Conserved Chromatin-Mediated Gene Silencing

In Yeast and Plants

by

Hazel Anne Cruickshanks

Supervisor – Dr. S. Pennings

University of Edinburgh

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Abstract

Cells must regulate gene expression to control development, differentiation, and to respond to changes in the environment. The simplistic view of gene regulation states that an activator or repressor molecule binds to a specific sequence in DNA and exerts its effects upon transcription. However, the situation becomes more complicated when we consider that eukaryotic DNA is packaged into chromatin. Chromatin must be modified in order to control gene expression. Cells have adopted many ways of achieving this including: chromatin remodelling, DNA methylation and histone modifications. The exact contributions of each of these need to be elucidated in order to fully understand gene regulation.

Many common themes run through gene regulation between species, suggesting there are conserved mechanisms of gene control. Using the simple model organism, *Saccharomyces cerevisiae*, I have studied two types of gene repression found in plant species to compare and further determine their molecular bases. A repetitive DNA fragment previously found to induce *de novo* methylation and expression variegation in *Petunia hybrida*, was found to cause gene silencing in *S. cerevisiae* in a methylation independent manner. The possible mechanisms of this were dissected using gene replacements and protein expression studies.

In a separate series of experiments, putative homologues of the *S. cerevisiae* transcriptional co-repressor, TUP1, were tested for chromatin remodelling ability in yeast. A TUP1 homologue from *Arabidopsis thaliana* was shown to repress

transcription in *S. cerevisiae* but in a different manner from TUP1 indicating mechanistic similarities and differences between their functions.

By using yeast as a tool to study gene regulation in higher eukaryotes, the principles of gene repression can be explored and we can speculate the roles of the individual features such as chromatin remodelling and DNA methylation.

Chapter 1 – Introduction

1.1 Chromatin Structure

The genetic information that determines the basic characteristics of an organism is encoded by DNA. Cells organise the DNA into chromosomes, which must be stably maintained and inherited following each cell division. The regulation of DNA expression into protein is essential for all processes that a cell faces during its lifetime. DNA contained within the cell nucleus must be folded and packaged into a nucleoprotein complex known as chromatin. This compacted structure containing the DNA, must be overcome to allow processes such as replication, transcription and repair.

Chromatin consists of a fundamental repeating unit known as the nucleosome. The nucleosome core particle consists of an eight histone proteins with 147bp of DNA wrapped around the octamer in one and three-quarter turns. Histones are small basic proteins consisting of a globular domain and a flexible N-terminal tail, which protrudes from the nucleosome. Histones fall into five classes: H1, H2A, H2B, H3 and H4 (reviewed by Wolffe, 1995). Each class contains histone variants, which are responsible for gene-specific or tissue-specific chromatin structure (Franklin and Zweidler, 1977). H2A, H2B, H3 and H4 are known as the core histones and two of each type comprise the histone octamer. These proteins are highly conserved throughout evolution, with H3 and H4 being the most conserved. Indeed, there are only eight amino acid changes between the yeast *S. cerevisiae* H4 and human H4,

which suggests histones have an important cellular role. This is further demonstrated by the observation that budding yeast are not viable without a complete set of core histones (Kim *et al.*, 1988). Post-translational modifications occur on N-terminal tails, including acetylation, methylation, and phosphorylation can influence gene expression and chromosome behaviour (reviewed by Jenuwein and Allis, 2001).

The complete nucleosome particle consists of the nucleosome core, histone H1 and linker DNA, which is the DNA between nucleosome core particles. H1 is the largest of the histones and associates with the linker DNA (Allan *et al.*, 1980). In higher eukaryotes, there is approximately one H1 molecule for each core particle; however, there may not be an association at every nucleosome core. Studies suggest H1 is required for the stabilisation, but not the establishment of higher-order structures (Schwarz and Hansen, 1994). *S. cerevisiae* contains a gene *HHO1*, which encodes an H1-like protein, but whether this protein functions as mammalian H1 remains unknown (Ushinsky *et al.*, 1997).

The nucleoprotein complex generated by histone association with DNA produces a "beads on a string" structure (fig. 1.1). This structure can be observed at low salt concentrations. However, in higher eukaryotes, at physiological salt concentrations electron micrographs show a more compact 30nm fibre (Thoma *et al.*, 1979). This is believed to be the chromatin structure that is present during most of the cell cycle. Further compaction of the chromatin fibre occurs during mitosis and meiosis (fig 1.1). The 30nm chromatin fibre is not rigid; it is believed to be a dynamic structure and in equilibrium with less compact states. These states can be observed by

differential staining of the nucleus. The more compact state is known as heterochromatin (Heitz, 1928) and is generally transcriptionally repressed. It corresponds mainly to centromeres and telomeres and is enriched in repetitive DNAs. The fibres that are less densely stained are euchromatin, which contain mainly active genes and make up the majority of chromatin. The molecular mechanism by which the equilibrium between states is controlled may influence gene expression. For example, acetylation of H4 slightly influenced the dynamics of chromatin folding suggesting histone modifications play a role in gene expression and chromatin structure (Tse *et al.*, 1998).

Naked DNA		2 nm
Nucleosome cores		10 nm
Folded nucleosomes		30 nm
Chromosome loops		300 nm
Folded chromosome		700 nm
Mitotic chromosome		1400 nm
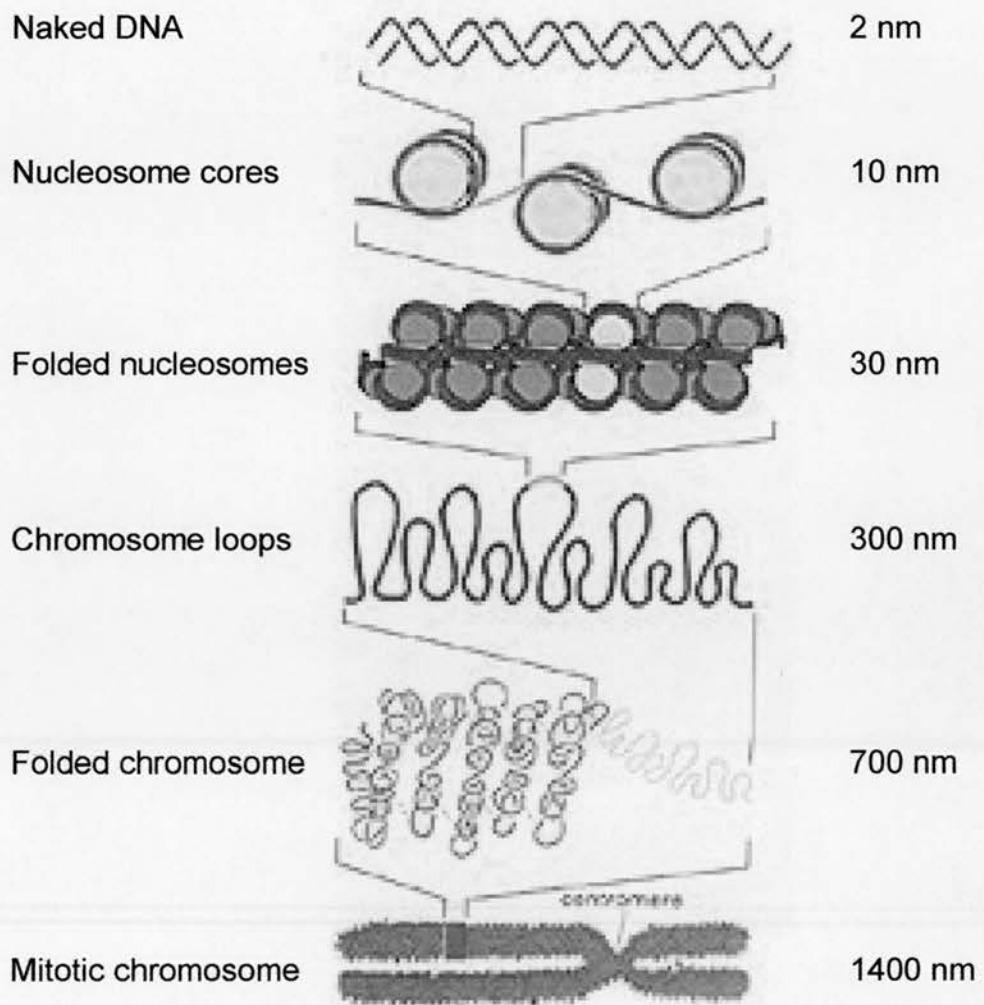
(Adapted from Alberts *et al.*, 1998)

Figure 1.1: Folding of chromatin into higher order structures. This schematic diagram shows the transition from naked DNA into the chromosomes observed during metaphase in mitosis, with intermediates shown.

1.2 Chromatin and Gene Expression

Transcription of genes in eukaryotic cells requires a number of basal and gene-specific regulatory proteins. General transcription factors are responsible for promoter recognition; for example, transcription by RNA polymerase II requires the binding of TFIID at the TATA box. This recruits other basal transcription factors, which position RNA polymerase II at the promoter, so that it can transcribe the gene. Regulation of genes also requires gene-specific proteins binding to *cis*-acting sequences to enhance DNA transcription. This model works very simply on naked DNA, but when we consider that eukaryotic DNA is packaged into chromatin the situation becomes more complicated. Chromatin is generally repressive to transcription since DNA binding sites for proteins involved in transcription may be obscured by nucleosomes. Therefore changes must be made to the chromatin to allow transcription or repression. Such alterations include covalent modifications to histone tails and chromatin remodelling (fig. 1.2)
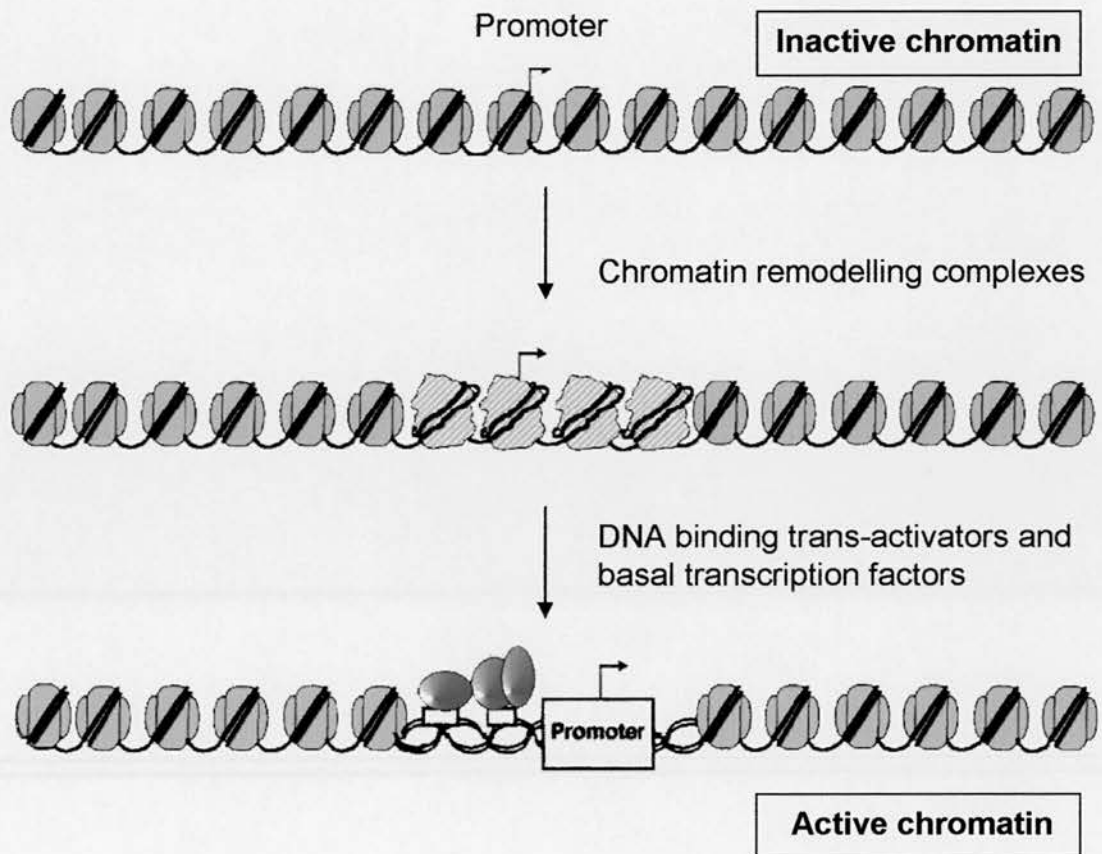
Figure 1.2: Influence of chromatin remodelling complexes on general transcription. Chromatin generally has a repressive structure if positioned nucleosomes occlude binding sites for DNA-binding proteins. Chromatin remodelling complexes alter nucleosomes and allow access of the basal transcriptional machinery and trans-activating factors to the promoter and relevant binding sites.

1.2.1 Chromatin Remodelling

Chromatin remodelling is carried out by multi-protein complexes, which mobilise or modify nucleosomes in order to activate or repress gene transcription in a targeted manner. Chromatin remodellers include the SWI/SNF, ISWI, and NURD complexes. A well-characterised example is SWI/SNF, which was originally identified in *S. cerevisiae* in a screen for sucrose fermentation and mating switch defects (reviewed by Peterson and Tamkun, 1995). SWI/SNF is a multi-subunit complex comprising at least 11 proteins and is responsible for the regulation of round 6% of genes in *S. cerevisiae* including, *SUC2* and *FLO1* (Hirschhorn *et al.*, 1992; Fleming and Pennings, 2000). Homologues are found in *D. melanogaster*, *A. thaliana* and mammalian cells.

SWI/SNF chromatin remodelling complexes are ATP-dependent, that is, they utilise the energy generated from the hydrolysis of ATP to remodel nucleosomes. They also contain a highly conserved helicase domain, although the associated helicase activity has never been shown (Laurent *et al.*, 1993). SWI/SNF interacts with DNA in a non-specific fashion; therefore, it is recruited to genes by specific DNA-binding proteins (Côté, *et al*, 1994). SWI-SNF alters nucleosome positioning, allowing activators to bind to their sites previously occluded by nucleosomes, thus activating transcription.

The exact mechanism by which SWI/SNF remodels nucleosomes and alters their positions is not fully understood. End-labelling analysis of DNaseI digested nucleosomal DNA that had been incubated with SWI/SNF, revealed different

digestion patterns of chromatin. This implies that SWI-SNF is capable of altering the interactions between histones and DNA, perhaps by inducing some conformational change in the nucleosome (fig. 1.3). SWI-SNF complexes are capable of a process known as octamer transfer. This is the directed movement of a histone octamer from one molecule of DNA to another (Phelan *et al.*, 2000). However, this is a very inefficient process. Another activity of SWI-SNF that is more efficient and perhaps relevant is sliding (Whitehouse *et al.*, 1999). Sliding occurs when SWI/SNF directs the movement of nucleosomes along a segment of DNA from one site to another (fig. 1.3). Sliding and conformational changes in nucleosomes may be important in revealing DNA binding sites at promoters for gene activation. Indeed, studies at the *SUC2* promoter in *S. cerevisiae*, show activation of *SUC2* transcription upon remodelling of nucleosomes which previously occluded the TATA box and upstream activating sequences (Gavin and Simpson, 1997). However, chromatin remodellers generally do influence transcription alone but in conjunction with complexes that modify chromatin, such as histone acetyltransferases.

A. Sliding

B. Conformational Change

Change in both DNA
and histone structure

Change in histone
conformation

Intermediate
structure

Change in
conformation of DNA
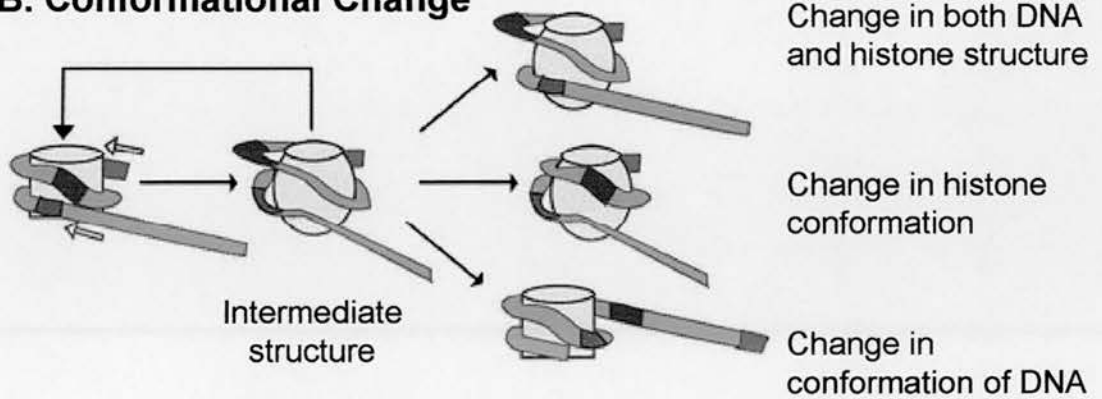
(Derived from Narlikar *et al.*, 2002)

Figure 1.3: Models for the mechanism of ATP-dependent chromatin remodelling. (**A**) shows sliding where chromatin remodellers direct the movement of nucleosomes from one area of DNA to another. (**B**) shows the putative conformational changes imposed by chromatin remodellers on histones, DNA or both to facilitate access of basal transcriptional factors to sites within the DNA.

1.2.2 Histone Modifications

The N-terminal tails of histone proteins are subject to a wide variety of post-translational modifications (fig. 1.4). These distinct changes provide specific binding sites for chromatin-associated proteins, which determine the transcriptional regulation of the locus. Indeed, differences in histone modifications can reflect whether or not a locus is "heterochromatic" or "euchromatic". The diverse array of possible modifications, which can occur on any one nucleosome, led to the histone code hypothesis (reviewed by Jenuwein and Allis, 2001). This hypothesis predicts that a particular set of alterations to histones can dictate the transcriptional activity of a locus. Modifications imposed upon histones may be interdependent and various combinations can be applied to any one nucleosome. The balance of these interactions determines the proteins recruited and ultimately the transcriptional activity. The histone code provides an additional level of regulation to the underlying DNA sequence and the regulatory nature of these modifications must be deciphered to fully understand this.

Histone acetylation correlates with an increase in gene expression. For example, it has been documented in *Saccharomyces cerevisiae,* where most of the genome is active; histones are hyperacetylated (Clark *et al*, 1993). However, at inactive loci, hypoacetylated histones are found (Braunstein *et al*, 1993).

Acetylation occurs on all four core histones on positively charged lysine residues. This reduces the net charge by neutralising the positive charge on the lysine. It is

thought that the loss of this positive change reduces the affinity of the histone for the negatively charged DNA backbone, thus giving a more open chromatin structure (Hong *et al.*, 1993). This reaction is carried out by histone acetyltransferases (HAT), which covalently attach an acetyl group from acetyl co.A to the histone. This process can also be reversed by histone deacetylases (HDAC) that remove the acetyl group from the lysine residue. The equilibrium between these two enzyme activities determines the number of acetylated lysine residues per histone. Many general transcription factors have HAT activity, for example, $TAF_{II}250$ (Mizzen *et al.*, 1996). HATs and HDACs tend to form part of larger transcriptional regulatory complexes and are recruited to the DNA to activate or repress transcription. The Sin3 complex requires the HDAC, RPD3 for full repression of genes (Kadosh and Struhl, 1997). GCN5, a HAT is found associated with the SAGA chromatin-remodelling complex (Grant *et al.*, 1997). HATs and HDACs are found to be conserved in a wide variety of organisms where they carry out similar functions underlining their importance through evolution.

The acetylation mark on histones acts as a beacon for proteins to bind. The SWI/SNF complex contains a protein with a bromodomain, which has the ability to bind acetylated lysine residues (Dhalluin *et al.*, 1999). Conversely, repressors such as SIR proteins and TUP1-SSN6 preferentially bind hypoacetylated histone tails (Edmondson *et al.*, 1996; Carmen *et al.*, 2002). Thus, the acetylation status of nucleosomes directs specific protein binding, which influences gene expression.

Methylation of histones can occur on arginine and lysine residues. Arginine methylation is a rare modification associated with activation of genes and has been implicated in signal transduction cascades in response to hormones (Wang *et al.*, 2001). Methylation of different lysine residues, by a family of histone methyltransferases, can have either activating or repressive effects on gene transcription. Methylation of lysine-4 on histone H3 tends to be a mark of euchromatin (Strahl *et al.*, 1999) whereas lysine-9 methylation of the same histone is associated with heterochromatin and repressed genes (Nakayama *et al.*, 2001). Methylation of lysine-9 is carried out by the chromodomain protein SUV39H1 also known as Su(var)3-9 in *D. melanogaster* or Clr4 in *S. pombe* (Rea *et al.*, 2000). This protein contains a SET domain, which is conserved among histone methyltransferases. Lysine-9 methylation of H3 creates a binding site for heterochromatin protein 1 (HP1) (Bannister *et al.*, 2001; Lachner *et al.*, 2001). HP1 is an important protein involved in gene silencing, which is conserved in fission yeast, flies, mammals and plants. Binding of HP1 to lysine-9 can lead to the polymerisation of HP1 along the chromatin by virtue of its self-association ability (Cowell and Austin, 1997) and silencing of the locus in question (fig. 1.5). HP1 and lysine-9 methylation are found at centromeres and are important for the maintenance of the heterochromatic state (Peter *et al.*, 2001).

No lysine-9 methylation has been detected in *S. cerevisiae*, however the yeast genome is generally transcriptionally active with few areas of heterochromatin. Notably, lysine-4 methylation is found in *S. cerevisiae* where the situation is more complex. Dimethylated lysine 4 residues are associated with either active or inactive

genes but trimethylated lysine 4 residues are exclusively associated with active genes (Santos-Rosa *et al.*, 2002). Moreover, proteins containing SET domains have been identified in *S. cerevisiae*; one such protein SET1 is required for full rDNA silencing (Briggs *et al.*, 2001).

Histone modifications play an important role in gene regulation and may also have a role in organising chromosome structure. Phosphorylation of serine 10 on H3 has been implicated in inducing chromosome condensation during mitosis (reviewed by Cheung *et al.*, 2000).
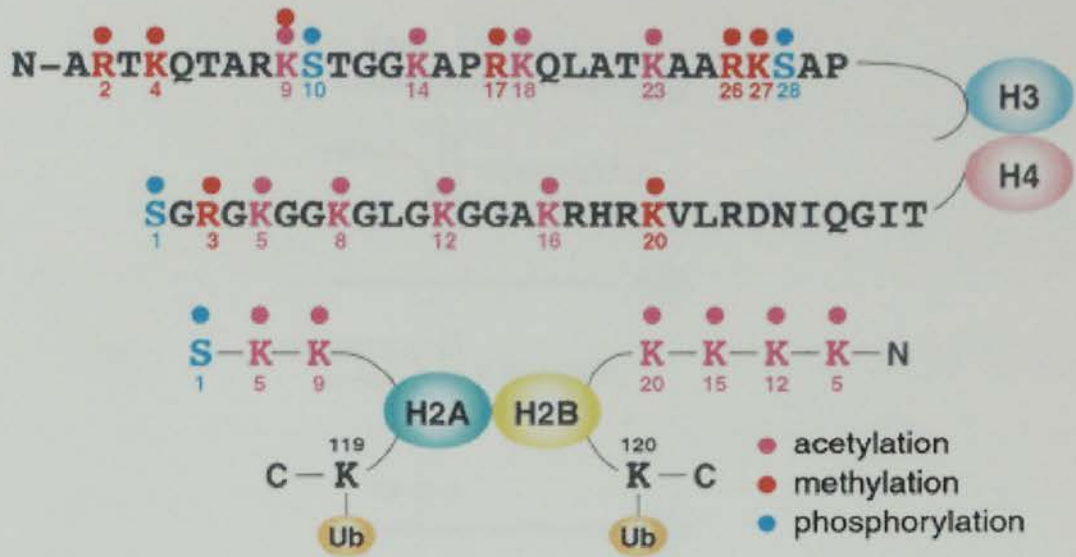
Derived from Zhang and Reinberg, (2001)

Figure 1.4: Sites of post-translational modifications on histone tails. The modifcations shown are: acetylation (purple), methylation (red), phosphorylation (blue) and ubiquitination (orange)

K9-Ac   K14-Ac

H3

HDAC → K Deacetylation

H3

Suv39h1 or clr4 → K Methylation

K9-CH3

H3

HP1 → K9-CH3 Recognition

H3

HP1 Self-association
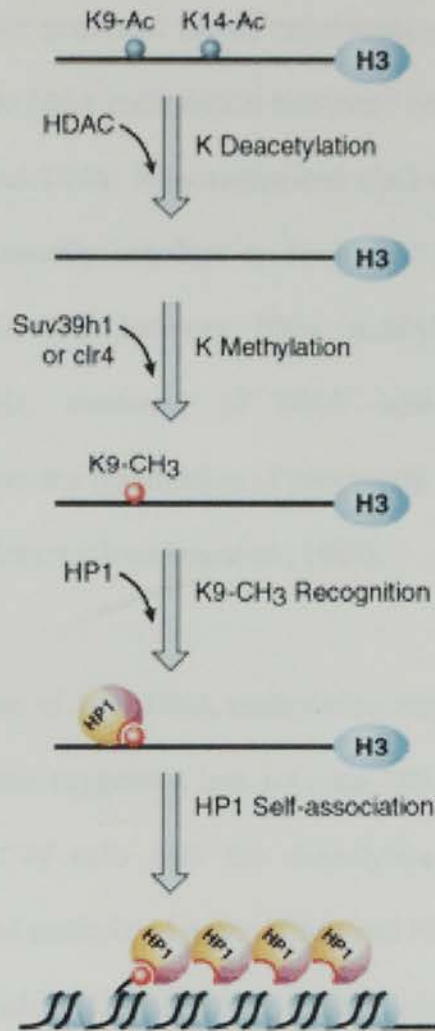
Derived from Zhang and Reinberg (2001)

Figure 1.5: Model of HP1-mediated silencing. Acetylation of lysine-9 on histones is removed by and HDAC. The lysine-9 methylase, SUV39H1, then methylates this residue. The methylated lysine 9 is recognised and bound by HP1. HP1 then spreads along the locus by virtue of its self-association ability inducing a heterochromatic state.

1.2.3 DNA Methylation

Methylation of the carbon-5 position of cytosine residues in CpG dinucleotides is a feature of many eukaryotic genomes. Lower eukaryotes such as *S. cerevisiae* and *S. pombe* have no detectable DNA methylation however; vertebrate and plant genomes contain mainly methylated DNA. Non-methylated CpG dinucleotides are restricted to CpG islands, which usually correlate to functional promoters. There is much evidence supporting the link between DNA methylation and transcriptional repression. For example, treatment of DNA with 5-azacytidine, a DNA demethylating agent causes the reactivation of previously repressed genes (Jones and Taylor, 1980) and retroviruses (Groudine *et al.*, 1981).

The molecular mechanism of how DNA methylation represses transcription is still unclear, however, evidence suggests a link between DNA methylation and histone deacetylation. Treatment of cells with the deacetylase inhibitor, trichostatin A, increases the expression of methylated genes (Chen and Pikaard, 1997). Furthermore, the MeCP2 complex, which specifically binds methylated DNA, can bind to the Sin3 complex that contains an HDAC (Nan *et al.*, 1998). This suggests MeCP2 recruits the HDAC complex to DNA to bring about transcriptional repression. DNA methylation may therefore provide an epigenetic mark, which allows areas of DNA to be silenced.

1.2.4 Position Effect Variegation

Position effect variegation (PEV) is characterised by change in levels of gene expression upon the integration or translocation of a gene to another region of the genome. PEV was first observed in *D. melanogaster* when a euchromatic gene was translocated to a heterochromatic region. This relocalisation caused silencing of the translocated gene in some cells, resulting in a variegated phenotype (Muller, 1930). PEV is a heritable but can be reversed by moving the gene away from the heterochromatic locus (Henikoff, 1990). The mosaic phenotype that results from PEV is due to the variation in spread of heterochromatin over the gene. Screens for modifiers of position effect variegation have been invaluable in determining the molecular players in the formation of heterochromatin, such as HP1 (Eissenberg *et al.*, 1990).

1.2.5 Epigenetic Regulation

The previously discussed mechanisms of histone modification, chromatin remodelling and DNA methylation can all be regarded as examples of epigenetic gene regulation. Epigenetics is defined as a heritable change in gene expression that occurs without a change to the DNA sequence. All of the epigenetic marks discussed are intrinsically linked to provide intricate and accurate gene regulation. As discussed, DNA methylation is associated with histone deacetylation but also histone methylation. In the filamentous fungus, *N. crassa*, disruption of lysine-9 methylation led to a loss of DNA methylation (Tamaru and Selker, 2001). Furthermore, a

methyltransferase has been identified in *A. thaliana*, which contains a chromodomain linking chromatin structure and DNA methylation (Henikoff and Comai, 1998). Putative chromatin remodelling proteins such as DDM1 from *A. thaliana*, which contains homology to SWI/SNF, confirm this (Jeddeloh *et al.*, 1999). Plants mutant for *DDM1* have disrupted DNA methylation patterns and lose gene silencing. These findings amongst others confirm that the field of epigenetics is important for our understanding of gene regulation, and interpreting how these processes relate to each other should expand it.

1.3 TUP1/GROUCHO Family of Transcriptional Co-repressors

Transcriptional co-repressors generally form part of a multi-protein complex brought to promoters to repress genes. Co-repressors adopt many mechanisms for gene repression, including chromatin remodelling and histone deacetylation. One such group of co-repressors is the TUP1/GROUCHO family, which have been grouped into a family due to their partial sequence and structural similarities, and conservation in their methods of repression (fig. 1.6). The family comprises members from different species, all of which contain several WD repeats namely: TUP1, GROUCHO, LEUNIG and Transducin-like enhancers of split (TLE).

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Figure 1.6: Structural comparison of the GROUCHO/TUP1 family of transcriptional co-repressors.(A) Shows a schematic comparison of domains from TUP1 homologues. Numbers above and in parentheses correspond to amino acids. Shown are ScTUP1 from *S. cerevisiae*, CaTUP1 from *C. albicans*, LEUNIG and BP1 from *A. thaliana*, and GROUCHO from *D. melanogaster*. The Q-rich domain corresponds to a region rich in glutamine and SP-rich domains correspond to regions rich in serine and proline. (B) shows an alignment of amino acid residues comparing *S. cerevisiae* TUP1 (blue) and BP1 from *A. thaliana* (green). (C) shows an alignment of amino acid residues comparing *S. cerevisiae* ARC40 (red) and *A. thaliana* BP1. (green).

1.3.1 WD Proteins

WD proteins are found in all eukaryotes and are involved in a wide variety of cellular processes, including: cell signalling, transcriptional repression, cytoskeletal assembly, mitotic spindle formation, and vesicle trafficking (reviewed by Smith *et al.*, 1999). The best characterised of these proteins is the G_β subunit of hetero-trimeric G-proteins.

The WD repeat is a 44-60 amino acid sequence that generally has a GH dipeptide 11-24 residues from its N-terminus and a WD dipeptide at its C-terminus, with a conserved core sequence in between these dipeptides. A formulaic representation is given below:

$$\{X_{6\text{-}94} - [GH - X_{23\text{-}41} - WD]\}$$

Variable   **Constant core**

length   **length**

This repeat adopts a β-propeller fold (fig. 1.7), which is a symmetrical structure thought to create a stable platform for forming multi-protein complexes and allowing simultaneous interactions between multiple proteins. This may reflect why the WD repeat is found in such a diverse range of proteins, including the TUP1/GRO superfamily of transcriptional repressors.
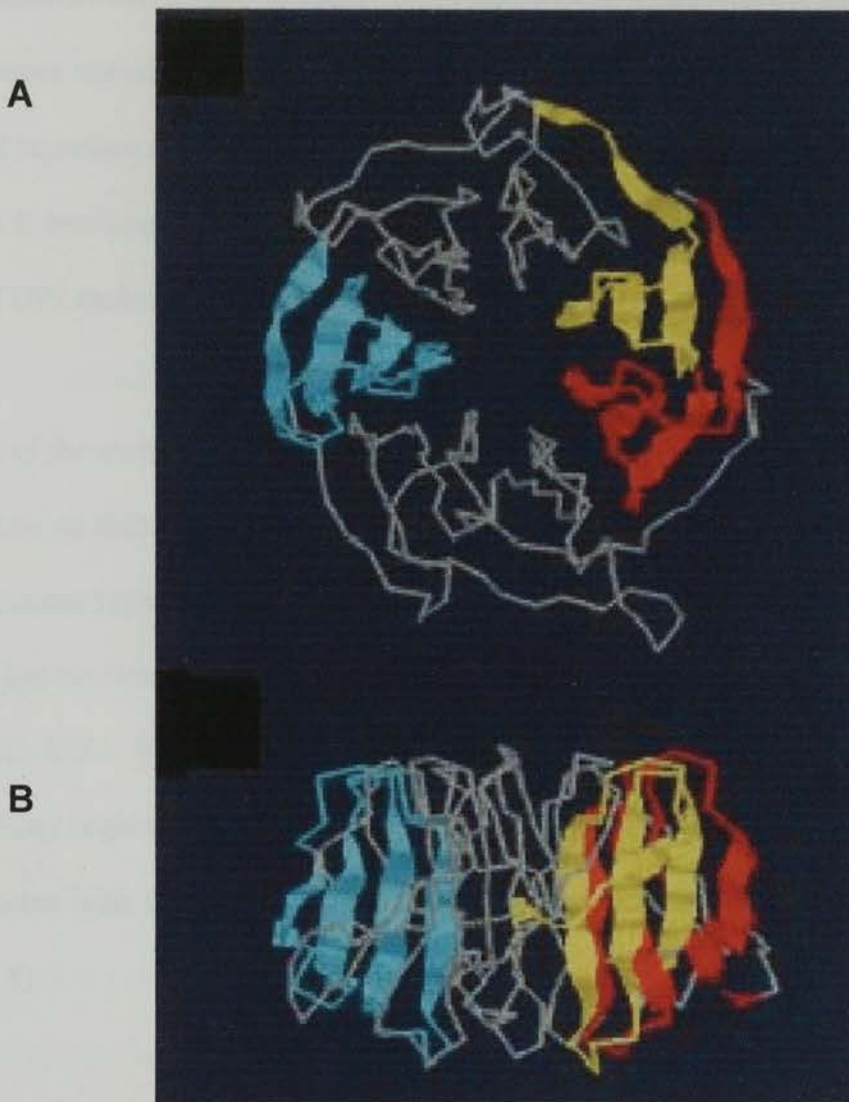
Figure 1.7: Structure of the WD repeat of the G_β subunit of a heterotrimeric G-protein. (**A**) shows the top view and (**B**) the side view. The α-carbon backbone is shown in grey, the N and C termini of the protein are coloured by red and yellow respectively. Each blade (shown in blue) consists of four single-stranded antiparallel β-sheets, which combine to form the β-propeller structure.

1.3.2 TUP1-SSN6 Co-repressor

The TUP1-SSN6 co-repressor complex, found in *S. cerevisiae,* is responsible for the repression of many diverse genes. It is a member of a large family of transcriptional repressors conserved in flies, worms, mammals and plants. It exemplifies how a global repressor can be part of a system that allows it to be highly selective about the genes it acts upon. The TUP1/SSN6 complex consists of one SSN6 molecule with four TUP1 molecules associated (Williams *et al.*, 1991).

Some of the many genes that TUP1-SSN6 regulates can be grouped into families on the basis of their function. These include flocculation genes, oxidative stress genes, and glucose repressive genes (reviewed by Smith and Johnson, 2000). TUP1-SSN6 itself has no intrinsic DNA binding ability; it represses genes by interaction with a specific DNA binding protein (Keleher *et al.*, 1992). Each set of genes has a regulatory region that the specific DNA binding protein will bind, TUP1-SSN6 then associates with the specific protein, localising it next to the gene to be repressed (fig.1.8).

TUP1-SSN6 interacts with the DNA binding protein using the evolutionary conserved WD repeat domain. SSN6 contains a different type of repeat known as a tetratricopeptide repeat. SSN6 has 10 of these repeats which form a right handed helical structure also involved in protein-protein interactions, including its interaction with TUP1. These functional domains both interact with α2/MIG1 complex when regulating **a**-cell specific genes (Komachi *et al.*, 1994; Smith and Johnson, 2000).

However, in most circumstances it is SSN6 that associates with the DNA binding molecule, leading to the proposal that SSN6 acts as an adapter protein (Tzamarias and Struhl, 1995). Furthermore, it has been shown that repression of genes is TUP1 and not SSN6 dependent (Tzamarias and Struhl, 1994).

There are several models of TUP1 mediated repression, which all potentially play a role in its function. For example, TUP1 is known to act by altering the local chromatin structure around the genes it regulates. Positioned nucleosomes are found upstream of both α2 and *FLO1* promoters (Shimizu *et al*, 1991 and Fleming and Pennings, 2001). TUP1 also has been found to interact with chromatin itself by specific interaction with the N-terminal tails of histones H3 and H4 *in vitro* (Edmondson *et al*., 1996). Removal of these histone tails activates genes formerly repressed by TUP1-SSN6. Furthermore, the histone-binding domain of TUP1 overlaps its repression domain, suggesting the interaction with histones is functionally relevant. TUP1 binding of H3 and H4 is specific for hypoacetylated histones suggesting that histone acetylation may modulate TUP1 activity. The finding that TUP1–SSN6 interacts directly with RPD3 and HOS1 reinforces this idea. Yeast mutant in class I histone deacetylases (HDAC) RPD3, HOS1, and HOS2 have hyperacetylated histones H3 and H4 and exhibit a loss of TUP1-SSN6 mediated repression at *MFA2* and *SUC2* genes (Watson *et al*., 2000). Other studies suggested that class II HDACs were also involved in TUP1-SSN6 repression. Deletion of the HDA1 histone deacetylase led to hyperacetylation of histones H2B and H3 producing a phenotype similar to a *tup1* mutant (Wu *et al*., 2001). Therefore, it is

likely that TUP1-SSN6 represses different genes using different types of HDAC or there is some functional redundancy between HDACs.

The ability of TUP1 to associate with hypoacetylated histones and HDACs suggests the co-repressor can nucleate an altered chromatin structure, which spreads along the template. Indeed, TUP1-SSN6 has been shown to remodel nucleosomes up to 5 Kb upstream of the *FLO1* promoter (Fleming and Pennings, 2001). There is conflicting evidence as to whether TUP1 itself spreads along the region it represses in a manner similar to SIR proteins. At the *STE6* gene, ChIP analysis showed a high density of TUP1 over the whole locus (Ducker and Simpson, 2000), however other studies showed TUP1 localisation was limited to the α2 binding site (Wu *et al.*, 2001).

In addition to affecting chromatin structure, TUP1-SSN6 also represses genes by interacting with the basal transcriptional machinery. These interactions alone are sufficient for repression since studies have shown TUP1-SSN6 activity on naked DNA *in vitro*. Genetic screens for genes affecting TUP1-SSN6 repression have identified proteins associated with RNA polymerase II, for example, SRB7. TUP1 has been shown to associate with SRB7 both *in vitro* and *in vivo* (Gromöller and Lehming, 2000). Yeast carrying a mutant allele of *SRB7*, which disrupts its ability to bind TUP1, exhibited a phenotype similar to a *tup1* mutant, causing de-repression of genes. TUP1 and MED6, a holoenzyme protein, which interacts with activators to stimulate transcription, both compete for SRB7 binding to determine gene expression. It is also thought that TUP1 alters the local chromatin structure and

inhibits TATA-binding protein (TBP) from associating with DNA, therefore allowing gene repression (Kuras and Struhl, 1999).

The mechanisms of repression described are not mutually exclusive and are likely to vary between genes. Other members of the TUP1/GRO family of co-repressors employ similar strategies for gene repression.

A
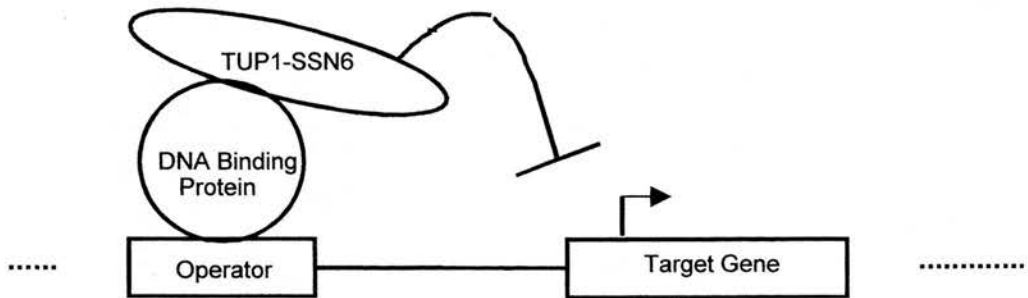
B

Gene Families Repressed by TUP1-SSN6	DNA-Binding Protein
a-cell specific genes	$\alpha 2$ and MCM1
DNA damage induced genes	CRT1
Flocculation genes	?
Glucose repression genes	MIG1
Haploid-specific genes	$\alpha 1$ and $\alpha 2$
Meiosis specific genes	?
Osomtic stress induced genes	SKO1
Oxygen utilisation genes	ROX1
Sporulation specific genes	?
Starch degrading enzymes	NRG1

(Adapted from Smith and Johnson, 2000)

Figure 1.8: Gene repression by TUP1/SSN6. (**A**) shows the mechanism of TUP1/SSN6 mediated repression. The co-repressor binds to a specific DNA-binding protein, usually via interactions with SSN6. This brings the TUP1/SSN6 complex in proximity to the promoter, where it orders nucleosomal arrays or interacts with RNA polymerase II to induce repression. (**B**) shows the families of genes regulated by TUP1/SSN6 and the equivalent DNA binding protein.

1.3.3 GROUCHO

Initially, groucho (gro) was identified in a screen for genes affecting neurogenesis in Drosophila, with one mutation resulting in a phenotype of thick sensory bristles over the eyes resembling the bushy eyebrows of Groucho Marx. Groucho was shown to be part of the enhancer of split complex (E (spl)), this complex mediates neurogenesis via Notch signalling. Cells that have initiated neurogenesis emit a signal that is received by Notch receptors of neighbouring cells and signal transduction prevents these cells initiating neurogenesis (reviewed by Parkhurst, 1998). Further studies have shown roles for groucho in development including segmentation, dorsal/ventral pattern formation, and sex determination (Paroush *et al.*, 1994).

Like the TUP1-SSN6 co-repressor, groucho lacks a DNA-binding domain and is recruited to the DNA by protein-protein interactions with specific repressors. Groucho interacts with a variety of such repressors including the hairy family of transcription factors, runt domain factors, engrailed and dorsal (Parkhurst, 1998). It acts as part of a large nucleoprotein complex to achieve repression, which is illustrated by its action at the zerknüllt gene (fig. 1.9).

The zerknüllt gene is regulated by dorsal, which binds a regulatory sequence upstream of the promoter where it acts with another transcription factor called dead ringer. Both bind groucho and bring it in proximity to RNA polymerase II and the gene to be repressed (Valentine et al., 1998). The complex also contains a protein

known as capicua, which has an HMG-box (Jimenez *et al.*, 2000). HMG domains are involved in bending DNA, which allows the protein complex to make interactions that would otherwise not be possible. Similarly to TUP1, groucho mediates these protein-protein interactions via its WD repeats, as disruption of these leads to loss of binding (Jimenez *et al*, 1997). However, in *S. cerevisiae*, in most circumstances it is SSN6 that is involved in protein interactions, but no SSN6 homologue has been identified yet in Drosophila.

The mechanism by which groucho represses genes, is still poorly understood. There is no evidence to suggest it interacts with the basal transcriptional machinery in the same manner as TUP-SSN6. However, similarities in the function of two co-repressors may arise at the local chromatin level. Groucho has the potential to form oligomeric structures (Chen *et al.*, 1998) and may impose a repressive chromatin environment by spreading along the locus like TUP1 at the *STE6* gene.

Groucho, like TUP1, interacts with HDAC1 encoded by the *rpd3* gene in Drosophila (Chen *et al.*, 1999). This interaction is mediated by the glycine-proline (GP) rich domain of groucho. Experiments using trichostatin A (TSA) and mutant forms of *rpd3* showed this interaction is functional, since repression of genes by groucho was compromised. However, the phenotype given by rpd3 mutation is not as severe as groucho mutation, indicating either functional redundancy of HDACs or, as with TUP1, groucho may interact with multiple HDACs to achieve repression depending on the particular gene. In addition, groucho binds hypoacetylated histone tails; mutations affecting this binding also weaken its repression (Flores-Saaib and Courey,

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2000). Groucho interacts with histones via its N-terminus, like TUP1, and although the interacting regions share no sequence homology it is plausible that they both form similar structures to facilitate histone binding. Interestingly, groucho also associates with histone H1, which may suggest that groucho is involved in influencing higher order chromatin structure (Chen *et al.*, 1999).
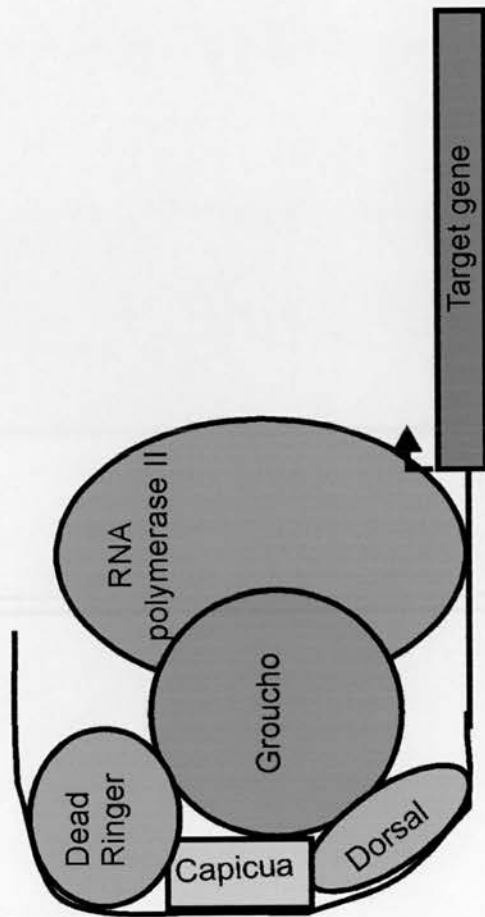
Figure 1.9: Nucleoprotein complex involved in groucho-mediated repression. Groucho is brought into the DNA via interaction with dorsal and dead ringer. This means groucho is positioned near the promoter of the target gene and the RNA polymerase II complex. The Capicua protein is also present within the complex to bend the DNA allowing multiple DNA-protein interactions.

