Factors affecting telomere structure and function in the fission yeast *Schizosaccharomyces pombe*

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I declare that :

1) This Thesis was composed by myself

2) Unless otherwise acknowledged this work is my own

Clare E. Drakeford
January 2002

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Abstract

Telomeres are complex nucleoprotein structures formed at the ends of linear eukaryotic chromosomes, which consist of a repetitive short nucleotide repeat and associated proteins. Telomeres are important for the complete replication of the ends of chromosomes and the stability of the genome, forming a protective cap that prevents the ends of the chromosome from being recognised as double-strand breaks or forming end-to-end fusions. Failure to maintain telomeres may result in genetic instability and uncontrolled cell division and is implicated as a major factor in tumourigenesis. Chromatin at telomeres is assembled into a transcriptionally repressed structure, which leads to the reversible silencing of genes placed in their vicinity. The functions of telomeres are conserved across many species. In this thesis I use a genetically tractable organism, the fission yeast *Schizosaccharomyces pombe* to perform screens for factors affecting the telomere.

Two genetic screens were performed using alleviation of repression of telomeric marker genes to isolate mutants of interest. In one screen, spontaneous mutagenesis and UV induced mutagenesis were used to create an extensive collection of mutants. Genetic crosses between a subset of these mutants identified six complementation groups, one of which contained novel alleles of *taz1* that showed silencing defects but not the telomere length regulation defect of a *taz1*Δ strain. These alleles may be useful in future experiments to dissect the function of *taz1* at the telomere. Expression of the telomeric marker genes in the mutant stains produced by this screen variegated, thus identification of the gene of interest using complementation by a genetic library was not possible. A second screen was performed using insertion of a marker gene into the genome as a mutagen. Over 10,000 colonies that tested positive for an insertion event were produced. Further study revealed that the insertion event appeared to cosegregate with expression of a telomeric marker gene in only two of these strains. Isolation of the sequence surrounding the insertion site revealed that the insertion event had occurred at a telomere and that expression of the telomeric marker gene was caused by a secondary mutation event in *rap1*. The screening techniques and phenotypic analysis of mutants are discussed.

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Introduction

I.1. Telomere structure and function

Telomeres are nucleoprotein complexes located at the ends of linear eukaryotic chromosomes. The DNA component consists of multiple tandem repeats of a short nucleotide sequence in most organisms and is essential for telomere function, acting as a scaffold to which telomere specific proteins bind to perform a number of functions; assembling the tertiary structure of the telomere, forming a protective cap at the end of the chromosome, allowing for complete replication of the telomere and controlling transcriptional repression of the telomeric DNA. Telomeres perform a protective function, shielding the ends of the chromosome from nucleolytic degradation, from being recognised as double strand breaks and from forming end-to-end fusions.

In most eukaryotes the telomeric DNA is formed from multiple tandem repeats of a short nucleotide sequence. The exact sequence and number of repeats varies between species but is consistent within species, as shown in Table 1.1. The strand that runs towards the end of the chromosome in the 5' to 3' direction has a high proportion of guanine residues and is known as the G-rich strand. Telomeres end with a 3' G-rich overhang that is generated in a cell cycle dependent manner by enzymatic degradation of the 5' strand (Wellinger, *et al.,* 1993). The 3' overhang is necessary for the recruitment of telomerase as the enzyme is unable to add repeats to a blunt end (Wang and Blackburn, 1997).

The two strands of duplex DNA are arranged in an antiparallel manner. DNA polymerase III synthesises DNA in the 5' to 3' direction only, thus as a replication fork passes along the DNA molecule the leading strand is synthesized continuously whilst the lagging strand is copied in short Okazaki fragments, as shown in Figure

Species	Telomere repeat consensus sequence	
Vertebrate	TTAGGG	(Moyzis *et al.*, 1988, Meyne *et al.*, 1989)
Saccharomyces cerevisiae	TG_{1-3}	(Blackburn and Greider, 1995)
Schizosaccharomyces pombe	$TTACAG_{1-8}$	(Hiroaka *et al.*, 1998)

Table 1.1. The consensus sequence of the telomeric repeats for the four species; mouse, human, budding yeast and fission yeast, on which this introduction will focus.

1.1.. The synthesis of each fragment is primed from a ~10nt RNA molecule that is removed at the end of the fragment's synthesis, leaving a gap between one fragment and the next, which is subsequently filled in by DNA polymerase I. The gap left by removal of the RNA primer at the end of the synthesised strand cannot be completed, leaving a small 3' overhang at the end of the lagging strand. The leading strand can be copied all the way to the end so finishes in a blunt end. Thus over a number of cell divisions the ends of linear molecules shorten due to incomplete replication.

It has been suggested that cells employ a counting mechanism that determines the number of cell divisions they have passed through (Olivkanov, 1973). This mechanism is proposed to be based on available telomere repeats being bound by proteins, such as Rap1p in *Saccharomyces cerevisiae* and TRF1 in mammalian cells. These are used to count the number of binding sites present and thus allow the cell to monitor the length of telomeres. Cell lines have a predetermined proliferative capacity called the Hayflick limit (Hayflick, 1975) and the proliferative capacity of any one cell is dependent on the organism, the age of the organism and the tissue type from which it originates. This can be seen in the limited proliferation of cells taken from patients and maintained in tissue culture. According to the telomeric clock theory this limited number of divisions is determined by the cumulative amount of telomere repeats lost. Successive rounds of replication shorten the telomeric repeats and cells cease dividing and enter a state known as senescence after a certain number of divisions. It is likely, however, that other cellular processes are also involved in determining the age and proliferative capacity of cells. Occasionally

