - = $p > 0.05$; * = $0.05 > p > 0.01$; ** = $0.01 > p > 0.001$. The size are in comparision to *WHI1/ whi1* heterozygotes (size 3), *WHI1* haploids (size 2) and *whi1* haploids (size 1). The values for *ade4* are high because *ade2* was also in the cross.

Chromosome	Marker	Number of Cells of Size			Predicted Number of Size Two Cells	Significance of Difference
		Three	Two	One		
1	*ade1*	50	45	2	51.19	*
2	*lys2*	57	70	4	69.65	-
	his7	71	101	7	85.34	*
3	*leu2*	73	82	8	80.50	-
4	*trp1*	138	143	9	138.08	-
5	*ura3*	104	109	3	104.13	-
	met6	9	13	0	11.69	-
6	*his2*	8	20	0	15.08	-
7	*trp5*	43	65	4	65.50	-
	leu1	25	42	2	36.10	-
	ade5	51	42	1	41.05	-
8	*arg4*	58	103	6	84.94	* *
9	*his6*	29	33	5	36.26	*
10	*ilv3*	114	167	11	147.53	*
11	*met1*	12	27	1	23.05	-
12	*ura4*	28	18	2	23.28	*
13	*ade4*	42	73	8	61.14	-
14	*met2*	15	23	0	22.50	-
15	*ade2*	16	29	2	25.04	-
	arg1	23	24	2	24.99	-
16	*aro7*	17	30	1	25.33	-
Totals		995	1286	79		

TABLE 4.10 Co-ordinate Expression Data

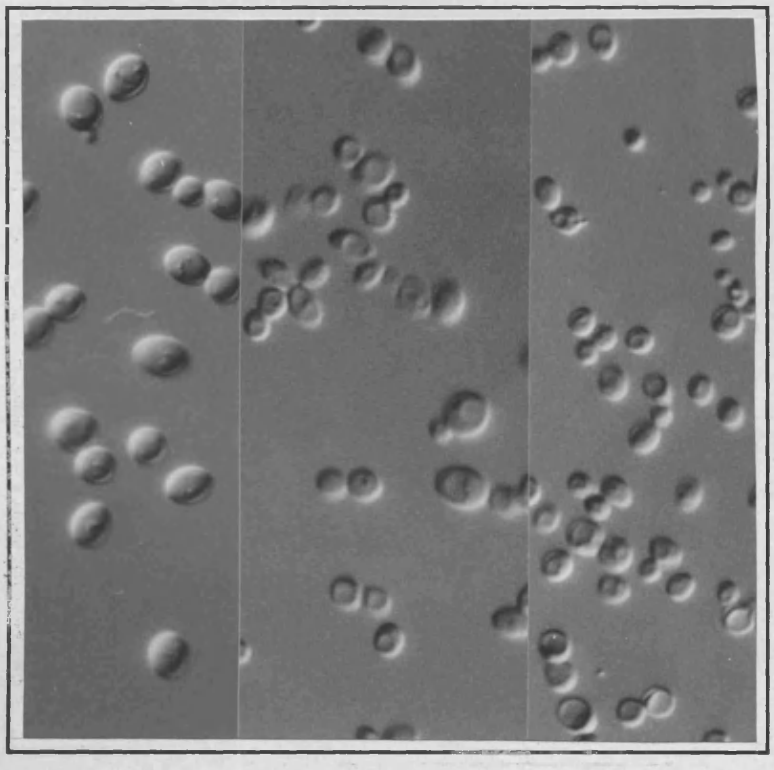
When the data were examined as a whole a number of points emerged. First, it was possible to unambiguously assign in excess of 97% of the cells to one of three size classes comparable to size units one, two and three. The remaining 3% of cells were assigned to all three size categories after accurate size measurements were made. A number of the results of examination by electronic particle counter (which gave an estimate of the actual size of cells) is shown in table 4.11. Also shown in plate 4.2 is a comparison of cell size by direct examination. All photographs were taken at the same magnification and care was taken to ensure printing at the same enlarger value. Secondly, the ratio of frequency of size cells 3, 2 and 1 was approximately 15:17:1 (data not shown). Thirdly the results show that all chromosomes were not lost with the same frequency. Although the sample size was only 2058 loss events, there seemed to be a clear indication that some chromosomes were lost with greater frequency than others. An examination of chromosome length showed that the frequency of loss did not seem to be related to size. Nevertheless, chromosomal markers were lost at different rates which were up to five fold different. Similar results were reported by Hanic-Joyce (1985). This anomaly may invalidate the use of binomial expressions in the calculation of probable number of chromosomes lost which relies on p, the probability that any chromosome is lost, being equal for all chromosomes.

Calculation of the probable numbers of chromosomes lost and the theoretical distribution of population chromosome loss

Using the expression:

$$Z = K + B(n-K, p) + B(m, 2p/1+p)$$

MAG X400



WHI1whi1	*WHI1*	*whi1*
Heterozygotes	Haploids	Haploids
Size 3	Size 2	Size 1

PLATE 4.2

The Three Size Classes Found After Irradition to Induce Chromosome loss

Eamples of the three size classes found after irradiation of the *WHI1whi1* heterozygous diploids which are homozygous for the *rad52* mutation. Size difference was due to the loss of chromosomes which carried the *WHI1* or *whi1* genes. All pictures were taken and printed at the same magnification.

SAMPLE SIZE BY EYE	READING 1	READING 2	MEAN READING	SIZE (μm^3)
WT STANDARD	271	277	274	28
whi STANDARD	317	315	316	53
WT/*whi* DIPLOID STANDARD	343	337	340	76
whi	285	285	285	33
whi	282	290	286	34
WT	327	323	325	61
WT	308	316	312	51
WT	318	315	316	54
WT	317	313	315	52
WT/*whi* DIPLOID	341	347	344	81
WT/*whi* DIPLOID	352	350	351	91

TABLE 4.11 Analysis of Eye Scored colonies Size by Electronic Particle Sizer

Colonies which had originally scored for their size by eye were grown up in liquid culture until mid-exponential phase and analysed by an electronic particle sizer. Using parental strains as standards a accurated assessment of size was made. Although some variation was seen, all sizes were assessed correctly by eye.

where Z= total number fo chromosomes lost

K= number of marked chromosomes

n= number of marked chromosome pairs

m= number of unmarked chromosome pairs

p= probability that any chromosome is lost

the probability of any given number of chromosomes being lost can be calculated for any given number of marked chromosomes appearing after irradiation. Table 4.12 shows examples of values obtained from the use of this binomial expression. Values were calculated from cross three data using either 0 or 3 additional markers appearing in strains after irradiation. The binomial calculates the probabilities for the actual number of chromosomes loss in these strains. This gives an idea of the value which one needs to know; how many chromosomes these strains contain. Both the estimated values of p have been used to calculate the totals of the number of chromosomes lost. The table shows that although no or a low number of markers may be revealed a large number of chromosomes may be lost. This is particularly true if the probability of a chromosome being lost is 0.57. At this higher value the mean number of chromosomes lost differs less between 0 and 3 markers revealed than it does at the lower value.

As well as individual cases the equations in Appendix 2 enable the calculation of total chromosome loss to be made. Expected values for the number of markers shown are obtained by inserting the value of p calculated into the binomial expression:

$$P(8, p/1+p)$$

This is then expanded and multiplied by the number of members in the distribution (N) which in this case is cross 3 (442). Figure 4.2

TABLE 4.12

Probability Distributions for a Given Number of Markers Revealed

The table represents a set of expanded binomial expressions where a given number of markers are revealed. Also two different values of p have been used. In cases 1 and 3 $p = 0.57$ and in cases 2 and 4 $p = 0.19$. These represent the values calculated by rate of death after irradiation and by calculation from distribution of markers revealed respectively. The probabilities represent the probability for that number of chromosomes having been lost. These probabilities are multiplied by the the number (N) showing the number of markers revealed (either 0 or 3). The difference in means is greater between different values of p than differing numbers of markers revealed. Even with the low value for p the mean number of chromosomes lost in a cell with no markers revealed is 4.1.

CHROMO. NO.	CASE 1 PROBS.	X323	CASE 2 PROBS.	X323	CASE 3 PROBS.	X42	CASE 4 PROBS.	X42
0	0	0	8.6x10-3	2.8	0	0	0	0
1	1.2x10-6	0	4.8x10-2	15.6	0	0	0	0
2	1.7x10-5	0	1.3x10-1	40.7	0	0	0	0
3	1.6x10-4	0.1	2.0x10-1	65.8	5.0x10-7	0	1.6x10-2	0.6
4	1.0x10-3	0.3	2.3x10-1	73.5	1.3x10-5	0	7.9x10-2	3.2
5	4.7x10-3	1.5	1.9x10-1	60.2	1.7x10-4	0	1.8x10-1	7.2
6	1.6x10-2	5.3	1.2x10-1	37.2	6.6x10-4	0	2.4x10-1	9.8
7	4.4x10-2	14.3	5.5x10-2	17.9	6.7x10-3	0.3	2.9x10-1	11
8	9.4x10-2	30.3	2.1x10-2	6.7	2.5x10-2	1.0	1.5x10-1	6.0
9	1.6x10-1	50.3	6.1x10-3	1.9	6.8x10-2	2.7	7.2x10-2	2.9
10	2.0x10-1	65.2	1.4x10-3	0.5	1.4x10-1	5.6	2.6x10-2	1.0
11	2.0x10-1	65.3	2.5x10-4	0.1	2.1x10-1	8.4	6.9x10-3	0.3
12	1.5x10-1	49.6	3.3x10-5	0	2.3x10-1	9.3	1.4x10-3	0.1
13	8.5x10-2	27.6	3.3x10-6	0	1.8x10-1	7.3	1.9x10-4	0
14	3.3x10-2	10.6	2.0x10-7	0	9.8x10-2	3.9	1.8x10-5	0
15	7.8x10-3	2.5	0	0	3.2x10-2	1.3	1.1x10-6	0
16	8.6x10-4	0.3	0	0	4.6x10-3	0.2	0	0
MEAN		10.4		4.1		11		6.6

TABLE 4.12 Probability Distributions for a Given Number of Markers Revealed

CASE 1 Z= 0 + B(8, 0.57) + B(8, 0.756)

CASE 2 Z= 0 + B(8, 0.19) + B(8, 0.319)

CASE 3 Z= 3 + B(5, 0.57) + B(8, 0.756)

CASE 4 Z= 3 + B(5, 0.19) + B(8, 0.319)

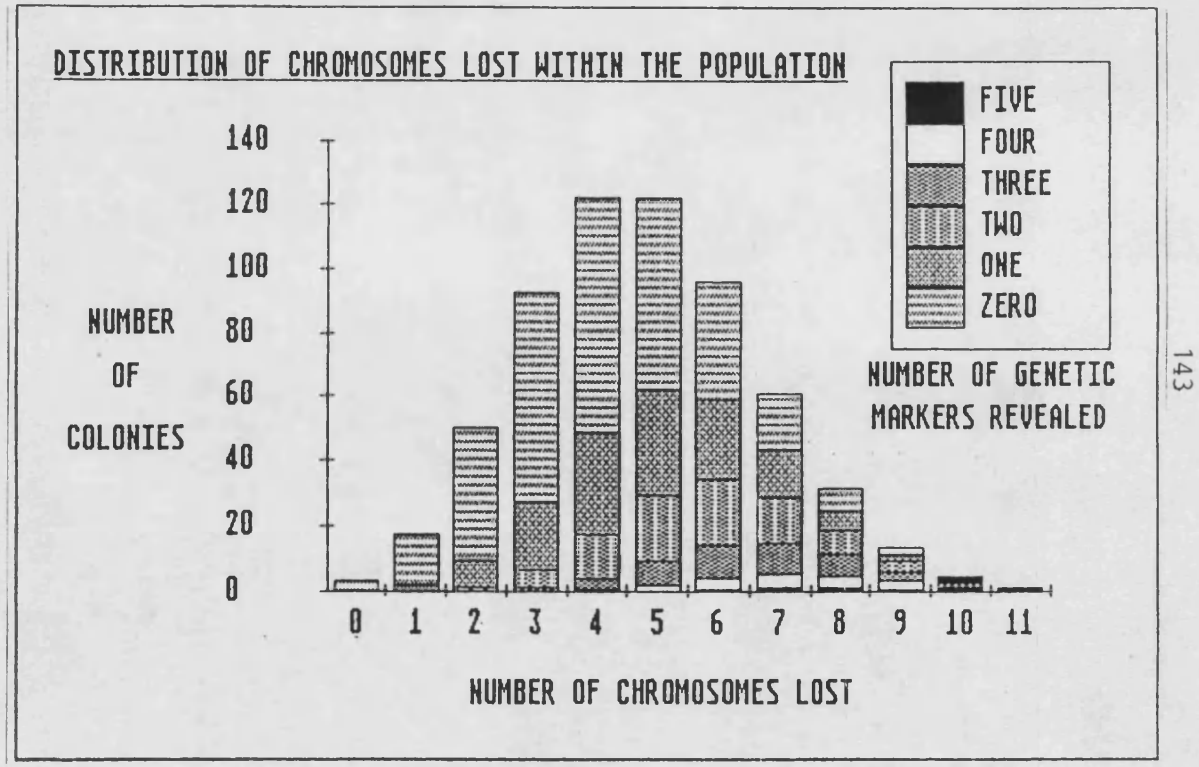
MEAN IS THE MEAN NUMBER OF CHROMOSOMES LOST

FIGURE 4.2

The binomial expression:

$$P(8_{p}p / 1 +_{p}')$$

was expanded using a value of p calculated from a distribution of markers revealed. The expanded values were multiplied by the number of members in that distribution (N) which was 442. This gives the distribution of the number of chromosomes lost in the population.



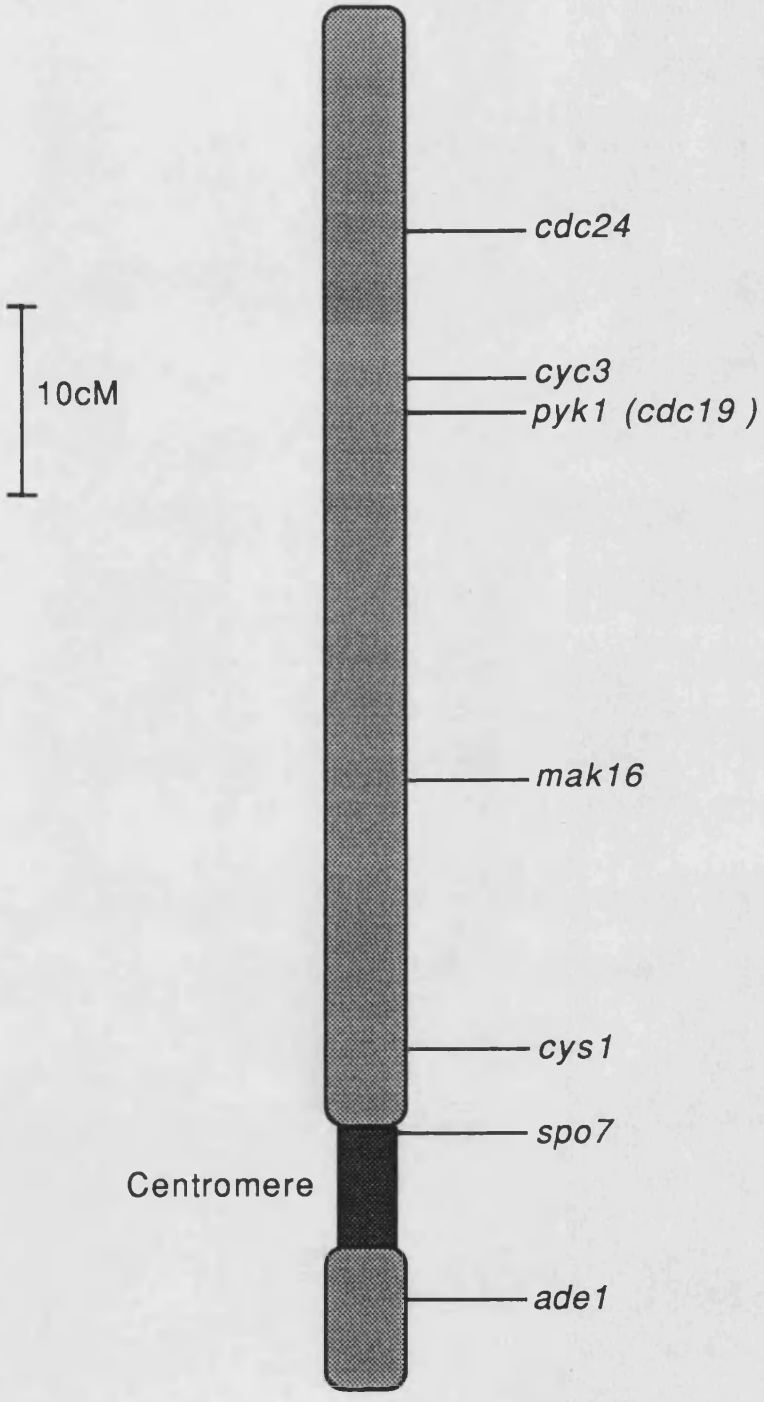


FIGURE 4.3 The left arm of chromosome I showing the location of known mutations referred to in this thesis

TABLE 4.13 Tetrad Data From Crosses Involving Mutations on Chromosome I

Data from all crosses made is shown. Calculations were only made on results where all four spores germinated. Asci with two or three spores germinating were ignored. The map distances (X') are the distances between the two mutations involved in the cross and were calculated by maximum likelihood methood (Snow, 1979). Errors for all these distances are shown. The cross involving *cdc24* gave only two spored asci, even though several alleles were tried.

