ANALYSIS OF HOST/PLASMID INTERACTIONS IN YEAST

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Abstract

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The 2μm plasmid of Saccharomyces cerevisiae is an autonomously replicating, multicopy, nuclear DNA plasmid. The plasmid requires both plasmid and host-encoded functions to ensure its stable maintenance. To date, no positive phenotype has been associated with the presence of the 2μm plasmid in the host cell. It is proposed, therefore, that host proteins involved in 2μm plasmid stability will have general DNA maintenance roles within the nucleus.

The role of the host in 2μm plasmid replication has been well documented. It has been demonstrated that the 2μm ARS element interacts with host replication proteins in the same way as chromosomal replicator sequences. Prior to this study, host genes involved in replication have been identified by the study of mutants defective in plasmid and minichromosome maintenance. More recently, the host has been implicated in the 2μm plasmid partitioning mechanism. One mutant defective specifically in 2μm plasmid partitioning has been isolated (plm2; plasmid maintenance).

This study describes the isolation of four further 2μm plasmid maintenance mutants. These are temperature-sensitive and were shown to represent two complementation groups, plm2 and plm3. Plasmid stability and copy number analyses suggest that plm2 is not defective in the replication of plasmid molecules.

Genetic complementation of the plm2 mutation with a genomic library was successfully carried out. PLM2 is a previously unidentified ORF on chromosome IV, 1.6kb in length encoding for a protein of 521 amino acids. Sequence analysis revealed that PLM2 has a homologue on chromosome XII, also of unknown function. A strain carrying a disrupted allele of plm2 created by integration of a Tn1000::HIS3 cassette is viable. Analysis of the Plm2p sequence demonstrated homology with nuclear proteins involved in DNA binding, either as DNA-directed RNA polymerases or as transcription factors. It is proposed that Plm2p has a general role in DNA maintenance within the yeast nucleus, perhaps as a protein of the nuclear scaffold.
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ABBREVIATIONS

A
ATP
bp, kb, Mb
BSA
C
DAPI
dATP
dCTP
dGTP
DNA
DTT
dTTP
EDTA
G
g, mg, μg, ng
IMS
I, ml, ml
M, mM, μM, nM
MAT
min
nt
OD
PCR
PEG
RNA
rpm
SDS
sec
SSC
T
Tris
UV

adenine
adenosine 5′-triphosphate
base pair; kilo-, mega-
bovine serum albumin
cytosine
4′, 6-diamidino-2-phenylindole
2′-deoxyadenosine 5′-triphosphate
2′-deoxycytosine 5′-triphosphate
2′-deoxyguanosine 5′-triphosphate
deoxyribonucleic acid
dithiothreitol
2′-deoxythymidine 5′-triphosphate
ethylenediaminetetra-acetic acid
guanine
grams; milli-, micro-, nano
industrial methylated spirits
litre; milli-, micro-
molar; milli-, micro-, nano-
mating type locus
minute
nucleotide
optical density
polymerase chain reaction
polyethylene glycol
ribonucleic acid
revolutions per minute
sodium dodecyl sulphate
second
standard saline citrate
thymine
Tris-(hydroxymethyl)-methylamine[2-amino-(2-hydroxymethyl)-propan-1,3-diol]
ultra violet

Abbreviations
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Abstract
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Abbreviations

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Chapter One

INTRODUCTION

The bakers yeast *Saccharomyces cerevisiae* is a eukaryotic organism with extremely well characterised genetics. *S. cerevisiae* has a haploid genome of 14Mb, which consists of sixteen chromosomes and a number of extrachromosomal elements including mitochondrial DNA (Piskur, 1994), double-stranded RNA viruses (Wickner, 1996) and the 2µm DNA plasmid (Broach, 1982). With the completion of the sequencing of the entire *Saccharomyces cerevisiae* genome, and the release of this sequence into easily accessible databases, its importance as an experimental organism and a model eukaryote is beyond question.

*S. cerevisiae* has a well characterised life cycle. It can exist as both a haploid and a diploid and so undergoes both mitosis and meiosis. Haploid cells exist in two mating types, a and α. Cells of opposite mating type can mate to form a diploid which, though able to go through the mitotic cell cycle, is also able to enter a meiotic cell cycle under conditions of nitrogen starvation to create four haploid spores. This greatly facilitates genetic study because mutant phenotypes of interest can be followed through meiosis. This enables the establishment of whether the phenotype is due to a single gene mutation, and linkage analysis with other known genes can be carried out. Also, essential genes can be mutated and maintained in diploid cells. The advantages of working with a simple unicellular organism has meant that yeast has become an important model system for many areas of biological research. Yeast has a typical eukaryotic cell structure, with a defined nucleus, actin cytoskeleton, vacuoles and organelles associated with cells of higher eukaryotes. The control of the life-cycle and other important physiological functions is carried out via a complex array of signal-transduction pathways. The main difference between *S. cerevisiae* and other eukaryotes as relates to the work presented in this Thesis, is that it undergoes a closed mitosis and meiosis, in that the nuclear membrane remains intact. This, however, has not prevented important parallels between the cell-cycle in yeast and higher eukaryotes being discovered. Indeed, the study of mitosis and meiosis in yeast remains a very important and informative field of research.

Molecular analysis of *S. cerevisiae* is relatively simple. DNA can be introduced into yeast by the process of transformation, DNA can be prepared from yeast for the construction of genomic libraries and genes can be cloned by rescue of recessive mutations. Due to the high level of homologous recombination that occurs in *S. cerevisiae*, gene disruption experiments can be carried out. Gene disruption, or allele replacement, is when a linear fragment of DNA, previously manipulated *in*
To contain a gene of interest and often with an integrated marker gene which destroys gene activity, is allowed to recombine with the genomic copy. This creates a well-defined null allele of the gene of interest to facilitate further genetic and physiological analysis, such as determining whether or not the gene is essential. Because *S. cerevisiae* can exist as a diploid, gene disruptions of recessive genes can be carried out in a diploid strain to avoid creating a lethal mutation.

The conservation of many of the basic cellular functions between yeast and higher eukaryotes has led to the discovery of genes in yeast that have human homologues. Some of these homologues have been identified by virtue of sequence comparison, such as the homologue for the determinant of adrenolukodystrophy found on yeast chromosome XI. More significant though is the identification of human gene homologues by the ability of yeast heterologous cDNA libraries to complement mutations. This cross-species complementation has also meant that yeast is a valuable tool for studying other organisms that are less amenable to molecular analysis, such as the yeast *Candida albicans*, an obligate diploid that is an important human pathogen.

Recombinant DNA manipulation in yeast is facilitated by the presence of the 2μm plasmid, a naturally-occurring, stable, multi-copy plasmid found in most natural populations of *S. cerevisiae*. The 2μm plasmid is the basis for many of the vectors used in yeast molecular analysis. It is an endogenous, multicopy plasmid which requires both plasmid and host encoded functions for its stable maintenance. The role of the host in its replication is well characterised (Zakian *et al.*, 1979) because of the presence on the plasmid of an ARS element. Less well characterised is the role of the host in plasmid partitioning. It has been demonstrated via biochemical studies and the isolation of a host mutant that a host factor is involved with the segregation of 2μm plasmids into daughter cells (Cashmore, 1984; Meacock *et al.*, 1989; Hadfield *et al.*, 1995), though exactly how these interactions facilitate plasmid partitioning and the possible implications for this in terms of chromosomal inheritance is unknown. The 2μm plasmid is therefore a useful tool to study processes of DNA metabolism and maintenance in the yeast nucleus.

The work described in this Thesis investigates the role of the host in 2μm plasmid maintenance during the mitotic cell cycle. The approach chosen involves the isolation and analysis of temperature-sensitive plasmid maintenance mutants. This introduction reviews current understanding of the biology and maintenance of the 2μm plasmid, plus an overview of the yeast mitotic cell-cycle and current understanding of the chromosomal elements required for stable chromosomal inheritance. The control of DNA replication is also dealt with in some detail because the MCM family of proteins, essential for this process, were isolated as a
consequence of a study involving mutants unable to maintain minichromosomes (Maine et al., 1984).

1.1 The mitotic cell cycle of Saccharomyces cerevisiae

The mitotic cycle can be divided into four main stages; G1, S, M and G2. G1-phase precedes the initiation of DNA replication and is followed by S-phase when DNA synthesis occurs. After replication and the first appearance of the daughter bud cell comes G2-phase. This is stage of growth during which the cell prepares itself for mitosis, M-phase, when the nucleus divides (Figure 1.1; Pringle and Hartwell, 1981). At several stages during the cell cycle, progression is subject to controls. Some reflect the action of extracellular growth factors and hormones. Others, termed check points, originate from within the cell and arise from the need to spatially and temporally co-ordinate cell cycle events, and to halt cell cycle progression in response to irregularities such as DNA damage (Nigg, 1995).

Much has been learnt about the genetic control of the cell-cycle from the study of cell-division-cycle mutants (cdc). The first mutants isolated were all temperature-sensitive and it was observed that, at the non-permissive temperature, the populations of mutant cells arrested with the same morphology. This suggested that they had all stopped at a specific point in the cell-cycle. This original screen resulted in the identification of 32 different cdc mutants (Hartwell et al., 1970), but since then the number has grown to over fifty.

The advantage of using yeast to study the cell-cycle is that at each stage the dividing cell has a characteristic morphology. It is therefore possible to determine at what specific stage the cdc mutants arrest. Detailed analysis of the cdc mutants in this way have produced a temporal map of the cell-cycle. For example, it was found that if a cdc mutant that blocked DNA replication was shifted to the non-permissive temperature, the cells arrested in S-phase and did not perform nuclear division or cytokinesis. It was therefore deduced that mitosis and cell division require the completion of DNA replication (Hartwell et al., 1974).

One of the most dramatic phenotypes of the early cdc mutants was that of cdc28. At the restrictive temperature cdc28 cells cannot bud, duplicate their spindle pole bodies or replicate their DNA (Reid and Hatwell, 1977). For a single mutation to have such an effect, it was deduced that Cdc28p must operate downstream from all the other mutations identified at this time. The point at which Cdc28p is important was found to be the point in the cell-cycle when the cell commits itself to going through the mitotic cell-cycle and has been defined as 'Start'. It is now known that
The mitotic cell-cycle

The mitotic cell-cycle is divided into four main stages. G1 is a period of growth when a series of events prepares the cell for Start and commitment to the mitotic cell-cycle. After Start, the cell prepares itself for the initiation of DNA replication. S-phase is the stage when initiation of DNA replication occurs and replication is completed. This also coincides with the emergence of the daughter bud. During G2 the daughter bud increases in size leading to M-phase and the transfer of genetic information from the mother to the daughter cell. The nuclei are represented by shading.
Figure 1.1

G1 pre Start / G1 post Start / G2

M

S

G2
Cdc28p is a serine/threonine protein kinase (Lorincz and Reed, 1984; Reed et al., 1985) and acts as the catalytic sub-unit of the mitosis-inducing protein kinase, referred to as maturation promoting factor (MPF; Gould and Nurse, 1989). The homologous protein in the fission yeast *Schizosaccharomyces pombe*, Cdc2p, has also been well characterised (Hindley and Phear, 1984; Booher and Beach, 1986; Carr et al., 1989). There seems to be a common pathway of cell-cycle control based on the association between Cdc28p/Cdc2p and cyclins to form multiple kinases, which, in turn, govern both the Start (G1-S), and G2-M transitions (Booher and Beach, 1987; Richardson et al., 1989; Nurse and Bissett, 1981). Key cell cycle events such as DNA replication, mitotic spindle formation, and exit from mitosis, are thought to be triggered by the appearance and disappearance of particular kinase forms.

Attainment of a critical cell size, or some other parameter related to this, seems to be an important control operating close to Start in *S.cerevisiae* (Johnston et al., 1977). Cells can arrest at this point of minimal cell size and make a decision to commit to another stage of growth. This can either be a decision to commit to another round of the mitotic cell-cycle if conditions prove favourable, or to enter stationary phase if the cell is starved for nutrients. Haploids can commit to mate and diploids can commit to the meiotic cell cycle at this point if starved of nitrogen and restricted to a non-fermentable carbon source.

If a cell has committed to Start, late in G1-phase the spindle pole body (SPB; the microtubule-organising centre in the nuclear envelope) begins to divide. At about the same time as completion of SPB duplication, the initiation of DNA replication occurs. During S-phase, a discrete ring of chitin appears on the wall of the mother cell and the daughter bud starts to emerge. Towards the end of S-phase, separation of the SPBs occurs, a complete spindle is formed and finally DNA replication is completed. Migration of the nucleus towards the neck connecting the mother and daughter bud occurs late in G2, this is followed by mitosis itself with the elongation of the spindle and the division of the nucleus (Pringle and Hartwell, 1981). Cytokinesis and separation of the mother and daughter cytoplasms follows shortly after nuclear division. As a definition, M phase ends after nuclear division. This means that cytokinesis occurs at the beginning of G1.

The most important stages in the mitotic cell cycle for studying chromosomal inheritance are S-phase, when the DNA is replicated and M-phase, when the DNA is partitioned into the daughter cell. It is known that the 2μm plasmid is under the same cell-cycle controls as chromosomal DNA, with a single initiation of replication during S-phase (Zakian et al., 1979). What is not known is whether or not the 2μm plasmid partitions at the same time, or under the same constraints as the chromosomes. It seems likely that the 2μm plasmid interacts with host proteins other than those normally associated with chromosome transmission, and different
interactions may occur as the cell-cycle progresses. One model for 2μm plasmid partitioning suggests that plasmid molecules are attached to an actively partitioning structure within the nucleus, perhaps components of the nucleoskeleton, that provide dispersed anchorage sites for plasmid association. It may be that the important stage in the cell cycle for plasmid inheritance is when the nuclear envelope migrates into the daughter cell rather than the stage of chromosome segregation itself.

In all eukaryotes the nuclear envelope is a double membrane layer that separates the nucleus from the cytoplasm (Burke, 1990). In many eukaryotes the nuclear envelope breaks down, allowing access of the mitotic spindle to the centromeres of the chromosomes. In *Saccharomyces cerevisiae*, however, the spindle is intranuclear and the nuclear envelope remains intact throughout the cell cycle (Byers, 1981). Cell cycle mutants *cdc14* and *cdc15* arrest in late mitosis. In these mutants, separation of the nuclear envelope has not taken place, although the bulk of the DNA has segregated (Adams and Pringle, 1984). Indeed, in *cdc15* mutants, cells complete nucleolar segregation even at the restrictive temperature (Granot and Snyder, 1991). Also antibody staining experiments, using antibodies directed against the nuclear pore complex, demonstrate that in *cdc14* and *cdc15* mutants, though the nuclear envelope fails to separate, the envelope is elongated and stretched between mother and daughter cells (Copeland and Snyder, 1993). This suggests that nuclear envelope separation may indeed be regulated independently from chromosome or nucleolar segregation. The study of 2μm plasmid replication and inheritance may therefore identify host proteins with general DNA maintenance or nuclear integrity roles in the cell.

1.2 Replication of DNA

1.21 Autonomously replicating sequences

Replication initiates during S-phase in the yeast cell cycle at well-defined autonomously replicating sequences (ARSs) positioned at intervals along each of the chromosomes. These sequences were first identified by Stinchcomb *et al.* (1979) and others by their ability, when present on plasmids, to confer high plasmid transformation efficiency of yeast. Importantly, these ARS-containing plasmids were shown to be maintained extrachromosomally. Initial estimates suggested that there were the same amount of ARS elements present in the genome as replication origins, previously calculated by determining average inter-origin distances by fibre
autoradiography (Pettes and Williamson, 1975; Newlon et al., 1974). However, it is now known that there is a high degree of redundancy of their replicator function, with only a subset colocalising with origins of replication in the chromosomes (Fangman and Brewer, 1992). Electrophoretic analysis has demonstrated that replication of DNA occurs at or near this active subset of ARS elements (Beach et al., 1980; Chan and Tye, 1980).

The sequences of various ARS elements that do function as origins of replication have been analysed. One of the most detailed analyses has been carried out on ARS1 and has revealed three separate domains (Figure 1.2). The most conserved of these domains between this and other ARS elements, including that of the 2μm plasmid, is domain A. Within domain A is the ARS consensus sequence (ACS) \( (A/\gamma)TTTA(C/G)(A/G)TTTA(A/\gamma) \) (Broach et al., 1982), an 11bp element that has been shown to be essential for replicator function on both chromosomes and plasmids (reviewed by Rowley et al., 1994). Indeed, the 14bp domain A of ARS1 has the ability to act as a replicator on a plasmid containing no other ARS1 flanking sequences (Srienc et al., 1985). Flanking domain A in ARS1 are domains B (containing elements B1, B2 and B3) and domain C. These domains are less well conserved between different ARS elements, but by mutational analysis have been shown to be important for full origin activity (Broach et al., 1982; Bouton and Smith, 1986; Walker et al., 1991; Huang and Kowalski, 1993). Comparisons of B domains within other origins identified has revealed that they tend be AT-rich and are sensitive to alterations in distance and orientation relative to the ACS. Detailed analysis of a second yeast replicator, ARS307, reveals a similar organisation to ARS1. ARS307 contains two elements within domain B which are necessary for full origin function (Theis and Newlon, 1994). Although these two elements show little sequence homology with B1 and B2 sequences from ARS1, they can be functionally substituted (Rao et al., 1994). More recently a further chromosomal replicator sequence, ARS305, has been analysed (Huang and Kowalski, 1996). Again, this sequence has homology with ARS1 in all the elements important for protein binding, though downstream sequences and the organisation of elements adjacent to the ACS is different in all three. Point mutations within element B1 can destroy origin function but no single mutation has been found to affect B2. Element B3 of ARS1 has homologues in a subset of other ARS sequences and contains a binding site for Abf1p (ARS binding factor 1) which functions as a transcription activator at a variety of promoters. Element B3 can be substituted by the binding sites of other transcription activators without affecting origin activity (Marahrens and Stillman, 1992).

A study of ars1 deletion mutants showed that the ARS1 replication origin was not essential for premitotic or premeiotic DNA replication, as no reduction in viability or growth rate of these mutant strains was identified (Kirpekar et al., 1994).
Anatomy of the yeast ARS element

The ACS found within Domain A is essential for replicator function and is found in all ARS elements. Functions 3' to the T-rich strand of the ACS comprise Domain B, functions 5' to the T-rich strand of the ACS comprise Domain C. Domains B and C are important for full replicator function, but are not essential. The elements within Domains B and C are variable in number and sequence between ARS elements.

Proteins that interact with ARS elements include Abf1p binding at element B3, and ORC binding at the ACS. Rap1p is also proposed to interact with ARS elements either directly, or indirectly via ORC binding to Sir1p, a protein involved in silencing.
Figure 1.2

Domain B

B3 B2 B1

Abf1p binding site

Domain A

ACS

Site of ORC binding

Domain C
It is concluded that the removal of ARS1 is compensated for by replication forks emanating from neighbouring origins. A more detailed study carried out on the ARS elements of chromosome III (Newlon, 1995) revealed that active chromosomal replication origins are associated with only a subset of chromosome III ARS elements. Even within this subset of active origins, it was determined by fork-direction analysis that some of these origins are only active in a small proportion of cell cycles. ARS308 is only active in 10-20% of cell cycles, an efficiency of use comparable with that observed for replication origins located in the tandemly repeated ribosomal DNA locus (Brewer and Fangman, 1988; Linskens and Huberman, 1988). One theory for the apparent high redundance of ARS elements during mitosis was that cells may utilise different chromosomal origins during premeiotic S-phase. However, the use of two-dimensional agarose gel electrophoresis demonstrated that the pattern of usage of premeiotic replication origins was identical to that in mitotic S-phase (Collins and Newlon, 1994).

To answer the question of what controls the efficiency of use of an ARS element more fully, studies of constructs containing tandemly repeated copies of ARS1 have shown that chromosomal replication initiates at only one of the copies of ARS1 in any particular chromosome (Brewer and Fangman, 1993; Marahrens and Stillman, 1994). In the study by Marahrens and Stillman, they went on to show that when two ARS1 elements were placed together, the inactive ARS1 element could be completely activated by the introduction of an inactivating point mutation in the active copy. It was concluded that the presence of an active replicator somehow suppresses the activity of nearby replicators. The precise mechanism of this suppression has yet to be fully understood.

One theory for the differential use of ARS elements in the genome, is the availability of the specific ARS elements to the DNA replication machinery. Evidence that there are fixed sites for DNA replication within the nucleus come from the visualisation of pulse-labelled Xenopus egg nuclei, which has demonstrate that replication is confined to a few hundred discrete foci. Further evidence has shown that replication forks are associated with the nuclear scaffold (Vaughn et al., 1990). This has led to the theory that active DNA polymerases are bound to the nuclear skeleton, with replication occurring as DNA is passed through a fixed complex (Jackson, 1990).

In yeast, it has been proposed that ARS activity may be dependent on attachment to the nuclear scaffold (Amati et al., 1990; Brun et al., 1993). The nuclear scaffold, or matrix, is a structural framework depleted of histones and most soluble proteins. Specific DNA sequences have been identified that bind to the nuclear scaffold (Laemmli et al., 1977; Paulson and Laemmli, 1977). These SARs (scaffold associated regions), or MARs (matrix attachment regions), are high AT-rich regions.
of variable size and have been proposed to be DNA elements that may serve as cis-elements of chromosome dynamics (Laemmli et al., 1992). Interactions of SARs with the nuclear scaffold is not determined by a specific sequence. It appears that it is the topology of the DNA in these regions, possibly due to the numerous A-tracts producing narrow minor grooves and bends in the DNA, that is the essential factor for scaffold binding. SARs may represent regions of chromatin that are easily unfolded, this would facilitate the entry of proteins necessary for transcription, chromosome condensation, or DNA replication (Zhao et al., 1993). Topoisomerase II is a major scaffold protein required for many aspects of DNA metabolism such as replication (Heck and Earnshaw, 1986; Gasser et al., 1986) and in vitro binding studies have shown that TopIIP selectively binds to and aggregates SARs.

In both S.cerevisiae and S.pombe, restriction fragments containing genomic ARS elements have been shown to be scaffold attached (Amati and Gasser, 1988; Amati et al., 1990). Moreover, SARs identified in Drosophila have been shown to have ARS activity in yeast (Amati et al., 1990). There appears to be a dual role for ARS elements, both as replicator sequences and sequences that have a structural or nuclear localisation role. Proteins that interact at ARS elements may serve to stabilise higher order DNA structures in the nucleus. At ARSI, the SARs flank the ACS and this attachment has been shown to be independent of Abf1p (Amati and Gasser, 1988). A candidate protein for the mediation of scaffold attachment of ARS elements is Rap1p (repressor/activator protein; Wright et al., 1992). Rap1p is an abundant transcriptional activator but is also necessary for transcriptional silencing at the mating-type loci (Buchman et al., 1988). Study of HML silencers, which are scaffold-associated, demonstrated that Rap1p was able to direct the formation of specific DNA loops between the silencer elements (Hofmann et al., 1989). Moreover, Rap1p has been identified as a component of the scaffold itself.

A region of the 2μm plasmid containing both the ARS and STB sequences has been shown to attach to the nuclear scaffold (Amati and Gasser, 1988). The investigation of the interactions between the 2μm and the nuclear matrix, with its associated replication proteins, may prove to be important for our understanding of both 2μm plasmid maintenance and of the processes involved in scaffold attachment.

1.22 Origin Recognition Complex

The identification and analysis of factors that bind to replication origins is required to fully understand origin function. The binding of Abf1p to element B3 was shown to be important for origin function in vivo, but mutational analysis indicates that the ABF1-binding sites are not essential (Marahrens and Stillman,
1992). Two lines of evidence support the idea that there is another important cellular factor involved in ARS function. Firstly, analysis of the chromatin structure around ARS suggests that the ACS (ARS consensus sequence) is protected by a protein factor in vivo (Lohr and Torchia, 1988). Also, the ACS is sensitive to point mutations (Broach et al., 1982; van Houten and Newlon, 1990) which is expected for a site required for a sequence-specific DNA-binding protein.

The origin recognition complex (ORC) was first purified by Bell and Stillman (1992) and is a six subunit protein that specifically recognises the ACS both in vitro and in vivo (Diffley and Cocker, 1992) and more recently has been shown to bind to element B1 (Rowley et al., 1995). This binding also occurs to replicator sequences on plasmids, including the 2μm (Diffley and Cocker, 1992; Diffley et al., 1994). ACS mutations that affect origin function in vivo have also been shown to affect ORC binding in vitro, suggesting that ACS function is mediated through ORC (Bell and Stillman, 1992). Further evidence that ORC is indeed a replication initiator protein comes from genetic analysis. Mutations in both the ORC2 and ORC5 genes, encoding the 72kDa and 53kDa subunits of ORC respectively, cause defective chromosomal replication and a high instability of plasmids (Foss et al., 1993; Fox et al., 1995; Micklem et al., 1993; Loo et al., 1995). Finally, 2-D gel techniques have demonstrated that both orc2 and orc5 mutants show a marked reduction in the efficiency of initiation of DNA replication from chromosomal origins. These results indicate that ORC is required for the initiation of DNA replication at replication origins, however, ORC binding by itself is not sufficient for this initiation to take place. Genomic footprinting experiments show that ORC remains bound to replicator sequences for a substantial part of the cell cycle, including G2 and mitosis, though re-replication remains blocked. There must be other factors rather than the presence of ORC involved in the actual control of DNA replication itself. Evidence that further proteins are involved comes from genomic footprinting experiments. Late in mitosis, the characteristic footprint seen with ORC alone becomes enlarged (Diffley et al., 1994). The binding of ORC by itself is termed the post-replicative complex (post-RC), and the extended footprint seen is termed the pre-replicative complex (pre-RC).

Cdc6p is a good candidate for a protein associated with the pre-RC and involved in the control of DNA replication. cdc6 mutants arrest in S-phase (Hartwell, 1976) and Cdc6 is expressed in late mitosis, the time of pre-RC formation (Zwerschke et al., 1994; Schwob et al., 1994). A clone of CDC6 was isolated from a screen to look for multicopy suppressors of the orc5-1 phenotype (Liang et al., 1995) and Cdc6p was then shown to interact physically with ORC via an immunoprecipitation assay. Cdc6p expression occurs at the end of mitosis (Platti et al., 1995; Zwerschke et al., 1994) when pre-RCs form, and de novo synthesis of both Cdc6p and its Schizosaccharomyces pombe homologue Cdc18p is required for entry into
S-phase (Piatti et al., 1995; Kelly et al., 1993). This role of Cdc6p in the establishment and maintenance of pre-RCs has recently been confirmed (Cocker et al., 1996).

Another role of ORC is in silencing. The HMR-E silencer represses transcription of silent mating-type genes by blocking site-specific interactions between proteins and their recognition sequences in the vicinity (Brand et al., 1985). Within the HMR-E is an A element containing the 11bp ACS found in replication origins (Rivier and Rine, 1992). Mutations in ORC2 and ORC5 have been found to affect the silencing function of this locus, thereby providing evidence that there is a link between the mechanism of silencing and DNA replication (Foss et al., 1993; Micklem et al., 1993; Fox et al., 1995; Loo et al., 1995). There is evidence that protein components of the ORC are bifunctional. Alleles of ORC5 have been isolated that are specifically defective in silencing but not DNA replication (Fox et al., 1995). Also, the N-terminal of the Orc1p is highly related to Sir3p, a regulator of transcriptional silencing. Studies of Orc1p/Sir3p chimeric proteins indicate that this N-terminal domain functions only in the silencing property of the ORC complex and plays no role in its replication function (Bell et al., 1995). Recently, two-hybrid studies have demonstrated a direct interaction between Orc1p and Sir1p, a further protein important for silencing (Triolo and Sternglanz, 1996). A model has been proposed by these authors to relate the two functions of ORC in both DNA replication and silencing. Sir1p has been shown to interact with Sir4p, which in turn forms dimers with Sir3p (Laurenson and Rine, 1992). Sir3p is able to interact with Rap1p, a protein proposed to mediate scaffold attachment of ARS elements as well as having a role in transcriptional silencing. Therefore, the interaction between Orc1p and Sir1p may be part of a whole complex of protein interactions which act to stabilise higher order DNA.

1.23 Control of DNA replication

Chromosomal DNA replication occurs only during S-phase and mitosis does not begin until replication is completed. The precise timing and fidelity of S-phase is achieved through multiple regulatory mechanisms including positive and negative feedback controls. Negative feedback signals from actively replicating DNA suppress the activation of maturation promoting factor (MPF), resulting in the prevention of mitosis until the completion of DNA replication (Dasso and Newport, 1990; Smythe and Newport, 1992). These regulatory mechanisms also ensure that re-replication of DNA during S-phase is prevented. This re-replication block is not removed until after mitosis, when the cell enters the next cell-cycle.
In prokaryotes, this regulation of DNA replication is brought about by a cis-acting negative control mechanism. Sequences near the origin of replication in newly replicated DNA may become altered by changes in methylation or superhelicity (Messer et al., 1985; Russell and Zinder, 1987). DNA replication in prokaryotes is different in that initiation of DNA replication can occur more than once per cell cycle and DNA isn't packaged into chromatin, however, a negative regulation process could be in operation in eukaryotes. Passage of replication forks might allow chromosome condensation events to take place, thereby preventing the passage of further replication forks. Also, the association of sister chromatids may play a part since the separation of sister chromatids does not occur until mitosis.