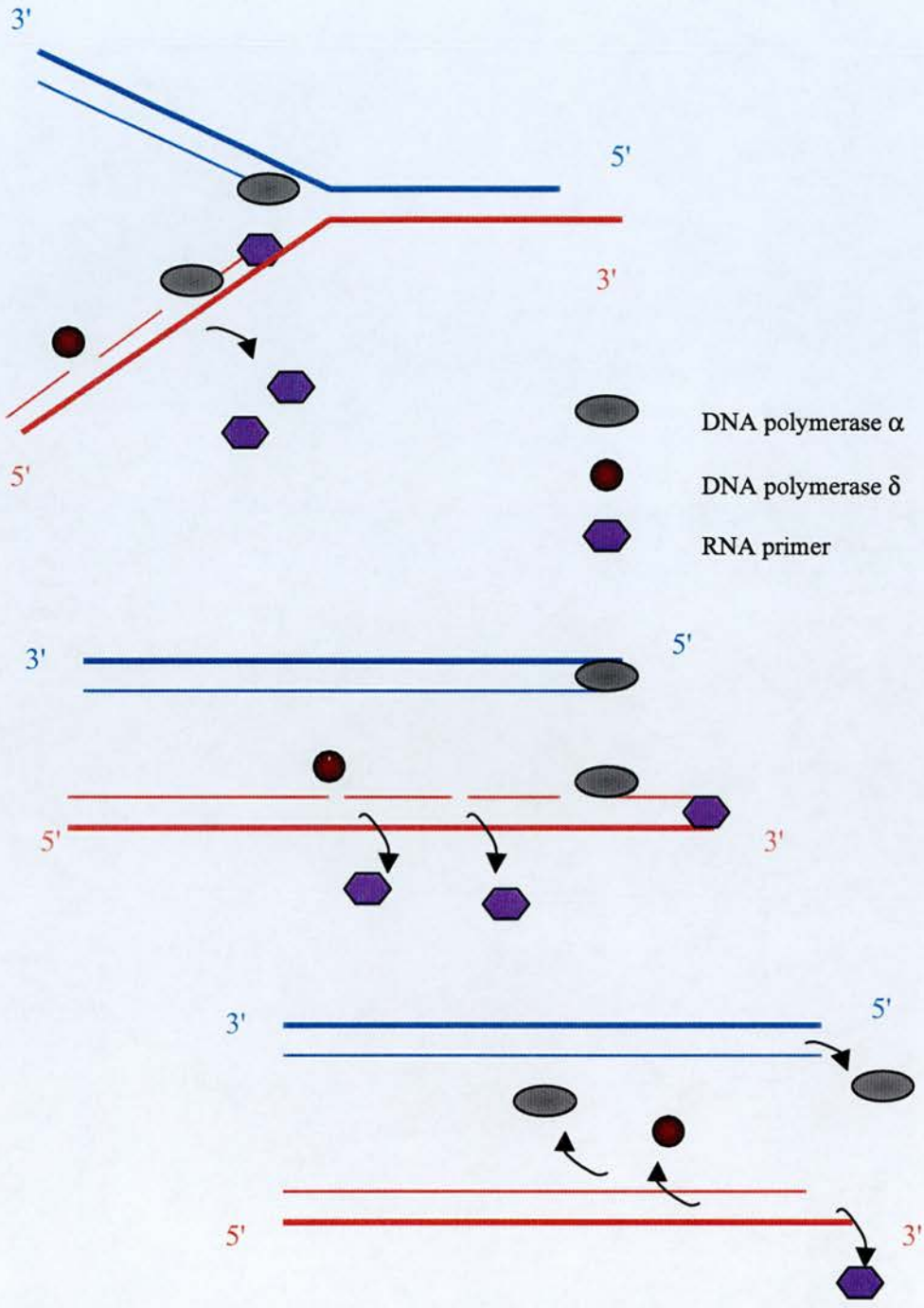

Figure 1.1 The end replication problem of linear DNA: DNA polymerase III synthesises DNA in a 5' to 3' direction only. Thus as a replication fork progresses the 3' to 5' strand is copied continuously and ends when the end of the chromosome is reached, resulting in a blunt end. Synthesis of the lagging strand is primed from a temporary RNA and is copied in a discontinuous manner by DNA polymerase α. The short fragments produced by this method are joined together by DNA polymerase δ. When synthesis reaches the end of the chromosome the last RNA primer is displaced and leaves an unfilled gap, creating a small 3' single stranded overhang. In this way over successive cell divisions the ends of the chromosomes are slowly lost.

factors such as defective proliferation control or immortalisation by a viral infection enable cells to overcome senescence and recommence cell division. In humans the abnormal progression of cell division beyond senescence and through crisis is an important step in tumour formation. In such cells telomeres initially continue to shorten past senescence, leading to an increase in genomic instability, chromosome fusions and aneuploidy. Gross chromosomal abnormalities caused by genomic instability lead to a second DNA damage checkpoint dependent halt in cell division called crisis, resulting in cell death and apoptosis. Occasionally a cell passes through crisis and the ability to synthesis telomeres is regained. This cell then has a selective advantage over the other cells in the vicinity and proliferation causes a clone of such cells to arise resulting in tumour formation (Counter *et al.,* 1992).

In immortal cell lines, germ cells and single cell organisms the telomeric repeats lost at each cell division have to be replaced in order to prevent the erosion of chromosome ends leading to a loss of ability to divide or genomic instability. In most organisms telomere repeat units are replaced by synthesising them *de novo* using an RNA template. This is accomplished by a ribonucleoprotein complex called telomerase, which consists of a catalytic subunit containing reverse transcriptase motifs (Ligner *et al.,* 1997a), an RNA template and associated proteins that mediate the activity of the enzyme complex at the telomere. The activity of telomerase at telomeres is tightly controlled to maintain the length of the telomere repeats. Telomerase is thought to synthesise additional repeats at the end of the $5'$ to $3'$ stand by aligning a small portion of the RNA templating region with the sequence of the $3'$ overhang and copying along the template. The RNA template is then disengaged from the synthesised DNA and moved along to generate the next part of the telomere. This slippage mechanism of *de novo* telomere repeat synthesis by telomerase, described in Figure 1.2, is reflected in the repetitive structure of the telomeric DNA. The exact method by which the activity of telomerase at telomeres is controlled is unknown but it is likely to involve regulating telomerase activity itself, the access of the telomerase enzyme to the ends of chromosomes or a combination of such processes. The balance between loss of repeats and their addition maintains an average number of telomere repeats at the ends of the chromosomes in a cell