CROSS NUMBER	STRAINS INVOLVED	ALLELES IN CROSS	% WITH FOUR SPORES GERM.	% FOUR SPORED ASCI	TOTAL TETRADS	PARENTAL DITYPES	NON-PARENTAL DITYPES	TETRATYPES	X' MAP DISTANCE
1	ISO19 JW4-5C	*whi1* *cys1-3*	50	<50	13	5	1	7	63.66 +57.19 -
2	IS019 395	*whi1* *cdc19-1*	99	80	89	9	8	72	78.51 +17.39 -
3	ISO19 B-614	*whi1* *cyc3-10*	<50	70	19	4	1	14	56.27 +21.88 -
4	ISO19 182-6-3	*whi1* *cdc24-1*	0	/	/	/	/	/	/ /
5	ISO19 2597	*whi1* *mak16*	99	>50	73	38	2	33	32.38 + 8.13 -
6	ISO8B B-614	*whi1mak16* *cyc3-10*	99	90	24	7	4	13	75.23 +53.47 -

shows the distribution for each value of known markers revealed and the overall chromosome loss distribution within the population.

Mapping of chromosome one by tetrad analysis

Although all crosses involving known mutations on the left arm of chromosome one (see Figure 4.3) were carried out (except *spo7*), results from some crosses were at best unsatisfactory and at worse unusable. The cross with strains containing the *cdc24* mutation (two different strains were used) resulted in only two-spored asci. These could not therefore be used for tetrad analysis. Although the results appeared clear-cut from certain markers, later discoveries put them in doubt. In all crosses only the results from true tetrads were taken. This was indicated by the segregation of markers under examination and confirmed by mating types and auxotrophic markers. Results of all crosses can be seen in table 4.13. There was no clear result from the mapping data. Although some results suggested that the *WHI1* gene was present on this chromosome others suggested it was not. It was decided on the the strength of these data to try and confirm if *WHI1* was to be found on chromosome I or elsewhere in the *S. cerevisiae* genome

4.3 DISCUSSION

The frequency with which there was spontaneous loss of chromosomes, as revealed by the appearance of recessive auxotrophic markers, was between 7% and 23% for the strains used. Chromosome loss was shown to be a progressive phenomenon during growth and diploids heterozygous for the *whi1* mutation appeared to lose chromosomes spontaneously at a rate comparable to strains in other investigations (Mortimer *et al.*, 1981; Schild & Mortimer, 1985). Any differences could be attributed to the inherent differences between the non-isogenic strains rather than the influence of the mutant *whi1* gene being expressed. The gamma radiation dose required to effect chromosome loss was also comparable to previously determined values.

The mathematics has enabled us to explore the process of chromosome loss in more detail. As the radiation dose resulting in less than 1% survival had to be known for the strains used, an initial death rate experiment had to be carried out. The data from this gave one of two independent values of p (the probability of a chromosome being lost). After the initial experiment, using a randomly chosen strain, death rate estimates were made for all strains used in mapping. Death of cells in these experiments was thought to be solely due to the loss of both homologues of a pair of chromosomes. The range of percentage survival found can again be due to the inherent differences found in non-isogenic strains. This is borne out by the small range in the values of p calculated from these data. As a common mechanism is present in all strains for recovery from this type of damage and all strains have comparable numbers of auxotrophic markers, this is perhaps not surprising. The

comparability of the p values indicates that this is a valid method of calculation for this parameter.

The other method of calculating p is to estimate it from the chromosome loss frequency distributions obtained from the mapping data which is shown in tables 4.6-4.10. A comparison of the two different estimates of p indicates a three fold discrepancy in the values. Since the lower value of 0.174 was obtained directly from the frequency distribution of revealed markers this is the value which gives a direct estimate of the probability of chromosome loss. The higher value of 0.57, the mean value from the death rate experiments, was calculated on the assumption that death was due entirely to both homologues in a pair being lost in cells homozygous for the *rad52* mutation. If this value is used to calculate the frequency distribution of revealed markers it is found that i) the calculated distribution is not in agreement with the predicted distribution and ii) on average the population has lost at least 10 (out of 32) chromosomes with the fraction of cells revealing no markers being vanishingly small compared with the observed value of approximately 50%. All this indicates that the value calculated from the death rate experiment is too high. The number of survivors is too small therefore to be accounted for entirely by nullisomy. Death must be occurring for other reason(s). Control data also showed that unmarked *RAD52/rad52* heterozygotes also died following irradiation. This was presumed to be due to DNA damage. It is thought that the *RAD52* gene is involved in DNA repair, especially in the repair of double strand breaks. As the function of the *RAD52* was not impaired in the control strain, death must have occurred because of damage which could not be repaired by the *RAD52* system. Another reason could be that cells that had lost many homologues become so weakened

that they could not recover after irradiation even on complex media. The addition of amino acid supplements was seen as a way of aiding these weakened cells, allowing them to initiate growth. The number of cells recovering did increase, as did the proportion of cells exhibiting auxotrophic markers as indicated by an increase in the percentage of red colonies (expressing *ade2*). However the increase was not enough to account for the discrepancy between the two calculated values of p. Hanic-Joyce (1985) noted that many suppressed temperature sensitive alleles are present in normal populations and chromosome loss of the suppressor reveals the temperature sensitive defect. If there are other suppressed subvital mutations present then these will also be a cause of death after irradiation and perhaps contribute to the high value of p deduced from viability experiments.

The chromosome mapping gave no clear indication of the location of the *WHI1* gene. This was in part a lack of knowledge of the exact nature of the mutation and in part a failure of the technique to cope with a parameter which might have been affected by many other factors than just the expression and action of a single gene. This was of course not evident until the mapping had been carried out. The gene being mapped was a dose dependent gene and this type of gene had not been mapped by this method before. The mathematical models arrived at to investigate this type of gene allow some conclusions to be made from the data. The frequency distribution of chromosome loss of both marked and unmarked chromosomes for cross three shows that cells having no revealed markers have a modal chromosome loss of four chromosomes and that an appreciable fraction of the population of 442 have lost 10 chromosomes. The average

chromosome loss from all six crosses by revealed markers is 0.85 per cell whereas the calculated mean is 4.5. This information is essential when evaluating the phenotypic effect of genes which have dose dependent effects.

Once the mapping had been carried out the data were examined by looking at coordinate expression. This should have revealed a single case of cosegregation which would have located the gene to an individual chromosome. This was not the case. There were two cases of cosegregation which appeared significantly different from chance. However, the great shortage of cells with a size one (*whi1*) phenotype implies that the initial assumptions were incorrect or that there were restrictions on the appearance of size one cells. On the basis of previous analysis of euploids, equal numbers of cells expressing the *whi1* and wild phenotype were expected. This is because for any given chromosome, both homologues are retained with a frequency of q^2 (size three cells), and one homologue is retained with a frequency of $2pq$ divided equally between size one and size two cells (depending on which homologue is lost and p^2 are dead). The actual ratio of cell sizes noted was 15:17:1 (where the size order is three: two: one).

There are two possibilities for the shortage of *whi1* type cells, (1) cells which should show *whi1* phenotype no longer do and appear in other classes or (2) they failed to produce colonies and died. *Whi1* size cells could become larger for several reasons. Hanic-Joyce (1985) noted that many mutations appeared in post radiation cells which made them temperature sensitive. It is possible that mutations are also revealed by the loss of suppressors which delay mitosis and therefore result in larger cells. This may also be true

of cells which delay DNA synthesis. Additional auxotrophic mutations may cause cells to change size independently of *WHI1* as is seen with adenine auxotrophy.

Whi1 is thought to be a hyperactive mitotic inducer (Nash *et al.*, 1989). In weakened cells it is possible that this is lethal. Cells may not be physiologically up to being pushed into early division due to the effects of chromosome loss and therefore die. Spores containing *whi1* and specific temperature sensitive mutations do not germinate (see chapter 3). It is possible that the revealed additional temperature sensitive alleles known to occur with this technique coupled with the hyperactive activator combine to kill cells which would otherwise survive.

Unfortunately non of these hypothesis is provable. It may not be any one of these reasons but a combination of all of them which reduce the numbers of *whi1* type cells producing scorable colonies. Whatever caused the shortage of *whi1* type cells it could not be ascribed to mis-scoring, since there was a clear demarcation between the smallest (size one) and the intermediate (size two) cells. This was shown clearly by the direct examination (shown in plate D2) and electric particle counter results.

As well as allowing further investigation of the mapping system, the methods used also allowed an examination of the *WHI1* gene function to be made in a novel way. All previous investigations of the dose-dependent operation of the *WHI1* gene have been done in euploids. Since the mode of action of the *WHI1* product was not known at this stage it was possible that there was a chromosome/*WHI1* interaction rather than just a ploidy/*WHI1* interaction. Cells which expressed

just a single cell size control gene but had n + x chromosomes (where x is an integer) might have been intermediate sizes especially when the cell size control gene was defective. Since over 97% of the cells fitted into one of the three size categories we concluded that a) more elaborate models to account for other size classes did not have to be constructed and b) that there was no evidence for a general quantitative gene dosage effect of the other chromosomes which would have tended to blur the size class distinctions.

In the models arrived at before this study was carried out it was assumed that the *whi1* mutation used (one of 5 alleles known with similar phenotypes) led to loss of function and was equivalent to *WHI1* gene loss. It was then predicted that *whi1* haploids would be identical to *whi1* aneuploids. However, this assumption appears to have been incorrect since the mutation coded for a hyperactive protein (Cross, 1988; Nash *et al.*, 1988). The phenotype of the hyperactive *whi1* allele depended on the rest of the genome. If a single copy of the cloned *whi1* allele was put on a plasmid and placed into a *WHI1* haploid, the cell exhibited a size of one. This compared with a size of three in a similar *whi1/WHI1* cell made heterozygous by diploidy. The only difference was the ploidy level of the rest of the genome. This indicated that the *WHI1* gene and its alleles acted upon or were acted upon by other gene(s) in the genome in a stoichiometric fashion such that the ploidy level of the rest of the genome was important in determining phenotype. Consequently, in near diploid aneuploids, *whi1* cells did not necessarily show a size of two or three depending on whether the other interacting gene or genes were present in one or two copies (i.e. mono- or disomic for that chromosome). This would have reduced the frequency of size

one cells and produced a proportionate increase in numbers of size two and size three cells. With p known and equal to 0.174 it is possible to calculate the probabilities of phenotypes occurring if the genotype which is required to give that phenotype is known. In the case of cell size the number of genes required to give each of the three phenotypes (sizes 1, 2 and 3) are known. From this it is possible to calculate the frequency with which each phenotype should occur. Using the p value above, the ratio of three:two:one size cells should be 4:2:1 if the interaction was with genes on only one other chromosome. This compared with the observed ratio of 15:17:1.

Another point which was clear from the data was that all chromosomes were not lost with the same frequency. Even with the relatively small number of loss events noted, there was, in some cases, a five fold difference in the the number of times specific markers were revealed. This discrepancy was too large to be due to chance alone. Loss of a specific chromosome did not correlate with the size of that chromosome. As loss of a homologue would be due to damage by radiation, it was thought that the larger the chromosome the more likely it was to be 'hit', and therefore the more likely it was to sustain damage and therefore to be lost. It was obvious from the results that this was not the case. Some other unknown factor was involved. Whether there was selection for or against cells missing certain chromosomes was unknown but if suppressors and subvital genes were present on specific homologues when the other of a pair was lost the cells might not survive to be scored. This might also have affected the number of cells showing a *whi1* phenotype, especially if the homologue carrying the mutant gene carried a lethal gene.

Chromosome mapping was carried out due to the failure of linking *whi1* with a known mutation on the left arm of chromosome I. Although relatively high numbers of tetrads were examined for some of the crosses, the data were inconclusive. The failure of this mapping was due mainly to strains which did not behave as expected. Two strains carrying *cdc24* produced diploids when crossed with IS019 but resulted only in two spored asci on sporulation. This allele was therefore not used in mapping. The *cyc3* mutation initially appeared useful. Strains containing it would grow on chlorolactate media whereas wild type strains normally do not. Unfortunately the IS019 strain was resistant to chlorolactate. The *whi1* mutation did appear to be linked to *mak16*, but a three point cross failed to confirm this. No linkage was indicated with *cdc19* (*pyk1*). Although the *cdc19* and *mak16* mutant phenotypes were easily scored this was no so with the other phenotypes. Had further stains been available, especially containing a *cdc24* mutation which performed normally during meiosis, this mapping may have been more successful. The *whi1* mutation was finally mapped before cloning by linkage to *cdc24* (B. Futcher, *pers. comm.*).

The *rad52* mapping method is proven in mapping auxotrophic mutations and temperature sensitive *cdc* mutations. Its failure to give a decisive answer here was not due to the failure of the method but the phenotype involved. The method will map dose dependent genes, where it fails is in mapping a dose dependent gene which affects size. This only became apparent on analysis of the results. Irradiation and chromosome loss may combine to affect cell size by growth defects , delaying mitosis or affecting DNA synthesis. A mapping method which may have less of an effect on cell size may have been more successful, although this is difficult to assess

before experimentation begins.

CHAPTER 5: THE CLONED *WHI1* GENE

5.1 INTRODUCTION

Although the mapping experiments had thrown up many questions about the nature of the *WHI1* gene product and the way it interacted with other gene products or the rest of the genome, the gene was not successfully mapped. The phenotype of the mutant was not good enough for cloning by direct selection and unknown effects or interaction with unknown factors had made mapping by the *rad52* mapping method inaccurate. Other phenotypic effects which were easily selectable and a consequence of the *whi1* mutation, were not found.

Dr. Fred Cross working at the Fred Hutchinson Cancer Research Center in the United States of America had been looking for yeast which, once mutated, showed dominant resistance to the mating pheromone alpha factor. Having selected cells which were resistant, the mutation was cloned due to its dominant phenotype.. This mutation also caused the cells to be small. Hybridisation studies located the mutation to chromosome I very close to *CDC19*. Dr. Cross suspected that the mutation was in the *CDC24* gene and therefore acquired that particular section of chromosome I which had been cloned by Drs Pringle and Kaback. The cloned fragment also contained several open reading frames which had no known function. A comparison of the restriction map of the cloned fragment and that chromosome I fragment as well as deletion mapping data located the fragment to one of the open reading frames with no assigned function adjacent to *CDC24*. The cloned gene, named *daf1* (dominant alpha factor resistance) now appears to be *WHI1* (personal communication; Dr Fred Cross and Dr John Pringle). At about the same time the gene was also cloned as *WHI1* by Dr. Bruce Futcher and his group at the Cold Spring Harbor Laboratory. They used classical mapping methods

and transposon tagging to clone the gene. Again the gene mapped to a region of chromosome one close to *CDC24*, and later identified as the *FUN10* reading frame (personal communication; Dr.Bruce Futcher). Comparisons of the amino acid sequence of both *daf1* and *FUN10* (*WHI1*) show that they are allelic (Cross, 1988; Nash *et al.*, 1988).

All the following data are from personal communications with both Fred Cross and Bruce Futcher and with reference to Cross, 1988 and Nash *et al.*, 1988. All the models for the number of genes involved in the control of cell size and the nature of the mutation were based on the supposition that the mutant gene was a non functional or only partially functioning gene. This would give the same effect as having no gene present or a null phenotype. With the gene cloned it was possible to create this artificially by disrupting the gene with a DNA insert (in this case the *URA3* gene). This disrupted gene was then used to replace the genomic copy by transplacement (Rothstein, 1983). When this was carried out it was found that the null phenotype was larger cells. This indicated that the mutant genes in both cases were in fact hyper-active and equivalent to having further copies of the wild type gene present. Cells containing no functional copy of the *WHI1* gene had sizes 25% and 50% larger than wild type cells. The null phenotype experiment also showed that *WHI1* was not essential for viability and suggests therefore that it is either duplicated or a regulatory type gene.

The wild type gene appeared to code for a polypeptide of around 580 amino acid residues. Both mutant forms coded for a truncated protein which was due to a stop codon around base pair 1200. This resulted in the loss of the C-terminus of the protein, confirmed by gap repair experiments. From the results of the null phenotype

investigation it would seem that the loss of the C-terminus resulted in a hyperactive protein. It may be that the C-terminus down-regulated the protein. The coding sequences were run through the Genbank Sequence library and the Protein Identification Research Data Bank and no homology found with any sequence. Also the sequence was searched for known structures and none were reported. The gene appeared to have three distinct domains, a basic well structured N-terminus, an acidic well structured middle third and a non structured basic C-terminus which was rich in serine, threonine and proline. The sequence had a codon bias of around 0.27 which suggested that the protein was not abundant (Bennetzen & Hall, 1982). The gene was constitutively transcribed throughout the cell cycle with no bursts or peaks of message.