A positive control model in eukaryotes involves the accumulation of a limiting diffusable nuclear factor during G1. DNA replication may result in the rapid degradation of this factor, with the result that re-replication is prevented until the next S-phase when the signal has been allowed to accumulate again. Observations from experiments with *Xenopus* eggs favour this model of positive regulation. *Xenopus* nuclei replicated in vitro are unable to re-replicate until they have passed through mitosis. The only mitotic change found to be essential to permit re-replication is the permeabilisation of the nuclear envelope (Blow and Laskey, 1988). It was proposed that there is an essential replication factor necessary for the initiation of DNA replication that can only gain access to the DNA during mitosis when the nuclear envelope breaks down. This factor is then immediately inactivated after initiation of replication, thus preventing re-replication from taking place. Factors thought to positively regulate the initiation of DNA synthesis in this way are termed 'replication licensing factors' (RLFs).

In *S.cerevisiae*, the MCM family of proteins are good candidates for the yeast licensing factor. The MCM (minichromosome maintenance) gene family originated with genes MCM2, MCM3 and MCM5 (also known as CDC46). These were identified as a result of the isolation and analysis of mutants that were unable to maintain both linear and circular minichromosomes in an ARS-dependent manner (Maine et al., 1984). The ability of the mutants to maintain minichromosomes containing a range of different ARS sequences was studied, including that from the 2μm. The term MCM now also refers to two other MCM-related genes, CDC54 and CDC47 plus a putative MCM6 that has not yet been isolated.

The first evidence that these genes may be important in licensing came from the analysis of CDC46 (MCM5). Cdc46p was found to be an essential replication protein that undergoes cell cycle changes in nuclear localisation, as predicted in the licensing factor model. The protein appears to persist in the cytoplasm throughout the cell cycle but accumulates in the nucleus at a late stage in mitosis and is then apparently degraded or exported at the start of S-phase (Hennessy et al., 1990). Next
followed the identification described above of mutants defective in minichromosome transmission which fell into three complementation groups (Maine et al., 1984). MCM2 and MCM3 were later shown to be related to CDC46 (Yan et al., 1991; Gibson et al., 1990) whereas a third, MCM5, turned out to be identical to it (Chen et al., 1992).

The mcm2 and mcm3 mutants have phenotypes similar to mcm5/cdc46 mutants. In addition to the ARS-specific mcm defect, these mutants exhibit a premitotic cell cycle arrest and an increase in chromosome loss and recombination (Yan et al., 1991; Hennessy et al., 1991; Gibson et al., 1990). The most convincing in vivo evidence that MCM2 and MCM3 play a part in the initiation of DNA replication came from two-dimensional gel analysis. These studies showed that the use of chromosomal replication origins in mutants of these genes is selectively reduced. This is supported by the study of 2μm plasmid maintenance in mcm2. The native 2μm was shown to be present at a 7-10 fold reduction in copy number, although a high fraction of cells still remained [cir+]. The low copy number of the plasmid was therefore proposed to be due to impaired replication resulting from a failure to efficiently initiate DNA replication from all the origins, rather than being due to a segregation defect (Maiti and Sinha, 1992). Cell-cycle arrest of conditional mcm mutants occurs in late S-phase, rather than at the G1-S boundary as in some cdc46 mutants. This later arrest can be attributed to incomplete replication from an insufficient number of replication origins. It has been demonstrated via immunofluorescence microscopy of yeast cells with antibodies against Mcm2p or Mcm3p that, as is the case with Mcm5p/Cdc46p, the nuclear and subnuclear localisations of these proteins is regulated with respect to the cell cycle. Mcm2p and Mcm3p enter the nucleus at the end of mitosis, persist throughout G1 phase, and then disappear from it at the beginning of S-phase. Once inside the nucleus, a fraction of the Mcm2p and Mcm3p proteins becomes tightly associated with DNA (Yan et al., 1993). Genetic studies also suggest that the MCM2 and MCM3 gene products play interacting or complementary roles in DNA replication. Overproduction of Mcm3p reduces the viability of conditional mcm2-1 mutants at the permissive temperature and also disrupts minichromosome replication in wild-type cells, whereas overproduction of Mcm2p partially complements the mcm3-1 mutant. An mcm2/mcm3 double mutant is inviable at the permissive temperature of the single mutants (Yan et al., 1991).

The next members of the MCM family to be characterised were CDC47 and CDC54. Conditional mutations in CDC47 and CDC54 cause the cells to arrest just prior to DNA synthesis at the non-permissive temperature. Moreover, immunofluorescence and protein fractionation experiments to look at the cellular localisation of Cdc47p revealed that it enters the nucleus at mitosis where it remains until soon after the initiation of DNA replication, when it is rapidly exported back
into the cytoplasm (Dalton and Whitbread, 1995; Hennessy et al., 1991; Whitbread and Dalton, 1995).

The predicted polypeptides encoded by all the MCM genes have three regions of high homology (Yan et al., 1991; Hennessy et al., 1991; Gibson et al., 1990; Whitbread and Dalton, 1995). The region of most homology is a conserved domain of 145 amino acids which is internal to each polypeptide. This conserved domain resembles the DEAD box of RNA helicases and is similar to the conserved domain associated with a group of transcription and replication factors with known or assumed DNA-dependent ATPase activity, suggesting it may be involved in nucleic-acid recognition. This domain is of interest because a specific function of replicator-initiator proteins is to unwind replication origins (Stahl et al., 1986). Homologues of the MCM family have been found in many other organisms including S. pombe (Takahashi et al., 1994; Coxon et al., 1992), Drosophila (Treisman et al., 1995; Su et al., 1996; Feger et al., 1996), Xenopus (Chong et al., 1995; Kubota et al., 1995; Kubota and Takisawa, 1993) and humans (Hu et al., 1993; Kimura et al., 1994; Thommes et al., 1992).

In summary, all the MCM genes are essential, display similar phenotypes and mcml and mcmS mutations have been shown to reduce the efficiency of replication origin function in the chromosome and in plasmids (Yan et al., 1993). Moreover, all of the MCM proteins show the same periodic association with the nucleus in G1 as originally shown for Cdc6p (Yan et al., 1993; Dalton and Whitbread, 1995), and also inter-relate by a complicated pattern of genetic suppression and synthetic lethality. Taken together, it seems likely that the MCM proteins interact to form a complex in order to carry out their function in DNA replication, and the temporally and spatially restricted localisation of the MCM proteins in the nucleus may serve to ensure that DNA replication occurs once and only once per cell cycle. However, recent evidence from Chong et al. (1995) has implicated the need for a further protein fraction to ensure DNA replication, at least in Xenopus. This casts doubt on the MCM protein family being the only proteins involved in the licensing of S-phase. The factors present in this second protein fraction are yet to be identified.
1.3 Sequences important for maintaining chromosome stability

1.3.1 Telomeres

Telomeres are specialised protein/DNA structures found at the ends of eukaryotic chromosomes, necessary for stable chromosome transmission, the prevention of end-to-end fusions and, perhaps more importantly, for the prevention of degradation of sequences due to semi-conservative DNA replication. The structure and function of yeast telomeres are similar to those in other species studied.

The telomere region in *S.cerevisiae* consists of a terminal G-rich repeat with an adjacent sub-telomeric region containing further repetitive sequences, the X and Y' elements (Figure 1.3a). The terminal telomere repeats in *S.cerevisiae* consist of a variable ((TG)$_{1,6}$TG$_{2,3}$)$_n$ sequence. The number of these repeats, though variable between strains, is actively maintained and crosses between strains results in segregants with varying lengths of arrays. This indicates that there is a genetic component controlling telomere length (Shampay and Blackburn, 1988; Horowitz et al., 1984; Walmsley and Petes, 1985). A number of genes involved in the control of telomere length have been identified. They fall broadly into three categories, the first being genes involved in general DNA metabolism such as *CDC17, CDC8* and *TOP3* (Carson and Hartwell, 1985; Lustig and Petes, 1986; Kim et al., 1995). Other genes are important for chromatin structure, for example *SIR3, SIR4* and *RAP1* (Conrad et al., 1990; Falladino et al., 1993; Gottschling et al., 1990). Finally, there are genes identified which have a specific function in telomere maintenance, for example *EST1, TEL1, TEL2* and *KEM1* (Lundblad and Szostak, 1989; Lustig and Petes, 1986; Liu and Gilbert, 1994), but the precise role of the majority of these isn't fully understood.

The length of telomeres has been shown to vary even within a clonal population (Shampay and Blackburn, 1988). This variation is not genetically controlled, and is due to the addition of (C$_{1,3}$A) repeats. This has led to the proposal of a mechanism of telomere replication involving the addition of telomere repeats to the 3' end of the chromosome. An enzyme, telomerase, that carries out this function has been identified in *Tetrahymena* (Greider and Blackburn, 1985). The telomerase is a ribonucleoprotein with an RNA component complementary to the telomere repeat unit. It is proposed that the RNA component acts as a primer, enabling the telomerase to extend the 3' end of the chromosome without the need for a DNA template. In yeast, cells carrying mutations in the *EST1* gene progressively lose telomeric DNA with each cell division and eventually undergo cellular senescence (Lundblad and Szostak, 1989). The *EST1* gene is a good candidate for a protein component of the yeast telomerase but there are conflicting reports as to this.
(a) Structure of the yeast telomere

The termini of yeast chromosomes consist of a simple repeat array that varies in length between different strains from an average of 250 to 620 bp. Telomeres may also contain zero to four copies of the highly conserved middle repetitive element Y'. The Y' elements are followed by further telomere repeats and then another repetitive element, X.

(b) Consensus sequence of the yeast centromere

The yeast centromere (CEN) consists of three elements, CDEI, CDEII and CDEIII. CDEI and CDEII are highly conserved, whereas CDEII is an AT-rich region with little sequence homology.
Figure 1.3

(a) telomere repeats Y' element X element

ARS ARS to centromere

(b) CDE I CDE II CDE III

PuTCACPuTG TGT(T/A)TGNITTCCGAANNNAAAAA

78-86bp AT-rich
likelihood. Evidence for a telomerase role of Est1p is that telomerase activity is absent in est1 cells (Lin and Zakian, 1995), however, another study revealed that EST1 activity was not required for telomerase activity (Cohn and Blackburn, 1995).

The exact role of EST1 in telomere maintenance is yet to be determined. The RNA template for telomerase, TLCl, has been identified (Singer and Cottschling, 1994). Deletion of TLCl results in similar shortening of telomeres and cellular senescence as identified in est1 mutants.

Telomeric DNA in Saccharomyces cerevisiae is organised into a non-nucleosomal chromatin structure called the telosome, in contrast to the subterminal repeats X and Y' which are assembled in nucleosomes (Wright et al., 1992). The major protein component of the telosome is Rap1p (repressor/activator protein; Wright et al., 1992), an abundant DNA binding protein involved in the transcriptional control of several genes. Rap1p also has a role in the control of expression at the silent mating type loci (Buchman et al., 1988). This property of Rap1p to control gene expression may be achieved by the ability of Rap1p to unwind double-stranded DNA (Gilson et al., 1994).

Rap1p binds directly to the telomere repeat array approximately every 18bp (Gilson et al., 1993; Graham and Chambers, 1994). The binding of Rap1p to telomeric DNA renders it resistant to DNase I and introduces 90° bends which could have an effect on the chromatin structure (Gilson et al., 1993). Temperature sensitive mutations in the RAPI gene demonstrate its role in the maintenance of telomeres. When these mutants were grown at the semi-permissive temperature the length of the telomere arrays shortened and then stabilised (Conrad et al., 1990). Rap1p is also a major component of the nuclear scaffold in yeast (Cardenas et al., 1990) and this localisation to the nuclear periphery is associated with the clustering of the 64 telomeres in a diploid to 5-8 foci (Palladino et al., 1993; Klein et al., 1992). The association of telomeres to the nuclear periphery in both human cell lines and in S.pombe is cell-cycle dependent (de Lange, 1992; Scherthan et al., 1994). It is not yet known if this is the case for S.cerevisiae. This could be an important mechanism involved in the segregation of chromosomes. The importance of Rap1p and its possible role in mediating the attachment of ARS elements to the nuclear scaffold has been discussed earlier.

It was demonstrated that over-expression of the C-terminus of Rap1p in a strain with normal Rap1p activity resulted in changes in telomere length and chromosome stability (Conrad et al., 1990). This suggested that there was a component interacting with Rap1p, which was subsequently identified to be Rif1p (Rap1p interacting factor; Hardy et al., 1992). Strains carrying disruptions of the RIF1 gene are defective in transcriptional silencing and telomere length regulation but grow normally (Hardy et al., 1992). It has not been determined whether Rif1p binds
DNA directly or is a part of the telomeric heterochromatin via interactions with Rap1p.

Interestingly, a protein which binds to the higher eukaryote telomere repeat unit, TTAGGG, has been identified in yeast (Brigati et al., 1993). This is the TTAGGG binding factor (Tbf1p). Though TTAGGG repeats are not found in the telomeres of yeast, they are found in one or two copies proximal to yeast telomeres and it has been shown by footprinting that it is to these that Tbf1p binds (then identified as Tbfop; Liu and Tye, 1991). The function of Tbf1p in telomere maintenance is unclear. TBF1 is an essential gene, but temperature-sensitive mutants grown at a semi-permissive temperature show no detectable change in telomere length (Brigati et al., 1993). It is proposed that Tbf1p may play an essential role in the regulation of gene expression or chromosome structure, perhaps comparable to the duel role that Rap1p plays as an essential telomere element, but also as an important transcriptional regulatory protein. Other Tbf1p binding sites may be present throughout the yeast genome.

Adjacent to the terminal telomere repeats are the highly conserved Y' elements. These elements fall into two major size classes of 5.2 and 6.7kb and are found at half to two thirds of chromosome ends in highly diverged strains of yeast (Jager and Philippson, 1989; Louis and Haber, 1990). Upto four Y' elements have been detected on a single chromosome, with a short stretch of yeast telomere repeats between adjacent elements (Chan and Tye, 1983; Walmsley et al., 1984). The Y' elements contain open reading frames encoding proteins with no strong homology to any known proteins (Louis and Haber, 1992). Though highly conserved, sequence variation between Y' elements exists resulting in Y' elements within strains up to 1.13% diverged and up to 1.75% diverged between strains. The majority of this variation disrupts the open reading frames (Louis and Haber, 1992). The sequence of these elements indicates that they were derived from mobile elements, however, it appears that now transposition between chromosome ends is by mitotic and meiotic exchanges (Horowitz et al., 1984; Louis and Haber, 1992; Louis and Haber, 1990). Chromosomes that lack Y' elements have been identified (Zakian and Pluta, 1989) and a fully functional yeast chromosome III has been created that lacks Y' elements (Murray and Szostak, 1986). This suggests that the subterminal repeats identified in yeast, are not required in yeast cells for normal telomere maintenance. However, the presence of these elements may have a secondary function in providing a buffer between the transcribed regions and the telomere. This could be useful for two reasons. Firstly, in the event of telomere repeat array shortening such a buffer would delay the deletion of the transcribed sequences and secondly, the buffering effect could avoid the transcriptional silencing effect of yeast telomeres (Gottschling et al., 1990).
The X elements are found either immediately adjacent to the telomere repeats, or next to the Y' elements (Chan and Tye, 1983). The X element is mosaic in nature and highly variable between chromosome ends. The only region of homology is a 475bp region called 'core X' which contains an ARS sequence and, in most cases, an Abf1p binding site (Louis et al., 1994; Pryde et al., 1995). The presence of the core X region at all chromosome ends is consistent with it having an essential function, though this is yet to be identified. A role in chromosome stability is one possibility. Having a core X region on a plasmid containing telomere sequences and a centromere improves plasmid stability by 2-3 fold. This improved plasmid segregation is dependent on the genes involved in telomere position effect and on the presence of intact ARS and Abf1 sites within the X element (Enomoto et al., 1994).

Mutants have been identified that specifically affect linear chromosome maintenance (Runge and Zakian, 1993). These linear chromosome stability (lcs) mutants are specifically defective in their ability to maintain both YACs and natural linear chromosomes. However, the sequence and length of the telomeres in these mutants remains the same, indicating that there is a role for telomeres in chromosome stability separate from its replication and chromosome-end protection roles.

1.32 Centromeres

Centromeres are the major structures that direct eukaryotic chromosome segregation in mitosis and meiosis. They have multiple functions, briefly outlined below. The centromere is the site of kinetochore formation, the structure that binds microtubules and regulates chromosome movement during mitosis. The centromere is also the final point at which sister chromatids pair and so therefore must be the locus that receives the signal that triggers the release of sister chromatids at the metaphase-anaphase transition (Spencer and Hieter, 1992; Li and Nicklas, 1995). Another function of the centromere is to group together the proteins that transfer from the chromosomes to the mitotic spindle during metaphase or anaphase. The mechanism by which the centromere directs the segregation of chromosomes to opposite poles during mitosis is essential for the equal distribution of genetic material to each new daughter cell. The DNA sequence that specifies centromere location on the chromosome is referred to as the CEN locus. There are two major classes of centromeres, point centromeres and regional centromeres. Point centromeres are the type found in S.cerevisiae and are compact loci, whereas regional centromeres, best described in S.pombe, are much larger and less well characterised.
In *S. cerevisiae*, centromeric DNA was first isolated from chromosome III (Clarke and Carbon, 1980). Hybrid plasmids containing overlapping DNA fragments from around the centromere-linked LEU2, CDC10 region, plus an ARS1 sequence, were tested for increased mitotic stability. A 1.6kb fragment, when present on an ARS plasmid, permitted those plasmids to behave mitotically and meiotically as minichromosomes. It has since been found that the minimal functional centromere unit in *S. cerevisiae* is approximately 125bp, encompassing three highly conserved DNA elements, CDEI, CDEII and CDEIII (Fitzgerald-Hayes et al., 1982; Murphy and Fitzgerald-Hayes, 1990). CDEII is a 78-86bp region greater than 90% AT flanked by the 8bp CDEI and CDEIII, a 28bp element with partial symmetry (Figure 1.3b). The whole of the centromere appears to be packaged into a nuclease-resistant chromatin structure of approximately 250bp (Bloom and Carbon, 1982) that binds a single microtubule (Peterson and Ris, 1976).

The CDEI sequence is found in the promoters of a number of genes and binds to a protein factor, Cpf1p (centromere and promoter factor). Cpf1p is an abundant protein and when bound to non-centromeric sequences acts as a transcriptional activator (Bram and Kornberg, 1987). Cpf1p is also known as CP1 (centromere protein) and CBFI (centromere-binding factor). Deletion of CDEI causes a 10-30 fold increase of mitotic chromosome loss (Niedenthal et al., 1991) but it is not an essential centromere element. It appears that CDEI has a more important function in meiosis II. Small deletions in CDEI dramatically elevates sister chromatid non-disjunction (Sears et al., 1995; Gaudet and Fitzgerald-Hayes, 1989).

The sequence of CDEII is less well conserved and the length of the AT-rich sequence seems to be the important determinant for centromere function (Clarke and Carbon, 1980). The length of CDEII can be doubled and the centromere still maintains some ability to assemble into a partially functional centromere. Deletions have a more deleterious affect, stopping centromere function, but deleted regions can be replaced with segments of DNA with little similarity to the AT arrangement and a partially functional mitotic centromere will be formed (Gaudet and Fitzgerald-Hayes, 1987).

CDEIII is absolutely required for centromere function, and is the binding site for an essential protein complex, CBF3 (Lechner and Carbon, 1991). Small point mutations in CDEIII can completely abolish centromere function on both chromosomes and plasmids (McGrew et al., 1986). The 25bp sequence of CDEIII forms an imperfect palindrome, but mutations in the left and right halves have different affects on centromere activity. Also, the orientation of CDEII and CDEIII with respect to each other seems important. This suggests that proteins bind to CDEIII in an asymmetric fashion (Hegemann et al., 1988; Jehn et al., 1991; Murphy et al., 1991). CBF3 is a complex consisting of three major proteins of 58, 64 and 110 kDa,
as well as a number of minor species (Ng and Carbon, 1987; Lechner and Carbon, 1991). The footprint of CBF3 is asymmetric, consistent with the affect of point mutations observed, as described above. Some of the genes encoding CBF3 subunits have been cloned. CBF2/NDC10/CFT14 is an essential gene encoding the 110 kDa subunit (Goh and Kilmartin, 1993; Jiang et al., 1993; Doheny et al., 1993). CTF13 and CEP3/CBFb are also essential genes, encoding the 58 kDa and 64 kDa CBF3 subunits respectively (Lechner, 1994). A fourth essential component of the CBF3 complex is encoded by SKP1, isolated as a high-copy suppressor of cft13. Skp1p is a 23 kDa protein and antibodies directed against epitope-tagged Skp1p shifts the CBF3-DNA complex in band-shift gel assays (Connelly and Hieter, 1996). Identification of Skp1p homologues from C. elegans, A. thaliana, and humans indicates that SKP1 is evolutionarily highly conserved.

A further yeast protein that shares homology with the centromere-associated proteins of other eukaryotes is Cse4p (chromosome segregation). This is an essential protein that is similar in structure to human CENP-A in that it contains a histone H3-like domain. It is proposed that Cse4p is an integral component of yeast chromatin that might act to form a modified nucleosome core particle necessary for the assembly of centromeric chromatin (Stoler et al., 1995).

Many other genes have been identified that play a role in chromosome transmission, but the majority of these are components of the mitotic spindle or involved in DNA metabolism pathways. Mutants that have been isolated that affect these aspects of chromosome stability include mif (mitotic fidelity; Meeks-Wagner et al., 1986), cft (chromosome transmission fidelity; Spencer et al., 1990), cin (chromosome instability; Hoyt et al., 1990) and chl (chromosome loss; Kouprina et al., 1993). Recently, a novel histone H4 gene mutation has been identified that arrests cells at nuclear division (Smith et al., 1996). This emphasises the role that the changing chromatin structure of sister chromatids, as they attach and subsequently disassociate from the mitotic spindle, plays in chromosome transmission.

There are many proteins yet to be identified that interact directly with centromere sequences. It is known that CDEII interacts with proteins but these, as yet, remain elusive. Also, further components of CBF3 remain to be identified. Before yeast centromere function can be fully dissected and understood the remaining proteins associated directly with the centromere need to be characterised.
1.4 The 2μm plasmid

The 2μm plasmid of the budding yeast Saccharomyces cerevisiae is an autonomously replicating, double stranded DNA molecule of 6.3kb in length. The plasmid is present at about 50-100 copies per haploid cell (Gubbins et al., 1977; Seligy et al., 1980) accounting for approximately 1-3% of the genome. The plasmid seems to offer no selectable advantage to the host cell and yet requires host functions for its replication (Livingston and Kupfer, 1977), with one initiation of replication per cell cycle occurring at the beginning of S-phase (Zakian et al., 1979). This means that the 2μm plasmid exerts extra replication pressure on the host replication machinery when present at a high copy number. Indeed, plasmid-free cells [cir+] have been demonstrated to have a 1.5-3.0% growth rate advantage over plasmid-containing cells [cir+] (Mead et al., 1986). For this reason, the 2μm plasmid is regarded as an example of parasitic or selfish DNA (Futcher and Cox, 1983; Mead et al., 1986a) and so must encode functions necessary for its maintenance, including partitioning during cell division as well as for copy number control. The 2μm plasmid is also efficiently inherited during meiosis, passing into all four haploid spores even if only one of the parental strains was [cir+] (Livingston, 1977).

The entire length of the 2μm plasmid has been sequenced, revealing that the plasmid consists of two unique regions separated by two 599bp inverted repeats (Hartley and Donelson, 1980). Site-specific recombination between these two repeats results in the plasmid being present in the cell as two forms, A and B (Broach et al., 1982). The 2μm plasmid has four major open reading frames A, B, C and D and two cis-acting sequences ori and STB (Figure 1.4). The A gene product (FLF) has been identified as a trans-acting recombinase, and is necessary for the 'flipping' reaction that interconverts the two forms of the plasmid. This reaction plays a role in the copy number control mechanism of the plasmid. Gene products B and C, along with STB have been shown to be necessary for stable inheritance of the 2μm plasmid during cell division (Kikuchi, 1983; Cashmore et al., 1986). The function of the D gene product is slightly less clear, but over-expression studies on this gene have implicated a role in the regulation of FLP expression (Murray, 1987). D protein has also been implicated in the regulation of expression of C (Cashmore et al., 1988). The two cis acting sequences are necessary for plasmid stability. Ori is the site of origin of replication and is a 75bp sequence containing an ARS element in the larger of the two unique regions, extending into one of the inverted repeats. STB is the stability locus which is characterised by a group of five-and-a-half 62bp direct repeats. As well as the four major open reading frames (ORFs), a number of other RNA transcripts have been identified. From S1 nuclease protection experiments and primer extension
The 2μm plasmid

The two isomeric forms of the 2μm plasmid are shown with the four open reading frames A-D and the cis-acting loci ORI and STB indicated. Also shown are the XbaI and EcoRI sites. The locations of the FRT sites are indicated by the boxes surrounding each of the XbaI sites.
Figure 1.4

FORM A

FORM B
analysis, it seems that most of the 2µm plasmid is transcribed into at least one polyadenylated species (Sutton and Broach, 1985) (Figure 1.5). The role of these other transcripts remains unknown, although a regulatory function has been proposed.

Evidence for the nuclear location of the 2µm plasmid comes from a variety of sources. Micrococcal digestion of 2µm DNA reveals a pattern that indicates that 2µm DNA is condensed with histone proteins into nucleosomal subunits, similar to chromosomal DNA (Nelson and Fangman, 1979). Hybrid plasmids containing 2µm sequences and auxotrophic markers are able to integrate into the genome via homologous recombination (Kielland-Brandt et al., 1980). More recently, the nuclear location of the 2µm plasmid has been directly demonstrated via an in situ hybridisation technique, using a non-radioactive digoxigenin probe specific to 2µm sequences (Albury, 1992).

Cells can be cured of 2µm plasmids due to incompatibility that exists between native 2µm and 2µm-based hybrid vectors. When a yeast/E.coli hybrid plasmid containing 2µm sequences and a selectable marker is introduced into a yeast cell via transformation, there is a significant increase in the number of 2µm-free segregants (Dobson, 1980). It is has also been demonstrated that the stability of 2µm-based vectors is greatly increased in (cir⁺) compared to (cir⁻) hosts (Cashmore, 1984). The exact nature of this plasmid incompatibility is not known, but competition for a limited host factor important for 2µm plasmid maintenance could be an important factor.

1.4.1 2µm-like plasmids from other yeasts

Several yeast strains other than S.cerevisiae harbor plasmids that resemble the 2µm plasmid in their structure and basic maintenance mechanisms. Those identified include plasmids pSR1 and pSR2 identified in Zygosaccharomyces rouxii (Toh-e et al., 1982), pSB1 and pSB2 from Saccharomyces bailii, pSB3 and pSB4 found in Zygosaccharomyces bisporus (Toh-e et al., 1984), pKD1 from Kluyveromyces drosophilae (Chen et al., 1986) and pKW1 from K.walii (Chen et al., 1992). All these plasmids have two unique regions separated by two inverted repeat sequences, and each are also present in two isomeric forms. Each of the plasmids encode three or four open reading frames, an origin of replication and a further apparently cis-acting sequence, each with functions analogous to those found on the 2µm plasmid. Plasmid pSR1 has two ARS sequences, one in each inverted repeat, either of which is sufficient for plasmid replication (Araki and Oshima, 1989).
Figure 1.5

Transcription map of the 2μm plasmid

The locations of the major transcripts are indicated on a map of the A form of the plasmid. The direction of transcription is indicated by the arrows. The length of each transcript is indicated in bases. Data from Broach et al. (1979); Sutton and Broach (1985) and Murray et al. (1987).
Each of the plasmids encodes a functional homologue to the 2μm FLP-recombinase which acts on two inverted repeat sequences specific to that plasmid (Toh-e and Utatsu, 1985; Matsuzaki et al., 1988; Araki et al., 1992; Chen et al., 1986). Although there is a high level of functional homology between the recombination systems of the plasmids, the FLP-recombinases and the IRs that they recognise are very specific (Utatsu et al., 1986). In pKD1, it has also been demonstrated that, unlike the 2μm plasmid, ORFs B and C involved in pKD1 plasmid partitioning are necessary for efficient site-specific recombination (Bianchi, 1992).