1.3.4 Transducin-Like Enhancer of Split Proteins (TLE)

Homologues of the Drosophila protein GROUCHO were identified in mammalian cells. The human genome encodes at least four Groucho homologues called transducin-like enhancer of split proteins 1-4 (Stifani *et al.*, 1992). These have significant homology to groucho, having similar Q-rich domains and WD repeats. A further subclass of groucho homologues was also identified, called AES (amino enhancer of split), which contain the conserved Q-domain but lack the WD repeats. The GP and SP domains identified in groucho are poorly conserved between TLEs.

Further research showed that mammalian homologues perform functions, which are similar to those in Drosophila, such as: neurogenesis, Notch signalling, and cell fate decision. However, the situation in mammalian cells is more complex, given the identification of multiple homologues and that each TLE has its own expression pattern (Stifani *et al.*, 1992). Despite these differences, a number of studies have shown TLEs to behave in a manner similar to TUP1 and GROUCHO: they associate with themselves allowing oligomeric structures (Palaparti *et al.*, 1997). Therefore, gene repression may be mediated by TLE spreading along the template. They interact specifically with histone H3 (Palaparti *et al.*, 1997), and an SSN6-like protein, encoded on either X or Y-chromosomes in mice and humans, UTX or UTY respectively. UTX and UTY have been shown to bind TLEs, suggesting they are a mammalian SSN6 homologue. This implies that TLEs function in a manner analogous to TUP1-SSN6 (Grbavec *et al.*, 1999).

These similarities in interactions lead to an understanding of how mechanisms in transcriptional repression are conserved. They also allow speculation that processes that occur in one functional homologue may also happen in others. For example, it has been shown that TLE1 and TLE2 are associated with the nuclear matrix, allowing concentration of these regulatory factors and facilitating transcriptional regulation (Javed *et al.*, 2000). This could suggest that TUP1 and GROUCHO also function in this way, although no studies have shown this yet.

Much more is known about the actual regulation of TLEs than the other homologues. Phosphorylation of TLE1 by cdc2 reduces their ability to repress genes. This occurs during the G2/M phases in the cell cycle. In these phases TLE1 appeared to be excluded from the nuclei; indeed, one phosphorylation site is located near the nuclear localisation sequence of TLE1 which may "mask" it, making it difficult for the protein to pass through the nuclear pores (Nuthall *et al.*, 2002). TUP1 is known to be a phosphoprotein, therefore a mechanism such as this is plausible for TUP1 regulation.

1.3.5 LEUNIG

LEUNIG (LUG) was first identified in a screen for modulators of flower development in *Arabidopsis thaliana* (Liu *et al.*, 1995). This protein was shown to be a negative regulator of *AGAMOUS (AG)*, a floral homeotic gene, the expression of which specifies stamen and carpel development. *AGAMOUS* expression is established by LEUNIG and maintained by the polycomb protein, CURLY LEAF

(Goodrich *et al.*, 1997) . Mutations in *LUG* caused ectopic expression of *AG* mRNA in petals and sepals resulting in carpel-like structures in the outer whorl and petals or stamens being absent.

Cloning of the *LEUNIG* gene showed that it encoded a protein with two N-terminal Q – rich regions and seven WD repeats at the C-terminal (Conner and Liu, 2000). These motifs have significant homology to the GROUCHO/TUP1 family of co-repressors. Furthermore, stamen and carpel development is a process similar to segment identity organisation in *D. melanogaster*, suggesting LUG is involved in similar processes to groucho. Therefore *LEUNIG* has been proposed as the *Arabidopsis* homologue of TUP1.

Further evidence of this comes from the discovery of an *Arabidopsis* protein called *SEUSS* (*SEU*), which is a candidate for an *Arabidopsis* SSN6 homologue (Franks *et al.*, 2002). *SEU* is also a negative regulator of *AGAMOUS*, moreover, phenotypes of *SEUSS* mutations are similar to those in *lug* mutants and the double mutant shows an enhanced phenotype. Seu mutations also cause ectopic expression of *AG* mRNA. The *SEUSS* gene encodes a glutamine rich protein with a putative protein-protein interaction domain, which is consistent with *SEUSS* being functionally homologous to SSN6 as the *S. cerevisiae* protein also has glutamine rich domains and is involved in protein-protein interactions in the cell. Furthermore, yeast-2-hybrid analysis revealed that *SEU* interacts with *LUG*. Both *SEU* and *LUG* have no apparent DNA binding motifs, implying an adapter protein would be required to recruit the complex to the DNA.

One hypothesis for the action of *LUG* and *SEU* is similar to the mechanism of the TUP1/SSN6 complex in yeast: SEU may bind a protein known as APETALA 2. APETALA 2 is thought to recruit the *SEU/LUG* complex to the *AGAMOUS* gene in the way that MIG1 will recruit TUP1/SSN6 to the *SUC2* gene, although further analysis is necessary to confirm this.

1.4 Gene Silencing in Plants

When transgenes were first introduced into plants, it became clear that they are subject to unpredictable silencing and variable expression patterns. This epigenetic silencing falls into two categories. One type of silencing is due to the chromosomal location of the integrated transgene where it is subject to position effects of its chromatin environment (discussed in section 1.2.4). The other type of silencing became known as homology-dependent gene silencing. When multiple copies of DNA sequences are present within a genome they interact and become silenced. This is somewhat counter-intuitive; if multiple copies of a gene were present, a higher level of expression would be expected. The endogenous genes are also subject to this silencing in a manner similar to paramutation, where an interaction between two alleles results in the heritable alteration in the expression pattern of one of the alleles (reviewed by Martienssen, 1996). Homology-dependent gene silencing is a troublesome phenomenon for genetic engineering, but it has brought to light many epigenetic features of gene regulation and has uncovered an ancestral mechanism for the suppression of transposable elements and viruses.

1.4.1 Homology-dependent Gene Silencing (HDGS)

Homology-dependent gene silencing can occur at different stages in the expression of genes. This allows HDGS to be separated into two categories: transcriptional gene silencing (TGS) and post-transcriptional gene silencing (PTGS). The most notable differences being that during TGS no transcripts are detected but for PTGS messenger RNAs are produced but not translated. TGS and PTGS share some common features such as *de novo* methylation of the specific DNA sequences (Matzke, 1989), but also differ mechanistically. For TGS it is the homology between the promoters, which is essential for silencing, but in PTGS, the coding regions of the gene are necessary for silencing. Indeed, it is the promoters of genes silenced by TGS, which are methylated, but methylation is found in the coding regions of genes silenced by PTGS. TGS is a heritable epigenetic state but PTGS can be lost after meiosis. However, common themes run through both mechanisms and the pathways may overlap to some extent.

Similar processes to TGS and PTGS occur in the filamentous fungi *Neurospora crassa* and *Ascobolus immersus*. These have been used as model organisms for repeat-induced gene silencing and much of the information generated from such experiments can be applied to plants.

1.4.2 Gene Silencing Mechanisms in Filamentous Fungi

Much of the evidence supporting the relationship between repeated DNA sequences and silencing comes from phenomena noted in two filamentous fungi. Methylation induced premeiotically (MIP) and repeat-induced point mutation (RIP) are processes that occur in response to repeated DNA sequences in the fungi, *Ascobolus immersus* and *Neurospora crassa*, respectively (Goyon and Faugeron, 1989; Selker and Garrett, 1988). These are virtually identical silencing mechanisms, where repeated DNA becomes methylated and silenced. In RIP however, this silencing becomes irreversible due to the spontaneous deamination of methylated cytosine residues generating C→T transitions. Once a sequence has been mutated by RIP, it becomes a target for *de novo* methylation of the remaining cytosine residues further, reinforcing the silenced state.

The exact mechanisms of these silencing events remain unclear. The recognition of the repeated sequences is likely to involve DNA: DNA pairing. This is strongly implied by the fact that the DNA modifications are exclusively confined to the duplicated sequences. Furthermore, closely linked repeats are discovered more readily by the RIP machinery than duplications that are separated (Selker, 1999). Intricate links between chromatin structure and DNA methylation have been established in *N. crassa*. Inhibition of histone deacetylases causes a loss of DNA methylation and RIP gene silencing, showing that two are closely linked and play a role in silencing (Selker, 1998). In addition, the discovery that mutation of a lysine-9 histone methyltransferase causes loss of DNA methylation further connects the roles

of chromatin structure and DNA methylation (Tamaru and Selker, 2001). This implies that the genes silenced by RIP are likely to have an altered chromatin structure.

This change in chromatin conformation has been hypothesised to allow the production of so called aberrant RNA. This is RNA, which is somehow different in structure to RNAs produced by genes that are not subject to silencing. This suggests a link with another silencing mechanism found in *N. crassa*, known as quelling (Romano and Macino, 1992). Quelling has similar mechanistic properties to PTGS in plants and is used as a model system. Quelling is triggered by duplicated coding sequences, which become silent. This is partially separate from RIP, as it is reversed once transgenes are removed. Mutants defective in quelling have been observed and one gene identified in this screen, *qde-1*, was shown to encode an RNA-dependent RNA polymerase (RdRP) (Cogoni and Macino, 1999). It is hypothesised that this is produced in response to aberrant RNAs produced by a highly active transgene or by a silent locus. The RdRP synthesises complementary RNAs generating double stranded RNA species that are known to promote gene silencing (Mette *et al.*, 2000). This process may also be used to reinforce silencing already achieved by RIP, suggesting mechanistic links between transcriptional and post-transcriptional gene silencing.

Much of our knowledge of plant transcriptional gene silencing and post-transcriptional gene silencing has been based on observations from these filamentous fungi, indicating an evolutionary conserved mechanism for silencing repeated DNA.

1.4.3 Post-transcriptional Gene Silencing

PTGS genes are silenced due to a sequence-specific RNA degradation process that affects homologous transcripts. RNA directed silencing or RNA interference (RNAi) has been observed in many other organisms including *C. elegans* and *D. melanogaster*. For example, multiple copies of a transgene caused gene silencing in *D. melanogaster*. This silencing was found to be dependent on a polycomb protein (Pal-Bhadra *et al.*, 1997). Polycomb proteins are involved in homeotic gene expression and cellular memory. Our understanding of PTGS like phenomena in other organisms can contribute to understanding the mechanisms in plants.

In fact, much of our understanding of PTGS comes from quelling in *N. crassa* and plants infected by viruses that have RNA intermediates in their life cycle. Like quelling, it is believed that transgenes and viruses produce aberrant RNA. What constitutes aberrant RNA is unknown; it may be a result of the high expression of these genes. Another hypothesis suggests that it is DNA: DNA pairing between homologous sequences interferes with transcription. DNA: DNA pairing produces an RNA species, which is recognised by the cell as different, perhaps due to the introduction of a premature stop codon. This aberrant RNA induces the production of an RdRP that produces small complementary RNAs (Hamilton and Baulcombe, 1999; Mourrain *et al.*, 2000). These small RNAs pair with the mRNA transcribed by the gene and produce dsRNA species, which are believed to be degraded by a double strand dependent RNase (Hammond *et al.*, 2000). This prevents the expression of proteins from these mRNAs, thus silencing the gene (fig. 1.10). Silencing of this type

can spread throughout the plant by a signalling molecule, popularly believed to be double stranded RNA, which travels through the plasmodesmata and phloem (Palauqui, *et al*, 1997).

The double stranded RNA not only allows the degradation of messenger RNA; it acts to reinforce the silencing at the DNA level. RNA has been shown to trigger the DNA methylation found in the coding sequences of PTGS genes (Wassenegger *et al.*, 1994). Indeed, the methylation found in these genes is found at both symmetric and asymmetric cytosine residues, which is frequently found at transgenic loci (Meyer *et al.*, 1994), and is indicative of RNA-directed DNA methylation (Pélissier *et al.*, 1999). The function of the DNA methylation may not be primarily for gene silencing but rather for maintenance of the silenced state, as the *MET1* gene, homologous to the mammalian DNA methyltransferase, *DNMT1*, seems to be required for the maintenance of the PTGS state (Morel *et al.*, 2000).

The role of double stranded RNA in gene silencing seems to have wider implications for heterochromatin formation. It has long been established that the Xist RNA produced from the inactive X chromosome in mammalian cells coats the inactive chromosome. This is followed by the introduction of a repressive histone code and DNA methylation (reviewed by Lyon, 1998). More recently, Maison *et al.* (2002) noted that pericentric heterochromatin contained an RNA moiety; removal of this meant the loss of a higher order chromatin structure. Furthermore, constituents of the RNAi machinery were found to be involved in the organisation of centromeric heterochromatin in *S. pombe* (Volpe *et al.*, 2002).

Deletion of components of the RNA interference-silencing complex (RISC), such as RdRP, caused loss of silencing of transgenes integrated at heterochromatic centromeres in *S. pombe*. These centromeres were also depleted in methylated lysine 9 residues on histone H3 tails. This prevents the recruitment of SWI6, the *S. pombe* homologue of HP1, and subsequent silencing. Repeats found at the centromere are believed to produce RNA species, which are manipulated by the RISC complex to produce RNAi. It is thought that RNAi may recruit the histone H3 methyltransferase to impose a repressive chromatin code, which is recognised by other proteins. This is reinforced by the finding that chromodomains of the type found in the H3 methyltransferase are known to interact with RNA molecules (Akhtar *et al*., 2000). Such repression only occurs on one strand, so the other is actively producing RNA, which leads to a self-reinforcing silent state. Much of the machinery, required for this type of silencing, has homologues in many other organisms. To date none of the RISC complex proteins have been identified in *S. cerevisiae*, which uses the species-specific silent information regulator (SIR) proteins for heterochromatin-induced gene silencing.

These observations show a broader spectrum of RNA function within a cell. With complexes involved in PTGS also involved in heterochromatin formation, it is feasible that double stranded RNA also may induce a repressive histone code and heterochromatic state at PTGS genes. A similar mechanism of one strand of DNA producing transcripts to reinforce silencing may also occur at these genes. The chromatin-related silencing once thought to be more applicable to TGS may also be involved in PTGS and the two may be mechanistically related.
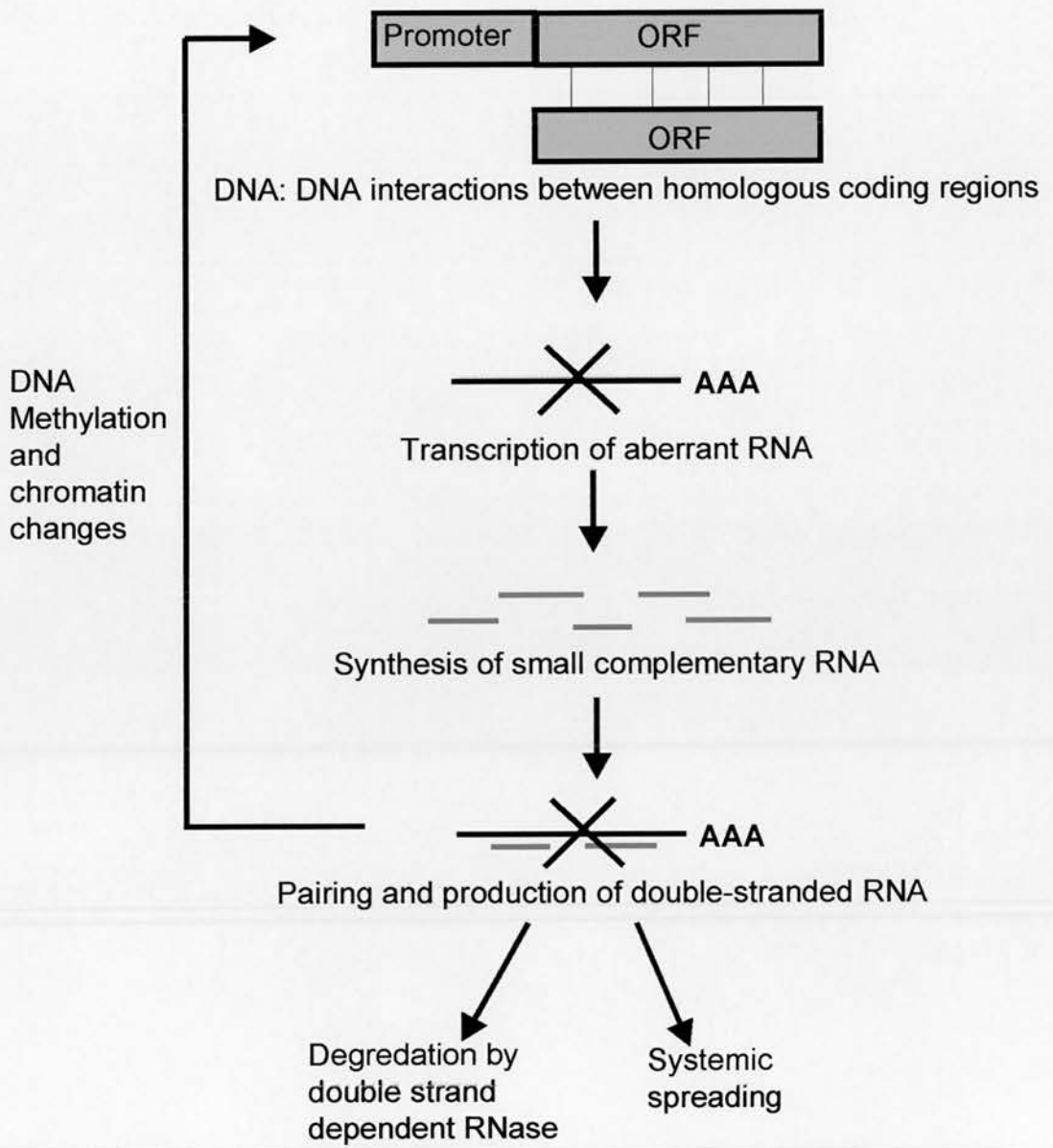
Figure 1.10: A schematic representation of post-transcriptional gene silencing (PTGS).

1.4.4 Transcriptional Gene Silencing

Genes silenced by TGS acquire epigenetic states associated with hypermethylation of promoters and alteration in the local chromatin environment (Matzke *et al*, 1989; Ye and Signer, 1996). Like PTGS, it is believed that this gene silencing and DNA methylation is triggered by DNA: DNA interactions between homologous sequences. Studies have shown that double-stranded RNA can also trigger promoter methylation in TGS, implying that TGS and PTGS may have some mechanistic similarities (Mette *et al.*, 2000).

Various studies suggest that it is unlikely that methylation alone causes gene silencing in TGS but may act to assist the chromatin components and mark it as a silent locus for inheritance purposes. Much of our understanding of the TGS phenomenon, and in particular TGS mutants, has led to a greater knowledge of how DNA methylation and chromatin structure are intrinsically linked.

Mutations in the *Arabidopsis* gene *DDM1* (decrease in DNA methylation) gene cause a global decrease in DNA methylation and reactivation of TGS silenced genes (Jeddeloh *et al.*, 1998) and transposable elements (Miura et al., 2001). This gene was shown to encode a protein with similarity to the SWI/SNF family of ATP-dependent chromatin remodelling complexes (Jeddeloh *et al.*, 1999). This suggests DDM1 plays an indirect role in DNA methylation and may remodel chromatin to allow the access of DNA methyltransferases. DDM1 is also required to maintain histone H3 methylation at lysine 9, which marks silent chromatin (Gendrel *et al.*, 2002). Since

DNA methylation has been shown to depend on H3 methylation (Tamaru and Selker, 2001), it is possible that the DNA methylation of transgenes and transposable elements is guided by H3 methylation at lysine 9. It is tempting to hypothesise a role of the lysine-9 binding protein HP1 (Bannister *et al.*, 2001; Lachner *et al.*, 2001) and other chromodomain proteins in TGS. Indeed, homologues of HP1 have been identified in *Arabidopsis* (Gaudin *et al.*, 2001). Moreover, a protein isolated from *Arabidopsis*, Chromomethylase, contains a chromodomain and a putative DNA methyltransferase domain, directly linking chromatin structure with DNA methylation (Henikoff and Comai, 1998).

Mutations in the *Arabidopsis MOM* (Morpheus molecule) gene have shown that DNA methylation and gene silencing are not always linked. *Arabidopsis* cell lines mutant for *MOM*, showed activation of previously silent gene (Amedeo *et al.*, 2000). However, unlike *ddm1* cells, there was no demethylation of the silent loci. Sequence analysis of the *MOM* gene revealed it has similarities to the SWI/SNF helicase proteins suggesting that, like DDM1, MOM may function as part of a chromatin-remodelling complex. MOM may act as a mediator which works downstream of DNA methylation to induce gene silencing, analogous to mammalian methyl-CpG binding proteins which recruit other proteins to methylated DNA to alter the chromatin structure. Alternatively, MOM may act in another pathway, which is completely independent of DNA methylation.

These gene-silencing mutants from *Arabidopsis* have shown that DNA methylation is not a pre-requisite for silencing genes. It is clear that chromatin structure plays a

significant role in the silencing of genes. Indeed, many organisms such as *S. cerevisiae* and *D. melanogaster* have the ability to confer epigenetic states onto genes without the extensive methylation seen in plant and mammalian cells. The discoveries linking chromatin structure and DNA methylation may have wider implications for all species. Mutations in the *ATRX* gene in humans (Gibbons *et al.*, 2000) and *LSH* in mice (Dennis *et al.*, 2001) also cause alterations in genomic methylation levels, and these genes both encode SWI/SNF like proteins. However, whether these proteins actually have the proposed chromatin remodelling activities remains to be seen. The relationship regarding gene silencing, DNA methylation, and chromatin remodelling is complex and may be situation dependent.

1.4.5 Ancestral Function of Homology-Dependent Gene Silencing

The evolutionary basis of homology-dependent gene silencing is as a protective mechanism against invasive exogenous DNA such as transposable elements. The mechanisms of transgene silencing share similarities to those of repression of repetitive DNA (which may cause deleterious effects through recombination), viruses, and transposable elements. Therefore, PTGS and TGS are important protective mechanisms for genomes against recombination and transposition events. Transgenes share common features with transposable elements given their invasive and often repetitive nature and are therefore subject to TGS and PTGS.

Like transgenes, transposable elements are generally heavily methylated for silencing and limiting their spread throughout the genome (Miura *et al.*, 2001). The inverted

repeats, produced as a result of transposition by transposable elements, are thought to act as a signal for *de novo* methylation to the host genome and are believed to be silenced by TGS. Further evidence linking transgene and transposable element silencing comes from the *ddm1* mutants. Loss of DDM1 from *Arabidopsis* leads to activation of both silenced transgenes and transposable elements suggesting a common pathway.

Whilst TGS seems to be a mechanism for silencing transposable elements, PTGS is known to be involved in the repression of viruses in plants. Plant viruses are both targets and inducers of PTGS. Many of the proteins involved in PTGS are also essential for viral suppression (Di Serio *et al.*, 2001; Xie *et al.*, 2001). Viroids have been shown to induce RNA-directed DNA methylation in a similar manner to non-pathogenic sequences (Wassenegger *et al.*, 1994). Indeed, any virus with an RNA genome or an RNA replication intermediate has the potential to induce PTGS. Furthermore, the systemic spread of PTGS throughout the plant could be seen as a mechanism to prevent other cells from being damaged by viral infection.

The need to silence parasitic DNA sequences is essential for the integrity of the genome. Transposable elements and viruses have the potential to transpose and integrate at potentially deleterious locations within the host genome. The cell has evolved TGS and PTGS mechanisms to minimise the prospective damage. Whilst these selfish DNA elements exploit the host genome, the host has also evolved to utilise the propensity for transposable elements to be silenced to its advantage. The Lyon hypothesis suggests that the enrichment and non-random distribution of LINE-

1 elements on the mammalian X-chromosome is a method of harnessing the cells ability to silence these elements to propagate X-inactivation and silencing along the chromosome (Lyon, 1998). This suggests that these silencing mechanisms, although evolved for one purpose, may have wider implications for gene regulation in the cell.

1.4.6 Position Effects and Gene Silencing

As discussed in section 1.2.4, the genomic context of where transgenes are integrated influences their expression patterns. Genes juxtaposed within heterochromatin are often silenced, which is a common cause of transgene silencing in plants. In addition, position effects may be responsible for the transfer of a repressive chromatin structure to a homologous gene not previously silenced, by DNA: DNA pairing or by the spread of heterochromatin. It has been shown that transgenes can adopt the methylation status of the locus where they are integrated (Prols and Meyer, 1992).

Recurring sequence motifs have been observed in DNA that flanks silenced genes in plants (Matzke and Matzke, 1998). These include matrix attachment regions (MARs); however, these have also been shown to promote stable expression of transgenes. The other category of flanking DNA is repetitive sequences including microsatellites. A repetitive sequence isolated from *P. hybrida* was shown to destabilise the expression of an adjacent reporter gene (ten Lohuis *et al.*, 1995). The sequence was proposed to induce a repressive chromatin structure, which spread along the reporter gene. A third category of flanking DNA is retroelement remnants. These have previously been shown to influence expression patterns of adjacent

regions (Cambareri *et al.*, 1996). The fact that transposable elements are also subject to transcriptional silencing and are often associated with heterochromatin reinforces the hypothesis that these can induce position effect variegation.

The different types of gene silencing observed in plants have similar mechanistic properties and have revealed many functions of chromatin that have wider implications for the cell. Many of these processes occur in other organisms suggesting there may be a conserved mechanism of chromatin-mediated gene silencing.

1.5 Gene Silencing in Yeast

Transcriptional gene silencing occurs in *S. cerevisiae* by the formation of a heterochromatin-like structure at discrete loci. The yeast genome is generally transcriptionally active compared to mammalian cells, and silencing is confined to the silent mating type loci (*HM* loci), telomeres and rDNA repeats. Placing genes next to any of these loci causes position effect variegation and silencing of the adjacent gene (Rine and Herskowitz, 1987; Gottschling *et al.*, 1990, Smith and Boeke, 1997). The SIR proteins (silent information regulators), SIR1, SIR2, SIR3, and SIR4 mediate this process. These SIR proteins do not directly bind DNA, but form complexes at silent loci by interacting with specific DNA-binding factors. This gives the SIR proteins added functionality at different loci in a manner similar to TUP1/SSN6 and its homologues.

A model for SIR silencing is that SIR3 and SIR4 bind to hypoacetylated N-terminal tails of histones H3 and H4 (Hecht *et al.*, 1995; Carmen *et al.*, 2002). This forms a self-reinforcing structure given that SIR3 and SIR4 are capable of homodimerisation and heterodimerisation. It is thought this forms a higher order structure that spreads along the silenced locus and stabilises a heterochromatin-like structure (Hecht *et al.*, 1996). Given that SIR2 is an NAD-dependent deacetylase, it may facilitate SIR3 and SIR4 binding by inducing hypoacetylation of the relevant histones (Imai *et al.*, 2000; Landry *et al.*, 2000). Mutational analysis shows that this HDAC activity is required for silencing, as mutations affecting this enzymatic ability also cause silencing defects. The structure generated by SIR protein binding is resistant to nucleases and displays features of heterochromatin such as localisation at the nuclear periphery and epigenetic inheritance of the silenced state (Palladino *et al.*, 1993).

1.5.1 Silencing at the Silent Mating-Type Loci (*HM* loci)

The two silent mating-type loci, *HMR* and *HML* confer information to the *MAT* locus and are responsible for the mating-type switch in *S. cerevisiae*. The *HM* loci are flanked by *cis*-acting elements, silencers *E* and *I* (for review see Laurenson and Rine, 1992). These silencers limit the spread of SIR proteins to adjacent regions and impose silencing in a directional manner. The *E* and *I* silencers contain arrays of binding sites for DNA-binding proteins which are required to initiate the assembly of the SIR complex. The proteins that are responsible for the recruitment of the SIR proteins by binding these sites are ABF1 (ARS-binding factor 1), and RAP1 (Repressor-activator protein 1), which recruit SIR3 and SIR4 (Sussel and Shore,

1991; Boscheron *et al*, 1996). The silencers also contain binding sites for ORC (origin recognition complex) that recruits SIR1, which in turn binds SIR4 (Triolo and Sternglanz, 1996). Multiple mutations in these binding sites are required to affect silencing, indicating some functional redundancy between the proteins involved. Once the recruitment is completed, SIR3 and SIR4 are believed to spread along the locus inducing a repressive chromatin structure.

SIR1 appears to have a different role in silencing than the other SIR proteins. SIR1 is only involved in silencing at the *HM* locus, not at the telomeres. Moreover, deletion of SIR2, SIR3 or SIR4 abolishes silencing at the *HM* loci, whilst disruption of SIR1 only causes mild defects in silencing. Whilst SIR2, SIR3 and SIR4 are evenly distributed throughout the silent loci, SIR1 remains localised at the *E* and *I* silencers Rusche *et al*., 2002). Yeast bearing functional mutations in *SIR1* lack stable inheritance of the silent mating type locus, and are composed of mixed populations of silenced and non-silenced cells. Studies showed once silencing was established it was stably maintained, suggesting maintenance and establishment of silent chromatin is essentially different. This suggests a role for SIR1 in the establishment of silent chromatin and not maintenance like the other SIR proteins.

1.5.2 Transcriptional Silencing at Telomeres

S. cerevisiae telomeres are composed of $C_{1-3}A/TG_{1-3}$ repeats of around 300bp in length (reviewed by Tham and Zakian, 2002). In addition, subtelomeric regions contain a series of repeats known as X repeats. Genes placed adjacent to long tracts

of telomeric DNA become silenced by position effect variegation, specifically called telomere position effect (TPE) (Gottschling *et al.*, 1990). The TPE phenomenon has been observed in other organisms such as *D. melanogaster* and humans. Silent chromatin found at telomeres has no obvious regulatory role as genes in this region are sparse, but exists to preserve the integrity of chromosome ends. Indeed, the loss of SIR2, SIR3 or SIR4 causes a decrease in telomere length and chromosome instability.

RAP1 is recruited to the telomere by multiple DNA-binding sites embedded amongst the telomeric repeats. SIR4 spreading along the telomeric tract is thought to be facilitated by binding to multiple RAP1 molecules situated along the telomere (Moretti *et al.*, 1994). SIR2 is recruited by virtue of its interaction with SIR4, which also recruits SIR3. SIR2 is believed to deacetylate histone tails, which reinforces SIR3 and SIR4 binding and allows their spread (fig. 1. 11). HDF1, which is the yeast homologue of the DNA damage repair protein Ku, is also required for telomeric silencing, and may play a role in SIR protein recruitment (Mishra and Shore, 1999). ORC, SIR1 and ABF1 are not required for the assembly of SIR proteins onto telomeres although binding sites have been identified in the subtelomeric X-repeats.

The composition of silent chromatin at telomeres is not uniform, there is a "core" region which contains SIR2, SIR3 and SIR4, and an extended region furthest from the chromosome end. This extended region mainly consists of SIR3 and limiting amounts of the other SIR proteins (Renauld *et al.*, 1993). This observation reinforced

by the fact that the subtelomeric X-elements act as proto-silencers, which direct chromatin formation towards the end of chromosomes (Lebrun *et al.*, 2001).

In both the silent mating-type loci and telomeres, there are definite demarcation lines of what is silent chromatin and active chromatin. This is shown by the presence of silencer elements, and is reflected by local histone modifications. Suka *et al* (2002) demonstrated that acetylation of histone H4 at lysine 16 prevented the spread of SIR3 at yeast telomeres. Such acetylation is carried out by the acetyltransferase SAS2; yeast with mutated SAS2 exhibited SIR3 spreading from 3 Kb to 15Kb with hypoacetylation of adjacent chromatin previously maintained active by SAS2. This implies that H4 lysine 16 acetylation provides a barrier for the spread of SIR proteins indicating it may be a mark for euchromatin. SIR2 has been shown to deacetylate H4 lysine 16 in vitro, suggesting SAS2 and SIR2 have opposing roles in acetylation and SIR3 spreading to induce a silenced chromatin state at yeast telomeres.

Further evidence for the role of histone modifications determining heterochromatic and euchromatic regions comes from the methylation of lysine 79 on histone H3 in *S. cerevisiae*. Methylation of this residue is mediated by the methylase DOT1 and mutations in *DOT1* disrupt silencing at both telomeres and silent mating-type locus (van Leeuwen *et al.*, 2002). One model suggests that methylation of H3 lysine 79 prevents SIR proteins binding to the histone tails. Indeed, H3 lysine 79 methylation is associated with active chromatin in both yeast and human cells (Ng *et al.*, 2003). All silenced loci in *S. cerevisiae* show hypomethylation of this residue. Loss of SIR2, SIR3 and SIR4 results in increased methylation of this residue, but not to the same

extent as euchromatin indicating only a partial dependence. The relationship between SIR proteins and this methylated residue is complicated. However, these observations combined the acetylation studies, suggest an intricate association between SIR proteins and modifications on histones, which in turn determines the chromatin structure of the locus.
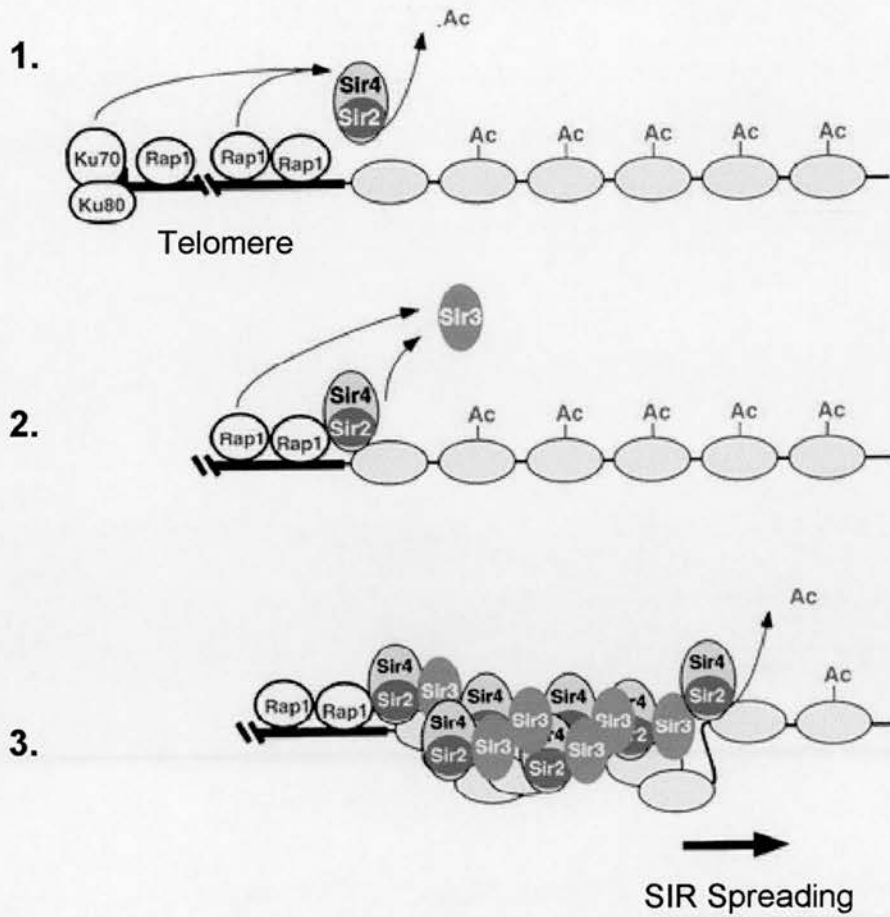
Figure 1.11: Model for SIR-mediated silencing at telomeres. Nucleosomes are shown as pink ovals and Ac is an abbreviation for acetyl groups. **1.** DNA-binding proteins yKu and RAP1 associate with the telomeric DNA. These recruit SIR2 and SIR4, SIR2 deacetylates histone tails. **2.** Following deacetylation SIR3 is recruited via its association with the hypoacetylated histone tails and also by its interactions with SIR4 and RAP1. **3.** SIR3 and SIR4 form multimers and spread due to multiple rounds of deacetylation by SIR2.

1.5.3 Transcriptional Silencing at the Ribosomal DNA Locus

The rDNA locus in *S. cerevisiae* consists of 100-200 tandemly repeated copies of rDNA genes, which form the nucleolus at the nuclear periphery. Each repeat contains a 5S rRNA gene and a 35S pre-rRNA gene transcribed by RNA polymerases III and I respectively. Some rDNA genes are repressed and some are expressed in order to produce ribosomes. The rDNA sequences are potentially recombinogenic; therefore, SIR2 represses mitotic and meiotic recombination events (Gottlieb and Esposito, 1989). SIR1, SIR3 and SIR4 do not play a role in silencing at this locus. The association of SIR2 with the rDNA locus requires the DNA-binding protein NET1, although the mechanism of NET1 recruitment is unclear, since no NET1 binding sites have been identified (Straight *et al.*, 1999). Certain RNA polymerase II transcribed genes are subject to SIR2 dependent position effect variegation when integrated into this locus (Bryk *et al.*, 1997; Smith and Boeke, 1997). This is surprising since RNA polymerase I and III can transcribe rRNA efficiently at this locus. This may suggest a specialised form of chromatin at this locus.

Psoralen cross-linking experiments revealed that the chromatin structures of active and inactive rDNA copies are different. Inactive genes have a regular nucleosomal array whilst active copies are nucleosome free (Dammann *et al.*, 1993). *SIR2* mutants had increased accessibility to psoralen cross-linking indicating that this structure is dependent upon SIR2 (Fritze *et al.*, 1997). This specialised chromatin structure may have originated not to prevent transcription but to exclude recombinational machinery, which may have deleterious effects.

1.5.4 Diverse Functions of Silent Information Regulators (SIR)

SIR proteins have additional functions within the cell that appear not to have a direct relationship with silencing. As previously mentioned, SIR4 associates with HDF1 or yKu at telomeres. Ku is a DNA-repair protein involved in non-homologous end joining in double-strand break repair. Upon DNA damage, yKu and SIR4 dissociate from the telomere and are directed to the site of DNA damage (Martin *et al.*, 1999). It is unknown whether SIR4 plays a direct role in DNA repair, or whether it is moved to the site of damage by virtue of its interaction with yKu. However, it has been hypothesised that SIR4 induces a heterochromatic-like state that aids end joining and DNA repair.

SIR3 and SIR4 have no known homologues in other species and appear to be specific for gene silencing in *S. cerevisiae*. However, the SIR2 protein has homologues from bacteria to humans. Bacteria have essentially no chromatin; suggesting the enzymatic properties of SIR2 may play other pivotal roles in cellular processes. Seven putative SIR2 homologues have been identified in humans. Indeed, four additional SIR2-like proteins occur in *S. cerevisiae*. These homologues (HST1-4) may also share a role in gene silencing (Brachmann *et al.*, 1995). HST2 has histone deacetylase activity and influences gene silencing at both telomeres and rDNA repeats (Perrod *et al.*, 2001).

One interesting role for SIR2 is its involvement in cellular ageing. Yeast ageing is based on the number of cell divisions undergone by a mother cell. The accumulation of extrachromosomal rDNA circles (ERC), generated by recombination of the rDNA

repeats was shown to cause ageing in *S. cerevisiae* (Sinclair and Guarente, 1997). ERC molecules have origins of replication, and therefore multiply at each cell division. Segregation of ERCs is biased towards the mother cell, and when they reach numbers of around 500-1000 per cell (after roughly 20 cell divisions), the mother cell can no longer divide. SIR2 directly controls the levels of ERCs by preventing homologous recombination of rDNA repeats (Kaeberlein *et al.*, 1999). In addition, caloric restriction in rodents and *S. cerevisiae* has been shown to increase life span (Lin *et al.*, 2000). The increase in life span by this mechanism requires SIR2 and NPT1, an enzyme involved in the synthesis of NAD. Since the ability of SIR2 to deacetylate histones is dependent upon NAD, this provides a link between cellular energy levels and chromatin structure. It is likely that the increased levels of NAD induced by caloric restriction increase the HDAC activity of SIR2, which ultimately influences chromatin structure and reduces ERC production.

1.5.5 Other Proteins Involved In Gene Silencing in *S. cerevisiae*

There are a number of other proteins that play a role in silencing functions, whilst not as well characterised as the SIR proteins, they mainly consist of proteins influencing chromatin structure. Histone modifications have been shown to play a role in silencing; this is reinforced by the finding that a mutation in the SET1 gene (encoding a histone methylase) alleviates telomeric silencing. The role of histones in silencing is furthered by the observation that a histone H2A variant, HTZ1, is required for silencing at HM loci and telomeres (Dhillon and Kamakaka, 2000).

Chromatin assembly factor-1 (CAF1) mediates the assembly of histones onto DNA. It comprises a 3 subunit complex, consisting of CAC1, CAC2, and CAC3. Deletion of any of these genes results in reduced silencing at *HM* loci, rDNA repeats, and telomeres (Enomoto and Berman, 1998). The CAF1 complex is also involved in gene silencing mediated by the HIR (Histone information regulators) proteins. HIR proteins control the level of histone expression and their deletion has little effect upon silencing at HM loci and telomeres, however when combined with a deletion of a *CAC* gene, the silencing defects at these loci become exacerbated (Kaufman *et al.*, 1998).

All of the proteins discussed demonstrate the dependence of gene silencing upon chromatin structure and chromatin-related proteins. Many of the proteins discussed have homologues in other species, so knowledge of gene silencing in yeast has implications for gene regulation in organisms with more complex genomes.

1.5.6 Transcriptional Co-suppression in *S. cerevisiae*

Transcriptional co-suppression, the silencing of genes in response to increased copy number, has been well documented in plants but until recently not in *S. cerevisiae*. This was likely to be due to the low numbers of repetitive sequences in yeast. However, transposable elements such as *Ty1* elements are repeated and dispersed making them good candidates for co-suppression. Jiang (2002) noted that Ty1 elements in yeast are subject to repeat-induced gene silencing at the transcriptional level. Expression of *Ty1* elements was found to occur in two states; all genes were

switched on or all genes were switched off, with rapid switches between the two states. There was no mosaic expression of the *Ty1* elements as seen for genes influenced by position effect variegation. This is the first example of co-suppression observed in *S. cerevisiae*, and the mechanisms still remain elusive. One could hypothesise that chromatin structure might play a role in the repression. Moreover, this is a clear example of repeat-induced gene silencing that is independent of DNA methylation, which is often observed in plant systems.

1.6 Thesis Aims

Current knowledge of gene silencing and gene repression mechanisms suggests common themes running through species. Many proteins involved in repression are conserved or have conserved mechanisms such as the TUP1/Groucho family of co-repressors. The way that cells silence and respond to repetitive DNA is conserved in species from fungi, plants and mammals. In most of these cases the role of chromatin structure is critical.

In chapter 3, I address silencing of repetitive DNA. A repetitive fragment from *Petunia hybrida* found to silence genes was introduced into the yeast *Saccharomyces cerevisiae*. I observed if this sequence caused gene silencing in yeast and studied the mechanisms responsible. This established if similar gene silencing mechanisms exist in plants and yeast. By using the non-methylating organism, *S. cerevisiae,* as a tool for studying repetitive DNA, the processes of DNA methylation and chromatin structure can be dissected.

In chapter 4, I studied similarities between the TUP1/Groucho family of co-repressors. Members of this family, Groucho, Leunig, and BP1, were tested for their ability to repress genes in *S. cerevisiae*. Since the TUP1/SSN6 co-repressor acts as a paradigm for the other repressors in this family, I wanted to observe if the other members could repress genes in the same manner with respect to nucleosome positioning. This would help establish how functionally related these proteins are and elucidate their mechanisms of repression.

This thesis should give an insight into different methods of repression from different species and similarities and differences can be observed and discussed.

Chapter 2 - Materials and Methods

2.1 Reagents and Solutions

Agarose Gel Loading Buffer – the buffer consisted of 0.208% orange G, 12.5% ficoll type 400, and 100mM EDTA.

Antibiotics – Ampicillin was dissolved in distilled water to give a stock solution of 50 mg/ml. Geneticin (G418) was dissolved in distilled water to give a stock solution of 25 mg/ml.

Chloroform:Isoamyl alcohol (IAA) consisted of chloroform and iso-amyl alcohol (IAA) mixed at a ratio of 24:1

Diethyl pyrocarbonate (DEPC)-water was prepared by diluting diethyl pyrocarbonate to 0.1% in distilled water with agitation, followed by incubation at 37°C for 1 hour and autoclaving.

Dithiothreitol (DTT) was prepared by dissolving solid dithiothreitol at 1M in distilled water, which was stored in small aliquots at -20°C.

Drop-out mix – this is used in the preparation of synthetic complete medium, it consists of amino acids and nucleotide bases in the following amounts with the nutrient that is selected for omitted from the mixture.