As well as producing cells which contained no functional copies of the gene, Dr Cross also produced strains which had two and four extra copies of the mutant . These showed more extreme phenotypes as far as pheromone resistance was concerned but did not show any further reduction in size. Dr Futcher constructed strains with two mutant genes which again showed no further reduction in size but had virtually no G1 growth phase. By placing the mutant gene on a multi-copy vector he also managed to increase the mutant gene by 5-10 fold, but this did not make the cells any smaller. With the wild type gene also cloned it was possible to give cells extra copies to see if this had any effect on phenotype. Two doses of the wild type gene did reduce size but only by 20% and resulted in a shorter G1. A 5-10 fold increase in wild type gene dosage again resulted in a small cell size but still not as small as the mutant phenotype. Indeed, up to 20 doses of the wild type gene still did not reduce the cells to mutant size. The mutant allele *DAF1* was assumed to be

dominant because its affect on resistance to pheromone could be seen even with a wild type gene present. The mutant *whi1* was assumed to be dominant by Bruce Futcher because when a single copy of the mutant gene was placed in wild type haploid cells they exhibited the mutant phenotype. This is not the only conclusion which can be drawn from this result. In these haploid cells the only gene of which there is an extra copy is the *WHI1*, albeit mutant. If the gene product of the *WHI1* alleles interacts directly with another component to control size and both act in a dose dependent way, increasing one without increasing the other would not demonstrate dominance of either *WHI1* allele but that direct interaction. Only when two copies of all genes involved are present can it be clear if one allele is dominant over the other. When diploids are heterozygous for the wild type and mutant alleles, the size seen is intermediate between both diploids. It is not the size of a *whi1* haploid, which is what would be expected if this allele were truly dominant as with, for example, auxotrophic mutations and their wild type counterparts.

The resistance to arrest by alpha factor was confirmed in both cases although there seemed to be subtle differences. Fred Cross reported a complete resistance to alpha factor, which was more profound the more copies of the mutant gene there are present. Bruce Futcher reported that mutant cells transiently arrested but recovered and continued to proliferate. This difference could be due to the method by which the two mutations were isolated; one because of its size and one because of its resistance to alpha factor. This transient arrest and resistance to arrest led to the idea that the system which allowed recovery from arrest by alpha factor was faulty in the

cells containing the mutation. This was investigated in both cases by crossing cells containing the mutant alleles with cells containing the mutant allele of *SST2*. *sst2* has the effect of making cells supersensitive to alpha factor (Chan & Otte, 1982). The double mutant was found to be no longer supersensitive to alpha factor arrest although the alpha factor did lead to expression of genes induced by the pheromone. This indicated that the fault lay in the division response and not at the level of pheromone receptors or in signal transduction. In fact one gene whose expression was found to be affected by alpha factor was the *WHI1* gene itself; its transcription was increased 2-3 fold by exposure to alpha factor. As well as relieving the block caused by *sst2* the mutant allele also relieved the block cause by the mutation *scg1*. This mutation causes cells to arrest as if they had a constitutive pheromone response. The double mutant grows like wild type cells. Again this led to the idea that the *DAF1* and *whi1* mutations are causing a hyperactive adaptation pathway.

One of the main conclusions of their work is that size control and pheromone response seem to be mechanistically coupled. The data also suggested that G1 can be dispensed with, without adversely affecting cells. The data also suggested that there may be a second cryptic control point which occurs at nuclear division. This would be the opposite to the situation found in *Sc. pombe* which has its major control point at nuclear division and a cryptic control in G1. In order to confirm some of the data mentioned above and carry out further investigations a copy of the *WHI1* gene was obtained in a lambda vector, as well as diploids containing single, 2 and 4 copies of the mutant *DAF1* gene. Vectors were also obtained containing wild type and mutant *WHI1* gene.

Although homology with other genes was looked at, the question of homology with other proteins was not fully addressed. Homology between the translated protein sequence and other proteins in the ProtSeq library was examined using the FastP program. This is a rapid system which compares sequences using algorithms developed by Wilbur and Lipman (1983) and Lipman and Pearson (1985). The system initially screens sequences for similarity by looking for aligned identical amino acids. As a system it is highly sensitive as it then considers naturally occurring conserved amino acid substitutions. Not all amino acids are considered with the same weight. Aligned identical amino acids which are rare (e.g. cysteine and trptophan) receive higher scores than the more common amino acids. Replacements which are known to occur in evolution receive a positive score, while unlikely substitutions receive a negative (Lipman & Pearson 1985). Sequences are examined using a Lookup Table (Dumas & Ninio 1982). This involves giving each amino acid in the sequence a position in the table. The sequence to be compared is then looked up in the table with regards to position. Regions of similarity are found when a run of amino acids is located which match when the sequences are aligned or offset. At this stage the similarity is scored. A further algorithm is then used to optimise the match by allowing gaps in the regions which already have a high score. Two scores are therefore obtained, an initial score and an optimised on. Sequences which show real functional homology usually have a higher optimised score.

On comparison of a sequence with a database many similar protein sequences are found. As well as addressing significance of the scores by magnitude and the relationship between the initial and

optimised score, statistical significance can be calculated. As the method does not follow a normal distribution the values are expressed as a z value where:

$$z = \frac{\text{(similarity score - mean of the random scores)}}{\text{standard deviation of the random scores}}$$

Significance is rated as:

z > 3 possible significance

z > 6 probable significance

z > 10 significant

All these data give an indication of the importance of the similarity of the two proteins but what must be kept in mind is the greater importance of the biological context of any similarity noted.

The folowing section deals with the cloning of the gene, the question of homology with other genes, the interaction of excess copies of the mutant gene with *cdc* mutations and clues to the action of *WHI1* as shown by the protein coding sequence analysis.

5.2 RESULTS

CLONING OF THE *whi1* GENE FROM A LAMBDA VECTOR

The *whi1* gene, located on the left arm of chromosome I, was found to have been cloned in 1988. This was carried out as part of the complete cloning of chromosome I (Coleman *et al.*, 1986). The *whi1* gene was present in a lambda vector which was made available to us. The clone also contained *CYC3*, *PYK1* and most of *CDC24* (see Figure 5.1). To allow the gene to be investigated fully it was necessary to sub-clone it into a yeast expression vector i.e. a vector which could be maintained in a *S. cerevisiae* strain of the correct genotype by advantageous selection and allow normal expression of the gene. A series of centromeric vectors were available which would be maintained in a single copy and allow expression of genes cloned into the correct site (see Appendix 1). Before the gene could be cloned into these vectors it had to be excised from the virus-based vector. As the site into which the gene was to be placed was a *Bam*H1 site, it was decided to cut the gene from the vector using *Bgl*II. This produces ends which are compatible with *Bam*H1 ends although on ligation neither site would be reconstructed. The *Bgl*II sites present and the fragment sizes produced on digestion of the vector are shown in figure 5.2. The 7.2Kb fragment which contains the *WHI1* gene also contained the whole *CYC3* gene and part of the *PYK1* gene. It was necessary to clone beyond the boundaries of the gene as it was not clear in which direction transcription occurred or where the control sequences for transcription were located. It was hoped that the other genes cloned in this initial fragment could be sub-cloned out later. On digestion of lambda C1a with *Bgl*II the fragments shown in plate 5.1 were seen. The band which was cut from the gel and sub-cloned is shown. Although large quantities of this DNA were

FIGURE 5.1 LAMBDA C1a VECTOR

The whole vector is shown although the lambda DNA(shown as ) is not to scale. All genes from the cloned S. cerevisiae chromosome I are shown to scale. They are as follows:

CDC24 = Cell division cycle gene

CYC3 = Cytochrome C

PYK1 = Pyruvate kinase

FUN10
FUN11
FUN12
= Function Unknown Now, although *FUN10* is now known to be *WHI1*.

R = *Eco* RI

B = *Bgl* II

H = *Hin* dIII

S = *Sal* I

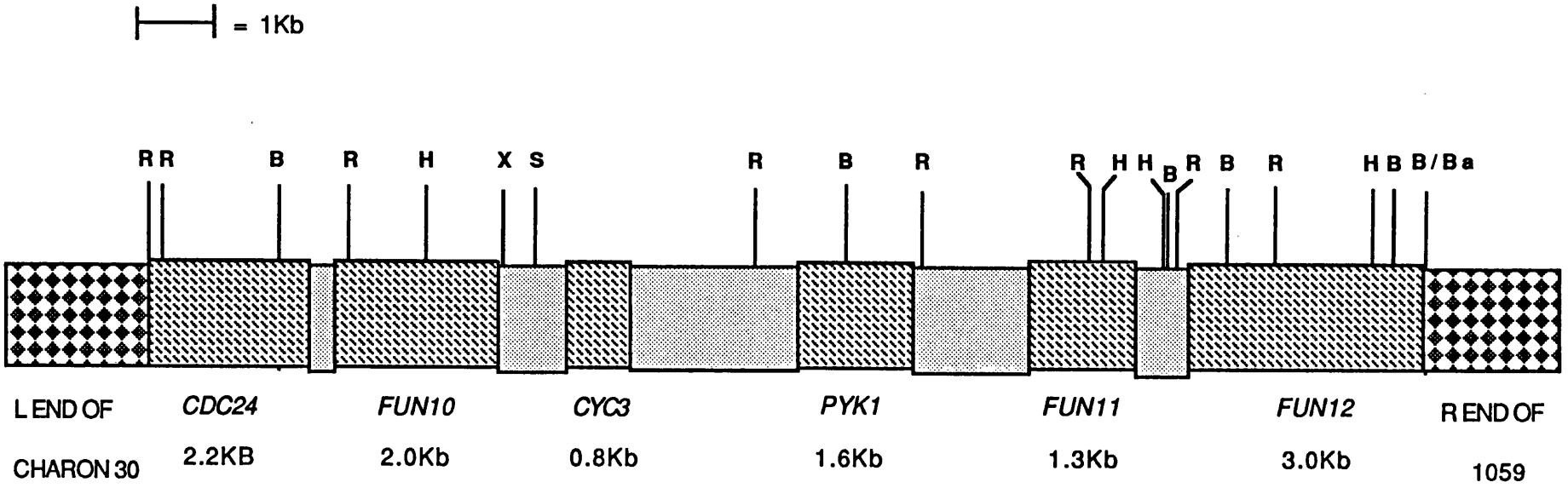
X = *Xho* I

B/Ba = *Bgl* II/ *Bam* HI join

 = EXONS

 = NON CODING DNA

 = LAMBDA DNA


= 1Kb
R R
B
R
H
X S
R
B
R
R H H B R B R
H B B/Ba
L END OF CHARON 30
CDC24
2.2KB
FUN10
2.0Kb
CYC3
0.8Kb
PYK1
1.6Kb
FUN11
1.3Kb
FUN12
3.0Kb
R END OF
1059

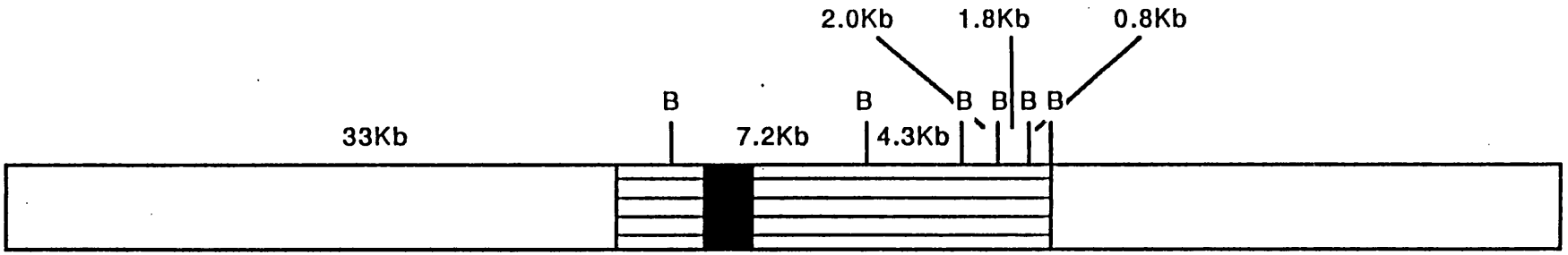


FIGURE 5.2 The Expected *Bgl*II Sites in Lambda C1a

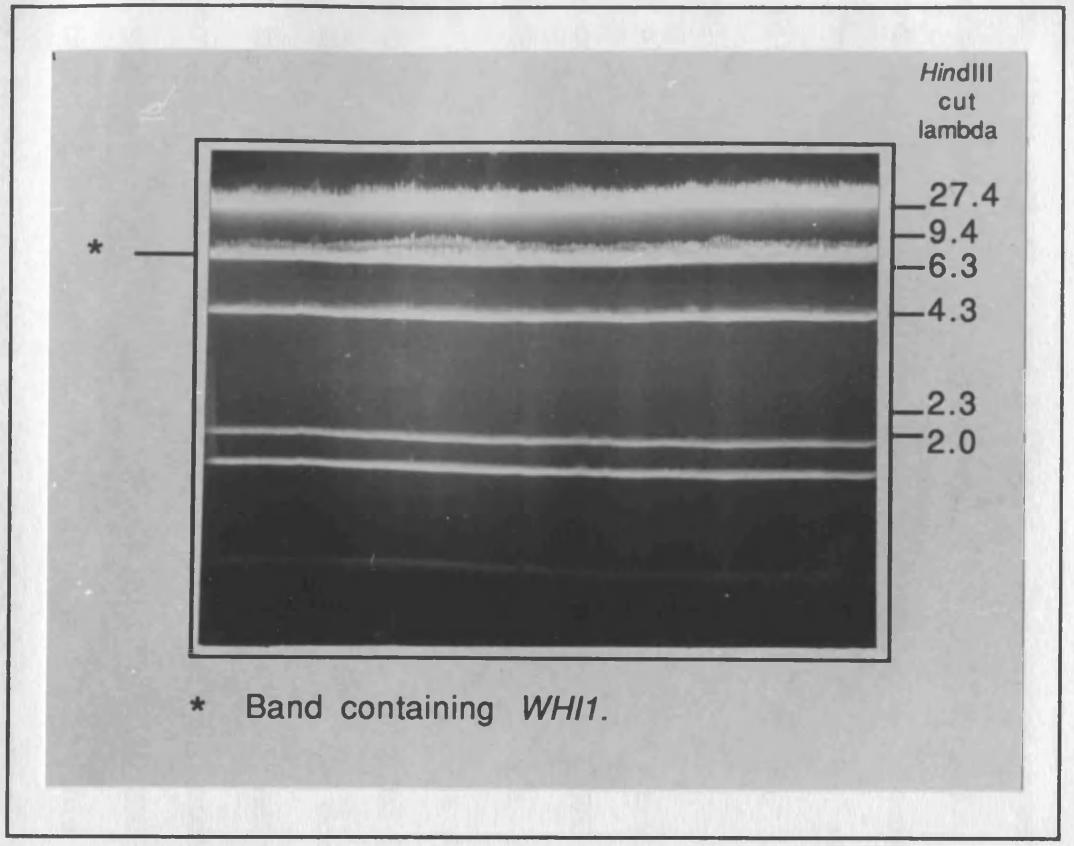
All expected *Bgl* I I fragments are shown with their sizes in kilobases. The 33Kb fragment occurs due to the sticky ends of the lambda vector. The sections are:

= Lambda DNA = *S. cerevisiae* DNA = *WHI1* gene

PLATE 5.1

Digest Pattern of the Lambda C1a Vector

A large pure preparation of lambda C1a DNA was made and digested with the *Bgl* II restriction enzyme. The resultant band pattern is shown. The band marked is approximately 7.2Kb in length and contains the *WHI1* and *CYC3* genes with portions of other genes. The *WHI1* gene was to have been sub cloned out. of this fragment.



made and the *Bgl*II digestion reliable, subsequent cloning of the fragment into other vectors proved difficult. The vector chosen for the first attempt at cloning was p*CEN-URA3* (see appendix 1). This plasmid contained the whole *URA3* gene as well as the complete centromere from yeast chromosome III. It is maintained in a single copy and will be faithfully passed on to daughter cells. Large caesium chloride preparations were made of this and other centromeric plasmids. All underwent normal digestion by restriction enzymes and could be successfully dephosphorylated. Both p*CEN3-URA3* and p*CEN3-ARG4* contain a single *Bam*H1 site into which it was hoped to clone the lambda C1a *Bgl*II fragment. Repeated attempts to clone in the lambda fragment all failed (see plates 5.2, 5.3 and 5.4). Plate 5.4 shows two clones which appeared to contain the correct fragment on initial digestion. This subsequent redigestion indicated that both contained no fragment. Many of the transformants appeared to contain vectors which were smaller than the original p*CEN3-URA3* which was used in ligations with the *whi1* fragment. This was confirmed by further digestions of DNA isolated from examples of these transformants. Sub-cloning from this particular lambda vector had proved difficult in other hands (Fred Cross; John Pringle; pers. comms.). It was decided to compare the ligation of any lambda C1a *Bgl*II fragment into the p*CEN3* based vectors, with that of commercial lambda fragments produced by digestion with the same enzyme. Both types of lambda were digested with the same enzyme in the same restriction buffer. All fragments from these digests were to be ligated into the yeast vectors. Aliquots of each were ligated into p*CEN3-URA3* and p*CEN-ARG4*, both of which had been digested with *Bam*H1 and dephosphorylated. All ligations were carried out in the same way, as were all transformations. Plate 5.5 shows samples of