Each of these plasmids also encodes for two reading frames which appear to be important for plasmid partitioning. In pKD1, the products of the ORFs B and C have been shown to act with a CSL sequence (cis-acting stability locus) to ensure correct partitioning (Bianchi et al., 1991). More detailed analysis has been carried out with plasmid pSR1. In pSR1 it is the products of ORFs P and S which act independently on the cis-acting sequence Z that ensure correct partition of the plasmid (Jearnpipatkul et al., 1987a; Jearnpipatkul et al., 1987b). Further analysis of the partitioning mechanism of pSR1 has revealed by gel retardation experiments a host factor able to bind at Z (Araki et al., 1993). Moreover, mutants of S. cerevisiae that are able to maintain pSR1 plasmids have been isolated (Irie et al., 1991a). The genes involved in this have been characterised, SMP1, SMP2 and SMP3. Smp1p and Smp2p are thought to be involved in mitochondrial maintenance as well as plasmid partitioning, as both smp1 and smp2 mutants are respiration deficient, and smp1 is actually rho° (Irie et al., 1992). A deletion of SMP3 is lethal and Smp3p has been show to have some homology to yeast protein kinases (Irie et al., 1991b).

Despite all the functional homology between these plasmids, the only sequence homology is found between plasmids pSB4 and pSR1 (Toh-e et al., 1984; Murray et al., 1988) which hybridise to each other. All the plasmid encoded functions have diverged so much that they function only in conjunction with their encoding plasmid, and with the exception of pKD1, only in the host in which they were first identified. (pKD1 was originally identified in Kluyveromyces drosophilarum (Chen et al., 1986) but is stably maintained at a high copy number in K. lactis.). This suggests that the plasmid proteins, cis-acting sequences and their native hosts have all co-evolved together (Murray et al., 1988). The consistency between the plasmids for sequence organisation, when the sequence itself is so diverged, must also indicate the importance for the spatial arrangement of plasmid sequences as well as the functions that they encode.
1.42 Copy number control

The 2μm plasmid is under the same stringent replication controls as the yeast chromosomal DNA and so is limited to one initiation of replication per cell cycle (Zakian et al., 1979). This initiation is at the ori site, and proceeds bidirectionally until the two replication forks meet and replication is terminated. It was observed, however, that when a single copy of the plasmid is introduced into a previously plasmid free nucleus [cir°] by cytoduction (Sigurdson et al., 1981), its copy number eventually rises to the normal copy level of 50-100 copies per cell. The 2μm plasmid must therefore be able to overcome the tight replication-initiation control when its copy number is low. Also, the partitioning mechanism of the 2μm plasmid is unequal, resulting in a variety of plasmid copy numbers in a population of dividing cells. This unequal partitioning has been demonstrated by in situ fluorescence microscopy of mother and daughter buds (Albury, 1992) and also by the use of a 2μm plasmid variant. This 2μm variant was found to be unable to amplify in vivo (Morrissey and Cashmore, 1992). The average copy-number of the plasmid in a population of cells was found to be unaffected, however copy number per cell was found to be variable. This demonstrates that the plasmid partitioning mechanism results in an unequal distribution of plasmid molecules, and that the role of the plasmid amplification mechanism may be to compensate for this.

A model to explain this amplification mechanism was proposed by Futcher, 1986 in which the plasmid can over-ride the strict cellular control of replication, so allowing multiple rounds of replication from a single initiation event (Figure 1.6). Ori is situated in the larger of the two unique regions and extends into one of the inverted repeat sequences (Broach and Hicks, 1980; McNeal et al., 1980). It is proposed that after initiation of replication, when only one of the replication forks has proceeded past the first inverted repeat sequence, FLP-mediated site-specific recombination occurs at the FLP-recombination target sequences (FRTs) so converting the plasmid into its other isomeric form. This flipping now also has the effect of changing the direction of one of the replication forks, with the result that both now travel in the same direction round the plasmid, thus changing the mode of replication from theta to double rolling circle. A subsequent recombination event causes the forks to converge, resulting in termination of replication. This double rolling circle model results in the formation of a multimer which can then be resolved by subsequent site-specific recombination events. The dependence of amplification of plasmid copy number on an intact FLP-FRT system was proved by Volkert and Broach (1986). A 2μm plasmid, mutated in FLP and flanked by FRT sites in direct orientation, was integrated into a chromosome. FLP protein was then provided on an inducible promoter at a different chromosomal location, with the result that the
The \( 2\mu m \) copy number amplification mechanism

(1) Initiation of replication occurs at the origin (O).

(2) Replication proceeds bidirectionally.

(3) After the replication of the inverted repeat nearest to the origin, site-specific recombination can occur between a replicated inverted repeat and the unreplicated inverted repeat.

(4) This causes a change in the direction of the replication fork. This results in the changing of the mode of replication from 'theta' to rolling circle, and the formation of a multimer.

(5) Further site-specific recombination changes the direction of a replication fork again.

(6) Replication is completed when the forks converge.

(7) Subsequent recombination events resolve the multimer.
2μm plasmid was excised from the chromosome as a single copy. If both FRT sites on the 2μm plasmid are functional, the plasmid is able to amplify under continued influence of FLP. If, however, one or both of the FRT sites are mutated, no copy number amplification is observed. It was also observed that this amplification occurred during S-phase, consistent with the single initiation of DNA replication at this time.

A novel form of the 2μm plasmid has recently been identified after isolation of DNA from S.cerevisiae incubated under conditions that enrich for DNA replication intermediates (Petes and Williamson, 1994). This form of the 2μm plasmid consists of two monomeric or dimeric circles joined by a linear double-stranded segment of variable length. This molecule could be formed as a consequence of site-specific recombination within a dimeric DNA molecule during DNA replication, and provides physical evidence for the FLP-mediated recombination process.

The FLP-recombinase is the only gene product required for copy number control via the recombination of the two FRT sites (Reynolds et al., 1987). FLP-mediated recombination occurs readily in vivo (Broach et al., 1982) and in vitro (Vetter et al., 1983; Senecoff et al., 1985). Moreover, the expression of a cloned FLP gene in E.coli results in efficient recombination within the bacterial cell (Cox, 1983). FLP gene expression, though, is under very tight transcriptional regulation. Copies of 2μm genes were integrated into the host genome so that their expression could be regulated. The effect of these gene products on targeted 2μm genes, present in the strains as lacZ fusion constructs, could then be deduced by measuring levels of β-galactosidase. Both FLP and B gene expression were shown to be repressed at least 100-fold by the combined activity of B and C proteins (Som et al., 1988). Previous work has also implicated the product of ORF D in this regulatory process (Murray et al., 1987).

The high stability of the 2μm plasmid is not only due to the ability of the plasmid to amplify when at a low copy number, but is also dependent on the ability of the plasmid to overcome maternal bias and so segregate into daughter cells at cell division. This partitioning of the plasmid appears to be unequal, but is efficient enough to enable the 2μm to be stably inherited. Gene products from ORFs B and C, as well as controlling expression of FLP, are also involved in this latter process of plasmid partitioning (Cashmore et al., 1986), showing that the functions of the 2μm plasmid are closely associated.
1.43 Partitioning mechanism of the 2μm plasmid

The 2μm plasmid requires the trans-acting products of ORFs B and C, as well as the cis-acting locus STB, for its effective partitioning at cell division (Kikuchi, 1983; Murray and Cesareni, 1986; Cashmore et al., 1986). The partitioning mechanism developed by the 2μm plasmid compensates for the maternal bias seen at cell division displayed by ARS plasmids. Plasmids containing an ARS sequence can partition efficiently if the plasmid also contains the STB locus and if this plasmid is present in a [cir+] cell (Murray and Szostak, 1983).

Evidence for the requirement of B and C gene products and the STB locus in the partitioning process has come from a variety of sources. The earliest work was carried out by Kikuchi (1983). It was demonstrated that in a [cir-] strain, a plasmid containing the STB locus and both B and C genes was partitioned correctly at cell division, but a construct lacking one or more of these elements was unstable. It was also found that a plasmid containing the STB element alone was stable provided that the B and C gene products could be provided in trans from a resident 2μm plasmid in the host strain. These results have been confirmed, and it has also been demonstrated that partitioning is dependent on the dose of gene B, but independent of the dose of gene C (Cashmore et al., 1986). Stability of 2μm-based plasmids was shown to increase as the number of B genes integrated into the chromosomes increased. An interaction between proteins B and C has been demonstrated via the 2-hybrid assay, a system developed in yeast to study protein-protein interactions (M. Albury, personal communication). An interaction was also observed between B protein with itself, but not C protein with itself.

Using micrococcal nuclease cleavage assays, it has been shown that mutations in the B gene affects organisation at STB. The same affect is not seen for genes A, C or D (Veit and Fangman, 1985). This suggests that protein B interacts directly with STB. In this model, C protein could be involved by interacting with another domain on the B protein, changing its conformation so that the B/STB interaction is made possible. Interaction between STB and both B and C has been demonstrated biochemically via gel-retardation assays, and also via biosensor experiments (Hadfield et al., 1995).

Unlike the B gene, the C gene product is not required in multiple copies. A 2μm-based plasmid carrying a mutation in the C gene and present at 50-100 copies per cell can be complemented by one chromosomally integrated copy of C, providing that the D gene is intact (Cashmore et al., 1988). This is also further evidence of a role for D in plasmid maintenance.

STB has been well characterised, and has two domains lying either side of a HpaI site. These domains have been designated STB-proximal and STB-distal.
The entire STB locus lies between an *AvaI* site that is proximal to *ori*, and a *PstI* site that is distal to it (Figure 1.7). STB-proximal lies between the *HpaI* and *AvaI* sites, and STB-distal lies between the *HpaI* and *PstI* sites. STB-proximal is characterised by the five-and-a-half 62bp repeats, and STB-distal includes the 3' end of the *D* ORF and a transcription terminator (Sutton and Broach, 1985). Work by Jarayam and colleagues (1985) and supported by Cashmore *et al.*, (1986) suggests that only the proximal, *HpaI*- *AvaI*, STB domain is required for effective partitioning. STB-distal is thought to have some controlling effect needed for the correct functioning of STB-proximal (Murray and Cesareni, 1986). Sequence analysis of the STB region has identified a 26bp element that has some homology with elements both between the *A* and *C* genes and within the *A* gene itself. This sequence in STB-distal acts as a silencer by reducing transcription through the STB region from upstream promoters (Murray and Cesareni, 1986). These authors proposed a model in which the *B* gene product is responsible for this silencing effect and that this protein also has an effect on *FLP* expression. The repression of *FLP* gene expression by *B* gene product has since been demonstrated (Som *et al.*, 1988).

The silencing of transcription through STB, along with the strong transcription termination sequence also in this region, could be necessary to ensure the correct interaction of the *B* and *C* gene products with the STB-proximal repeated elements. A further model for plasmid partitioning has been proposed in which transcription through STB disrupts the B/C protein interaction, possibly preventing attachment of the plasmid to some host nuclear structure via the STB/protein complex.

Evidence for the interactions between STB and B protein has come from sequence analysis of the 2μm and 2μm variant plasmids found in industrial strains of yeast. The 2μm plasmid of common laboratory strains is highly conserved, in contrast, 2μm plasmids isolated from industrial yeast strains exhibit extensive nucleotide diversities (Xiao *et al.*, 1990; Xiao *et al.*, 1991a). These 2μm variants consist of two types, differing in the number of repeats at the STB locus and the consensus sequence of these repeats (Xiao, 1991b). DNA sequencing of these variants at the B gene locus has indicated that there is a molecular co-evolution of the *cis* and *trans* acting elements required for 2μm plasmid partitioning (Xiao *et al.*, 1991b). Significantly, this co-evolution also includes the *D* gene, providing further evidence for the involvement of the *D* protein in the partitioning mechanism (Xiao *et al.*, 1990).
The STB-ORI region of the 2μm plasmid

The STB-ORI region of the B form of the 2μm plasmid is shown. The two domains of the cis-acting loci, STB, are indicated relative to the origin of replication (ORI). The inverted repeat is shown as an open box, whereas open reading frames A and D are shown as arrows.
Figure 1.7
1.44 Co-ordination of 2μm gene expression and plasmid maintenance

Underlying the maintenance of the 2μm plasmid is the tight co-ordination of both the copy number amplification and the partitioning mechanisms of the plasmid. B and C proteins act together to inhibit B gene expression (Som et al., 1988), and also appear to be required for the repression of FLP (Reynolds et al., 1987). This provides a role for B and C in copy number control. An increase in FLP expression elevates copy number but would also increase levels of B and C proteins in the cell. This would then result in repression of FLP expression and the turning off of the amplification process (Reynolds et al., 1987; Murray et al., 1987). The repression of FLP appears to be at the transcription level. FLP mRNA levels drop approximately 100-fold in the presence of high levels of B and C protein (Som et al., 1988), and it has also been demonstrated that both B and C proteins affect chromatin organisation 5' to FLP (Veit and Fangman, 1985).

D protein has also been implicated in the co-ordination of 2μm maintenance, as levels of D protein induce expression of FLP and B genes (Murray et al., 1987). This induction acts antagonistically to the repression of FLP and B carried out by B and C proteins. D has also been implicated in the control of C gene expression (Cashmore et al., 1988). Plasmids lacking the D gene have an increased stability in the presence of elevated C gene dosage. D protein has also been shown to interact with STB (Hadfield et al., 1995). However, loss of D gene activity has only a minor affect on 2μm stability (Cashmore et al., 1988; Veit and Fangman, 1985), suggesting that its transcriptional de-repression and STB-binding activities act to fine tune the other maintenance controls in place (Figure 1.8). There are two other large transcripts detectable from the 2μm plasmid in the large unique region that contains ORFs B and D (Murray et al., 1987). These transcripts may encode for proteins that have not yet been identified, or it may be possible that the transcripts themselves are involved in transcription or translation control.

1.45 Host involvement in 2μm plasmid maintenance

The models for 2μm plasmid maintenance presented suggest a role for host proteins in the partitioning process. It is known that the 2μm plasmid requires host functions for its replication, and there is evidence for some interaction between plasmid molecules and a cellular structure during cell division.

A chromosomal mutation, nib1, has been isolated which confers sensitivity to the presence of the 2μm plasmid (Holm, 1982a; Holm, 1982b). The phenotype of nib1 is a nibbled colony morphology in [cir+] cells. In contrast, [cir-] cells have a smooth,
A simple model for the coordination of the 2μm-encoded gene products to ensure plasmid maintenance

The figure depicts the various roles of each of the 2μm-encoded gene products. *FLP* encodes a site-specific recombinase which acts at the FLP recombination target site (*FRT*), indicated by the box. The B and C proteins are thought to act in concert to mediate plasmid partitioning via an interaction with the *STB* loci. They also negatively regulate the expression of the *B*, *FLP* and *D* genes. The B and C repression of *FLP* is antagonised by the *D* gene product.
The reason for the nibbled phenotype is the presence of a subpopulation of abnormally large sized cells found at the perimeter of the colonies. These large cells are formed spontaneously from the smaller ones and fail to divide, producing buds the same size as the mother cell which can’t easily be removed, even by micromanipulation. The large cells also contain the 2μm plasmid at an elevated copy number (Holm, 1982b). A high proportion of nibl cells, when grown in liquid media, also have an abnormal morphology. Cells became elongated and DAPI staining revealed that these elongated buds often lacked nuclei though spindles were intact (J. Murray, personal communication). The nibl gene was originally observed by mating [cir°] strains of S.carlsbergensis with S.cerevisiae [cir+] karl-1 mutants. Strains with the karl-1 mutation are able to mate normally, but are deficient in nuclear fusion. This results in the formation of unstable heterokaryons. The main progeny of such heterokaryons have the nuclear genotype of one of the parental strains (Conde and Fink, 1976) however, there is a low frequency of genetic transfer of chromosomes as well as plasmids between nuclei in the heterokaryon (Dutcher, 1981; Livingston, 1977). In the cross described above, some progeny expressing the S.carlsbergensis genotype received the 2μm plasmid and displayed the nibbled phenotype. This sensitivity to the 2μm plasmid was shown to segregate as a single recessive allele that mapped to chromosome XVI (Holm, 1982b). It was proposed that the nibl gene plays a role in the control of plasmid copy number, possibly by preventing plasmid amplification in some way. Sweeney and Zakian (1989) have since demonstrated the requirement of ORFs A and D for the expression of the nibl phenotype, giving weight to this hypothesis. However, this report also suggests that the nibl mutation confers a general sensitivity to extrachromosomal elements. The NIBl gene has yet to be successfully cloned and characterised, and its exact role in 2μm maintenance isn’t yet fully understood. This sensitivity of extrachromosomal elements conferred by nibl is perhaps significant in the light of analysis of smp1 and smp2 mutations in S. cerevisiae. These mutations enable this host to efficiently maintain the 2μm-like plasmid pSR1 from Zygosaccharomyces rouxii. smp1 mutants are rho° and smp2 mutants are respiration deficient, suggesting a function for SMP1 and SMP2 in mitochondrial as well as plasmid maintenance (Irie et al., 1991a; Irie et al., 1992).

A second host mutant defective in 2μm plasmid maintenance has been isolated. A spontaneous [cir°] isolate was identified during continuous batch culture of the strain MC16 (Cashmore, 1984). This is the plm1 mutation (plasmid maintenance), and host strains carrying this allele cannot be successfully transformed with whole 2μm or 2μm-based plasmids, though they are transformable with ARS, CEN and YIp plasmids. The only way to introduce the 2μm into the plm1 mutant strain is by mating with a 2μm-containing strain. After meiosis, the spores show a
segregation of 2:2 transformability against untransformability. The plm1 spores that receive the 2μm plasmid have a wildtype copy number, though the plasmid is then rapidly lost once the spores germinate and during successive generations. This demonstrates that the plm1 mutation is partitioning specific, rather than being involved in replication or amplification of the plasmid. It is proposed that the wildtype PLM1 allele is a karyoskeletal protein, possibly part of the nuclear scaffold to which the 2μm may bind during part of the cell cycle. Other candidate proteins include the lamins, which have been demonstrated to interact directly with chromatin (Nigg, 1992; Bridger et al., 1993). One lamin in particular, lamin B1, has been shown to bind directly to DNA sequences (MARS) that are normally found in association with components of the nuclear matrix (Luderus et al., 1992). By raising antibodies against a B-β-galactosidase fusion protein it has been found that B protein co-purifies with a karyoskeletal subfraction directly equivalent to the nuclear matrix lamina fraction of higher cells (Wu et al., 1987). One possibility is that protein B may promote plasmid partitioning by interacting with the nuclear lamina of the host cell to provide dispersed anchorage sites for attachment of plasmid molecules. Antibody staining experiments using antibodies directed against the nuclear pore complex demonstrate that, though the nuclear envelope is dynamic and flexible during cell division, the nuclear pore complexes remain evenly distributed around the nucleus (Copeland and Snyder, 1993).

Recent biochemical evidence also supports the presence of a host factor necessary for 2μm plasmid partitioning. STB binding studies have demonstrated a direct interaction of the STB proximal repeat sequences with an insoluble yeast protein fraction obtained from [circ] cells (Hadfield et al., 1995). The presence of a host binding factor in this particular fraction also supports the model of 2μm binding in some way to proteins of the host karyoskeleton. Evidence for a direct interaction between the host and the plasmid has also come from the study of 2μm-like plasmids. Analysis of the pSR1 plasmid of Zygosacharomyces rouxii by gel retardation experiments has revealed that there is a host factor able to bind at the Z locus, the cis-acting sequence involved in the partitioning of this plasmid (Araki et al., 1993).

The protein binding experiments carried out by Hadfield et al. (1995) have suggested that plasmid protein and STB interactions with the host may not only mediate partitioning, but may play an important role in its regulation. It is proposed that the STB-binding host factor is a subnuclear structure, and when bound, the interaction physically blocks replication and FLP-mediated amplification of the plasmid. However, the presence of plasmid molecules in the nucleus in an unbound state, and able to replicate, implies that the association and dissociation of the plasmid from the host factor is a key regulation mechanism. By regulating sub-
nuclear structural association at STB, possibly via the B/C protein interaction, plasmid molecules may be attached for active partitioning and dissociate for copy number amplification. A passive model for plasmid partitioning however suggests that the STB-host interaction occurs during DNA replication. The ARS of the 2μm is associated with the replication machinery at this time, and these protein-DNA interactions may also involve the STB sequence (Amati and Gasser, 1988). In this model, the interaction of B protein with STB would act to lever the plasmid off the replication machinery and so allow for random diffusion of plasmid molecules. This would result in their passive diffusion into daughter cells at cell division (Figure 1.9).

B protein, at levels equivalent to that present in [cir+] cells, appears to act to block host factor binding to STB. However, this blockage is removed by high levels of C protein. This control of host binding by intercellular concentrations of C protein may be a dosage-dependent effect. C gene expression isn’t as high as B gene, indeed one chromosomally integrated copy of C is enough to complement a plasmid-borne mutation (Cashmore et al., 1988). Therefore, high levels of C protein may only be present when plasmid copy-number is sufficiently increased. When high plasmid numbers are achieved, B and C proteins may then act co-operatively to mediate host binding at STB and subsequent plasmid partitioning.

FLP protein was demonstrated to enhance host factor dissociation from STB. This would presumably have a role once cell division has occurred and levels of B protein are reduced resulting in de-repression of FLP. This would then allow for the release of plasmid molecules and their replication and copy number amplification. D protein binding at STB was also demonstrated to reverse host binding. Expression of D, like FLP, is also induced at low levels of B protein.

By what appears to be a complex series of positive and negative control mechanisms co-ordinating the expression of ORFs involved in all aspects of 2μm maintenance, the model outlined above suggests that the plasmid is able to monitor its copy number and inhibit attachment to the host for partitioning until the correct copy number is achieved. The elucidation of the exact processes involved in the maintenance of the 2μm plasmid, with the identification of host functions involved in this, may provide us with a handle for studying the interactions between nuclear structures and DNA during the eukaryotic cell cycle.
Models for the partitioning mechanism of the 2µm plasmid

(a) Passive

Plasmid molecules are attached to the host replication machinery via ORI during DNA replication. Plasmid-encoded proteins B and C form a complex which then binds at STB, effectively levering the plasmid molecule away from the host attachment, resulting in the random diffusion of plasmid molecules at cell division.

(b) Active

This model demonstrates the possibility that, rather than random diffusion of plasmid molecules as in the previous model, the 2µm plasmid is attached to a nuclear structure via an interaction between proteins B and C, the STB locus and the host. This host factor could provide dispersed anchorage sites for the attachment of plasmid molecules at cell division.
Figure 1.9

(a)

(b)
1.5 Aims of the Project

Both host and plasmid-encoded functions are involved in the stable maintenance of the 2μm plasmid during haploid vegetative growth. The 2μm plasmid encodes functions for its copy number amplification and partitioning mechanisms which together help it to overcome maternal bias experienced by ARS plasmids. The involvement of the host during replication of the plasmid is well characterised. The 2μm plasmid utilises the host replication machinery during S-phase, and is also under the same replication control as the chromosomes in that replication is initiated from an ARS sequence only once per cell cycle (Livingston and Kupfer, 1977; Zakian et al., 1979). The ARS of the 2μm has been extensively studied as a model yeast replicator sequence. This has been vital to the elucidation of the sequences important within the ARS as well as to identify the proteins involved in DNA replication and its initiation (Broach et al., 1982; Rowley et al., 1995). The MCM family of proteins that have a central role in the control of the initiation of DNA replication, were isolated specifically for their inability to maintain minichromosomes with a variety of ARS sequences, including that from the 2μm (Maine et al., 1984; Maiti and Sinha, 1992).

Less well understood is the role of the host during plasmid partitioning. There is strong evidence to suggest that the host plays a direct part in this mechanism, from biochemical studies (Hadfield et al., 1995) and the isolation of a mutant specifically defective in 2μm plasmid partitioning rather than replication (Cashmore, 1984). The precise mechanism for 2μm plasmid partitioning is unknown, even to the extent of whether it is an active mechanism, with plasmid molecules remaining attached to a host nuclear structure during mitosis, or a passive mechanism, with plasmid molecules being released from a host structure to enable diffusion into a daughter cell. The identification of host proteins involved in this event, and the elucidation of their interactions with 2μm plasmid molecules or the proteins encoded by them, will greatly advance our knowledge of this process.

The identification and analysis of host proteins involved in 2μm plasmid maintenance will further our understanding of the proteins involved in general DNA interactions during the cell-cycle. The 2μm plasmid is also the basis for many yeast vectors used in molecular biology and industry, so to understand its mode of inheritance more completely will be important to create more stable, high copy number plasmids for a variety of applications.
The aim of the work described in this Thesis was to investigate the mechanisms taking place during the partitioning of the yeast 2μm plasmid, and more specifically to identify the host proteins and their interactions involved in this process. This was to be addressed in the following way:

1. The isolation of temperature-sensitive host mutants defective in 2μm plasmid maintenance. Genetic analysis will then determine whether the mutations are dominant or recessive. This is followed by the assigning of the mutants into complementation groups to determine how many different genes are represented.

2. A phenotypic analysis will determine the specificity of the mutation analysed, both in terms of general growth and viability of the mutant, and the affect of the mutation with respect to the maintenance of other plasmids.

3. Complementation of one mutant allele with a genomic library will identify a host gene involved in plasmid maintenance. Detailed sequence analysis may suggest a function for the protein. The creation of a disrupted allele will then provide a defined allele for further analysis.
Chapter Two

MATERIALS AND METHODS

2.1 Standard buffers, solutions and materials

0.5M EDTA
0.5M ethylenediaminetetraacetate disodium salt
The pH was adjusted with sodium hydroxide.

1M Tris-HCl
1M Tris base
pH was adjusted with concentrated hydrochloric acid.

TE Buffer (TE)
10mM Tris-HCl pH8.0
1mM EDTA pH8.0

50 x Denhardt's Solution
1% Ficoll
1% Polyvinylpyrrolidone
1% BSA (Pentax Fraction V)
Made up in distilled water and stored in aliquots at -20°C.

20 x Standard Saline Citrate (20 x SSC)
3M NaCl
0.3M Sodium citrate

Tris-Equilibrated Phenol
Water saturated phenol containing 0.1% 8-hydroxyquinolene was
equilibrated with an equal volume of 1M Tris-HCl pH8.0 then twice
with an equal volume of 0.1M Tris-HCl pH8.0. The phenol was
finally equilibrated with TE buffer and stored at 4°C.

Salmon Sperm DNA
10mg/ml salmon sperm DNA (TypeIII sodium salt) was made up in
distilled water and sheared by sucking through an 18 gauge needle
at least 20 times. The solution was boiled for 10 minutes and stored
at -20°C.

RNase
10mg/ml pancreatic RNase (RNase A) was made up in 10mM Tris-
HCl pH7.5; 15mM NaCl. The solution was boiled for 15 minutes to
inactivate any contaminating DNase activity and stored at -20°C.
2.2 Growth conditions and maintenance of S. *cerevisiae*

The *Saccharomyces cerevisiae* strains used in this study are shown in Table 2.1

2.2.1 Media

**YPED**: (1% yeast extract, 2% Bactopeptone and either 2% glucose, 2% glycerol or 2% galactose).

**Synthetic medium (SD)**: (0.6% Difco yeast nitrogen base, 2% glucose with added amino acid supplements).

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Stock concentration</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>uracil</td>
<td>2 mg/ml</td>
<td>0.02 mg/ml</td>
</tr>
<tr>
<td>L-histidine</td>
<td>8 mg/ml</td>
<td>0.02 mg/ml</td>
</tr>
<tr>
<td>adenine sulphate</td>
<td>2 mg/ml</td>
<td>0.02 mg/ml</td>
</tr>
<tr>
<td>L-leucine</td>
<td>12 mg/ml</td>
<td>0.04 mg/ml</td>
</tr>
<tr>
<td>L-tryptophan</td>
<td>8 mg/ml</td>
<td>0.02 mg/ml</td>
</tr>
</tbody>
</table>

**Presporulation medium**: 0.8% Bacto-yeast extract  
0.3% Bactopeptone  
10% glucose

**Sporulation medium**: 1% Potassium acetate  
0.1% Bacto-yeast extract  
0.05% glucose

**Culture storage media**: 1% Bacto-yeast extract  
1% Bacto-peptone  
2% glucose  
25% (v/v) glycerol

**Glucose**: A stock solution of 40% glucose was autoclaved at 10 p.s.i. for 15 minutes.

For solid media, Bacto agar was added at a concentration of 2% prior to autoclaving.
Table 2.1 *Saccharomyces cerevisiae* strains

<table>
<thead>
<tr>
<th>STRAIN</th>
<th>GENOTYPE</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>S150-2B</td>
<td>MATa, leu 2-3, 112, his 3-D, trp 1-289, ura 3-52</td>
<td>McLeod et al., 1984</td>
</tr>
<tr>
<td>MC16</td>
<td>MATa, leu 2-3, his 4-712fs, ade 2-1, lys 2-1, SUF 2</td>
<td>Beggs, 1978</td>
</tr>
<tr>
<td>8HB</td>
<td>MATa, leu2-3, 112, ade2-1, ura3-52, trp1-289</td>
<td>M. Pocklington</td>
</tr>
<tr>
<td>C1</td>
<td>MATa, trp1, lys1</td>
<td>Cold Spring Harbor Laboratories</td>
</tr>
<tr>
<td>C2</td>
<td>MATa, leu2-3, lys1</td>
<td>Cold Spring Harbor Laboratories</td>
</tr>
<tr>
<td>842</td>
<td>MATa/MATa, leu2/leu2, ade1/ade1, his3/his3, ura3/ura3, trp1-1/trp1-1</td>
<td>K. Nasmyth</td>
</tr>
</tbody>
</table>
2.22 Sterilisation

All media without a carbon source was autoclaved at 15 p.s.i. for 15 minutes. Media containing a carbon source and supplements for SD media were autoclaved at 10 p.s.i. for 15 minutes.