Adenine – 0.125g	Leucine – 10g
Alanine – 2g	Lysine – 2g
Arginine – 2g	Methionine – 2g
Asparagine – 2g	*para*-aminobenzoic acid – 0.2g
Aspartic acid – 2g	Phenylalanine – 2g
Cysteine – 2g	Proline – 2g
Glutamine – 2g	Serine – 2g
Glutamic acid – 2g	Threonine – 2g
Glycine – 2g	Tryptophan – 2g
Histidine – 2g	Tyrosine – 2g
Inositol – 2g	Uracil – 2g
Isoleucine – 2g	Valine – 2g

EDTA – ethylene diamine-tetraacetic acid (disodium salt) was dissolved at 0.5M in distilled water and the pH was adjusted to 8.0 with NaOH.

Ethidium bromide stock solution was prepared by dissolving ethidium bromide to 10mg/ml in distilled water; the solution was stored in a light proof bottle at room temperature.

Phenol was prepared as follows: 250g of solid phenol was dissolved in 127ml of 2M Tris.HCl (pH 7.5) and the phases left to settle. The aqueous phase was removed and discarded. To the phenol phase, 55ml 2M Tris.HCl (pH 8), 13.75ml m-cresol, 550μl β-mercaptoethanol and 275mg 8-hydroxyquinoline were added. The solution was mixed well and left to settle. The phenol layer was retained, aliquoted and stored at -20°C. Buffered phenol chloroform was prepared by mixing phenol, chloroform and iso-amyl alcohol at a ratio of 25:24:1 respectively.

PMSF – phenyl-methyl-sulphonyl-fluoride was dissolved in isopropanol to a final concentration of 250mM.

RNase A was dissolved in water to 2mg/ml and boiled for 30 minutes to inactivate any DNases present in the preparation, then stored in small aliquots at -20°C.

Salmon sperm DNA was dissolved at 2mg/ml in TE (pH 8) overnight on a roller drum at 4°C, and stored in small aliquots at -20°C.

Sodium Acetate – 3M sodium acetate was prepared by dissolving powder in water and adjusting to pH 5.2 using concentrated acetic acid.

Sodium dodecyl- sulphate (SDS) – 10% (w/v) stock prepared in distilled water and pH adjusted to 7.2 with concentrated hydrochloric acid.

Sephadex G-25 solid was swelled with TE buffer overnight before use, and stored at room temperature.

Tris.HCl – 1M Tris.HCl was prepared by dissolving powder in water and adjusting to the appropriate pH using hydrochloric acid.

Tris/EDTA (TE) buffer – 10mM Tris.HCl (pH 8), 0.1mM EDTA.

2.2 *Escherichia coli* Culture and Manipulation

2.2.1 Culture Media

In liquid cultures, *E. coli* cells were grown in Luria-Bertani (LB) broth (1% tryptone, 0.5% yeast extract, and 0.5% NaCl). Agar plates were prepared by supplementing LB broth with 1.5% agar. For plasmid selection after transformation, ampicillin (final concentration of 50μg/ml) was added to either broth or solid media. Bacterial cultures on both solid and liquid media were left to grow at 37°C overnight or as indicated.

2.2.2 Bacterial Strains

E. coli JM110 (F' *traD36 lacI^{q}Δ(lacZ)M15 proA$^+$B$^+$/rpsL(Str^r) thr leu thi lacY galK galT ara fhuA dam dcm supE44 Δ(lac-proAB)*)) was used for cloning plasmids involved in RPS experiments. This strain is, *dam, dcm*, therefore deficient in methyltransferases which may methylate the RPS sequence. For other routine cloning *E. coli* DH5α (F'/*endA1 hsdR17(r^K.m^K+) supE44 thi-1 recA1 gyrA (Nal^r) relA1 Δ(lacIZYA-argF) U169 deoR (Φ80dlacΔ(lacZ)M15)*)) cells were used.

2.2.3 Bacterial Glycerol Stocks

1 ml of a saturated culture was added to an equal volume of glycerol solution (65% glycerol, 0.1M MgSO$_4$, and 0.025M Tris.HCl pH 8), and stored at -70°C.

2.2.4 Transformation of Plasmids into *E. coli*

Plasmids can be transformed into cells by either electrical or chemical transformation; electrical transformation was used as generally it has a much higher transformation efficiency.

2.2.5 Preparation of Electro-competent cells

2 ml of an overnight starter culture of E. coli cells was used to inoculate a 500ml culture of LB medium. This was left to grow, with good aeration until an OD_{600} reading of 0.6 was reached and the cells were left to cool on ice for 30mins. All steps following this were carried out in a cold room (4°C). The cells were then harvested by centrifugation at 4000rpm for 20 minutes at 4°C using a JA-14 rotor in a Beckman centrifuge. Cells were then washed twice in ice-cold sterile water and centrifugation was repeated. Cells were finally harvested by centrifugation at 5000rpm in a JA-20 rotor and resuspended in an ice-cold solution of 10% glycerol (500μl). Aliquots were either used immediately or frozen in dry ice and stored at -70°C.

2.2.6 Transformation of Electro-competent Cells

An appropriate amount of plasmid DNA was added to 50μl of competent cells, and transferred to a 2mm electroporation cuvette, which had been chilled on ice for 5

minutes. The cuvette was then placed in the electroporator and pulsed at 240v. Immediately after 1ml of SOC medium (2% tryptone, 0.5% yeast extract, 10mM NaCl, 2.5mM KCl, 10mM MgCl$_2$, 10mM MgSO$_4$, 20mM glucose) was added. The cell suspension was removed and placed in a 50ml falcon tube. This was incubated at 37°C, with gentle agitation, for 45 minutes. Aliquots were spread onto LB plates supplemented with the appropriate antibiotic.

2.3 *Saccharomyces cerevisiae* Culture and Manipulation

2.3.1 Culture Medium

Routinely, yeast cells were grown in YPD medium (2% peptone, 2% glucose, 1% yeast extract), for solid media, broth was supplemented with 2% agar. For selection of kanamycin resistant yeast, YPD was supplemented with geneticin G418 (Sigma) at a final concentration of 300mgl^{-1}. Selection with zeocin was performed on complete synthetic medium lacking appropriate amino acids with zeocin at a concentration of 100mg/ml. For selection or maintenance of plasmids or selection of integrants, synthetic - complete medium (0.067% Yeast nitrogen base, 2% glucose, and 0.2% drop-out mix, for plates this was supplemented with 2% agar) was used dropping out the appropriate amino acids or nucleotide bases to maintain selection. All yeast were incubated at 30°C for optimal growth.

2.3.2 *Saccharomyces cerevisiae* Strains

For experiments involved in RPS work, strains BY4733 (*MAT*a, *his3*Δ200, *leu2*Δ0, *met15*Δ0, *trp1*Δ63, *ura3*Δ0) and FY2 (*MAT*a, *ura3-52*) were used as specified. For experiments with TUP1 homologues, strain BY4742 (*MAT*α, *his3*Δ1, *leu2*Δ0, *lys2*Δ0, *tup1*Δ0, *ura3*Δ0) was used.

2.3.3 Yeast Glycerol Stocks

An overnight culture was mixed with an equal volume of 30% glycerol and stored at -70°C.

2.3.4 Transformation of Yeast Cells

Yeast were transformed by the method of Gietz and Woods (1994). Cells were counted from a saturated overnight culture using a hemacytometer and a fresh 50ml culture was inoculated to a cell density of 5×10^6 cells/ml. Yeast cell culture was left to grow at 30°C with aeration until a cell density of 2×10^7 cells/ml had been reached. Cells were harvested by centrifugation (5000 rpm for 5 minutes), washed in sterile water to remove any residual medium and harvested by repetition of the centrifugation. Cells were resuspended in 1 ml of lithium acetate (100mM), harvested and resuspended in 400μl of lithium acetate (100mM). The cells were split into 50μl aliquots, harvested and the lithium acetate was removed. Cells were resuspended in transformation mix (240μl 50% polyethylene glycol, 36μl 1M lithium

acetate and 50μl of single-stranded DNA) with the desired plasmid. Cells were incubated at 30°C for 30 minutes followed by heat shock at 42°C for 30 minutes. Cells were harvested by centrifugation (6000 rpm for 1 minute). Cells were resuspended in sterile water and an appropriate dilution was plated onto agar plates.

For transformations involving antibiotics, an outgrowth was performed in non-selective media (YPD or synthetic complete) at 30°C for 5 hours. Subsequently, cells were harvested and resuspended in sterile water and plated onto medium with appropriate antibiotics. Colonies from transformations were then picked and replated onto the same media to ensure loss of any false positives.

2.4 DNA Purification

2.4.1 Phenol/Chloroform Extraction and Ethanol Precipitation

The solution containing the DNA was adjusted to 200μl with water if appropriate. An equal volume of phenol/chloroform/IAA (25:24:1) was added and mixed by vortexing. The phases were separated by centrifugation (13,000 rpm for 1 minute). The aqueous phase was isolated and an equal volume of chloroform/IAA (24:1) was added to remove any residual phenol from the sample.

The DNA was precipitated by adding sodium acetate (pH 5.2) to a final concentration of 0.3M with 2-2.5 volumes of ethanol and incubated at -70°C for 30 minutes or at -20°C overnight. The DNA was collected by centrifugation (13,000

rpm for 10 minutes). The pellet was washed in 1ml of 70% ethanol to remove any residual salt. The liquid was removed and the pellet was dried under a vacuum. The DNA was resuspended in an appropriate volume of water or TE (pH 8).

2.4.2 Gel Extraction

The DNA fragments were resolved on an agarose gel and the band of interest was excised using a razor blade. The DNA was recovered using the Qiaex II gel extraction kit (Qiagen) and further purified by phenol/chloroform extraction and ethanol precipitation.

2.5 Preparation and Manipulation of DNA

2.5.1 Preparation of Plasmid DNA

Small quantities of plasmid DNA (<10μg) were isolated from a 5 ml overnight bacterial culture using a QIAprep spin mini-prep kit (Qiagen). Larger quantities of DNA were obtained from 200 ml of an overnight culture. Cells were centrifuged at 4000 rpm in a JA-14 rotor. Cells were resuspended in 6 ml GTE (50mM glucose, 25mM Tris.HCl, 10mM EDTA) buffer, to this 12 ml of lysis buffer (200mM NaOH, 1% SDS) was added to lyse the cells and denature chromosomal DNA, then 9 ml of acidic potassium acetate solution (3M potassium acetate, 2M acetic acid) was added to neutralise the solution. At all stages the cell suspension was mixed by inversion. The mixture was centrifuged at 5000 rpm for 20 minutes using a JA-20 rotor. The

supernatant was removed and a phenol/chloroform and ethanol precipitation was carried out as previously described. The pellet was resuspended in 1 ml of heat-inactivated RNaseA (1mg/ml) and incubated at 37°C for 30 minutes. 300µl of PEG solution (20% PEG-6000, 5M NaCl) was added and the sample was incubated on ice for 1 hour to allow selective precipitation of plasmid DNA. The DNA was isolated by centrifugation (13,000 rpm for 5 minutes), washed with 70% ethanol and dried under a vacuum. The pellet was resuspended in 400µl of a TE/lithium chloride solution (400mM LiCl, 1mM Tris.HCl, 0.2mM EDTA), and phenol/chloroform extractions were carried out until the interphase was clear. A final ethanol precipitation was performed and the pellet was resuspended in 200-400µl of TE. The concentration was determined spectrophotometrically by measuring absorbance at A_{260} using the conversion factor 1 absorbance unit = 50µg DNA/ml.

2.5.2 Restriction Enzyme Digestion

DNA was cut by restriction enzymes according to the manufacturer's instructions. The products from the digestions were analysed directly by agarose gel electrophoresis or purified by phenol/chloroform extraction followed by ethanol precipitation and resuspended in water or TE buffer.

2.5.3 Dephosphorylation of DNA Fragments

The DNA fragments generated by restriction enzyme digestion are 5'-phosphorylated. These phosphates can be removed by alkaline phosphatase to

facilitate further manipulation such as inhibiting self-ligation of a vector backbone in ligation reactions.

A known amount of DNA was incubated with calf intestinal alkaline phosphatase (1 unit per 1 pmole DNA ends) with appropriate buffer and incubated for 1 hour at 37°C. Alkaline phosphatase was removed from the DNA by phenol/chloroform/IAA extraction, followed by ethanol precipitation.

2.5.4 DNA Ligation

DNA ligation reactions consisted of approximately 100ng of vector DNA and insert DNA at a 1:3 molar ratio in 1X ligation buffer and 6 Weiss units of T4 DNA ligase. Sticky - end ligations were incubated at room temperature for 2 hours. Blunt - end ligations were carried out at 16°C overnight. The ligation products were purified by phenol/chloroform/IAA extraction and ethanol precipitation. Samples were then resuspended in 10µl of water; 5µl of this would then be used to transform electro-competent cells.

2.5.5 Polymerase Chain Reaction

DNA fragments were amplified using the polymerase chain reaction (PCR). Routinely, PCR reactions were performed using 1X Taq buffer, 200µM dNTPs, 0.25µM primers, an appropriate amount of template DNA and 2 units of Taq polymerase. For other applications, Vent polymerase was also used. As standard, the

PCR reaction consisted of 3 minutes at 95°C followed by around 30 cycles of denaturation at 95°C for 1 minute, annealing temperature (determined by average melting temperature of the two primers) for 1 minute. This was followed by an extension at 72°C, the extension time was determined by the length of fragment to be amplified, generally 1 minute per kilobase of DNA. The resulting PCR product was purified from an agarose gel as described.

2.6 Radio-labelling of DNA fragments

2.6.1 5'-end Labelling

This technique was used for labelling DNA size ladders.

Approximately 300ng of DNA ladder was incubated with 1X polynucleotide kinase buffer, 10 units of polynucleotide kinase and 4 picomoles of $[\gamma\text{-}^{32}P]ATP$ at 37°C for 1 hour. The reaction was stopped by heating to 68°C for 20 minutes. Any unincorporated label was removed by passing the sample through a G-25 Sephadex column.

2.6.2 Random Prime Labelling

This technique was used for evenly labelling DNA fragments to use as probes in Southern and Northern hybridisations.

Approximately, 1µg of DNA and 1.5mg/ml random hexamers were boiled for 5mins to denature the DNA; the sample was then chilled on ice. To this, 1X Klenow polymerase buffer, 200µM dNTPs (except dCTP), 5 units of Klenow polymerase and 8 picomoles of [α-^{32}P]dCTP were added. The reaction was incubated at room temperature for 4 hours. The reaction was stopped by adding 1µl of EDTA (0.5M). To purify the DNA, 100µl of TE buffer and 125µl of phenol/chloroform/IAA were added. The sample was centrifuged (13,000 rpm for 10 minutes) and the aqueous phase removed and passed through a G-25 Sephadex column to remove any unincorporated nucleotides. The sample was boiled for 5 minutes before adding to the hybridisation solution to denature the DNA.

2.6.3 Removal of Unincorporated Label

A 1 ml syringe was plugged with glass wool and filled with Sephadex G-25 slurry. The Sephadex was packed by centrifugation (1,500 rpm for 2 minutes). The column was washed through with TE buffer, which was then removed by centrifugation (1,500 rpm for 5 minutes). The labelled sample was applied to the column and recovered by centrifugation (1,500 rpm for 5 minutes).

2.7 Preparations from *S. cerevisiae* Cultures

2.7.1 Genomic DNA

Genomic DNA was prepared from a 10 ml overnight culture of yeast. Cells were harvested by centrifugation (5000 rpm for 5 minutes). The pellet was then resuspended in 1 ml of water and centrifuged (13,000 rpm 30 seconds) to remove any remaining medium. The harvested cells were resuspended in 200µl of breaking buffer (2% triton X-100, 1% SDS, 100mM NaCl, 10mM Tris.HCl pH 8, 1mM EDTA pH 8). Glass beads (0.3g) and 200µl of phenol/chloroform/IAA were added to the tube and the mixture was vortexed at maximum speed for 3 minutes to lyse the cells. After vortexing, 200µl of TE buffer was added and the sample was briefly vortexed before centrifugation (13,000 rpm for 10 minutes) to separate the phases. The aqueous phase was removed, 1 ml of ethanol was added and centrifuged (13,000 rpm for 5 minutes). The pellet was resuspended in 400µl of TE buffer and 30µl of RNase A (1mg/ml) was added and incubated at 37°C for 5 minutes. To precipitate the DNA, 10µl of ammonium acetate (4M) was added an ethanol precipitation was carried out as described.

2.7.2 Total RNA

A 10ml culture was grown to exponential phase (around $2x10^7$ cells). The cells were harvested by centrifugation (6000 rpm for 3 minutes). The pellet was resuspended in 1 ml of ice-cold water and centrifuged (15 seconds at 13,000 rpm) to remove any

remaining medium. 400µl of TES solution (10mM Tris.HCl pH 7.5, 10mM EDTA, 0.5% SDS) was added to resuspend the pellet; to this, 400µl of acid phenol was added and the sample was vortexed at full speed for 10 seconds. The mixture was incubated at 65°C for 45 minutes with occasional vortexing. The sample was placed on ice for 5 minutes and then centrifuged (13,000 rpm, for 5 minutes). The aqueous phase was removed and another 400µl of acid phenol added Vortexing and centrifugation were repeated. The sample was further purified by adding 400µl of chloroform, followed by vortexing, and centrifugation as before. The aqueous phase was removed and 40µl of sodium acetate (3M, pH 5.3) and 1 ml of ethanol were added. This was centrifuged (13,000 rpm for 5 minutes). The RNA pellet was washed in 1 ml of ice-cold 70% ethanol, and centrifuged as before to isolate the pellet. The pellet was finally resuspended in 50µl of water and concentration determined spectrophotometrically by measuring A_{260} using the conversion factor of 1 absorbance unit = 42µg RNA/ml .

2.7.3 Nuclei

Yeast nuclei were prepared for chromatin studies; the protocol was divided into 2 stages:

(i)   Preparation of spheroplasts

2 litres of culture, grown in appropriate medium, were grown to mid-log phase. All proceeding steps were carried out at 4°C. Cells were harvested by centrifugation at

3000 rpm for 5 minutes, and the wet weight of the pellet determined. The cells were then resuspended in 3 volumes of ice-cold water and centrifuged at 3500 rpm for 5 minutes and the supernatant removed. The pellet was resuspended in 1 volume of pre-treatment buffer (50mM Tris.HCl pH 7.5, 10mM EDTA, 1M sorbitol, 30mM DTT, 5mM β-mercaptoethanol) and incubated at room temperature for 15 minutes, to facilitate breakage of disulphide bonds. The sample was centrifuged at 5,000 rpm for 5 minutes and the pellet was resuspended in 3 volumes of spheroplasting buffer (50mM Tris.HCl pH 7.5, 10mM $MgCl_2$, 1M sorbitol, 1mM DTT). Yeast lytic enzyme was added and the sample incubated for 40 minutes at 30°C with slow shaking (110 rpm). To determine if the conversion to spheroplasts had been completed a sample was placed in distilled water and analysed under the microscope to see if cells were bursting. Once spheroplasting was completed, the sample was centrifuged for 5 minutes at 3,500 rpm. The supernatant was removed and the spheroplasts were washed in 2 volumes of spheroplast buffer and the sample centrifuged at 3,500 rpm for 5 minutes; this washing and centrifugation step was repeated twice.

(ii)   Preparation of nuclei by differential centrifugation.

Nuclei were prepared from the spheroplasts by lysis in the presence of Ficoll. The final pellet from the washes was resuspended in 0.5 volumes of spheroplast buffer. The cells were dropped, using a pasteur pipette, into 6 volumes of ice-cold Ficoll buffer (18% Ficoll-400, 10mM Tris.HCl pH 7.5, 20mM KCl, 5mM $MgCl_2$, 1mM EDTA, 3mM DTT, 1mM PMSF) and left stirring for 20 minutes at 4°C. The

suspension was centrifuged at 5000 rpm for 5 minutes, to pellet any cell debris and unlysed spheroplasts. The supernatant was removed and centrifuged for 20 minutes at 13,500 rpm. The pellet, which contains the nuclei, was resuspended in 1 volume of storage buffer (20mM Tris.HCl pH 7.5, 0.1mM EDTA, 10% glycerol, 100mM KCl, 1mM DTT, 1mM PMSF). The nuclei were aliquoted and stored at -70°C.

2.8 DNA Analysis

2.8.1 Agarose Gel Electrophoresis

DNA fragments were separated according to size by agarose gel electrophoresis. Different buffers and percentage gels were used depending on the size of fragments to be resolved.

Fragments smaller than 200bp were separated on a 1.5% agarose gel, fragments larger than 2Kb were isolated on a 0.7% gel. Anything in between these values was isolated using a 1% gel.

All routine electrophoreses were carried out using 1X TBE (8.9mM Tris, 8.9mM boric acid, 2mM EDTA) as a running buffer. Fragments larger than 8Kb were electrophoresed using a 1X TAE buffer (40mM tris-acetate, 2mM EDTA) to allow better separation. Prior to electrophoresis, 6X orange G loading buffer (0.25% orange G, 15% Ficoll-400, 120mM EDTA) was added to samples to a final concentration of 1X loading buffer.

All gels were then stained for 10 minutes in a 3μg/ml ethidium bromide solution and de-stained for 10 minutes in distilled water.

2.8.2 Southern Blotting

Southern blotting (Southern, 1975) was used to identify specific DNA sequences within a population of DNA fragments. Three steps were involved:

(i)   Separation of DNA fragments on an agarose gel

As in section 2.8.1.

(ii)   DNA transfer and immobilisation onto a membrane

After electrophoresis the gel was stained with ethidium bromide as before and photographed. The gel was washed in denaturation solution (1.5M NaCl, 0.5M NaOH) for 40 minutes followed by washing in neutralisation solution (1mM ammonium acetate, 20mM NaOH) for 50 minutes. The DNA was transferred onto the membrane by upward capillary transfer in 20X SSC (3M NaCl, 0.3M tri-sodium citrate). After overnight transfer, the membrane was washed in 2X SSC and the DNA immobilised by baking in a vacuum dryer at 80°C for 1 hour.

(iii)   Hybridisation

The membrane was placed in a hybridisation bottle with a mesh support and pre-hybridised at 65°C in pre-hybridisation buffer (3X SSC, 10mM EDTA, 0.2% PVP, 0.2% Ficoll-400, 0.2% BSA, 0.1% SDS, 0.04 mg/ml salmon sperm DNA, 0.02mg/ml heparin) for around 2 –3 hours. The boiled probe was added with fresh hybridisation buffer (25 ml) supplemented with 2.25g of dextran sulphate and the hybridisation continued overnight at 65°C. The membrane was washed for 4X 20 minutes in 2X SSC with 0.1% SDS and again for 2X 20 minutes in 0.1X SSC with 0.1% SDS. The membrane was finally washed in 2X SSC and exposed to a phosphorimager screen (Fuji).

2.9 RNA Analysis

2.9.1 Agarose Gel Electrophoresis

RNA was separated by size using a denaturing 1.5% agarose gel. These gels were prepared by adding agarose to DEPC-treated water and boiling. After the solution had cooled to 60°C 10X MOPS (0.4M MOPS, 0.1M sodium acetate, 0.01M EDTA) was added to give a final concentration of 1X, followed by the addition of 40% formaldehyde (500μl). The gel was poured in a gel tray, which had been pre-treated with 3% hydrogen peroxide and rinsed in DEPC-treated water to remove RNases. RNA gels were electrophoresed in a 1X MOPS running buffer in a tank also treated with hydrogen peroxide.

RNA samples (15-20µg) were prepared by adding 25µl of MMF solution (500µl formamide, 162µl 40% formaldehyde and 100µl 10x MOPS) and ethidium bromide was added to a final concentration of 0.1 mg/ml. These were incubated at 60°C for 15 minutes and 5µl loading dye was added prior to electrophoresis. The gel was scanned using a phosphorimager in fluorescent mode and the ethidium bromide image saved to use as a loading control.

3.9.2 Northern Blotting

Northern blotting was used to identify specific RNA sequences within a population of RNA fragments. There were three steps involved:

(i)   RNA size separation on an agarose gel.

As in section 3.9.1.

(ii)   RNA transfer and immobilisation on an inert membrane.

Agarose gels were washed in DEPC-treated water twice, each for 20 minutes. This was followed by transfer onto a nylon membrane by upward capillary transfer in 20X SSC. After overnight transfer, the membrane was rinsed in 2X SSC and RNA immobilised by baking in a vacuum dryer at 80°C for 1 hour.

(iii)   Hybridisation

As section 2.8.2.

2.10 Chromatin Analysis

2.10.1 Micrococcal Nuclease Digestion of Nuclei

Nuclei were washed in 1ml of micrococcal nuclease digestion buffer (1M sorbitol, 15mM Tris.HCl, 1mM $MgCl_2$, 50mM NaCl, 0.5mM PMSF) twice. Nuclei were harvested by centrifugation (11,000 rpm, 1 minute) and resuspended in 400µl of digestion buffer. Nuclei were incubated at 37°C for 2 minutes, after which 0.5-1U of micrococcal nuclease was added and the reaction started by the addition of $CaCl_2$ to a final concentration of 1.25mM. Aliquots (90µl) were removed and placed into 10µl of 10X termination solution (250mM EDTA, 5% SDS, 50mM Tris.HCl pH 8) at appropriate time intervals, generally between 1 and 8 minutes.

The DNA was purified by adding 30µl of RNaseA (2mg/ml) and incubation at 37°C for 45 minutes. This was followed by proteinase K treatment (2mg/ml) at 50°C for 30 minutes. The DNA was further purified by phenol/chloroform/IAA extraction followed by ethanol precipitation.

2.10.2 Indirect End Labelling

DNA which had been subjected to micrococcal nuclease digestion as in section 2.10.1 was digested with an appropriate restriction enzyme overnight. Samples were ethanol precipitated, resuspended, and run on a 1.5% agarose gel at 90V. The gel was then manipulated as a Southern blot (section 2.8.2).

Chapter 3 – Analysis of the Repressive Effects of a Repetitive DNA Fragment from *Petunia hybrida* in *Saccharomyces cerevisiae.*

3.1 Introduction

Repetitive DNA can consist of nucleotide sequences of varying lengths and composition that occur in tandem, inverted and dispersed organisations. There has long been an association between repetitive DNA and silent heterochromatin; indeed constitutive heterochromatin consists mainly of repetitive DNA with few active genes. In recent years, the phenomenon of repeat-induced gene silencing has become known in plants and filamentous fungi, where multiple copies of genes lead to silencing. This is often associated with hypermethylation (Matzke *et al.*, 1989) and changes in chromatin conformation at the silenced locus (Ye and Signer, 1996).

The RPS sequence randomly isolated from *Petunia hybrida* reinforces the idea that a repetitive sequence can act as a signal for DNA methylation and local chromatin condensation (tenLohuis *et al.*, 1995). The RPS is a 1.6 Kb fragment that is 60.3% AT-rich, which is of a similar composition to most repetitive sequences found in higher eukaryotes. It consists of a mixture of direct and indirect repeats; at its 3' end there are 3 direct repeats of a 57 bp motif, which consist of two 13-mers interrupted by a 9-mer and a 22-mer.

The RPS was shown to enhance expression variegation of a GUS reporter gene in both *P. hybrida* and *N. tabacum*. This was associated with hypermethylation of the RPS sequence at both symmetric and asymmetric sites, a phenomenon associated with silenced transgenic loci (Meyer *et al.*, 1992). One particular methylation site, located within a *HhaI* restriction site, is part of a larger 40 bp palindromic sequence that has the potential to form a cruciform structure. Stem-loop structures are known to attract DNA methyltransferases, and methylated cytosine residues in single stranded DNA can signal *de novo* methylation of adjacent regions (Christman *et al.*, 1995). Therefore, secondary structures formed due to the repetitive nature of the RPS may allow it to act as a hot spot for *de novo* DNA methylation. Indeed, inverted repeats have been shown to trigger DNA methylation in *Arabidopsis* (Luff *et al.*, 1999) by virtue of hairpin RNA production (Melquist and Bender, 2003).

The RPS potentially functions as an initiation region for heterochromatin formation, which spreads to the adjacent reporter gene by a mechanism akin to position effect variegation. This is supported by the fact that although the RPS becomes heavily methylated, the promoter of the reporter gene does not, implying that DNA methylation is not the primary cause for gene inactivation. Furthermore, the RPS is methylated in lines that do not show enhanced expression variegation. Other studies in *Arabidopsis* have shown that mutations in the *MOM* gene allow expression of previously repressed genes although they remain heavily methylated. This suggests that, in these instances, methylation is a secondary effect after the initial silencing as a consequence of a repressive chromatin structure (Amedeo *et al.*, 2000).

Another feasible hypothesis is that the RPS is subject to homology-dependent gene silencing. Southern blotting analysis revealed 10^3 and 10^4 endogenous RPS homologues in Tobacco and Petunia respectively; therefore pairing of these DNA sequences may "transfer" a repressive chromatin structure to the other. This is emphasised by the fact that when the RPS is integrated into *A. thaliana*, which has no sequence homology to the RPS, there is no expression variegation of a reporter gene attached to the RPS (Müller *et al.*, 2002). Moreover, the RPS becomes methylated at similar sites in *Arabidopsis*, indicating again that methylation is not the initiating factor in gene silencing in this instance. However, the RPS is not associated with constitutive heterochromatin in Petunia cells, suggesting that if it does induce a restrictive chromatin environment, it most likely has structural plasticity and may revert between states. The type of homology-dependent gene silencing associated with the RPS is not like co-suppression, as no RNA transcripts were identified from any ORFs within the sequence, although it is possible that small, rapidly degraded RNA fragments are produced (ten Lohuis *et al.*, 1995).

The RPS may also contain specific sequences for DNA binding proteins, which function to create a repressive chromatin environment. A protein isolated from *Arabidopsis* called BP1 can bind to the RPS sequence (P. Meyer, personal communication). Although a BP1 homologue has not yet been identified in *P. hybrida*, it is plausible that one exists. BP1 contains seven WD repeats and has significant homology to the *S. cerevisiae* TUP1 co-repressor. If BP1 functions in the same manner as TUP1, it potentially creates an ordered nucleosome array which can extend at long range (Fleming and Pennings, 2001), thus inducing silencing of a

reporter gene. If BP1 is a member of the TUP1/GRO family of transcriptional co-repressors, it is unique in its DNA binding ability. BP1 is also homologous to another *S. cerevisiae* protein, ARC41. This is a member of the actin related complex (Arp2/3) complex, which is involved in cytoskeletal organisation and actin filamentation. Indeed, actin-related proteins have been implicated in epigenetic gene regulation in *S. cerevisiae* (Jiang and Stillman, 1996). With the position of genes in the nucleus influencing their expression (for review see Gasser, 2001) and silent genes often being found at the nuclear extremities, it is possible that BP1 associates with the RPS as part of a larger protein complex at the nuclear periphery, where it is maintained in an inactive state. For instance, the SIR proteins, which mediate gene silencing in *S. cerevisiae,* are localised at the edges of the nucleus (Palladino *et al.*, 1993).

There are many possible explanations as to why the RPS causes expression variegation of an adjacent reporter gene, and these may or may not be mutually exclusive. In order to understand the mechanism, all of these points must be considered in turn. This is difficult to achieve in a system such as *P. hybrida*, where gene knockouts are difficult and large amounts of other repetitive sequences are present. An interesting candidate for studying the action of the RPS would be the budding yeast, *S. cerevisiae*. This is an organism that can be manipulated easily. *S. cerevisiae* has few repetitive DNA sequences, which are confined mainly to the ribosomal DNA repeats and the telomeres. In addition, a BLAST search of the *S. cerevisiae* genome revealed no endogenous sequences with significant homology to the RPS.

Budding yeast also has proteins with homology to the RPS binding protein BP1; any interactions that occur between them can be studied by way of gene knockouts. The question of the importance of DNA methylation in RPS-induced expression variegation can also be addressed. As previously mentioned, the methylation status of the RPS did not necessarily correlate with enhanced expression variegation, suggesting DNA methylation may not be the primary cause of RPS-mediated silencing. Since *S. cerevisiae* does not methylate its DNA, the RPS can be studied so that the roles of chromatin structure and DNA methylation can be observed separately.

3.2 Analysis of RPS Function in *S. cerevisiae* on Plasmids

3.2.1 Techniques and Materials for Study

In order to observe the action of the RPS sequence in *S. cerevisiae*, the RPS sequence was cloned next to a reporter gene in a vector that can be maintained in yeast. The reporter gene selected was *ADE2*. The *ADE2* gene has previously been used in various silencing assays because of the simple and instantly observed phenotype of *ade2* cells (for example see Gottschling *et al.*, 1990). The gene encodes the enzyme phosphoribosylimidazole carboxylase, which is involved in purine biosynthesis. When the *ADE2* gene is mutated or silenced, an intermediate in the purine biosynthesis pathway cannot be broken down in the normal fashion. This intermediate has a distinctive red colour, which can be seen in the yeast colony (see fig. 3.1). The *ADE2* gene is particularly useful in silencing assays. If there is expression variegation where some cells within the colony are expressing *ADE2* and some have silenced the gene, a sectored colony results from the outwards growth of the yeast cells (fig. 3.1). If *ADE2* is active, the colonies or sectors will be white and if *ADE2* is silenced, colonies or sectors should be pink to red.

The *ADE2* gene was cloned into vectors next to the RPS sequence (for details of cloning see table 6.1). The vectors selected were pRS414 (fig. 3.2) and pRS424 (fig. 3.3). These vectors contain the *TRP1* selectable marker and yeast carrying the plasmids were selected for and maintained on SC-trp medium. The pRS414 vector has a *CEN6* origin of replication and is present in the yeast cell at 1-2 copies. The

pRS424 vector has a 2μ origin of replication and is present at higher numbers, usually between 20-50 copies per cell. A comparison of results of yeast transformed with each of these vectors allows us to establish if multiple copies of the RPS are required for its silencing in yeast, as appears to be the case in *Arabidopsis* (Müller *et al.*, 2002). Alternatively, if a yeast factor is required for silencing, it may be "diluted out" by the multiple copies of the RPS. As shown in figures 3.2 and 3.3, different orientations of the RPS and *ADE2* constructs were cloned and tested to see if a particular orientation has more of an effect on the reporter gene. This is important since PEV can be influenced by gene orientation (Feng *et al.*, 2001).

Figure 3.1: Colours of *ADE2* and *ade2* cells. The plate shown has a mixture of colonies. White colonies are expressing the *ADE2* gene, pink colonies have silenced the *ADE2* gene and sectored colonies have some cells expressing the *ADE2* gene and some cells with a silent *ADE2* gene.
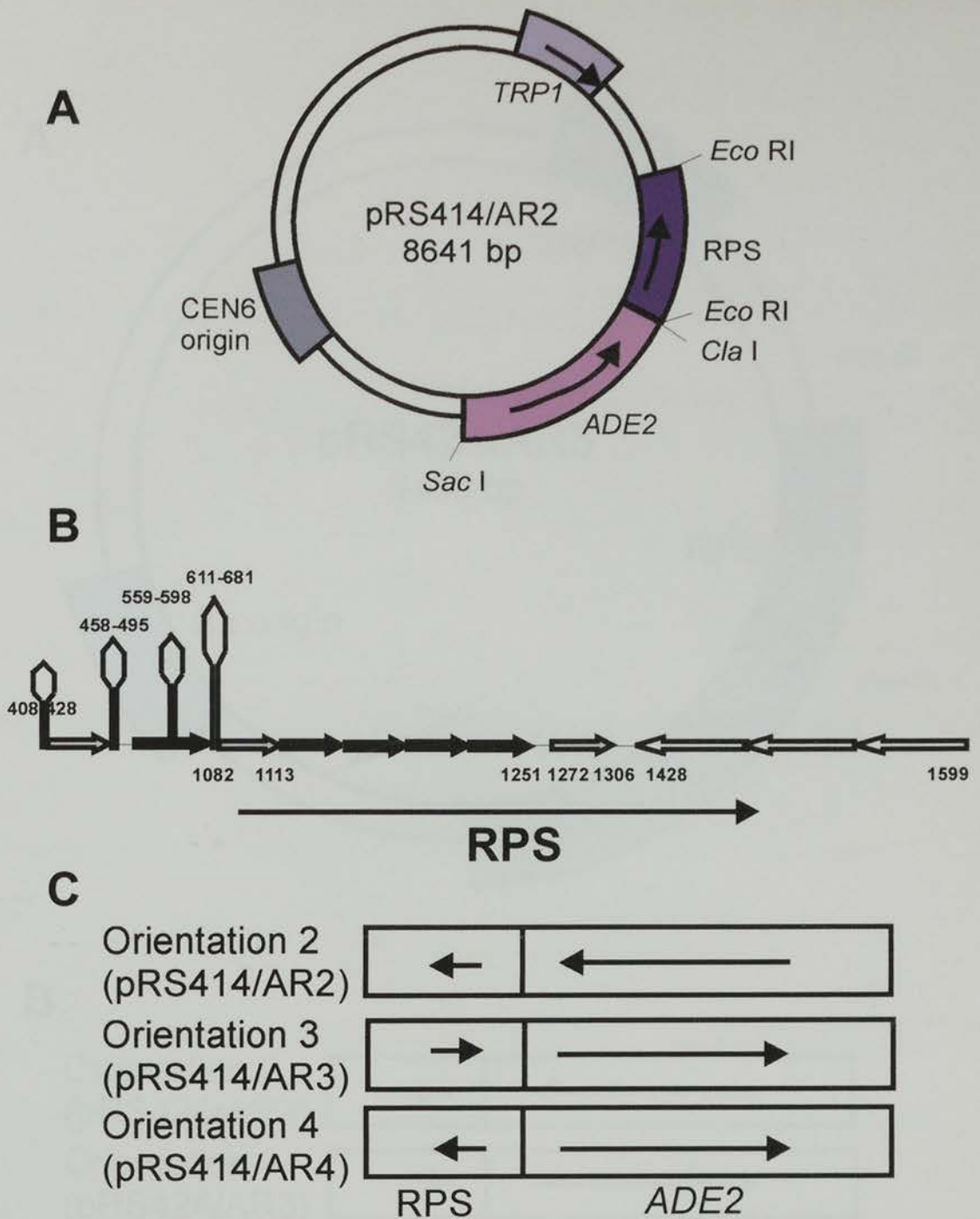
Figure 3.2: Features of the low copy number plasmid pRS414. (**A**) shows an example of one plasmid used in the study. Notable features of pRS414 are the *TRP1* selection marker and the CEN6 origin of replication (Christianson *et al.*, 1992). The *ADE2* and RPS sequences were cloned 11 bp apart in the multiple cloning site. (**B**) is a schematic diagram of the repeat structure in the RPS. Numbers indicated are nucleotide positions. Inverted repeats are denoted by lollipops and direct repeats are shown as arrows. Homologous repeats are shown by the same colours. Grey arrows are indicative of 32-33 bp repeats, black arrows show 34-35 bp repeats and white arrows indicate 57 bp repeats. (**C**) shows a schematic diagram of the different orientations of the *ADE2* gene and RPS cloned into the vector, which were analysed in this study.
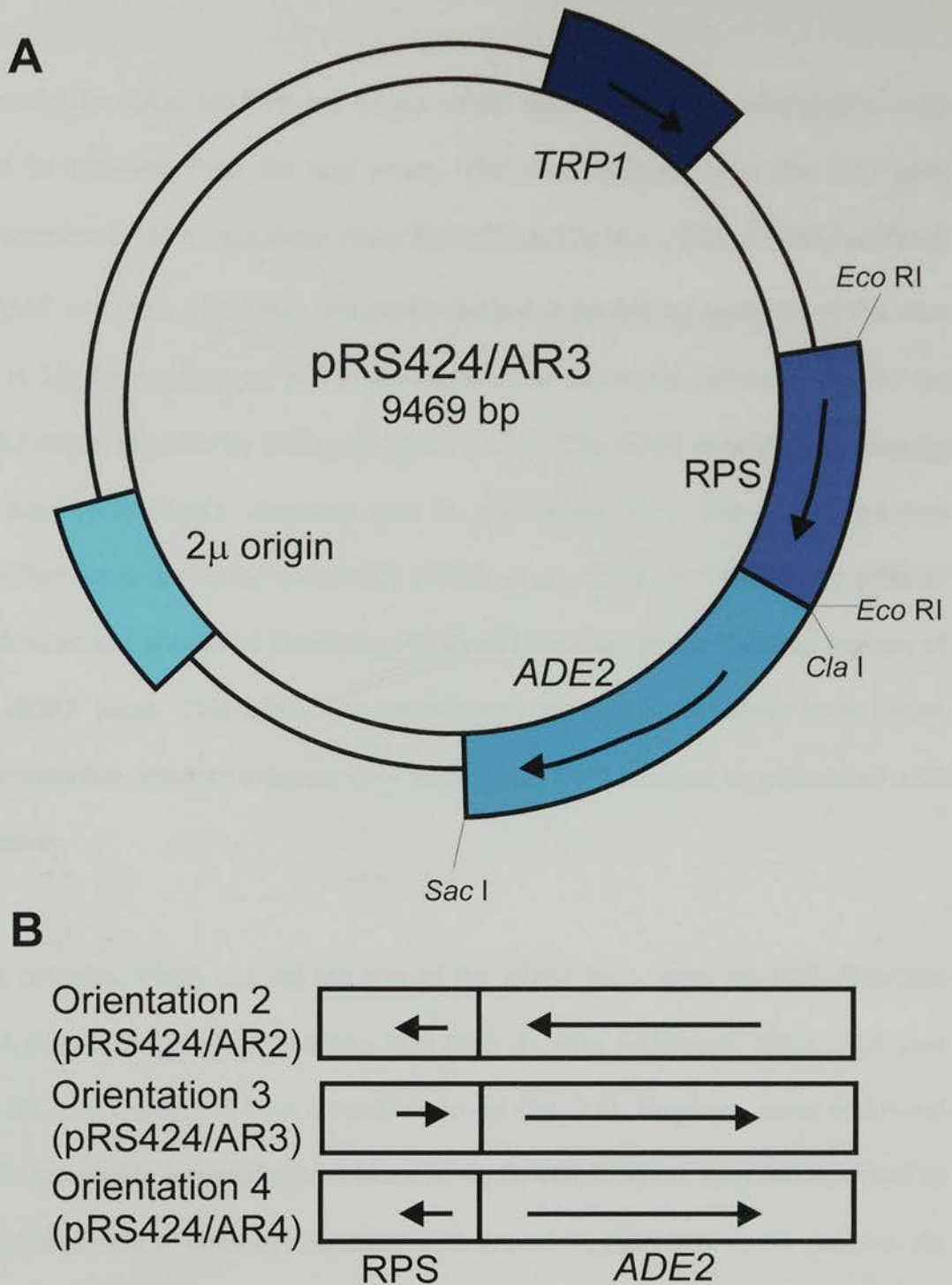
Figure 3.3: Features of the high copy number plasmid pRS424. (**A**) shows an example of one plasmid used in the study. Notable features of pRS424 are the *TRP1* selection marker and the 2µ origin of replication (Christianson *et al.*, 1992). The *ADE2* and RPS sequences were cloned 11 bp apart in the multiple cloning site. (**B**) shows a schematic diagram of the different orientations of the *ADE2* gene and RPS. cloned into the vector, which were analysed in this study.

3.2.2 Knockout of *ADE2* Gene

To study the effect the RPS has on the *ADE2* reporter gene, the endogenous copy must be removed from the host strain. This was achieved by a one step gene replacement in the *S. cerevisiae* strain BY4733 (*MATa, his3Δ200, leu2Δ0, met15Δ0, trp1Δ63, ura3Δ0*). *ADE2* was completely deleted from 602 bp upstream of the start site to 239 bp downstream of the stop codon; this was based approximately on the *ADE2* sequence noted by Stotz and Linder (1990). The *ADE2* gene was replaced by the kanamycin (Kan^r) resistance gene to give strain HC1. The Kan^r gene was amplified from the vector pKanMX4 (Wach *et al.*, 1994) by PCR, using primers adedelstart and adedelend containing 40 bp of homology to the flanking regions of the *ADE2* locus. This DNA was transformed into yeast to allow homologous recombination. Positive colonies were selected on YPD medium supplemented with geneticin.