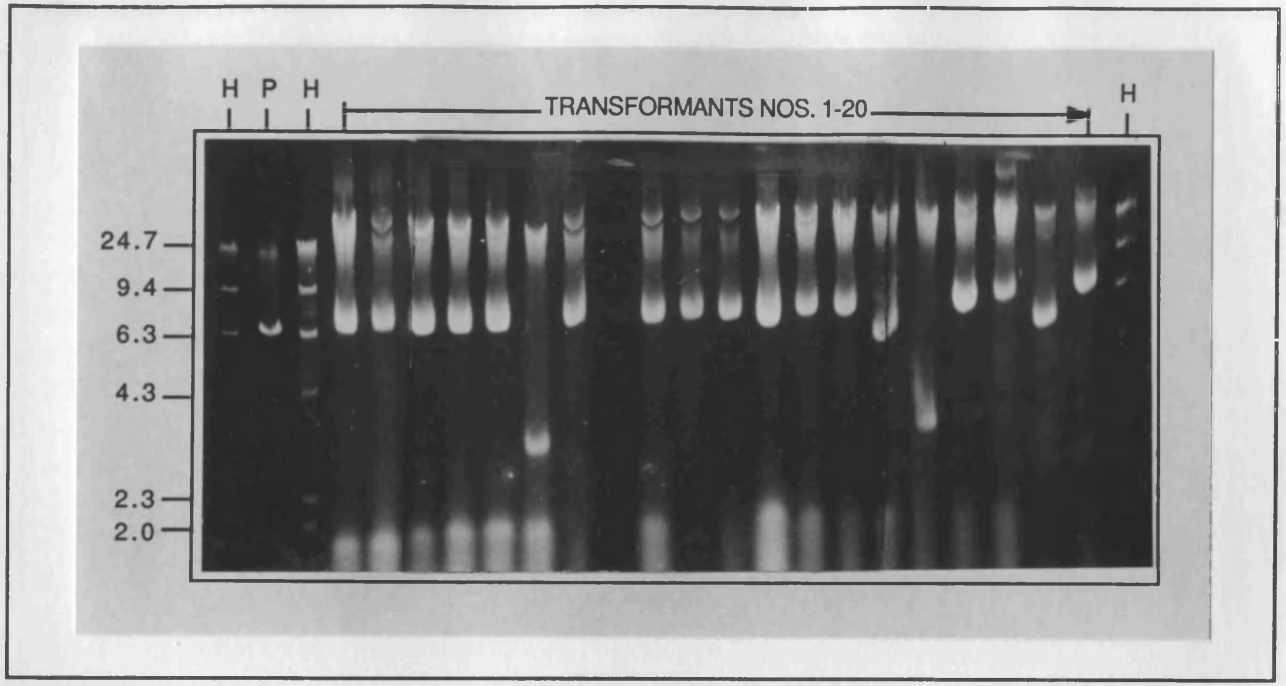


PLATE 5.2 Analysis of Transformants by Digestion with *Eco* RI

These are the initial transformants from the ligation of pCEN-URA3 cut with *Bgl* II and the 7.2Kb *Bgl* II fragment of Lambda C1a. H =*Hin* dIII digest lambda DNA as markers with P = to the parental vector, pCEN-URA3, being cut with *Eco* RI. All transformants either resemble the parent or contain a deletion except 32 and 40 which will be looked at in greater detail.

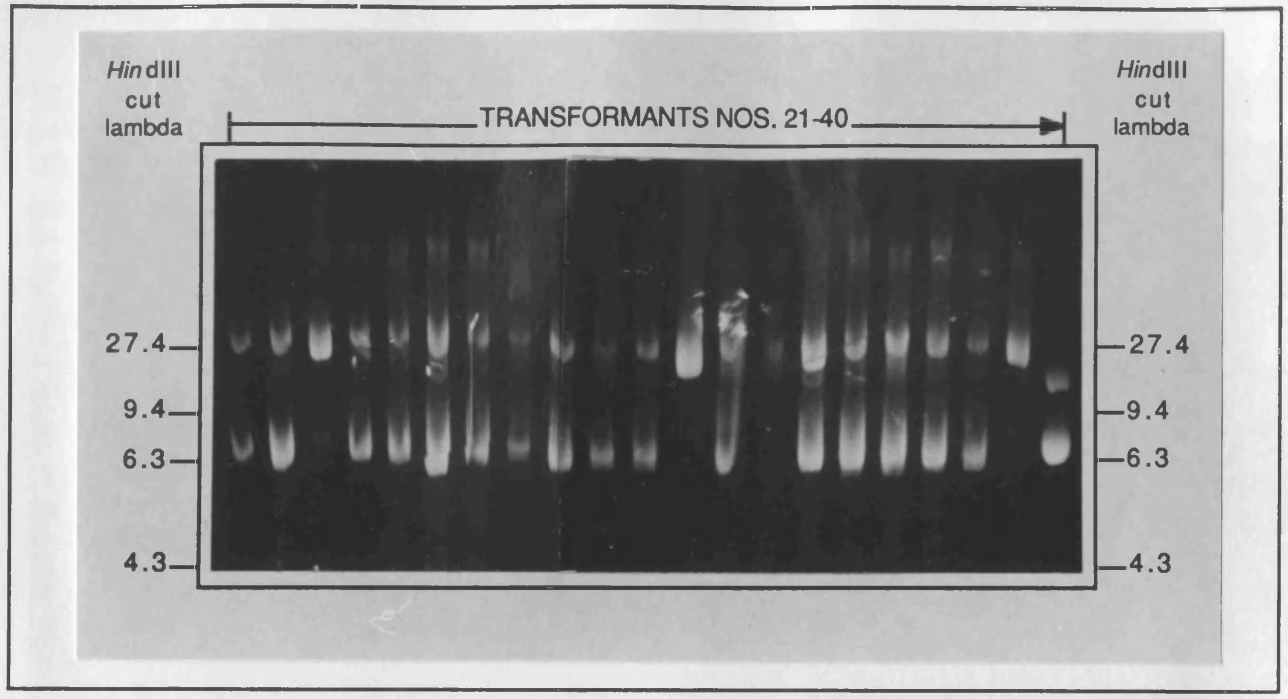


PLATE 5.3 Analysis of Transformants by Digestion with *Eco* RI

The transformants are the result of the ligation of pCEN-URA3 cut with *Bgl* II and the 7.2Kb *Bgl* II fragment of Lambda C1a. The final lane contains parental pCEN-URA3 cut with the same enzyme. All transformants resemble the parent and are probaly religations.

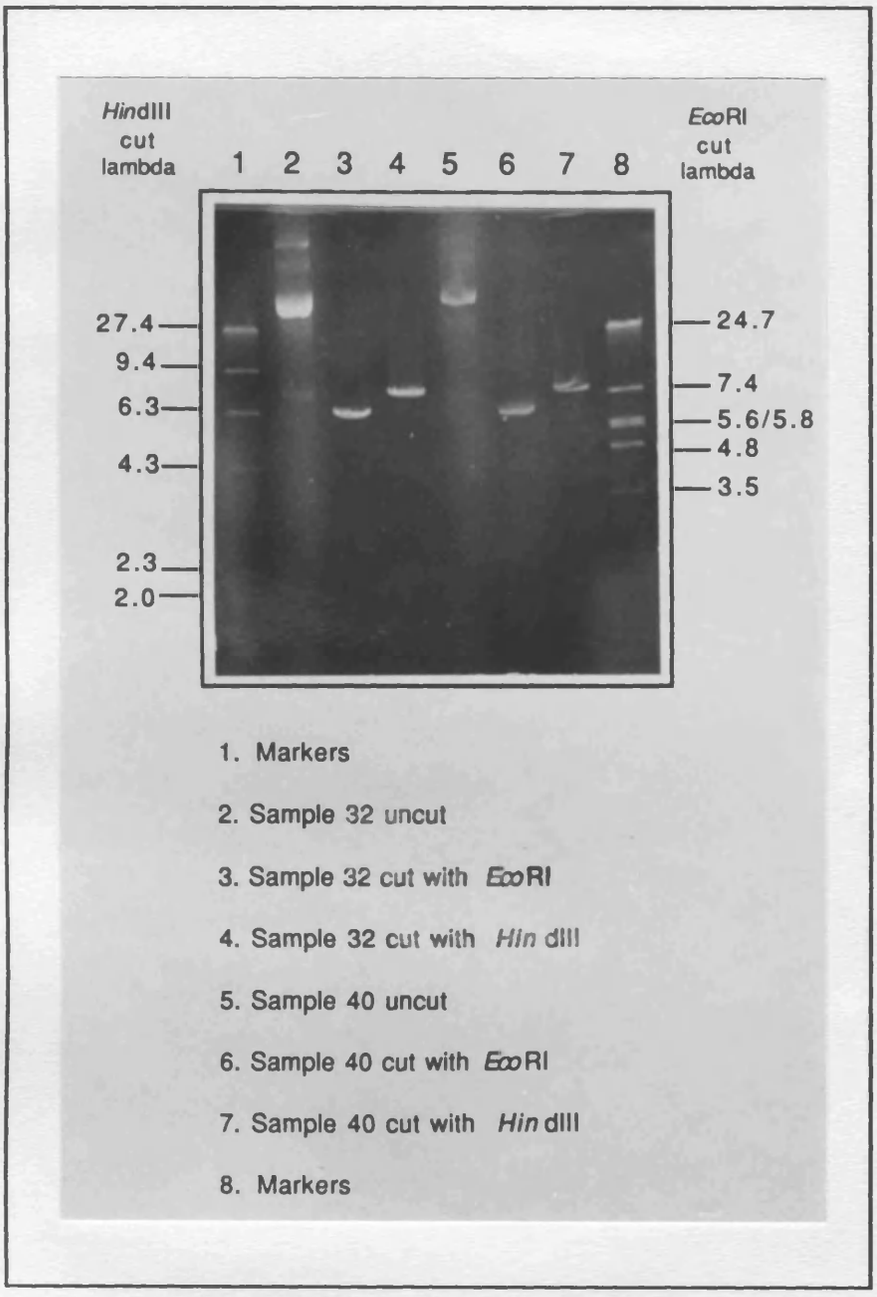


PLATE 5.4 Further Digests of Possible Positive Clones

Pure DNA was made from clones 32 and 40 (lanes 2 and 5 repesctively) and cut to investigate size and the possibility of inserts. Both gave parental patterns when digested with the restriction enzymes shown.

PLATE 5.5 Digest Patterns of Transformants From Lambda Shot gun Cloning Experiment

The lanes contain mini prep. DNA from twenty transformants which have been digested with *Eco*RI. Two types of lambda DNA were digested with *Bgl* II and all fragments cloned into centromeric DNA based vectors. Lanes 1-5 are lambda C1a fragments in pCEN-URA3, 6-10 are C1a in pCEN-ARG4, 11-15 are commercial lambda fragments in pCEN-URA3 and 16-20 are commercial lambda in pCEN-ARG4. Analysis of bands is shown in Table E.1.

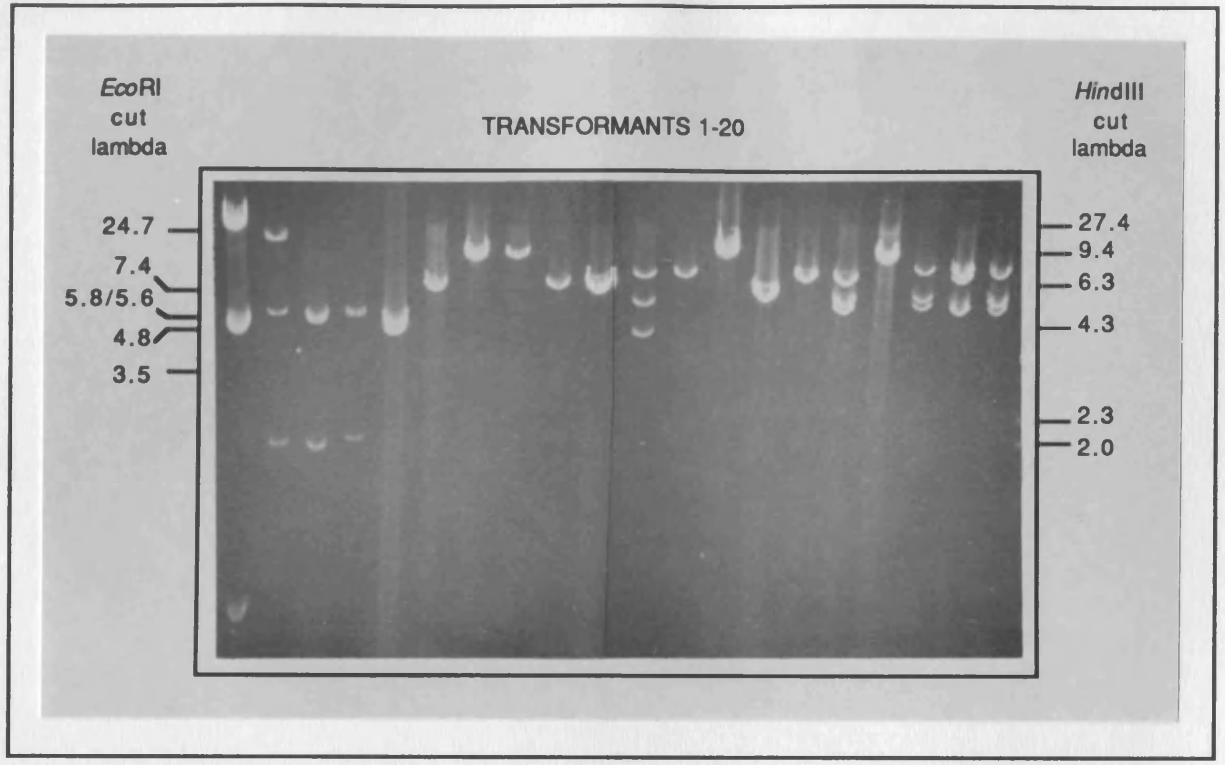

EcoRI
cut
lambda
TRANSFORMANTS 1-20
HindIII
cut
lambda
24.7
7.4
5.8/5.6
4.8
3.5
27.4
9.4
6.3
4.3
2.3
2.0

TABLE 5.1

Analysis of Digest Fragments Shown in Plate 5.5

Lane No. refers to the lane number of the gel in Plate E.5. The constructs were either lambda C1a *Bgl* II fragments ligated into pCEN-URA3 (C1a + URA3) and the same fragments ligated into pCEN-ARG4 (C1a + ARG4) or Commmercial lambda *Bgl* II fragments ligated into pCEN-URA3 (Comm + URA3) or PCEN-ARG4 (Comm + ARG4). The size from the gel represents the total size of all fragments in any one lane added together to give the size of the new constructed vector. An insert has a positive value while a deletion has a minus sign before it. The final column indicates the size of any of the possible lambda fragments inserted into the original vector, a deletion (D) or a religation (R). Where there are two numbers there are possibly two fragments. Deletions are assumed to have occured when the total size of the recombinant vector is either smaller than the original or cannot be achieved by addition of any fragments from the lambda digests. Deletions do not occur with any one of the parent vectors but in both and with both types of lambda DNA.

LANE NO.	CONSTRUCT	SIZE (KB FROM GEL	SIZE OF VECTOR	SIZE OF INSERT OR DELETION	ANALYSIS
1	C1a + URA3	28.18	8Kb	20.18	22Kb
2	"	27.10	"	19.10	22Kb
3	"	11.68	"	3.68	4.Kb
4	"	11.68	"	3.68	4Kb
5	"	8.7	"	0.7	1Kb
6	C1a + ARG4	6.5	10Kb	-3.5	D
7	"	9.1	"	- 0.9	D
8	"	9.1	"	-0.9	D
9	"	6.5	"	-3.5	D
10	"	6.5	"	-3.5	D
11	Comm + URA3	15.2	8Kb	7.2	D
12	"	7.1	"	-0.9	D
13	"	9.1	"	1.1	D
14	"	5.6	"	-2.4	D
15	"	7.1	"	-0.9	D
16	Comm + ARG4	20.1	10Kb	9.0	10Kb
17	"	9.8	"	-0.2	R
18	"	20.1	"	10.1	10Kb
19	"	21.7	"	12.0	10 + 2
20	"	20.1	"	10.1	10Kb

miniprep DNA from transformants digested with *Eco*R1 and run out on a 0.75% gel. Of the 20 samples investigated in this way 12 of them contained deletions. The details of this are shown in table 5.1 Further cloning from the lambda vector became unnecessary as vectors containing *DAF1* became available. These could be used in the same way as the *WHI1* gene present in the lambda vector and were also available as integrated vectors. Strains produced from these vectors were used to investigate the interaction of *whi1* with cell cycle mutations. The lambda vector was used to investigate any homology between the *WHI1* gene of *S. cerevisiae* and genes involved in size control in the fission yeast *Sc. pombe.*

Homology between *WHI1* and the *WEE1*

Investigations had previously been carried out to assess any structural homology between the *CDC25* gene of *Sc. pombe* and *WHI1* by Southern blot (J. Milner, 1988). The same type of investigation was carried out to assess any homology between *WEE1* and a portion of the *WHI1* gene (J. Shea, 1988). Further Southern blot analysis was carried out using the full *WHI1* gene from the lambda C1a vector. Direct homology between the two genes was looked for as well as the presence of *WHI1* homologues in *Sc. pombe* genomic DNA. Probes were made using non-radioactive labelling methods of both the *WHI1* and *WEE1* genes. Genomic DNA from *Sc. pombe* (4ug digested with *Hin*D III and *Pst* I), was run out on a 0.75% agarose gel with digests of plasmids (0.5ug) which contained the whole *WEE1* and *WHI1* genes. After blotting onto nylon membranes, hybridisations were carried out with the two probes. Washing of the blots was carried out at high and low stringency after hybridisations followed by development. The final blots are shown in plates 5.6 and 5.7 Both positive and

PLATE 5.6

Southern Blot Analysis of *Sc. pombe* Genomic DNA (Low Stringency Wash)

This plate shows two nylon membranes from a Southern Blot analysis of *Sc. pombe* genomic DNA. The membrane on the left has been probed with a vector containing the *WHI1* gene, the one on the left with a vector containing the *WEE1* gene. After hybridisation both membranes were given identical low stringency washes. The *WHI1* probe picks up vector fragments containing *WHI1* as well as the negative control. There is also a faint band picked up in the *WEE1* lane. The blot shows no homology between *WHI1* and sequences in either of the *Sc. pombe* genomic DNA lanes.