2.23 Growth Conditions

All wild-type *S.cerevisiae* strains were grown at 30°C. When necessary, temperature-sensitive mutant strains were grown at the permissive temperature of 24°C, or the non-permissive temperature of 35°C. Liquid cultures were grown with continuous shaking.

Matings were carried out between haploid strains of the opposite mating-type on YEPD plates, cells were then replica-plated onto SD medium selective for vegetatively growing diploid cells.

Presporulation was carried out by patching diploid cells onto presporulation plates and incubating at 30°C for 2-3 days.

Sporulation was carried out by patching cells from presporulation plates onto solid sporulation medium. After incubation for 3-5 days at 30°C, spore development was checked microscopically.

2.24 Determination of Cell Numbers

The cell density of a liquid culture was measured by counting the number of cells in a known volume using a haemocytometer with modified Fuch's Rosenthal ruling. A 25μl sample of the liquid cultures, diluted if necessary, was placed over the grid and covered with a counting chamber cover glass. The density of the culture was calculated from the average of 10 readings.

2.25 Preservation of *S.cerevisiae* Strains

All strains were viable for 6-8 weeks when kept on YEPD or SD agar plates at 4°C. Strains remained viable for several years when stored on YEPD slants at 4°C. For long term preservation of strains a fresh colony was picked into 1.5 ml of CM medium and shaken thoroughly before freezing in a dry ice/ IMS bath, then stored at -80°C.

2.26 Genetic analysis

Tetrad dissections

Tetrad dissections were carried out using the Singer MSM system. A loop full of sporulated cells were resuspended in 150μl of sterile distilled water. 5μl of β-glucuronidase (Sigma) was added and the cell suspension incubated at room
temperature for 5 minutes. A loop full of the suspension was then streaked across one side of a YEPD plate, as outlined in the instructions for the Singer MSM system. Up to twenty dissections can be carried out on one plate. Once finished, plates were incubated at 30°C until growth of the haploid spores was observed. The plates were poured on an adjustable stand, made flat by the use of a spirit level.

2.3 Growth conditions and maintenance of *E.coli*

The strains of *Escherichia coli* used in this study are shown in Table 2.2

2.3.1 Media

**Luria-Bertani Medium (LB):**
- 1% Bacto-tryptone
- 0.5% Bacto-yeast extract
- 0.5% Sodium chloride

pH was adjusted to 7.2 with sodium hydroxide.

**Antibiotic Media:**

**L-Ampicillin:** (LB medium containing ampicillin at a final concentration of 0.1mg/ml). A 10mg/ml stock solution of ampicillin was made up in 50% ethanol and stored at 4°C.

**L-Methicillin:** (LB medium containing methicillin at a final concentration of 0.1mg/ml). A 10mg/ml stock solution of methicillin was made up in 50% ethanol and stored at 4°C.

**L-Tetracycline:** (LB medium containing tetracycline at a final concentration of 75µg/ml). A 7.5mg/ml stock solution of tetracycline was made up in 50% ethanol and stored at 4°C.

**L-Streptomycin:** (LB medium containing streptomycin at a final concentration of 0.2mg/ml). A 200mg/ml stock solution of streptomycin was made up in distilled water and stored at 4°C.

For solid medium Bacto agar was added to 2% (w/v).

2.3.2 Growth Conditions

All *E.coli* strains were grown at 37°C. Liquid cultures were grown with continuous shaking.
<table>
<thead>
<tr>
<th>STRAIN</th>
<th>GENOTYPE</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>HB101</td>
<td>F(^{-}), recA13, hsdS20 (rB(^{-}), mB(^{-})), ara-14, proA2, lacY1, galK2, xyl-5, mtl-1, supE44, rpsL20 (Sm(^{R}))</td>
<td>Boyer and Roulland-Doussiox, 1969</td>
</tr>
<tr>
<td>DH5(\alpha)</td>
<td>recA1, endA1, hsdR17(rB(^{-}), mB(^{-})), thi-1, gyrA96, relA1, supE44</td>
<td>Hanahan, 1983</td>
</tr>
<tr>
<td>DH1</td>
<td>ecA1, endA1, hsdR17(rB(^{-}), mB(^{-})), thi-1, gyrA96, relA1, supE44</td>
<td>Hanahan, 1983</td>
</tr>
<tr>
<td>MH1578</td>
<td>As DH1 plus rpsL20 (Sm(^{R}))</td>
<td>Sedgewick and Morgan, 1994</td>
</tr>
<tr>
<td>MH1598</td>
<td>As DH1 plus R388::TnHIS3</td>
<td>Sedgewick and Morgan, 1994</td>
</tr>
</tbody>
</table>
2.33 Preservation of *E.coli* Strains

All strains were viable for several weeks when stored at 4°C on LB agar plates containing the appropriate antibiotic if necessary. For long term storage, 1ml of a fresh culture was mixed with an equal volume of 40% sterile glycerol and frozen in a dry ice/IMS bath before storing at -80°C.

2.34 Determination of *E.coli* Cell Numbers

The growth of a liquid culture was monitored by withdrawing an aliquot and measuring the optical density (OD) at a wavelength of 600 nm. An OD₆₀₀ of 1.0 is approximately equal to a cell density of 6 x 10⁸ cells/ml.

2.4 Extraction of DNA

2.41 Preparation of Total Yeast DNA

An exponentially growing culture was pelleted and washed in 0.5 ml of 1.2μm sorbitol, 50mM potassium phosphate buffer pH 7.5, 50mM dithiothreitol (DTT). The pellet was suspended in 0.5ml of the same buffer but with the addition of 50 μg/ml zymolyase and incubated at 37°C, until the formation of spheroplasts was complete. 0.5ml of 100mM NaCl, 50mM Tris HCl pH7.4, 100mM EDTA, 1% SDS and 10μg/ml protease K were added and incubated at 65°C for 20 minutes. The lysates were extracted with an equal volume of phenol/chloroform and precipitated with 1/4 volume of 10.5M ammonium acetate and 2 volumes of ethanol. The pellet was resuspended in 200μl of sterile water and 5μl of RNase then incubated at 37°C for 30 minutes. The DNA was recovered with a further treatment of 1/4 volume of 10.5M ammonium acetate and 2 volumes of ethanol. The resulting pellet was resuspended in 50μl of sterile distilled water and stored at -20°C.

2.42 Small-scale yeast DNA Preparations

The following method, essentially that of Hoffman and Winston (1987), was used for the preparation of yeast genomic DNA enriched for plasmids for transformation into *E.coli*.

1ml of an overnight culture grown in YEPD was spun in a microfuge and resuspended in 200μl of lysis solution (100mM NaCl; 10mM Tris-HCl pH8.0; 1mM EDTA; 0.1% SDS). Sterile glass beads (Sigma, acid washed 0.45 mm) were then added to just below the level of the liquid. Samples were then vortexed for 2 mins. 200μl of phenol/chloroform was added followed by a further 2 min vortex. Samples were then microfuged for 2 mins ad the aqueous layer transferred to a clean eppendorf tube. A further 200μl of phenol/chloroform was added, the sample
briefly vortexed and microfuged for a further 2 mins. The aqueous layer was again transferred to a clean eppendorf and 1/10th volume 3M sodium acetate, 2xvolumes ethanol added. The DNA was precipitated for 10 mins on ice. The samples were microfuged for 10 mins, the pellet washed with 70% ethanol and resuspended in 50μl TE. 2.5μl of the DNA solution was then used in each subsequent *E.coli* transformation. Approximately 1-5x10⁵ transformants per μg of DNA were obtained for each transformation using this method, though the efficiency depended on the size of the plasmid to be rescued. This method was less efficient for plasmids of over 15kb in size.

2.43 Plasmid DNA Extraction

Small-scale plasmid DNA preparations

Minipreps were performed from *E.coli* by alkaline lysis based on the method of Birnboim and Doly (1979).

5ml overnight cultures were spun down in a microfuge and the pellet resuspended in 100μl of 50mM glucose; 25mM Tris HCl pH8 and 10mM EDTA. 200μl of 0.2M NaOH and 1% SDS were then added and the cell suspension left on ice for 5 minutes. 150μl of potassium acetate was added and the solution was left for a further 10 minutes to allow a precipitate to form. After spinning for 5mins in a microfuge, the clear phase was transferred to an eppendorf containing an equal volume of phenol/chloroform. The mixture was vortexed and then microfuged and the upper phase was transferred to a clean eppendorf and an equal volume of isopropanol added. The tube was incubated on ice for 10 mins and then spun in a microfuge for 10 mins. The pellet was washed in 70% ethanol, air dried and resuspended in 30μl TE/RNase.

Large-scale plasmid DNA preparations

To obtain a large amount of pure plasmid DNA, Qiagen midi columns were used as outlined in the manufacturers instructions. From a 100ml overnight culture, approximately 50-100μg of plasmid DNA was regularly obtained.

2.5 Analysis of DNA

2.51 Restriction Analysis

All restriction endonuclease digestions were carried out at 37°C using enzymes and their recommended React® buffers from Bethesda Research Laboratories Ltd. The React® buffers were supplied as 10 x concentrates. TAB
universal buffer (O'Farrell et al., 1980) was used for digestions involving two or more enzymes requiring different buffers.

Plasmid DNA was digested for 1 to 2 hours with 1 to 2 units of enzyme per reaction. Yeast genomic DNA digestions were carried out in volumes of 20μl for 3 hours with 15 to 20 units of enzyme. This was followed by further digestion with an additional 10 units for 2 hours. The reaction volume was increased to 30μl with restriction buffer and distilled water.

2.52 Agarose Gel Electrophoresis

DNA was identified and fragments were purified from gels made up and electrophoresed in 1 x Tris-acetate electrophoresis buffer (TAE) containing 0.5μg/ml ethidium bromide.

\[
\text{50 x TAE} : \\
2 \text{M Tris-base} \\
1 \text{M Sodium acetate (trihydrate)} \\
0.5 \text{M EDTA} \\
pH \text{ was adjusted to 8.2 with glacial acetic acid.}
\]

The percentage of agarose used depended on the size of the DNA being analysed. The type of agarose in the gel depended on whether the DNA was used for further analysis. For most purposes, such as Southern analysis and restriction mapping, a 0.7-1.2% (w/v) Seakem HGT agarose gel was used. For probe preparation a 0.6-0.8% (w/v) Seakem LMT agarose gel was used.

DNA sample sizes of between 10 and 30μl were run after adding 0.1 volumes of 10 x loading buffer. Gels were run overnight 15-25 Volts or for 2 to 3 hours at 70-90 Volts.

\[
\text{10 x Loading Buffer} : \\
0.4\% \text{ Bromophenol Blue} \\
0.4\% \text{ Xylene Cyanol} \\
25\% \text{ Ficoll}
\]

2.53 Recovery of DNA Fractionated on Agarose Gels

The amount of DNA digested was calculated so that there was between 250 and 500ng present in the band of interest.
Preparation of DNA fragments for use as probes

The DNA, after digestion with appropriate restriction enzymes, was electrophoresed in a low melting temperature agarose gel. The DNA fragment of interest was recovered after visualization using UV light. The band was cut out with a scalpel and placed into an Eppendorf tube together with distilled water to produce a final DNA concentration of approximately 1.5μg/ml. The tube was placed in a boiling water bath for 5 minutes to melt the agarose. The DNA was aliquoted and stored at -20°C.

Isolation of DNA for ligation reactions

The digest reaction was loaded into a high gelling temperature agarose gel and electrophoresed until adequate separation of the restriction fragments was achieved. The isolation of the band of interest was carried out by electroelution of the DNA onto a dialysis membrane ("Deathwishing"). The membrane was then rinsed in 2x40μl volumes of distilled water and the DNA precipitated in 1/4 volume 10.5M ammonium acetate, 2 x volume 100% ethanol and washed in 80% ethanol. The air-dried DNA was then resuspended in 10μl distilled water.

2.5.4 Ligations

The vector into which the fragment of interest was to be cloned was restricted with the appropriate restriction enzyme. Included in the restriction digestion was 0.2units of shrimp alkaline phosphatase. After 1-2hours the reaction was phenol extracted and precipitated as outlined previously. The molar amounts of vector and insert purified was calculated and ligations set up with differing ratios of the two, for example 1:1 (vector : insert) and 1:3 or 1:6. The reactions were carried out in ligase buffer (0.1M Tris pH7.6; 0.1M MgCl₂; 0.1M DTT; 20mM spermidine; 10mM ATP; 5mg/ml BSA) and 0.5 - 1.0 units of T4 ligase and incubated overnight at 15°C.

2.5.5 Polymerase chain reaction (PCR)

Primers

Primers were synthesised by D. Langton (protein and nucleic acid laboratory, University of Leicester) and provided in a 35% ammonia solution. 200μl was aliquoted into a fresh eppendorf tube and to it was added 1/10th volume 2M sodium acetate, 4xvolume ethanol. The oligonucleotides were precipitated at room temperature for 10 mins and then microfuged for 15 minutes. The pellet was washed in 80% ethanol and resuspended in 200μl of distilled water. Concentrations of primers were determined by absorbance at OD₂₆₀. Primers were diluted to a concentration of 50ng/μl for standard PCR applications, and 3.2pmol for automated sequencing reactions. Approximate annealing temperatures (Tₘ) for the primers
Table 2.3 Oligonucleotides used in this study

<table>
<thead>
<tr>
<th>NAME</th>
<th>SEQUENCE</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>TetA</td>
<td>5' CGATCATGGCGCCCACA 3'</td>
<td>This Study</td>
</tr>
<tr>
<td>TetB</td>
<td>5' ACGATGCGTCCCGGTA 3'</td>
<td>This Study</td>
</tr>
<tr>
<td>δ</td>
<td>5' AGGGGAACTGAGAGCTCTA 3'</td>
<td>Sedgewick and Morgan, 1994</td>
</tr>
<tr>
<td>γ</td>
<td>5' TCAATAAGTTATACCAT 3'</td>
<td>Sedgewick and Morgan, 1994</td>
</tr>
</tbody>
</table>
were calculated using the equation of 4°C for every purine plus 2°C for every pyrimidine. Primers used in this study are in Table 2.3.

**Standard PCR**

The standard PCR protocol was based on that of Saiki et al., 1985. PCR was carried out on DNA prepared from both minipreparations and Qiagen preparations. Reaction conditions and cycles varied according to the template DNA, primers used and the subsequent use of the PCR products. All reactions were carried out using 50-100ng of DNA in a standard PCR buffer (50mM KCl; 10mM Tris-HCl pH8.4; 1.5mM MgCl₂) and 2.5 units of Taq polymerase.

**2.5.6 Sequencing**

Sequencing was carried out using the Perkin Elmer PRISM™ Ready Reaction DyeDeoxy™ Terminator cycle sequencing kit as per instructions. 0.2-1μg of template DNA and 0.8pmol of appropriate primer were added to the terminator premix provided with the kit. The final reaction volume was made up to 20μl with distilled water and overlayed with mineral oil. The cycling reaction consisted of 25 cycles of 96°C for 30 seconds denaturing; 50°C for 15 seconds annealing; 60°C for 4 minutes elongation. The reaction mix was then taken from under the oil and transferred to a clean eppendorf tube, to which 35μl of 95% ethanol was added. The reaction products were then allowed to precipitate for 10 minutes on ice and were subsequently microfuged for 30 minutes. The ethanol was removed and the pellet allowed to air-dry. The pellet was then stored at -20°C until loaded onto an ABI sequencing gel. The results from the sequencing was analysed using the Sequence Navigator (v1.0; Applied Biosystems) programme for Apple Macintosh.

**2.6 Southern transfer and hybridization of DNA**

**2.6.1 Southern Transfer**

The method used to transfer DNA to a nylon membrane was essentially that of Southern (1975) with a depurination step before alkaline denaturation to facilitate the transfer of large DNA fragments (Wahl et al., 1979).

After visualisation the gel was washed briefly in distilled water. The gel was washed twice for 10 minutes in depurinating solution, then twice for 15 minutes in denaturing solution and finally twice for 15 minutes in neutralizing solution. The gel was washed briefly in distilled water in between the different solutions. The gel was mounted on a glass sheet, previously covered with Whatman® 3MM to act as a wick over a reservoir of 20 x SSC. The edges of the gel were covered with plastic film to...
prevent "short circuiting". The Hybond Nfp membrane, wetted in 2 x SSC, was placed on the gel and covered with 5 sheets of 'Quick draw' blotting paper and all kept in place with a weight. After overnight elution of DNA, the DNA was crosslinked onto the membrane by UV light with a dose of 70Jm. For elution of the DNA in one hour, 10 sheets of 'Quick draw' blotting paper were used.

**Depurinating Solution:** 0.25M HCl

**Denaturing Solution:** 0.5M NaOH
1M NaCl

**Neutralising Solution:** 0.5M Tris-HCl pH7.4
3M NaCl

'Quick draw' blotting paper was obtained from Sigma Chemical Company Ltd.

### 2.62 Preparation of hybridization probes using random primers

Radioactive DNA probes for filter hybridization were prepared using the random oligo-priming method of Feinberg and Vogelstein (1983). The labelled nucleotide was incorporated into the DNA in the presence of the other three unlabelled nucleotides.

The DNA fragment was re-boiled for 3 minutes and stored at 37°C before use. The labelling reaction was carried out at room temperature by the addition of the following reagents in the stated order.

<table>
<thead>
<tr>
<th>Volume</th>
<th>Reagent</th>
</tr>
</thead>
<tbody>
<tr>
<td>5μl</td>
<td>OLB C Buffer (5 x concentration)</td>
</tr>
<tr>
<td>1μl</td>
<td>10mg/ml BSA (Enzyme grade)</td>
</tr>
<tr>
<td>Up to 16.5μl</td>
<td>DNA Fragment (25ng)</td>
</tr>
<tr>
<td>0.5μl</td>
<td>Klenow Polymerase I (2 units)</td>
</tr>
<tr>
<td>2.5μl</td>
<td>32P a-dCTP (10μCi/ml)</td>
</tr>
</tbody>
</table>

The reaction was either incubated at room temperature for 5 hours or overnight or incubated at 37°C for 30 minutes. The reaction was stopped by adding 2μl of 2mM EDTA pH8.0. Unincorporated trinucleotides were removed by precipitation of the probe in the presence of high molecular weight herring DNA.

**Oligo-labelling Buffer C (OLB C)**

Solutions A, B and C were mixed in the ratio 10 : 25 : 15 and dispensed into 5μl aliquots which were stored at -20°C.
Solution A
- 1000µl 1.25M Tris-HCl; 0.125M MgCl₂ at pH 8.0 (Stored at 4°C)
- 18µl 2-mercaptoethanol
- 5µl each of 0.1M dATP, dTTP, dGTP in 3mM Tris-HCl; 0.2mM EDTA at pH 7.0 (Stored at -20°C)

Solution B
- 2M HEPES titrated to pH 6.6 with 4M NaOH (Stored at 4°C).

Solution C
- Hexadeoxynucleotides. 50 OD units were resuspended in 550µl of TE to give a final concentration of 90 OD units/ml (Stored at -20°C).

2.63 Filter Hybridization
The nylon membrane was soaked in 6 x SSC for 2 minutes then transferred to prehybridization solution and incubated at 65°C for 1 to 2 hours. The prehybridization solution was replaced with hybridization solution and the membrane was incubated at 65°C for 12 to 16 hours. After hybridization the filter was washed in 2 x SSC; 0.5% SDS at room temperature for 5 minutes and then washed in 2 x SSC; 0.1% SDS at room temperature for 15 minutes with occasional agitation. The membrane was washed 3 or 4 times in 0.1 x SSC; 0.5% SDS at 65°C over a period of 2 - 3 hours. The membrane was dried briefly before detecting the signal. Temperatures used for the hybridisation and washing steps was altered according to the stringency of the hybridisation. The higher the temperature, the more stringent the reaction. Salt to detergent ratios in the washes were also altered for the same reasons. Increased salt in the washes reduces the stringency of the hybridisation.

Prehybridization Solution:
- 6 x SSC
- 0.5% SDS
- 5 x Denhardt's Solution
- 100mg/ml denatured salmon sperm DNA

Hybridization Solution:
- 6 x SSC
- 0.5% SDS
- 5 x Denhardt's Solution
- 100mg/ml denatured salmon sperm DNA
2.64 Detection of $^{32}$P-labelled DNA Probes

The membrane, mounted on 3MM paper and wrapped in polythene film, was applied to X-ray film with intensifying screens and exposed at -80°C. If necessary, the filter was marked with radioactive ink in order to align it with the autoradiograph.

2.7 Transformation of \textit{S.cerevisiae} strains

The procedure used was based on the method of Gietz \textit{et al.} (1992). An overnight culture grown in liquid YEPD media to a concentration of 1-2 x $10^7$ cells/ml was diluted to 2 x $10^6$ cells/ml in fresh, warm YEPD and regrown to 1 x $10^7$ cells/ml. The cells were washed twice in sterile water, resuspended in 1.0ml of sterile water and transferred to 1.5ml Eppendorf tubes. The cells were then washed in 1.0ml 1xTE/LiAc (made fresh from 10xTE and 1M LiAc stocks: 10mM Tris-HCl pH7.5; 1mM EDTA; 0.1M LiAc adjusted to pH7.5 with dilute acetic acid) and resuspended at a concentration of 2 x $10^9$ cells/ml in the same. 50\mu l of the yeast cell suspension was mixed with 1\mu g of transforming DNA and 50\mu g of single-stranded salmon sperm carrier DNA. 300\mu l of sterile 40\%PEG 3350 (made fresh: 40\%PEG3350; 1xTE; 0.1M LiAc) was added and the suspension mixed thoroughly. The mixture was then incubated at 30°C for 30 mins, followed by a heatshock at 42°C for 15mins. The cells were then pelleted in a microcentrifuge for 5 secs, resuspended in 1.0ml 1x TE and plated out onto selective media.

2.8 Transformation of \textit{E.coli} Strains

The preparation of chemically competent cells and the transformation by a heat-shock procedure was essentially that of Mandel and Higa (1970).

An overnight culture grown in LB media was diluted 100-fold into the same medium and grown to an \textit{OD}_{600} of between 0.45 and 0.55. The culture was chilled on ice and the cells harvested at 3K for 5 minutes at 4°C. The cells were washed in 1/2 volume of ice cold 0.1M MgCl$_2$, then resuspended in the same volume of ice cold 0.1M CaCl$_2$ and left on ice for 20 minutes. The cells were then spun down and for immediate use resuspended in 1/20 of the original volume of ice cold 0.1M CaCl$_2$.
For long term storage, the cells were resuspended in 0.1M CaCl$_2$; 12.5% glycerol. One ml aliquots were frozen in a dry ice/IMS bath and stored at -80°C. Frozen cells were thawed on ice for approximately 30 minutes before use.

For each transformation reaction between 0.01 and 1μg of plasmid DNA in 10μl was added to 0.2ml of competent cells and incubated on ice for 30 minutes. The cells were heat shocked at 42°C for 2 minutes then incubated on ice for a further 20 minutes before adding 2ml of LB media. The cells were incubated at 37°C for between 30 and 90 minutes to allow expression of antibiotic resistance before plating onto selective media. Plates were incubated overnight at 37°C.

Transformation frequencies of between 10$^5$ and 10$^7$ per microgram of intact plasmid DNA were obtained using this protocol.

2.9 Conjugation

Filter matings were carried out in this Thesis because only plasmids based on the IncW group were used. Donor and recipient cells were grown overnight in LB with appropriate antibiotics. Cells were then diluted to an OD$_{600}$ of 0.05 in 10ml LB and allowed to grow with aeration until an OD$_{600}$ of 0.5 was reached. 0.5ml of both donor and recipient were then mixed prior to applying them to a nitrocellulose filter (25mm diameter, 0.45μm pore) attached to a vacuum. Nitrocellulose filters were then placed on a pre-warmed LA plate and incubated at 37°C for one hour. The filters were then transferred to 5ml of phosphate buffer (3mg/ml KH$_2$PO$_4$; 7mg/ml NaHPO$_4$; 4mg/ml NaCl; 0.1mg/ml MgSO$_4$·7H$_2$O).

2.10 Plasmid analysis

Copy number determination in yeast

The copy numbers of yeast / E.coli hybrid plasmids were measured using Southern hybridization techniques to determine the amount of a plasmid-borne gene relative to that of a single copy chromosomal gene.

Total DNA from transformant cells, grown exponentially in liquid media, was treated with a restriction endonuclease to linearize the plasmid DNA outside the yeast marker gene. Southern blot analysis was then carried out using a 32P-labelled probe fragment. For hybrid plasmids the probe used was the yeast marker gene. The amounts of radioactivity that hybridized to the plasmid and chromosomal bands were measured by a Phospho-Imager (Molecular Dynamics). The filter is exposed against a radiation-sensitive screen for 24-48 hours. This screen is then scanned by
the Phospho-Imager and a computer image of the filter and regions of radioactivity is produced by the Image-Quant™ package (Johnston et al., 1990). Regions of radioactivity can be selected and the intensity of the signal produced quantified. The intensity of the chromosomal band is compared against the intensity of the plasmid band to quantify number of plasmid copies.

**Determination of plasmid stability**

The stabilities of autonomously replicating plasmids were obtained by plating from exponentially growing cultures onto non-selective media. The colonies were replica plated onto selective media and colonies prototrophic for the marker on the plasmid were scored. For each sample approximately 500 colonies were screened.

### 2.11 Staining nuclei with DAPI

A 1ml aliquot of an exponentially growing yeast culture was harvested and resuspended in an equal volume of 70% ethanol for 30 mins. DAPI (4',6-diamidino-2-phenylindole; Sigma) was then added to a final concentration of 0.5μg/ml. 30μl of the suspension was then allowed to air dry on a microscope slide. Once fully dry, 30μl of 50% phosphate-buffered glycerol was added and a coverslip applied on top. Slides were viewed immediately with a high magnification fluorescence microscope. If photographs were to be taken, slides were pre-soaked in 50μg/ml poly-L-lysine to ensure the yeast cells adhered to the slide surface.

### 2.12 UV Mutagenesis

A description of the development of this technique is found in Chapter Three along with a summary of the selection procedure used. The UV mutagenesis protocol is based on a procedure outlined by Lee et al. (1988).

A 30ml culture was grown to a concentration of 0.5-1.0 x 10^9 cells/ml, washed twice in sterile water and resuspended in 0.9%(w/v)KCl. The suspension was transferred to a lidless 9cm diameter petri-dish and exposed to a UV dose of 90J/m. During exposure, the suspension was agitated by a magnetic flea. 100μl of the irradiated suspension was then used to inoculate 50ml of liquid YEPD which was then incubated overnight in the dark at 26°C to prevent photoreactivation. The selection for mutants was carried out as detailed in Chapter Three, with the use of 5-fluoro-orotic acid (5-FOA) as a positive selection for uracil auxotrophs. Each plate contained the following:
5-FOA plates: 0.5mg/ml 5-fluoro-orotic acid
0.01mg/ml uracil
2% glucose
0.6% Difco yeast nitrogen base

Amino acids and bases, other than uracil, were added as required by the strain, plus water agar up to a final volume of 25mls.

2.13 Plasmids

The plasmids used in this study are shown in Table 2.4.