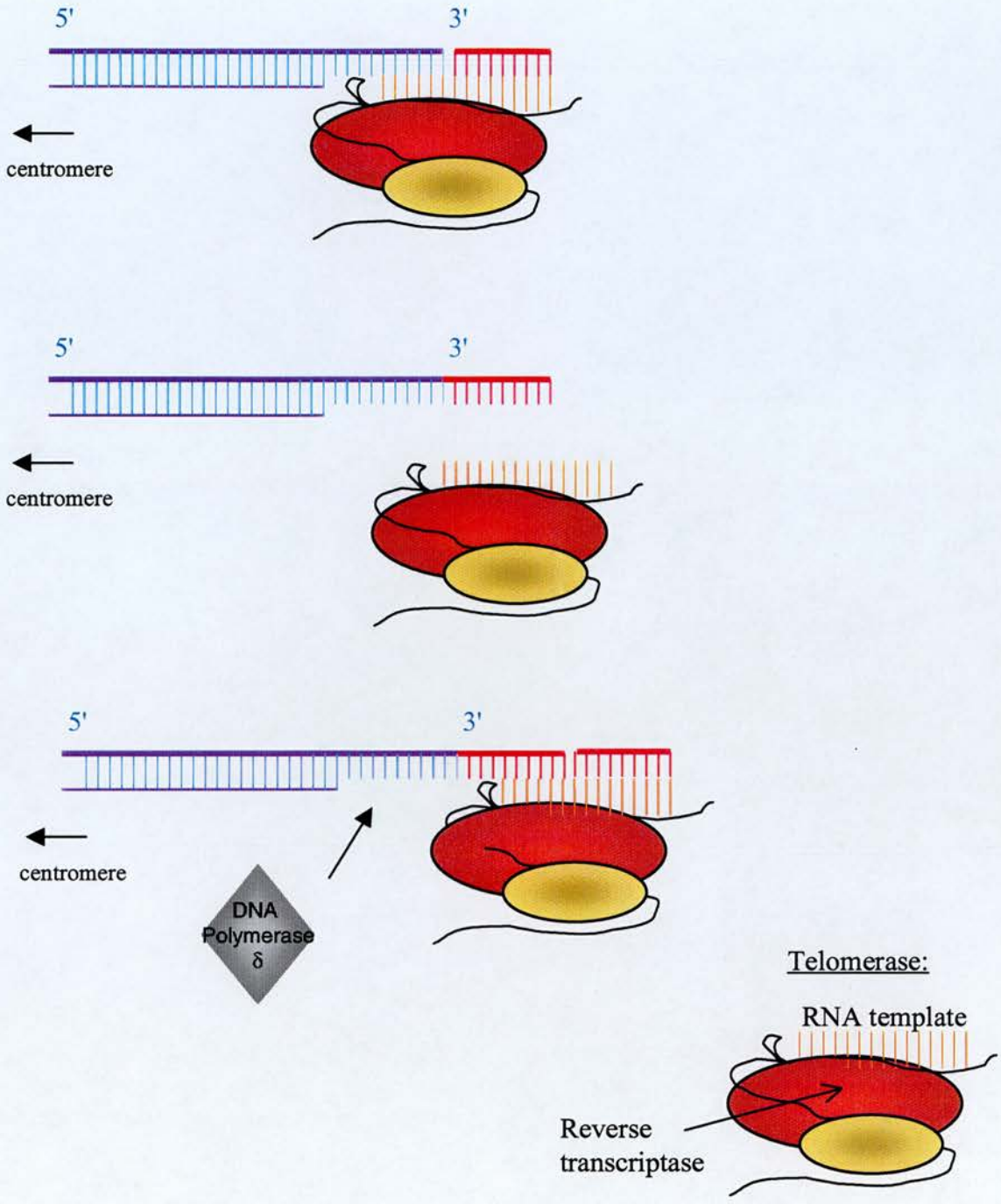

Figure 1.2 The *de novo* synthesis of telomere repeats at the ends of linear DNA by telomerase. Telomerase is recruited to the end of the chromosome, a portion of the RNA is aligned with the 3' single stranded overhang and the reverse transcriptase catalytic subunit copies new repeats from the template region. Alterations in the template region of the RNA lead to alterations in the telomere repeat sequence. Lagging strand synthesis is completed by DNA polymerase δ.

population. In cells lacking telomerase activity telomeres progressively shorten and senescence occurs.

In budding yeast lacking telomerase activity a small proportion of the cells survive by extending their telomeres using alternative lengthening of telomere (ALT) pathways (Lundblad and Blackburn, 1993). These increase the telomere length rapidly using methods such as unequal recombination between telomeres and by rolling circle replication. Methods of ALT investigated require the ability of telomeres to recombine with homologous sequences or invade strands of DNA with regions of homology. In budding yeast deletion of mismatch repair factors that prevent recombination between regions of low homology, such as *MLH1* and *PMS1*, allows for the recombination between telomeres that is necessary for telomere elongation in the absence of telomerase (Rizki and Lundblad, 2001). Genes, such as *RAD50, RAD51* and *RAD52*, that are involved in homologous recombination are important for the generation of survivors from populations unable to activate telomerase, and deletion of *RAD52* leads to an inability of cells deficient for telomerase to escape senescence (Lundblad and Blackburn, 1993). Telomere length changes indicative of ALT have also been observed in human cells (Perrem *et al.*, 1999).

The repetitive nature of telomeric DNA creates an array of tandemly repeated motifs for protein binding and can therefore form the basis for a counting system with which the cell can monitor telomere length. The telomeric repeats are bound by a number of proteins with varying roles in telomere protection, length regulation, replication and silencing. A myb-like DNA binding domain, called the telobox, that binds preferentially to double stranded telomeric DNA has been identified in telomeric proteins in yeast, plants and humans (Bilaud *et al.*, 1996). These proteins bind as homodimers and form the basis of a counting mechanism that determines the size of the telomere and therefore regulate the activity of telomerase relative to the amount of repeats lost by erosion. Proteins such as Cdc13p in budding yeast and Pot1p in fission yeast, that bind to single stranded G-rich overhangs are purported to

be involved in recruiting telomerase to telomeres and may also have a role in end protection. (Nugent, *et al.,* 1996, Baumann and Cech, 2001).

End-to-end joining would be undesirable at telomeres, and therefore it would appear to be strange that proteins involved in non-homologous end joining (NHEJ) should be found here. Deletion of the Ku proteins leads to telomeric shortening in both budding yeast and fission yeast (Nugent *et al.,* 1998, Manolis *et al.,* 2001) and when combined with other mutations affecting the telomere can result in an increase in the frequency of end-to-end fusions and the rate of recombination between telomeres (Bailey *et al.,* 1999, Baumann and Cech, 2001, Ferreira and Cooper, 2001). This indicates that these proteins, which bind to double strand breaks, are not at the telomere in a NHEJ role but instead are involved in end protection and therefore have a modified role in the context of the telomere. Several more proteins that are involved in the response to and repair of DNA damage are present at telomeres, such as Sir2p, Sir3p and Sir4p in budding yeast (Gottschling *et al.,* 1991). A proportion of these proteins are disassociated from telomeres and relocated to double strand breaks in response to DNA damage. (Martin *et al.,* 1999, McAinsh *et al.,* 1999, Mills *et al.,* 1999). Other protein complexes involved in homologous recombination and the single strand invasion of duplex DNA during DNA damage repair, such as the Mre11/Rad50/Xrs2 complex in budding yeast and the RAD50/MREll/NBS1 complex in mammalians cells, and ATM homologues have also been found to have an effect on telomeres (Nugent *et al.,* 1998, Zhu *et al.,* 2000, Ritchie *et al.,* 1999, Naito *et al.,* 1998).

The tertiary structure of the telomere is important in mediating telomere function and may be important for controlling access of telomerase to the telomere. Mammalian chromosomes end in large loop structures, termed T-loops, which are also present at trypanosome telomeres (Griffith *et al.,* 1999, Muñoz-Jordán *et al.,* 2001). It is suggested that these telomeres fold back on themselves and the single stranded overhang invades the preceding duplex DNA to form a D-loop, providing a method by which the single stranded overhang can be sequestered away from telomerase (Griffith *et al.,* 1999). It is unclear whether a shorter telomere such as those found in

budding yeast and fission yeast would be able to form such a structure, although it is known that in *S. cerevisiae* Rap1p is capable of bending DNA (Hofmann *et al.*, 1989).