PLATE 5.7

Southern Blot Analysis of *Sc. pombe* Genomic DNA (High Stringency Wash)

This plate shows exactly the same as above except that the membranes shown underwent higher stringency washing than those in plate E.6. The probes now only pick up their own positive control DNA. There is still some slight homology picked up between *WHI1* and the negative control lambda DNA. Neither manages to pick any sequences in the genomic samples.

For both plates:

A. Lambda cut with *Hin*dIII

B. *Sc. pombe* genomic DNA digested with *Pst* I

C. *Sc. pombe* genomicDNA digested with *Hin* dIII

D. pUC18- *FUN10* digested with *Hpa* I & *Xho* I

E. pWEE1-12 digested with *Bgl* II & *Kpn* I

PLATE 5.6

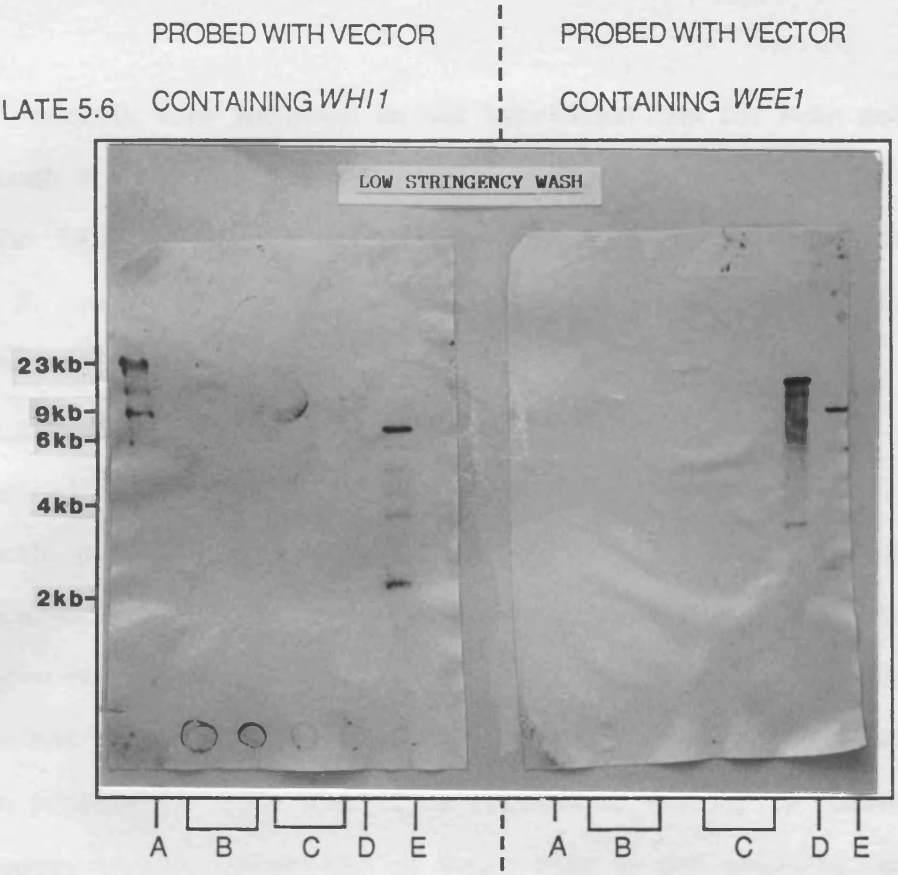
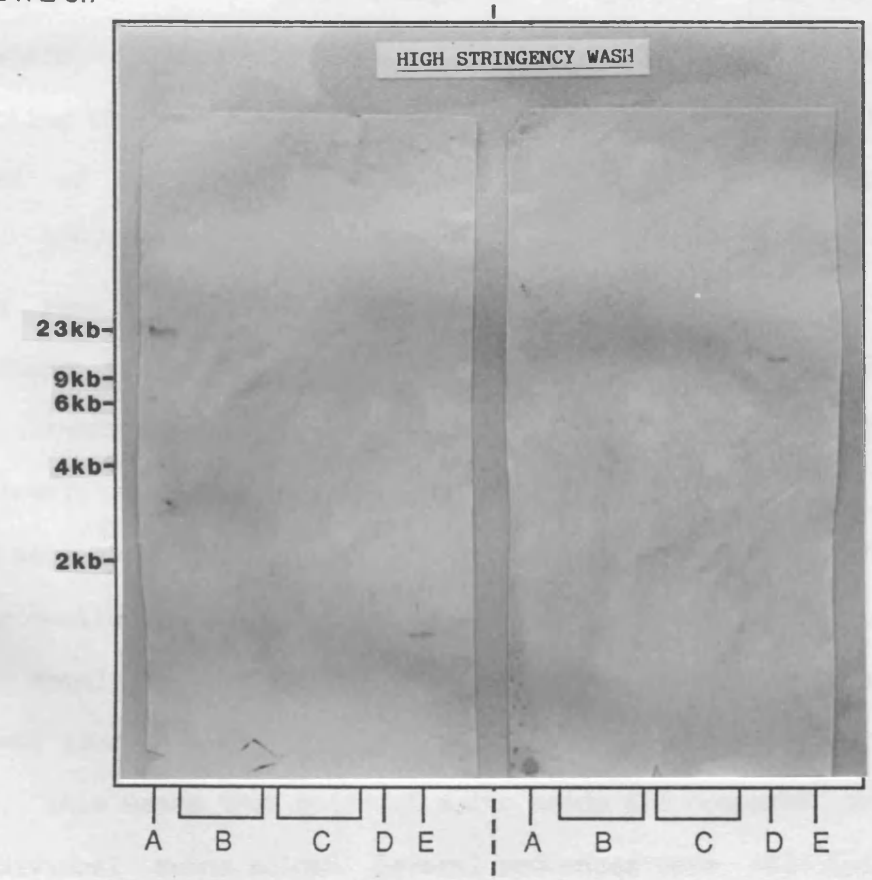


PLATE 5.7



negative controls were included in the hybridisations for both genes and although a *WHI1* homologue was looked for in a genomic spread of *Sc. pombe* DNA no homologue of the *WEE1* gene was sought in genomic DNA of *S. cerevisiae* as this had been checked in a previous investigation (J. Shea, 1988).

The gene sequence of *WHI1*.

The original *whi1* mutation was cloned at about the same time as *DAF1*. Both sequences became available and although they had both been compared with data banks in the search for homology with other gene sequences, it was decided to compare the sequences with genes which may not have been on the data bases used. This was done using a Diagon programme. This identifies regions of similarity between two seqences by the comparision of every base in one sequence with that in the other. It locates segments of perfect identity or regions where, using a scoring matrix, a minimum value is exceeded. By selecting the size of segment and the statistical significance, the level of sensitivity of a given comparision can be altered (Staden, 1982). No significant homology was found when *whi1* sequences were compared with *ARD1*, *CDC25*(*S. pombe.*), *WEE1*, and *CDC28*. These results confirmed the results of the Southern blot analysis already carried out. As well as no significant homology at the DNA level, two data banks (Protein Identification Resource and Genbank) were searched for homology at the protein level using FastP (see introduction). Again this showed no proteins with a significant level of homology. Despite this it was decided to run a search of the Protseq library using the Fast P system. The system was run with ktup = 2, this means that pairs of amino acids are compared rather than individual amino acids. Several sequences were selected as having greater than 45% homology over small regions. These included

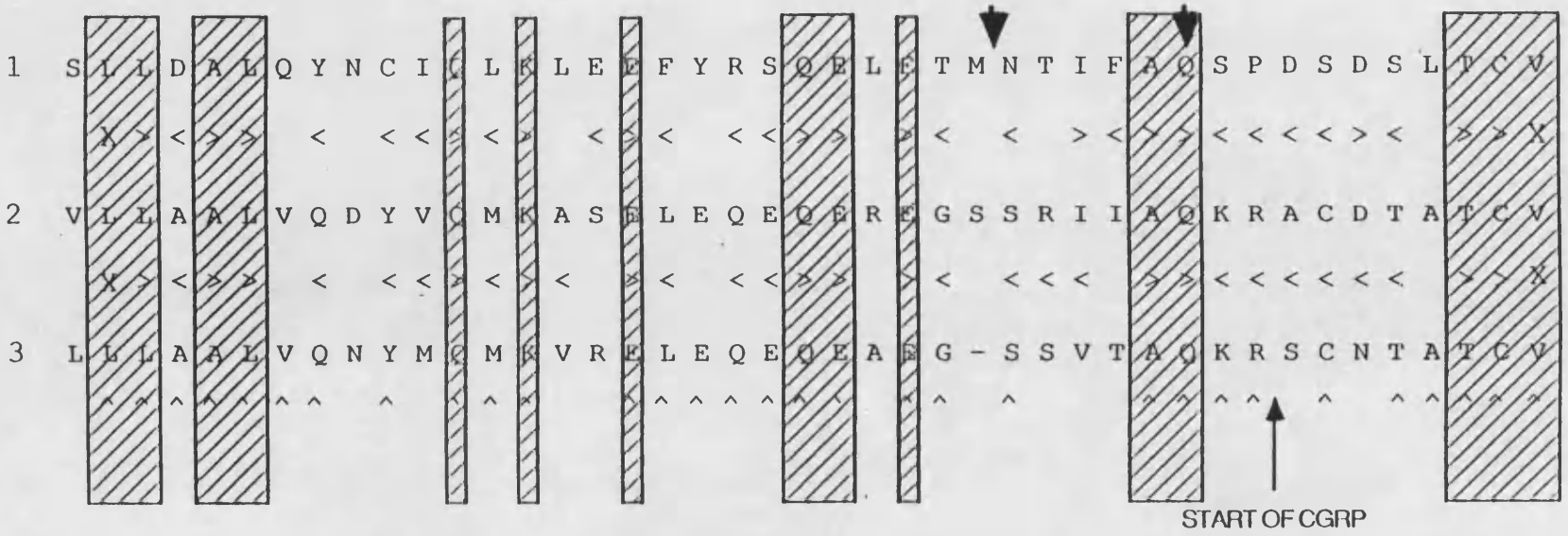


FIGURE 5.3 Simularity Between a Small Region of *WHI1* and the Pre-region of Two CGRP Proteins.

Shown are regions from :1 the *WHI1* protein, 2 rat pre-CGRP and 3 chicken pre-CGRP.

are marked as follows:

< = Homology between any two proteins

= Known *whi1* mutations

> = Homology between all three proteins

two heamagglutinin precursors and most strikingly calcitonin gene related peptide (CGRP).This gave a score of 70 which was 3 standard deviations above the value of the other nearest protein. The similarity appeared to be over a small region (see figure 5.3) . The z value for the homology was 7.55 but there was no increase in the optimised score of similarity. A point which appeared to be of some significance was that the region of homology was in the same area as both the mutations which caused gain in function in the mutant alleles.

As well as sequence comparison, probable structures, based on the properties of the amino acids in the sequence, were calculated. This was carried out using the Pheonix Mainframe computer at Cambridge University with the assistance of Dr J. Beeching, University of Bath. The program used was Rpredict. This system uses the simplest possible statistical proceedures to predict the secondary structures of proteins. It is able to predict structures such as alpha helicies, beta sheets and turns (Garnier *et al.*, 1978). This revealed that both the basic N-terminus and the acidic middle third of the protein appear to be highly structured being made up of a mixture of alpha-helices and beta-pleats (portion shown in figure 5.4). The basic C terminus portion which was lost in both mutations, appears to be unstructured with many turns (due to the prolines) which prevent any real ordering of structure. The significance of this lack of structure is not known but this portion of the protein must be involved in regulating the function of the protein in some way as loss leads to hyperfunction.

Another way of examining protein sequences was to look for the presence of sequences which were thought to be involved in a specific process such as DNA binding or phosphorylation. Again this

FIGURE 5.4 Prediction of Structural Features of a Portion of the *WHI1* Protein.

Shown is the predicted structure of the *WHI1* protein based on its amino acid sequence. The lines represent the probability of a structure occuring. A peak above the centre line represents a high probability of that structure occuring and below it a low probability. The figure shows a region towards the C terminus of the protein (amino acids 335-529) which has high probability of random turns and low probaility of any ordered structure (alpha helix or beta sheet).