Plasmids pYRG23 (Figure 2.1), pJDB248 (Figure 2.2), YCp50 (Figure 2.3) and YRp7 (Figure 2.4) are all shuttle vectors and were used in this study as experimental plasmids. The two YDp plasmids were used solely as a source of the yeast genes LEU2 and TRP1 which were subsequently used in copy number analyses.
Table 2.4 Plasmids used in this study

<table>
<thead>
<tr>
<th>PLASMID</th>
<th>SIZE</th>
<th>FEATURES / COMMENTS</th>
<th>REFERENCE / SOURCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>pYRG23</td>
<td>5.4kb</td>
<td>2μm ori, STB, ORFSB and C. URA3. AmpR, ori</td>
<td>Peter Meacock</td>
</tr>
<tr>
<td>pJDB248</td>
<td>13.6kb</td>
<td>whole 2μm interrupted in ORFD. LEU2 TetR, ori</td>
<td>Beggs, 1978</td>
</tr>
<tr>
<td>YCp50</td>
<td>7.8kb</td>
<td>ARS1, CEN4, URA3 AmpR, TetR, ori</td>
<td>Rose et al., 1987</td>
</tr>
<tr>
<td>YRp7</td>
<td>4.9kb</td>
<td>ARS1, TRP1 AmpR, TetR, ori</td>
<td>Struhl et al., 1979</td>
</tr>
<tr>
<td>YDp-L</td>
<td>4.3kb</td>
<td>LEU2 AmpR, ori</td>
<td>Berben et al., 1991</td>
</tr>
<tr>
<td>YDp-W</td>
<td>3.5kb</td>
<td>TRP1 AmpR, ori</td>
<td>Berben et al., 1991</td>
</tr>
</tbody>
</table>
Figure 2.1

pYRG23 carries the XbaI/EcoRI fragment of the 2μm plasmid which contains the origin of replication and the STB locus.
pJDB248

pJDB248 contains the complete sequence of the 2\(\mu\)m plasmid interrupted in \(D\), and the bacterial sequences from the vector pMB9.
**Figure 2.3**

YCp50

YCp50 contains \textit{CEN4} and \textit{ARS1} sequences for its maintenance. The bacterial \textit{ori} sequence is from the bacterial vector pBR322.
YRp7

YRp7 consists of an EcoRI fragment, containing the chromosomal loci ARS1 and TRP1, inserted into the bacterial vector pBR322.
Chapter Three

ISOLATION AND GENETIC ANALYSIS OF TEMPERATURE-SENSITIVE PLASMID MAINTENANCE MUTANTS

3.1 Introduction

The aim of this work was to identify host proteins involved in 2μm plasmid maintenance, both during replication and partitioning, by the isolation and characterisation of host mutants defective in 2μm plasmid maintenance. Because the plasmid confers no known positive phenotype to the host, it is unlikely that any proteins identified through a screen of this type will only be utilised by plasmid molecules. It is proposed that by analysing the 2μm's maintenance in this way, proteins involved in the general maintenance of DNA can be identified.

Candidate proteins that can be expected to be identified by a screen for 2μm plasmid maintenance mutants are discussed below.

3.11 Host proteins proposed to have a role in 2μm plasmid maintenance

Replication proteins
The host's involvement in 2μm plasmid replication has been well documented. The 2μm ori is an ARS sequence and the 2μm plasmid is under the same replication constraints as the chromosomes. Protein binding studies have demonstrated the positioning of the origin recognition complex over the 2μm ARS throughout the cell cycle (Rowley et al., 1995). Mutations in genes that affect any aspect of DNA replication in the nucleus can be expected to affect plasmid replication in the same way.

Host proteins important for DNA replication have been identified by studying mutants defective in their ability to maintain plasmids or minichromosomes. The majority of the minichromosomes used in these screens contain the 2μm ARS only and so contain a centromere sequence for their partitioning. Mutants identified through screens of this type include the mcm (minichromosome maintenance) mutants (Maine et al., 1984). These mutants were isolated specifically for their inability to effectively initiate DNA replication at ARSs. Subsequent study of the 2μm plasmid in the mcm2 mutant revealed that this mutation does indeed only affect replication, and not segregation or amplification of the plasmid (Maiti and Sinha, 1992). A role has been proposed for the MCM protein family as DNA replication
licensing factors, the presence or absence of a subset of the MCM proteins being necessary for the correct timing of origin firing during S-phase. Homologues of the MCM protein family have since been identified in other eukaryotes (Takahashi et al., 1994; Feger et al., 1996; Kubota et al., 1995; Kimura et al., 1994).

One host gene, MAP1 (maintenance of plasmid), has been identified by studying the stability 2µm-based plasmids containing the ori and STB sequences. Mutations in the MAP1 gene result in poor maintenance of the 2µm and ARS-CEN plasmids (Kikuchi and Toh-e, 1986). This suggests that Map1p acts through the ARS, and support for this came from the isolation of plasmid suppressors of map1 that carried additional chromosomal ARS elements on the plasmids.

Host proteins that may have a role in partitioning

The host’s involvement in the correct partitioning of the 2µm plasmid is not as well documented as its involvement in replication. It is known that the 2µm plasmid requires the plasmid-encoded protein products of ORFs B and C, together with the cis-acting STB sequence for its correct partitioning (Kikuchi, 1983; Cashmore et al., 1986). There is, however, evidence to suggest that the host also plays a role in this maintenance mechanism.

Genetic evidence includes the isolation of a host mutant, plm1 (plasmid maintenance) (Cashmore, 1984). This was identified as a spontaneous [cir°] isolate after continuous batch culture. The plm1 mutant was shown to be defective in plasmid maintenance rather than replication by copy number experiments. 2µm plasmids were introduced into the plm1 strain via mating with a wild-type strain. Upon dissection of the tetrads, the plm1 spores rapidly lost plasmid molecules but copy number experiments showed that the number of 2µm plasmids in the plm1 plasmid-containing cells was at the wild-type level (50-100 copies/cell).

A further host mutation affecting 2µm plasmid maintenance is nibl (nibbled phenotype) (Holm, 1982a). nibl colonies exhibit a "nibbled" colony morphology caused by sub-populations of abnormally large cells. It was found that these large cells contained twice the normal copy number of the 2µm plasmid. The NIB1 allele was therefore thought to play a role in 2µm copy number control (Holm, 1982b). However, later work has suggested that the nib1 phenotype isn’t due specifically to the presence of 2µm plasmids, but that the nib1 allele confers a general sensitivity to extrachromosomal elements (Sweeney and Zakian, 1989).

There is biochemical evidence for host protein binding at the STB locus (Hadfield et al., 1995). Gel-shift experiments using the 2µm STB sequence against protein prepared from [cir°] yeast cells produces a band shift indicating protein binding. The STB locus has a definite role in the partitioning mechanism of the 2µm
plasmid, and no function for it in the replication of the plasmid has been shown. This suggests that any interaction by the host at this locus could be to facilitate partitioning.

Because the 2µm plasmid has a unique partitioning mechanism, this may utilise host proteins not normally directly associated with chromosome transmission, such as components of the nuclear scaffold. How host/plasmid interactions affect plasmid stability is unknown, and speculation as to the nature of the interaction will depend on the mode of plasmid segregation in the cell. There are two broad categories that models for 2µm plasmid partitioning fall into, and these are discussed more fully in Chapter One (see also Figure 1.9). The active models suggest that the 2µm plasmid is attached to an actively partitioning nuclear structure, binding sites for which are at regular intervals around the nucleus. Candidate proteins for this include those of the karyoskeleton such as the actins, myosins and lamins. B protein co-purifies with a karyoskeletal subfraction corresponding to the nuclear matrix lamina fraction of higher cells (Wu et al., 1987). One possibility is that B may promote plasmid partitioning by interacting with the nuclear lamina of the host cell to provide dispersed anchorage sites for attachment of plasmid molecules. It is suggested that B protein also interacts with the STB locus (Hadfield et al., 1995), and it has been shown via the 2-hybrid system that B protein interacts with C protein (Albury, in preparation). A more elaborate model therefore is that all these factors may combine together to form the anchor that the 2µm plasmid needs during segregation.

Passive models for the partitioning of the 2µm plasmid suggest that plasmid molecules are attached to a nuclear structure during part of the cell cycle. This structure could be the replication machinery, or some other site in the nucleus from which plasmids are released prior to cell division. This would allow for the random diffusion of plasmid molecules in the nucleoplasm, and so allow for its random segregation into the daughter cell.

3.2 Results

3.2.1 Isolation of temperature-sensitive plasmid maintenance mutants

Optimisation of UV mutagenesis conditions

The strain chosen for the optimisation experiments was MC16 [cir+]. MC16 was chosen because it is a URA3 strain, and therefore ura3 mutations can be induced and selected on plates containing 5-fluoro-orotic acid (5-FOA) (Boeke et al., 1984). This is used as an indication of mutation frequency.
The UV mutagenesis procedure was adapted from Lee et al., 1988, and is described in detail in Chapter Two. UV mutagenesis was carried out at room temperature on log-phase cells at a concentration of 5x10^7/ml. These cells were dosed at 0.5Jm/sec and at set time points two 100µl aliquots were taken. One aliquot of cells was diluted and plated immediately onto YEPD plates for cell viability calculations, the second aliquot was inoculated into YEPD broth at a concentration of 5x10^5 cells/ml. The cultures were then incubated for six generations in the dark to prevent photoreactivation, diluted and plated directly onto plates containing 5-FOA for subsequent calculations of mutation frequency. All incubations were carried out at 30°C.

As UV dose increased, cell viability decreased and mutation frequency increased (Figures 3.1a and 3.1b). The optimum dose for the UV mutagenesis was chosen as 90Jm^-2. This produced a cell viability of approximately 50% and a mutation frequency of 1.5x10^-3 mutations/cell. Though the mutation frequency increased further with increased dose rate beyond that chosen, the viability decreased dramatically.

It has been reported (Boeke et al., 1984) that, following a UV mutagenesis procedure, only 5-10% of colonies that grow on 5-FOA are actually ura3 mutants, the majority being 5-FOA resistant mutants. However, later data obtained by Lee et al. (1988) showed that at a UV dose of 90Jm^-2, the dose chosen for this experiment, the frequency of 5-FOA resistant ura3 mutants was found to be 70-75%. The recovery of uracil auxotrophs on 5-FOA by Lee et al. (1988) was optimised by inoculating mutagenised cells into uracil omission medium for at least three generations immediately following mutagenesis. It is proposed that a phenotypic lag is involved, requiring time for the enzyme orotidine-5'-monophosphate decarboxylase to be diluted out of the population. This optimisation step was added to the final mutant screen. To estimate the numbers of 5-FOA resistant uracil prototrophs isolated in the optimisation procedure, twenty colonies that grew on 5-FOA after a dose of 90Jm^-2 were patched onto media with and without uracil. In all cases the colonies picked were shown to be uracil auxotrophs. This sample size isn’t large enough to be taken as significant but, because of the intended use of 5-FOA as an enrichment step for plasmid-free isolates, any 5-FOA resistant mutants present at the initial stage of the screen will be selected against later. Subsequent experimental design used the mutation rates calculated in this series of experiments as an approximation of the mutation frequencies expected in the screen itself.
Figure 3.1

Cell viability decreases and mutation frequency increases with increased UV dose

Exponentially growing cells were exposed to UV light at a dose of 0.5Jms⁻¹. At set time points, aliquots of cells were taken to test for cell viability and mutation frequency.

(a) Cell viability
Cells were diluted and plated onto YEPD plates. These were then incubated in the dark at 30°C until colonies appeared. Numbers of colonies on the experimental plates were compared to the numbers of colonies on the control plate, plated at time zero with no exposure to UV light.

(b) Mutation frequency
Cells were inoculated into YEPD broth and incubated, in the dark, at 30°C. After an overnight period of growth, cells were plated onto media containing 5-FOA. The number of colonies on the 5-FOA plates were compared to the numbers of cells plated, and mutation frequency was calculated.
Figure 3.1

(a) Proportion of viable cells

(b) Mutation frequency
Screen for temperature-sensitive plasmid-maintenance mutants

The UV mutagenesis procedure was carried out on a culture of S150-2B [cir+], transformed with the plasmid pYRG23 (Figure 2.1). pYRG23 is a 2μm-based plasmid, containing the 2μm ori and STB sequences. This plasmid requires the presence of native 2μm to provide the necessary plasmid proteins in trans for its stable maintenance. pYRG23 also contains the URA3 gene, so enrichment for plasmid-free isolates can be carried out by positive selection of colonies on 5-FOA.

The UV dose for the experiment was chosen as 90Jm⁻² which results in a mutation rate of approximately 1.5x10⁻³ mutations/cell. Using this figure, culture sizes and numbers of colonies to be screened were calculated in order to optimise the chance of successfully isolating the desired mutants. An overnight culture of S150-2B [cir+, pYRG23] was harvested and washed in sterile distilled water. Cells were then resuspended in 0.9%KCl at a density of 5x10⁶ cells/ml. The cells were then subjected to a UV dose of 90Jm. Figure 3.2 describes the screen used to select for plasmid-maintenance mutants.

Firstly, an aliquot (100μl; 5x10⁶ cells) of the UV-mutagenised population of cells was inoculated into 50ml of medium lacking uracil at 24°C in the dark. This was to enrich for plasmid-containing cells and thereby reduce the proportion of cells that had spontaneously lost plasmids as a result of the UV-mutagenesis procedure, or as a result of the plasmid’s inherent instability. Also, cells carrying lethal mutations may be lost. The temperature of 24°C is the permissive temperature chosen for the screen, so the temperature-sensitive mutants that are to be isolated will still have their plasmids at this first stage.

Once the culture reached 1x10⁷ cells/ml (approximately seven doublings), 1ml of culture was inoculated into 50ml of uracil-containing medium and incubated at the non-permissive temperature of 37°C. This stage was to remove from the population any lethal temperature-sensitive mutations, and to allow any plasmid-maintenance mutants to begin to lose their plasmids. The cells underwent five doublings and twelve aliquots of 100μl (2.5x10⁶ cells) were then plated onto a medium containing 5-FOA and again incubated at 37°C. A yeast cell containing the plasmid pYRG23 has a probability of 0.03 of losing the plasmid at each generation (Morrissey, 1993). After the number of generations that the population of cells in this experiment had undergone, only 70% of the cells can now be expected to contain pYRG23 compared to the native 2μm’s stability of over 99.99% in the same population. For this reason, the growth of colonies on 5-FOA will represent a population of cells that have naturally lost pYRG23 as well as those that have been induced to lose it as a result of mutations incurred during the mutagenesis procedure. However, the selection for uracil auxotrophs on 5-FOA represents an enrichment for the mutations affecting plasmid stability. Confluent growth was
observed on the 5-FOA medium, and the colonies from four of the twelve plates were pooled and retransformed with pYRG23. Approximately 3000 transformants were picked into microtitre dishes and inoculated onto medium lacking uracil.

The plate screen carried out for temperature-sensitive plasmid maintenance mutants is summarised in Figure 3.2. The transformant plates were replica-plated onto one plate without uracil (selective for the pYRG23), and two plates with uracil (non-selective for pYRG23). All plates without uracil were incubated at 24°C, to act as master plates, and of the duplicated plates with uracil one replica was incubated at 24°C and the other incubated at 37°C. The plates incubated at 37°C allowed the patches to have a period of growth at the non-permissive temperature in medium conditions which allowed for loss of plasmids from the population. Both non-selective plates were then replica plated onto selective medium. The amount of growth of the patches on the selective media after their period of growth at both permissive and non-permissive temperatures was recorded. Each of the uracil-containing plates that had been incubated at 37°C were also replica-plated onto more non-selective plates and incubated again at the non-permissive temperature. This replica-plating regime was repeated three times, and at each stage the non-selective plates that had grown at 37°C were replica-plated onto selective plates. Any gradual deterioration of growth that would be consistent with uracil auxotrophy, i.e. loss of pYRG23 from the patches, was noted and the transformants rescreened.

This first screen resulted in 300 transformants being rescreened, using the same replica-plating regime. This 300 was then further reduced to 59 possible temperature-sensitive plasmid-maintenance mutants. These 59 isolates fell broadly into two categories. There were 34 that were identified as losing pYRG23 gradually over the course of the replica-plating screen, but a further 25 that failed to grow on uracil omission medium after the first non-selective growth at either permissive or non-permissive temperatures.

The first category of 34 isolates were screened further by a quantitative liquid assay. In this assay each of the pYRG23 transformants were grown firstly in uracil omission medium at 24°C, and then log phase cells were transferred to uracil-containing medium at 37°C. The cells were grown until late log-phase, 1-4x10^7 cells/ml, diluted and plated onto complete medium to a concentration of 200 cells/plate in triplicate. These plates were then incubated at 24°C until the appearance of colonies 3-4 days later, and then replica-plated onto plates with and without uracil. The plates were scored for colony growth and the percentage plasmid-containing cells in the population was calculated. The initial 34 isolates were reduced to five based on their plasmid stability phenotypes after a further repeat of this screen.
pYRG23 transformants were exposed to UV light at a dose of 90 Jm. Cells were inoculated into media selective for plasmid molecules (+uracil) and incubated at 24°C in the dark. After an overnight period of growth, cells were inoculated into fresh media non-selective for pYRG23 (+uracil) and incubated overnight at 37°C. Cells were then plated onto 5-FOA and colonies pooled and retransformed with pYRG23. Transformants were picked into microtitre dishes and seeded onto selective plates at 24°C. These plates were then replica-plated onto media both with and without uracil, at 24 and 37°C. The non-selective plates at 37°C underwent successive rounds of replica-plating onto selective plates at 24°C and further non-selective plates at 37°C. The appearance of the patches on the selective plates was scored. Any patches that grew poorly were repicked and screened again.
Figure 3.2

3000 pYRG23 transformants picked into microtitre dishes and seeded onto selective plates

plated onto 5-FOA 37°C

colonies pooled and retransformed with pYRG23

37°C o/n

selective media 24°C o/n

non-selective media 37°C o/n

colonies that show reduction in growth, repicked onto non-selective plates and screened again

replica plated

selective at 24°C

non-selective at 37°C and 24°C
One possibility was that the second category of mutants displayed their increased plasmid instability due to loss of the native 2μm. Once native 2μm is lost from a population of cells, pYRG23 can no longer receive necessary plasmid proteins in trans and so behaves as an ARS plasmid, demonstrating strong maternal bias, and so is unable to be efficiently maintained. Absence of 2μm plasmids from these isolates was confirmed by colony hybridisation with a sequence from the 2μm B gene (data not shown). This analysis was also carried out for the five isolates identified from the first category. All of these five hybridised weakly to 2μm sequences, possibly representing a gradual loss of the 2μm from these isolates resulting in the much more gradual loss of pYRG23 observed in the plate screen. The identification of 2μm-free isolates from this screen is highly significant. Spontaneous [cir°] isolates occur very rarely, at a frequency of approximately 1x10⁻⁷/cell (Futcher and Cox, 1983; Cashmore, 1984) therefore, to obtain 25 such isolates from a starting population of 3000 represents approximately a 100,000 fold increase of this event. It was decided to continue the rest of the analysis on the 25 [cir°] isolates initially identified. It was considered likely that these would have the most significant loss of plasmid stability and so be easier to study using the plate screen.

Stability analysis of putative temperature-sensitive plasmid maintenance mutants

Each of the remaining 25 [cir°] isolates were transformed with the plasmid pJDB248 (Figure 2.2). This is a whole 2μm-based plasmid and so can be maintained in the absence of the native 2μm. This plasmid is also more stable than pYRG23, so any differences in stabilities between the putative mutants and the wild-type S150-2B [cir°] strain should be more easily identifiable. For ease of analysis, it was necessary to develop a plate assay that would demonstrate any plasmid loss phenotype of these isolates with pJDB248. In the growth of a colony on a plate, a cell can be expected to go through approximately 20 generations, compared to the six or seven usually achieved in an overnight period of growth in liquid culture. A plate assay should therefore prove to be more sensitive, as well as enabling large numbers of isolates to be screened with relative ease.

The plate screen developed is summarised in Figure 3.3. Individual transformants were streaked to single colonies on selective medium (lacking leucine) at 24°C. A single colony was then streaked to single colonies on non-selective medium and incubated at the non-permissive temperature of 37°C. Four colonies from the non-selective plates were then placed into microtitre wells and plated again onto non-selective medium and placed at 37°C. The resulting patches were then replica-plated onto selective and non-selective media and incubated at 24°C.
Figure 3.3

Plate screen developed for assay of temperature-sensitive plasmid maintenance mutants

Transformants are streaked to single colonies on plates selective for the plasmid and incubated at 24°C. Single colonies are then streaked on non-selective plates and incubated at 35°C. Multiple colonies are then picked from these plates into microtitre dishes and seeded onto further non-selective plates. After an incubation at 35°C, the non-selective plates are replica-plated onto both selective plates at 24°C and non-selective plates at 35°C. The selective plates are scored for a visible difference in patch growth between possible mutants and wild-type controls. If there is no clear difference after the first round of replica-plating, the non-selective plates are replica-plated again as described above for further rounds.
Figure 3.3

Selective 24°C

Non-selective 35°C

Streaked

Colonies picked into microtitre dishes and seeded onto non-selective plates at 35°C

Replica-plated

Selective at 24°C

Non-selective at 35°C

Wild-type colonies on each plate as controls
Differences in patch growth on the leucine omission medium was then observed. Figure 3.4 shows patches of cells after a screen of this type. Differences in plasmid stability can be observed after non-selective growth at 37°C between the isolates shown and the wild type S150-2B [cir°] transformants.

A range of plasmid stabilities was observed between the 25 [cir°] isolates. The screen was repeated and only four mutants showed a consistently high instability of pJDB248. These four mutants were then screened for plasmid loss after an overnight period of growth in non-selective liquid cultures. Single colonies from plates selective for pJDB248 were inoculated into selective media at 24°C. Log-phase cultures were then inoculated into fresh non-selective media at both 24 and 37°C. Cells were diluted and plated in triplicate from each of the cultures onto YEPD plates to densities of 200 colonies/plate and incubated at 24°C. These plates were then replica-plated onto plates both with and without leucine and numbers of plasmid-containing cells as a percentage of the population were scored. The results of this analysis are shown in Table 3.1. The plasmid instability phenotype of these four isolates was confirmed.

It was observed that cultures of the mutants, and S150-2B [cir°], grown in SD media at 37°C over more than seven generations became slow growing and lost viability. To be able to study the kinetics of plasmid loss over longer periods of time in liquid culture conditions, it was therefore necessary in subsequent experiments to study the plasmid-loss phenotype at the lower temperature of 35°C. The plate screen described above was repeated at 35°C for the four mutants isolated and differences in plasmid stability relative to S150-2B [cir°] were still observed.

3.22 Genetic analysis of mutants

Genetic analysis was carried out to determine whether the mutations isolated were in single genes. Also, the mutations could then be isolated in the opposite mating type for complementation group analysis. Finally, any segregation of the mutant phenotype with known genetic markers would imply linkage of the mutation with known genes and so enable placement onto a genetic map.

For the genetic analysis it was first necessary to create diploids by mating the isolates with another strain of the opposite mating type. It was necessary that this second strain chosen had certain characteristics. Most importantly it had to be leu2 and able to maintain pJDB248 to the same extent as the parental MATα S150-2B [cir°] strain at both permissive and non-permissive temperatures. For ease of analysis it was also preferred that the strain had an ade2 mutation, resulting in a pink colouration of the colonies due to the accumulation of an intermediate in the adenine
Differences in plasmid stability are observed after a plate screen for temperature-sensitive plasmid maintenance mutants

pJDB248 transformants were streaked to single colonies on plates selective for the plasmid (-leucine) and incubated at 24°C. Single colonies from these plates were streaked onto media non-selective for the plasmid (+leucine) and incubated at 35°C. Four colonies from each streak were then picked into microtitre dishes, seeded onto non-selective plates and incubated at 35°C. These plates were then replica-plated onto plates selective for pJDB248 and incubated at 24°C. Growth of the patches was scored for plasmid loss.
Figure 3.4
Table 3.1

Stability analysis of pJDB248 after an overnight period of non-selective liquid growth at 37°C

<table>
<thead>
<tr>
<th>ISOLATE</th>
<th>% plasmid - containing cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24°C s</td>
</tr>
<tr>
<td>S150-2B [cir°]</td>
<td>85.1 (2.4)</td>
</tr>
<tr>
<td>RF1(plm3-1)</td>
<td>82.0 (3.2)</td>
</tr>
<tr>
<td>RD4(plm2-3)</td>
<td>82.0 (4.3)</td>
</tr>
<tr>
<td>RB5(plm2-2)</td>
<td>88.1 (4.7)</td>
</tr>
<tr>
<td>RA3(plm2-1)</td>
<td>85.7 (1.9)</td>
</tr>
</tbody>
</table>

Transformants of S150-2B [cir°] and each of the mutant strains with pJDB248 were given an overnight period of growth at 24°C selective for the plasmid (-leucine). Cells were then subbed back to $1 \times 10^5$ cells/ml into media non-selective for the plasmid (+leucine) and incubated overnight at 35°C. At each stage, cultures were plated in triplicate at approximately 200 colonies/plate on YEPD. These plates were incubated at 24°C and replica-plated onto both selective and non-selective media to determine numbers of plasmid-containing cells in the population.

s = growth in media selective for pJDB248 (-leucine)

ns = growth in media non-selective for pJDB248 (+leucine)

Numbers in brackets indicate standard deviations.

The names of the isolates refer to the coordinates on the plate from which they were identified in the final screen for plasmid maintenance mutants.
biosynthetic pathway. This makes genetic analysis easier because complete tetrads can be identified visually based on the 2:2 segregation of this pink colour. Two candidate MATα laboratory strains were chosen, 8HB [cir⁰] and MC16 [cir⁰]. Both of these strains are leu2, ade2 and were transformed with pJDB248.

Initial stability analysis on these two strains was by the plate assay described above. 8HB [cir⁰] showed a significant decrease in plasmid stability and it was decided to try to quantitate this further by a liquid assay. The results of this are shown in Table 3.2. The reduced plasmid stability displayed by this strain is confirmed. The reasons for this variability is not known and this strain was not studied further.

MC16 [cir⁰] was chosen as the strain with which to carry out the genetic analysis. Heterozygous, plm/PLM diploids were made by crossing MC16 [cir⁰, pJDB248] with each of the mutants, and also a PLM/PLM diploid was created by mating S150-2B [cir⁰] with MC16 [cir⁰, pJDB248].

Analysis of plasmid stability in plm/PLM diploids

To determine whether the mutations present in the mutant isolates are recessive or dominant, a plate assay to study the stability of pJDB248 in the heterozygous diploids was carried out. This screen was identical to the plate screen described above for the characterisation of the haploids. In all cases, the appearance of the patches on selective plates, after a period of growth at 35°C on non-selective plates, was identical to the wild-type diploid. This indicates that the plm mutation in each of the strains is complemented by the presence of the wild-type copy of the gene in the heterozygous diploid. It was therefore concluded that each of the mutations isolated is recessive.

Tetrad analysis

Each of the heterozygous plm/PLM diploids were patched onto pre-sporulation media at 30°C. After 2-3 days of growth, cells were taken from these plates and patched onto sporulation media at 30°C. The formation of tetrads was checked microscopically after 7-14 days. There was no difference in the time of formation of tetrads, or the appearance of them between the diploids.

Tetrad dissections were carried out using the Singer MSM system onto YEPD plates, after incubation of tetrads in β-glucuronidase to degrade the ascus wall. After dissection, the plates were incubated at 35°C for 4-5 days until colony growth was observed.

Up to fourteen tetrads were dissected for each of the diploids. Complete tetrads were scored for auxotrophic requirements, mating type and plasmid loss. Colonies were initially picked from the dissection plates into microtitre dishes. From
Table 3.2

Stability of pJDB248 in three [cir⁰] laboratory strains at 24 and 35°C

<table>
<thead>
<tr>
<th>STRAIN</th>
<th>% plasmid-containing cells</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24°C s</td>
<td>24°C ns</td>
<td>35°C ns</td>
<td></td>
</tr>
<tr>
<td>S15-2B [cir⁰]</td>
<td>91.3 (2.3)</td>
<td>68.7 (4.8)</td>
<td>70.1 (2.6)</td>
<td></td>
</tr>
<tr>
<td>MC16 [cir⁰]</td>
<td>92.2 (1.4)</td>
<td>70.3 (5.5)</td>
<td>75.8 (3.7)</td>
<td></td>
</tr>
<tr>
<td>8HB [cir⁰]</td>
<td>76.8 (3.2)</td>
<td>62.6 (2.8)</td>
<td>56.6 (0.7)</td>
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</tbody>
</table>

Transformants of each of the strains with pJDB248 were given an overnight period of growth at 24°C selective for the plasmid (-leucine). Cells were then subbed back to 1x10⁵ cells/ml into media non-selective for the plasmid (+leucine) and incubated overnight at both 24 and 35°C. At each stage, cultures were plated in triplicate at approximately 200 colonies/plate on YEPD. These plates were incubated at 24°C and replica-plated onto both selective and non-selective media to determine numbers of plasmid-containing cells in the population.

s = growth selective for the plasmid (-leucine)
ns = growth non-selective for the plasmid (+leucine)

Numbers in brackets indicate standard deviations.
these, cells were inoculated onto a series of SD plates lacking tryptophan, histidine, uracil or leucine, as well as onto YEPD plates. Growth of colonies on each of these plates was scored, and the auxotrophic requirements of each of the colonies determined. The adenine requirement of each of the colonies was inferred from the presence of the pink colouration. One of the YEPD plates was incubated at 35°C and then replica-plated onto plates with and without leucine to score for increased plasmid loss. Mating type was determined by crossing each of the colonies with both \textit{MATa} C1 and \textit{MATa} C2 strains. The successful creation of diploids with one or other of these strains was scored, and mating-type of the colonies determined.