In budding yeast and fission yeast, genes in the vicinity of telomeres are transcriptionally repressed in a reversible manner so that only a proportion of cells in a genetically homogenous population express the gene. This is referred to as Telomere Position Effect (TPE) (Gottschling *et al.*, 1990, Nimmo *et al.*, 1994). The transcriptional status is stable over several generations as seen in colony sectoring experiments in budding yeast (Gottschling *et al.*, 1990). The terminal telomeric repeats are packaged into a non-nucleosomal structure, similar to that seen at complex centromeres in higher eukaryotes (Wright *et al.*, 1992) and histones in the telomeric region are hypoacetylated, a feature of transcriptionally repressed chromatin (Braunstein *et al.*, 1993). It has been suggested that TPE may be due to encroachment of heterochromatin like structures into the neighbouring region of the genome (Gottschling, 1992).

In *S. cerevisiae* sequestration of DNA to the nuclear periphery facilitates transcriptional repression of genes in the surrounding region (Andrulis *et al.*, 1998). In many organisms telomeres locate to the nuclear periphery and form a cluster at the centrosome prior to meiosis (von Wettstein *et al.*, 1984). This is important for chromosomal alignment and pairing. When the localisation of meiotic telomeres is disrupted, as for *S. pombe* cells with mutations in *kms1* or *ndj1*, the premeiotic arrangement of chromosomes in the nucleus is defective, the efficiency of homologue pairing decreases and the number of recombination events between sister chromatids is drastically reduced (Shimanuki *et al.*, 1997, Trelles-Sticken *et al.*, 2000). Misalignment of chromosomes and failure to pair homologues may lead to chromosome missegregation, resulting in genomic instability and aneuploidy.

In 85% of human tumours studied expression of the telomerase catalytic subunit has been reactivated allowing cells to regain a proliferative capacity. In mice, however, telomerase is normally present in somatic cells and it has been shown in tissue

culture that although the activation of telomerase allows immortalisation of cells it is not sufficient to cause the genomic instability found in many tumours (Bodnar *et al.*, 1998, Varizi and Benchimol, 1998, Jiang *et al.*, 1999, Zhu *et al.*, 1999). This suggests that the activation of telomerase itself is not mutagenic and does not cause genomic instability, thus telomerase activity in a cell alone is not carcinogenic. When combined with other factors, however, such as loss of function of p53 or cell transformation by a virus such as the human papilloma virus that disrupts checkpoints, telomerase activity is an important factor in the continued uncontrolled division of damaged cells resulting in tumour formation. In tumour cells without telomerase activity another method of telomere maintenance must be adopted. Inactivation of missmatch repair mechanisms, which normally act to prevent recombination between sequences that share rough homology, is an important factor in telomerase independent cell proliferation (Rizki and Lundblad, 2001, reviewed in Fishel, 2001).

1.2 Vertebrate telomeres

1.2.1 Overview of telomere function in vertebrate systems

Discrepancies in the distribution of the active telomerase catalytic subunit in mice and humans may reflect differences in their mechanisms of controlling the access of telomerase to the telomere. In both organisms telomeres are shortened upon each cell division and cells senesce after a predetermined number of divisions. The continued division of cells beyond senescence may lead to gross chromosomal abnormalities before the cells enter crisis. Occasionally survivors arise from crisis and go on to form clones of cells. These cells have short but stable or elongated telomeres and telomerase activity is detectable in many cases (Counter *et al.*, 1992). The growth advantage of cells able to activate telomerase at crisis may lead to tumour formation, however the activity of telomerase alone does not result in changes typically associated with malignant transformation and is not the sole cause of carcinogenesis (Bodnar *et al.*, 1998, Varizi and Benchimol, 1998, Jiang *et al.*, 1999, Zhu *et al.*, 1999). Other constraints on cell division such as DNA damage checkpoints and apoptotic pathways also have to be overcome before carcinogenesis occurs.

1.2.2 Telomeric DNA in vertebrate systems

All vertebrates share a common telomere repeat subunit composed of six nucleotides with a consensus sequence of 5'-d(TTAGGG)$_n$-3' (Moyzis *et al.* 1988, Meyne *et al.* 1989). In humans the telomere repeat length is up to 10kb in size depending on the tissue type studied and the age of the individual from which it is derived (de Lange *et al.*, 1990). In mice the telomere repeats are longer, ranging between 20kb and 150kb and with an average size of 100kb (Kipling and Cooke, 1990, Starling *et al.*, 1990). At the end of vertebrate telomeres the 3' G-rich strand forms a single stranded overhang that can be up to 45nt long. The 3' overhang is found both in dividing cells and senescent cells that have arrested in G_0 (McElligott and Wellinger 1997). Electron microscopy of cross linked telomeric DNA from both human and mouse cells has shown that mammalian chromosomes end in a large loop of DNA in which the telomere bends back on itself and the 3' single stranded overhang invades the preceding duplex telomeric repeats to form a D-loop structure. This structure was shown to occur much more frequently than predicted to happen by chance in randomly sheared DNA of the same size and has been termed a T-loop (Griffith *et al.*, 1999). It is proposed that incorporating the 3' single stranded overhang into such a structure may protect the end of telomeric DNA from degradation and control the access of telomerase to the end of the chromosome (Griffith *et al.*, 1999). A diagram of the T-loop structure and interacting proteins is shown in Figure 1.3.

1.2.3 Proteins interacting with telomeric DNA in vertebrate systems

In human cells the duplex telomeric DNA repeat is bound by two proteins that contain telobox repeat domains but have distinct functions at the telomere; TRF1 and TRF2. Both proteins bind the telomeric DNA as homodimers but the dimerization domains of TRF1 and TRF2 do not interact with one another and so these proteins would appear not to form heterodimers (Broccoli *et al.*, 1997). TTAGGG repeat binding factor 1, TRF1, was discovered by using TTAGGG repeats as a probe in mobility shift assays (Zhong *et al.*, 1992). TRF1 binds as a homodimer to double