Pink colonies, which suggest the loss of the *ADE2* gene, were selected. Genomic DNA digests followed by Southern blot analysis were performed; these confirmed that the *ADE2* gene had been entirely removed (fig. 3.4). However, some additional bands are visible in transformant lanes A, C, D, and E. These may be attributed to partial digestion or cross-hybridisation with sequences elsewhere in the genome. As transformant B lacked these extra bands it was selected for further experiments as strain HC1. To further substantiate these results, a complementation test was carried out where Δ*ade2* cells (HC1) were transformed with pRS412, a single-copy number plasmid containing the full-length *ADE2* gene. This test was employed to ensure the

new strain had the potential to change colour from pink to white, as is critical for this silencing assay. Sample colonies are shown in fig. 3.5, demonstrating that the *ADE2* deletion can be rescued by supplying the full-length *ADE2* gene on a plasmid. The Southern blot and complementation tests confirm that the endogenous *ADE2* gene was removed and these strains and plasmids could be used to analyse the RPS function in yeast.
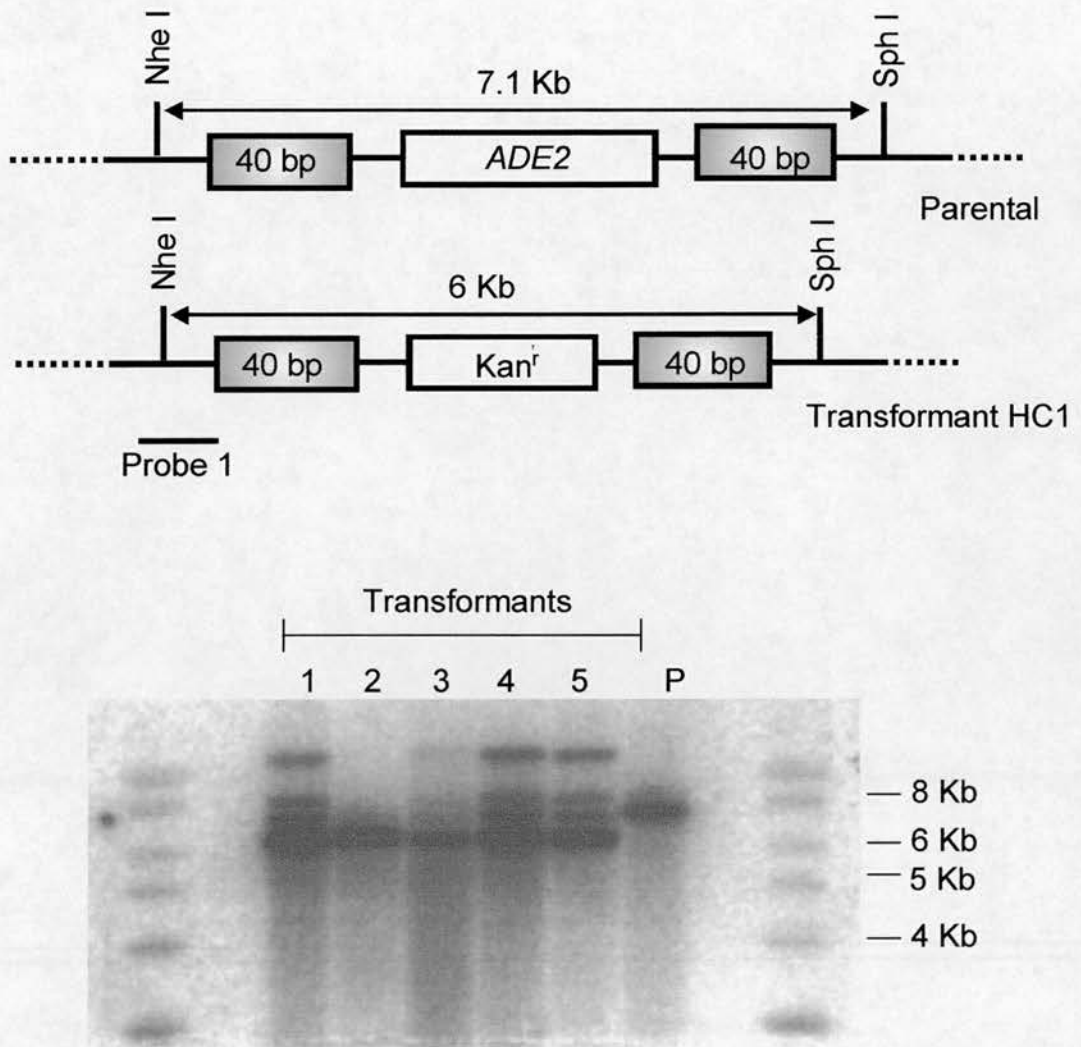
Figure 3.4: Strategy for knockout of *ADE2* gene and Southern blot confirming gene replacement. P denotes the parental strain (BY4733) and shaded boxes indicate the 40 bp of homology within the disruption fragment allowing homologous recombination. The *ADE2* gene was deleted from 602 bp upstream of the trancriptional start site of 239 bp downstream of the transcriptional stop site. The PCR-generated disruption fragment was produced with primers 'adedelstart' and 'adedelend' using the pKanMX4 as a template (Wach et al., 1994). The probe was used to visualise size differences in the restriction digests and was generated by PCR using primers 005a and 005b (see table 6.2). The blot shows the correct replacement of the *ADE2* gene with the kanamycin resistance gene.
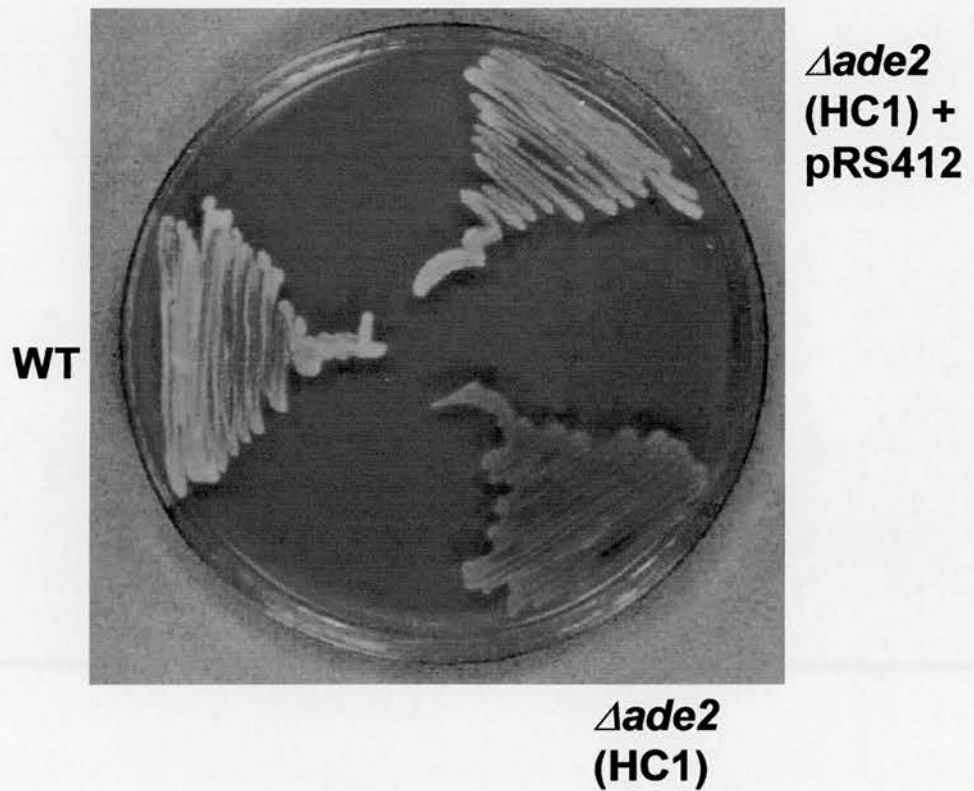
Figure 3.5: Complementation of *ade2* mutant. Shown are the WT parental strain (BY4733) and my *ade2* knockout strain (HC1). This strain was transformed with pRS412, which carries the *ADE2* gene; this allows the strain to return to its white colour.

3.2.3 Analysis of RPS Function on Plasmids

The three centromeric pRS414 plasmids containing various orientations of the RPS and *ADE2* sequences (fig. 3.2) were independently transformed into strain HC1 in triplicate. As controls, the parental plasmid pRS414 and pRS414 with an *ADE2* gene but no RPS (pRS414/A) were used. An identical procedure was followed for the pRS424 series of multi-copy vectors. The same number of moles of DNA of each were transformed: 0.7 picomoles and 0.1 picomoles for the pRS414 and pRS424 series respectively, as estimated by the A_{260} of the plasmid preparations. This step was carried out to ensure the numbers of colonies returned were comparable. Differences in colony numbers could reveal if the RPS mediates long-distance silencing in yeast. If the RPS silences the *TRP1* marker gene it would prevent the growth of colonies on SC-trp medium and the numbers of colonies returned from the transformation would be lower than expected. Tables 3.1 and 3.2 show numbers of colonies from each transformation with fig. 3.6 showing average transformation efficiencies. Interestingly, pink and sectoring colonies were found on some plates; examples are shown in figures 3.7A and 3.7B.

Tables 3.1 and 3.2 show that pink colonies (*ADE2* off) occur at a higher level on vectors with the RPS. This indicates that the RPS may function in yeast to enhance expression variegation. The pink colonies found in the control plates that have the *ADE2* gene alone may represent the level for natural mutation of the *ADE2* gene or may be due to false positives or mutations may have been generated within the *ADE2* gene. However, no sectored colonies were found on control plates indicating that the

sectored colonies observed are examples of true *ADE2* expression variegation. In the case of the pRS414 series of vectors (low copy number), this was up to two fold higher than the control pRS414/A vector. Interestingly, the pRS424 series (high copy number) study showed consistently less pink cells than the pRS414 series and levels were close to background on control plates.

However, the results between separate transformations are also variable and therefore it is difficult to deduce how reliable they might be. For example, the percentage of pink colonies observed in orientation 3 (pRS414/AR3) in table 3.1 varies from 1%-2.6%, over a two and a half fold increase. However, orientation 4 (pRS414/AR4 and pRS424/AR4) seems to give consistently less pink colonies than the other orientations, implying that this particular orientation is not as efficient in silencing as the other orientations.

The hypothesis that the RPS could potentially repress the *TRP1* marker gene on the plasmid was addressed by comparing the transformation efficiencies between vectors (fig. 3.6). There is a large difference between the average transformation efficiencies of the parental vector, pRS414, and the vectors containing the RPS (fig 3.6 A). This may be due to size differences, with smaller plasmids being more easily transformed, although, this is less obvious with the pRS424 series (fig. 3.6 B). There are small differences in transformation efficiencies between the different orientations in both series of vectors. However, due to the large variations in numbers between transformations, these results are difficult to interpret. For example, pRS424/AR3 shown in table 3.2 has an almost 6-fold difference in transformation efficiency

between seemingly identical transformations. Despite these variations, a general rule seems to follow between the orientations where 3>2>4 with respect to transformation efficiencies, suggesting the RPS may influence the expression of the *TRP1* marker gene especially in orientation 4. This observation may explain why less pink colonies were found for this orientation; if silencing occurs, in this instance it spreads to both *ADE2* and *TRP1* genes.

Vector	No. of colonies/ml	No. of pink colonies/ml	% Pink colonies
pRS414	5.0×10^6	5.0×10^6	100
	2.4×10^6	2.4×10^6	100
	1.5×10^6	1.5×10^6	100
pRS414/A	8.8×10^3	40	0.5
	5.1×10^3	50	1
	4.9×10^3	0	0

Vector	No. of colonies/ml	No. of pink colonies/ml	% Pink colonies
pRS414/AR2	4.6×10^3	70	1.5
	1.0×10^4	200	2
	2.9×10^3	30	1
pRS414/AR3	8.4×10^3	150	1.8
	7.9×10^3	80	1
	1.3×10^4	340	2.6
pRS414/AR4	4.2×10^3	0	0
	5.1×10^3	60	1.1
	2.6×10^3	0	0

Table 3.1: Transformation efficiencies of pRS414 series. The table shows three sets of numbers, which correspond to three separate transformations of which the cells were counted.

100

Vector	No. of colonies/ml	No. of pink colonies/ml	% Pink colonies	Vector	No. of colonies/ml	No. of pink colonies/ml	% Pink colonies
pRS424	3.7×10^3	3.7×10^3	100	pRS424/AR2	2.7×10^3	20	0.7
	7.1×10^3	7.1×10^3	100		3.6×10^3	30	0.8
	3.3×10^3	3.3×10^3	100		8.3×10^2	0	0
pRS424/A	5.1×10^3	20	0.4	pRS424/AR3	5.7×10^3	20	0.4
	6.2×10^3	10	0.2		1.2×10^3	0	0
	5.5×10^3	10	0.2		8.9×10^2	10	1.1
				pRS424/AR4	1.6×10^3	0	0
					2.0×10^3	10	0.5
					1.3×10^3	0	0

Table 3.2: Numbers of colonies from transformations of pRS424 series vectors. The table shows three sets of numbers these correspond to three separate transformations of which cells were counted.
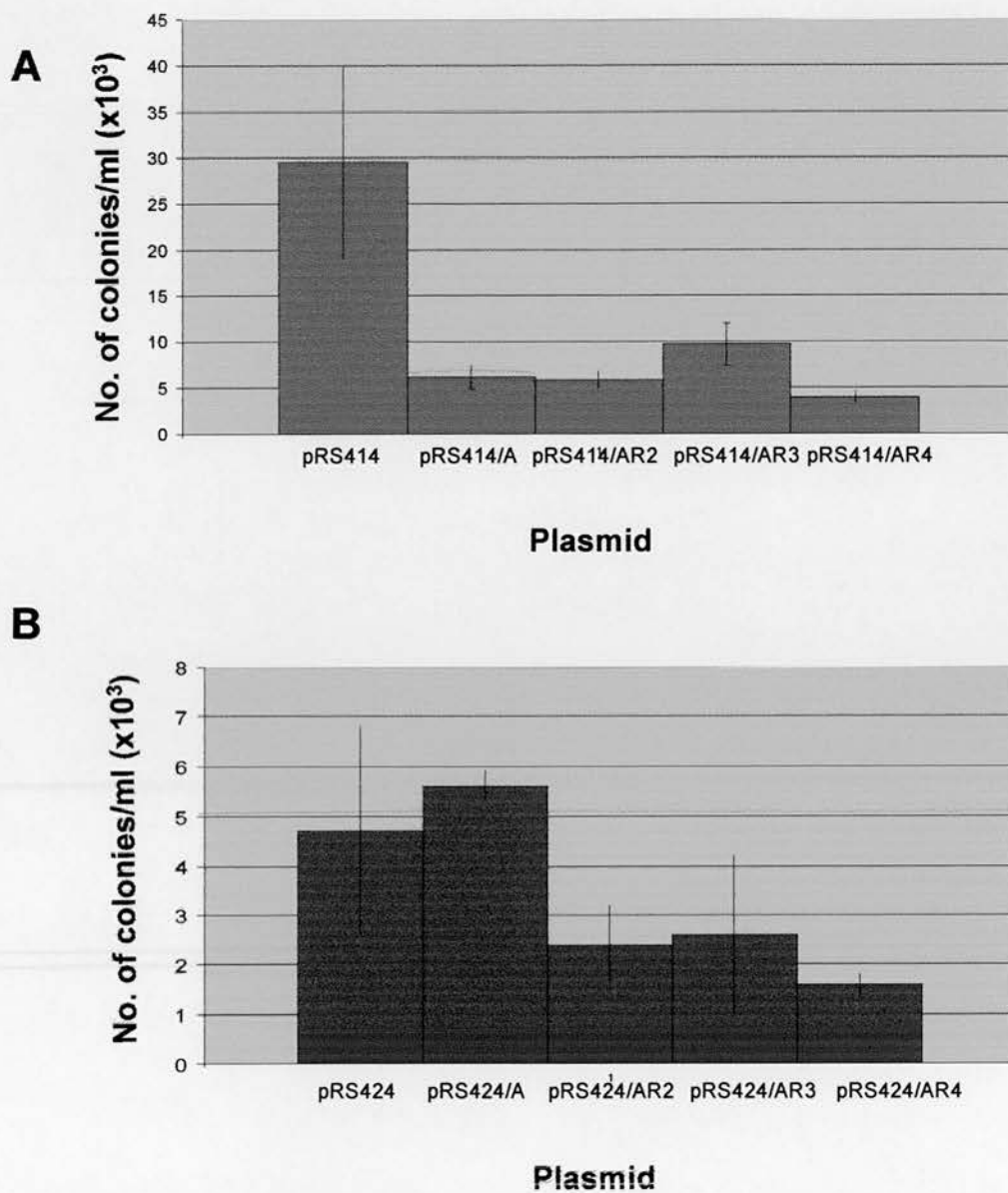
Figure 3.6: Average transformation efficiencies. (**A**) Shows the average transformation efficiency of cells transformed by pRS414 series. (**B**) Shows the average transformation efficiency of cells transformed with pRS424 series of vectors. Error bars were calculated using standard error.

A
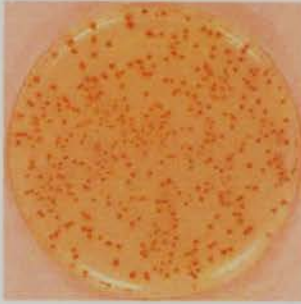
pRS414   pRS414/*ADE2*
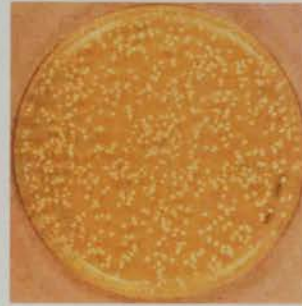
Sectoring colony

Pink colony

Figure 3.7 A : Examples of pink and sectoring colonies found in pRS414 series of experiments. Shown are the control plates, yeast transformed with the parental pRS414 vector are pink in colour as expected. Yeast transformed with the pRS414/*ADE2* vector are white in colour as expected. Representative pink and sectoring colonies are from yeast transformed with the RPS and *ADE2* on the pRS414 plasmid.

B
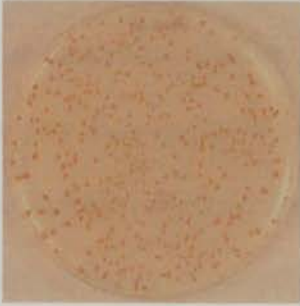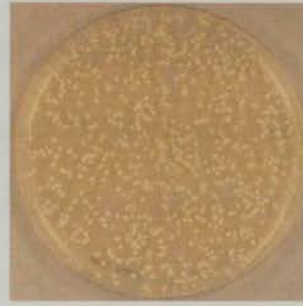
Figure 3.7 B : Examples of pink and sectoring colonies found in pRS424 series of experiments. Shown are the control plates, yeast transformed with the parental pRS424 vector are pink in colour as expected. Yeast transformed with the pRS424/*ADE2* vector are white in colour as expected. Representative pink and sectoring colonies are from yeast transformed with the RPS and *ADE2* on the pRS424 plasmid.

3.2.4 Analysis of RPS Function on Plasmids in a *tup1* Background

Since BP1, the RPS binding protein, has homology to the yeast transcriptional co-repressor, TUP1, it would be interesting to transform plasmids into both *TUP1* and *tup1* deletion strains. Differences in transformation efficiencies or numbers of pink colonies may indicate a role for TUP1/SSN6 in RPS-mediated repression. Therefore, the same number of molecules of DNA was transformed into a *TUP1* strain and a *tup1* strain of the same genetic background. In this series of experiments, the pRS414 series of low copy-number plasmids was used, as it showed the biggest difference in pink colonies between experimental and control plasmids.

In order to carry out this set of experiments the *TUP1* gene was deleted in the HC1 strain to obtain strain HC3 (see table 6.4). This was achieved by PCR-mediated disruption, replacing the *TUP1* gene from 2 bp upstream and downstream of the start and stop codons with a *MET15* selectable marker gene. The *MET15* gene was amplified from plasmid pRS401 with primers PRSTUP1A and PRSTUP1B containing homology to the flanking regions of the *TUP1* gene (table 6.2). The disruption fragment was transformed into yeast (strain HC1) and positive colonies selected on SC-met medium. A Southern blot was performed on genomic DNA digests, using probe 5, which confirmed the complete deletion of the *TUP1* gene (fig. 3.8).

After successful deletion of the *TUP1* gene, the pRS414 series of vectors was transformed into the *tup1* strain (HC3) and the parental strain (HC1), with the same

number of picomoles DNA (0.7 pmoles) added to each. The results are shown in fig. 3.9.

Figure 3.9 (A and B) shows an approximately 10-fold difference between the transformation efficiencies of the *TUP1* (HC1) and *tup1* (HC3) strains. This is more likely to be due to intrinsic strain differences, as flocculation phenotype of the *tup1* cells makes them difficult to count accurately so there may have been a difference in the actual number of cells transformed. Furthermore, *tup1* cells have different proteins on their cell walls (reviewed by Stratford, 1992) that may make them more resistant to the transformation process. Therefore, if we normalise the levels of the transformation efficiencies between the strains by dividing the transformation efficiencies of the *TUP1* strain by around 10-fold (the approximate difference between the control parental pRS414 transformation efficiencies) then transformation efficiencies are within the same order. This shows any silencing of the *TRP1* marker gene that may occur is strain independent and is not affected by TUP1.

There are differences between the numbers of pink colonies between the two strains (fig. 3.9). Indeed no pink or silenced colonies are found in the *tup1* mutant whereas some are found in cells expressing *TUP1*. This may suggest that TUP1 plays a role in RPS-mediated silencing, as silencing is lost when TUP1 is absent . However, these differences may to be due to the lower transformation efficiencies of the *tup1* mutants rather than TUP1/SSN6 playing a role in the silencing of the RPS and

reporter gene. Having comparable transformation efficiencies in both *TUP1* and *tup1* strains would confirm this.
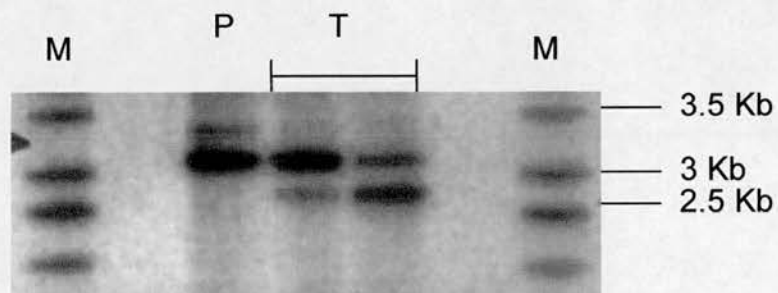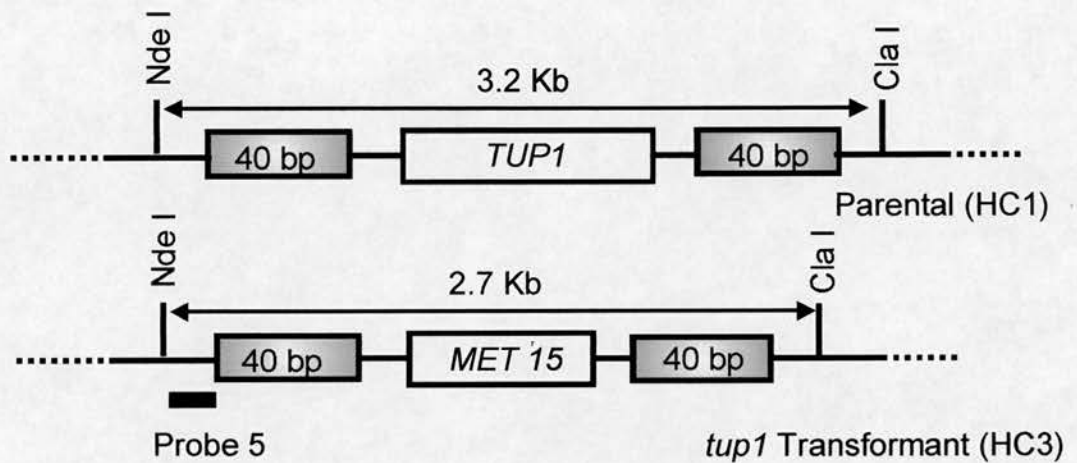
Figure 3.8: Strategy and Southern blot of *TUP1* knockout. The *TUP1* gene was deleted in its entirety from 2 bp upstream and downstream of start and stop codons. The disruption fragment was amplified by PCR from plasmid pRS401 using primers PRSTUP1A and PRSTUP1B. Probe 5 was used to visualise size differences between genomic restriction fragments. P denotes the parental strain (HC1) and T denotes transformants. Shaded boxes indicate the 40 bp of homology allowing homologous recombination. The blot shows bands of approximately 2.7 Kb in the transformant lanes confirming *TUP1* has been successfully deleted.

A

Strain	*TUP1*			*tup1*		
Vector	No. of colonies/ml	No. of pink colonies/ml	% Pink colonies	No. of colonies/ml	No. of pink colonies/ml	% Pink colonies
pRS414	2.3×10^3	2.3×10^3	100	2.5×10^2	2.5×10^2	100
pRS414/ A	2.4×10^3	20	0.8	2.3×10^2	0	0
pRS414/ AR2	2.3×10^3	40	1.7	1.4×10^2	0	0
pRS414/ AR3	1.6×10^3	40	2.5	1.0×10^2	0	0
pRS414/ AR4	1.6×10^3	0	0	9.0×10^1	0	0

B
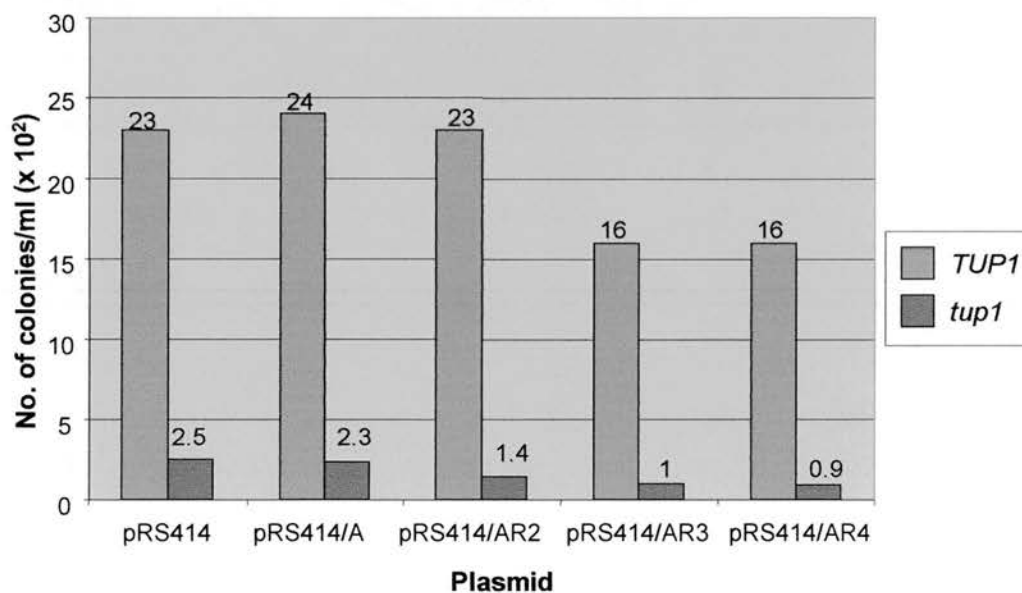
Figure. 3.9: Transformation efficiencies of *TUP1* and *tup1* cells. (**A**) shows a table of numbers of colonies returned from a single transformation. (**B**) represents a bar chart of these results.

3.2.5 Discussion

The results from plasmids suggest the RPS may have a silencing function in *S. cerevisiae*. The pink silenced colonies and especially the sectoring colonies observed in fig. 3.7 demonstrate this, although the overall incidence of silencing is low. A comparison of results between high and low copy number vectors shows subtly increased levels of silencing in the low copy number vectors (compare tables 3.1 and 3.2). This is contrary to the findings in *Arabidopsis* where one copy did not silence a reporter gene and the mechanism was thought to involve the recognition of multiple copies, which induces silencing (Müller *et al.*, 2002). My results could suggest a factor, at low abundance in yeast, may bind to the RPS and repress the adjacent reporter gene, as multiple copies of the RPS would dilute this effect. This may be a different mechanism to RPS-induced silencing in *P. hybrida*. Nevertheless, this does not exclude the possibility that, at some level, the molecular players may be homologous or conserved.

Comparing the transformation efficiencies between experiments should reveal if the RPS has a silencing effect on the *TRP1* marker gene. The large deviations in transformation efficiencies between similar experiments make this difficult to conclude with certainty. However, it seems unlikely that the RPS had an effect on the *TRP1* marker gene although it cannot be excluded, as orientation 4 consistently returned less colonies in both pRS414 and pRS424 vectors. Comparison of transformation efficiencies of *TUP1* and *tup1* strains was carried out to determine the role of TUP1 in RPS-mediated silencing. However, the small number of colonies

returned from the transformation of the *tup1* mutant makes it difficult to conclude whether *TUP1* had an effect on RPS silencing.

Whilst these plasmid experiments have given an insight into RPS silencing in yeast they have their limitations. The possibility that the *TRP1* gene was silenced could lead to the loss of silenced colonies and the different chromatin environment in plasmids compared to chromosomes suggest stable integration of the RPS and *ADE2* construct into yeast chromosomes would be a more appropriate method of study.

3.3 Integration of RPS into *S. cerevisiae* BY4733 Using 5-FOA Counter-Selection

3.3.1 Techniques and Materials for Study

As established by studying the effects of the RPS on plasmid gene expression, observing the action of the RPS when integrated into yeast chromosomes would be preferable. In order to integrate the RPS and *ADE2* into the genome, an appropriate mechanism for the selection of colonies with the integrated RPS and *ADE2* constructs had to be considered. Selection for transformants on SC-ade medium using the *ADE2* gene as a selectable marker is not desirable, as this is the gene being tested for silencing. Therefore, cells that have silenced the *ADE2* gene would be lost upon its selection on this medium. A chemical called 5-fluoro-orotic acid (5-FOA) has been used to counter-select for cells expressing the *URA3* gene (Boeke *et al.*, 1984). Cells that have a functional *URA3* gene degrade 5-FOA to a toxic compound and die. Only cells that have a non-functional *URA3* gene can survive on this medium. Therefore, prior integration of the *URA3* gene into the locus of interest, followed by the introduction of the RPS and *ADE2* sequences to the same locus, inducing the loss of the *URA3* gene, would allow selection for transformants positive for the RPS and *ADE2* by 5-FOA (further discussed in section 3.3.3)

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3.3.2 Integration of the *URA3* Gene at the *ADE2* Locus

To utilise 5-FOA selection, the *URA3* gene was introduced at the locus of interest. The locus chosen was the native *ADE2* locus. This is a housekeeping gene, which should generally have an open chromatin conformation. The *URA3* gene was amplified from plasmid pBlue/*URA3* (table 6.1) using primers RpsadeD and RpsadeE that contain 40 bp of DNA flanking the region of interest. The knock-in was performed in the HC1 strain to generate strain HC2. Positive colonies were selected on SC-ura medium and the correct integration was confirmed by a genomic digest and Southern blot analysis (fig. 3.10). The Southern blot confirms the production of yeast strains positive for the *URA3* gene. This strain can be used for 5-FOA counter-selection of strains containing the RPS and *ADE2* construct.
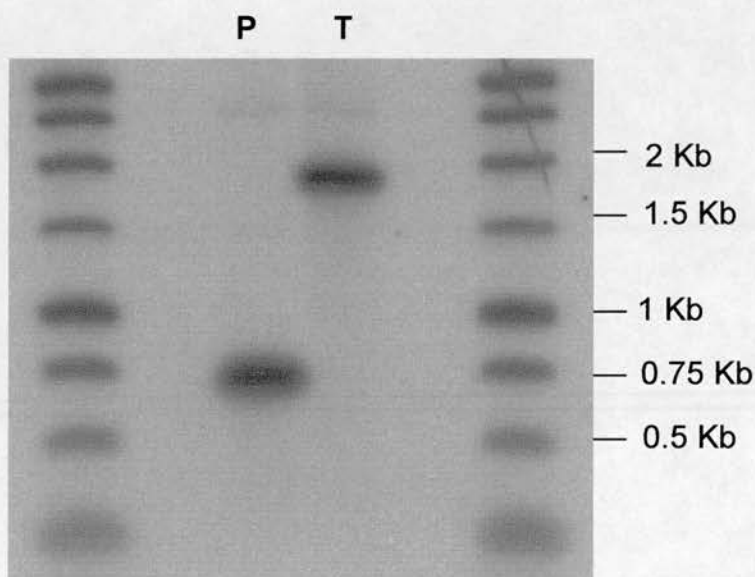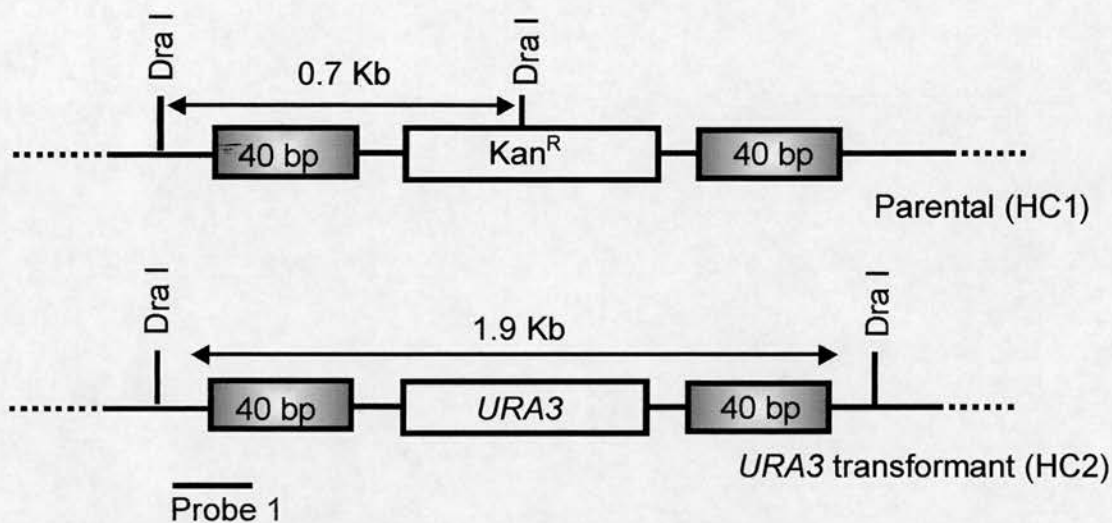
Figure 3.10: *URA3* integration at *ADE2* locus. The strategy for the Southern blot is shown with the actual blot below. P denotes parental strain and T denotes transformant, HC2. Primers 'RpsadeD' and 'RpsadeE' were used for amplification of the disruption fragment from vector pBlue/*URA3* (see appendices). Probe 1 was used to visualise size differences in genomic restriction digests between strains. The expected size of 1.9 Kb was found for the transformant indicating a successful replacement. The areas of homology for homologous recombination are shown by shaded boxes.

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3.3.3 Selection of RPS Integration by 5-FOA

In order to integrate RPS and *ADE2* constructs into the genome, disruption fragments containing these sequences were prepared. The strategy is simplified in fig. 3.11. To promote homologous recombination, larger areas of homology to the locus of interest of approximately 80 bp and 200 bp were amplified using primer pairs 007a and 007b, and 008a and 008b respectively. These regions of homologous DNA were cloned into pBluescript and sequenced to confirm their correct identity. The RPS and *ADE2* sequences of each orientation (1-4) were cloned between the arms of homology to prepare plasmids pBlue78/AR (for details of cloning see table 6.1). This allowed easy production of the disruption fragment by plasmid restriction enzyme digestion. A control plasmid (pBlue78/A) with the *ADE2* gene only was cloned in a similar fashion. The disruption fragments of each orientation and control were transformed into yeast strain HC2 and positive colonies were selected on minimal media supplemented with amino acids and bases for which the strain is auxotrophic, and 5-FOA (1 mg/ml).

Colonies returned from the selection were re-plated onto 5-FOA medium and SC-ura medium to confirm 5-FOA resistance and the loss of the *URA3* gene respectively. It was noted that often colonies would grow on both SC-ura medium and 5-FOA medium indicating the initial selection was not as stringent as expected. Furthermore, similar numbers of colonies were found on control plates with no DNA added and plates of transformations with disruption fragments, suggesting unsuccessful transformations. Despite this, colonies that behaved as expected (growth on 5-FOA

and no growth on SC-ura) were selected for Southern blot analysis to confirm the presence of the RPS/*ADE2* construct (fig. 3.12).

The Southern blot shows that all seventeen colonies selected were false positives. The same experiment was repeated three times from the transformation stage. Despite optimising the transformations and increasing the amount of DNA added, the same result was obtained. All of the 51 colonies tested that were growing on 5-FOA were false positives. The reason for this is unclear. Firstly, these colonies have the *URA3* gene and therefore should not survive on medium containing 5-FOA. Survival of these colonies may occur due to repression of the *URA3* gene and utilisation of the small amount of uracil provided by the medium. Secondly, even if there are false positives on the plates, it does not explain why no colonies containing the correct integration were found. This may suggest that the sequence does not integrate in the chromosomes despite having longer than standard arms of homology. Increasing the amount of DNA added to the transformations did not remedy this. Alternatively, the RPS may kill the cells although this is unlikely, as previously, cells transformed with the RPS sequence on a plasmid survived and did not show any growth defects and no *ADE2* only control integrations were found. However, in a chromosomal environment, the RPS may exhibit different effects. As no correct integration was found, it would appear that yeast bearing the new integration are disadvantaged and are subject to competition by false positives in the medium. Therefore, perhaps if 5-FOA selection were optimised to prevent the growth of false positives it would allow the growth of cells positive for the RPS and *ADE2*.

A

pBlue78/AR2
7073 bp

Sac I

217bp flanking
target gene

ADE2

RPS

Xho I

79 bp flanking
target gene

Digest plasmid, purify region
of interest and transform into yeast

RPS   *ADE2*

Homology arms   Homology arms

URA3

URA3 gene integrated at *ADE2* locus

Homologous recombination and
selection for transformants on
5-FOA medium

RPS   *ADE2*

ADE2 and RPS integrated into yeast chromosome at *ADE2* locus

B

Orientation 1
(pBlue78/AR1)

Orientation 2
(pBlue78/AR2)

Orientation 3
(pBlue78/AR3)

Orientation 4
(pBlue78/AR4)

RPS   *ADE2*

Figure 3.11: Vectors and strategy for integration into BY4733 *ADE2* locus. (**A**) shows the strategy for integration. The two sequences which, function as arms of homology were cloned into the multiple cloning site of pBluescript after being amplified by PCR and sequenced. The *ADE2* and RPS sequences were cloned adjacent to these sequences. The whole construct was excised by restriction digest, purified and transformed into the *S. cerevisiae* HC2 strain. This allows selection of transformants on 5-FOA medium, which selects against cells expressing the *URA3* gene. (**B**) shows the orientations of the RPS and *ADE2* sequences used in this study, which were cloned into the vector in the same manner (table 6.1).
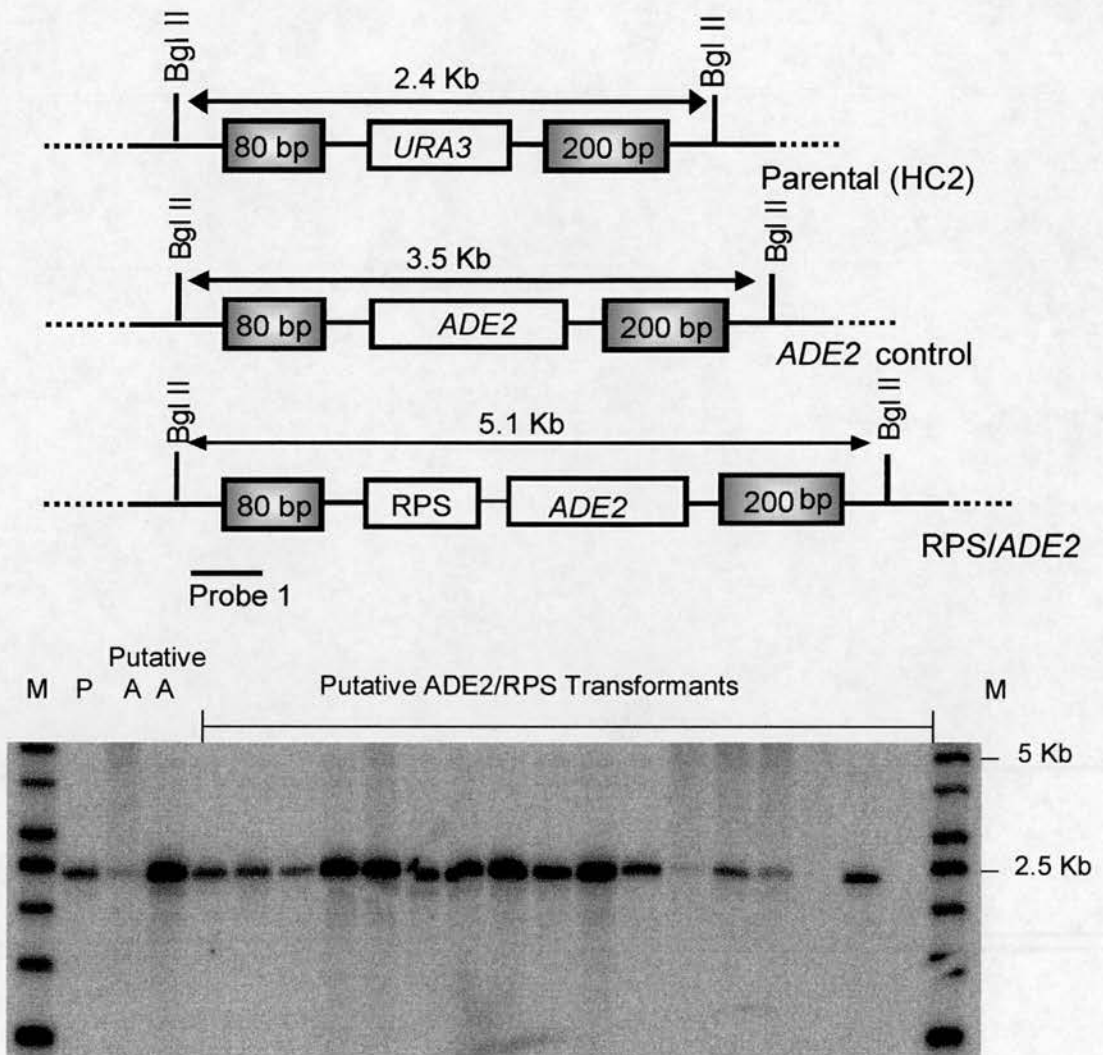
Figure 3.12: Southern blot showing selection of RPS integration by 5-FOA. M denotes markers, P, the parental strain (HC2) and A, the putative *ADE2* only control. Probe1 was used for visualisation of size differences between genomic restriction fragments. The expected size of the parental strain was observed, however, this 2.4 Kb band was also identified in the lanes of all putative transformants indicating that the integration was unsuccessful.

3.3.4 Optimisation of 5-FOA Selection

The standard amount of 5-FOA added to medium for *URA3* counter-selection is 1 mg/ml. Transformations of the RPS/*ADE2* disruption fragments were spread on plates containing increasing amounts of 5-FOA (1.5 mg/ml and 2 mg/ml) in an attempt to minimise the number of false positive colonies. Southern blot analysis on colonies growing on these higher 5-FOA concentrations showed that only false positives were present (fig. 3.13). Since 5-FOA is quite expensive, higher concentrations of the chemical could not be used. McCusker and Davis (1991) noted that when proline was used as a nitrogen source, instead of standard ammonium sulphate, *S. cerevisiae* cells become hypersensitive to 5-FOA allowing lower concentrations of it to be used in the medium. Therefore, this approach with proline-containing medium was adopted. Concentrations of 5-FOA were tested ranging from 25μg/ml to 1 mg/ml. Colonies were transformed and selected as previously and analysed by Southern blot as before (fig. 3.13).

The Southern blot of putative positive colonies shows the same pattern as the parental strain demonstrating that the *URA3* gene has not been replaced. Despite attempts to optimise 5-FOA selection, no positive colonies were obtained. This lead to the hypothesis that perhaps the RPS/*ADE2* construct was not integrating into the yeast chromosomes for reasons unknown or that the constructs were toxic to the yeast cells at this integration site.
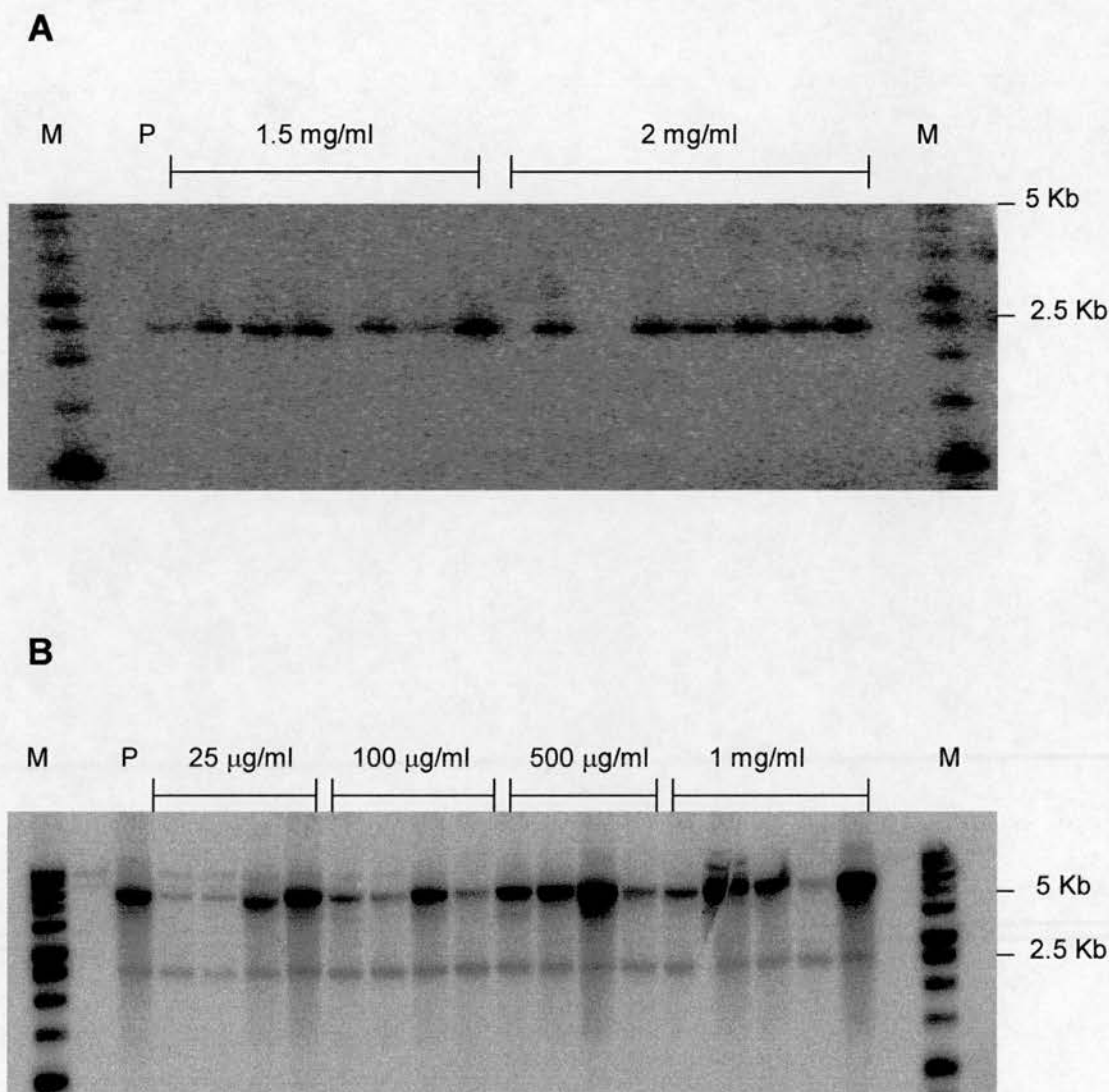
Figure 3.13: Southern blots showing optimisation of 5-FOA strategy. In both blots M denotes markers, P denotes the parental strain (HC2). The concentrations indicated are the final concentrations of 5-FOA in the medium used for selection of transformants. The same Southern blotting strategy (*Bgl* II digest) was used as is shown in fig. 3.12. (**A**) shows increasing concentrations of 5-FOA on the standard plates. (**B**) shows increasing 5-FOA concentrations on plates containing proline as a nitrogen source. In all cases a 2.4 Kb band, indicating the same structure at the *ADE2* locus as the parental strain was found. This suggests the integration was unsuccessful.