In each case segregation of all phenotypes tested, except for histidine requirement which is discussed below, was 2:2. This includes the segregation of the plasmid-loss phenotype. The 2:2 segregation of the \textit{plm} phenotype in all tetrads analysed suggests that the \textit{plm} mutations in each of the mutants is in a single gene. No linkage between the \textit{plm} phenotypes and any of the auxotrophic markers was observed. Histidine requirement in the haploids doesn’t segregate 2:2. The two parental strains in each case have different mutations in the histidine biosynthetic pathway. SI50-2B, and so each of the mutants, carries a mutation in the \textit{his3} gene, whereas MC16 carries the \textit{his4} mutation. This is made more complicated by the presence in MC16 of a suppressor for the \textit{his4} mutation, such that this strain and diploids created from it do not require histidine in the media to grow. The suppressor of the \textit{his4} mutation isn’t linked to \textit{his4} and so these loci segregate randomly with respect to each other in a diploid undergoing meiosis. This results in only spores receiving both \textit{his4} and the suppressor having a \textit{his+} phenotype. This resulted in the segregation of the \textit{his+} phenotype in ratios of 2:2, 1:3 or 0:4 in the tetrads studied. A summary of the complete tetrads obtained and their phenotypes is found in Appendix 1.

\textbf{Complementation group analysis}

To determine how many genes are represented in the mutants isolated, strains carrying each of the mutations in both mating-types were mated with each other in all possible pairwise combinations. The plate assay for loss of pJDB248 was then performed on these diploids. Increased plasmid loss resulting from lack of complementation of \textit{plm} alleles in diploids indicates that the two \textit{plm} loci present are allelic, and the mutations present in those haploids can be inferred to be in the same gene. The results of this analysis are summarised in the complementation grid in Figure 3.5. Two complementation groups were identified; The group defined as \textit{plm2} consists of isolates RA3 (\textit{plm2-1}), R85 (\textit{plm2-2}) and RD4 (\textit{plm2-3}) while RF1 complements each of the mutants in this group and so is designated \textit{plm3}. 

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Figure 3.5

(a) Strains used for complementation group analysis

All MATα strains used were the original plm isolates from the mutagenesis screen. The MATα strains were chosen from the segregants obtained after tetrad dissection of each strain. The same MATα and MATα strains for each plm allele were used for all matings. The MATα strains are listed below:

RA3 B3  MATα  trp⁻  his⁺  ura⁻  ade⁻  plm⁻
RB5 B3  MATα  trp⁻  his⁺  ura⁺  ade⁻  plm⁻
RD4 B3  MATα  trp⁻  his⁺  ura⁺  ade⁻  plm⁻
RF1 G4  MATα  trp⁺  his⁻  ura⁺  ade⁻  plm⁻
S150-2B A3  MATα  trp⁻  his⁻  ura⁺  ade⁻

(b) Complementation group table

Matings were carried out in all pairwise combinations. The MATα strains used in the matings were transformed with pJDB248 prior to conjugation. Diploids were screened for plasmid loss.

<table>
<thead>
<tr>
<th>MATα</th>
<th>RA3</th>
<th>RB5</th>
<th>RD4</th>
<th>RF1</th>
<th>S150-2B</th>
</tr>
</thead>
<tbody>
<tr>
<td>MATα</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RA3</td>
<td>plm⁻</td>
<td>plm⁻</td>
<td>plm⁻</td>
<td>plm⁺</td>
<td>plm⁺</td>
</tr>
<tr>
<td>B3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RB5</td>
<td>plm⁻</td>
<td>plm⁻</td>
<td>plm⁻</td>
<td>plm⁺</td>
<td>plm⁺</td>
</tr>
<tr>
<td>B3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RD4</td>
<td>plm⁻</td>
<td>plm⁻</td>
<td>plm⁻</td>
<td>plm⁺</td>
<td>plm⁺</td>
</tr>
<tr>
<td>B3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RF1</td>
<td>plm⁺</td>
<td>plm⁺</td>
<td>plm⁺</td>
<td>plm⁻</td>
<td>plm⁺</td>
</tr>
<tr>
<td>G4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S150-2B</td>
<td>plm⁺</td>
<td>plm⁺</td>
<td>plm⁺</td>
<td>plm⁺</td>
<td>plm⁺</td>
</tr>
<tr>
<td>A3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 3.5
3.3 Discussion

The development and use of a UV mutagenesis procedure has resulted in the successful isolation of nine temperature-sensitive mutants defective in 2μm plasmid maintenance. Four of these demonstrated the most striking plasmid loss phenotype and so were selected for further analysis. The decreased stability of the 2μm-based plasmid pJD8248 has been observed for these mutants by both the development of a plate assay and through a period of growth in liquid media. Genetic analysis of these mutants has shown that each mutation is recessive and in a single gene. Complementation group analysis has placed these mutations into two complementation groups, \textit{plm}2 and \textit{plm}3.

The successful isolation of \textit{plm} mutants is an important step in the analysis to further elucidate the role of the host in 2μm plasmid maintenance. The analysis so far however doesn't give any indication as to the function of the wild-type \textit{PLM} alleles. They may be important for either the replication or partitioning mechanisms of the 2μm plasmid, or may reveal an as yet unknown role in another aspect of plasmid maintenance or general DNA metabolism such as control of transcription or translation. Phenotypic analysis of the mutants may go some way in the elucidation of gene function, but the eventual cloning and characterisation at both the DNA and protein level of the wild-type \textit{PLM} alleles will answer these questions more fully.

The UV mutagenesis procedure resulted in the isolation of 25 [\textit{cir}0] isolates and five other putative plasmid maintenance mutants. The identification of [\textit{cir}0] isolates is highly significant, and it was on these that the analysis focused, resulting in the eventual characterisation of four mutants. The fact that these isolates lost the native 2μm plasmid over a very short period of growth, suggests that the \textit{plm} alleles in these are more likely to be involved in plasmid partitioning rather than replication. If the \textit{plm} mutants are replication deficient, a more gradual loss of plasmids may be observed, as is perhaps the case with the other five mutants isolated. If, however, the partitioning mechanism of the 2μm plasmid is affected, the plasmids may be expected to behave as \textit{ARS} plasmids, with a more dramatic plasmid-loss phenotype. The frequency of spontaneous [\textit{cir}0] cells in a population is normally extremely low, approximately 1x10^{-7} but it is not known if this event can be induced by exposure to UV light at the doses used.

Variation in the stability of the 2μm-based plasmid also was observed between standard laboratory strains. It is not known if the creation of spontaneous [\textit{cir}0] isolates between S150-2B and 8HB is also increased, but this is unlikely given the successful transformation and use of the 8HB [\textit{cir}+] strain in our laboratory and others. The 8HB [\textit{cir}0] isolate used in this analysis was created by chasing out native
2µm plasmid with a 2µm-based hybrid plasmid (Morrissey, 1993). It is important to note this because it has been reported that there are differences in the behaviour of induced and spontaneous [cir^7] strains (Mead et al., 1987). It would therefore be necessary to study the maintenance of pJDB248 in a spontaneous 8HB [cir^7] isolate, and also in 8HB [cir^+7] to determine the significance of this observation. The presence of strain variations in plasmid maintenance is further evidence outlining the importance of the host in these mechanisms.
Chapter Four

PHENOTYPIC ANALYSIS OF plm2-1

4.1 Introduction

The isolation of mutants defective in plasmid maintenance has been an important tool in the investigation of replication and maintenance of DNA in the yeast cell. The MCM family of genes, important in the initiation of DNA replication, were initially identified through a screen that identified mutants defective in minichromosome maintenance (Maine et al., 1984). One mutant, mcm2, was identified by its inability to maintain a minichromosome that carried the 2μm ARS sequence. This mutant was later demonstrated to be defective in replication, rather than segregation or amplification of native 2μm plasmids (Maiti and Sinha, 1992).

The analysis of plasmid behaviour in mcm2 proved to be an important step in understanding the role of the wild-type gene. A further host mutant, plm1, was identified as a spontaneous [cir°] isolate after continuous batch culture of [cir+] yeast cells (Cashmore, 1984). The PLM1 gene has not been cloned. The plm1 allele isolated is non-conditional and so the nontransformability phenotype of the strain makes it difficult to work with. Phenotypic analysis has demonstrated that the mutation is 2μm-specific which suggests a role for PLM1 in partitioning, rather than replication, of the plasmid.

Chapter Three describes the isolation, after UV mutagenesis, of four yeast mutants with a temperature-sensitive 2μm plasmid maintenance phenotype. The mutations are all recessive, in single genes and genetic analysis placed them into two complementation groups, plm2 and plm3. The plm2-1 allele (isolate RA3) demonstrates the most striking phenotype of plasmid loss and is therefore the one chosen for further analysis. The ultimate aim is to identify the wild-type PLM2 gene, but an important step towards understanding the role of this gene in the host is to study the phenotype of the mutant.

An analysis of the stability of plasmids with different maintenance mechanisms in plm2-1 is important. If CEN-ARS and ARS plasmids are poorly maintained, the mutation is not 2μm-specific and may suggest a role for PLM2 in DNA replication. Copy number analysis can also be used as an indication as to the function of PLM2. If the copy number per cell of 2μm-based plasmids is unchanged in the plm2-1 mutant, then the defect is likely to be partitioning. If the copy number per cell is lower than wild-type however, then the mutation is likely to be replication specific.
A host mutation, *nib1*, that is defective in 2μm plasmid maintenance has a nibbled colony morphology. This phenotype is due to the presence of large cells containing elevated numbers of the 2μm plasmid (Holm, 1982a; Holm, 1982b). Staining of *nib1* cells with DAPI reveals aberrant nuclear migration, with DNA remaining in the neck of budding cells (J. Murray, personal communication). Staining of mitotically dividing nuclei with DAPI will go some way to identifying whether or not the integrity of the nucleus, and the DNA within it, is maintained in the *plm2-l* mutant.

Further important analysis of *plm2-l* includes growth and viability experiments. This will reveal whether or not the *plm2-l* mutation affects more than plasmid maintenance. These experiments may also identify an enrichment step which can be incorporated into the screen for *PLM2* from a genomic library.

### 4.2 Results

#### 4.21 Visualisation of *plm2-l* nuclei using DAPI

The appearance of *plm2-l* under phase contrast in both minimal and complete, YEPD, media is the same as wild-type. This is true of log phase and stationary phase cultures of all four *plm2* alleles. The *PLM2* gene may play a role in the general maintenance of DNA, or the integrity of the nucleus. Exponentially growing cultures of S150-2B [cir⁰] and *plm2-l* strains, grown in liquid YEPD media, were stained with DAPI and viewed by fluorescence microscopy. The results are shown in Figure 4.1a and b. There was no difference observed in the staining patterns of *plm2-l* compared to wild-type. The general integrity of the nucleus appears to be unchanged under DAPI staining, and the division of the nucleus between mother and daughter cells can be clearly observed.

#### 4.22 The growth rate and viability of *plm2-l* is unchanged compared to wild-type

A series of experiments were carried out to determine whether or not the growth rate of *plm2-l* was different to that of the wild-type, S150-2B [cir⁰] strain. Overnight YEPD cultures of these strains were inoculated into fresh YEPD media at a concentration of 5x10⁴ cells/ml, at 24 and 35°C. At time points of 20-40 mins, the density of the cultures was measured until the cultures entered late log-phase (approximately 3-5x10⁷ cells/ml). This growth experiment was repeated for the
Figure 4.1

Appearance of plm2 and wild-type cells after DAPI staining

Exponentially growing cultures of both plm2-1 and S150-2B [cir°] were stained with DAPI. Photographs were taken of cells viewed with phase contrast and fluorescence.

(a) S150-2B [cir°]

(b) plm2-1
Figure 4.1

(a) S150-2B

(b) \textit{plm2}
strains transformed with pJDB248. The media used in these experiments was selective for the plasmid. All experiments were repeated three times and the average doubling times obtained are shown in Table 4.1. There appears to be no difference in growth rate in these conditions. Both strains have a doubling time in YEPD media at 35°C of approximately 80 minutes. This doubling time is increased when cells are grown at 24°C, and when cultures are grown in media selective for pJDB248 at both temperatures.

At the time points taken in each of the growth experiments, cells were diluted and plated to an expected cell density of 200 colonies per YEPD plate. There was no significant difference between the expected number of colonies plated and those observed between the strains (data not shown). It was concluded that there is no observable phenotype conferred by plm2-1 that affects growth and viability under these growth conditions.

The growth curves only cover approximately six generation of growth, so any small difference in growth rate may not be detected. It is estimated that a single cell however goes through twenty generations to produce a colony. The morphology of colonies of the strains tested above on selective and complete media was therefore examined for any size differences or difference in morphology. No difference in growth rates or morphology of colonies was observed between the strains on the media tested, or at 25 or 35°C. Interestingly, colonies of plm2-1 [pJDB248] on YEPD plates at 35°C, if allowed to grow for 5-6, days showed visible sectoring of slow and fast-growing cell lines. This sectoring is also visible to a lesser extent with strain S150-2B [cir°, pJDB248]. When replica-plated onto plates selective for pJDB248, the regions of faster growth corresponded to plasmid-containing sectors. Cell lines containing pJDB248 are able to synthesise their own leucine. This process appears to be more efficient than taking leucine up from the surrounding media, therefore enabling plasmid-containing cells to outgrow plasmid free cells.

4.23 Stability of pJDB248 in plm2-1 over fifty generations of non-selective growth

Cultures of plm2-1 and S150-2B [cir°] containing pJDB248 were first grown in selective media at 24°C. Cell densities after an overnight period of growth were then calculated and the cultures inoculated to 5x10^4 cells/ml in fresh non-selective media. Identical cultures were set up at both 24°C and 35°C. Cultures were grown to between 8x10^6 and 2x10^7 cells/ml and then re-inoculated to 5x10^4 cells/ml in non-selective media to keep the cultures in log phase. This was repeated, with cultures being subbed back into fresh non-selective media until up to 50 generations of growth was achieved. At certain points throughout the experiments, aliquots of cells
Table 4.1

Doubling times obtained for plm2-1 and S150-2B [cir^6] with and without pJDB248

<table>
<thead>
<tr>
<th>Strain</th>
<th>Doubling time (mins)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24°C</td>
</tr>
<tr>
<td>S150-2B [cir^6]</td>
<td>105 (4.5)</td>
</tr>
<tr>
<td>plm2-1</td>
<td>111 (6.3)</td>
</tr>
<tr>
<td>S150-2B [cir^6, pJDB248]</td>
<td>124 (5.9)</td>
</tr>
<tr>
<td>plm2-1 [pJDB248]</td>
<td>130 (5.2)</td>
</tr>
</tbody>
</table>

Overnight cultures were innoculated into fresh media at a concentration of \(0.5-1.0 \times 10^5\) cells/ml. Samples were taken every 30-60 minutes and cell densities calculated. The growth of the cultures was plotted for each experiment and doubling times calculated.

Strains without plasmids were grown in YEPD media, strains with pJDB248 were grown in SD media lacking leucine.
were diluted and plated in triplicate onto YEPD plates at a concentration of approximately 200 colonies/plate. These plates were incubated at 24°C and after 3-4 days were replica-plated onto selective and non-selective plates to look for plasmid loss as indicated by leucine auxotrophy. The results for this experiment are shown in Figure 4.2.

The instability of pJDB248 at 35°C in plm2-1, previously observed in the plate screen, was confirmed. There is an approximately 50% reduction of plasmid-containing cells after 50 generations of growth of plm2-1 compared to the wild-type. It is interesting to note, however, that pJDB248 appears to be significantly more stable at 24°C in plm2-1 compared to S150-2B [cir°]. This observation was consistent in repetitions of the experiment.

4.24 Copy number of pJDB248 in plm2-1

It is important to determine plasmid copy number per plasmid-containing cell in the population of cells analysed in the stability experiments. If the copy number of pJDB248 in the plm2-1 strain is lower per cell than that observed for the wild-type strain, this would suggest that the instability of the plasmid is due to inefficient plasmid replication. If, however, plasmid copy number is higher or the same, then the instability may be due to incorrect plasmid partitioning.

Genomic DNA was prepared from cells harvested at time points throughout the plasmid stability experiments. This was then restriction digested with BamHI and electrophoresed on 0.8% agarose gels until separation of the expected band sizes was achieved. Genomic DNA prepared from plasmid-free isolates, and plasmid DNA only were also digested and electrophoresed in the same way. A Southern blot procedure was then carried out on the gels and the filters were probed with a radioactively-labelled LEU2 gene, obtained from plasmid YDp-L by BamHI restriction. An example of an autoradiograph is shown in Figure 4.3. Copy numbers were calculated by comparing the intensity of the band corresponding to the chromosomal copy of LEU2 in each lane, with the plasmid band on a Phospho-Imager, as described in Chapter Two. This calculation determines the average copy number of the plasmid throughout the entire population. To obtain a more informative copy number for the plasmid, the data obtained were corrected for the number of plasmid-containing cells within the population at that time point, as calculated previously in the stability analysis. The copy number per plasmid-containing cell of pJDB248 in plm2-1 compared to wild-type does not appear to be significantly different over the course of the experiment (Table 4.3).
Stability of pJDB248 over fifty generation of non-selective growth in \textit{plm2-1} and S150-2B [cir°]

Strains S150-2B [cir°, pJDB248] and \textit{plm2-1} [pJDB248] were grown exponentially for approximately 50 generations in media not selective for the plasmid (+ leucine). At time points, samples were taken from the cultures and plated onto YEPD. These plates were then replica-plated onto plates with and without leucine to determine numbers of plasmid-containing cells. The graphs show the average data obtained from two experiments.

This experiment was repeated at 24°C (Figure 4.2a) and 35°C (Figure 4.2b).
Figure 4.2

(a)

![Graph showing percentage of plasmid-containing cells over generations for two different conditions.

(b)

![Graph showing percentage of plasmid-containing cells over generations for two different conditions.

Legend:
- S150-2B [cir, pJDB248]
- plm2-1 [pJDB248]
Copy number gel

Genomic DNA, prepared from exponentially growing liquid cultures was digested with BamHI and electrophoresed. A Southern blot procedure was then carried out and the filter probed with a $^{32}$P-labelled LEU2 gene obtained by BamHI restriction of the plasmid YDp-L. Copy number is determined by calculating the intensity of the plasmid LEU2 band compared to the chromosomal LEU2 using a Phosphor-Imager.

Lanes

2. plm2-1 [pJDB248] after seven generations of growth at 24°C selective for the plasmid.
4. plm2-1 [pJDB248] after six generations of growth at 24°C, non-selective for the plasmid.
7. S150-2B [cir°]
8. pJDB248

Two bands are observed on this gel corresponding to cut and un-cut plasmid molecules. The intensity of both plasmid bands was calculated together to give copy number data.
Figure 4.3
Strains S150-2B [cir°, pJDB248] and plm2-1 [pJDB248] were grown exponentially for approximately 50 generations in media not selective for the plasmid (+ leucine). At time points, samples were taken from the cultures and plated onto YEPD. These plates were then replica-plated onto plates with and without leucine to determine numbers of plasmid-containing cells. At the same time points, cells were harvested and genomic DNA was prepared. The average plasmid copy number was calculated by comparing the radioactivity of the plasmid bands compared to chromosomal using a Phospho-Imager, as described in the Chapter Two. The copy number data was then corrected for the plasmid-containing cells in the population, as calculated previously. The experiment was repeated twice, the Tables show the complete set of data obtained for one of the experiments.

This experiment was repeated at 24°C (Table 4.2a) and 35°C (Table 4.2b).
Table 4.2

(a) 24°C

<table>
<thead>
<tr>
<th>Strain</th>
<th>No. of generations</th>
<th>% plasmid-containing cells</th>
<th>Average copy no.</th>
<th>Copy no. / plasmid-containing cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>S150-2B [cir°]</td>
<td>0</td>
<td>87</td>
<td>15</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>92</td>
<td>17</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>78</td>
<td>14</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>55</td>
<td>40</td>
<td>7</td>
<td>18</td>
</tr>
<tr>
<td>plm2-1</td>
<td>0</td>
<td>89</td>
<td>14</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>90</td>
<td>15</td>
<td>17</td>
</tr>
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<td>22</td>
<td>87</td>
<td>14</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>55</td>
<td>78</td>
<td>12</td>
<td>16</td>
</tr>
</tbody>
</table>

(b) 35°C

<table>
<thead>
<tr>
<th>Strain</th>
<th>No. of generations</th>
<th>% plasmid-containing cells</th>
<th>Average copy no.</th>
<th>Copy no. / plasmid-containing cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>S150-2B [cir°]</td>
<td>0</td>
<td>87</td>
<td>15</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>75</td>
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<td>9</td>
<td>17</td>
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<td>57</td>
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<td>8</td>
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</tr>
<tr>
<td>plm2-1</td>
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<td></td>
<td>57</td>
<td>20</td>
<td>3</td>
<td>17</td>
</tr>
</tbody>
</table>
4.25 The \textit{plm2-1} mutation is 2\mu m plasmid specific

To determine whether or not the plasmid-instability phenotype of \textit{plm2-1} is 2\mu m-specific, the stability of the ARS plasmid YRp7 (Figure 2.4) and the CEN, ARS plasmid YCp50 (Figure 2.3) was studied. The analysis was performed in the same way as described for the stability analysis of pJB248.

The results obtained for the stability of YCp50 is shown in Figure 4.4. The stability of YCp50 in \textit{plm2-1} is not significantly different from that in S150-2B [cir\textsuperscript{o}] strain. YRp7 data is incomplete because this plasmid was unable to be maintained extrachromosomally in the \textit{plm2-1} mutant during selective growth at 24\degree C (Figure 4.5). Genomic preparations of cultures containing YRp7 were digested with \textit{BamHI} and electrophoresed. A Southern blotting procedure was carried out and the filter was then probed with the \textit{TRPL} gene obtained by \textit{BamHI} restriction of plasmid YDpW. The absence of plasmid YRp7 is confirmed in \textit{plm2-1}.

4.3 Discussion

The phenotype of the plasmid maintenance mutant, \textit{plm2-1}, has been further characterised by a series of growth, plasmid stability and plasmid copy number experiments.

The results of the growth and viability experiments do not reveal any significant differences between the mutant and wild-type strains, with or without plasmid pJB248. One of the aims of these experiments was to identify a set of conditions under which enrichment for wild-type alleles may occur in a mixed population. This would be important for the cloning of \textit{PLM2} by complementation with a genomic library. However, the growth and viability of \textit{plm2-1} observed in these experiments demonstrate that enrichment for \textit{PLM2} in this way is not feasible. DAPI staining of mitotically dividing nuclei in both \textit{plm2-1} and wild-type cells suggests that the integrity of the nucleus, and the division of DNA between mother and daughter cells is unaffected by the mutation.

The main aim of the plasmid stability and copy number experiments was to determine whether or not the \textit{plm2-1} mutation is 2\mu m-specific, to enable further conclusions to be drawn as to the role of \textit{PLM2} in the host. The instability of pJB248 seen in the original plate screens was confirmed over 50 generations of liquid growth at 35\degree C. There were approximately 50\% less pJB248-containing \textit{plm2-1} cells than wild-type at the end of the experiment. The stability of YCp50, a low
Stability of YCp50 in \textit{plm2-1} and S150-2B \textit{[cir^0]}

Strains S150-2B \textit{[cir^0]}, YCp50 and \textit{plm2} \textit{[YCp50]} were grown exponentially for approximately 50 generations in media non-selective for the plasmid (+ uracil). At time points, samples were taken from the cultures and plated onto YEPD. These plates were then replica-plated onto plates with and without uracil to determine numbers of plasmid-containing cells. The graphs show the average of the data obtained for two experiments.

This experiment was repeated at 24°C (Figure 4.4a) and 35°C (Figure 4.4b).
Figure 4.4

(a)

(b)
YRp7 is not successfully maintained in plm2-1

Transformants of S150-2B [cir0, YRp7] and plm2-1 [YRp7] were grown in selective media (- tryptophan) overnight at both 24 and 35°C. The cells were harvested and genomic DNA was prepared. The DNA was restricted with BamHI and electrophoresed. A Southern blot procedure was then carried out and the filter probed with a 32P-labelled TRP1 gene obtained by restriction of plasmid YDp-W with BamHI.

Lanes

1  S150-2B [cir0]
2  YRp7
3  S150-2B [cir0, YRp7] grown at 24°C
4  plm2-1 [YRp7] grown at 24°C
5  S150-2B [cir0, YRp7] grown at 35°C
6  plm2-1 [YRp7] grown at 35°C

The autoradiograph demonstrates that the plm2-1 strain has no Yrp7 plasmid molecules maintained extrachromosomally. There is no band shift at the chromosomal locus, or appearance of further bands that may be indicative of integration of Yrp7 by a single recombination event. The trp+ phenotype of this strain may therefore be due to reversion of the chromosomal trp1-289 mutation, integration of TRP1 from Yrp7 by a double recombination event or perhaps gene conversion. No conclusions as to the behaviour of YRp7 in plm2-1 can be drawn from this data.
Figure 4.5
copy number CEN ARS plasmid, is not significantly different between the wild-type
and plm2-1 strains. This suggests that PLM2 has a role in 2μm plasmid maintenance
other than replication. The copy number data obtained for pJDB248 in plm2-1 over
the course of the stability experiments was not significantly different from that
observed in the wild-type population at 24 or 35°C. This also suggests that
replication of plasmid molecules is unaffected in the plm2-1 mutant. The stability
analysis of YRp7 in plm2-1 is incomplete so no conclusion as to the ability of the plm2-
1 strain to maintain an ARS plasmid can be made from this data.

Interestingly, the stability of pJDB248 at 24°C in plm2-1 was found to be
significantly increased compared to the stability over the equivalent number of
generations in S150-2B [cir^c]. This observation was reproducible. The plasmid
instability phenotype is only observed at 35°C, but these observations suggest that
the kinetics of Plm2p in the mutant is also altered at the permissive temperature. An
interaction of Plm2p with either DNA sequences or proteins important for its
function may be reduced at one temperature, but increased at the other.
Chapter Five

CLONING AND ANALYSIS OF PLM2

5.1 Introduction

Plasmid maintenance mutants (plm) have been isolated that show a reduced stability of 2μm-based plasmids (Chapter Three). One of the mutants, plm2-1, shows a clear phenotype of plasmid loss during plate screens, and also over forty culture doublings in liquid medium. Copy number analysis has revealed that the number of plasmid molecules per plasmid-containing cell in an exponentially growing population is not significantly different. This suggests that PLM2 is not involved in 2μm plasmid replication, a theory supported by the ability of the plm2-1 mutant to maintain a CEN-ARS plasmid at wild-type levels.

Identification of the PLM2 gene, or a gene that interacts with it, will further elucidate the mechanism of 2μm plasmid maintenance and the role of the host in this. Sequence and protein analysis may reveal the likely cellular location of Plm2p and sequence homology with families of genes or proteins with known function should give some indication as to its role.

5.2 Results

5.21 Complementation of plm2-1 with a yeast genomic library

The library chosen was that of Rose et al. (1987). This is a low copy number yeast genomic library based on the shuttle vector YCp50 (Figure 2.3). This library was chosen because YCp50 is a CEN-based vector and so has a copy number of 1-2 plasmid molecules per yeast cell. YCp50 carries the auxotrophic marker URA3 so that both a library plasmid and the LEU2-containing 2μm-based plasmid pJDB248 (Figure 2.2) can be selected for in the same strain. The library has been constructed by ligating partial Sau3AI genomic digests into the unique BamHI site within the TelR gene of YCp50. The published average size of the inserts in this library is 14.5kb, however previous use of this library in our laboratory had shown that insert sizes in our population of library plasmids were between 7-13kb (J. Morrissey, personal communication; personal observation). This reduction in insert size is probably due to selection against large inserts during repeated periods of growth of the E.coli
strain. It was calculated that to ensure the screening of the entire yeast genome, approximately 9200 colonies would need to be assayed (Figure 5.1).

The strategy for screening the library is summarised in Figure 5.2. The plate screen was based on that employed when isolating the original mutants. This time the stability, rather than the instability, of the 2μm-based plasmid pJDB248 was screened for to indicate a possible rescuing clone. The plm2-1 mutant was first transformed with pJDB248 and then a large-scale transformation of plm2-1[pJDB248] with the library was carried out. This resulted in approximately 10 000 successful transformants, each containing pJDB248 and a library plasmid, spread over 100 plates. These colonies were screened in comparison with plm2-1 [circ, pJDB248, YCp50] and PLM2 [circ, pJDB248, YCp50] transformants as controls.