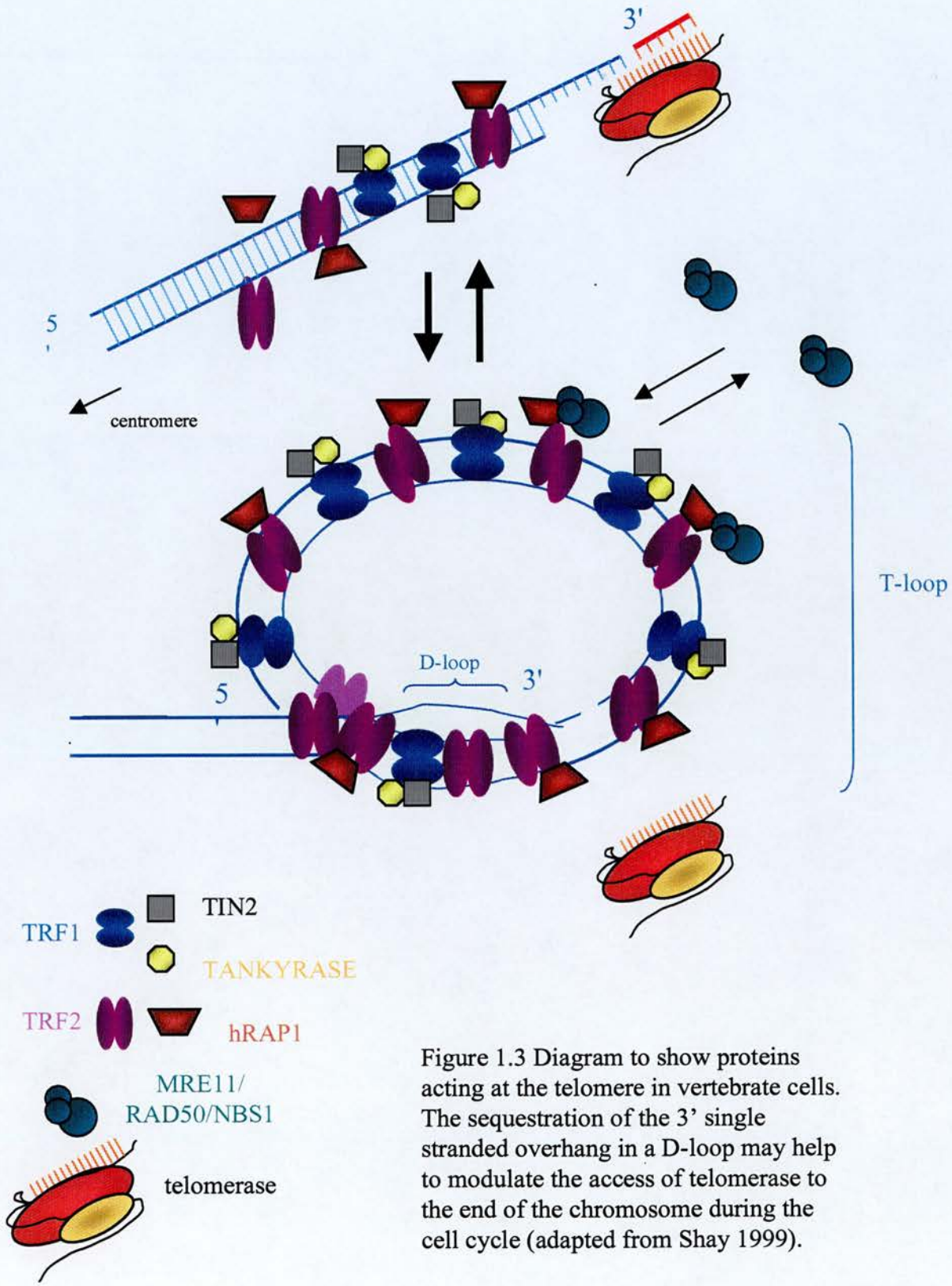

Figure 1.3 Diagram to show proteins acting at the telomere in vertebrate cells. The sequestration of the 3' single stranded overhang in a D-loop may help to modulate the access of telomerase to the end of the chromosome during the cell cycle (adapted from Shay 1999).

stranded but not single stranded DNA and binds preferentially to multiple tandem repeats of the telomeric TTAGGG sequence via a myb-like DNA binding domain. TRF1 inhibits the access of telomerase to the telomere and therefore acts as a suppressor of telomerase activity (van Steensel and de Lange, 1997). In accordance with this role it has been shown that a truncated version of TRF1 that is no longer able to suppress telomerase activity causes inappropriate telomere lengthening in a telomerase dependent manner. Mutations in the homodimerisation domain of TRF1 impair its ability to bind to telomeric DNA and disrupt telomere localisation (Fairall *et al.*, 2001).

TRF2 was identified as an open reading frame containing a telobox domain similar to that found in TRF1. It was found by immunofluorescence that the product of *TRF2* encodes a protein that binds specifically at the telomeres of human chromosomes *in vivo* (Bilaud *et al.*, 1997, Broccoli *et al.*, 1997). Like TRF1, TRF2 homodimers bind to DNA via myb-like DNA binding domains. TRF2 would appear to have a capping role at the telomere, with its deregulation causing deprotection of the telomere ends and the overexpression of a dominant negative form leading to inappropriate telomere-telomere fusions (van Steensen *et al.*, 1998). Inhibition of TRF2 leads to loss of the 3' single stranded overhang and inappropriate activation of DNA checkpoint pathways mediated by p53, leading to ATM-dependent apoptosis. This indicates that in the absence of TRF2, telomeres are recognised as double strand breaks and elicit a DNA damage response (Karlseder *et al.*, 1999). TRF2 is necessary for the formation of T-loops at the end of the chromosome (Griffith *et al.*, 1999).

Both TRF1 and TRF2 interact with a number of other factors, which mediate facets of their role at the telomere. TIN2 is a TRF1 interacting protein that was cloned by two hybrid analysis using TRF1 as bait. Immunofluorescence experiments to investigate TIN2 localisation detected weak but distinct foci at the telomeres of human chromosomes (Kim *et al.*, 1999). TIN2 is unable to bind to the telomeric DNA and requires to be bound by TRF1 for its localisation. A TIN2 protein lacking the amino terminal sequence causes inappropriate telomere lengthening in a telomerase dependent manner, which is a similar phenotype to that of a truncated

version of TRF1, but it does not displace TRF1 from the DNA. This suggests that TRF1 localisation alone is not enough to inhibit telomere elongation and that TIN2 mediates the TRF1 function in negatively regulating telomere length (Kim *et al.*, 1999).

Tankyrase is another TRF1 interacting protein that was also found in a yeast two hybrid screen and has a similar pattern of nuclear localisation to that of TRF1 by immunofluorescence (Smith *et al.*, 1998). Tankyrase interacts with TRF1 through a domain containing 24 ankyrin repeats. The C-terminus of Tankyrase has ADP ribosylation activity which ribosylates both TRF1 and itself, leading to a reduction in the DNA binding activity of TRF1 *in vitro,* thus enabling telomerase to access the telomere (Smith *et al.*, 1998).

A two hybrid screen for TRF2 interacting factors found a human homologue of the *S. cerevisiae* telomere binding protein Rap1p. Unlike the *S. cerevisiae* protein, hRAP1 does not bind directly to telomeric DNA but instead is recruited to telomeres by TRF2. The human protein shares three domains with scRap1p including a region unique to Rap1, a BRCT domain and a Myb-like domain. Overall hRAP1 has 25% identity with scRap1 (Bibo *et al.*, 2000).