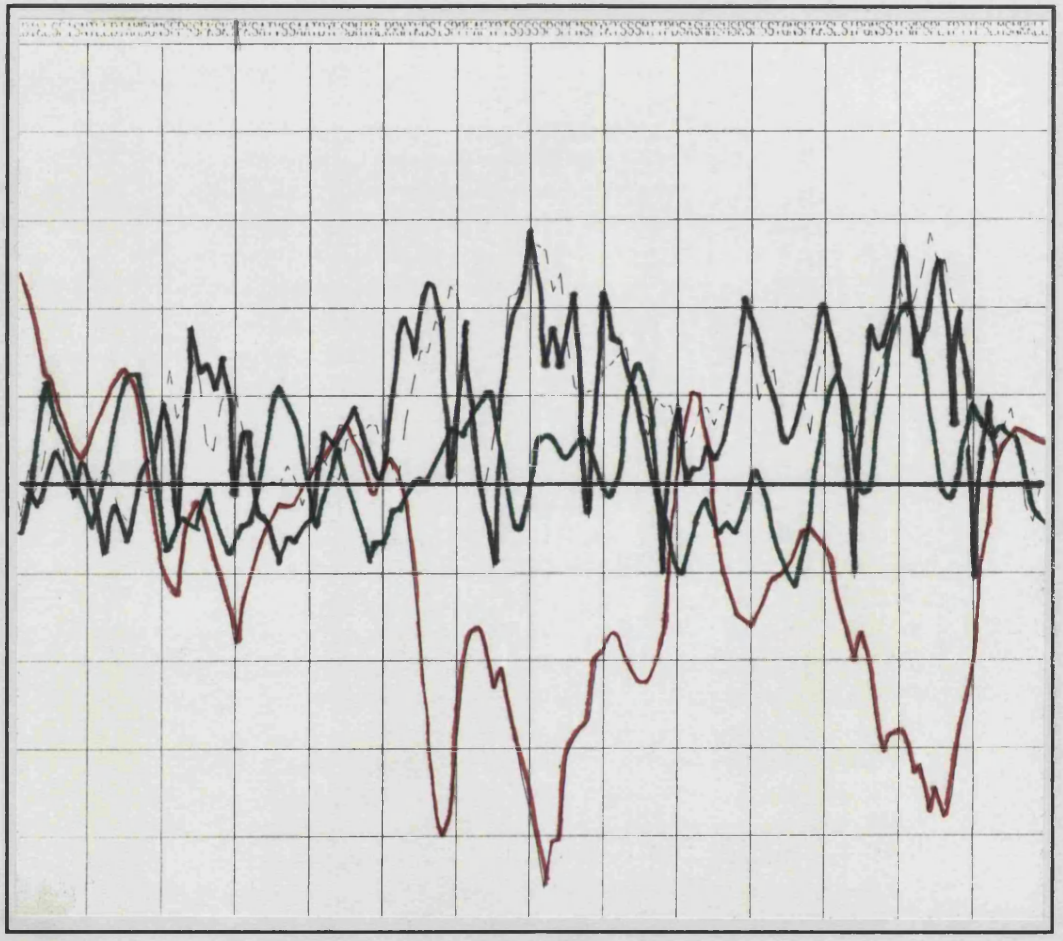
RED = ALPHA HELIX

GREEN = BETA SHEET

SOLID = TURN

DASH = RANDOM COIL

FIGURE 5.4



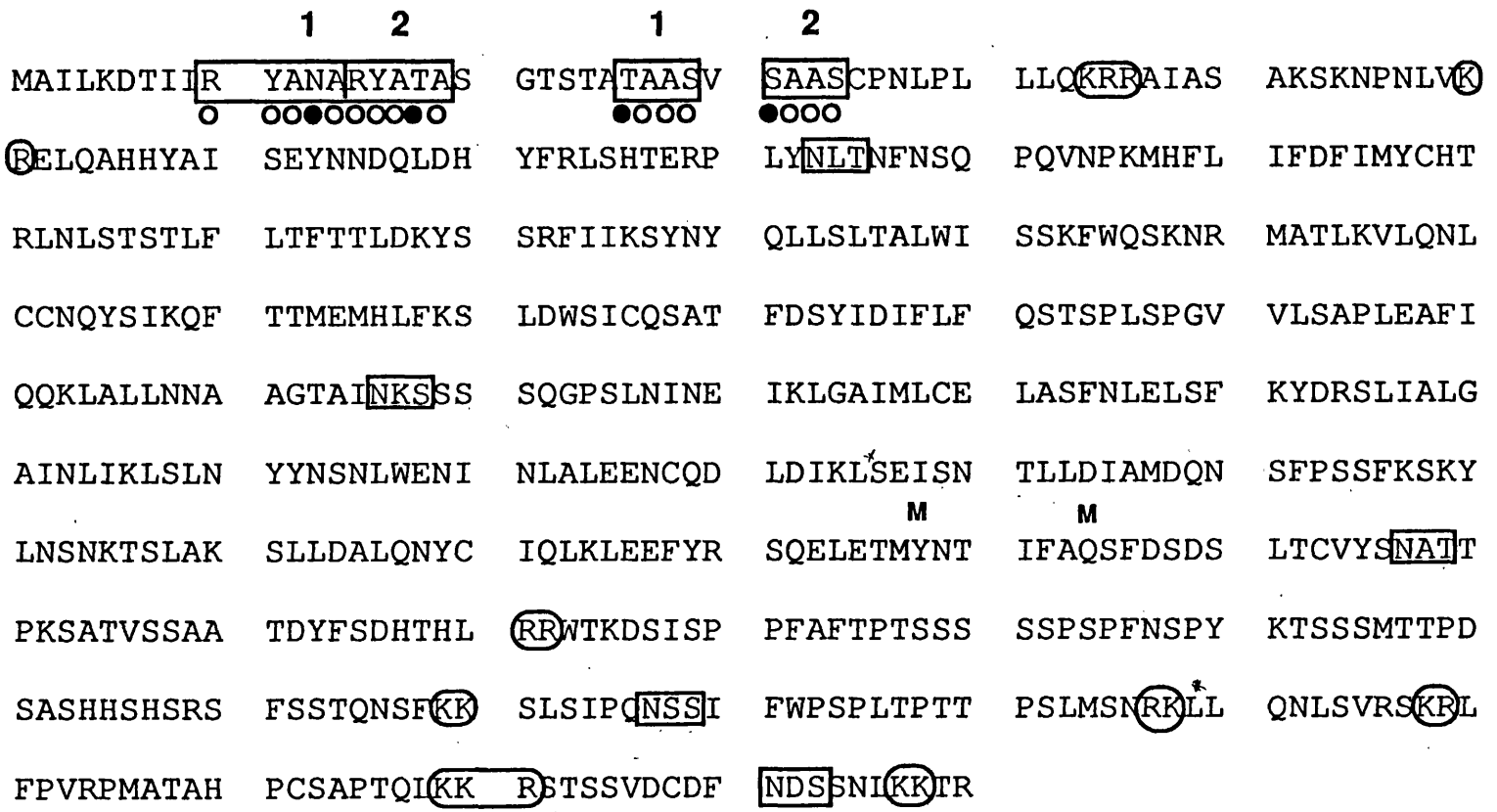


FIGURE 5.5 Protein Sequence of *WHI1* Showing Defined Structures

1 = Internal Short Repeats

= Possible Phosphorylation Target Sites

= N-Glycosylation Sites

M = Sites of Known Mutations

O = A.A. Matches

● = Related A.A.

had been carried out to a certain extent by those who had initially sequenced the gene. On close inspection several structural features appeared to be present, dibasic amino acid residues which could mark cleavage sites or may represent target sites for phosphorylation, possible glycosylation sites and internal short repeats. These are shown in full in figure 5.5 Although these amino acids were known to be present, as were the structural features, there was still no complete consensus on what the sites indicated. It was known for example that two basic amino acids in the sequence of alpha factor in a certain position signalled cleavage by the protein product of *KEX2* (Julius *et al.*, 1983). It is also known that they signal the point at which phosphorylation occurs in other proteins when associated with serines (S) or threonines (T), for example in cAMP-dependent protein kinase, smooth muscle myosin light chain and liver pyruvate kinase (Edelman *et. al.*, 1987). The presence of the N-glycosylation sites and a leader sequence of sorts (the abundance of alanines, A, in the first 30 amino acid residues), both point to the protein being exported, although the codon bias indicates that the protein is translated at low levels. Another feature which is present but has an unknown function is a short internal repeat in the N-terminal end of the protein. This may be a binding site for a protein with which the *WHI1* protein interacts directly in control of cell size or pheromone response. Although the protein sequence does have the above features there are just as many which are absent. Although the protein may have phosphorylation target sites it has no homology with any of the protein kinases known and no sites for tyrosine kinase phosphorylation. The protein does not have zinc fingers present, known to be involved in the binding of DNA, or Ca^{2+} binding loops.

5.3 DISCUSSION

Although the *WHI1* gene remained unmapped in this investigation, another researcher mapped and cloned the gene (Bruce Futcher). While this was being carried out another worker (Fred Cross) had cloned it by a completely different route, revealing another unknown facet of this gene. Once the gene was cloned many new avenues of experimentation were open. These can complement those already explored to give more insight into the function of *WHI1* and the mechanism by which it regulates cell size. The cloned gene was made available for experimentation just as the mapping was completed. As it was initially supplied in a lambda phage vector the first job was to clone it into vectors which could express the gene in yeast cells.

Although some information was available about the vector and the genes it contained it was not known in which direction the *WHI1* gene was expressed. To overcome any problems with promoter sequences and because of the available *Bgl*II sites, attempts were made to clone a much larger fragment than was necessary. All of these attempts failed. The complete fragment was never recovered from the centromeric plasmids into which it was cloned. The vectors had been used in other investigations (Futcher & Carbon, 1986) and were used here although there were some problems with yeast cells becoming sickly with the addition of extra centromeres, as in these vectors. No references were made to *E. coli* becoming sick or excising segments of DNA from these vectors, therefore it was decided investigate the DNA which was being cloned into the vectors. As the nature of control of the *WHI1* gene was unknown it was possible that the gene may have had some deleterious effect on the *E. coli* cells

which resulted in cells surviving only if fragments of the ligated vector were excised. As the promoter sequences of prokaryotes and eukaryotes are not the same, the cloned genes would not have been expressed efficiently. Still the virus DNA sequences were being removed. It was of course possible that it was one of the other gene sequences present in the fragment which was causing the problems. To try and clarify this matter shotgun lambda DNA cloning was tried. As a control, commercial lambda DNA was also cut and ligated into the centromeric vectors. Most of the recovered constructs appeared to contain deletions. The sizes of the fragments resulting from digestion with restriction enzymes was calculated by construction of a standard curve from the distance run by fragments of known size run on the same gel. The distances run by the fragments from transformants could have been affected by the presence of large amounts of RNA. Although RNAse was added to the digests there may still have been effects on the gel. This could not explain all the results. Several of the plasmids recovered appear to be smaller than the vector into which the fragment was cloned , in some cases by up to 3Kb. These must have contained deletions. The vector which remained must have contained the ampicillin resistance gene and an origin of replication as it was maintained in cells which grew on media containing an inhibitory concentration of ampicillin. The vectors used in this experiment were 8 and 10Kb in size. Approximately 50% of this was yeast DNA and the remainder plasmid pBR322. The smallest recovered vector was of a size which could contain all the genes necessary for survival in *E. coli* but it would have not survived in yeast cells. If intact *WHI1* genes in yeast expression vectors had not arrived in the form of the *DAF1* alleles further digests would have been carried out to investigate exactly

which DNA remained in these deletion vectors. It seems likely that only pBR322 remained with some yeast DNA but little or no lambda DNA. The deletions must have been due to a restriction system within the *E. coli* strain used although this was not fully investigated. This strain of *E. coli* was not used to raise lambda particles but only as a cloning vehicle. It was possible that a restriction system directed against lambda DNA was intact in the strain and therefore any vectors containing DNA from this source suffered deletions. This could have been tested with the use of pure pCEN- vectors as controls or through a further transformation.

With the cloned gene it was possible to investigate homology between it and other genes which were involved in size control in other yeasts. The system in *Sc. pombe* involving the mitotic activator *CDC25* and mitotic inhibitor *WEE1* had been well documented. Although this system operated at the end of mitosis rather than at its start the two systems did have a common link, namely the homologues *CDC28* in *S. cerevisiae* and *CDC2* in *Sc. pombe*. These genes are structural as well as functional homologues and operate at the times in these yeasts cell cycles when size control is exhibited. In *Sc. pombe* the *WEE1* gene product, a protein kinase homologue, may inhibit the function of the *CDC2* gene product while *CDC25* seems to have a positive effect on its activity. Structural homology between *CDC25* and *WHI1* had already been shown not to exist. The possibility of homology between *WHI1* and *WEE1* was only partially explored. The Southern blot analysis carried out showed no structural homology existed between these two genes but as the controls did not give the expected results some questions were still not answered. The one control which should have shown a positive result was *Sc. pombe* genomic DNA digested with *Hin*D III or *Pst*I and probed with *WEE1*. At

least a single band should have appeared , especially with low stringency washes although the amount of DNA on the blots may not have been sufficient to allow efficient hybridisation So although the question of direct homology between the two genes was clear, whether *S. cerevisiae* contained any structural homologues of *WEE1* was still not resolved. It was possible that the cryptic size control in *S. cerevisiae* at the end of mitosis may have been of the same type as seen in *Sc. pombe* and therefore may be controlled by the same type of protein.

As well as comparison by Southern Blot analysis, direct comparison of the sequences could be carried out once the sequence of *WHI1* was revealed. Diagon plots of *WHI1* against *WEE1* and *CDC25* showed only small insignificant regions of homology. As well as having large or small regions of homology, genes, which are controlled in the same way, will have structures acting as target sites for the controlling mechanism. These may be too small to be picked out by Diagon plots but may be visible on examination by eye. This was true of *CDC25* and *WHI1*. Both gene products were the same size, being proteins of MW 67,000. They both contained the same number of paired basic amino acid residues (arginine and lysine) and had large regions which were rich in serine (S), threonine (T), proline (P) and glutamic acid (E)/ aspartate (D). The paired dibasics were associated with serines and threonines which indicated that they might be target sites for phosphorylation. The regions which were rich in P, E(D), S and T might be regions which were liable to degradation by proteolysis. This was known in *S. cerevisiae* (Rogers *et al.*, 1986) but had not yet been shown to be the case in *Sc. pombe*. Although both proteins may have acted in a different way or on different substrates, they

may have been controlled in the same way and shown a common link for size control. If indeed the dibasic sites were targets for phosphorylation they might be dependent on protein kinases for control as well as the action of proteolytic enzymes. In fact the time when these proteins could act might be a short "window" in the cell cycle when they had been phosphorylated yet not degraded. The two proteins did share common features and when it is clearer how these proteins act and are acted upon perhaps the question of size control will be better understood.

Some of the features that the *WHI1* protein exhibited have been mentioned above. The consequences of these and other features as far as producing models for control of cell size, have been discussed by other investigators (Cross, 1988 & Nash *et al.*, 1988). The models have been proposed on the data they had available. Here other features have been recognised in the *WHI1* protein as present or absent. The significance of these are discussed below.

One of the most surprising features of the predicted protein sequence was the number of dibasic sites. In other proteins these are target sites for proteolytic cleavage (as in the production of mature alpha factor). This indeed may be the case here but basic amino acid residues may also be target sites for phosphorylation (Hardie, 1988. Edelman, *et al.* , 1987) especially when associated with serines or threonines. The dibasic sites were not spread throughout the entire protein but were found mainly in the C-terminus which was lost in both hyperactive mutants of *WHI1* isolated. The loss of these sites in particular, may be significant as far as control of protein function is concerned. Another feature of the C-terminus was a number of regions rich in P, E (D),S and T

residues. The number of these residues not only meant that this end of the protein was highly likely to be devoid of any organised structures but would be open to degradation by proteolytic attack (Nash *et al.*, 1988). This may in fact be the controlling factor, missing in the mutants isolated, which coded for prematurely terminated proteins.

Another feature which was present in the C-terminus but also in other regions of the *WHI1* gene product, was what appear to be N-glycosylation sites. These have the structure asparagine (N) followed by any amino acid, followed by either serine (S) or threonine (T). There are eight such sites present in the protein sequence. Four are in the C-terminus and would be lost in both mutants. Three remain, spread throughout the rest of the sequence. Could these be candidates for loss of control due to loss of the C-terminus? This question apart, the problem of whether they do actually function in this protein as sites for glycosylation must be addressed. Proteins are glycosylated when they are transported within and exported from the cell, but proteins which undergo this fate also have leader sequences which guide the protein through the transport system.

The major feature of a leader sequence is a high concentration of alanine residues in the first thirty residues. Examination of the *WHI1* gene product showed a high concentration of alanine residues, eight of thirty, in the N-terminus. These two pieces of data taken together seemed to indicate at least transport, if not export, of this protein, however closer examination of the sequence indicated that this might not be the case. Although the alanine residues were present they were not correctly positioned and there was a lack of

other amino acid residues required in a true leader sequnce. If a leader sequence was present it was in a very primitive form. Leader sequences, once they have completed their function, are cleaved from the functional polypeptide. Although there was a possible cleavage site within twenty residues of the putative leader it lacked the correct signal for cleavage found in other yeast proteins such as alpha factor. Further features of the N-terminus of the *WHI1* protein are two small direct repeats. These were of four and five residues separated by eleven residues and seem to be real structures. It is unlikely that these would be present in a portion of the protein that was cleaved off. Although they match no known structure they might represent an "active site". Control of this protein may reside in the C-terminus with the N-terminus being involved in interactions with other components of the size control system.

Another feature of this protein which indicated that it was not exported was the codon bias of the gene. The amount of protein produced by a cell can be estimated by the codon bias in the base sequence of the gene. The codon bias for *WHI1* indicated that it was made in small amounts (Nash *et al.*, 1988). Proteins which are transported and have an effect outside of the cell are made in much higher quantities than those which control events inside the cell. When all this data was taken into account, it seemed unlkely that the *WHI1* protein was exported. The N-glycosylation sites might still have a function during transport within the cell. Perhaps *WHI1* is made in one part of the cell but must be transported to complete its function e.g. produced in the cytoplasm but functioning in the nucleus. These sites do seem to be real and unless another function is attributed to them this seems to be the most reasonable

explanation.

As well as having these structural features the protein sequence also shows homology to another protein, unrelated in any respect. The region of homology of the *WHI1* protein is the region in which both the known mutations are found. The protein which shares the homology is the calcitonin gene related peptide (CGRP). This is one of the proteins which is translated from calcitonin genes. Homology is found between calcitonin peptides from several species. The significance of the homology is difficult to understand. CGRP is produced in both pro- and pre- forms and is known to play several roles in cells. The region of homology in CGRP does not cover the functioning structural part of the peptide but mainly the pre- region of the protein. The dibasic (KR) in the protein sequence of CGRP is the signal for cleavage (see figure 5.3). This is missing in the *WHI1* protein. The homologous region may be coding for cleavage of the *WHI1* peptide resulting in it becoming active. The mutant genes achieve this with a terminated protein which is hyperactive. Although this is an attractive possibility is unlikely to be correct due to the absence of a cleavage signal in an appropriate position. While the homology does appear real, it is over a relatively small region of both proteins. When the z value, the increase in similarity when optimized and the biological significance are taken into account the significance of the similarity of these sequences is reduced. The z value for the comparison is only 7.5. Using guidelines laid out (Lipman & Pearson 1985) this is only probably significant. There is no increase in the similarity score when gaps are allowed. This usually increases when a true homology is found. Finally real biological significance must be taken into account.

Although it is possible that cleavage may occur at this point, there is much greater evidence that the C-terminus is removed by proteolytic degradation signalled by P, E, S and T residues. Also the fact that the sequence appears in the middle of the *WHI1* protein while, being a pro- region, it appears at the front of the CGRP peptides makes the reality of any functional homology unlikely. It is more likely to be an intriguing coincidence.

All of these structural features do give us hints of how this protein and the other proteins involved in the control of cell size and the pheromone response function. The fact that, for example, the protein has no zinc fingers means that it probably does not interact with DNA, it may therefore control mitosis by interaction with other proteins. The presence and position of the presumptive phosphorylation sites indicate that control of the protein may be exerted by cAMP via a cAMP dependent protein kinase. If the PEST theory applies in this case control may also be dependent on the C-terminus of this protein being degraded. This protein must have some function at *start*. It is a protein which affects the size of cells, this being one of the parameters important at *start*, but it is also involved with the pheromone pathway. As size of cells is also dependent on growth rate, requiring information on nutrient status, then the function of the *WHI1* protein seems to be dependent on all of the parameters of *start*. If this is the case then perhaps there is more than one control mechanism for this protein, although ultimately affecting only the one characteristic, size, and through this mitosis.

CHAPTER 6: CONCLUSIONS

All the data gathered during the project have been presented and discussed in the previous chapters. The data do not suggest the way in which the *WHI1* gene functions. The interactions suggest that other genes may be involved directly or indirectly in the mechanism controlling cell size but not how the mechanism itself operates. Although data which reveals how the protein functions have not been produced, other investigators recent work does give clues which they themselves have not mentioned. These come mainly from the predicted protein sequence already mentioned. The actual sequence has been covered in Chapter 5 as have the structures it contains. Their significance as an indicator of function will be covered here.

Both independently isolated *whi1* mutations have the same physical defect as far as the protein is concerned, namely the loss of the C terminus. This single alteration to this particular protein has two substantial effects on the individual cell, altering its size and preventing the cell cycle arrest normally induced by mating pheromones. Both these effects are due to a hyperactive protein as demonstrated by the null phenotype for this gene. This fact leads one to look closely at the lost C terminus and the structures it contains for clues as to how the normal protein is controlled. The C terminus contains five PEST regions bounded by dibasic amino acid residues (Nash *et al.*, 1988). These regions are thought to be specific signals for proteolysis (Rogers *et al.*, 1986). Although this particular region has a very high PEST ranking it is thought that phosphorylation would enhance proteolysis (Bruce Futcher pers. comm.). It is possible that dibasic sites are targets for phosphorylation and this, coupled with proteolysis, may be the way in which the activity of this protein may be controlled. The dibasic residues that do occur are not spread throughout the protein

but are found only within or adjacent to PEST regions. They are not a general feature of the whole protein.