3.4 Integration of RPS into Yeast Genome by Adenine Selection.

To test the hypothesis that the RPS was either toxic to cells at this chromosomal locus or that the construct could not integrate, the same yeast strain (HC2) was transformed with the same disruption fragments but positive colonies were selected on SC-ade medium. This positive selection is not ideal: if the RPS induces silencing of the reporter gene, these colonies will be lost on SC-ade medium. However, if colonies appear it shows that the construct can integrate and that the constructs are not toxic to the cells in a chromosomal context. Moreover, studies have shown that genes can alternate between active and repressed states (Gottschling *et al.*, 1990). Therefore, after selection on SC-ade medium, colonies were picked and grown overnight in non-selective YPD medium. A loop of this culture was then placed in fresh YPD and grown overnight. This was repeated again and a sample was plated on YPD medium and the colour of colonies was noted. This takes advantage of the fact that the integration should be stable and plating onto non-selective medium will not cause the loss of the cassette. Although this method of selection is not ideal, it may give an insight into the mechanism and the stability of RPS-induced silencing.

3.4.1 Integration of RPS into *S. cerevisiae* BY4733

Using the same constructs and strains as shown in fig. 3.11, the RPS/*ADE2* constructs were transformed into yeast and selected for on SC-ade plates. Two colonies from each orientation were picked and grown in non-selective medium as described along with two *ADE2* gene only controls. These are shown in fig. 3.14

after the outgrowth. As shown, two colonies from the group were pink in colour (orientations 3 and 4, colonies 3a and 4b). Before we could conclude that these colonies contain a silenced *ADE2* gene, a Southern blot was performed to check the constructs were integrated in the genome correctly (fig. 3.15).

The blot shows that all the white colonies and controls have bands of the correct sizes and that the constructs are integrated correctly. However, the two pink colonies (3a and 4b) have a different pattern of bands from the expected length. No band is found for colony 3a, the reason for this may be insufficient DNA loaded into the lane. Colony 4b has the same size band as the parental strain suggesting that the construct has not integrated at this locus. However, since the colony grew on SC-ade medium, it implies that the constructs have integrated at the wrong locus. To test this hypothesis, two Southern blots were performed on the pink colonies (3a and 4b) and one white colony as a control (3b), although, no real theory can be extrapolated from 3a since no band was found in the localisation Southern blot (fig. 3. 15). The blots were probed with full-length RPS and *ADE2* sequences to observe if the RPS or *ADE2* sequences were present within the genome (fig.3.16).

The Southern blots confirm that the full-length RPS and *ADE2* sequences are present within the yeast genome. It also appears that some rearrangement of the RPS has occurred in 4b since a smaller band was found in the Southern blot. From my own observations, I have seen rearrangements of the RPS when placed in an *E. coli* host, and indeed repetitive DNA has the potential to recombine and rearrange. Studies have shown that other repetitive sequences, namely the Alu repeats from humans,

become unstable when placed in *S. cerevisiae* (Lobachev *et al.*, 2000) suggesting the rearrangement of the RPS sequence is a feasible hypothesis. As both colonies were pink, it is possible that the *ADE2* gene contained a point mutation. Therefore, all colonies were re-plated on SC-ade medium to see if the *ADE2* gene was functional (fig. 3.17). Figure 3.17 shows that all colonies grow on SC-ade medium except the pink colonies. This suggests that for the pink colonies the gene is silenced by the new locus, the RPS or that the *ADE2* gene contains a point mutation.

The band patterns of the Southern blots suggest that the RPS and *ADE2* construct has integrated at a site other than the endogenous *ADE2* locus. To verify if the sequence has been wrongly targeted, cells were plated onto SC-ura medium (fig. 3.17). As the *URA3* gene is present at the integration locus, colonies with the correct integration should have lost the *URA3* gene and would not grow on this medium. However, if the construct is elsewhere in the genome, the *URA3* gene would still be present and cells would be able to grow on this medium. The outcome of this plate assay indicates that all the white colonies were correctly targeted and that the pink colonies have retained the *URA3* gene, confirming that the construct went to the wrong location.
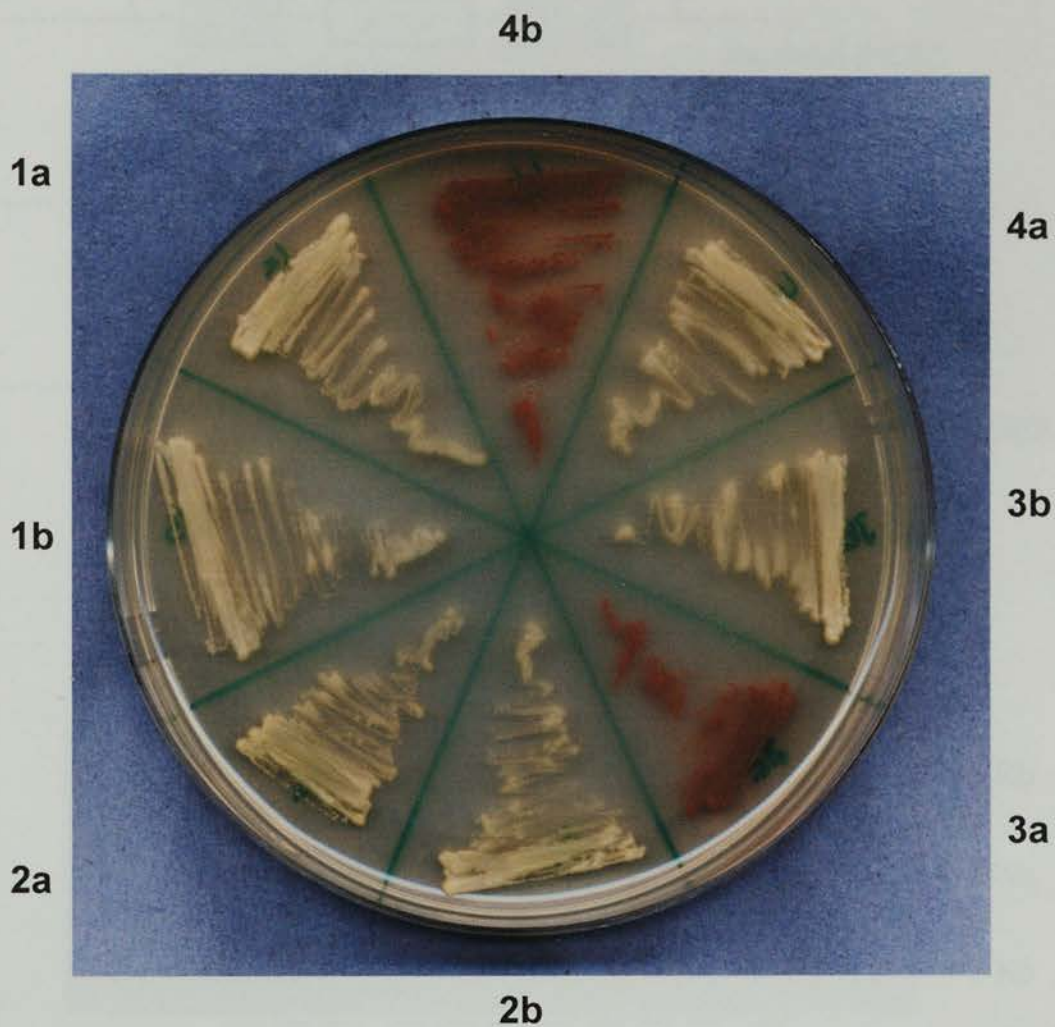
Figure 3.14: Colours of colonies after adenine selection and outgrowth. Shown are selected colonies plated onto YPD, non-selective medium. These colonies were originally selected for on SC-ade medium, and subjected to 3 sequential overnight outgrowths in YPD. Shown is the final plating. The numbers annotated to each colony represent the construct orientation integrated into the yeast. All colonies are white except 3a and 4b, which are pink in colour.
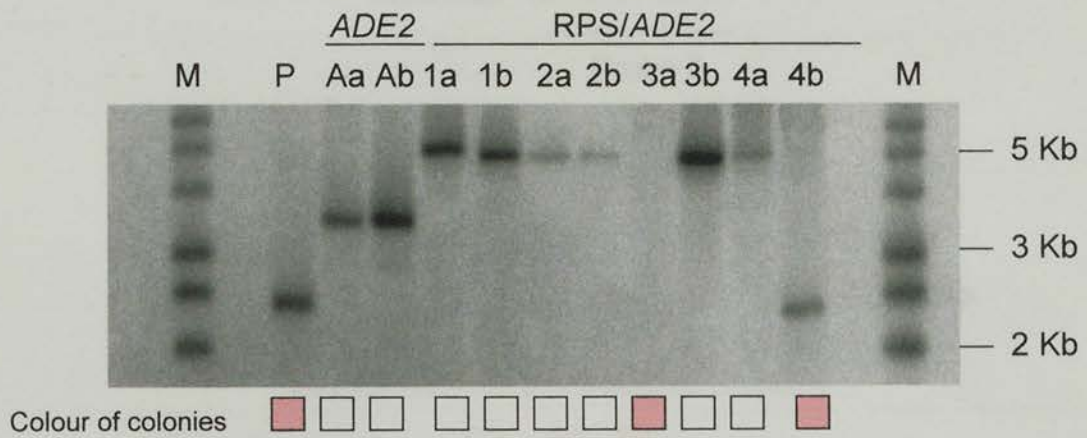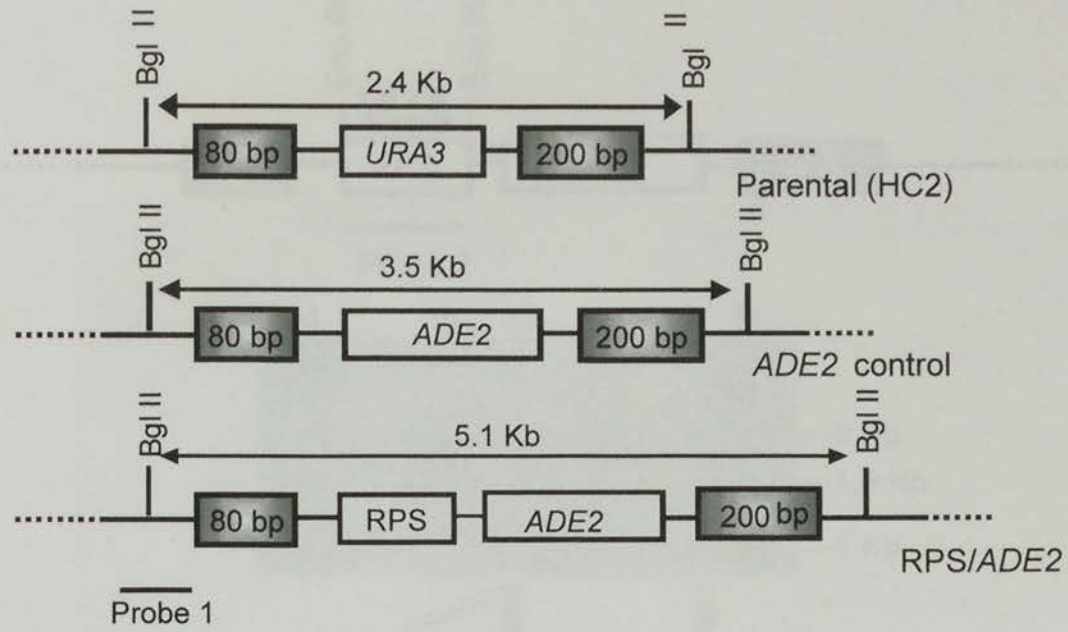
Figure 3.15: Southern blot showing integration of RPS/*ADE2* constructs into *S. cerevisiae* genome. P denotes the parental strain (HC2). Control strains with *ADE2* alone are called Aa and Ab. The RPS and *ADE2* strains are denoted by the number of the orientation used and a letter to distinguish between strains. Areas of homology for homologous recombination are indicated by shaded boxes. Coloured boxes beneath the Southern blot indicate the colour of the strain, pink for pink cells and white for white cells.
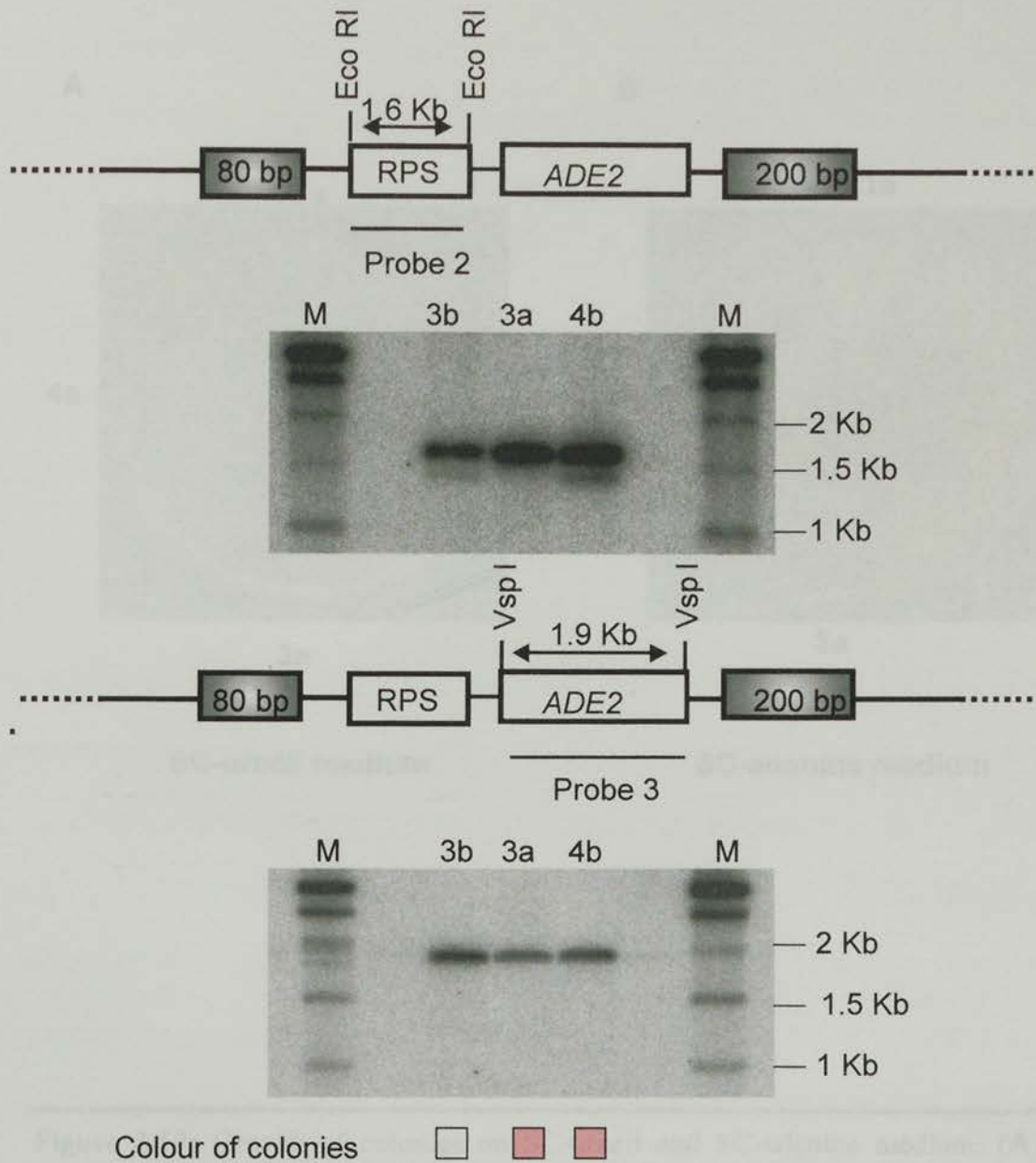
Figure 3.16: Southern blots confirming intact RPS and *ADE2* sequences. Shaded boxes indicate regions of homology allowing homologous recombination. Colours of colonies are indicated with pink squares being pink colonies and white squares denoting white colonies. Probes were used to visualise the correct size of sequences. These give the predicted sizes, indicative of bands showing that both *ADE2* and RPS sequences are of the correct length within the genome.
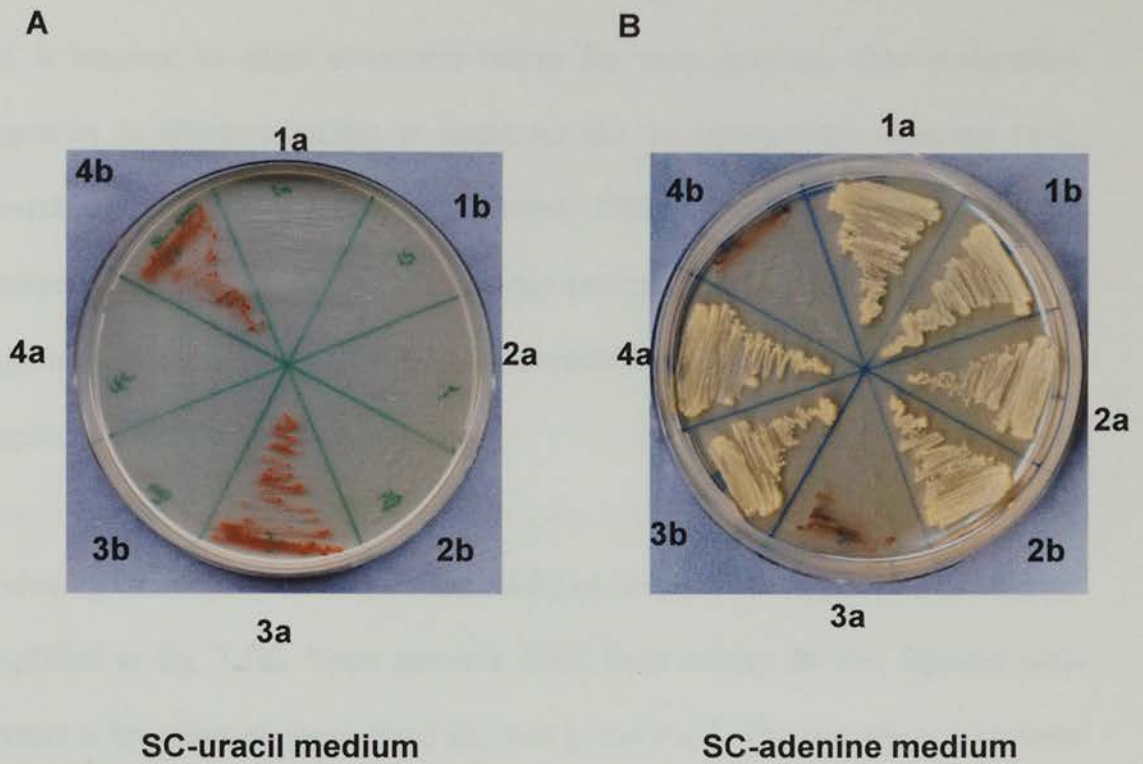
Figure 3.17: Growth of colonies on SC-uracil and SC-adenine medium. (**A**) shows only colonies 3a and 4b can grow on medium lacking uracil, implying the URA3 gene is still intact. (**B**) shows only the white colonies can grow on SC-adenine medium, although slight growth is detected for 3a and 4b.

3.4.2 Localisation of RPS Sequence

The observation that the RPS sequence was wrongly targeted at a high frequency, despite having longer than standard arms of homology, led to the hypothesis that the RPS is targeted to silent chromatin within the yeast genome. This preferential integration to silent chromatin is found for the Ty transposable elements in *S. cerevisiae* (reviewed by Boeke and Devine, 1998). Moreover, the fact that the incorrectly targeted colonies are pink, whilst correctly integrated colonies are white suggests the silenced state of the *ADE2* gene could be attributed to integration next to a region of silent chromatin.

To identify the location of the RPS and *ADE2* an inverse PCR approach was adopted (simplified in fig. 3.18). Yeast genomic DNA from colony 4b was digested with different 6 bp cutter enzymes *Hind* III, *Xba* I, and *Pst* I. The restriction fragments were self-ligated and divergent primers complementary to sequences in the *ADE2* gene was used to amplify the unknown DNA. An approximately 1.7 Kb fragment was amplified from *Hind* III digested genomic DNA. PCR of the other genomic restriction digests did not generate any bands; this may be because the sequences generated by ligation were too long for the PCR conditions used. The 1.7 Kb band was excised, cloned and sequenced.

The sequence revealed that the *ADE2* gene, and presumably the RPS had been wrongly integrated at a locus on chromosome XII, upstream of the co-ordinate 231381 (fig.3.19). This corresponds to an insertion in the *TRX1* and *PDC1* genes.

The *TRX1* gene encodes a thioredoxin, which is required for protection against reductive stress (Trotter and Grant, 2002). *PDC1* encodes a decarboxylase involved in amino acid metabolism (Dickinson *et al.*, 2000). Little is known about the chromatin structure of this locus, other than it is not one of the classic silent yeast loci, it is not sub-telomeric, it is gene-rich, and should therefore have an active chromatin conformation. Subsequent BLAST searches comparing the DNA transformed into yeast and the locus of integration revealed no homologous sequences to this locus in any of the sequences transformed into the yeast. Therefore, the construct must have integrated by non-homologous recombination. One notable feature of this locus however is that there is a high proportion of tRNA genes and δ-sequences from Ty elements upstream of the integration site (fig. 3.19).
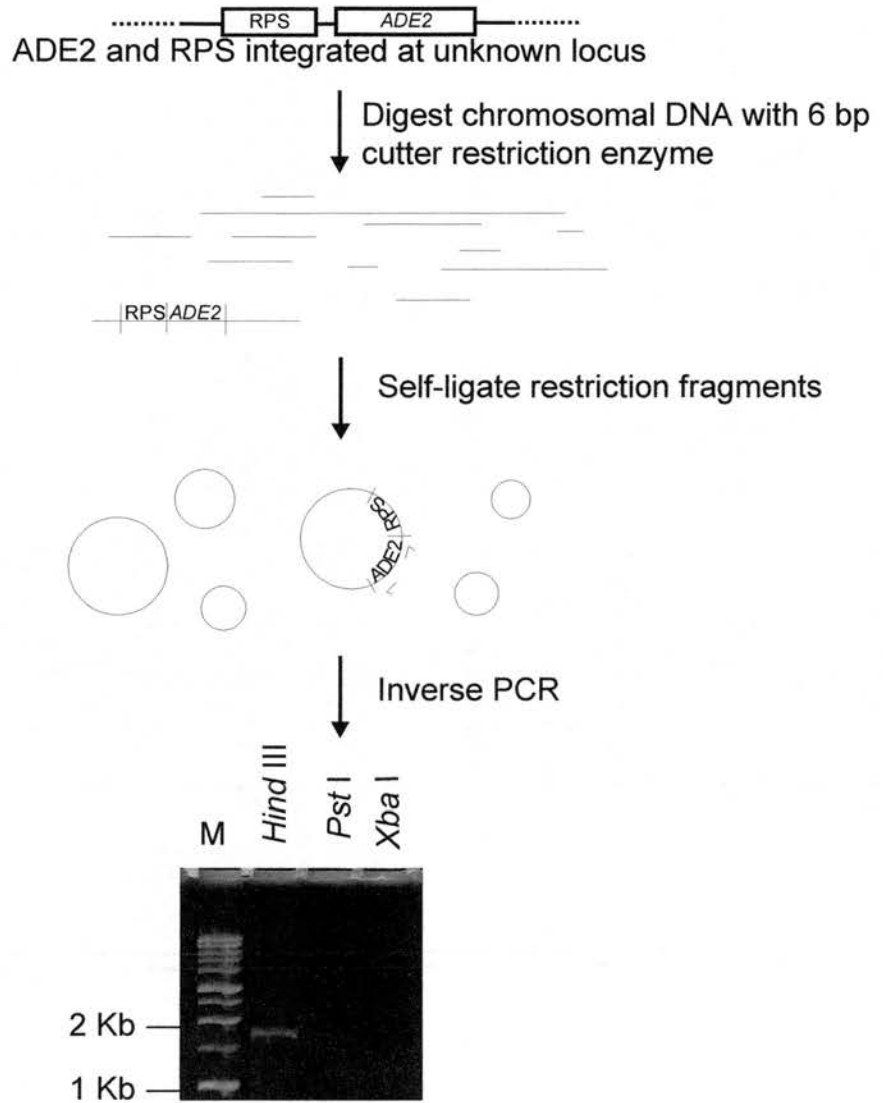
The band in the lane from amplification of *Hind* III digest, was purified, cloned, and sequenced.

Figure 3.18: Inverse PCR strategy for localisation of RPS and ADE2 sequences in the yeast genome. The gel shows the approximately 1.7 Kb band successfully amplified from *Hind* III digested genomic DNA by primers 019a and 019b (see appendices), which was cloned and sequenced. The other restriction digests did not yield products.
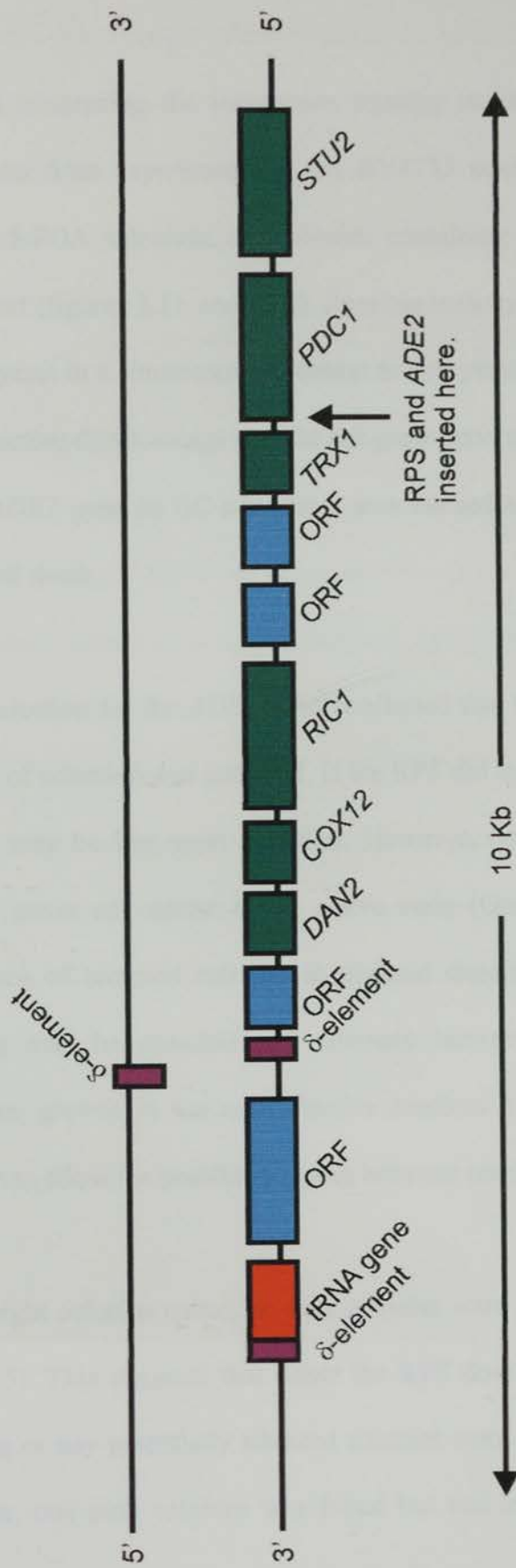
Figure 3.19: Features of the new chromosomal locus of the misplaced RPS and *ADE2* sequences. Shown is a schematic diagram of the region. Known genes are shown as green boxes, putative open reading frames (ORF), are shown as blue boxes. Genes encoding tRNA genes are represented by red boxes whilst *Ty* δ-elements are demonstrated by purple rectangles.

3.4.3 Discussion

Problems concerning the integration strategy make it difficult to establish valid conclusions from experiments in the BY4733 strain. Despite several attempts to optimise 5-FOA selection, no colonies containing correctly integrated constructs were found (figures 3.11 and 3.12). Possible reasons for this were that the RPS was lethal to yeast in a chromosomal context or that yeast carrying the RPS sequence had some selective disadvantage and did not grow upon competition. Therefore, selection for the *ADE2* gene on SC-ade plates was utilised to confirm that the RPS did not induce cell death.

Whilst selection for the *ADE2* gene confirmed that the RPS was not lethal to yeast; this type of selection was not ideal. If the RPS did induce *ADE2* gene silencing these colonies may be lost upon selection. However, previous studies have shown that silenced genes can revert to an active state (Gottschling *et al.*, 1990) and the appearance of sectored colonies in plasmid experiments suggested RPS-mediated silencing may be unstable and alternate between repressed and active states. Therefore, growth in the non-selective medium YPD was employed after initial selection to allow the possible flipping between conditions.

Of the eight colonies tested, no pink colonies were found at the correct *ADE2* locus (fig. 3.15). This suggests that either the RPS does not mediate chromosomal gene silencing or any potentially silenced colonies were lost at the initial selection stage. However, one pink colonies was found but had localised elsewhere. Inverse PCR

demonstrated that this construct had integrated at a locus on chromosome XII. The reason why the wrongly placed colony is pink is unclear, the novel locus of integration was not one of the classical silent yeast loci and therefore, the *ADE2* gene was not subject to known endogenous position effect variegation. It was hypothesised that the pink colour may be due to further recombination between the RPS and *ADE2* sequences resulting in mutations in the *ADE2* gene. However, Southern blot analysis proved that the full-length DNA sequences were present (fig. 3.16). As the presence of the full-length sequences does not account for any point mutations that may occur, the pink colonies were plated on SC-ade medium to observe if the *ADE2* gene was functional (fig. 3.17). This plate assay shows that the pink colonies did not grow on this medium, however the lack of growth could also be attributed to RPS-induced silencing.

The difficulties in selection with adenine warrant caution in interpretation of this data. If any conclusive results on RPS-mediated silencing are to be generated an appropriate selection must be found to allow stable integration of the RPS and *ADE2* sequences into the genome. Such stable integration would potentially allow the yeast cell to be genetically manipulated to determine the mechanism of RPS silencing.

3.5 Analysis of RPS in *S. cerevisiae* FY2 Strain

The limitations of the adenine selection in the previous experiments make it difficult to extrapolate conclusive answers about RPS function in yeast. Therefore, some means of selection of transformants other than the *ADE2* gene had to be devised. This involved changing *S. cerevisiae* strain from BY4733 to FY2. The *S. cerevisiae* FY2 strain contains a defective *URA3* allele known as *ura3-52*. The *ura3-52* allele contains a *Ty1* insertion in the *URA3* gene at codon 121 (Rose and Winston, 1984). In conjunction with using this strain, yeast-integrating vectors were employed. These vectors have no origin of replication and are not supported in yeast unless they integrate into the genome. The vector selected was pRS406, which contains a functional copy of the *URA3* gene. When the FY2 strain is transformed with pRS406, the functional *URA3* will recombine with the *ura3-52* allele and give one functional copy of *URA3* and one *ura3-52* allele. When the *ADE2* and RPS sequences are cloned into this vector, they will become integrated into the genome with the rest of the vector (fig. 3.20). This means the positive integrants containing the RPS and *ADE2* sequences can be selected for on SC-ura medium. In this case, the locus at which the RPS will be studied is *URA3*. It is a similar locus to *ADE2*, as it is a housekeeping gene and should have "normal" chromatin structure.
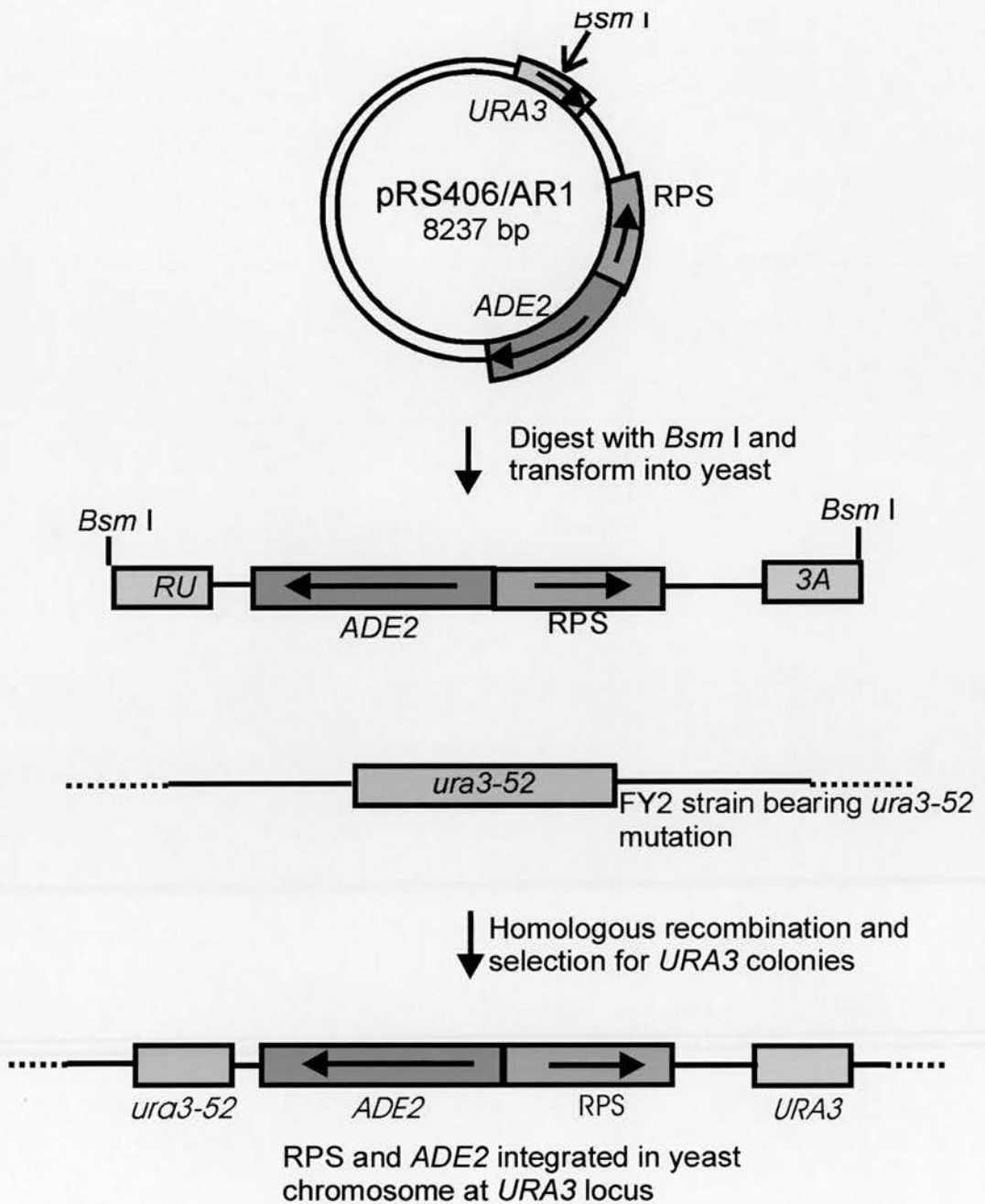
Figure 3.20: Vectors and strategy for integration in FY2 strain. RPS and *ADE2* were cloned into the multiple cloning site of pRS406 integrating vector (Christianson *et al.*, 1992) in the various orientations shown in fig. 3.11 (B). The vector was linearised by digestion with *Bsm* I for optimal recombination. This was transformed into *S. cerevisiae* FY2 strain to allow homologous recombination and positive colonies were selected on SC-ura medium. The nature of the recombination is shown and produces one functional copy of *URA3* and one mutated copy, with the construct of interest, RPS and *ADE2* also integrated at the locus of interest.

3.5.1 Knockout of *ADE2* Gene in *S. cerevisiae* FY2

As previously achieved for the BY4733 strain, the endogenous *ADE2* gene was deleted in the FY2 strain in order to utilise it as a reporter gene. The same strategy was performed as in section 3.2.2. The Southern blot of the knockout is shown in fig. 3.21, which confirms the successful deletion of the *ADE2* gene to generate strain HC4. A complementation test was also carried out and confirmed the strain would change from pink to white when the *ADE2* gene was supplied on a plasmid (not shown).
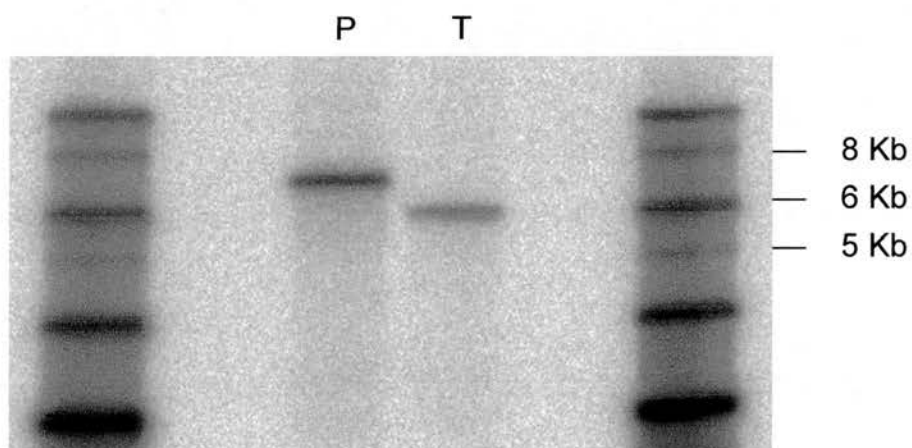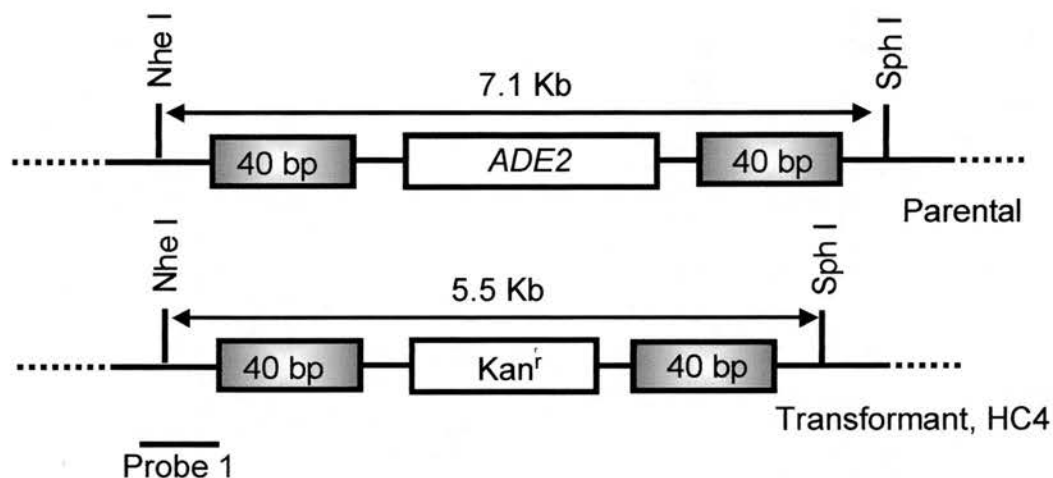
Figure 3.21: Southern blot showing knockout of *ADE2* gene in FY2 strain. The *ADE2* gene was deleted from 602 bp upstream and 239 bp downstream of the start and stop codons. Primers 'adedelstart' and 'adedelend' were used to generate a disruption fragment by PCR from plasmid pKanMX4 (see appendices). The hybridisation probe was used to visualise size differences between strains . The Southern blot shows the expected 7.1Kb parental band and the transformed strain, HC4, has the expected 5.5 Kb band. P denotes the parental strain and T denotes the transformed strain with the kanamycin resistance gene. DNA sequences used for homologous recombination are indicated by shaded boxes.

3.5.2 Integration of RPS into *S. cerevisiae* Chromosomes

All orientations (1-4) of the RPS and *ADE2* constructs were integrated into *S. cerevisiae* FY2 using the pRS406/AR series of vectors (description of cloning given in table 6.1) by the mechanism shown in fig. 3.20. A control with the *ADE2* gene only was also transformed, pRS406/A (see table 6.1). Positive transformants were selected on SC-ura medium. Colonies and numbers of pink colonies were counted and are shown in table 3.3. The results in this table show, no pink colonies on the control plates, which were transformed with the linearised control *ADE2* only plasmid. Cells transformed with vectors containing the RPS sequence have pink and sectoring colonies, which are most prevalent in colonies containing the construct in orientation 4. The occurrence of pink and sectoring cells on the plates suggests the RPS has induced gene silencing when integrated at a chromosomal locus. However, the integrations must be confirmed by Southern blot analysis.

Of the colonies listed in table 3.3, sixteen putative RPS/*ADE2* integrants were selected for further analysis. For each orientation one pink colony, one sectoring colony and two white colonies were chosen. Colonies were annotated according to their phenotype and the orientation integrated: the number (1-4), being the number of the orientation, 'a' denotes a sectoring colony, 'b' is a pink colony and 'c' and 'd' denoting white colonies. For example, 3b would be a colony with the RPS and *ADE2* orientation 3 construct integrated and would be sectoring. A representative sample of the plates and colonies are shown in fig. 3.22.

The correct localisation of these sequences was ascertained by Southern blotting (fig. 3.23). The Southern blot shows that most sequences have the expected length of bands suggesting the constructs are correctly integrated at the *ura3-52* locus. Colony 1a has no band, this may be due to insufficient DNA levels on the original gel. Therefore, as no positive conclusions can be made; 1a will not be considered any further. Some colonies show different banding patterns (2a, 2b, 3b and 4b) from the expected length. As the length of bands is also different from the parental strain, it suggests that some recombination of the transformed sequences has occurred but this region has undergone further aberrant recombination.

Southern blot analysis on genomic DNA digests probed with full length *ADE2* and RPS sequences was performed to determine if the different banding patterns could be attributed to recombination of the repetitive RPS sequence or *ADE2* gene. Figure 3.24. shows the Southern blots and confirms that full length *ADE2* and RPS sequences are present in all of the correctly integrated colonies as established by the previous Southern blot (fig. 3.23). No bands corresponding to the *ADE2* and RPS sequences were identified in colonies 2a and 4b suggesting these are false positives or have undergone further recombination at the *ura3-52* locus, which has resulted in the exclusion of these sequences from the genome. The Southern blots show that colonies 2b and 3b have full-length RPS and *ADE2* sequences present within the yeast genome. If we consider that for these strains to grow on SC-ura medium an integration event must have occurred at the *ura3-52* locus to complement the mutation and the different banding patterns compared to the parental strain, it suggests integration has happened at this locus. However, the full-length RPS and

ADE2 sequences are not integrated in the expected manner predicted in fig. 3.20. Therefore this locus may have undergone further recombination leaving the *ADE2* and RPS sequences intact at this locus, or the full-length sequences have moved to another location within the genome.

Interestingly, in fig. 3.24, for colonies 1b, 1c, 1d and 2b, bands of slightly shorter length to the *ADE2* gene are found. This suggests some recombination of the *ADE2* gene has occurred. However, since some of these colonies are white, it is evident that this does not affect the colour of colonies. In addition, in fig. 3. 23, only one band is found that corresponds to the insertion at the *ura3-52* locus, implying that if there is an additional truncated *ADE2* gene within the genome it is not present at this locus.

All other colonies studied reveal that the targeting and sequence integrity are correct. This shows that in the majority of cells that are white, the construct is correctly targeted, implying that in these cases there is no silencing of the *ADE2* reporter gene. However, in two out of the sixteen colonies studied, constructs were correctly localised and colonies were pink or sectored implying that the RPS induces gene silencing in yeast. Therefore, the average number of pink (1b) or sectored colonies (4a) over all experiments for all orientations was 10.6%. This figure would have to be amended to 5.3% for silenced colonies to take account of recombinations and wrongly targeted sequences. However, due to the small number of colonies studied in these experiments, these results would have to be repeated in order to determine the statistical significance of these figures. Nevertheless, the appearance of a sectored colony reveals something about the mechanism of silencing; it shows that throughout

colony development the expression of the *ADE2* reporter gene changed in some cells. This suggests that the *ADE2* gene may alternate between silent and active states when placed next to the RPS sequence, particularly in orientation 4.