1.2.4 DNA repair proteins with functions at the telomere

Proteins involved in the Non-homologous End Joining (NHEJ) pathway have also been shown to play a role at the ends of chromosomes. It was found that the KU proteins which normally bind to the ends of double strand breaks also bind to mammalian telomeres (Bianchi and de Lange, 1999). The KU70/KU86 heterodimer appears to play a role in preventing end-to-end fusions at the telomere, in contrast to its role in NHEJ. KU70 and KU86 deficient cells both have an increase in the frequency of chromosomal aberrations, including telomeric fusions (Bailey *et al.*, 1999). hKU70 was found to interact with TRF2 in a two hybrid assay, which was confirmed by co-immunoprecipitation experiments (Song *et al.*, 2000). *Scid* mice that are deficient in DNA-PK have a 1.5-2 fold increase in telomere length over wild

type mice and cells derived from such mice have an increase in the incidence of telomeric fusions (Bailey *et al.,* 1999). DNA-PK is related to the ATM protein, which is defective in the human disease ataxia telangiectasia. Patients with this disease suffer from immune deficiencies and a predisposition to cancers amongst other traits. Cells derived from these patients are sensitive to ionising radiation. Human cell lines carrying a dominant negative form of ATM have a normal level of telomerase but shortened telomeres, which is consistent with the phenotype of DNA-PK negative mouse cells (Thomas and Rothstein, 1989).

Proteins involved in double strand break (DSB) repair have also been shown to play a role at telomeres at interphase and may also be involved in telomere replication. Telomeric fusions have been detected in murine cells deficient for components of the DSB repair mechanism. In human cells the RAD50/MRE11/NBS1 complex associates with TRF2 in a cell cycle dependent manner. RAD50 and MRE11 associate with TRF2 throughout interphase but NBS1 only associates during S-phase, and not G1 or G2 (Zhu *et al.,* 2000). Transient recruitment of NBS1 at S phase may recruit helicase activity to "chew back" the 5' strand and create a 3' single stranded overhang, which is necessary for the recruitment of telomerase. There is no evidence that the interaction of the RAD50/MRE11/NBS1 complex with TRF2 is direct and it may occur through a third factor, such as hRAP1. The interaction of TRF2 with the RAD50/MRE11/NBS1 complex could be due to a role of TRF2 in DNA damage repair. However, whilst MRE11 accumulates at regions of DNA damage TRF2 remains localised at the telomeres, suggesting that this is not the case (Zhu *et al.,* 2000). Closure of the T-loop structure found at mammalian telomeres involves invasion of the 3' G-rich overhang into duplex DNA to form a D-loop structure, similar to those seen at sites of homologous DNA recombination for DNA repair. It is possible that the role of the RAD50/MRE11/NBS1 complex at the telomere is similar to that found at sites of homologous DNA recombination and either creates and/or maintains the D-loop structure at telomeres. MRE11 does not interact with TRF1 or Tankyrase (Zhu *et al.,* 2000).

The binding of TRF1and TRF2 to telomeric DNA and the interaction with them of TIN2, Tankyrase, hRap1 and the RAD50/MRE11/NBS1 complex may help mediate T-loop formation at vertebrate telomeres and provide a method by which the 3' single stranded G-rich overhang end is protected from degradation (Griffith *et al.,* 1999).

1.2.5 *De novo* synthesis of the telomere repeats by telomerase in vertebrate cells

The sequence of the human telomerase catalytic subunit was identified by homology to that of the *S. cerevisiae* gene (Nakamura *et al.,* 1997). Mutagenesis of conserved residues in the protein demonstrated that the gene product, hTERT, indeed has telomerase catalytic activity (Weinrich *et al.,* 1997). Ectopic expression of hTERT in post senescent virally transformed cells enables them to proliferate beyond crisis and immortalisation to occur (Counter *et al.,* 1998). In these cells telomeres become stably maintained at a length shorter than the size at which telomeres in control cells trigger crisis. The continued cell division with short telomeres is not accompanied by telomere fusions or an increase in the rate of aneuploidy, suggesting that telomerase may have some capping activity at the telomere (Zhu *et al.,* 1999). C-MYC is a proto-oncogene involved in cell proliferation and differentiation control and whose mis-expression is an important factor in cancers. Interestingly is has been found that the *hTERT* promoter has c-MYC binding sites and that c-MYC can activate transcription of TERT (Wu *et al.,* 1999).

Mice homozygous for a deletion of the telomerase RNA template (*mTR*$^{-/-}$) lack telomerase activity but are viable for 6 generations. In these mice telomeres shorten at a rate of 4.8±2.4kb per generation (Blasco *et al.,* 1997). Cells derived from the fourth generation of *mTR*$^{-/-}$ mice contain chromosomes lacking detectable amounts of telomeric repeats. The cells also displayed an increased rate of aneuploidy and chromosome abnormalities such as end-to-end fusions. Telomere shortening in later generations of these mice is associated with shortened life span, increased incidence of spontaneous malignancies, and a loss of fitness and well being that leads to reduced tolerance of stresses such as wound healing (Rudolph *et al.,* 1999). By the

sixth generation the *mTR*$^{-}$/ mice were suffering from problems in gametogenesis and much reduced litter sizes. Problems in development such as a failure to close the neural tube were also becoming apparent (Herrera *et al.,* 1999). Cells from all generations of these mice studied are able to form tumours when transformed with viral oncogenes, suggesting that telomerase activity is not essential for tumour formation.

Mice that are both deficient for the telomerase RNA template and of a cancer prone background may have one of two distinct phenotypes. *mTR*$^{-}$/ mice that are also deficient for the tumour suppressor gene *INK4a* produce fewer tumours than telomerase producing mice with a similar *INK4a* null background. Reintroduction of telomerase into these *mTR*$^{-}$/ *INK4a*$^{2/3}$ cells restores their oncogenic potential, suggesting that telomerase activity is necessary for the malignant transformation of *INK4a* cells and tumour formation (Greenberg *et al.,* 1999). Conversely in *mTR*$^{-}$/ mouse cells, senescence and crisis can be overridden by the loss of p53, leading to their enhanced carcinogenic potential (Chin *et al.,* 1999). The p53 mediated apoptotic pathway is normally activated by genomic instability resulting from telomere loss, and leads to growth arrest and cell death. In the absence of p53 there is no brake on the system to prevent the division of cells with gross chromosomal abnormalities thus increasing the risk of malignant transformation. In contrast, in *INK4a* null cells the telomeres need to be maintained for continued propagation of the cell line past crisis. The loss of the telomerase RNA subunit removes the possibility of *INK4a* null cells being able to escape crisis by activating telomerase, so reducing the carcinogenic potential of these cells.