Because there were approximately 100 transformants per plate, an initial replica-plating screen could be carried out directly. Colonies were replica-plated onto both plates with and without leucine, those with leucine were incubated at the non-permissive temperature of 35°C and those without leucine at 24°C. All plates lacked uracil so that selection for the library plasmid was maintained. This procedure was repeated up to four times, with each successive round of replica-plating being carried out on the non-selective plates. After this initial screen, approximately 200 colonies that grew successfully without leucine at the non-permissive temperature were picked into microtitre dishes and inoculated onto fresh leucine-containing plates, again at 35°C. The plates were replica-plated as described above for a further two rounds. This resulted in the identification of fifty-seven isolates that were still showing an increased plasmid stability inferred from their ability to grow without leucine. These remaining possible positive clones were rescreened by streaking the isolates to single colonies from plates that lacked leucine. These plates were then replica-plated onto fresh plates and incubated at 35°C and 24°C as before. This resulted in the number of possible rescuing clones being reduced to twenty-six.

The recombinant plasmids from the remaining transformants that still consistently rescued the plm2-1 phenotype were prepared from yeast and transformed into E.coli. Large-scale plasmid preparations were then carried out and the plasmids were then transformed back into plm2-1 [pJDB248]. The recombinant plasmids were also transformed into the wild-type strain S150-2B [circ]. The screen described above was based on the stability of a leu+ phenotype and so it was necessary at this stage to omit from the screen any isolates in which the library plasmid contained the LEU2 gene. The S150-2B [circ] strain transformed with the recombinant plasmids was selected for on plates without uracil and then replica-plated onto plates also lacking leucine. Twenty four of the remaining twenty six recombinant plasmids conferred a leu+ phenotype to S150-2B [circ].
Calculation of numbers of colonies to be screened for rescue of the \textit{plm2} phenotype

The probability of having any DNA sequence represented in a genomic library is given by the formula:

$$N = \frac{\ln(1 - P)}{\ln(1 - F)}$$

where

- $N =$ necessary number of recombinants
- $P =$ desired probability (for a 99% probability, $P = 0.99$)
- $F =$ fractional proportion of the genome in a single recombinant

The average size of inserts in the library used is between 7-13kb. The lower size of 7kb was used in the calculation:

$$N = \frac{\ln(1 - 0.99)}{\ln(1 - 7/14000)}$$

$N = 9208$
Figure 5.2

Strategy for screening a genomic library for recombinant plasmids that rescue the *plm2* phenotype

Strain *plm2 [pJDB248]* was transformed with a genomic library. 10 000 transformants were initially screened via a replica-plating regime. Colonies that demonstrated improved growth on plates lacking leucine were picked into microtitre dishes and seeded onto fresh non-selective plates. The replica-plating regime was followed again. Those patches that still consistently grew to a wild-type level in the absence of leucine were streaked to single colonies on plates with leucine at 35°C and then replica-plated onto plates without. Transformants that consistently grew to wild-type levels throughout this screen were selected for further analysis.
Figure 5.2

recombinant plasmids

\[ plm2 \text{ [pJDB248]} \]

10,000 transformants

replica plated

24°C - Ura - Leu

35°C - Ura + Leu

replica plated as before

patches with improved growth on
- Ura - Leu streaked to single colonies
on - Ura + Leu 35°C

replica plated

- Ura - Leu 24°C

transformants with improved plasmid stability identified
Fresh transformants of the two remaining clones, 27.5 and 31.3 in \textit{plm2-1} [pDB248] were rescreened and both still rescued the \textit{plm2-1} phenotype (Figure 5.3). Both inserts were the same size and had identical restriction maps with all enzymes used. It was therefore concluded that clones 27.5 and 31.3 were identical. Figure 5.4 shows the restriction map calculated for the insert within recombinant plasmid 27.5.

Clone 27.5 was also transformed into the \textit{plm2-2}, \textit{plm2-3} and \textit{plm3-1} strains. An identical plate screen was carried out as described above. Clone 27.5 rescued the \textit{plm2-2} phenotype in the \textit{plm2} strains, but not in the \textit{plm3-1} strain. This confirmed the placement of these alleles into their complementation groups (data not shown).

5.22 Clone 27.5 represents a region of chromosome IV

The majority of the yeast genome had been sequenced at this stage so it was decided to sequence only a small part of the insert, from each end. From these data, a homology search against sequences submitted to the EMBL database would make it possible to retrieve the rest of the sequence. Primers TetA and TetB were designed flanking the \textit{BamHI} site in the \textit{Tet^R} gene of YCp50 to enable sequencing into the insert from both ends (Figure 5.5). Obtaining sequence from both ends of the clone was important to ensure that the insert consisted of a sequence relating to a continuous stretch of DNA, rather than to more than one \textit{Sau3A1} fragment ligated together. Also, obtaining sequence from both ends of the clone could confirm the position of the insert sequence in the genome.

Approximately 500 base pairs of sequence was obtained from each primer (Figure 5.5). These sequences were compared to sequences submitted into the EMBL database from the yeast genome mapping project. A FASTA homology search (Pearson and Lipman, 1988) was submitted through the Stanford Genome Database accessed via the Internet at http://genome www.stanford.edu/Saccharomyces. Homologies to the two sequences were found 9.5kb apart in a region on the right arm of chromosome IV (cosmid U33057, accession number sc9719). This corresponded to the size of the insert as approximated from restriction mapping. Comparison of the restriction map that had been calculated previously to the one obtained from the database for this region suggested that these regions were the same. Moreover, PCR products were generated from clone 27.5 from primers designed to the cosmid sequence (data not shown). The restriction maps can be compared (Figures 5.4b and 5.6a). The main discrepancy between the two maps is on the left where there is a cluster of \textit{ClaI} and \textit{HindIII} sites in the map obtained from the database. These were not detected in the map of clone 27.5, probably because the restriction fragments
Clone 27.5 rescues the \textit{plm2} phenotype

Single colonies from strains \textit{plm2-1}[pJDB248, YCp50] and \textit{plm2-1}[pJDB248, clone 27.5] were picked from plates lacking leucine (selective for pJDB248) and streaked to single colonies on plates containing leucine (non-selective for pJDB248). These plates were then incubated at 35°C. Four colonies from these were then picked into microtitre dishes and seeded onto fresh non-selective plates at 35°C. These plates were then replica-plated onto selective plates. Uracil was omitted from all plates to maintain selection for YCp50 and clone 27.5 throughout the screen. On the plate shown opposite, a clear difference in patch growth is seen between the strain containing clone 27.5 and the strain containing the control plasmid YCp50.
Figure 5.3

$plm2$ [pJDB248, YCp50]

$plm2$ [pJDB248, clone 27.5]
Figure 5.4

Restriction mapping of clone 27.5

(a) An example of a gel analysed to determine the restriction map of the genomic insert in recombinant plasmid 27.5

Recombinant plasmid 27.5 was restricted with a variety of restriction enzymes. All reactions were electrophoresed through 0.8% agarose gels, and stained with ethidium bromide.

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(b) Restriction map calculated for the genomic insert in clone 27.5

C = ClaI
H = HindIII
X = XbaI
B = BglII
K = KpnI
E = EcoRI
S = SalII
Figure 5.4

(a)

kb  M  1  2  3  4  5

23.1
9.4
6.6
4.4
2.3
2.0
1.4
1.1

(b)

Insert

1kb
Figure 5.5

Homology search with sequence obtained for primer TetA

Sequencing reactions were performed using TetA and TetB as primers to generate sequence into both ends of the insert on clone 27.5. The sequence obtained was compared using a FASTA homology search against sequences in the Saccharomyces Genome Database. The results of the homology search for the sequence generated from primer TetA is shown opposite.
Figure 5.5

D1445 Chromosome IV Region from 1445001 to 145500

initn: 1815 initl: 1523 opt: 2007 Z-score: 1417.6 expect() 0
92.8% identity in 477 nt overlap

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Figure 5.6

The restriction map and position of open reading frames of clone 27.5 as determined by sequence analysis

(a) Restriction map

C H H C H H
C X H B K B X H B E S

Insert

1kb

C = Clal, H = HindIII, X = XbaI, B = BglII, K = KpnI, E = EcoRI, S = SalI

(b) Location and orientation of predicted open reading frames

SEC20 D9719.5 RPL35B D9719.7 SAM2

Insert

1kb
generated were too small for the sensitivity of the agarose percentage of the gels analysed.

A map of the predicted open reading frames in the insert is shown in Figure 5.6b. There are four complete ORFs plus the 3' end of SAM2, the gene for S-adenosylmethionine synthetase. Of the four complete ORFs, only SEC20 (integral membrane protein required for endoplasmic reticulum (ER) to Golgi transport in the yeast secretory pathway; Sweet and Pelham, 1992) had previously been characterised. The evidence for the presence of the other three ORFs was non-experimental, based only on sequence data.

**Tagged Tn transposition of clone 27.5**

To identify which ORF was that responsible for the rescue of the plm2-1 phenotype a transposon mutagenesis procedure was employed. This strategy was chosen because of the absence of suitable restriction sites for sub-cloning individual ORFs. Also, a PCR strategy for sub-cloning would carry a risk of introducing potential function-destroying point mutations into the ORFs. It was also not known how much of the sequence upstream of each potential ORF may be important for full gene function.

The procedure for tagged Tn transposition is outlined in Figure 5.7 (Sedgewick and Morgan, 1994). The aim of this mutagenesis procedure was to create a set of plasmids that contain Tn1000 cassettes in each of the ORFs present on the insert of clone 27.5. Transformants of plm2-1 containing mutagenised plasmids were then screened for a plm' phenotype. Loss of complementation of plm2-1 would suggest that the Tn1000 had inserted into the ORF responsible for the initial rescue seen with clone 27.5. There are two further advantages of using this procedure to identify ORFs of interest. Primers are available for PCR that enable sequence to be generated out of either 6 or y ends of the transposon. This enables easy placement of the cassettes by PCR and/or sequencing. Secondly, if the mutagenesis is carried out with a Tn1000 cassette carrying a yeast auxotrophic marker, this creates a disruption cassette for subsequent allele replacement experiments.

An R388 (IncW) based conjugative plasmid containing a Tn1000::HIS3 construct in a Sm-sensitive donor E.coli strain was chosen for this procedure. The HIS3 gene is important because further work with this construct in yeast would be facilitated if the cassette was able to be selected for, preferably with an auxotrophic marker different to those resident on plasmids that were frequently used throughout this work. After transformation of a target plasmid into the donor cell, in this case clone 27.5 under selection for ampicillin resistance, the Tn1000::HIS3 cassette on the conjugative plasmid is able to duplicate itself and one copy can insert randomly into
Figure 5.7

Tagged Tn1000 transposition (Sedgewick and Morgan, 1994)

A. Duplicative insertion links a conjugative plasmid carrying Tn1000 in a cointegrate with the target plasmid.

B. Conjugation transfers the cointegrate from the donor to the recipient cell.

C. Site-specific recombination between the two copies of the transposon in the cointegrate releases a transposed target plasmid and the original conjugative donor plasmid.

After mating, selection with streptomycin (Sm) eliminates the donor cells, and with additional ampicillin (Amp), all the recipient cells are also killed except for those which received a transposed target plasmid through conjugation.
Figure 5.7

**Sm\( ^{S} \)**

- Conjugative plasmid
- Target plasmid
- Amp

**A**

Donor Cell

**B**

Mating

**C**

Recipient Cell

**Sm\( ^{R} \)**

- Conjugative plasmid
- Amp
- Transposed plasmid
- Tn
target plasmid sequences. The two copies of the Tnl000::HIS3 are then able to recombine with each other, resulting in the formation of an intermediate. These intermediates are then able to transfer across to recipient cells during conjugation.

After a filter mating between the donor strain and a Sm<sup>R</sup> recipient E.coli strain, transconjugants can be selected for on plates containing both ampicillin and streptomycin. The streptomycin selects against donor cells and the ampicillin selects for only those recipients that receive the intermediate as described above. Once into the recipient cells, the intermediates are able to resolve themselves into the original conjugative plasmids and the target plasmids that now each contained a copy of the Tnl000::HIS3 cassette randomly inserted. This procedure was carried out successfully with clone 27.5 as the target plasmid.

The positions of the transposons within each plasmid was initially determined via restriction analysis. In the original 27.5 clone, EcoRI cuts once in the insert, 1.6kb from the vector sequence on the right, and once in YCp50, 300bp from the insert on the left. This produces two bands approximately corresponding to the insert and the vector (Figure 5.8a). Digests using miniprep DNA from the transconjugant colonies produced loss of one of these bands, and production of extra bands corresponding to the insertion of the 6kb transposon cassette in either vector or insert sequences. This initial analysis was carried out on thirty-six colonies. Insertion of the transposon into the smaller EcoRI fragment occurred in nineteen of these. Mapping of the transposon more precisely with Kpnl revealed that there was a hotspot for TnlOOO insertion in the SEC20 gene. Of the nineteen plasmids, sixteen had the transposon cassette inserted in SEC20 and three had the cassette inserted into ORF D9719.5. To identify disruptions of the other ORFs, a PCR strategy was employed. PCRs were set up to amplify miniprep DNA using the TetB vector primer only in conjunction with either δ or γ Tnl1000 primers. The reaction favoured amplification from transposons that had inserted no more than 2.5kb from the site of the TetB primer. This identified two transposons inserted into ORF D9719.7. The positions of these transposons was confirmed by restriction analysis. Ultimately, all ORFs on the insert except D9719.6 were disrupted. A summary of the positions of the Tnl1000::HIS3 cassettes in clone 27.5 that were screened for loss of rescue of the plm2-1 phenotype are shown in Figure 5.8b.

The screen for loss of rescue of the plm2-1 phenotype was a plate screen similar to that used for initially identifying clone 27.5. The plasmids mutated by the transposon, the initial clone 27.5 and YCp50 were transformed separately into strains plm2-1 [pDB248] and S150 [cir<sup>9</sup>, pDB248]. Transformants were plated onto media lacking both leucine and uracil, and in the case of the transposon-mutagenised plasmids, also lacking histidine. However, during the actual screen all plates contained histidine for accurate comparison of growth of the control strains.
Figure 5.8

Mapping and screening of transposons on mutagenised plasmids

(a) Restriction digestion with EcoRI

<table>
<thead>
<tr>
<th>Lane</th>
<th>Plasmid</th>
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<tr>
<td>1</td>
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<td>5K</td>
</tr>
<tr>
<td>9</td>
<td>clone 27.5</td>
</tr>
</tbody>
</table>

The restriction of 27.5 with EcoRI produces two bands of 7kb and 9.5kb. The 7kb band contains the majority of the insert sequence, and so transposed plasmids in which this band has disappeared were investigated further. Examples of transposon insertions into this 7kb band are plasmids 3P, 3L, 3P, 3R, 3T and 5K.

(b) Insert from recombinant plasmid 27.5 showing positions of transposons

All plasmids were mapped via restriction mapping with enzymes EcoRI and KpnI. Positions of transposons in plasmids 19 and 30 was confirmed by PCR.

(c) Table showing the ability of the transposed plasmids to rescue the plm2 phenotype

A screen for plasmid loss was performed on transformants of plm2-1 [pJDB248] with the transposed plasmids. The screen was that shown in Figure 3.4.
Figure 5.8

(a) kb lane

<table>
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</table>

(b)

Clone Rescue pim 2?

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<th>3C</th>
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<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

1kb
containing plasmids YCp50 or 27.5, and those containing the transposed experimental plasmids. All plates lacked uracil to maintain selection for the YCp50-based plasmids. The stability of the HIS3 phenotype conferred by the Tn1000::HIS3 construct was confirmed at the end of the screen by replica-plating onto plates without histidine.

Transformants were streaked to single colonies on plates containing leucine at 24°C. Single colonies were taken from these and streaked to single colonies on plates with leucine at 35°C. Up to eight colonies for each plasmid to be analysed were then picked from these plates into microtitre dishes. Cells were then inoculated onto further plates containing leucine and incubated again at 35°C. Once grown, these plates were replica-plated onto plates with and without leucine. At all times, the plates with leucine were incubated at 35°C and those without at 24°C. The replicating protocol was repeated on the plates that had allowed for the loss of pJDB248, followed by 35°C incubations, for up to four times until a clear difference in growth between the controls was observed.

The results are summarised in Figure 5.8c. The two plasmids that could now no longer rescue the plm2-1 phenotype were those that contained the Tn1000::HIS3 cassette in ORF D9719.7. It was therefore concluded that ORF D9719.7 was the gene responsible for the complementation or suppression of the plm2-1 mutation.

5.23 Disruption of ORF D9719.7

A wild-type gene of interest can be replaced by transforming into the yeast strain a linear piece of DNA that contains the relevant gene altered in some way to create a defined allele for further analysis. This alteration normally takes the form of an auxotrophic marker gene cloned into the gene of interest so that integration events can be selected for (Scherer and Davis, 1979). The ends of linear pieces of DNA are highly recombinogenic and recombination will be targeted specifically to sequences homologous to these ends (Orr-Weaver et al., 1981). Linearising the DNA in this way is also important to minimise the proportion of recombination events between the auxotrophic marker and its chromosomal homologue. Recombination between homologous sequences then occurs resulting in the gene disruption cassette being inserted into the chromosomal locus of the original gene.

The use of the tagged Tn1000 transposition to identify the ORF of interest on the insert meant that potential gene disruption cassettes had already been created. The cassette consists of a HIS3 gene inserted in place of a 1kb EcoRI fragment at the γ end of the Tn1000. The entire construct is approximately 6kb in length.
The transposon in construct 30 is positioned 300bp into the coding sequence of ORF D9719.7 and shows a more significant loss of rescue of plm2-1 than that in construct 19, nearer the 3' end of the gene. Gene disruptions were carried out using the disrupted D9719.7 allele from plasmid 30 (Figure 5.9). Plasmid 30 was restricted with XbaI and Eco72I. The digest was then transformed directly into both a haploid, S150 [cir°], and a diploid, 842 [cir°] strain. The transformation was performed in a diploid as well as a haploid in case the position of the transposons in ORF D9719.7 reduced cell viability. The transformants were plated onto media lacking histidine. Because the whole digest had been transformed into the strains, there was a possibility that the transformants contained whole, unrestricted, plasmid molecules rather than those in which targeted integration had taken place. The transformants were replica-plated or patched, depending on the density of colonies, onto plates lacking both uracil and histidine and also plates just lacking histidine. Approximately half of the transformants in each case were able to grow on both plates, indicating that intact plasmids had been transformed in these cases. Only transformants that were unable to grow without uracil, and were therefore lacking vector sequences, were considered likely to have had the correct integration event occurring.

To confirm the identity of the integrants, colonies were picked and genomic DNA was prepared. The DNA was restricted with HpaI, which doesn't cut within the cassette and cuts outside of ORF D9719.7, and electrophoresed on an agarose gel until size separation of the expected bands occurred. The genomic DNA restrictions were then probed with a radioactively-labelled HindIII fragment. This is a 2kb fragment that lies within the HpaI fragment containing ORF D9719.7 (Figure 5.10a). In those cases where ORF D9719.7 had been replaced with the disrupted version, a band shift is seen corresponding to the 6kb size of the Tn1000::HIS3 cassette (Figure 5.10b). This confirmed that the chromosomal ORF D9719.7 had been successfully replaced with that within construct 30. The transposon mutagenesis procedure doesn't create a null allele of the gene, but creates an allele that has a 6kb insert. In this case, the D9719.7 allele has an insert approximately 300bp into the translated sequence. This means that rather than no protein translation from this sequence, a truncated protein will still be produced that may retain some function.

Analysis of the disruptant strains

A plate screen was performed on the disrupted haploid transformed with pJDB248, identical to that performed previously during the transposon mutagenesis procedure. Periods of growth non-selective for pJDB248 were carried out at 24°C, 30°C and 35°C. It was determined that the disruptant has the same phenotype of
Figure 5.9

Strategy for targeted integration of the Tn1000::HIS3 cassette into D9719.7

Target plasmid 30 was restricted with enzymes XbaI and Eco72I. Linear fragments were then transformed into haploid strain S150-2B [cir°, pJDB248] and diploid strain 842 [cir°, pJDB248].

Recombination of the fragment containing the mutated ORF D9719.7 was expected to be targeted to the homologous site in the genome.
Figure 5.9

Eco72I

D9719.7::Tn1000::HIS3

XbaI

restriction with XbaI and Eco72I

targeted recombination with genomic

D9719.7

D9719.7::Tn1000::HIS3
Confirmation of targeted integration

(a)

Diagram showing the presence of D9719.7 on a HpaI fragment. The HindIII sites shown are those used for generating the probe fragment. Not all HindIII sites in this region are shown. Diagram not to scale.

(b) Autoradiograph

<table>
<thead>
<tr>
<th>Lane</th>
<th>DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>clone 27.5</td>
</tr>
<tr>
<td>2</td>
<td>S150-2B [cir(^o), D9719.7]</td>
</tr>
<tr>
<td>3</td>
<td>S150-2B [cir(^o), D9719.7::Tn1000::HIS3]</td>
</tr>
<tr>
<td>4</td>
<td>842 [cir(^o)]</td>
</tr>
<tr>
<td>5</td>
<td>842 [cir(^o), D9719.7::Tn1000::HIS3, D9719.7]</td>
</tr>
</tbody>
</table>

Genomic DNA was prepared from strains and digested with HpaI and electrophoresed. A Southern blot procedure was then carried out with \(^{32}\)P-labelled HindIII fragment shown in Figure 5.10a.
Figure 5.10

(a)

Tn1000::HIS3 cassette

HpaI HindIII HindIII HpaI

ORF D9719.7

(b)

lane

1 2 3 4 5

D9719.7::Tn1000::HIS3

D9719.7
plasmid loss as the original \textit{plm2-1} allele, however the disruptant is not temperature-sensitive (data not shown).

The disrupted strain 842 [\textit{cir}°, \textit{pJDB248}] had a \textit{plm+} phenotype, as would be expected from the previous genetic analysis that demonstrated that \textit{plm2-1} was a recessive mutation (see Chapter Three). The diploid was sporulated and eight complete tetrads were dissected. The \textit{his}+ phenotype co-segregated 2:2 with the \textit{plm}− phenotype in each of the tetrads, confirming the previous hybridisation experiment that demonstrated that the integration of the \textit{D9719.7::Tnl000::HIS3} cassette had been targeted to the genomic copy of the \textit{D9719.7} gene.

\textbf{Is ORF D9719.7 allelic to PLM2?}

The rescuing clone of the \textit{plm2-1} phenotype was isolated on a low copy number plasmid, however there was still a possibility that a suppressor of \textit{plm2-1} rather than the \textit{PLM2} gene itself had been cloned. A diploid was constructed that contained the original \textit{plm2-1} allele, plus a disrupted ORF D9719.7. If the two loci are allelic, no complementation would occur and the diploid will have the \textit{plm2-1} phenotype.

Strain 842 A4 [\textit{cir}°,\textit{MATa}, ORF D9719.7::Tnl000::\textit{F[IS3]}, obtained from the previous sporulation of the disrupted 842 diploid strain, was mated with the S150-2B [\textit{MATa}, \textit{plm2-1}, \textit{pJDB248}] strain. The plate assay was carried out on the diploid as before and compared with the stabilities shown for other diploids and haploids as controls. The stability of the plasmid in the diploid was reduced compared to the positive diploid controls (Figure 5.11). It was concluded that \textit{PLM2} is allelic to \textit{D9719.7}.

5.24 DNA sequence comparison of PLM2

The sequence of the \textit{PLM2} open reading frame, the translation start and the amino acid sequence is shown in Appendix 2. \textit{PLM2} is a 1.6kb gene encoding for a predicted protein of 521 amino acids. A putative TATA box is highlighted on the sequence 120bp upstream of the translation start. A search against Transfac, a transcription factor binding site database was carried out on the 500bp upstream of the translation start site. This revealed no strong homologies with any sequences other than the 6bp core sequence of the \textit{GCN4} binding site, a transcription factor important for regulation of amino acid biosynthesis (Arndt and Fink, 1986; Hinnebusch, 1992).

A FASTA homology search (Pearson and Lipman, 1988) against the rest of the yeast sequences in the EMBL database reveals that \textit{PLM2} has 62.9% homology at the
Figure 5.11

D9719.7 is allelic to PLM2

Diploid strains PLM2/PLM2, PLM2/plm2-1, plm2-1/plm2-1 and plm2-1/D9719.7::Tn1000::HIS3 were transformed with pJDB248. A plate screen was carried out as described in Figure 3.3. The plate opposite lacks leucine and demonstrates that the homozygous plm2-1 and plm2-1/D9719.7::Tn1000::HIS3 diploids have the same phenotype of plasmid loss.
DNA level, over 423bp, with a putative ORF, *L9470.22*, on chromosome XII of unknown function (Figure 5.12).

Homology searches at the DNA level are generally not as informative as homology searches at the protein level because of the redundancy of the third bases in codons. A match at the sequence level becomes more significant if the search reveals matches with a set of genes of similar function, thereby possibly identifying a sequence motif present in a known gene family. Homology searches against all sequences present on the EMBL database, both with yeast and all sequences submitted, reveals no significant homologies with any set of genes of known function.

5.25 Sequence comparison of Plm2p

Two analyses of the amino acid composition of Plm2p were carried out. The results are shown in Figure 5.13. A hydrophilicity plot (Kyte and Doolittle, 1982) shows Plm2p to be a strongly hydrophilic protein, and an analysis of charge over the length of the protein (Hopp and Woods, 1981) reveals areas of strong net positive charge. Both these features are indicative of putative DNA-binding proteins.

Homology searches using the amino acid sequence against a variety of databases proved to be more informative than the DNA sequence homology searches. A variety of protein homology searches are available. Three different ones were chosen in this case to avoid any bias of the algorithms used in any one of the searches to detect a particular set of homologies. All the homology searches were carried out against the SwissProt database.

A FASTA search (Pearson and Lipman, 1988) uses a fixed set of parameters to firstly detect strong regions of pairwise identity between sequences (identical amino acids) and then looks for sequence similarity (amino acids that can be substituted for each other). The program then allows for multiple regions of similarity to be joined, so increasing the score, and significance, of related sequences. This is a useful search for looking for distant, but biologically related, sequences. The majority of the significant homologies are in nuclear located proteins from a wide range of eukaryotes, prokaryotes and viruses. Many of these are DNA-binding proteins such as RNA polymerases and transcription factors, though the regions of homology are not generally within the expected DNA-binding domains of these. The best homologies were with two proteins that belong to the MHC class II of regulatory factors. The same region of Plm2p showed an identical homology with these proteins from both human and mouse; 19.1% identity (68.1% similarity) over 47 amino acids in both. The role of these factors is to bind to regulatory sequences.
PLM2 has homology with an open reading frame on chromosome XII

A FASTA analysis (Pearson and Lipman, 1988) was carried out with PLM2 against all sequences submitted in the EMBL database. The best match was with L9470.22, a putative gene of unknown function on yeast chromosome XII.

PLM2 is on cosmid U33057, from 13625-15190

L9470.22 is on cosmid U17246, from 22707-24176 (rev)
Figure 5.12

SCORES
62.9% identity in 423 bp overlap

U33057
TTATGCTATGGAATTTGCTTCTCGGAATTAATCTGCTTCTCGGCTTTTTCAAAATAACCTGT
15189 15179 15169 15160
15159 15149 15139 15129 15119 15109
U33057
TTATGTGCATATGGCTTCTCGGAATTAATCTGCTTCTCGGCTTTTTCAAAATAACCTGT
22740 22750 22760 22770 22780 22790
15099 15089 15079 15069 15059 15049
U33057
TTATCTCGGAATTTGCTTCTCGGAATTAATCTGCTTCTCGGCTTTTTCAAAATAACCTGT
22800 22810 22820 22830 22840 22850
14979 14969 14959 14949 14939 14929
U33057
GAATCTCGGAATTTTCTGGCTTCTCGGAATTAATCTGCTTCTCGGCTTTTTCAAAATAACCTGT
22920 22930 22940 22950 22960 22970
14859 14849 14839 14829 14819 14809
U33057
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23040 23050 23060 23070 23080 23090
14799 14789 14779 14769 14759 14749
U33057
TTATGTAATGAATTTTCTGGCTTCTCGGAATTAATCTGCTTCTCGGCTTTTTCAAAATAACCTGT
23100 23110 23120 23130 23140 23150
U33057
TTATATCGATGATAGTGAATCTTGTCTCAGGGTTTGTTCCTCGTGATCAACAGAGGAGAC
23160 23170 23180 23190 23200 23210
Figure 5.13

Analysis of hydrophilicity and charge over the length of Plm2p

(a) Hydrophilicity (Kyte and Doolittle, 1982)

(b) Analysis of charge (Hopp and Woods, 1981)

All analyses were carried out using the GeneJockey molecular biology application for the Apple Macintosh.
Figure 5.13

(a) Hydrophilicity value (Hopp-Woods, 1981)

(b) Hydropathy index (Kyte-Doolittle, 1982)
upstream of MHC class II genes (Reith et al., 1990). The most significant homologies with yeast sequences using this search were with Srb9p (suppressor of RNA polymerase B; Hengartner et al., 1995) with 25% identity (79.5% similarity) over 44 residues and Rad50p (a DNA repair protein; Alani et al., 1989) with 17.1% identity (64.8% similarity) over 193 residues.