Vector Integrated	No. of Colonies/ml	No. of Pink Colonies/ml	No. of Sectored Colonies/ml	% Pink/Sectored Colonies
pRS406/A	312	0	0	0%
	212	0	0	0%
pRS406/AR1	468	20	12	6.8%
	164	6	1	4.3%
pRS406/AR2	267	15	7	8.2%
	54	3	0	5.6%
pRS406/AR3	362	18	8	7.2%
	65	5	0	7.8%
pRS406/AR4	213	27	25	24.4%
	49	6	4	20.4%

Table 3.3: Transformation efficiencies of pRS406 series integrations. Shown are the results from two separate transformations. The numbers shown are the average numbers of colonies from four plates of the same transformation.
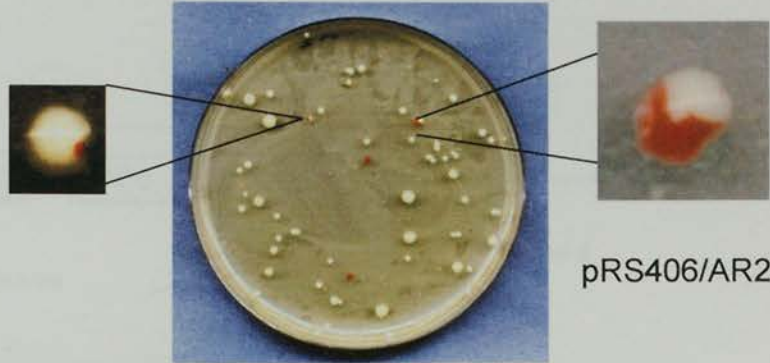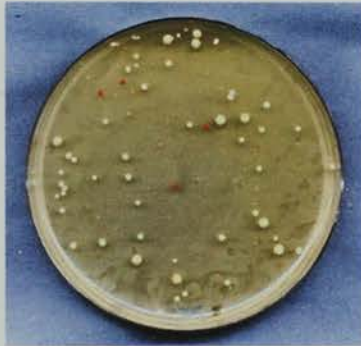
Figure 3.22: Colonies following pRS406 series transformations. Shown are a representative sample of plates following integration of the pRS406 series constructs. As expected, only white colonies are found on control plates, pRS406/A with the *ADE2* gene alone. Other plates with colonies containing the RPS and *ADE2* constructs contain a mixture of pink, white and sectored colonies some of which have been highlighted.
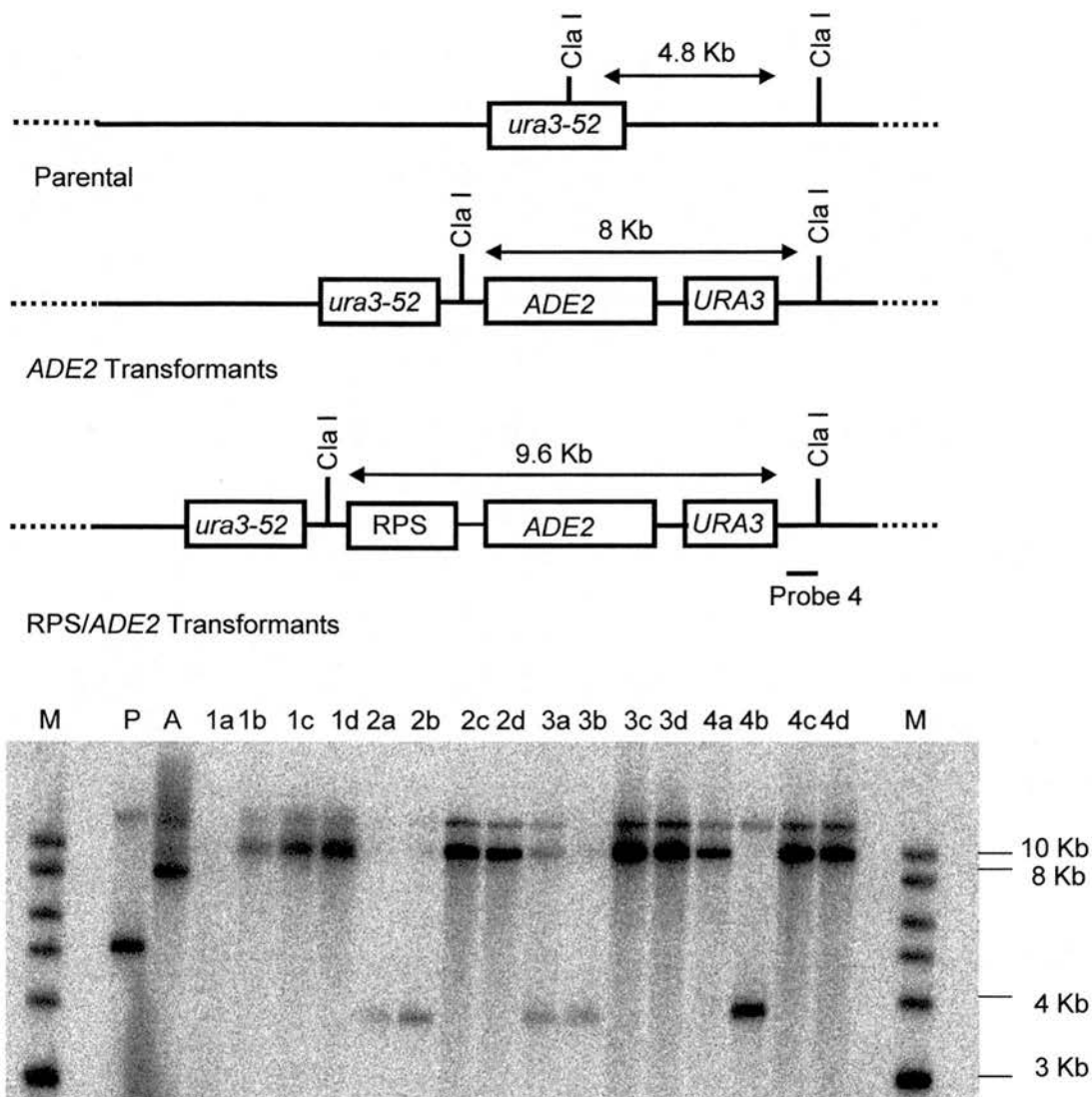
Figure 3.23: Southern blot confirming RPS and *ADE2* sequences at *ura3-52* locus. M denotes the marker, P denotes the parental strain, A denotes the *ADE2* only control strain. The other lanes are RPS/*ADE2* transformants. The nomenclature is as follows: the number denotes the orientation of the construct integrated. The letter **a** denotes a sectoring colony, the letter **b** denotes a pink colony and letters **c** and **d** refer to white colonies. Probe 4 was used to differentiate between sizes of bands and the expected sizes are shown above.
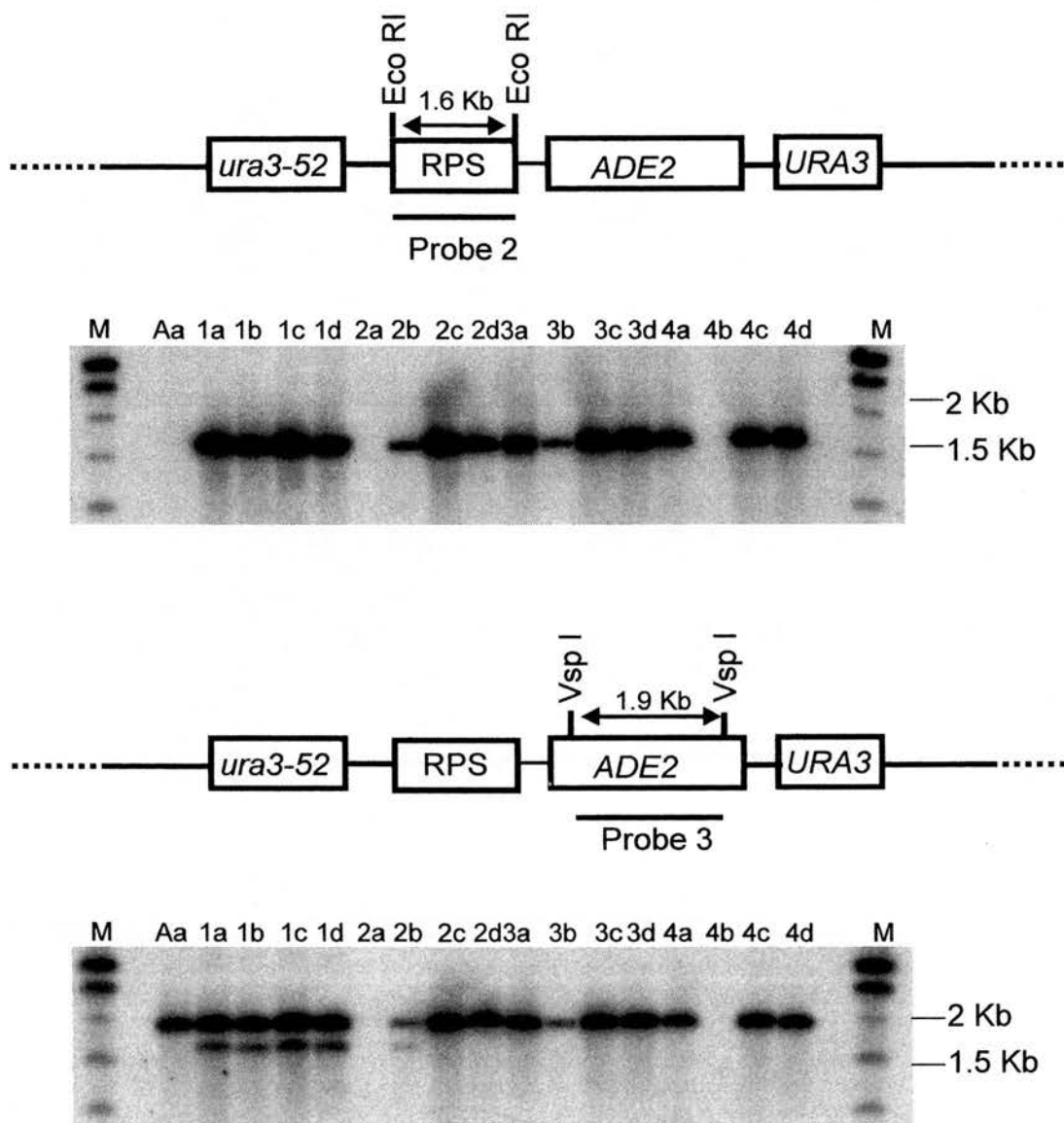
Figure 3.24: Southern blots showing full length *ADE2* and RPS sequences integrated into FY2 genomic DNA. Probes were used to detect full length RPS and *ADE2* sequences, and bands of expected sizes, shown in the schematic diagrams can be seen in both blots. The nomenclature is as follows: the number denotes the orientation of the construct integrated. The letter **a** denotes a sectoring colony, the letter **b** denotes a pink colony and letters **c** and **d** refer to white colonies.

3.5.3 Role of BP1 in RPS-mediated Silencing in Yeast

To examine how RPS-mediated silencing occurs in yeast and to elucidate the mechanisms of silencing in plants, effectively yeast is being used as a "test tube". A WD repeat protein from *Arabidopsis*, with homology to the yeast co-repressor TUP1 was found to bind the RPS. This may or may not be linked with RPS silencing. To investigate the role of BP1 in RPS-induced silencing in yeast, colonies that were white in colour but contained the RPS sequence were transformed with a vector expressing the BP1 protein (p415-*MET25*-BP1, see table 6.5). In order to utilise the *MET25* promoter, the methionine biosynthesis pathway must be intact. The strain used is a methionine auxotroph, therefore the *MET15* gene was restored to its original locus by PCR-mediated disruption. The *MET15* gene was amplified from vector pRS401 using primers 017a and 017b (see table 6.2).

Once the methionine biosynthesis was intact, colonies were transformed with p415-MET25-BP1. Northern blot analysis confirmed that BP1 transcription was being driven by the *MET25* promoter (see chapter 4). The colonies transformed were 1c, 2c, 3c, and 4c along with an *ADE2* only control. Any change in the colour of colonies from white to pink, which would indicate silencing, was noted (fig. 3.25). As can be observed from the plates, all of the colonies retained their white colour suggesting that the BP1 protein has no influence over gene silencing in yeast in the experimental conditions examined here.

This observation can be explained several ways. Perhaps the BP1 protein, like TUP1, needs to interact with other proteins to achieve repression. BP1 may be too distant, in evolutionary terms, from the machinery in *S. cerevisiae* to allow this (further discussed in chapter 4). Furthermore, BP1 may not act alone in RPS-mediated silencing; it may act as part of a larger protein complex, which is absent in yeast. Also, the mechanisms of RPS-mediated silencing in plants and yeast may differ in their mechanisms and where BP1 may be relevant in plants, it is redundant in *S. cerevisiae*. Alternatively, BP1 may not have any function in silencing the RPS and the RPS binding site may be coincidental. This is furthered by the fact that the RPS does not enhance expression variegation in *Arabidopsis*, which contains endogenous BP1 protein. It is also conceivable that once the decision is made for the *ADE2* gene to be active or silent it remains in that state; therefore the RPS/*ADE2* should have been transformed into cells already expressing the BP1 protein. However, the appearance of sectoring colonies in the previous experiments suggests that the silencing or expression of the *ADE2* gene can change through colony development.

In order to understand the mechanism of BP1-mediated silencing, the sequences were sub-cloned into a yeast expression vector that constitutively over-expresses BP1 proteins so the *ADE2* gene silenced by the RPS product. As previously described, BP1 protein itself is involved in the RPS-dependent silencing. This will help understand why *ADE2* gene silencing is observed. RPS could be removed second mutant deletions in the *ADE2* cassette could silence the observed. RPS could also be removed and the construct could also be followed by the above silencing. Construct in an expression vector which over-expresses the BP1 protein. The *ADE2* gene was inserted in all orientations to examine the direction and silencing of the RPS. In this silencing study, all the silencing construct ADE2 silencing were over-expressed by the BP1 protein. The RPS-mediated gene silencing as shown. The observed direction of the BP1 gene over-expressed in S. cerevisiae.

To test the role of BP1, we examined silencing of the ADE2 gene silencing and sub-cloned into a silencing vector that constitutively over-expresses BP1 proteins to the silencing. As previously the direction RPS all silenced the ADE2 gene over-expressed, BP1 protein in S. cerevisiae.

Figure 3.25: Effects of BP1 on RPS-mediated gene silencing. Shown are plates of white colonies containing RPS and *ADE2* constructs transformed with a plasmid expressing the BP1 protein. As shown all cells are white suggesting BP1 does not influence RPS-induced silencing in *S. cerevisiae*.

3.5.4 Role of TUP1 in RPS-mediated Silencing

In order to understand the mechanism of RPS-mediated silencing, the components that may be involved must be dissected. The two well-characterised gene repressors in *S. cerevisiae* are the TUP1/SSN6 co-repressor and the SIR proteins. As previously mentioned, BP1, a putative TUP1 homologue binds the RPS sequence. When BP1 was transformed into yeast there was no difference in silencing; this could be because it cannot interact with other yeast proteins necessary for repression. Since BP1 has homology to TUP1, TUP1 may be a candidate for inducing the observed RPS-mediated repression in yeast. Therefore, the *TUP1* gene was replaced by the zeocin antibiotic resistance marker in a strain showing silencing of the *ADE2* reporter gene (4a) and colonies were checked to see if silencing was alleviated and sectored colonies became white. *TUP1* was deleted by PCR-mediated disruption using the plasmid pPICZB (table 6.1), which contains the zeocin resistance gene and primers 020a and 020b that contain 40bp homology to the *TUP1* locus (table 6.2).

To test if colonies were mutant for *TUP1*, cells were grown in broth medium and any that displayed a severe flocculating phenotype, typical of a *tup1* mutant were considered positive for a *TUP1* deletion. The results are shown in fig. 3.26. As the colonies retain their sectoring phenotype, it indicates that, in this instance, TUP1 is not responsible for RPS-mediated silencing. However, this experiment would have to be repeated to validate these results.
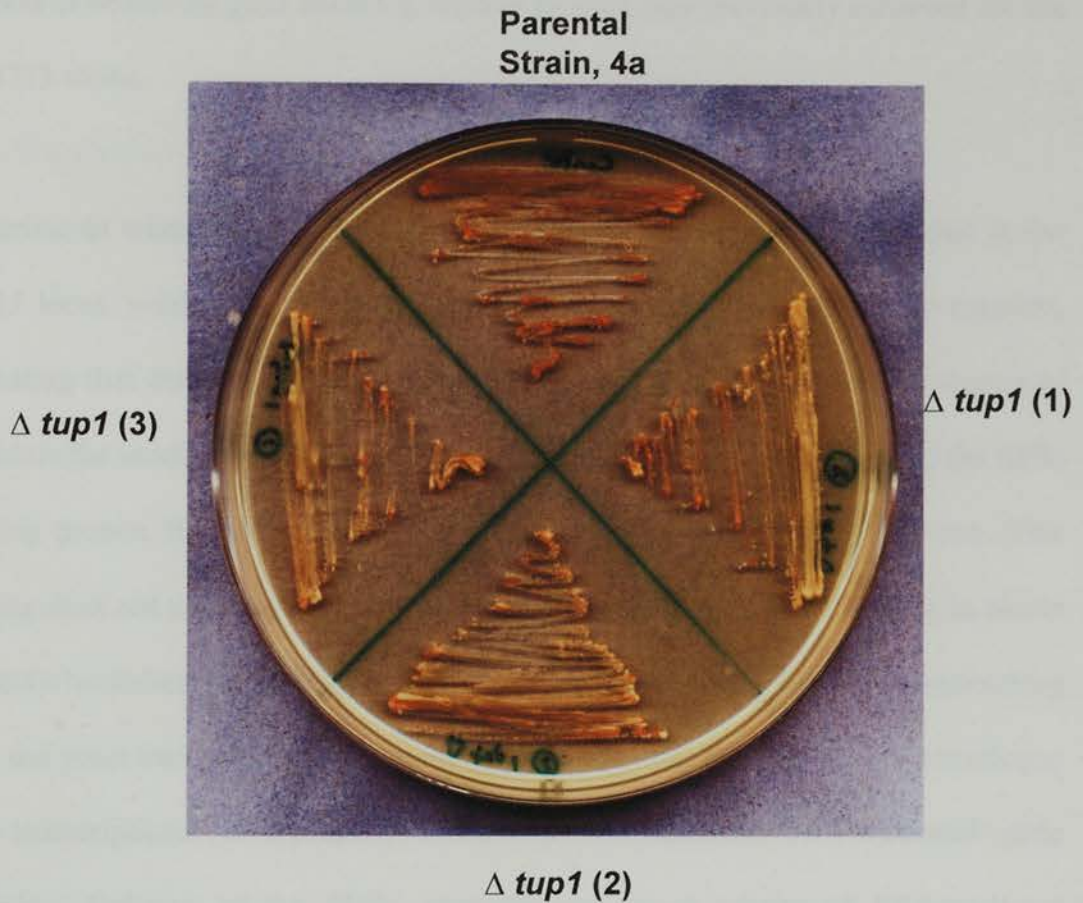
Figure 3.26: Effects of TUP1 on RPS-mediated silencing. Shown are examples of colonies following a *TUP1* deletion. The parental strain (4a) containing the *ADE2* and RPS sequences integrated at the *URA3* locus is sectoring as expected. The plate also contains 3 *tup1* mutants, which were selected from plates following replacement of the *TUP1* gene with the zeocin resistance marker. These colonies are also sectoring implying TUP1 has no effect on RPS-mediated silencing.

3.5.5 Discussion

The utilisation of the FY2 strain allowed positive selection for cells containing the RPS and *ADE2* constructs without selection of the *ADE2* gene. This improved the method of observing gene silencing relative to what was previously achieved for the BY4733 strain.

Experiments where the RPS and *ADE2* constructs were correctly integrated at the *URA3* locus yield pink and sectoring colonies, not seen in *ADE2* only controls, indicating that the RPS can mediate gene silencing in yeast. However, attempts to elucidate the mechanism of this silencing were inconclusive. Where tested, the RPS-binding protein BP1 did not enhance RPS-mediated gene silencing in yeast. This finding does not mean that BP1 is not involved in RPS-mediated silencing in plants but may be indicative of evolutionary divergence preventing BP1 from interacting with the yeast transcriptional machinery. The putative *S. cerevisiae* BP1 homologue and transcriptional co-repressor, TUP1 did not influence RPS-mediated gene silencing. Deletion of the *TUP1* gene did not cause release of RPS-mediated silencing where tested. Therefore, the mechanism of RPS-induced silencing remains unknown.

Interestingly, a high frequency of pink or sectoring colonies were found to have full-length RPS and ADE2 sequences integrated into the genome in an unexpected fashion. This may be due to further recombination at the *ura3-52* locus. It would be interesting to carry out a study on a variety of these colonies to determine the exact

localisation of these sequences and deduce if there are particular hotspots for integration or recombination.

These experiments clearly show epigenetic gene silencing of the *ADE2* gene as directed by the RPS can occur in both yeast and plants.

3.6 Discussion

The study of a repetitive sequence from *P. hybrida* in the yeast *S. cerevisiae* has revealed certain similarities in the way cells respond to repetitive DNA. As suggested by the experiments on plasmids, but further validated by experiments with the RPS integrated into yeast chromosomes, I have demonstrated that the RPS could mediate silencing of an *ADE2* reporter gene in yeast cells. This is a clear example of an epigenetic event, which is separate from the chromosomal locus and is induced entirely by the RPS sequence itself. Since the mechanism of RPS-induced silencing in yeast remains elusive, the evidence from yeast in conjunction with data already generated in plants must be used to speculate upon its method of silencing.

There are some notable differences in RPS-mediated silencing between the two systems. In yeast, comparisons between silencing induced by the RPS on low-copy plasmids and high-copy plasmids show that the frequency of silencing is elevated when a single copy of the RPS is present (compare table 3.1 and table 3.2). This differs from previous findings in *Arabidopsis*, the RPS did not mediate gene silencing as in *Petunia* where multiple endogenous copies are present (ten Lohuis *et*

al., 1995; Müller *et al.*, 2002). Therefore, it was concluded that multiple endogenous copies of the RPS were a pre-requisite for silencing. However, this result was not reproduced in yeast.

The fact that low-copy number plasmids show enhanced silencing in yeast may indicate the role for a low abundance factor in silencing. Multiple copies of the RPS provided by the high copy-number plasmid may dilute this factor out; thus, the incidence of silencing is reduced. The identity of the yeast factor possibly involved in this silencing remains unknown. The *A. thaliana* protein, BP1, a putative homologue of the TUP1 co-repressor, may be involved in RPS-mediated silencing by virtue of the fact it can bind the sequence. When expressed in yeast, BP1 had no effect upon chromosomal RPS-mediated silencing (fig. 3.25). However, the inability of BP1 to influence gene silencing in yeast could be due to sequence divergence throughout evolution, which prevents BP1 from interacting with the yeast transcriptional machinery to repress gene expression. Therefore, the finding that BP1 has no role in yeast does not exclude BP1 from being involved in RPS silencing in plants. However, in *Arabidopsis* where BP1 is natively expressed, no expression variegation of a reporter gene was noted implying that BP1 may not be involved in RPS mediated repression (Müller *et al.*, 2002). Since BP1 appears to be functionally redundant in *S. cerevisiae*, its putative yeast homologue, the transcriptional co-repressor TUP1 was deleted to observe its role in RPS-induced gene silencing (section 3.5.4). My findings show that TUP1 has no effect on RPS-mediated silencing in yeast. This again may indicate separate mechanisms of silencing in yeast and plants.

In addition to TUP1, BP1 has significant homology to the *S. cerevisiae* gene *ARC41*. It would have been beneficial to the study to knock out this gene and observe the effects, particularly since actin-related proteins have been implicated in epigenetic gene regulation (Jiang and Stillman, 1996). However, deletion of this gene is lethal in yeast. If BP1 is related to actin proteins it perhaps functions to silence the RPS by tethering the sequence at the nuclear periphery bringing it in contact with silencing complexes such as the SIR proteins (Palladino *et al.*, 1993). Experiments tagging the RPS sequence with GFP and determining its localisation within the nucleus would help resolve this hypothesis (for example see Straight *et al.*, 1996). Future experiments would attempt to elucidate the molecular mechanism of repression in yeast. Studies would include deletion of the SIR proteins, as these are involved in telomeric silencing and position effect variegation, making them possible candidates for RPS-induced silencing. Co-immunoprecipitation studies with the RPS would also establish which proteins bind to the RPS sequence in yeast and may contribute to the deduction of the molecular mechanism of RPS-induced silencing.

My experiments, consistent with findings in *Arabidopsis* and *Petunia* (ten Lohuis *et al.*, 1995; Müller *et al.*, 2002), confirm that DNA methylation is not essential for RPS-induced silencing. The *S. cerevisiae* genome contains no cytosine methylation and yet the RPS was shown to silence a reporter gene. In its native species *P. hybrida*, methylation is perhaps a secondary epigenetic mark of a silent locus, which is essential in organisms with a more complex genome than *S. cerevisiae*. The methylation-independent manner of RPS silencing is in parallel with other findings. For example, Amedeo *et al* (2000) showed that removal of DNA methylation from a

silenced locus was not sufficient to allow gene expression suggesting that DNA methylation is not the primary effecter of gene silencing. However, this is not seen in all cases and removal of DNA methylation can lead to the expression of previously silent genes (Miura *et al.*, 2001). These findings suggest the role of DNA methylation in gene silencing may be context and situation dependent.

Thus, the exact mechanism of RPS-induced silencing remains unknown. Since methylation is not pivotal to its mechanism, the role of chromatin structure is implicated. The RPS may nucleate an altered chromatin structure by virtue of its repeats or by interacting with specific proteins that allow the formation of silent chromatin. This state is likely to spread to the adjacent reporter gene, causing position effect variegation. The argument for position effect variegation is supported by the stochastic nature of the silencing observed in yeast. For example, although all *ADE2* and RPS constructs were at the same locus, some *ADE2* genes are silent and some are not, with other sectoring colonies confirming *ADE2* expression variegation. One might hypothesise that the variegation occurs because the reporter gene has adopted an altered, heterochromatic-like state. The appearance of variegating colonies reinforces this since the extent of heterochromatin-like structure spreading, joined with epigenetic inheritance patterns and would result in this variegating phenotype. Future experiments would explore the role of chromatin structure in RPS-mediated silencing. It would be interesting to determine the histone modifications found at the RPS and repressed gene to see if they are consistent with the histone code of silent chromatin. Chromatin immunoprecipitations (ChIP) would reveal if there was extensive hypoacetylation at the silenced locus, which would implicate the

role of histone deacetylases (HDACs) in RPS-induced silencing. It would also be beneficial to look at histone methylation in a similar manner to observe if residues such as lysine-79 are hypomethylated, as is found at silent loci in *S. cerevisiae* (Ng *et al.*, 2003). This would implicate SIR proteins in silencing (van Leeuwen *et al.*, 2002) and help elucidate the mechanism of silencing.

My results also reveal differences in the silencing capacity of the RPS in relation to its orientation to the reporter gene. As shown by the plasmid experiments orientations 2 and 3 gave similar levels of gene silencing whilst levels of silencing in orientation 4 were consistently lower (tables 3.1 and 3.2). However, this situation was reversed when the constructs were integrated into the genome, with all orientations giving comparable levels of pink colonies except for construct 4, which gave a substantially higher number of pink colonies (table 3.3). These apparent discrepancies in findings between plasmid and chromosomal silencing may be explained if for orientation 4 plasmids, the *TRP1* marker gene may also have been silenced by the RPS. This theory would account for the differences since colonies with a silenced *TRP1* marker gene would be lost or out-competed by white colonies on the particular selective medium. If indeed, in plasmids bearing the orientation 4 construct the *TRP1* and *ADE2* genes are silenced, it implies the RPS has the potential to silence in both directions. However, this finding was not observed in orientation 2, which has the same orientation of the RPS as construct 4. One might hypothesise that to achieve this type of silencing observed for orientation 4, the RPS must be in close proximity to the reporter gene promoter where there is some reinforcing function. However, as shown by the plasmid and integration experiments, the promoter of the

reporter gene does not have to be directly adjacent to the RPS to allow gene silencing as is found for orientations 1 and 2. The reason why orientation 4 should have a higher incidence of silencing compared to the others is unclear; perhaps the promoter being in close proximity to the inverted repeats has some influence in gene silencing.

Although homologous recombination experiments always show a background of wrongly integrated sequences, the fact that the RPS and *ADE2* constructs were found incorrectly integrated at a high frequency may give some understanding of the evolutionary origins of the RPS sequence. When selecting for transformants based on SC-ade medium, one wrongly inserted sequence was found (fig. 3.15). No homologies between the locus where the sequences were found and the integrating construct could be identified, implying that the sequence was directed there by non-homologous recombination. Further experiments with the FY2 strain also showed the presence of misplaced sequences (fig. 3.23), although the position of these sequences was never identified for this strain. In both strains, Southern blot analysis revealed that the RPS and *ADE2* sequences were found intact elsewhere within the genome, which suggests that these sequences were not subject to internal recombination events.

In order for colonies to grow on the SC-ura selective medium used in the FY2 strain experiments, the functional *URA3* gene transformed into yeast must recombine with the *ura3-52* allele at the endogenous *ura3-52* locus to generate a functional *URA3* gene (see fig. 3.20). The construct transformed into yeast could not support the growth of cells on SC-ura medium without this recombination event occurring.

Therefore, for the *ADE2* and RPS sequences to be found intact but elsewhere in the genome one could hypothesise that either the locus underwent further recombination leaving the RPS and *ADE2* sequences intact at the ura3-52 locus. Indeed the RPS may promote illegitimate recombination as palindromic sequences have the potential to carry out non-homologous recombination with other palindromic sequences (Müller *et al.*, 1999). This recombination may affect the *ADE2* gene giving rise to pink colonies. However, this could not be a gross recombination of the *ADE2* gene as frequently the full length gene was identified by Southern blotting. The red colonies generated by this type of scenario could be distinguished from silenced colonies by serial plating of the sectored colonies. If the red colour was attributable to epigenetic silencing we would expect to see reversibility of the state, whereas for colonies that have undergone recombination the red colour would never change as the *ADE2* gene has been irreversibly altered. A further possibility for the unexpected banding pattern found at the *ura3-52* locus is that the transformed sequences originally went to the *ura3-52* locus and then translocated to a separate locus.

The only group of DNA sequences with the ability to do this are transposable elements (TE). A putative ORF search of the RPS sequence revealed it had the potential to synthesise a 61 amino acid peptide. This protein shares homology (50% identity) with proteins of *Ty* elements in *S. cerevisiae* (fig. 3.27), gag-pol proteins from TEs in other species and a transposase protein found in *A. thaliana*. Although the ORF could not encode a full-length gag-pol protein, it implies that part of the RPS sequence may have evolved from a transposable element that has been corrupted throughout evolution. Indeed, the RPS has direct and indirect repeats,

which are indicative of transposable elements. The RPS may retain the structure of a transposable element, which are potentially recognised by proteins produced by active *Ty* elements that may allow it to transpose with the *ADE2* gene. Indeed, disrupted non-functional elements can be reactivated by enzymes produced from active TEs (see Lewin, 2000). Perhaps the heavy methylation of the RPS in *P. hybrida* and *A. thaliana* is to prevent the activation of the transposable element, as abolishing DNA methylation in Arabidopsis causes reactivation of transposons (Miura *et al.*, 2001). The fact that all colonies containing misplaced inserts are pink may be explained by the fact that transposable elements are known induce the repression of adjacent sequences (Cambereri *et al.*, 1996), which may explain the silencing effects of the RPS. This is a very primitive hypothesis and would have to be confirmed by a series of experiments to prove the RPS is a mobile element. Transposon display has been previously used in *P. hybrida* to discover integration events by TEs and would help to confirm if the RPS was capable of this in its native species (Van den Broek *et al.*, 1998; De Keukeleire *et al.*, 2001). A series of inverse PCR and Southern blots in *S. cerevisiae* would determine the exact nature of the recombination events at both the *ura3-52* locus and the sites of integration of the RPS and *ADE2* sequences and help to ascertain if the RPS is a mobile genetic element.

Of course, the possibility cannot be excluded that the rearrangements at the *URA3* locus activates the Ty1 element present in the *ura3-52* allele and that this is responsible for the transposition. This could be established by more inverse PCR and Southern blotting to confirm exactly what sequences are present at the new locus. It

is also feasible that some further recombination of the loci has occurred which causes the differences in expected lengths of bands identified. However, the fact that full-length *ADE2* and RPS sequences have been identified somewhat dismisses this idea.

The fact that all of the misplaced colonies are pink and potentially silenced cannot be simply explained. The possibility exists that some rearrangement of the *ADE2* gene occurred although the Southern blot confirming the full-length sequence somewhat dismisses this idea. The one locus that was identified, was not one of the typical *S. cerevisiae* silent loci although upstream of the region there were many δ-elements and tRNA genes (fig. 3.19), which Ty elements are known to favour as spots for integration (Boeke and Devine, 1998). Furthermore, δ-elements from Ty1 transposable elements are epigenetically regulated by the ACT3 protein (Jiang and Stillman, 1996). This is an actin-related protein similar to BP1, which may give some clues to the function of BP1.

Although the exact system of RPS-mediated gene silencing remains unknown, this study of the gene repressive properties of the sequence in yeast has demonstrated interesting features of its mechanism. A repetitive silencing sequence from *P. hybrida* was shown to repress genes in yeast in a plasmid at low frequencies and at higher frequencies in chromosomal contexts. It has been clearly shown that RPS-induced silencing can occur in yeast and that it is methylation independent. Further studies could look at histone modifications such as methylation and acetylation to see if a repressive chromatin code and structure has been introduced at the silenced locus. I have hypothesised on the possible evolutionary origin of the sequence as a

transposable element remnant. This would need to be confirmed by detailed studies in both *P. hybrida* and *S. cerevisiae*. This study has shown that there are many similarities and differences in how yeast and plants both recognise and respond to repetitive DNA.

```
14  YPQYDAMERN-PPAPRAE-SSYPQYDAMERNPPAPRAESSYPQYDAMEQN 61
    YP Y        PP  +++ + YPQY      N P+P + +S+P   + + N
112 YPPYQMSPMYAPPGAQSQFTQYPQYVGTHLNTPSPESGNSFPDSSSAKSN 161
```

15/50 positives = 30%
25/50 identities = 50%

Figure 3.27: Alignment of putative RPS protein and transposable element protein. The sequence of the putative RPS protein is shown in red and the yeast transposable element sequence is shown in blue. The numbers correspond to amino acid residues.

Chapter 4 - Function of TUP1 homologues in the yeast *Saccharomyces cerevisiae*

4.1 Introduction

Gene specific transcriptional repression plays a critical role in gene regulation. A well-characterised example is the WD repeat protein, TUP1, from *S. cerevisiae*. TUP1 functions with the adapter protein SSN6 to repress a wide-variety of genes, including the flocculation genes, glucose repression genes and oxidative stress genes. TUP1/SSN6 represses genes by organising an ordered, repressive nucleosomal array over promoters and by inhibiting the RNA polymerase II complex (reviewed by Smith and Johnson, 2000).

TUP1 acts as a prototype for a family of transcriptional repressors that includes, mammalian transducin-like enhancer of split proteins (TLEs), and Groucho (GRO) from Drosophila. Although these proteins are classified as a family on the basis of their C-terminal WD repeats they lack extensive sequence similarity out-with these motifs. The N-termini of these proteins is required for functions such as self-association (Chen *et al.*, 1998) and interaction with histones (Edmondson *et al.*, 1996; Palaparti *et al.*, 1997; Flores-Saaib and Courey, 2000). The divergent N-termini potentially form similar structures to facilitate these interactions, so despite the lack of sequence homology, there may be significant structural and functional homology between these proteins, suggesting a conserved mechanism of gene repression between species.

The parallels in the methods of action of these transcriptional repressors, and in particular their ability to interact with histones (Edmondson *et al.*, 1996; Palaparti *et al.*, 1997; Flores-Saaib and Courey, 2000) and histone deacetylases (Chen *et al.*, 1999; Watson *et al.*, 2000) suggest they influence chromatin structure to repress gene transcription. However, except in the case of TUP1, there are no studies showing chromatin remodelling activity associated with any of the other members of this family. Indeed, little is known about how this family of proteins exerts their effects upon transcription. Through our understanding of the mechanism of TUP1 action in yeast we can speculate and assess the activities of higher eukaryotic members of the family.

Putative members of the TUP1/GRO family have been identified in *Arabidopsis thaliana*. LEUNIG has significant homology to TUP1 but little is known about its method of repression (Conner and Liu, 2000). BP1 is another protein that was isolated from *A. thaliana*, which also contains seven WD repeats, suggesting it is another putative TUP1 homologue (P. Meyer, personal communication). BP1 differs from other homologues by having DNA binding activity. BP1 is implicated in the repression of repetitive sequences (P. Meyer, personal communication) and shares homology to TUP1 and the actin related protein (ARP) ARC41 from *S. cerevisiae*.

Previous studies have compared the functionality of TUP1 homologues between different species of yeast. A TUP1 homologue from *Candida albicans* repressed a *lacZ* reporter gene in *S. cerevisiae* (Braun and Johnson, 1997). The means of this repression were not established, so we cannot conclude that it behaves in the same

manner as TUP1 with respect to chromatin. In another series of experiments, a TUP1 homologue from *Schizosaccharomyces pombe* failed to repress genes in *S. cerevisiae*. It was thought to be due to sequence differences in the *S. pombe* SSN6 binding domain, which meant *S. pombe* TUP1 could not associate with *S. cerevisiae* SSN6 to allow repression (Mukai *et al.*, 1999). Surprisingly, the more evolutionary distant TLE1 from humans could form a functional interaction with *S. cerevisiae* SSN6 to repress genes in mammalian cells (Grbavec *et al.*, 1999).

In this study, I aim to establish whether functional orthologues of TUP1 (GROUCHO, LEUNIG, BP1, and CaTUP1) can work with the yeast transcriptional machinery to repress genes in a *S. cerevisiae tup1* mutant. Using the well-characterised nucleosome pattern at the TUP1-SSN6 dependent gene *FLO1* (Fleming and Pennings, 2001), I can observe if the foreign proteins can influence or re-establish this distinct local chromatin structure in a *tup1* mutant. This would give an insight into their mechanisms of repression, enabling deduction of how functionally related the proteins are, and ultimately help elucidate if there is a conserved pathway for gene repression.

4.2 Materials and Techniques for Study

To determine the action of TUP1 homologues in *S. cerevisiae*, each cDNA was cloned into a yeast expression vector (for details of cloning see table 6.5) and transformed into an *S. cerevisiae* strain (BY4742) with a *TUP1* gene deletion (*MATα, his3Δ1, leu2Δ0, lys2Δ0, tup1Δ0, ura3Δ0*). The vector chosen was p415-

MET25, which allows protein expression driven by the *MET25* promoter (fig. 4.1). TUP1 is thought to have no involvement in the regulation of this promoter. The vector allows inducible expression of genes depending on the media conditions (Mumberg *et al*., 1994). When cells are grown in SC-methionine medium they should express the protein, and when grown in SC+methionine (1mM methionine), expression from the *MET25* promoter should be shut off by feedback inhibition. This allows the use of the "off" conditions as a control. The vector used has a *CEN6* origin of replication, which means the plasmid is only present at 1-2 copies per cell. Endogenous *S. cerevisiae TUP1* DNA was cloned into these vectors as an additional control.
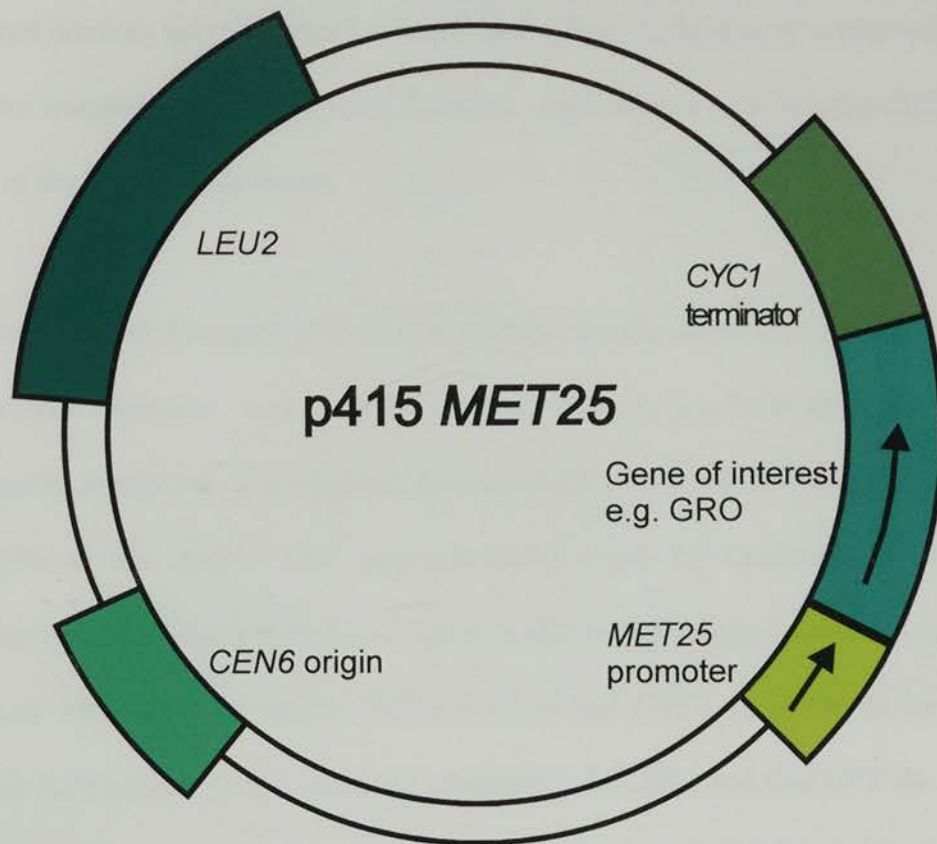
Figure 4.1: Features of the expression vector system p415 *MET25*. This plasmid contains a *LEU2* gene for selection and the CEN6 origin supports replication of around one plasmid per cell. The expression of cDNAs is driven by the *MET25* promoter (Mumberg *et al.*, 1994).

4.3 Confirmation of Homologue Transcription from p415-*MET25*

Northern blot analysis was performed on RNA extracts prepared from each of the strains containing homologues grown in either inducing or repressive conditions (fig. 4.2). Each was probed with the specific cDNA of the homologue to confirm that there was expression of the desired full-length RNA in the yeast cell. This in itself does not confirm that the protein was present. Western blotting of whole cell protein extracts would be more conclusive, however, antibodies are not readily available for most of the homologues tested.

The results show that each of the p415-*MET25* vectors transcribes the mRNA of the homologue. However, there is some residual transcription from cells grown under repressive conditions. This suggests the repression of the *MET25* promoter is not as stringent as was hoped. This was previously noted by Mumberg *et al* (1994). Furthermore, the repression of some genes under non-inducing conditions appears to be more efficient than others. Sc*TUP1*, *LUG* and *GRO*, all show an increase in mRNA transcription under inducing conditions, but *BP1* and Ca*TUP1* do not. The reasons for this are unclear but may be due to differences in RNA stabilities.
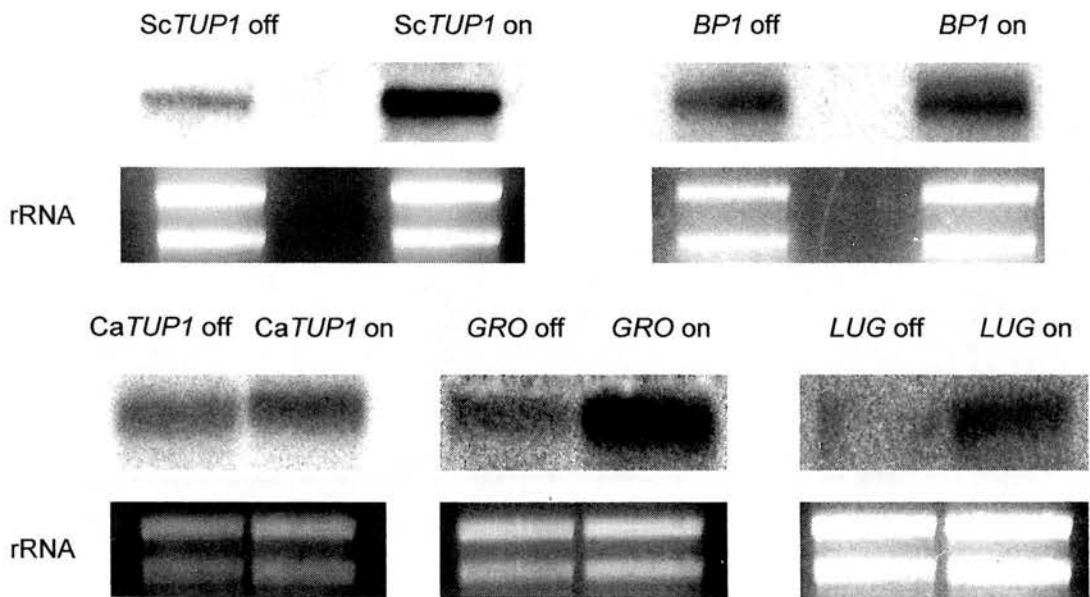
Figure 4.2: Northern blots showing transcription of TUP1 homologues from the p415-*MET25* promoter. Separate Northern blots were carried out on total RNA samples from yeast transformed with vectors containing the cDNA of each homologue. These were probed with the specific cDNA of the appropriate homologue. "Off" refers to yeast grown in SC+methionine medium, and "on" refers to yeast grown in SC-methionine medium. The ethidium bromide stained rRNA bands are shown as a loading control.