Dibasic residues do feature in many target sites where phosphorylation is known or thought to occur (Cherry *et al.*, 1989, Edelman *et al.*, 1987; Hardie, 1988). They are always associated with serine or threonine residues. This fact is true of the dibasic residues in the C terminus of the *WHI1* protein. So far the *WHI1* protein has not been shown to be phosphorylated, although as yet it has not been investigated. A protein kinase which is specific for this proposed target site has not been found, although the specificity of all protein kinases is unknown are as far as target sites are concerned. Phosphorylation at these sites in the G1 phase of the cell cycle, may control activation of the protein and would allow input of nutrient status into size control.

The PEST theory is well accepted, so it is not unreasonable to assume that the C terminus does undergo proteolysis. As the absence of the C terminus leads to a hyperfunctional protein which appears to function earlier (shortening G1) than the wild type protein, it is also not unreasonable to propose that the wild type protein must lose its C terminus to become active. If phosphorylation of this protein is a real event perhaps it occurs to allow proteolysis. The predicted structure of the C terminus shows that the probability of any secondary structures is very low. It consists mainly of turns (due to the proline residues). This may not be the ideal substrate for a proteolytic enzyme. Phosphorylation may alter the structure giving it enough order to allow degradation. Phosphorylation inducing structural changes has been noted in at least one case (

Sprang *et al.*, 1988). In this case phosphorylation induced structural changes which altered binding sites for allosteric effectors and substrates. These two activation steps mean that *start* would not only be dependent on the synthesis of sufficient *WHI1* protein but also on the fact that the cell could phosphorylate it sufficiently to allow proteolysis and final activation. The mutations which are known to occur would have the effect of making this protein independent of phosphorylation and the control exerted by this step. As *whi1* protein would be synthesised in its activated form, G1 would be shortened resulting in smaller cells. The phosphorylation step may also allow other factors which affect *start* such as nutrient availability and the pheromone system, to have an input in size regulation. In fact it is possible that this may be the way they feed information into *start* itself.

The number of dibasic residues found in the protein sequence of *whi1* is not common amongst yeast proteins. Yet on examination of a number of other yeast protein sequences two, of genes involved with the pheromone signalling system, were found to contain a large number of dibasic residues (see figure 6.1). Both *SST2* and *SCG1(GPA1)* have paired residues associated with serine and threonine residues. The *SST2* gene codes for a protein which is thought to be involved with recovery of the cell from arrest by mating pheromone. It is thought it may do this by uncoupling the signal from receptors which have bound pheromone (Dietzel & Kurjan, 1987). The *SCG1* gene encodes a G protein homologue which is thought to be involved with transduction of the signal from pheromone receptors to the cell cycle control mechanism. The dibasic sites are found in the middle third and C terminus of the *SST2* gene product but throughout the whole of the

FIGURE 6.1

Dibasics Within Yeast Genes

The dibasic sites are shown with their surrounding amino acid residues. Note the prevelence of serine and threonine residues. Isoleucine and leucine also occur with high frequency.

WHI1	*SST2*	*SCG1(GPA1)*
THLRRWTR	GTSKKIVI	IQARKLGI
MSNRKLLQ	RTDKKKFQ	FACKRILL
VRSKRLFP	TDKKKFQI	LQNKRAND
NSFKKSLS	TLSKRGWD	YQTRRRVQ
SNLKKTR-	RDEKKTLD	QTRRRVQS
LLQKRRAIS	YLFRRHLE	MFVRKTSE
LQKRRAIS	IEI KRFLK	GQNRRNLI
NLVKRELQ	TILKKLID	RSERKKWI
TQLKKRST	RFLRKMTI	SERKKWIH
QLKKRSTS	HCDKKSNT	MPIRKYFP
	CSFKKQGF	IYVKRTCA
	KVLRKLYP	QNLKKSGI

SCG1 protein. Although two pairs of dibasics are found in the regions homologous to all G proteins the other ten are not. The *SST2* gene product also shows a very high number of PEST residues, significantly more than most proteins. It is possible that whatever the function of this region in *WHI1* it might be the same in *SST2* and possibly *SGC1*, as this also has a significant number of PEST residues . It may be that all these residues indicate is a rapid degradation of all or parts of these proteins.

One must be careful of drawing too many conclusions from little or unproven data but other data available also suggest a link between phosphorylation and cell size. Recent data concerning genes which affect cellular levels of cAMP, and may ultimately control the amount of phosphorylation carried out by a cell, suggest that the amount of cAMP affects cell size. It is not surprising that cAMP has an indirect effect on size through growth but data on mutations in *CDC25* and *RAS2* suggest a more direct effect and that the mutations are in fact true cell size mutations (Baroni *et al.*, 1989). Where the *whi1* mutation is concerned cells seem to be smaller due to a reduced G1. Cells carrying a mutation in the *CDC25* gene the cells are smaller but have an extended G1 (as do cells growing on poor media). The mutation leads to a lower concentration of cAMP in the cell and this in turn reduce the function of cAMP dependent protein kinases. In the model proposed, reduced cAMP would mean that the time taken to activate the *WHI1* protein would be increased or that the *WHI1* protein would not become active. A mutant form of *RAS2*, *RAS2*^{val19}, leads to constitutive production of cAMP, thus leading to larger cells but with a shorter G1. The length of time taken to activate the *WHI1* protein is reduced and cells traverse *start* sooner. Why are these cells not smaller, as seen with the *whi1*

mutation? The *whi1* mutation affects only one protein which becomes independent of phosphorylation. In cells containing the *RAS2*^{val19} the amount of cellular cAMP is raised. This component of cells affects many proteins and processes, not least growth. Cells with this mutation will be gaining mass much quicker than *whi1* cells due to the deregulated cAMP production. The vital fact is the shortened G1.

If *WHI1* does carry out this vital function what happens when it is not present as with the null phenotype, or if it is not phosphorylated, as is possibly the case in cells containing the *cdc25* mutation? It is known that cells with no functional *WHI1* are large but healthy (Nash *et al.*, 1988). In this case some other protein must be carrying out the function of *WHI1*. The middle third of *WHI1* has homology with cyclins. Recently two other proteins have been isolated which have homology with cyclins. These are known to react with *CDC28* protein, a vital component of the *start* mechanism (Wittenberg *et al.*, 1989). If these proteins interact with *CDC28* protein it is not unreasonable to assume that *WHI1* may act similarly. The data presented in chapter 3 demonstrates that there is an interaction between the *cdc28* allele used and the mutant *whi1* genes. When the *whi1* gene was present the need for a functional *cdc28* gene was bypassed. This indicates that for *WHI1* to complete its function it requires a functional *CDC28* and only the hyperfunctional allele becomes independent of *CDC28*. Although cells may be able to bypass the need for *CDC28* they require at least one functional cyclin gene to survive. If all genes encoding the three cyclin homologues are destroyed or inactivated then cells are not viable (S Reed *pers. comm.*). Possibly there exists a system of

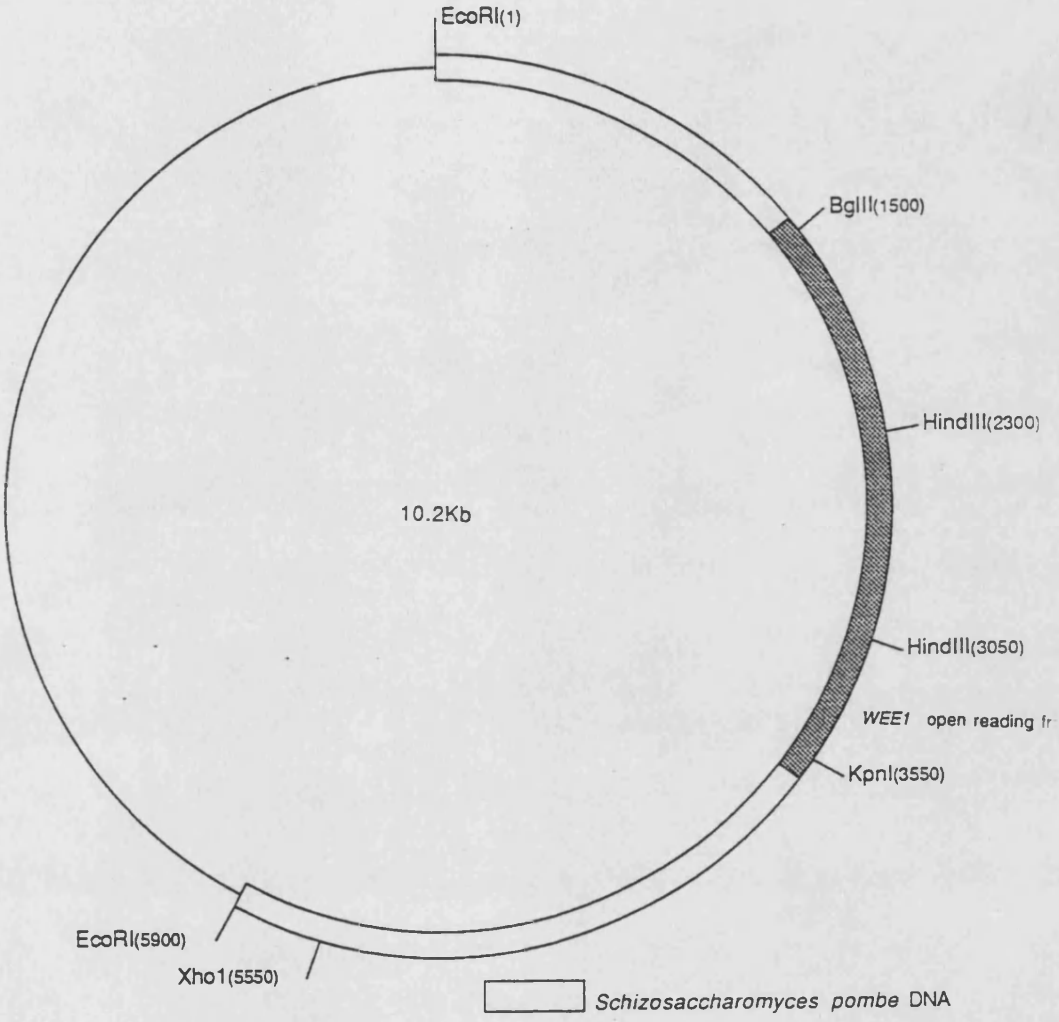
cyclins, of which *WHI1* represents the primary for a particular function, which together modulate size. The two additional cyclins have been isolated as suppressors of mutations in the *CDC28* gene. This seems to indicate that they interact directly with the *CDC28* protein or the mechanism by which it controls traverse of *start*. Again while it is dangerous to extrapolate what is known about other systems, especially where so little data are concerned, there are similarities here with other systems involving cyclins and *CDC28* homologues.

While much is known about the interactions of *CDC28* homlogues from other species at mitosis, how this protein functions at initiation of division is not clear. Data on *CDC28* itself indicates that phosphorylation of an interacting protein is vital. There is no reason to assume that the mechanism for control of entry into division is in any way the same as the one allowing mitosis although the number of functions a single protein can carry out is limited. One fact which does seem to be clear is that *CDC28* homologues require cyclins to function at mitosis. This seems to be the case at *start* as well. How this interaction occurs or what follows on from the interaction are yet to be investigated as are other areas which are raised by the propositions made here.

Several of the questions raised here could be answered by several relatively simple experiments. If the PEST region in the C terminus does signal partial degradation to an active form of *WHI1* then both forms should be isolatable from cells. Western blots of total cellular proteins against antibodies specifically raised against *WHI1* protein should be able to identify two specific bands depending on the stage of the cell cycle. The relative amounts of these

proteins would change with the phase of the cell cycle. Antibodies could also be used to assess binding of the *CDC28*/p40 complex. The question of phosphorylation should also be addressed. Phosphorylation of the *WHI1* protein shouldbe examined throughout the cell cycle. These two experiments would answer specific questions about this particular protein as well as giving vital clues to the involvement of the *WHI1* protein in the control at *start* and control of cell size.

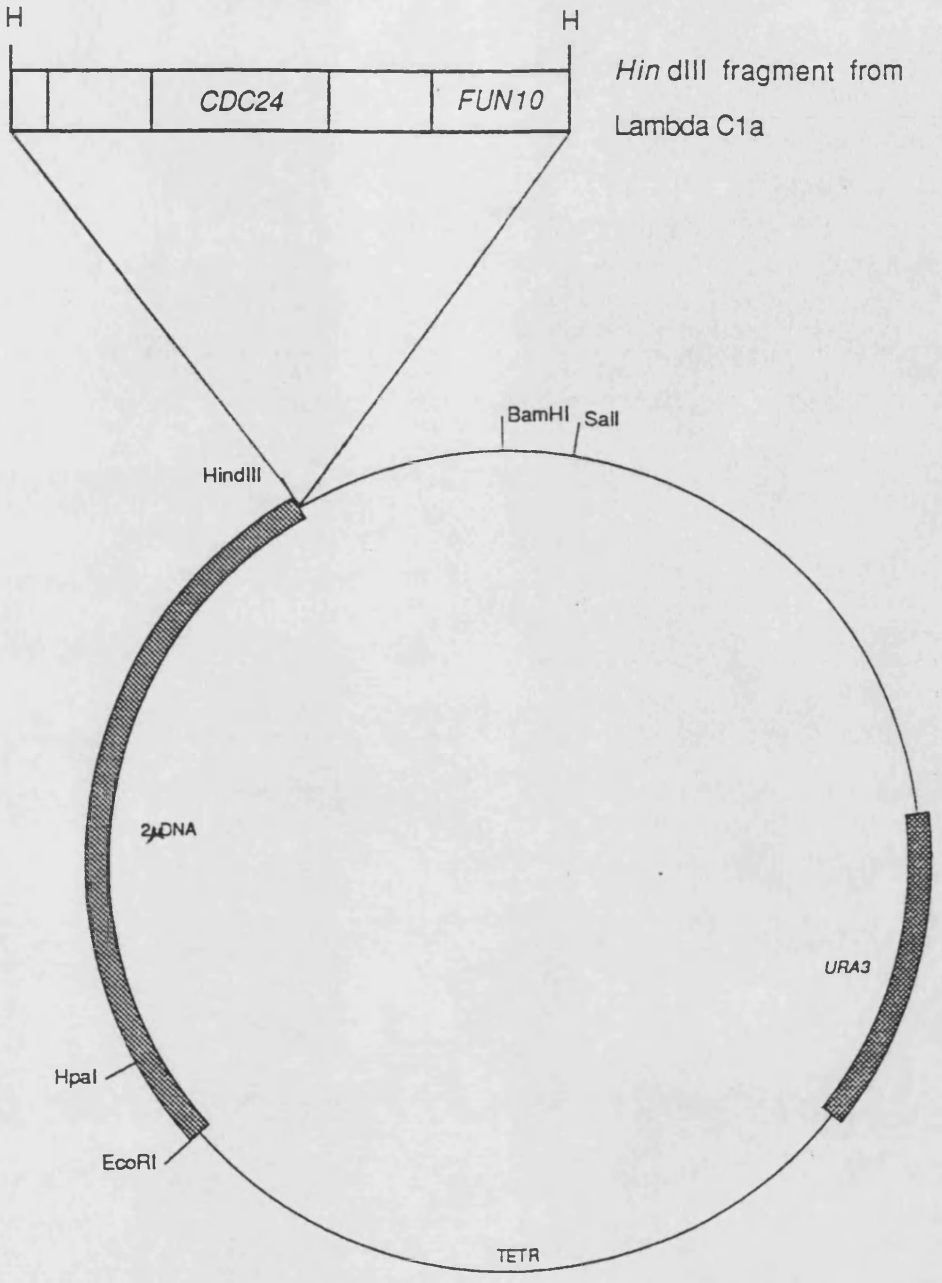
APPENDIX 1: VECTORS



VECTOR 1 pWEE1-12

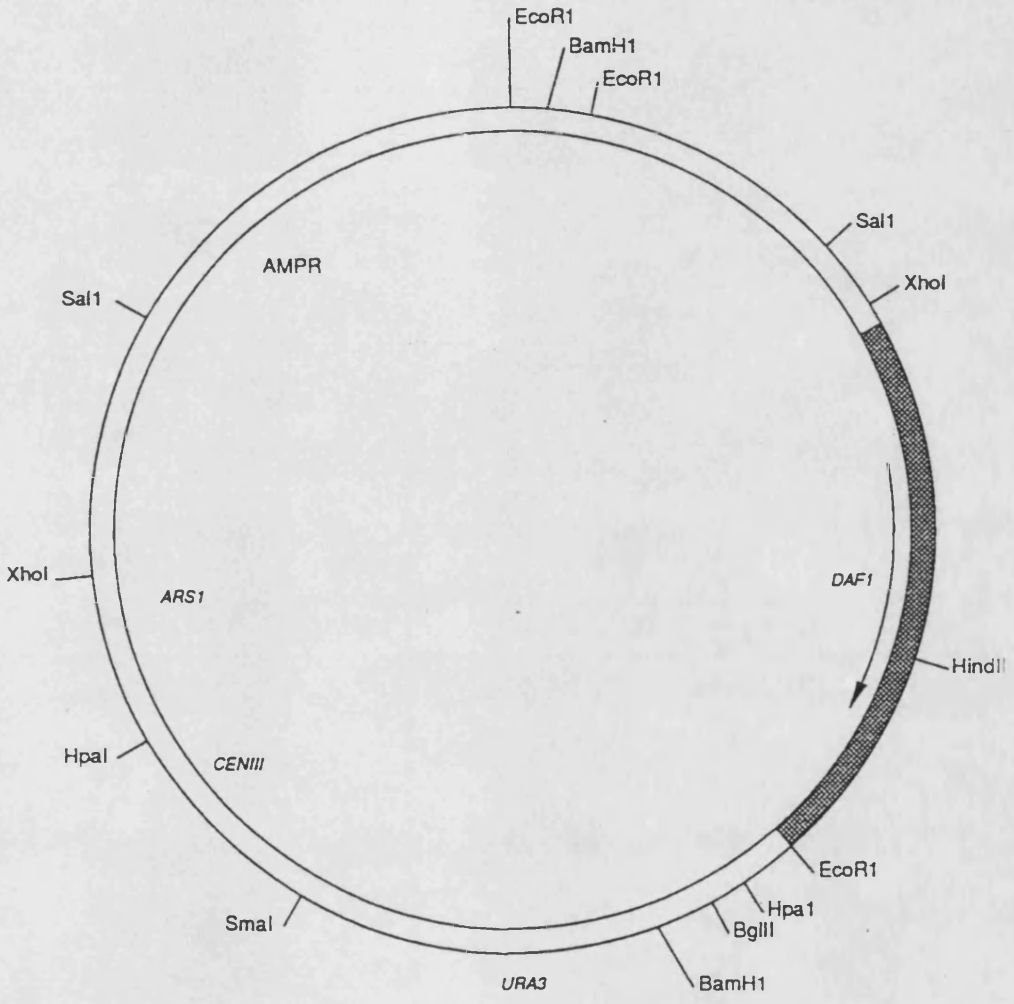
Used in the Southern Blot Analysis

(see Chapter 5).