A BLAST analysis (Basic Local Alignment Search Tool, Altschul et al., 1990) is different to the FASTA analysis in that weaker but biologically significant similarities can be detected. However, by increasing the sensitivity of the search, the selectivity is reduced so many chance matches are also identified. The program also seems unable to identify regions of similarity if gaps are abundant, resulting in most of the matches identified being over less than twenty amino acids. The parameters of the search can be altered to decrease the sensitivity and find stronger homologies, however in this search the default parameters were used. None of the sequence homologies detected in this search were the same as those from the FASTA search. Also, there didn’t seem to be much functional relationship between the proteins identified. The best alignment was with the anthranilate synthase component II (Trp1p, involved in tryptophan biosynthesis, Revuelta and Jayaram, 1987) from the yeast Phycocyes blakesleeanus with 28% identity (69% similarity) over 42 amino acids. The best match with budding yeast was with Npl1p (SEC63) with 22% identity (62% similarity) over 27 residues. Npl1p is essential for cell growth and germination, and there is evidence that Kar2p may physically interact with it in an ATP-dependant manner. Npl1p is localised to the inner nuclear membrane as well as the nuclear envelope and endoplasmic reticulum (Sadler et al., 1989).

The BLITZ search uses a different algorithm again from the other two searches (Smith and Waterman, 1981). The search is more comparable with the FASTA, in that multiple homologies along a sequence are considered together to give an overall score. This search also has an advantage in that it gives a probability score to each match, calculating the number of matches to the aligned sequence that would be expected to occur randomly. The two most significant matches were with yeast sequences. The most significant was against Msb2p (multicopy suppressor of a budding defect) with 24.1% identity (42.6% similarity) over 216 residues.

Two other homology searches were carried out against databases that are specific for functional domains within proteins, and good scores can often give a better idea of the actual function of the query protein. A BLAST analysis was carried out against the SBASE protein domain library (Murvai et al., 1996) and the Prosite database (Bairoch et al., 1996). The databases are both collections of annotated protein domain sequences representing various structural, functional, ligand-binding and topogenic segments of proteins as defined by their publishing authors. No
significant domain homologies were found, other than possible phosphorylation sites.

5.3 Discussion

This Chapter describes the successful cloning of PLM2, a gene involved in 2μm plasmid maintenance. PLM2 is a 1.6kb ORF encoding for a predicted protein of 521 amino acids. Analysis of the sequence upstream of the translation start codon reveals the presence of a TATA box, consistent with this ORF being transcribed, rather than being a pseudogene. PLM2 was found to be allelic to ORF D9719.7, located on the right arm of chromosome IV approximately 75kb from the telomere.

A haploid strain containing an insertion of a 6kb Tn1000::HIS3 cassette 300bp into the coding sequence of PLM2 is viable. This may be due to the presence of a homologue of PLM2 on chromosome XII. Alternatively, the 300bp of the gene downstream of the insertion may be translated into a partially functional peptide.

The homology searches carried out on the predicted protein sequence of Plm2p reveals significant matches with nuclear-located proteins. This is consistent with the phenotype of the plm2-1 mutation of plasmid loss, and the models proposed for 2μm plasmid maintenance. The phenotypic analysis of the plm2-1 mutant carried out in Chapter Three suggested that the plm2-1 phenotype of plasmid loss was not due to a replication defect. The copy-number of the 2μm-based plasmid pJD248 remains at wild-type levels in plasmid-containing cells. Also, the stability of the CEN-ARS plasmid YCp50 was unchanged in the mutant compared to the wild-type.

The proteins with the best homology scores all have DNA-binding functions, such as DNA-directed RNA polymerases and transcription factors. There is no significant homology with structural proteins, or proteins known to be part of the nucleoskeleton. The role of Plm2p in 2μm plasmid maintenance therefore, may be a regulatory role important for the correct expression of plasmid-encoded genes involved in partitioning. Alternatively, it may act as a chaperone, perhaps relocating plasmid molecules away from the replication machinery to other proteins that may then bind plasmids during cell division itself.
Chapter Six

ANALYSIS OF SEQUENCE HOMOLOGY BETWEEN CHROMOSOMES IV AND XII

6.1 Introduction

The completion of the yeast genome project, with the entire sequence of *Saccharomyces cerevisiae* now available in the public domain has enabled detailed analysis of the organisation of a eukarytic genome. The degree of redundancy uncovered in the genome has opened up major questions regarding function, organisation and evolution. There are a wealth of genes with sequence homologues and much of the sequence may have originated from duplications followed by divergence. If this homology is functional as well, this perhaps explains why so many genes aren’t essential with a minority of single gene disruptions being lethal to the cell.

The presence of duplicated regions offers insights into the mechanisms involved in the evolution of the yeast genome. These duplications occur among single genes and also among regions containing more than one gene. Duplications have also been identified between sub-terminal telomere elements, such as the Y’ elements.

The majority of gene families containing multiple homologues with similar functions are found adjacent to telomeres. These gene families are generally involved in carbon metabolism, or other functions important for fermentation. Well characterised families include *MEL, MAL, SUC, RTM* and *FLO*. The *MEL* family consists of at least ten structural genes for melibiose-hydrolysing enzyme α-galactosidase (Naumov *et al.*, 1991). All are located next to telomeres, but each is on a different chromosome (Turakainen *et al.*, 1993). Sequence homology has been confirmed by Southern hybridisation and by direct sequence comparison (Turakainen *et al.*, 1994). The presence of Ty delta sequences downstream of some of the *MEL* genes indicates that these duplications may have occurred via Ty-mediated recombination, a mechanism also suggested for the duplication of the *MAL* (maltose metabolism) family of genes (Charron *et al.*, 1989).

The *SUC* (sucrose metabolism) family consists of six identified genes, each present on separate chromosomes. These genes are situated between the X and Y’ elements of subtelomeric regions, apart from *SUC2* which is situated more centromere proximal (Carlson and Botstein, 1983; Carlson *et al.*, 1985).
Homologous recombination between large telomeric repeat sequences has been suggested as a mechanism for these, and other gene duplication events adjacent to telomeres (Carlson et al., 1985). A relatively new gene family, RTM, has also been identified in this region, always physically associated with the SUC telomeric loci (Ness and Aigle, 1995). The RTM genes confer resistance to molasses toxicity. Interestingly, the RTM genes are not always found in laboratory strains of S.cerevisiae, but can be found in multiple copies in the genomes of yeast used in industrial biomass or ethanol production where molasses is used as a substrate. This is an example of genomic rearrangements playing a role in adaption and evolution of the yeast to its environment.

One of the most extensive continuous sequence homologies so far reported involves the duplication of a region of approximately 30kb adjacent to the right telomere of chromosome VIII. This shares over 90% identity to the same region of chromosome I (Johnston et al., 1994). A smaller part of this region is also found duplicated on the left arm of this chromosome. Within this 30kb are six ORFs, which are duplicated in the same order and orientation. The only major difference is the presence of a Ty1 element in chromosome VIII, not present in chromosome I, but this is possibly due to a LTR (long tandem repeat) of Ty1 present in chromosome I at this location. The borders of the duplicated region coincide with coding sequence, but extends through non-coding sequence. There is a possibility that the duplication is ancient, with homology in the non-coding sequences being maintained by lack of pairing preventing homogenisation by gene conversion events. However, the duplication is more likely to be a recent event. Shorter duplicated segments of this subtelomeric region are also found in analogous positions of chromosomes III and XI. This homology varies from 54 to 94% and is largely limited to four of the six ORFs. Further subtelomeric duplications have been identified between the left and right arms of chromosomes XI and III (Dujon et al., 1994).

A large duplication has also been reported between chromosomes X and XI (Galibert et al., 1996). This is a duplication of a syntenic segment, where a syntenic segment is defined as two or more genes situated closely on the same chromosome which have their homologous loci positioned near each other on another chromosome in the same order and orientation with respect to each other. The physical distance and nucleotide sequence between the ORFs on a syntenic segment are not necessarily conserved. The duplication between chromosomes X and XI involves separate regions, one of three ORFs and another of five, separated by 30kb of non-homologous sequence. Chromosome X has less unique genes in this region than chromosome XI, suggesting that this is the ancestral locus from which the other was duplicated. However, the presence of
novel genes on both chromosomes in this region suggests that the duplication event is ancient.

A centromere duplication has been identified between chromosomes XIV and III (Lalo et al., 1993). The region of homology extends over 15kb and contains the centromere, five ORFs including two tDNAs, and a truncated Ty-delta element. These are all identical in order and transcriptional orientation relative to the centromere. Additional inserts bearing unique genes are present on the centromeric region of chromosome III which indicates an ancient duplication with chromosome XIV being the ancestral sequence. The level of silent substitutions in the third codon suggests that this duplication pre-dates the emergence of S. cerevisiae and S. douglasii as distinct species.

The duplications described above may have arisen by a variety of mechanisms. In the cases where only the coding sequences share homology, it is possible that processed genes have been inserted into the genome. However, this is unlikely because each ORF duplication would have had to occur by a separate integration event of a full length cDNA. Also, the homologous region of 30kb described between chromosomes VIII and I involve an ORF with a duplicated intron. A second explanation involves the duplication of the entire genomic region in a single duplication event. Because of the lack of homology outside of these regions, these duplication events would be ancient. This is consistent with the intergenic regions being diverged, but the high degree of sequence similarity, even among the third bases in the codons often seen, is at odds with this view.

Sequence analysis of the entire yeast genome identified 53 pairs of duplicated chromosomal regions, occupying at least half of the yeast genome (Wolfe and Shields, 1996). In many of the cases, the duplicated regions are immediately adjacent to each other. These duplicated blocks, with the exception of sequences adjacent to telomeres, never occur in three or more copies. The average block is 56kb, containing seven duplicated genes plus others unique to one or other of the chromosomes in which the blocks are present. In total, 708 different genes were identified as having homologues. It is proposed by these authors that S. cerevisiae is actually an ancient diploid, having undergone a duplication of the entire genome in its distant past, after the emergence of S. cerevisiae and K.lactis as distinct species. This theory suggests that, subsequent to this genomic duplication, many of the duplicated genes have been lost and a small number of chromosomal rearrangements have resulted in the fragmenting of the original duplicated chromosomes into the dispersed duplicated syntenic segments observed now.

Sequence analysis of PLM2 on chromosome IV described in Chapter Six revealed the presence of a homologue of this ORF on chromosome XII. Further
analysis revealed that this homology extends over 8kb, including four ORFs. All the genes are in the same order and orientation. The homology only extends over the coding sequences, the intergenic regions are highly diverged. The analysis of this apparent duplication is described below.

6.2 Results

The sequence analysis of the region of chromosome IV, and the duplication observed in chromosome XII, was carried out using FASTA homology searches on the DNA sequence, and TFASTA analysis of the predicted protein sequences of ORFs (Pearson and Lipman, 1988). The analysis was carried out by separately submitting each of the ORFs on a continuous 35kb stretch of chromosome IV to a FASTA search against cosmid U17246 from chromosome XII. A second search looked for homologies between the intergenic regions.

The region of duplication on chromosome IV extends over approximately 8kb, from D9719.6 to GIN5, adjacent to the right telomere. The entire region covers six ORFs, but only four of these have homologues on chromosome XII, all in the same relative orientations and order (Figure 6.1). There was no significant homology identified between the intergenic regions in this segment. The duplication on chromosome XII is positioned approximately central on the right arm. There is a reduced protein identity in most cases compared to sequence homology, however the similarity between proteins, where similarity is calculated taking conserved amino acid substitutions into account, is very significant. Protein similarity varies from 83.5% between GIN5 and DL9470.10 to 99.5% similarity observed between SAM2 and SAM1. This duplication was confirmed by using the MIPS ( Martinsried Institute for Protein Sequences) genome browser programme accessed at http://speedy.mips.biochem.mpg.de/mips/yeast/.

All the homology identified between the four pairs of genes occurs in the latter half of the sequences. In three of the cases, this homology extends up to the last base and then stops, the exception being the most telomere proximal gene pair of GIN5 and DL9470.10. The amino acid identity scores suggest that the duplication did not occur in-frame. Because of third base redundancy in codons, if the duplication had occurred in-frame a higher amino acid homology score compared to sequence homology would be expected. However, the similarity between the expected proteins over these regions is highly significant.
Homology observed between cosmids U33057 (chromosome IV) and U17246 (chromosome XII)

FASTA and TFASTA (Pearson and Lipman, 1988) analyses were carried out on ORFs and intergenic regions of cosmids U33057 and U17246.

(a) Homology data obtained for the two cosmids

nt = nucleotide
aa = amino acid

(b) Diagram showing regions of homology
Figure 6.1

(a)

<table>
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<tr>
<th>ORF on IV</th>
<th>Homologue on XII</th>
<th>nt identity %*</th>
<th>aa identity (similarity) %</th>
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<tr>
<td>D9719.6</td>
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<td>75.8 (95.5)</td>
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<td>L9470.22</td>
<td>62.9 (1150-1469)/1469</td>
<td>40.8 (88.2)</td>
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<tr>
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<td>SAM1</td>
<td>83.4 (17-1154)/1154</td>
<td>92.6 (99.5)</td>
</tr>
<tr>
<td>GIN5</td>
<td>L9470.10</td>
<td>69.7 (1603-2085)/2525</td>
<td>25.5 (83.5)</td>
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</table>

*numbers indicate % identity, boundaries of nt comparison (in brackets) and size of the ORF on chromosome IV.

(b)
The region of shared homology on chromosome XII is the region containing SWI6, an important cell-cycle regulatory gene (Moll et al., 1993). A homologue on the syntenic segment of chromosome IV wasn't identified, however, a further homology search with just this ORF against the entire U33057 cosmid sequence identified a 52.9% identity over 469bp with ORF D9719.19, a further 13kb towards the telomere from GIN5. However, similarity at the amino acid level isn't significant.

6.3 Discussion

The duplication observed between chromosomes IV and XII only extends over coding sequence and, apart from the homology seen between SWI6 and D9719.19, has defined boundaries. This suggests that this duplication is ancient. The comparative lack of novel ORFs on chromosome IV, compared to XII at this locus suggests that chromosome IV is the ancestral sequence. There are no Ty elements present near to either locus, and the position of the duplication midway along the right arm of chromosome XII discounts telomere recombination mechanisms as an explanation for the duplication event. Mechanisms of genome duplication involving the recombination of processed genes is also unlikely in this case. The conservation of the order and orientation of the genes involved, and the duplication of D9719.6 with its intron intact is evidence against this. Further analysis is needed to be able to postulate further on the exact mechanism involved in the creation of this particular duplication.

The apparent lack of amino acid identity, but high similarity between ORFs in this duplicated region suggests that the regions of homology between these genes may be functionally substituted. This is significant in terms of PLM2 and its homologue L9470.22. The insertion of a Tn1000::HIS3 cassette approximately 300bp into the coding region of PLM2 isn't lethal to the cell (Chapter Five). This plm2 allele may not be a null allele, however L9470.22 may be able to functionally substitute for PLM2 if this were the case.

The identification of a region of duplication between chromosomes IV and XII at this locus is consistent with previous analysis of the yeast genome, and the discovery of the high degree of redundancy. This redundancy explains the identification of relatively few essential genes in S.cerevisiae, and makes assigning functions to the many, as yet, unanalysed genes a huge problem.
Chapter Seven

GENERAL DISCUSSION

The 2μm plasmid requires both plasmid and host-encoded functions for its stable maintenance. The plasmid has two main strategies for its survival; it can amplify its copy number and partition plasmid molecules efficiently at cell division. The host is also required for plasmid replication. The identification of host proteins involved in 2μm plasmid maintenance may enable us to answer questions concerning the general organisation and maintenance of DNA in the host cell.

This work describes the successful isolation and genetic characterisation of four temperature-sensitive mutants defective in 2μm plasmid maintenance (plm). The mutations in each case are recessive and in single genes, and complementation analysis placed them into two groups, plm2 and plm3. The plm2-l mutation appears to be 2μm-specific; YCp50, a CEN-ARS plasmid, demonstrates the same stability in plm2-l as in a wild-type strain over fifty generations of growth. Moreover, the copy number of the 2μm-based plasmid pJDB248 is unchanged at the non-permissive temperature in the plm2-l strain. This suggests that Plm2p may have a role in an aspect 2μm maintenance other than replication per se. This role could be in the partitioning and stable inheritance of the plasmid.

Complementation of plm2-1 with a genomic library resulted in the successful isolation of the wild-type PLM2 gene. This is a previously uncharacterised ORF (D9719.7) on chromosome IV; 1.6kb in length, encoding for a predicted protein of 521 amino acids. Successful disruption of the PLM2 gene in a haploid, by integration of a 6kb Tnl000::HIS3 cassette 300bp downstream from the translation start codon created a defined plm2 allele. To determine whether or not PLM2 is an essential gene, creation of a null allele would be necessary. However, a homologue of PLM2 was identified on chromosome XII, L9470.22, which may functionally compensate for this. This possibility could be investigated further by the analysis of a plm2/L9470.22 double disruptant haploid strain.

Evidence to suggest that PLM2 is a transcribed gene comes from sequence analysis. There is a putative TATA box 130bp upstream from an ATG translation start codon. Also, the disruption of the PLM2 gene results in a mutant phenotype which suggests that this ORF is functional. RNA studies could investigate whether the transcription of this gene is cell-cycle specific.
The aim of this work was to identify a host protein involved in 2μm plasmid maintenance, and to be able to draw some conclusions as to the protein's function in the host cell. Candidate host proteins include those involved in DNA replication, or in the partitioning mechanism of the plasmid. Models for the partitioning mechanism of the 2μm plasmid fall broadly into two categories, active and passive (see also Figure 1.9). Both models propose that the 2μm plasmid is attached to the nuclear scaffold for DNA replication, and is then released at a later stage of the cell-cycle for partitioning. Active models propose that plasmid molecules subsequently become re-attached to an actively partitioning structure in the nucleus. This could be the nuclear envelope, and support for this comes from the co-precipitation of 2μm B protein with a nuclear lamina fraction (Wu et al., 1987). Passive models for plasmid partitioning propose that plasmid molecules become diffused in the nucleoplasm, and migrate randomly into the daughter bud. Host proteins may also be involved in 2μm maintenance in a more indirect way, by the transcriptional control of plasmid-encoded genes.

To determine more fully the role of Plm2p in 2μm plasmid maintenance, homology searches were carried out with PLM2 nucleotide and protein sequence against sequences submitted in the EMBL database. The significant homologies observed between Plm2p and protein sequences in the SwissProt database are all with nuclear-located, DNA-binding proteins. Moreover, a hydrophilicity plot (Kyte and Doolittle, 1982) suggests that Plm2p is hydrophilic, and an analysis of charge along the length of the protein indicates that Plm2p is positively charged (Hopp and Woods, 1981). Both these features are indicative of DNA-binding proteins. This is consistent with Plm2p having a role in 2μm plasmid maintenance. The location of Plm2p in the yeast cell can be determined by raising antibodies against the protein. A fluorescent label can then detect the exact location of the antibody, which is then viewed by fluorescence microscopy.

Recent models of 2μm plasmid partitioning suggest that the host interacts directly with STB. This is based on protein binding studies (Hadfield et al. 1995) using STB against a yeast insoluble fraction of proteins. It is possible, therefore, that Plm2p is this protein factor. Alternatively, Plm2p may interact with the ARS sequence. The origin recognition complex (ORC) has two defined functions. ORC proteins have been shown to be involved in both DNA replication and transcriptional regulation. An orc5 allele has been identified that specifically affects transcriptional silencing but not replication (Fox et al., 1995). DNA replication and transcription have a number of features in common. Both require an interaction between proteins and defined DNA sequence elements, and both involve the assembly of multi-protein complexes capable of opening the
duplex and catalysing template-directed nucleic acid synthesis. The homology identified between Plm2p with transcription factors and DNA-directed RNA polymerases may be significant in the light of this.

To determine whether Plm2p does interact with 2μm sequences in vitro, gel retardation experiments could be carried out. Purified Plm2p would be incubated with STB or ARS sequences and then electrophoresed. Protein binding to DNA sequences would result in a band shift, with the protein/DNA complex having a different mobility to the DNA sequences on their own. The homologies identified between Plm2p and transcription factors could suggest an alternative DNA-binding role for Plm2p in the regulation of transcription of the 2μm genes important for partitioning, B and C. The gel retardation experiment described above could be carried out with the upstream regulatory regions of these two plasmid genes.

If Plm2p interacts with 2μm plasmid ARS or STB sequences, this interaction is likely to occur at the nuclear scaffold, where replication takes place and from which the disassociation of plasmid molecules must occur for partitioning. As well as being the proposed site of DNA replication, the nuclear scaffold may be the site of other nuclear functions such as transcription and RNA processing (Carri et al., 1986; Xing and Lawrence, 1991). The nuclear scaffold is a complex of proteins that remain as an insoluble fraction after nuclei have been treated with salts, detergents and nucleases (Amati and Gasser, 1988; Cardenas et al., 1990). The protein composition of the nuclear scaffold is largely unknown. The main scaffold protein so far identified is topoisomerase II, an essential protein for DNA replication (Heck and Earnshaw, 1986; Gasser et al., 1986). Another scaffold-associated protein is Rap1p (repressor-activator protein; Wright et al., 1992; Cardenas et al., 1990). Rap1p is an abundant protein which acts in the transcriptional regulation of many genes. Rap1p also has a role in the control of expression at the silent mating type loci (Buchman et al., 1988) and in telomere maintenance (Conrad et al., 1990). To determine whether or not Plm2p is a part of the nuclear scaffold, a Western blot procedure could be carried out using a Plm2p antibody as a probe against scaffold proteins. Also, a two-hybrid assay could be performed to look specifically for an interaction of Plm2p with known nuclear scaffold proteins such as topoisomerase II. The two-hybrid assay is a system for analysing protein/protein interactions in yeast which was initially described utilising the GAL4 transcription activation system (Fields and Song, 1989). Gal4p has two functional domains, one that binds to a specific regulatory sequence upstream of a target gene, and an activation domain which enables RNA polymerase to position itself at the transcription start site. Both domains of Gal4p need to be connected by a protein bridge to enable gene transcription. The
two-hybrid system exploits this by having the two separate Gal4p domains expressed on different plasmids, each fused to a target protein of interest. If an interaction occurs between the two target proteins, the bridge between the two Gal4p domains is formed, and transcription of a reporter gene can be detected.

A mutant defective specifically in 2μm plasmid maintenance has previously been identified (Cashmore, 1984). This mutant, plm1, has a phenotype of non-transformability with whole 2μm or 2μm-based plasmids. Plasmid suppressors of the plm1 phenotype were isolated. A hydroxylamine mutagenesis procedure was performed on a population of the whole 2μm-based plasmid pJDB248. Eight plasmids from this mutagenised population were then successfully transformed into plm1 (S.Hawkins, personal communication). A further analysis of plm2-1 would be to determine whether or not any of these plm1-suppressing plasmids are also efficiently maintained in this mutant. If successful, analysis of the stability of these suppressor plasmids in plm2-1, perhaps by a sub-cloning procedure to identify the site on the plasmids conferring the suppression of the phenotype, could be carried out. This would suggest possible plasmid/Plm2p interactions.

In conclusion, this work has confirmed the presence of host factors important for 2μm plasmid maintenance. Two complementation groups have been identified, plm2 and plm3. Complementation of plm2 with a genomic library was successfully carried out. PLM2 is a novel gene and analysis of the nucleotide and expected protein sequence has revealed strong homologies with nuclear-located, DNA-binding proteins from yeast and higher eukaryotes. The plm2-1 mutation is 2μm-specific which suggests a role for Plm2p in 2μm maintenance other than replication, perhaps through a direct interaction with STB or proteins B and C. The homology to transcription factors may also suggest a regulatory role in either B and C gene expression, or expression of other host proteins important for plasmid maintenance. It is proposed that Plm2p may be a protein of the nuclear scaffold and as such may have a general role in the maintenance of DNA in the nucleus.
Appendix 1

PHENOTYPES OF TETRADS

Matings
Strain S150-2B and the S150-2B-based plm mutants (MATa, leu2, his3, trp1, ura3) were mated with MC16 (MATa, leu2, his4, ade2, [pJDB248])

Key
trp  tryptophan requirement
his  histidine requirement
ura  uracil requirement
ade  adenine requirement
plm  plasmid maintenance

Tetrads without plm phenotypes noted were isolated as plasmid-free segregants.

RA3 (plm2-1)

B1  MATa    trp+  his−  ura+  ade+  plm+
B2  MATa    trp−  his−  ura+  ade+  plm+
B3  MATa    trp−  his+  ura−  ade−  plm−
B4  MATa    trp+  his+  ura−  ade−  plm−
C1  MATa    trp−  his+  ura−  ade+
C2  MATa    trp+  his−  ura+  ade−
C3  MATa    trp+  his+  ura+  ade−
C4  MATa    trp−  his−  ura+  ade+
D1  MATa    trp+  his−  ura−  ade+  plm−
D2  MATa    trp−  his+  ura+  ade−  plm+
D3  MATa    trp−  his+  ura+  ade+  plm−
D4  MATa    trp+  his−  ura−  ade−  plm+
E1  MATa    trp+  his+  ura+  ade−
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<td>ade−</td>
<td>plm−</td>
</tr>
<tr>
<td>J1</td>
<td>MATα</td>
<td>trp−</td>
<td>his</td>
<td>ura</td>
<td>ade+</td>
<td>plm+</td>
</tr>
<tr>
<td>J2</td>
<td>MATα</td>
<td>trp+</td>
<td>his+</td>
<td>ura−</td>
<td>ade+</td>
<td>plm−</td>
</tr>
<tr>
<td>J3</td>
<td>MATα</td>
<td>trp+</td>
<td>his+</td>
<td>ura+</td>
<td>ade−</td>
<td>plm+</td>
</tr>
<tr>
<td>J4</td>
<td>MATα</td>
<td>trp+</td>
<td>his+</td>
<td>ura+</td>
<td>ade−</td>
<td>plm−</td>
</tr>
</tbody>
</table>
S150-2B [cir°]

A1  MATα  trp⁺  his⁺  ura⁻  ade⁺
A2  MATα  trp⁻  his⁺  ura⁺  ade⁺
A3  MATα  trp⁻  his⁺  ura⁺  ade⁻
A4  MATα  trp⁺  his⁻  ura⁻  ade⁻

B1  MATα  trp⁻  his⁻  ura⁻  ade⁻
B2  MATα  trp⁻  his⁺  ura⁺  ade⁺
B3  MATα  trp⁺  his⁻  ura⁺  ade⁺
B4  MATα  trp⁻  his⁺  ura⁻  ade⁻

C1  MATα  trp⁺  his⁺  ura⁺  ade⁺
C2  MATα  trp⁻  his⁺  ura⁺  ade⁺
C3  MATα  trp⁻  his⁻  ura⁻  ade⁻
C4  MATα  trp⁺  his⁻  ura⁻  ade⁻

D1  MATα  trp⁺  his⁺  ura⁻  ade⁺
D2  MATα  trp⁻  his⁻  ura⁻  ade⁺
D3  MATα  trp⁻  his⁺  ura⁺  ade⁺
D4  MATα  trp⁺  his⁺  ura⁻  ade⁻

E1  MATα  trp⁺  his⁺  ura⁻  ade⁺
E2  MATα  trp⁺  his⁺  ura⁻  ade⁺
E3  MATα  trp⁻  his⁻  ura⁺  ade⁺
E4  MATα  trp⁻  his⁻  ura⁺  ade⁺

G1  MATα  trp⁻  his⁻  ura⁺  ade⁺
G2  MATα  trp⁺  his⁻  ura⁻  ade⁺
G3  MATα  trp⁻  his⁺  ura⁻  ade⁺
G4  MATα  trp⁻  his⁻  ura⁺  ade⁺
Appendix 2

UPSTREAM AND CODING SEQUENCE OF PLM2

GCN transcription factor binding site and putative TATA sequence are highlighted in bold, upstream of translation start.

tagtttacctggctctctggtggaaaattttttcagtgcttctttctcatgtaatcct
tagtttacctggctctctggtggaaaattttttcagtgcttctttctcatgtaatcct
13000 +---------+---------+---------+---------+---------+---------
ctaatcagacggagttgattgaacgttgactcatcaattaataattcctgtacatact
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13060 +--------+--------+---------+---------+--------+----;----
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13180 + -------+----------+---------+---------+----------+---------
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tccttcgccgttgctgactgaatattcttccggcgtgctttccctggtgtc
13240 + -------+----------+---------+---------+----------+---------
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acccatctttacttatattcgtttacctctttgttctttatctacacatagtagtgct
13300 + -------+---------+---------+--------+---------+--------
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tcctcttcgcagctgttactagcacaagggaaaccatataataataagctttttttttt
13480 + -------+---------+---------+--------+---------+--------
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13540 + -------+---------+---------+--------+---------+--------
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tccttcaccgctcatttttcagtctcccttttcagcttctcttttgttttgcgatc
13600 + -------+---------+---------+--------+---------+--------
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