1.2.6 Alternative lengthening of telomere pathways of telomere maintenance in vertebrates

In 85% of human cancers telomerase is reactivated while in the remaining 15% the cells have a found a way to perpetuate division beyond crisis. In order for these cells to continue dividing and form a tumour, alternative pathways of telomere

vertebrate		
gene	function at the telomere	reference
TRF1	binds TTAGGG repeats, inhibits the access of telomerase to the telomere, telomere localisation	Zhong *et al.*, 1992
TRF2	Identified by homology to TRF1, binds TTAGGG repeats, protection of the telomere ends, required for T-loop formation, protects 3' overhang	Bilaud *et al.*, 1997, Broccoli *et al.*, 1997
TIN2	requires binding by TRF1 for localisation, inhibits telomere elongation	Kim *et al.*, 1999
TANK1	modifies TRF1 to reduce its DNA binding activity of TRF1 *in vitro.*	Smith *et al.*, 1998
TANK2	TRF1 associated protein, localises to the nuclear periphery	Kaminker *et al.*, 2001
hRAP1	binds to telomeres via interaction with TRF2	Bibo *et al.*, 2000
KU70	NHEJ protein that prevents end-to-end fusions at the telomere, forms part of the protective cap, forms a heterodimer with KU80	Bailey *et al.*, 1999
KU80	NHEJ protein that prevents end-to-end fusions at the telomere, forms part of the protective cap, forms a heterodimer with KU70	Bailey *et al.*, 1999
TERT	telomerase reverse transcriptase catalytic subunit	Nakamura *et al.*, 1997
TLC1	telomerase RNA template	Blasco *et al.*, 1995, Feng *et al.*, 1995
DNA-PK	telomere length regulation, prevents end-to-end fusions	Bailey *et al.*, 1999
MRE11	DNA repair protein	Zhu *et al.*, 2000
RAD50	DNA repair protein	Zhu *et al.*, 2000
NBS1	DNA repair protein	Zhu *et al.*, 2000

Table 1.2 A summary of proteins involved in telomere structure and function in vertebrate cells and their apparent role at the telomere

maintenance are necessary. The mechanisms of ALT are not understood in mammalian cells but the characteristic pattern of highly heterogeneous telomere lengths ranging from very short to very long has been observed in tumour cells lacking telomerase (Murnane *et al.*, 1994, Bryan *et al.*, 1995, Bryan *et al.*, 1997). Fusion of ALT cells to telomerase positive cells results in rapid deletion of the

elongated telomeres and a return to wild type telomere propagation pathways (Perrem *et al.*, 1999). Proteins with functions at the telomere in vertebrate cells are summarised in Table 1.2.

1.3 Telomere formation in *S. cerevisiae*

Vertebrate systems can be difficult to manipulate: creating mutations, deleting genes and transferring mutations into suitable backgrounds for further experiments are all lengthy procedures. There is wide conservation of telomere structure across eukaryotes from single cell to multicellular organisms, allowing for the use of a genetically tractable organism to study telomeres and telomeric pathways and produce data relevant to mammalian systems. Two yeasts which are divergent by 330 to 420 millions of years, *S. cerevisiae* and *S. pombe* (reviewed Sipiczki, 2000), are excellent model organisms for investigation of telomere dynamics and allow for comparison between two divergent systems.

1.3.1 Telomeric DNA in the budding yeast

Telomeres in the budding yeast *S. cerevisiae* are much shorter than those of vertebrate cells with an average length of between 350bp and 500bp. The basic structure of the telomere echoes that of vertebrate cells, with the telomeric DNA consisting of an array of short nucleotide repeats of consensus sequence $TG_{1\text{-}3}$, and a 3' G-rich single stranded overhang (Blackburn and Greider, 1995). The 3' single stranded overhang arises independently of telomerase activity and is cell cycle dependent, being produced in S-phase as a result of cell cycle regulated degradation of the complementary C-rich strand (Wellinger *et al.*, 1993, Wellinger *et al.*, 1996). The 3' single stranded overhang is necessary for telomerase activity as telomerase is unable to extend a blunt ended DNA molecule (Wang and Blackburn, 1997). Larger, less conserved, tracts of repetitive DNA called telomere associated sequences abut the telomeric DNA. The telomere associated sequences are formed from two types of array named X' and Y' elements. All yeast telomeres have one X' element, while only some have Y' elements, which may be present in multiple copies and may be

interspersed with TG_{1-3} repeats. (Chan and Tye, 1983). Telomeric DNA is packaged into a non-nucleosomal structure called the telosome (Wright *et al.*, 1992). The addition of telomeric sequence to the end of the chromosome is restricted to a few bases per generation and slows as the telomeres increase in length, while the rate of loss of telomere repeat sequence in the absence of telomerase is constant. This suggests that as telomeres become longer, the complex that builds upon the telomeric repeats inhibits telomerase activity (Marcand *et al.*, 1999).

1.3.2 The *S. cerevisiae* telomere forms a transcriptionally repressive complex

Silencing at telomeres in budding yeast was first described using a terminal truncation that placed a *URA3* gene adjacent to newly formed telomeric sequence. The gene placed in this environment was subject to reversible repression of transcription so that only a proportion of cells in a genetically homogenous population express the gene. This reversible repression was termed Telomere Position Effect (TPE) (Gottshling *et al.*, 1990). The ability to silence genes in close proximity to a telomere extends uninterrupted from the telomeric sequence with a gradual reduction in the level of transcriptional repression the further the gene is from the chromosome end. TPE is highly sensitive to the size or structure of the telomere repeats (Kyrion *et al.*, 1993). The expression of a gene in a region affected by TPE variegates and is affected by alterations in the levels of silencing proteins and the strength of a gene's promoter (Renauld *et al.*, 1993). The transcriptional state of a gene affected by TPE is maintained in repressive or active state for several generations. This perpetuation of the transcriptional state can be seen by inserting an ADE2 gene near to the telomere. In an *ade2*Δ background, colonies are red on media containing limiting adenine, colonies able to express the gene inserted at the telomere are white. When a change in the transcriptional state of the ADE gene at the telomere occurs a switch in the colour of daughter cells results in the growth of sectored colonies (Gottschling *et al.*, 1990). Cells can only swap silencing state at TPE during G2 (Aparicio and Gottschling, 1994). Transcriptional silencing in yeast is associated with a localised reduction in histone acetylation (Braunstein *et al.*, 1993) and a reduction in Dam methylase sensitivity is found in genes silenced by TPE,

suggesting that TPE may be due to encroachment of heterochromatin-like structures into the gene (Gottschling, 1992).

It has been observed that adding tracts of telomeric DNA on the telomere distal side of a telomeric marker has no effect on TPE at that locus, but that elongation of the telomere by telomerase does reduce TPE. This scenario predicts that there is a telomere terminus specific binding factor critical for TPE that is moved away from the site of the gene studied, thus reducing TPE (Wiley and Zakian, 1995).