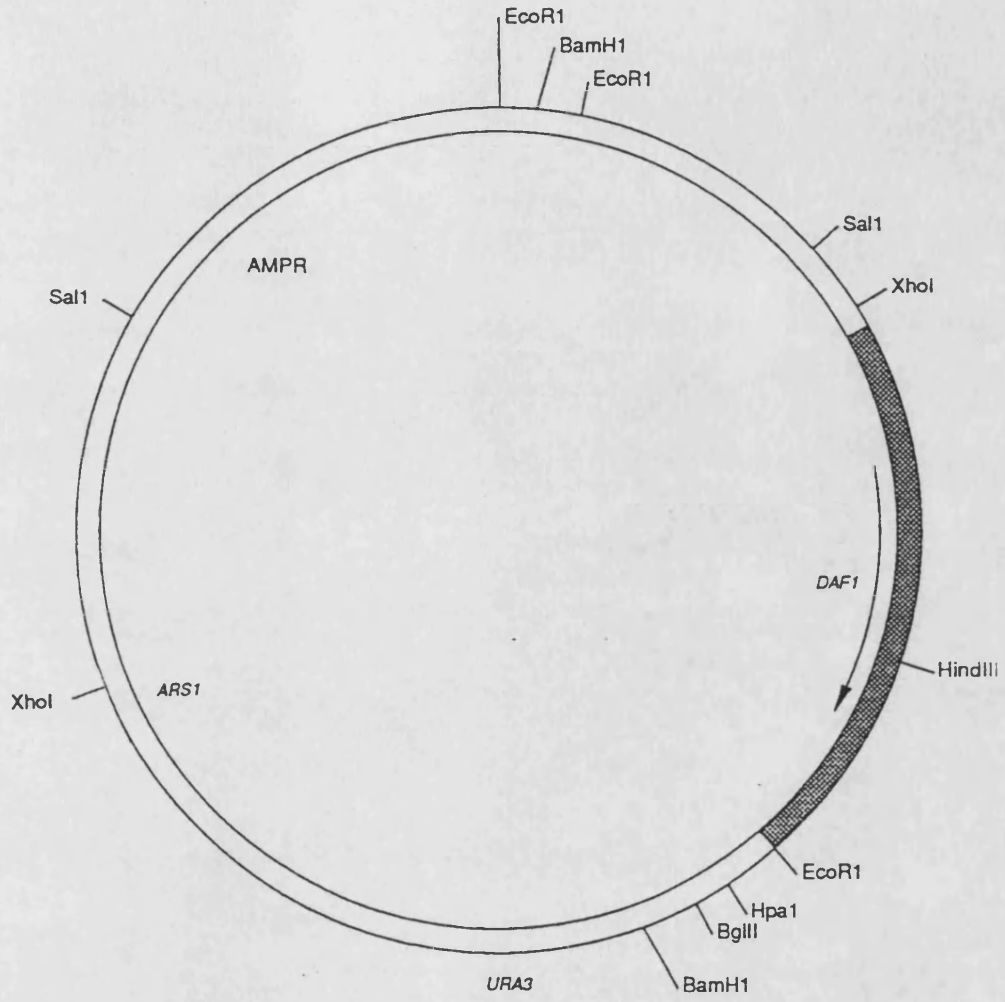
VECTOR 2 YEp103(CDC24)

This vector was used in Southern Blot analysis (see Chapter 5)



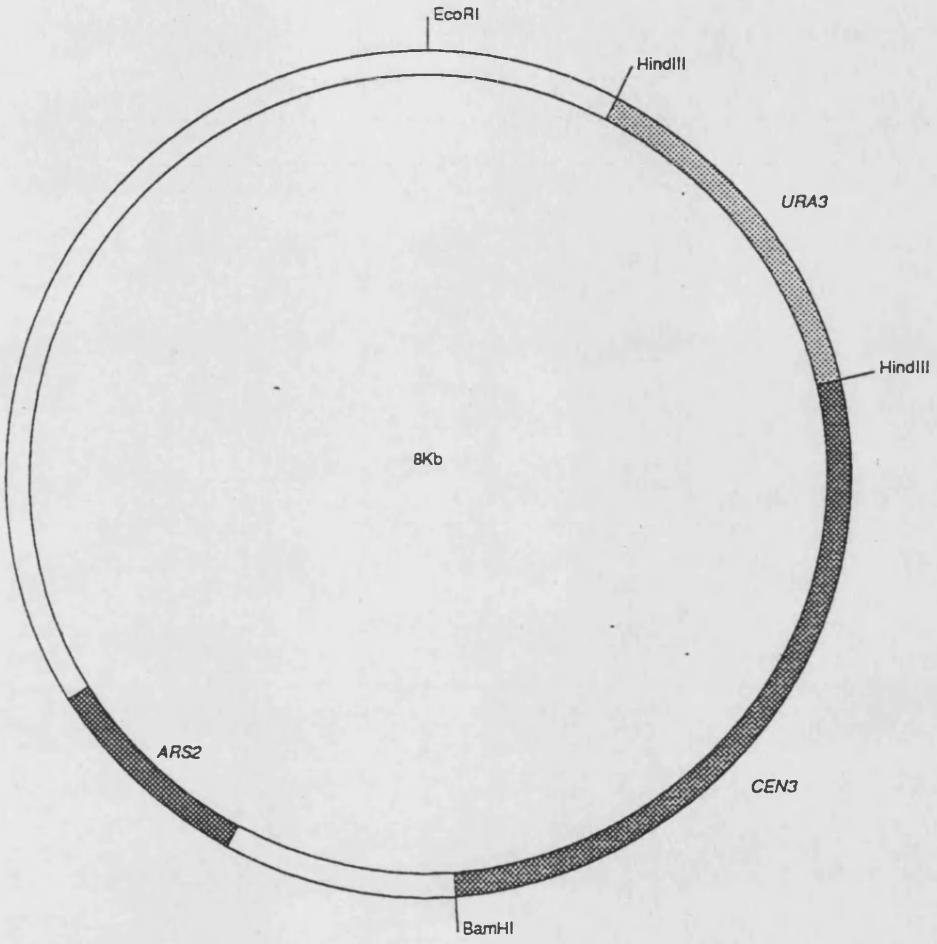
VECTOR 3 pFC101

Used to construct the 2 x *whi1*(*DAF1*) and 4 x *whi1* strains when linearized to form pFC101-1



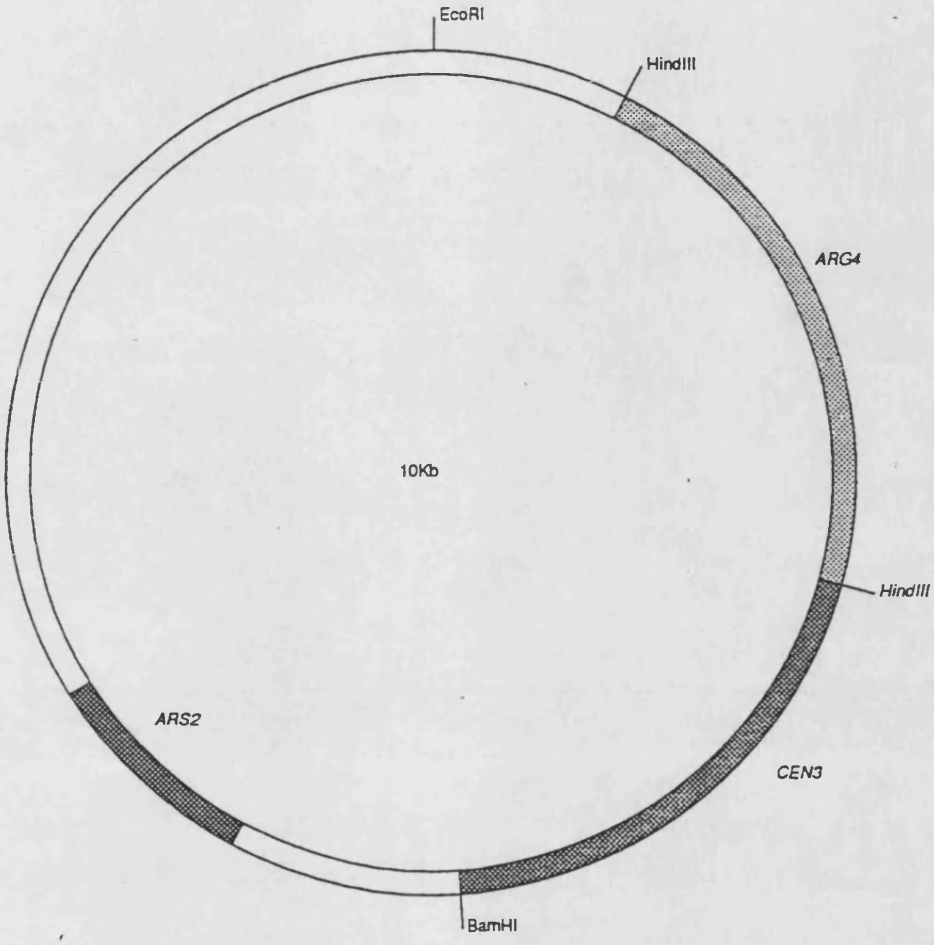
VECTOR 4 pFC101-1

The *Sma1-Hpa1 CEN* fragment was deleted from pFC101 to give the above which could intergate into the genome.



VECTOR 5 p*CEN3-URA3*

Used in the construction of vectors to express *whi1* from LambdaC1a



VECTOR 6 p*CEN3-ARG4*

Used in the construction of vectors to express *whi1* from LambdaC1a

APPENDIX 2: MATHEMATICAL MODELS

Assumptions:

i) After irradiation, chromosomes are lost at random with equal probability,

ii) there is no mitotic crossing over,

iii) loss of both homologues is lethal,

iv) loss of one homologue is non-lethal, and.

v) irradiation only enhances chromosome loss.

Let p = probability that a chromosome is lost.

$1-p = q$ = probability that a chromosome is not lost.

X_i= the number of chromosomes lost from chromosome pair i. X_i may equal 0 with a probability of q^2. X_i may equal 1 with a probability of $2pq$. X_i may equal 2 with a probability of p^2. The last case is lethal therefore the probability that a cell dies equals the probability that both chromosomes are lost in at least one pair

$$= 1-\left[(1-p^2)^n\right] \qquad [A1]$$

where $(1-p^2)$ is the probability that at least one chromosome per pair is retained and n is the number of pairs (which is 16). The probability of a chromosome being lost can be calculated by measuring the frequency of dead cells. If the viable cell number in a culture before irradiation is C and after irradiation is l, then the number of dead cells is $C - l = d$.

The fraction of dead cells is

$$\frac{d}{C} \qquad [A2]$$

which is in effect equal to (A1) therefore

$$\frac{d}{c} = 1-\left[(1-p^2)^n\right] \qquad [A3]$$

or

$$\left[(1-p^2)^n\right] = \frac{1}{c} \qquad [A4]$$

$$1-p^2 = \left[\frac{1}{c}^{1/n}\right] \qquad [A5]$$

$$p^2 = 1-\left[\frac{1}{c}^{1/n}\right] \qquad [A6]$$

$$p = \sqrt{1 - \left[\frac{1}{c}^{1/n}\right]} \qquad [A7]$$

The probability that a given recessive marker is revealed is:

$$\frac{pq}{1-\left[p^2\right]} \qquad [A8]$$

where $1-p^2$ is the total probability that the cell lives.

This is equal to $\frac{p}{1+p}$ [A9]

If h markers are involved, the mean number of chromosomes lost (k) will be equal to $h\pi$ where π is the estimate of the probability that a chromosome will be lost

$$h\pi = h \cdot \frac{p}{\left[1+p\right]} \qquad [A10]$$

Therefore $\frac{k}{h} = \frac{p}{\left[1+p\right]}$ [A11]

and $\frac{k}{h-k} = p$ [A12]

Therefore, from a sample of irradiated cells if the mean number of chromosomes lost and the number of markers are known, then an estimate of p can be made. From this the sample mean can be calculated.

This value of p can be used to give probabilities for the number of chromosomes present in irradiated cells. In any single cell the chromosomes can be divided into two groups; those in which at least one chromosome is marked (P) and those in which neither is marked (Q). Therefore the genome consists of:

P_1 u_1 v_n

. .

. .

P_n u_n v_n

where v chromosomes are all marked, u chromosomes are the unmarked homologues and n = the number of marked pairs.

Q_1 t_1 w_1

. .

. .

Q_m t_m w_m

where m = the number of unmarked pairs and t and w are the unmarked homologues.

Let Y_i be the number of chromosomes lost in a live cell Q_i. For a given chromosome pair Y_i may equal 0 with probability q^2 total is $q^2/1-p^2$. Y_i may equal 1 with probability $2pq$ total is $2pq/1-p^2$. Y_i may not equal 2.

$$q^2/1-\left[p^2\right] = 1-p/1+p \qquad \text{[A13]}$$

or $2pq/1-p^2 = 2p/1+p$ [A14]

With a total probability of 1.

Let Y be equal to the chromosome lost in all Q_i's such that

$Y = Y_i$ which is equivalent to the binomial $B(m, 2p/1+p)$ such that:

$$\text{Probability that } (Y=S) = \frac{m}{S} (2p/1+p)^2(1-p/1+p)^{m-s}$$

where S is the number of chromosomes lost, and

m is the number of unmarked chromosomes.

Having formed an expression for unmarked chromosomes the same must be done for marked chromosomes. The addition of these two probabilities will give a total probability for a given number of chromosomes.

Let K_I be the number of marked chromosomes lost in cell P_I with the condition that the cell lives. M is the marked homologue and M' is the unmarked. With any pair of homologues probabilities that single homologues of a pair are lost are:

M	M′	Probability	
0	0	q	
0	1	pq	(marked homologue shows)
1	0	pq	
1	1	p	(cell dies)

If the condition is that a marked chromosome is revealed then:

Probability that neither of a given pair of marked homologues is lost = 0

Probability that the unmarked homologue is lost = 1

If the condition is that no marker is revealed then:

Probability that neither of the homologues is lost is $\frac{q}{q} = q$

Probability that the marked homologue is lost is $\frac{pq}{q} = p$

If X_I is the number of chromosomes lost in P_I then:

Given that a marked homologue is revealed than $X_I = 1$ with probability = 1

Given that no marked homologue is revealed, then $X_I = 0$ with probability = q, and $X_I = 1$ with probability = p.

If there are many marked pairs in a live cell with K markers showing then Z, the total numbers of chromosomes lost, will be:

$Z = K$ (the number shown by the markers revealed)

+ $n-K$ (the number of chromosomes lost from marked pairs P, but revealing no marker)

+ the number lost from Q, the unmarked chromosomes which is:

$$Z = K + B(n-K, p) + B(m, 2p/1+p)$$

Using these expressions, knowing the number of auxotrophic markers expressed and the probability that a chromosome will be lost, the probability that a given number of chromosomes is lost can be calculated. For example the probability that $Z = K$ if

X = number lost in $(n-K)P$

and Y = number lost in Q

is equal to the:

Probability that $X = 0$ + Probability that $Y = 0$

or $P(Z=K) = P(X=0) + P(Y=0)$

In the case where $Z = K + 1$ the probability is equal to:

$$P(Z=K+1) = P(X=0).P(Y=1) + P(X=1).P(Y=0)$$

Using these binomial expressions and the data resulting from the irradiation of cells the actual number of chromosomes lost in a cell where a known number of auxotrophic markers are observed can be estimated.

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