4.4 Action of Homologues at TUP1-Regulated Genes

4.4.1 Effects of Homologues on Glucose Repression

Glucose repression is a universal mechanism also found in *S. cerevisiae* whereby carbon energy sources are utilised in the most energetically favourable manner. When glucose is present, the need to metabolise other carbon sources, such as galactose or sucrose, is superfluous. In yeast, many of the genes for uptake and metabolism of other carbon sources can be repressed by TUP1-SSN6 under these conditions. Mutations in either TUP1 or SSN6 cause constitutive derepression of these genes in the presence of glucose (Trumbly, 1992). I examined the glucose repression function of yeast expressing various TUP1-homologues to investigate if they could repress genes in the same manner as TUP1.

This was achieved by a plate assay whereby cells are challenged to grow on medium containing galactose and a non-metabolisable glucose analogue, 2-deoxyglucose (2-DG). In a wild type strain, where glucose repression is functional, galactose uptake is inhibited, cells are unable to metabolise 2-DG and do not grow. However, if glucose repression is abrogated, as in the case of *tup1* mutants, galactose uptake and utilisation will occur in the presence of 2-DG and the cells are able to grow.

Yeast expressing the homologues were plated onto medium containing 2-DG and galactose with an additional galactose-only plate as a control. Wild type and *tup1* strains were included as a reference (fig. 4.3). The plates clearly show growth of *tup1*

mutants and no growth of wild type cells as expected. The control experiment, with *S. cerevisiae TUP1* supplied on a plasmid, shows no cell growth indicating that the plasmid does not effect the outcome of this experiment. No growth on 2-DG was also noted with yeast expressing *TUP1* from *C. albicans*, indicating that it complements a *tup1* mutation and represses genes in the glucose repression pathway. Yeast cells expressing *GROUCHO*, *LEUNIG* and *BP1* all show growth on 2-DG medium. This suggests that, like a *tup1* mutant, they cannot repress genes involved in glucose repression and do not function to the same extent as TUP1.
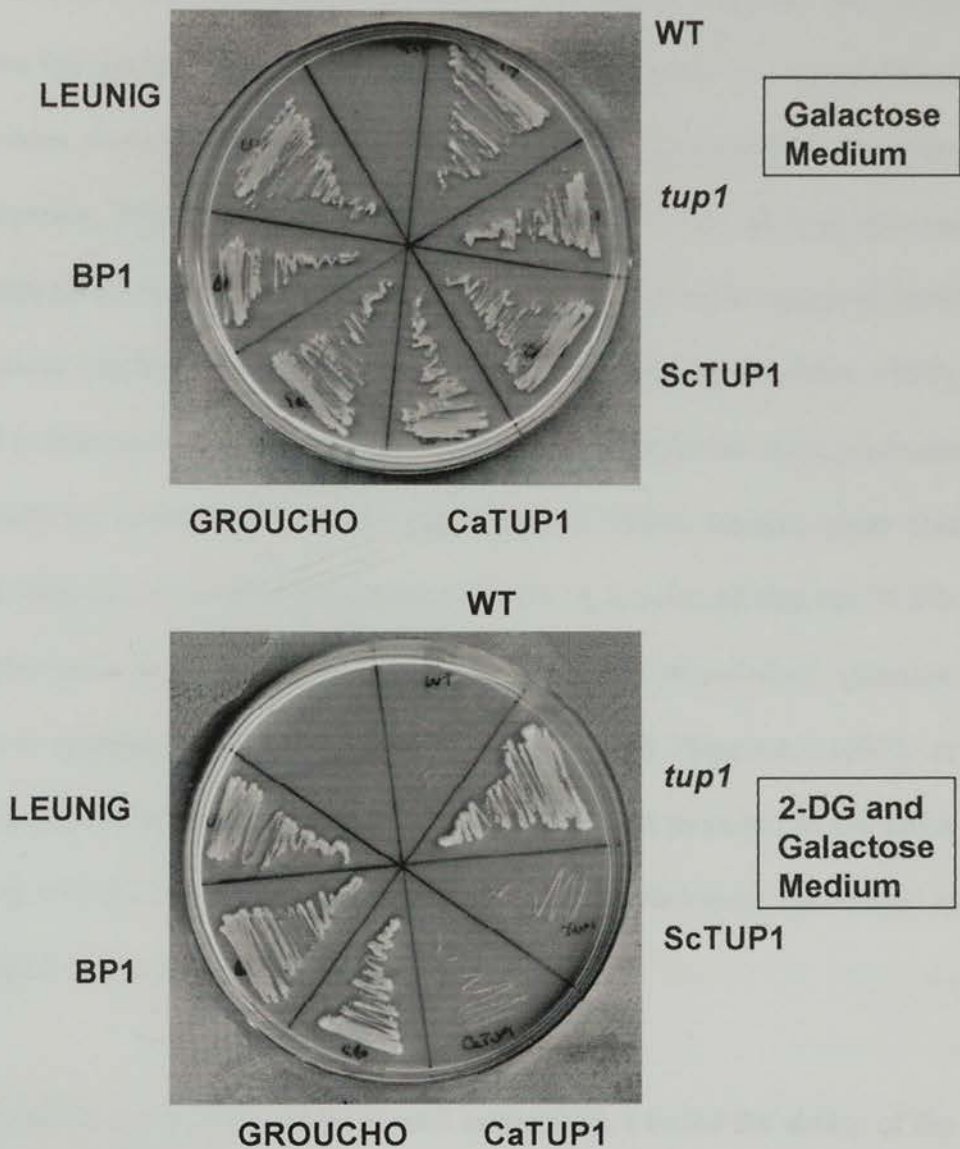
Figure 4.3: Growth of cells expressing TUP1 homologues on 2-deoxyglucose (2-DG) medium. As shown all cells can grow on the control galactose only plate. Wild type cells, cells expressing *S. cerevisiae TUP1* (ScTUP1) and cells expressing *C. albicans TUP1* (CaTUP1) all fail to grow on medium containing 2-DG and galactose indicating that their glucose repression pathways are intact.

4.4.2 Activity at the *SUC2* Promoter

To further confirm the results generated from the 2-DG plate assay in a more quantitative manner, the *SUC2* gene was selected for further analysis. The *SUC2* gene encodes the enzyme invertase, which hydrolyses sucrose to its constituent monosaccharides, fructose and glucose. *SUC2* is subject to TUP1-SSN6 dependent glucose repression. When yeast cells are grown in the presence of high glucose concentrations, *SUC2* transcription is repressed by TUP1-SSN6. Mutations in *SSN6* and *TUP1* cause constitutive derepression of *SUC2* (reviewed by Trumbly, 1992). When *SUC2* is repressed, there is an ordered chromatin conformation at the promoter region (Hirschhorn *et al.*, 1992; Gavin and Simpson, 1997). Studies show this nucleosomal array is disrupted by mutations in *TUP1*. It is believed that the TUP1-SSN6 complex acts by blocking the activating SWI-SNF remodelling complex, maintaining a repressive chromatin structure (Gavin and Simpson, 1997). A schematic representation is shown in fig. 4.4. TUP1-SSN6 is recruited to the DNA by associating with the zinc finger protein MIG1 and its homologues (Matallana *et al.*, 1992; Treitel and Carlson, 1995).

Since *SUC2* regulation by TUP1-SSN6 is well established, I tested the ability of the homologues to repress the *SUC2* gene in a *tup1* knockout strain of *S. cerevisiae*. Northern blot analysis was performed to measure the amount of *SUC2* mRNA being produced by yeast expressing the homologues grown in the presence of glucose (fig. 4.5). RNA was prepared from yeast grown in medium that induces expression of the homologues and medium that does not. This is indicated by "on" and "off" in fig.

4.5, respectively. The probes used for the analysis were SucPr, specific for *SUC2*, and ActPr, specific for *ACT1* mRNA (see table 6.7). *ACT1* was used as a loading control and RNA was quantitated by 2D densitometry using a phosphorimager. The *SUC2* mRNA transcription is presented as a percentage relative to a *tup1* mutant, which will have an active *SUC2* gene (100% *SUC2* transcription).

The results confirm that in wild type cells that express TUP1 and in which glucose repression is intact, *SUC2* transcription has decreased approximately 16 fold in relation to a *tup1* mutant. Although there is a dramatic difference between the amounts of *SUC2* mRNA, there is still a basal level of *SUC2* transcription in wild type cells. The control, with *S. cerevisiae TUP1* supplied on the p415-*MET25* plasmid, mirrors these results. When *TUP1* is expressed from the plasmid (Sc*TUP1* on), there is a decrease in *SUC2* transcription compared to the *tup1* mutant. This decrease is not as significant as in wild type cells but this may be due to the different expression patterns imposed by expression from a plasmid. When *TUP1* is supposedly not expressed from the plasmid (Sc*TUP1* off) there is still repression of the *SUC2* gene. This is due to the inefficient regulation of the *MET25* promoter, which allows expression of the homologues under non-inducing conditions, as observed in section 4.3.

The leakiness of the *MET25* promoter must be taken into consideration when looking at the results for *TUP1* from *C. albicans*. There is substantially more *SUC2* transcription from yeast expressing Ca*TUP1* (Ca*TUP1* on) than a wild type cell, although still less than a *tup1* mutant. Plasmid-borne *S. cerevisiae* TUP1 experiments

(Sc*TUP1* off and Sc*TUP1* on) reveal a 2-fold decrease in *SUC2* transcription between off and on states. We also see a 2-fold decrease in *SUC2* transcription between off and on states with *C. albicans TUP1* (compare Ca*TUP1* on and Ca*TUP1* off), this shows that the *C. albicans* TUP1 can repress the *SUC2* gene in *S. cerevisiae*. This is consistent with the plate assay (fig. 4.3) showing that *C. albicans* TUP1 can restore glucose repression in an *S. cerevisiae tup1* mutant.

The other, less conserved, homologues: GROUCHO, BP1, and LEUNIG do not repress *SUC2* transcription confirming their inability to restore glucose repression. These results suggest that these proteins do not function in the same manner as TUP1 with respect to the *SUC2* gene. Interestingly, GROUCHO showed an almost 2 fold increase in *SUC2* transcription when it was expressed from the plasmid (GRO on) compared to when GROUCHO expression was repressed (GRO off). This would suggest that GROUCHO, either directly or indirectly, has an activating effect on *SUC2* transcription. Ideally, this experiment would be repeated to ascertain the validity of this result.

SUC2 Repression
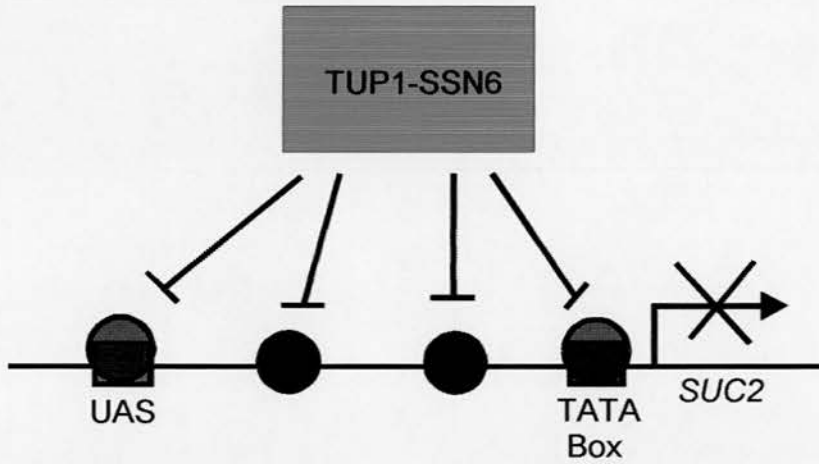
SUC2 Activation
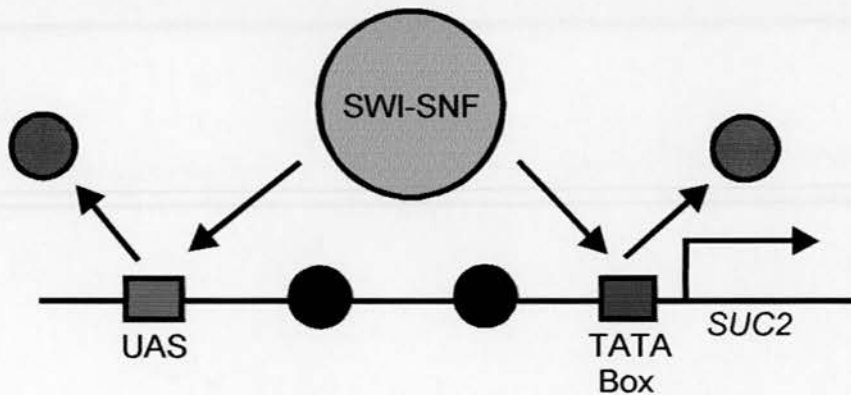
Figure 4.4: A schematic representation of the activation and repression of the *SUC2* gene. Nucleosomes are represented by black or grey circles. Grey nucleosomes are those which become mobilised during chromatin remodelling. UAS is an abbreviation for upstream activating sequence. TUP1-SSN6 is responsible for the maintenance of a repressive chromatin structure, where nucleosomes occlude the UAS and TATA box, preventing regulatory proteins from binding. Upon activation by SWI-SNF, the nucleosomes positioned on the TATA box and UAS are remodelled, and these sites become available for other proteins to bind allowing gene activation.
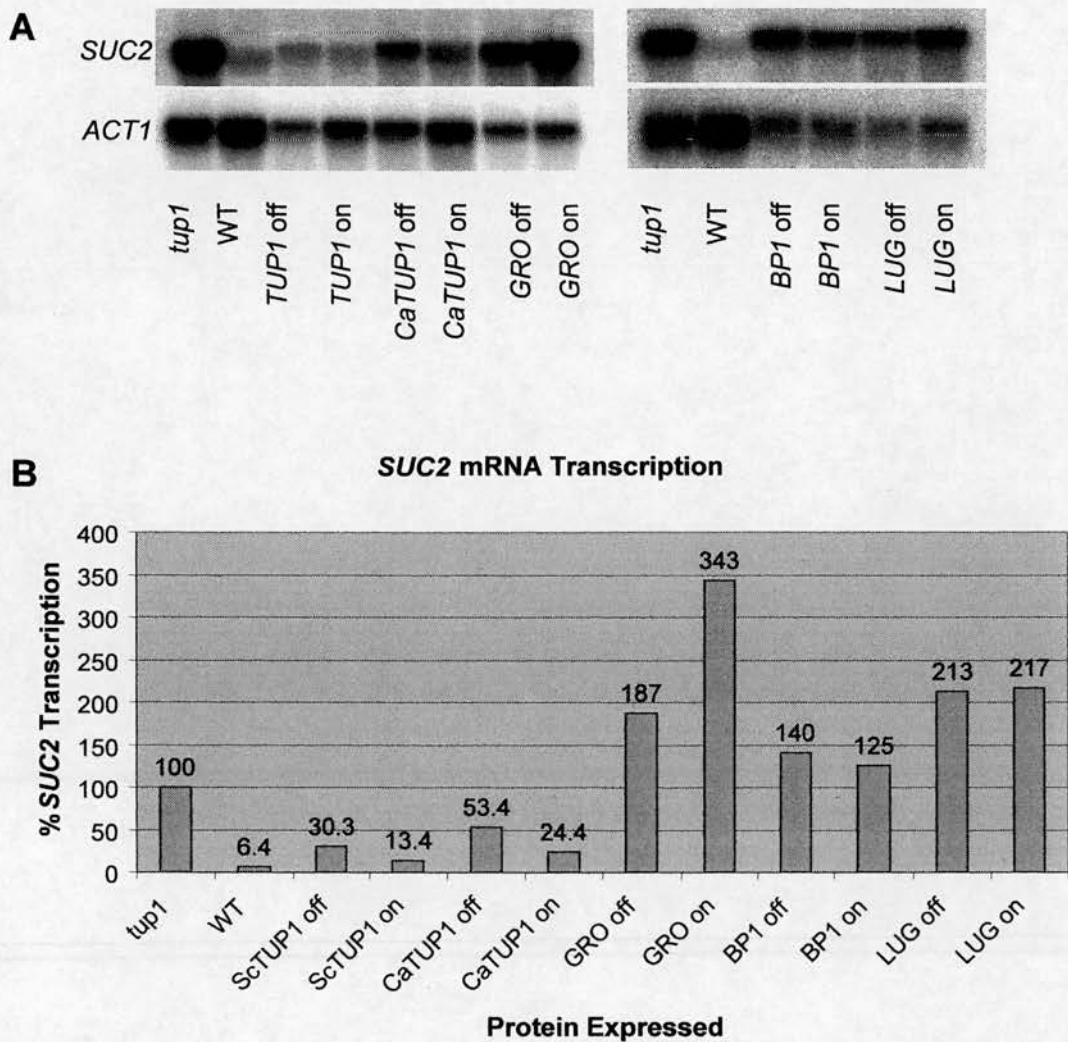
Figure 4.5: Northern blot analysis of *SUC2* transcription as affected by TUP1 homologues. "Off" denotes yeast grown in non-inducing medium, with respect to homologue expression. "On" denotes cells grown in inducing medium. All yeast were grown in medium containing 2% glucose as the carbon source. Sc*TUP1* and Ca*TUP1* refer to *TUP1* genes from *S. cerevisiae* and *C. albicans* respectively. (**A**) shows the Northern blot on which, *SUC2* mRNA was visualised using a DNA probe (SucPr). The blot was stripped and re-probed for *ACT1* mRNA as a loading control, using a DNA probe specific for *ACT1* (ActPr). (**B**) shows levels of SUC2 mRNA transcribed as a percentage of the *tup1* mutant normailised against the *ACT1* signal. This was determined by 2D densitometry. These results clearly show only *C. albicans* TUP1 functions to repress the *SUC2* gene in *S. cerevisiae*.

4.4.3 Activity at the *FLO1* Promoter

The *FLO1* gene encodes a lectin-like protein that is located on the cell wall. *FLO1* is the dominant member of a family of genes, which cause the flocculation phenotype (Miki *et al.*, 1982). Flocculation is a phenomenon where cells form clumps that result in their sedimentation in liquid medium. Yeast mutant for *tup1* or *ssn6* show this flocculation phenotype (Teunissen *et al.*, 1995). The *FLO1* gene is regulated by the TUP1-SSN6 and SWI-SNF chromatin-remodelling complexes (Fleming and Pennings, 2001). In a wild type strain, where the *FLO1* gene is not transcribed, the promoter region is occupied by five strongly positioned nucleosomes. However, when the SSN6-TUP1 complex is absent, the regular array of nucleosomes found at the *FLO1* promoter is completely disrupted (fig. 4.6). Furthermore, detailed nucleosome mapping analysis at *FLO1* shows that remodelling by TUP1-SSN6 and SWI-SNF extends up to 5 Kb upstream of the transcription start site (Fleming and Pennings, 2001). The DNA binding protein that recruits TUP1-SSN6 to the *FLO1* promoter remains unidentified.

TUP1 homologues were tested for their ability to repress the *FLO1* gene. The flocculation phenotype of cells was noted following overnight growth in SC-methionine medium, which induces expression of the homologues, or SC+methionine medium that should repress homologue expression. Flocculation is dependent upon Ca^{2+} ions; this can be used to distinguish flocculation from other processes that may induce cell clumps, such as mating or aberrant cell separation. Therefore, cells were harvested and resuspended in 250mM EDTA. If the cells

previously flocculating become dispersed in EDTA, then this is indicative of true flocculation (table 4.1).

Homologue	Flocculation (-EDTA)	Flocculation (+EDTA)
tup1 knockout	+	-
Wild Type	-	-
Sc*TUP1* off	+	-
Sc*TUP1* on	-	-
Ca*TUP1* off	+	-
Ca*TUP1* on	-	-
GRO off	+	-
GRO on	+	-
BP1 off	+	-
BP1 on	+	-
LUG off	+	-
LUG on	+	-

Table 4.1: Presence (+) or absence (-) of the flocculation phenotype in yeast cells expressing TUP1 homologues. Sc*TUP1* and Ca*TUP1* denote *TUP1* genes from *Saccharomyces cerevisiae* and *Candida albicans* respectively, GRO denotes GROUCHO and LUG denotes LEUNIG.

The results show that, as expected, *tup1* mutants display a flocculation phenotype and wild type cells do not. In all cases the flocculation phenotype was lost upon

resuspension in EDTA, proving that the cell clumps observed were true flocculation. Replacement of endogenous *TUP1* with *S. cerevisiae TUP1* or *C. albicans TUP1* on the p415-*MET25* plasmid causes cells to lose the flocculation phenotype. This indicates that both proteins are functional at repressing *FLO1* gene transcription. Yeast expressing GROUCHO, BP1, and LEUNIG all retain the flocculation phenotype of a *tup1* cell, suggesting they do not repress the *FLO1* gene. However, as there is a family of genes contributing to the flocculation phenotype, *FLO1* gene transcription was analysed specifically by Northern blotting.

Northern blot analysis was carried out on total RNA samples from yeast expressing each homologue (grown in inducing medium) and from yeast where homologue expression should be repressed (grown in non-inducing medium). The *FLO1* and *ACT1* mRNA were specifically probed using probes FloPr and ActPr respectively (table 6.7). *ACT1* mRNA was used as a loading control and the RNA was quantitated by a phosphorimager using 2D densitometry. The results are shown in fig. 4.7 and are presented as *FLO1* mRNA transcription normalised against *ACT1* mRNA, as a percentage relative to a *tup1* mutant, which will have an active *FLO1* gene (100%).

Northern blot analysis confirms that the *FLO1* gene is actively transcribed in *tup1* cells and repressed in wild type cells, although a basal level of *FLO1* transcription remains. When *TUP1* from *S. cerevisiae* is transcribed from the p415-MET25 plasmid, *FLO1* transcription is repressed (Sc*TUP1* on). When expression of *S. cerevisiae* TUP1 is repressed by growth on non-inducing medium (Sc*TUP1* off), the levels of *FLO1* transcription remain more comparable to wild type cells than *tup1*

cells, which is a consequence of the inefficient regulation of the p415-*MET25* promoter. This leaky repression can also be seen when LEUNIG and CaTUP1 expression is switched off (Ca*TUP1* off and *LUG* off).

TUP1 from *C. albicans* shows *FLO1* mRNA levels of 8% compared to 5.7% in wild type *S. cerevisiae* cells (Ca*TUP1* on). This indicates that *C. albicans TUP1* can complement a *tup1* mutation and repress the *S. cerevisiae FLO1* gene. Similar repression was observed by LEUNIG, where *FLO1* transcription was also repressed (*LUG* on). This implies that the flocculation observed in yeast cells expressing LEUNIG was a consequence of the other genes involved in flocculation (Table 4.1).

Expression of GROUCHO and BP1 do not result in a decrease in *FLO1* mRNA, and are comparable to the levels of an *S. cerevisiae tup1* mutant (*GRO* on and *BP1* on), suggesting they do not repress the *S. cerevisiae FLO1* gene. However, when transcription from the p415-*MET25* promoter is repressed, although we know there is residual expression, *FLO1* mRNA levels are lower, suggesting a more complicated mechanism of action. These experiments would need to be replicated to ensure the validity of these results.

FLO1 Repression
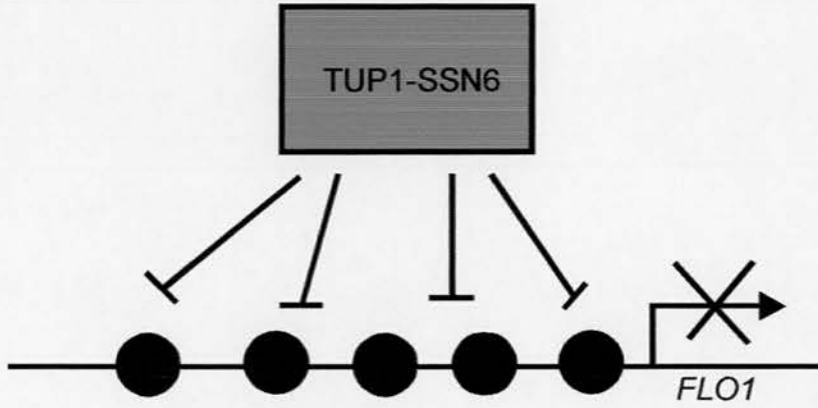
FLO1 Activation
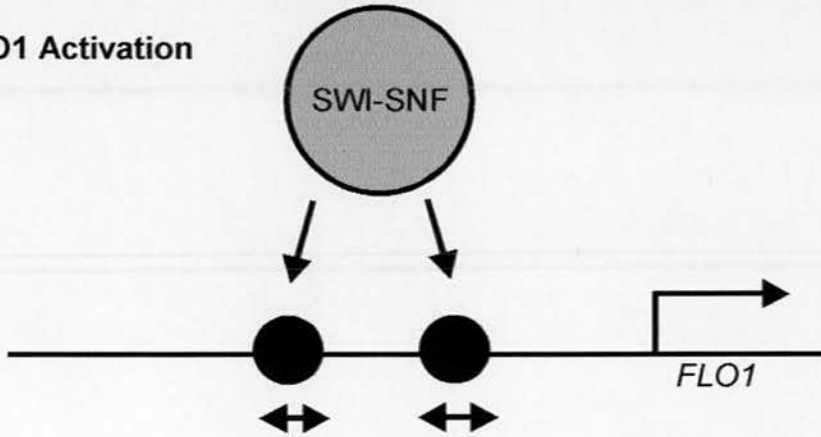
Figure 4.6: A schematic representation of the activation and repression of the *FLO1* promoter. Nucleosomes are indicated by black circles. *FLO1* repression is mediated by the TUP1-SSN6 complex, which maintains a regular array of nucleosomes over the *FLO1* promoter, potentially masking DNA binding sites for regulatory proteins. Upon activation by SWI-SNF, nucleosomes become remodelled; some are lost from the promoter, but others are more mobile (indicated by arrows), occupying different sites. The regular array of nucleosomes is disrupted and transcription can occur.

A

FLO1 mRNA Transcription

B

Protein Expressed
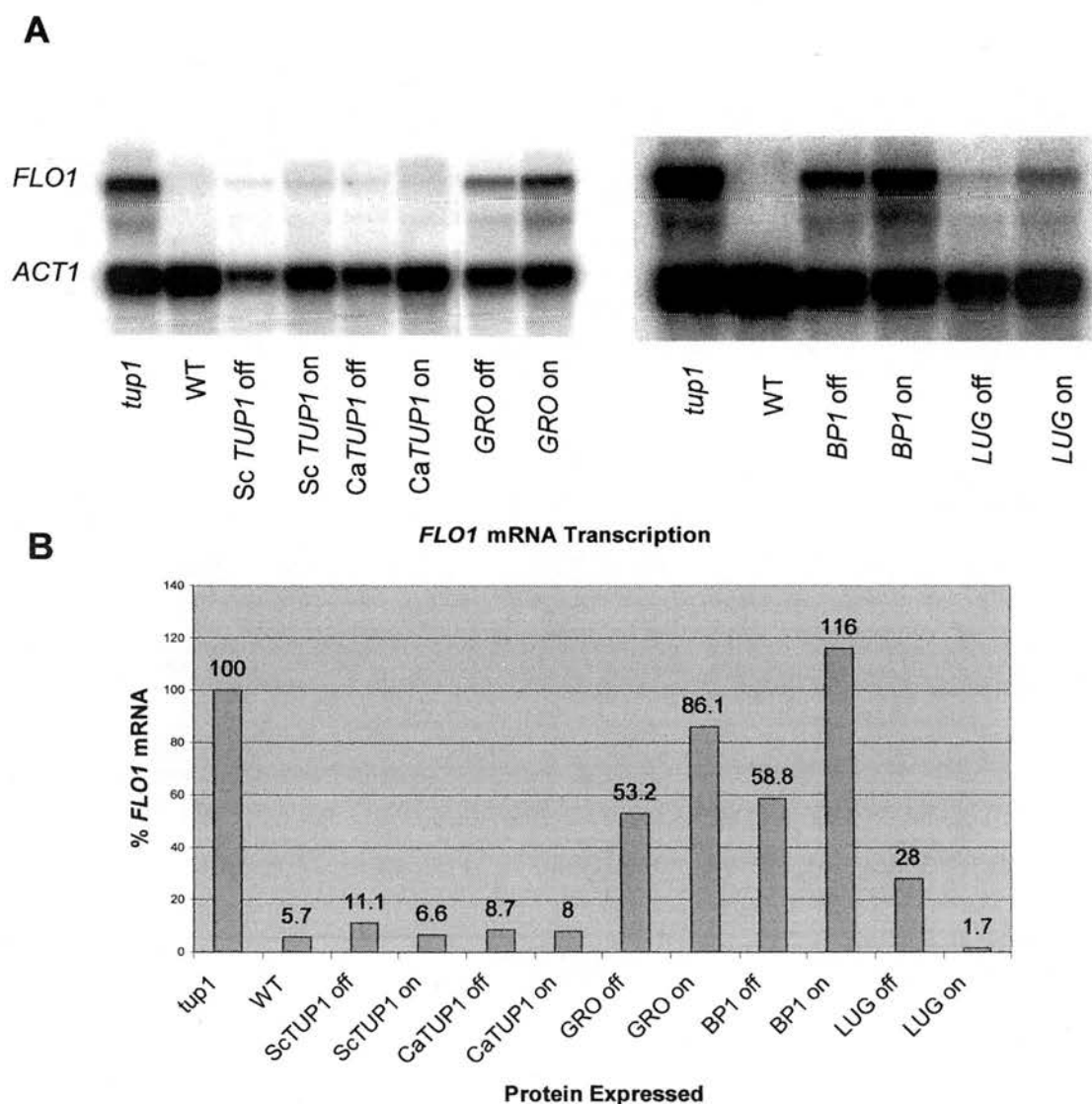
Figure 4.7: Northern blot analysis of *FLO1* transcription as affected by TUP1 homologues. "Off" denotes yeast grown in non-inducing medium, with respect to homologue expression. "On" denotes cells grown in inducing medium. Sc*TUP1* and Ca*TUP1* indicate *TUP1* genes from *S. cerevisiae* and *C. albicans* respectively. (**A**) shows the Northern blot on which, *FLO1* mRNA was visualised using a DNA probe (FloPr). *ACT1* mRNA as a loading control, using a DNA probe specific for *ACT1* (ActPr). (**B**) shows levels of *FLO1* mRNA transcribed as a percentage of the *tup1* mutant normalised against the ACT1 signal. This was determined by 2D densitometry. These results show LEUNIG and *C. albicans* TUP1 function to repress the *FLO1* gene in *S. cerevisiae*.

4.5 Activity of Homologues on Chromatin

4.5.1 Chromatin at the *FLO1* Promoter

Little is known about the actual mechanism of repression of BP1, CaTUP1, and LEUNIG, but because they share homology with *S. cerevisiae* TUP1, one could hypothesise that these proteins may share the chromatin-remodelling activity of TUP1. This was tested by examining chromatin at the TUP1-SSN6 regulated *FLO1* gene. Micrococcal nuclease digestion and indirect-end labelling on nuclei and naked DNA controls were employed to observe the nucleosomal array at the *FLO1* promoter in *tup1* mutants and wild-type cells. The region was probed (using probe FloPr2, see table 6.7) relative to a *Dra I* restriction site approximately 1 Kb upstream of the *FLO1* transcription start site. A representation of the indirect end labelling strategy is shown in fig. 4.8.

In wild-type cells where the *FLO1* gene is repressed, an ordered nucleosome array was evident (fig. 4.9 A). The DNA protection pattern from the micrococcal digest reveals five strongly positioned nucleosomes, which could obscure DNA binding sites for activating transcription factors. In contrast, the *tup1* mutant, which expresses *FLO1*, exhibits less defined cleavage sites and increased smearing between bands (fig. 4.9 B). This demonstrates the loss of the ordered nucleosomal array that correlates with gene activation. These differences in the nucleosome pattern at the *FLO1* promoter clearly implicate the TUP1-SSN6 complex in chromatin remodelling in accordance with previous studies (Fleming and Pennings, 2001).

This knowledge of nucleosome positions in wild type and mutant cells was applied to yeast cells expressing *C. albicans* TUP1, BP1 and LEUNIG to determine their effects on nucleosomes positioning at the *FLO1* promoter. Indirect end-labelling analyses were carried out (fig. 4.10). The chromatin analysis reveals that *C. albicans* TUP1 restores the nucleosomal pattern to the wild-type array. This is consistent with earlier findings that CaTUP1 represses TUP1 regulated genes in *S. cerevisiae* (Braun and Johnson, 1997). BP1 and LEUNIG show a smeared pattern at the *FLO1* promoter that is more associated with the *tup1* mutant. In the instance of BP1, this correlates with Northern blot analysis showing that BP1 does not repress the *FLO1* gene. However, LEUNIG also showed a smeared nucleosome pattern consistent with the *tup1* mutant despite Northern blot analysis revealing the *FLO1* gene was repressed. This suggests that LEUNIG may have another mechanism of repressing the *FLO1* gene other than chromatin remodelling, possibly by interaction with the RNA polymerase complex.
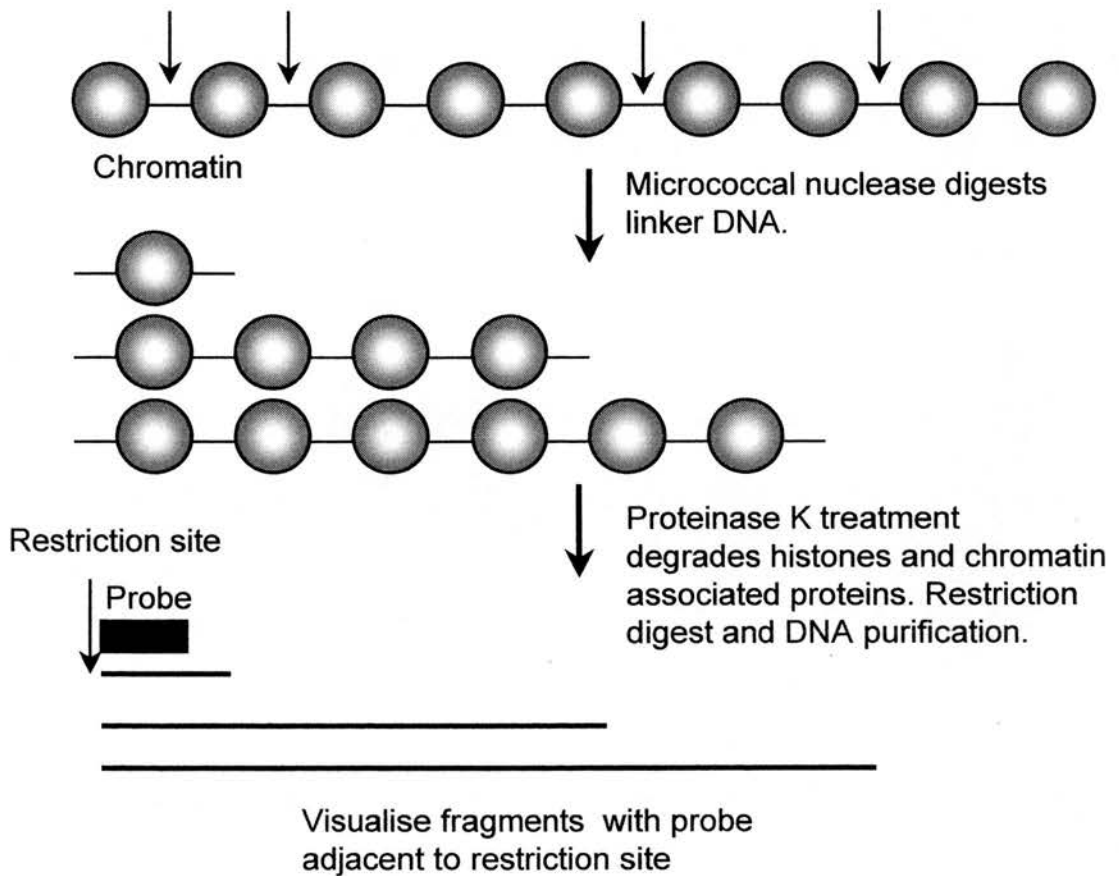
Figure 4.8: The principles of indirect end labelling. Chromatin is digested with micrococcal nuclease, which cuts DNA between nucleosomes. This generates different sized fragments with respect to time and the nucleosome pattern. Protein is removed from the extract, the DNA purified and digested with an appropriate restriction enzyme. The DNA fragment sizes are visualised using a probe adjacent to the restriction site in a manner similar to Southern blotting.
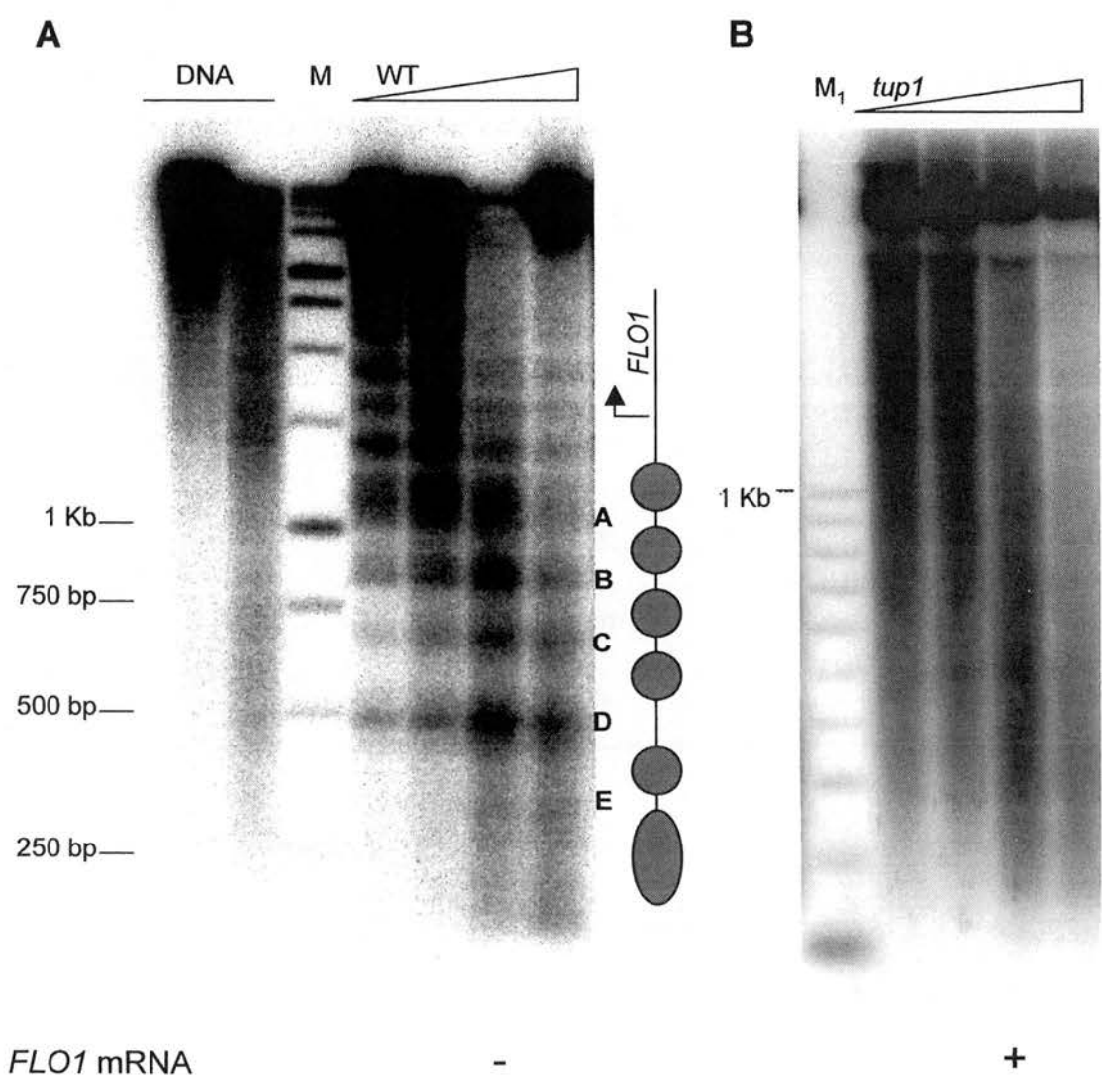
Figure 4.9: Indirect end labelling analysis of the *FLO1* promoter in wild type and *tup1* cells. In all cases M denotes marker, M_1 denotes a 100 bp ladder, and DNA is naked DNA, which was treated in the same manner as yeast chromatin. Triangles indicate increased time of microccocal digestion. (**A**) shows wild-type yeast chromatin with an ordered nucleosome array. Noted are nucleosome positions corresponding to sites protected from micrococcal nuclease digestion. These positions are consistent with previous findings at the *FLO1* promoter (Fleming and Pennings, 2001). (**B**) has a smeared pattern, which demonstrates loss of these positioned nucleosomes in a *tup1* mutant which allows expression of the *FLO1* gene.
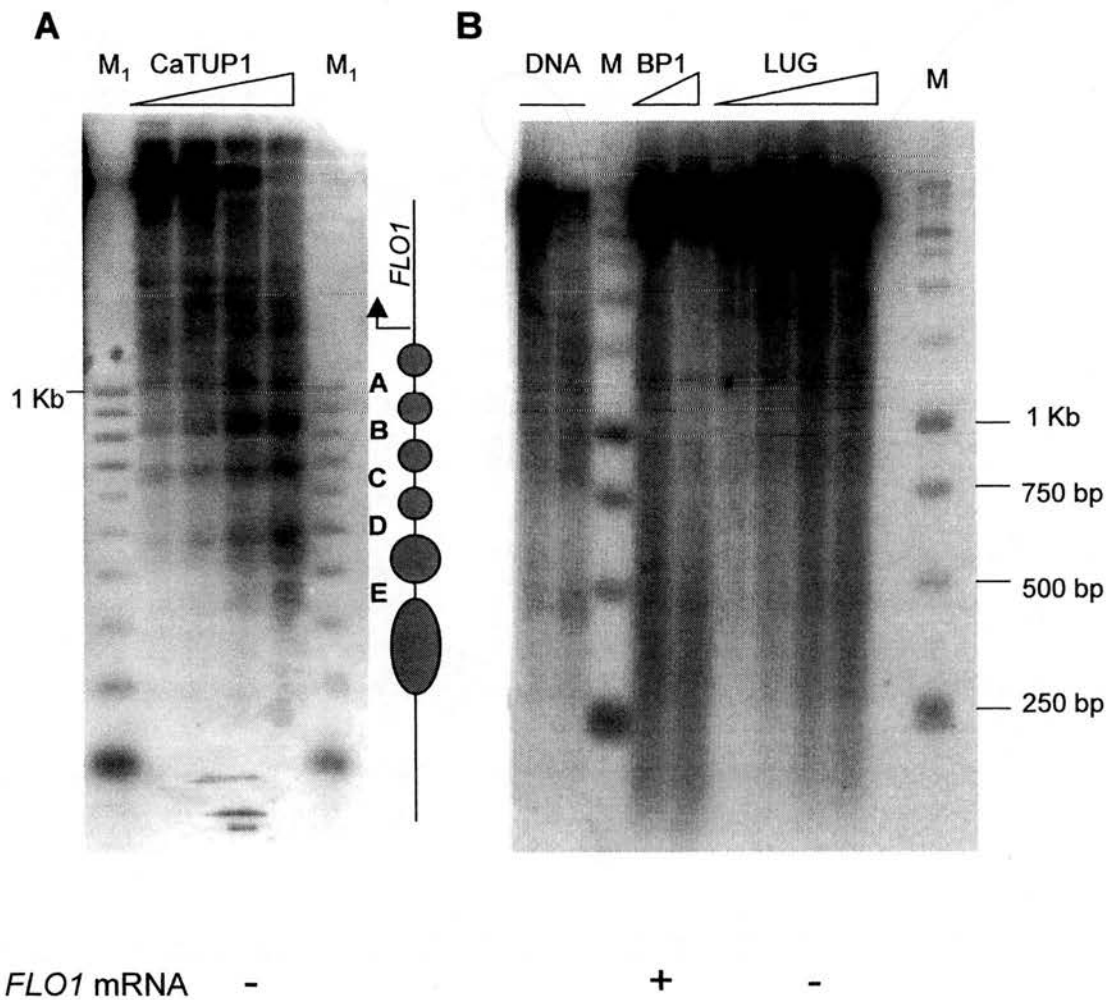
Figure 4.10: Indirect end labelling analysis of the *FLO1* promoter in yeast cells expressing: *C. albicans* TUP1 (CaTUP1), LEUNIG (LUG), and BP1. In all cases M denotes marker, M_1 denotes 100 bp ladder, and DNA is naked DNA, which was treated in the same manner as yeast chromatin. Triangles indicate increased time of micrococcal digestion. (**A**) shows chromatin from yeast expressing *C. albicans* sharing the same ordered nucleosome array as wild-type yeast cells. This shows that CaTUP1 has chromatin remodelling ability. Noted are nucleosome positions corresponding to sites protected from micrococcal nuclease digestion, as in fig. 4.6. (**B**) shows indirect end labelling analysis of yeast expressing BP1 and LEUNIG. Both have a smeared pattern, similar to *tup1* mutants, which confirms these proteins do not have chromatin remodelling activity in *S. cerevisiae*

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4.5.2 Bulk Chromatin

The effects of the homologues were also tested on the entire chromatin of the yeast cell to investigate their influence on nucleosome and chromatin structure in general. As the TUP1-SSN6 co-repressor is responsible for the regulation of around 3% of genes in S. cerevisiae, it may have a genome-wide effect on chromatin (Smith and Johnson, 2000). Therefore, the nucleosomal repeat length of chromatin from yeast expressing the different TUP1 homologues was estimated and compared to wild type and *tup1* mutants. Therefore, nuclei were digested with micrococcal nuclease and DNA fragments were visualised on agarose gels (fig. 4.11 and fig. 4.12). From these ethidium-stained gels the nucleosomal spacing of the chromatin was calculated by linear regression. This was determined by establishing the apparent base pair length per nucleosome from the digestion ladders. The repeat length values were finally resolved by extrapolation of curves to a time point near zero (fig. 4.13).

The nucleosomal repeat length of the wild type strain is estimated to be 165 bp, which is the expected length for yeast (van Holde, 1988). The *tup1* mutant has a similar repeat length of 167 bp suggesting deletion of the *TUP1* gene has very subtle, if any, effects on general chromatin structure. Expression of the *A. thaliana* protein, BP1 in yeast results in the same repeat length (167 bp) as the tup1 mutant that it was expressed in implying that it has no effect on overall chromatin structure. However, LEUNIG and *C. albicans* TUP1 both have repeat lengths of 175 bp which is different from both wild type and *tup1* yeast. This finding suggests that these proteins are capable of modifying general yeast chromatin structure in some manner. A similar

result was established for GROUCHO, which produced a repeat length of 163 bp, implying that GROUCHO can also influence yeast nucleosomal organisation. Whilst these results do not offer specific information on how these proteins interact and influence chromatin, they suggest that at some level they can direct the organisation of chromatin within the yeast nucleus.
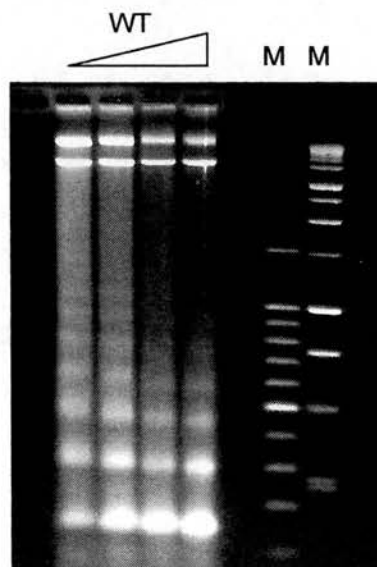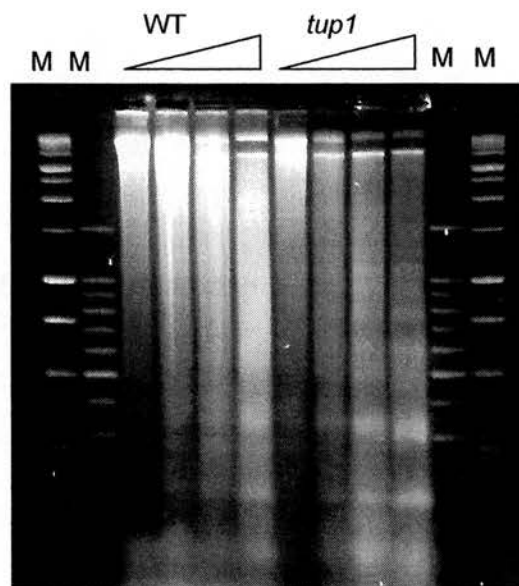
Figure 4.11: Micrococcal digests of wild type (WT) and *tup1* mutant cells. The gels show yeast nuclei incubated for different time periods (1, 2, 4 and 8 minutes) with micrococcal nuclease. The triangles denote increasing time of incubation.
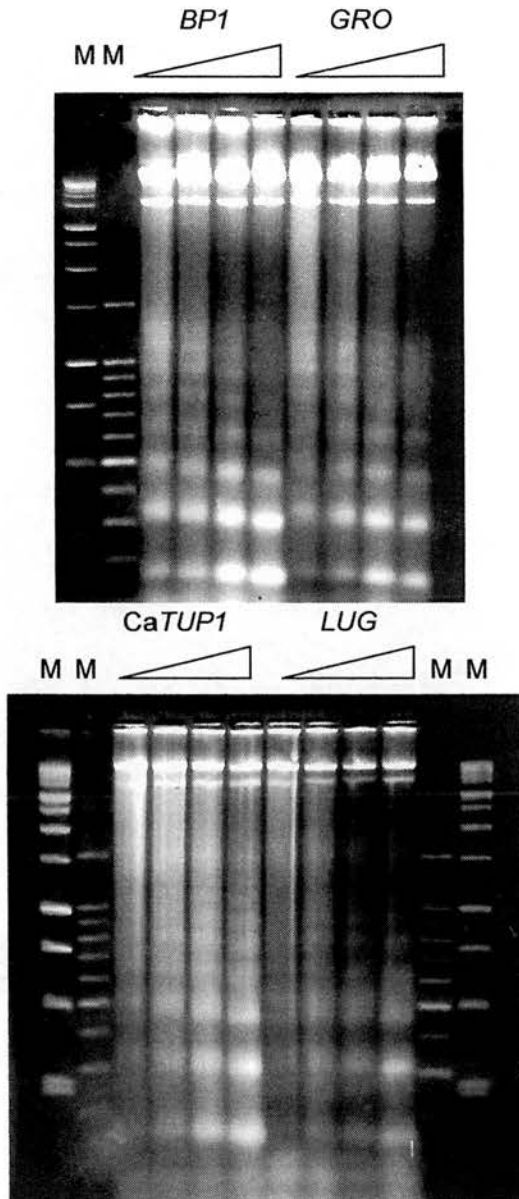
Figure 4.12: Micrococcal digests of *S. cerevisiae* nuclei expressing CaTUP1, LEUNIG and GROUCHO. The gels show yeast nuclei incubated for different time periods (1, 2, 4 and 8 minutes) with micrococcal nuclease. The triangles denote increasing time of incubation.

A
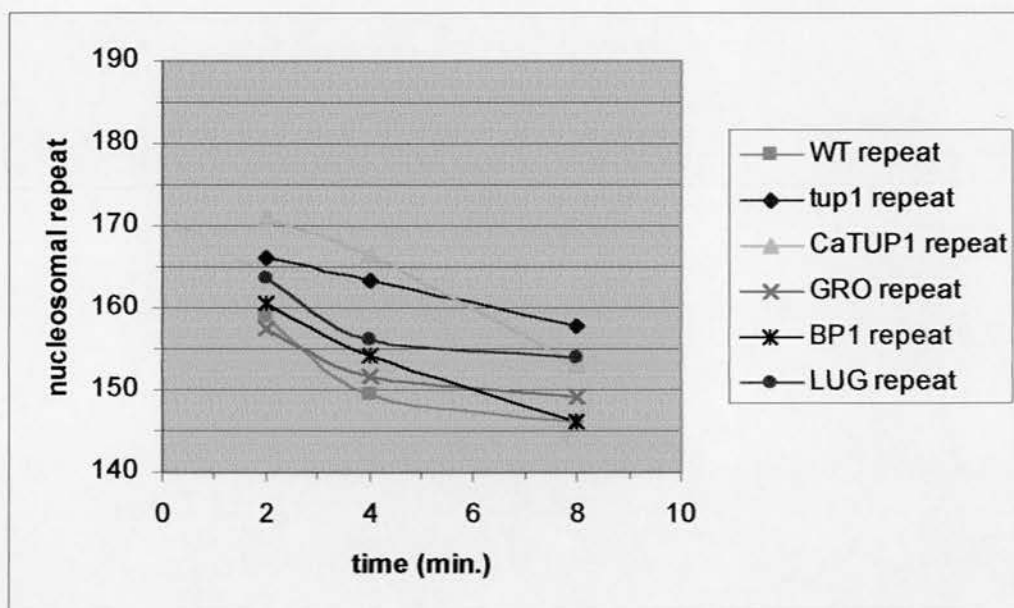
B

Strain	Estimated Repeat Length
WT	165 bp
tup1	167 bp
C. albicans TUP1	175 bp
GROUCHO	163 bp
BP1	167 bp
LEUNIG	175 bp

Figure 4.13: Nucleosomal repeat lengths cells expressing TUP1 homologues. (**A**) shows a graph of nucleosomal repeat lengths calculated by linear regression from the micrococcal digests shown in fig. 4.11 and 4.12. (**B**) shows the estimated repeat length from this graph determined by extrapolation of the curves to time points near zero.

4.6 Function of Histone Acetylation at *FLO1* Promoter

Previous studies have established that a an ordered nucleosomal array over the *FLO1* promoter is essential for gene repression. In addition, studies at other TUP1-SSN6 regulated genes, such as *SUC2*, have revealed that hypoacetylation of histones is also associated with TUP1-mediated repression (Watson *et al.*, 2000). Furthermore, TUP1 has been shown to interact with histone deacetylases (HDACs) leading to the hypothesis that TUP1 recruits HDACs which induces hypoacetylation and gene repression (Watson *et al.*, 2000). Therefore, I tested the ability of a histone deacetylase inhibitor, trichostatin A (TSA), to induce activation of the *FLO1* gene due to hyperacetylation of histones at this locus. This would determine if hypoacetylation of histones is a requirement for TUP1-SSN6 mediated repression of the *FLO1* gene.

The fission yeast *S. pombe*, has both SSN6 and TUP1 homologues, which are also involved in transcriptional repression (Mukai *et al.*, 1999). When the TUP1 genes, tup11p and tup12p, were deleted from *S. pombe*, flocculation occurred (personal communication). A BLAST search revealed putative *FLO* gene homologues in *S. pombe*. Therefore, both *S. cerevisiae* and *S. pombe* were treated with TSA to see if hypoacetylation of histones is a requirement for gene repression by TUP1 at the *FLO1* locus.

To test the importance of acetylation at the *FLO* genes a flocculation assay was performed. Differing amounts of TSA were added to exponentially growing cultures

to give final concentrations of 0, 5, 10, 20 and 50µg/ml. Flocculation was determined by first reading the absorbance of the culture at 600nm; the cuvette was then left for 15 minutes. Any flocculating cells will fall out of solution, which would result in a change in absorbance. The absorbance was measured again; any difference in the absorbance readings ($\Delta OD600_{nm}$) at the different time points is indicative of flocculation. The results are shown in table 4.2.

Species	[TSA] µg/ml	$\Delta OD_{600nm}/15min$
S. cerevisiae	0	0.010
	5	0.006
	10	0.001
	20	0.002
	50	0.012
S. pombe	0	0.002
	5	0.086
	10	0.104
	20	0.159
	50	0.446

Table 4.2: Trichostatin A (TSA) treatment of *S. cerevisiae* and *S. pombe*, and their effects on flocculation.

Little difference is observed in OD_{600} measurements in *S. cerevisiae* cells, showing the cells have remained in suspension; therefore flocculation has not occurred. This shows that the *FLO1* gene is inactive. This observation could be explained in several ways. Firstly, repression of the *FLO1* gene is not dependent upon the acetylation status of the locus. Secondly, the functional redundancy of the histone deacetylases plays a role. Watson *et al* (2000) showed that to alleviate TUP1-mediated repression of genes, several HDACs had to be deleted. Therefore, given that some HDACs are insensitive to TSA (Carmen *et al*., 1999), functional redundancy between these proteins may maintain a hypoacetylated state at the *FLO1* gene. Thirdly, the TSA may be unable to penetrate the cell wall of yeast. Therefore, whether histone acetylation plays a role in gene regulation at the *FLO1* gene in *S. cerevisiae* remains unclear.

With *S. pombe* cells there is an increase in ΔOD_{600nm} measurements in response to increasing TSA concentration. This shows that *S. pombe* cells flocculate in response to TSA treatment, suggesting that TUP1 regulation of the *FLO* genes is dependent upon deacetylation of the locus. These results, taken with other findings suggest that deacteylation plays an important role in TUP1-mediated repression. Considering these results in *S. pombe* and the fact that HDACs interact with GROUCHO and TUP1 in *D.melanogaster* and *S. cerevisiae* (Chen *et al*., 1999; Watson *et al*., 2000), it implies conservation in the mechanism across evolution, which highlights the importance of histone acetylation in TUP1-mediated gene regulation.

4.7 Discussion

This series of experiments was performed to examine the functionality of TUP1-like co-repressors from different species in *S. cerevisiae*. Using the simple budding yeast, I was able to look at the co-repressors' effects on gene transcription and chromatin structure with the aim of establishing if there is a conserved mechanism of gene repression between species.

TUP1 homologues were tested for their ability to repress the TUP1-regulated genes *SUC2* and *FLO1* in a *tup1* knockout background. Plate assays revealed a TUP1 protein from a related yeast *C. albicans* (CaTUP1) restored glucose repression in an *S. cerevisiae tup1* mutant (fig. 4.3). This finding was reiterated by the fact that CaTUP1 could repress both *SUC2* and *FLO1* genes to a level comparable with wild type cells when examined by Northern blotting (CaTUP1 on, fig. 4.5 and fig. 4.7). Indirect end-labelling analysis reflects these observations, showing chromatin extracted from cells expressing *C. albicans* TUP1 had an ordered nucleosome array at the *FLO1* promoter, with nucleosomes positioned in an identical manner to the wild type strain (compare wild type and Ca TUP1, fig. 4.9 and 4.10). This shows that the TUP1 protein from *C. albicans* can influence chromatin structure and is capable of directing nucleosome positioning to the same extent as *S. cerevisiae* TUP1. Although this protein has previously been shown to repress a LacZ reporter gene in *S. cerevisiae* (Braun and Johnson, 1997), this is the first instance of the TUP1 homologue from *C. albicans* repressing native genes in *S. cerevisiae* and the first

study where it is shown to exert effects on chromatin. These results suggest that *C. albicans* TUP1 regulates genes in its native species via a similar mechanism.

The effects of GROUCHO and BP1 on gene transcription in *S. cerevisiae* are somewhat less conclusive. Neither protein could restore glucose repression in a plate assay (fig. 4.3) and Northern blot analysis of the *FLO1* and *SUC2* genes showed that GROUCHO and BP1 did not reduce mRNA levels to the level of a wild type cell (fig. 4.5 and fig 4.7). Therefore, these proteins do not repress genes in *S. cerevisiae*. These findings are supported by indirect end labelling analysis where cells expressing BP1 showed the less structured nucleosome pattern of a *tup1* mutant expressing the *FLO1* gene.

However, an unexpected observation at the *SUC2* gene (fig. 4.5) suggests GROUCHO may have some influence over gene expression in *S. cerevisiae*. When we compare the *tup1* mutant, which we assume to have full de-repression of the *SUC2* gene, to low levels of GRO expression (GRO off), the amount of *SUC2* mRNA produced is almost 2-fold higher in GRO than a *tup1* mutant. The levels of *SUC2* mRNA are further augmented when GROUCHO is fully expressed (GRO on), where we see *SUC2* transcription rise to 3-fold higher than a *tup1* mutant. This implies that when GROUCHO is present, there is less of a repressive effect on the *SUC2* gene. Therefore, GROUCHO has an activating effect on the *SUC2* gene either by a direct or indirect mechanism. One hypothesis for this could be that GROUCHO binds and sequesters a native protein involved in the partial repression of the *SUC2* gene in the absence of TUP1. This would result in an increased level of *SUC2*

expression relative to a *tup1* mutant and levels of *SUC2* expression become consistently higher as GROUCHO expression increases. Given the fact that GROUCHO contains the WD repeat motif, which has the propensity to form protein-protein interactions (Smith *et al.*, 1999); multiple non-specific associations may occur between foreign proteins and native *S. cerevisiae* proteins. Future experiments could look at the effects of GRO on more genes both TUP1-repressed and not regulated by TUP1 to see if this is a general effect on transcription.

These findings are contradicted by the results concerning *FLO1* transcription. When GROUCHO and BP1 are expressed at low levels (BP1 off and GRO off) *FLO1* gene expression is approximately half the level of a *tup1* mutant, suggesting low levels of BP1 and GRO impose repression on the *FLO1* gene. However, when expressed at a higher level (GRO on and BP1 on) the expression levels of *FLO1* are roughly the same as a *tup1* mutant implying there is no repression. These results infer that at low levels of expression BP1 and GRO may influence the repression of the *FLO1* gene in *S. cerevisiae* and this repressive effect is diluted out at higher concentrations of these proteins. The reasons why this should occur remain unclear. Perhaps, at low concentrations, BP1 and GRO can form part of a functional complex to repress genes but at higher concentrations of BP1 and GRO they self-associate. GROUCHO is known undergo self-association (Chen *et al.*, 1998) and this process may cause the abolition of the functional complex.

Like BP1 and GROUCHO, LEUNIG did not restore glucose expression nor repress *SUC2* transcription (fig. 4.5). In fact, similarly to GROUCHO, LEUNIG expression

caused a 2-fold increase in *SUC2* transcription when compared to a *tup1* mutant, again suggesting a direct or indirect activating effect (fig. 4.5). However, at the *FLO1* gene, repression of transcription by LEUNIG was observed and was comparable to yeast cells expressing TUP1 (fig. 4.7). Indirect end-labelling revealed that cells expressing LEUNIG did not show the wild type ordered nucleosomal array associated with gene repression but instead showed the smeared pattern typical of a *tup1* mutant. Why should LEUNIG repress some genes in *S. cerevisiae* and not others and what is the apparent chromatin independent mechanism?

One hypothesis that may be tested, considers that repression by the TUP1-SSN6 complex is dependent upon a DNA-binding protein recruiting this complex to DNA. Therefore, for a TUP1 homologue to function in *S. cerevisiae*, it must have the ability to interact with yeast SSN6 or the appropriate DNA-binding protein in order to bring it in proximity to the promoter and allow repression. The LEUNIG protein has a region of homology to the *FLO1* activator protein FLO8 (Conner and Liu, 2000). There is no evidence showing that FLO8 directly binds to DNA although it is believed to act as part of a complex with DNA-binding proteins and SWI-SNF to activate transcription (Kobayashi *et al.*, 1996). If LEUNIG has enough homology to FLO8, it too has the potential to interact with this DNA-binding protein, but perhaps in a non-functional context (fig. 4.14). This could prevent the formation of the activating complex with SWI-SNF and thereby inhibit activation of the *FLO1* gene. Indeed, the default state of the *FLO1* gene is repressed when both TUP1-SSN6 and SWI-SNF are absent (Fleming and Pennings, 2001). Therefore, LEUNIG may

indirectly repress the *FLO1* promoter as is reflected by the fact that LEUNIG does not influence the nucleosomal structure at the *FLO1* promoter.

Another feasible possibility is that, like TUP1, LEUNIG has the ability to interact with the SRB7 component of the RNA polymerase II complex and represses gene transcription using that mechanism. These hypotheses could be tested by yeast-2-hybrid analysis to see if LEUNIG can interact with *S. cerevisiae* SRB7, chromatin immunoprecipitations (ChIP) to observe if LEUNIG is bound to the *FLO1* promoter and co-immunoprecipitations and yeast-2-hybrids, to observe what proteins, if any, are interacting with LEUNIG.

These possible indirect effects of non-specific protein binding potentially seen at *SUC2* and *FLO1* highlight the difficulties in studying a protein from one species in another. The expression of these proteins is not regulated, as it would be normally. The expression levels of the protein may be different and non-specific and non-functional interactions may occur, which may mask the true function of the protein. Therefore, although there was no gene repression observed for GROUCHO and BP1 in this study, it does not mean that they do not function as TUP1-like repressors in their own species. The reasons for their inability to repress genes in *S. cerevisiae* may be due to their inability to bind the adapter protein SSN6. Indeed, TUP1 from a more related organism, the fission yeast *S. pombe*, was unable to repress genes in *S. cerevisiae* due to differences in the SSN6 binding domain (Mukai *et al.*, 1999). Therefore, GROUCHO, LEUNIG, and BP1 may have been unable to influence gene expression, as they could not be localised at promoters. Experiments with lexA

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fusion proteins tethering the protein to the promoter may help to establish if this is the case. Perhaps co-expression of LEUNIG with its putative *A. thaliana* SSN6 homologue SEUSS (Franks *et al.*, 2002) would give a better representation of LEUNIG action in *S. cerevisiae*. However, these proteins would ideally be studied in their own environment.

The effects of TUP1 homologues on chromatin structure were tested by indirect end-labelling analysis of the *FLO1* promoter and on chromatin structure in general by looking at the repeat lengths of *S. cerevisiae* bulk chromatin. BP1 and LEUNIG did not restore the ordered nucleosomal array of a cell expressing TUP1, however, *C. albicans* TUP1 did, showing it to be a true homologue of TUP1. Analysis of the repeat lengths of bulk chromatin does not give us specific information on the effects that these proteins have on promoter nucleosome structure, but it can reveal if these proteins do indeed affect chromatin. Compared to the *tup1* mutant background, BP1 causes no change in nucleosomal repeat length; this correlates with the indirect end labelling analysis showing it to be redundant at the *FLO1* promoter. When considered together, this data suggests BP1 has no influence on chromatin structure. *C. albicans* TUP1, LEUNIG and GROUCHO all show subtle changes in the nucleosome repeat lengths suggesting they have some influence over chromatin structure (fig. 4.13). This is not surprising since TUP1 and GROUCHO are chromatin associated proteins by virtue of their interaction with the highly conserved histone H3 tails (Flores-Saaib and Courey, 2000; Edmondson *et al.*, 1996), and this in turn may influence chromatin structure. The nucleosomal repeat length data suggests that CaTUP1, GROUCHO and LEUNIG have the potential to affect

chromatin structure although looking at chromatin structures in their native species could validate these results.

The finding that TSA influences FLO gene expression in *S. pombe* suggests that the histone acetylation status of the locus is important for TUP1-mediated gene repression. Although the exact mechanism of FLO gene repression in *S. pombe* remains elusive, the *S. pombe* TUP1 homologue is involved and I have shown loss of HDAC activity by TSA treatment also causes de-repression. This is a similar situation to what occurs in *S. cerevisiae*, where deletion of HDACs leads to de-repression of TUP1- regulated genes (Watson *et al.*, 2000). However, these findings in *S. pombe* are very preliminary and would need to be substantiated by chromatin IP analysis to confirm the acetylation status of the locus and that TUP1 is also found at the FLO locus. These findings along with other results predict that HDACs co-operate with TUP1 homologues to achieve repression. Groucho interacts with rpd3 (Chen *et al.*, 1999); S. cerevisiae TUP1-repression involves various HDACs (Watson *et al.*, 2000) and now it has been shown *S. pombe* TUP1 repression requires HDAC activity. One could hypothesise that LEUNIG will also require HDACs for full repression of its target genes. These findings implicate the histone code in repression by TUP1-like proteins; further experiments should show if histone methylation also plays a role in repression. Not only is hypoacetylation required for TUP1 mediated silencing but it is employed for repression by SIR proteins (Carmen *et al.*, 2002) and methyl-binding proteins in mammals (Nan *et al.*, 1998) implying it is a pivotal mechanism of inducing gene repression throughout evolution.

In summary, a TUP1 protein from a related yeast *C. albicans* was shown to complement a *S. cerevisiae tup1* mutation at every level including chromatin structure. Hypoacetylation is important for TUP1-mediated repression in *S. pombe* as in *S. cerevisiae*. LEUNIG can cause repression of a TUP1-regulated gene in *S. cerevisiae*, and GROUCHO and LEUNIG have some influence over bulk chromatin structure in *S. cerevisiae*. Given these similarities and the known similarities between their function in their native organisms it is likely that although these proteins cannot complement a *tup1* mutation in yeast that they perform analogous functions in their native organisms. A possible reason why they cannot perform these functions in *S. cerevisiae* is due to sequence divergence throughout evolution.
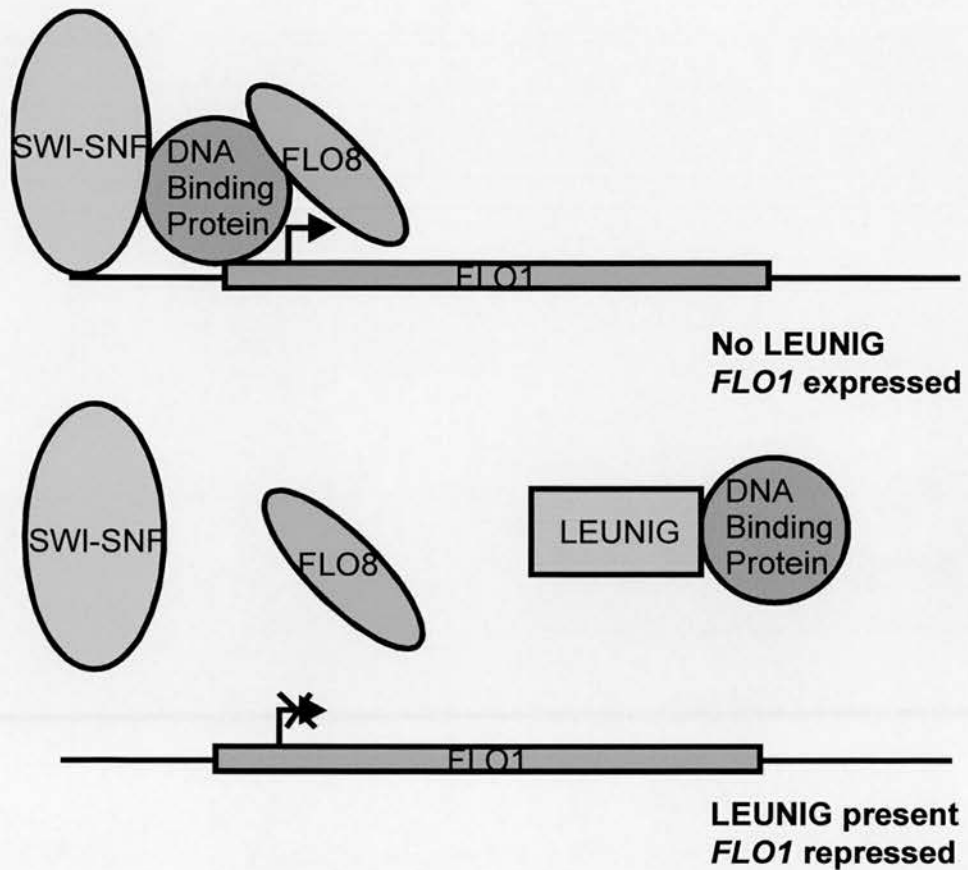
Figure 4.14: Putative mechanism of LEUNIG-induced *FLO1* repression. When there is no LEUNIG present a complex of proteins including SWI-SNF can assemble at the *FLO1* promoter and activate the *FLO1* gene. When LEUNIG is present it binds the DNA-binding protein, which prevents the activating complex assembling and the *FLO1* gene resorts to its default state of repression.

Chapter 5 – General Discussion

The aim of my thesis was to elucidate if there are conserved mechanisms of chromatin-mediated transcriptional repression between yeast and plants. I tested the ability of a repetitive DNA sequence isolated from *P. hybrida* to induce expression variegation in yeast and observed the function of various TUP1 homologues at genes in *S. cerevisiae*. My results show that a repetitive DNA fragment can silence genes in both *S. cerevisiae* and *P. hybrida* and that the ability of putative TUP1 homologues to repress genes in *S. cerevisiae* varies.

My data shows that the RPS sequence can induce expression variegation in *S. cerevisiae* on both plasmids and when integrated into chromosomes. This demonstrates a level of conservation in the way species respond and deal with potentially deleterious repetitive DNA sequences. It also confirms that, although yeast have few repetitive DNA sequences, they still have the capacity to silence them. This finding is in line with previous studies of repeated sequences in *S. cerevisiae* such as telomeres and Ty elements, both of which are subject to silencing (Gottschling et al., 1990; Jiang, 2002).

The mechanism of RPS-induced silencing remains unknown in both *P. hybrida* and *S. cerevisiae*. One hypothesis for RPS-mediated repression is that it induces a repressive chromatin structure by virtue of its repetitive nature or by binding a protein that induces a heterochromatin-like structure, which spreads to the adjacent gene by position effect variegation. In accordance with previous studies in *P. hybrida*

and *A. thaliana*, where the methylation status of the RPS did not correlate with gene silencing (ten Lohuis *et al.*, 1995; Müller *et al.*, 2002), the non methylating organism, *S. cerevisiae* was able to support RPS-mediated gene silencing. This suggests, that in this instance, DNA methylation is not the initial stage in gene silencing, and may be a secondary epigenetic mark that is useful in more complex genomes. Indeed, other studies have shown that disruption of a putative chromatin remodelling factor allows expression of heavily methylated genes (Amedeo *et al.*, 2000). Previous studies demonstrated that the RPS contained hotspots for DNA methylation (ten Lohuis *et al.*, 1995), since RNA from inverted repeats can trigger DNA methylation (Luff *et al.*, 1999; Melquist and Bender, 2003) it may be that the repetitive nature of the RPS induces this DNA methylation. However, since RPS-induced gene silencing has been shown in *S. cerevisiae*, it could be hypothesised that these inverted repeats are also recognised by some other chromatin associated protein that induces a repressive chromatin structure.

The fact that the RPS can trigger *de novo* methylation, it is repetitive in nature with, and has putative homology to a transposable element protein suggest that it may be a remnant of a transposable element. These studies in yeast also suggest that it has moved from one part of the genome to another. This hypothesis must be considered with caution and multiple experiments would need to confirm this. However, it is a feasible hypothesis and would help to explain the repressive nature of the sequence since transposable elements can induce position effects on the surrounding areas.

To appreciate fully the nature of the RPS sequence, the mechanism of action needs to be elucidated. Differences in levels of silencing between high-copy number and low-copy number plasmids suggest a low abundance factor in yeast is responsible for RPS-induced silencing. However, this is different to the situation in plants, where it was proposed that multiple copies of the RPS sequence were required to initiate gene silencing (Müller *et al.*, 2002). Moreover, it is known that the *A. thaliana* protein BP1 is capable of binding the RPS sequence, however deletion of the putative yeast homologue *TUP1* did not release the RPS-induced silencing. This potentially suggests that there are different mechanisms of RPS-mediated silencing in yeast and plants.

This theory is not unexpected when one considers the divergence of many proteins involved in silencing. However many of these diverse proteins share common themes in mechanisms of gene silencing. For example, the SIR3 and SIR4 proteins in *S. cerevisiae*, which are possible candidates for RPS mediated silencing have not been identified in other species to date. The SIR proteins induce a heterochromatin-like state at telomeres, silent mating-type loci and rDNA repeats (Laurenson and Rine, 1992). They achieve this by binding to specific histone modifications and by spreading along the template via interactions with histones and SIR-protein association (Hecht *et al.*, 1995). This method of silencing has many common mechanistic properties to HP1-induced silencing, which is not found in *S. cerevisiae*. HP1 is attracted to chromatin by its interaction with a methylated lysine-9 (Bannister *et al.*, 2001; Lachner *et al.*, 2001). HP1 induces gene silencing and spreads along the template via its interaction with histones and its self-association ability (Cowell and

Austin, 1997). Therefore, despite sequence divergence this mechanism between species are similar. This highlights the difficulties in studying sequences out with their own genomic context. Whilst the RPS did induce silencing in yeast there is no evidence suggesting it has the same mechanism as in plants since many different silencing proteins exist between the species. Perhaps a better system for the study of the RPS would be *S. pombe*, which still has relatively easy genetic manipulation techniques but also has many silencing factors in common with higher eukaryotes such as the RNA interference-silencing complex (RISC) complex responsible for PTGS. Furthermore, to date no constituents of the PTGS pathway have been identified in S. cerevisiae, suggesting it may not be an appropriate model to study certain aspects of plant gene silencing.

The experiments carried out expressing TUP1 homologues in yeast also echo the idea that despite sequence divergence many proteins have similar roles. Out with the WD repeats there is little sequence similarity between TUP1-homologues. However, they carry out similar functions such as self-association and interactions with histones and histone deacetylases. When studied in yeast only a closely related TUP1 from *C. albicans* could rescue a *tup1* mutation in *S. cerevisiae* in the same manner as the endogenous protein. However, my results suggest that GROUCHO and LEUNIG can both affect chromatin structure and in some manner may influence gene expression in *S. cerevisiae*. These clues imply that in their native species GROUCHO and LEUNIG may interact and manipulate chromatin to a similar extent as TUP1. Indeed studies on acetylation highlight its importance in TUP-mediated repression in *S. pombe*. This is in line with other findings in *D. melanogaster* and *S.*

cerevisiae indicating conservation in the mechanisms of repression (Chen *et al.*, 1999; Watson *et al.*, 2000).

My results do not conclusively tell if there is a conserved method of chromatin-mediated repression. The studies on the RPS suggest that yeast and plants both recognise and respond to repetitive DNA in a similar manner. Future studies would elucidate the mechanisms of RPS-induced repression in both yeast and plants. This could be achieved by a series of gene deletions, band-shift experiments to determine what proteins bind the RPS sequence in yeast and looking at the nucleosomal organisation of sequences which are silent and active when adjacent to the RPS. The studies of TUP1 homologues suggest GROUCHO and LEUNIG can affect nucleosomal structure, this could be followed up looking at the nucleosomal positioning of genes regulated by these proteins in their endogenous species. It would also be useful to elucidate the mechanism of LEUNIG repression at the *FLO1* gene to determine if it is direct or indirect repression. My studies have hinted at conservation in mechanisms of repression, future studies should determine this conclusively.

Chapter 6 – Appendix

6.1 Vectors, Strains and Plasmids used in Chapter 3

Plasmid	Parent	Description	Cloning Strategy
pKanMX4		Vector containing the kanamycin resistance gene (Wach *et al.*, 1994).	.
pPICZB		Vector containing the zeocin resistance gene (Invitrogen).	
pRS401		A yeast integrating vector containing the *MET15* marker gene.	
pRS402		A yeast integrating plasmid containing an *ADE2* marker gene	
pRS406		A yeast integrating vector containing the *URA3* marker gene.	
PRS412		A yeast low-copy number plasmid containing an *ADE2* marker gene, used for complementation test with *ade2* strain.	
pRS414		A yeast low-copy number plasmid containing a *TRP1* marker gene	
pRS424		A yeast multi-copy plasmid containing a *TRP1* marker gene	
pBlue/AR1-4	pBluescript	pBluescript containing the RPS and the *ADE2* gene in all orientations used for study	The *ADE2* gene was purified from vector pRS402 following a *Bgl* II digest and cloned into the *Bam* HI site of pBluescript. The RPS was purified from vector pBlue/RPS (kindly provided by Prof. P. Meyer) following an *Eco* RI digest. This was cloned into the *Eco*RI site of pBluescript containing the *ADE2* gene.
pRS414/A	pRS414	A yeast low copy number vector containing the *TRP1* marker gene and the *ADE2* gene	The *ADE2* gene was purified from vector pRS402 following a *Bgl* II digest and cloned into the *Bam* HI site of pRS414.

Plasmid	Parent	Description	Cloning Strategy
pRS414/AR1-4	pRS414	A yeast low-copy number plasmid containing a *TRP1* marker gene and RPS and *ADE2* sequences.	The *ADE2* and RPS sequences were removed from pBlue/AR vectors using a *Sac* I and *Cla* I digest. The construct was cloned into the same sites in pRS414.
pRS424/A	pRS424	A yeast multi-copy plasmid containing the *TRP1* marker gene and the *ADE2* gene	The ADE2 gene was purified from pRS402 following a *Bgl* II digest and cloned into the *Bam* HI site of pRS424.
pRS424/AR1-4	pRS424	A yeast multi-copy plasmid containing a *TRP1* marker gene and RPS and *ADE2* sequences	The *ADE2* and RPS sequences were removed from pBlue/AR vectors using a *Sac* I and *Cla* I digest. The construct was cloned into the same sites in pRS424
pBlue/*URA3*	pBluescript	pBluescript containing the *URA3* gene, used for gene knock-outs.	The *URA3* gene was amplified by PCR using primers Blura3A and Blura3B and plasmid pRS406 as a template. These primers contained restriction sites for *Cla* I and *Sac* I. The resulting PCR product was cloned into these sites in pBluescript. Plasmid was used as template for PCR mediated disruption to generate strain HC2.
pRS406/AR1-4	pRS406	Used for targeting RPS and *ADE2* sequences to *ura3-52* locus by homologous recombination	The *ADE2* and RPS sequences were released from pBlue/AR by digestion with *Sac* I and *Cla* I and cloned into these sites in pRS406.
pBlue78/AR1-4	pBluescript	Used for targeting RPS and *ADE2* sequences to *ADE2* locus by homologous recombination	Regions of homology were PCR amplified using primer pairs 007a and 007b and 008a and 008b. Primers 007a and 007b contain restriction sites for *Xho* I and *Cla* I and were cloned into these sites of pBluescript. Likewise 008a and 008b have restriction sites for Spe I and Sac I and were cloned into these sites. The *ADE2* and RPS sequences were released from plasmid pBlue/AR by digestion with *Spe* I and *Cla* I and cloned into these sites in between homologous DNA sequences. The whole cassette was released by digestion with *Xho* I and *Sac* I and was used for transformation.

Table 6.1: Plasmids used in chapter 3.

Primer	Sequence	Description
adedelstart	TGCTTATGGGTTAGCTATTTCGCCCAAT GTGTCCATCTGACAGCTGAAGCTTCGTA CGC	To amplify the kanamycin resistance gene from plasmid pKan-MX4. These primers contain homology to *ADE2* locus for PCR-mediated disruption. Used to generate strains HC1 and HC4.
adedelend	TCGAAACGTTATTTTTTTAATCGCAGACT TAAGCAGGTAAGCATAGGCCACTAGTG GATCTG	
Blura3A	TCCATCGATTACTGAGAGTGCACC	To amplify the *URA3* gene from pRS406, with *Cla* I and *Sac* I restriction sites.
Blura3B	ATTTAGGAGCTCGCGGTATTTCACACC	
PRSTUP1A	TAAGCAGGGGAAGAAAGAAATCAGCTT TCCATCCAAACCAATATGAGATTGTACT GAGAGTGCAC	To amplify the *MET15* gene from plasmid pRS401. These primers contain homology to *TUP1* locus for PCR-mediated disruption. Used to generate strain HC3.
PRSTUP1B	TAGTTAGTTACATTTGTAAAGTGTTCCTT TTGTGTTCTGTTCTTACTGTGCGGTATT TCACACCG	
RpsadeD	TCGAAACGTTATTTTTTTAATCGCAGACT TAAGCAGGTAAGTAATACGACTCACTAT AGG	To amplify regions of DNA cloned into multiple cloning site of pBluescript. Primers contain homology to *ADE2* locus for PCR-mediated disruption. Used to generate strain HC2 and for inserting *ADE2* and RPS sequences into *ADE2* locus.
RpsadeE	TGCTTATGGGTTAGCTATTTCGCCCAAT GTGTCCATCTGAGGAACAAAAGCTGGG TACCGb	
005a	CCAACACTTCCTCTACCATTGC	To amplify probe 1 used for Southern blot analysis at *ADE2* locus.
005b	TGGACACATTGGGCGAAATAGC	
006a	CAGAACCCTCTTACATTATCG	To amplify probe 5 used for Southern blot analysis at *TUP1* locus.
006b	TGCAGAAGGACAGATAGAGGG	
007a	CTTAATCGCTCGAGGAGTAACGCCGTA TCG	To amplify a region of DNA next to *ADE2* gene corresponding to 686-609bp upstream of the *ADE2* transcriptional start site.
007b	ATGAATCCATCGATGGACATTGGGCGA AATAGC	
008a	AACTATGGACTAGTCCGTCTTAAGTCTG CG	To amplify a region of DNA next to *ADE2* gene corresponding to 204-421bp downstream of the *ADE2* transcriptional stop site.
008b	TTTTGATCGAGCTCGGACGCTTTATAAT TTGGC	
017a	GATAGACAATAGTGGATTTTTATTCCAA CAGTGTCTTTGTAGATTGTACTGAGAGT GCAC	To amplify the *MET15* gene from pRS401. The primers contain homology to the *MET15* locus to allow use as a disruption fragment.
017b	GTTCAATTGTAGAATATCCTGTATAATTT GATACTGTCTGTGCGGTATTTCACACCG	
018a	AACGCGGTTTATTCTGCC	To amplify probe 4 used for Southern blot analysis at *URA3* locus.
018b	TGTGGTGCTTCAGGGGAT	
019a	CAACGCTTACAGGTCTCC	Utilised for inverse PCR on *ADE2* gene.
019b	TGTAATCATAACAAAGCC	

Plasmid	Sequence	Description
020a	TAAGCAGGGGAAGAAAGAAATCAGCTT TCCATCCAAACCAATATGACCTTCGTTT GTGCG	To amplify the zeocin resistance gene from plasmid pPICZB. These primers contain homology to the *TUP1* locus allowing the amplified fragment to be used for PCR-mediated disruption.
020b	TAGTTAGTTACATTTGTAAAGTGTTCCTT TTGTGTTCTGTTCTGTTCTTACACATGTT GGTCTCC	

Table 6.2: Primers used in chapter 3.

Probe	Description
Probe 1	Amplified by primers 005a and 005b. Corresponds to region of DNA 830-605bp upstream of the *ADE2* transcriptional start site.
Probe 2	Full length RPS sequence. Generated by *Eco* RI digestion of pBlue/AR.
Probe 3	Probe for *ADE2* sequence. Generated by *Vsp* I digestion of pBlue/AR.
Probe 4	Amplified by 018a and 018b. Corresponds to region of DNA 1652-2013bp downstream of the trancriptional stop site of the *ura3-52* locus.
Probe 5	Amplified by primers 006a and 006b. Corresponds to a region of DNA 120-359bp upstream of the *TUP1* transcriptional start site.

Table 6.3: Probes used in chapter 3.

Strain	Parent	Genotype	Description
HC1	BY4733	*MATa, ade2Δ::kanMX4, his3Δ200, leu2Δ0, met15Δ0, trp1Δ63, ura3Δ0*	Deletion of *ADE2* gene from 602bp upstream of transcriptional start site to 239bp downstream of transcriptional stop site. The *ADE2* gene was replaced with the kanamycin resistance gene.
HC2	HC1	*MATa, ade2Δ::URA3, his3Δ200, leu2Δ0, met15Δ0, trp1Δ63, ura3Δ0*	Contains deletion of *ADE2* gene from 602bp upstream of transcriptional start site to 239bp downstream of transcriptional stop site. The *ADE2* gene was replaced by the *URA3* gene.
HC3	HC2	*MATa, ade2Δ::URA3, his3Δ200, leu2Δ0, met15Δ0, trp1Δ63, tup1Δ::MET15, ura3Δ0*	Deletion of *TUP1* gene from 2bp upstream of transcriptional start site to 2bp downstream of transcriptional stop site. The *TUP1* gene was replaced by the *MET15* gene.
HC4	FY2	*MATa, ade2Δ::kanMX4, ura3-52*	Deletion of *ADE2* gene from 602bp upstream of transcriptional start site to 239bp downstream of transcriptional stop site. The *ADE2* gene was replaced by the kanamycin resistance gene.

Table 6.4: Strains made in chapter 3.

6.2 Primers and Plasmids Used in Chapter 4

Vector	Parent	Cloning Strategy
p415-*MET25*/GRO	p415-*MET25*	GROUCHO cDNA was amplified by PCR from vector, pCR4-GRO (kindly provided Dr. D. Ish-Horowicz) by, using primers 015a and 015b. These primers contain restriction sites for *Xho* I and *Spe* I and these sites were used for cloning into p415-*MET25*.
p415-*MET25*/Ca*TUP1*	p415-*MET25*	CaTUP1 cDNA was amplified by PCR from vector, pMH1 (a gift from Prof. A. Johnson, Braun and Johnson, 1997) using primers 016a and 016b. These primers contain restriction sites for *Xho* I and *Spe* I and these sites were used for cloning into p415-*MET25*.
p415-*MET25*/Sc*TUP1*	p415-*MET25*	Sc*TUP1* was amplified by PCR from genomic DNA purified from *S. cerevisiae* using primers 004a and 004b. These primers contained *Hind* III restriction sites, these sites were used for cloning into p415-MET25. The positive clones were analysed by restriction enzyme cleavage to select clones with the correct orientation of Sc*TUP1*.
p415-*MET25*/LUG	p415-*MET25*	LUG cDNA was purified after digestion of plasmid pAVA393 (a gift from Prof. Z. Liu, Conner and Liu, 2000) with *Bgl* II. The restriction fragment, containing LUG, was cloned into the *Bam* HI site of p415-MET25. Restriction enzyme digests confirmed the correct orientation of the LUG gene.
p415-*MET25*/BP1	p415-*MET25*	BP1 cDNA was purified after digestion of plasmid pBluescript-BP1 (kindly provided by Prof. P. Meyer) with *Xho* I and *Xma* I. BP1 was then cloned into the *Xho* I and *Xma* I sites of p415-*MET25*.

Table 6.5: Plasmids used in chapter 4.

Primers	Sequence	Description
004a	ATATATACCCAAGCTTGGGATCAGCTTT CCATCCAAACC	To amplify the *TUP1* gene from *S. cerevisiae* genomic DNA, with *Hind* III restriction sites on the ends.
004b	TTAAATCCCAAGCTTGGGGTTACATTTG TAAAGTGTTCC	
015a	ATTCCGCTCGAGTGCATGGTTTTGTGG	To amplify *GROUCHO* cDNA from plasmid pCR4-*GRO*, with *Xho* I and *Spe* I sites on the ends.
015b	ACATGGACTAGTATGTATCCCTCACCG	
016a	TAACCGCTCGAGAGAGTACATTGATGG	To amplify Ca*TUP1* cDNA from plasmid pMH1, with *Xho* I and *Spe* I sites on the ends.
016b	TATCAGACTAGTATGTCCATGTATCCC	

Table 6.6: Primers used in chapter 4.

Probe	Description
ActPr	Amplified by PCR, and contains *ACT1* ORF sequences between +411 and +1422.
FloPr	The full-length *FLO1* gene, which was isolated from plasmid pYY105 following an *Eco* RV digestion.
FloPr2	Amplified by PCR and corresponds to 775-1146 bp upstream of the *FLO1* transcriptional start site
SucPr	Amplified by PCR, and contains *SUC2* ORF sequences between +119 and +1222.

Table 6.7: Probes used in chapter 4.

Chapter 7 – References

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