1.3.3 Proteins involved in the establishment of transcriptionally repressed chromatin at telomeres

Several proteins including Rap1p, Sir2p, Sir3p, Sir4p, as well as two ribosomal amino terminal acetylases, Nat1p and Ard1p, are involved in silencing both at telomeres and *HMR* (Aparicio *et al.*, 1991). Expression of a gene in a region affected by TPE is thought to be affected by alterations in the levels of Sir2p, Sir3p and Sir4p. Sir2p was shown to be necessary for the silencing of transcription at telomeres (Gottshling *et al.*, 1990). Sir2p functions as a histone deacetylase whose over expression promotes the global deacetylation of histones (Imai *et al.*, 2000). Targets of Sir2p include the lysine 16 residue of histone H4, which is necessary for the recruitment of Sir3 (Hecht *et al.*, 1995). Sir3p and Sir4p are recruited to telomeres by interactions with the carboxy-terminus of Rap1p and interact with the N-terminal tails of histone H3 and H4 *in vitro* (Morretti *et al.*, 1994, Hecht *et al.*, 1995). Sir3p is a dose dependent enhancer of silencing whose recruitment to telomeres is a limiting factor in telomeric silencing (Renauld *et al.*, 1993, Lustig *et al.*, 1996). Sir3p has also been found to interact with itself and with the Sir4p C-terminus via a domain that is nonessential for silencing (Park *et al.*, 1998). Recruitment of Sir3p and Sir4p is thought to be an initiating event in establishing silencing at telomeres. Tethering a LexA-Sir3p or a LexA-Sir4p fusion protein to internal tracts of telomeric TG_{1-3} repeats and at telomeres nucleates the hyper repression of genes in the vicinity. Tethered LexA-Sir3p/4p can nucleate silencing at the telomere in a *rap1-17* mutant that is unable to recruit Sir3 or Sir4p (Lustig *et al.*,

1996, Maillet *et al.*, 1996). Telomeres may fold back so that Sir3p and Sir4p proteins can interact with histones in the subtelomeric chromatin prior to the non-nucleosomal structure. Rap1p does not interact with histones itself. Telomeric folding is required to give rise to stable TPE (Bruin *et al.*, 2000).

Factors important for TPE are also involved in silencing at the silent mating type loci in *S. cerevisiae*. Telomeric silencing and *HMR* are thought to compete for silencing factors, in a *rap1*^{s} mutant with elongated telomeres silencing at the mating type loci is reduced. This is proposed to be due to titration of a limiting amount of silencing factors away from *HMR* by the telomeres (Sussel and Shore, 1991, Buck and Shore, 1995).

1.3.4 Proteins that bind to duplex DNA at telomeres in *S. cerevisiae*

Repressor and Activator Protein, Rap1p, is a double stranded DNA binding protein that binds to the duplex telomere repeats. As its name suggests Rap1p also serves other functions in the cell making it an essential gene for mitotic growth. Since deletion of *RAP1* is lethal its function at telomeres has been elucidated from phenotypes resulting from mutations in different regions of the gene. *In vivo* Rap1p binds telomeric DNA as a monomer via a central DNA binding domain containing two myb-like DNA binding sub-domains (Conrad *et al.*, 1990). It has been shown by immunofluorescence that Rap1p co-localises with the ends of paired bivalent chromosomes, and that in interphase cells anti-Rap1p staining gives a punctate pattern of foci at the nuclear periphery (Klein *et al.*, 1992). Rap1p is required for telomere length control, negatively regulating telomere length possibly by blocking access of telomerase to telomeres (Palladino *et al.*, 1993). Binding sites for Rap1p occur approximately every 18bp of telomeric DNA giving approximately 25 to 30 Rap1p binding sites per telomere (Gilson *et al.*, 1993). The rate of telomere turnover is controlled by levels of Rap1p and disrupting the Rap1p binding sites in the telomere repeats by altering the telomerase RNA template leads to the uncontrolled elongation of telomeres. The severity of the phenotype depends upon the level of disruption conferred to Rap1p binding by the modified telomere repeats (Krauskopf

and Blackburn, 1996). Rap1p nucleates a protein complex that is required for TPE via interactions at its C-terminus (Aparicio *et al.*, 1991, Kyrion *et al.* 1993). It has been shown that Rap1p is capable of bending DNA *in vitro*, which may be important for the folding of a tertiary structure at telomeres (Muller *et al.*, 1994).

Another telomere binding protein from *S. cerevisiae* was identified through its ability to bind vertebrate telomere sequences *in vitro*. This was named Telomere Binding Factor 2, Tbf1p (Brigati *et al.*, 1993). The last 60 amino acids at the C-terminus show homology to myb-like DNA binding domains but it does not recognise the budding yeast $TG_{(1\text{-}3)}$ telomeric repeat. Tbf1p is essential for mitotic growth and its exact function in the cell is unclear although it would appear to be capable of binding DNA.

1.3.5 Rap1 interacting proteins

Sir3p and Sir4p were found by a two hybrid screen to interact with the carboxy-terminus of Rap1p. The C-terminus of Rap1p contains a domain of 28 amino acids that is essential for silencing at telomeres and *HMR*, within this domain is a group of 8 amino acids essential for telomeric silencing alone. *RAP1* alleles that cannot interact with Sir3p are deficient for silencing and a *rap1* mutant lacking the C-terminal 28 amino acids is immune to the effects of Sir3p overproduction (Moretti *et al.*, 1994, Lui *et al.*, 1994).

Rap1 Interacting Factor, Rif1p, was identified by two hybrid screen using Rap1p as bait. Rif1p interacts with Rap1p through the Rap1p Carboxy-terminus and strains with mutations in *RIF1* have moderately elongated telomeric tracts (Hardy *et al.*, 1992). The silencing of genes inserted into the vicinity of telomeres is improved in a *rif1* mutant (Kyrion *et al.*, 1993). Rif2p is a second Rap1p interacting factor that when mutated has a similar phenotype to a *rif1* mutant. A strain carrying a double *rif1*/*rif2* deletion has a telomere length similar to that of a $rap1^t$ mutant. Rif1p and Rif2p interact *in vivo* and overexpression of either protein leads to shortening of the telomere (Wotton and Shore, 1997). The improvement in telomeric silencing seen in

a Rif1 or Rif2 mutant could be due to competition between Rif and Sir proteins for binding sites at Rap1p C-termini.

1.3.6 Proteins that bind to the single stranded 3' G-rich overhang at the telomere in *S. cerevisiae*

CDC13 was isolated in a screen for mutations that abolished the activity of telomerase at telomeres (Lendvay *et al.,* 1996). Cdc13p binds to the 3' single stranded overhang and is involved in recruiting telomerase *in vivo* but is dispensable for telomerase activity *in vitro* (Lingner *et al.,* 1997b). The presence of Cdc13p at telomeres is important for the addition of telomere repeats and maintaining telomere length. Cdc13p loss leads to unregulated strand specific degradation of the C-rich strand at telomeres thus generating an extended G-rich overhang strand resulting in a *Rad9* dependent cell cycle arrest. The $cdc13^{EST4}$ mutation that was isolated in the original screen is deficient for recruitment of telomerase, leading to the gradual cumulative loss of telomere repeats, resulting in cell senescence and death (Lendvay *et al.,* 1996). Cdc13p also has a protective role at telomeres.

EST1 was isolated in a similar screen to that which identified Cdc13p as an important factor in the recruitment of telomerase *in vivo* (Lundblad and Szostak, 1989). Est1p is a telomerase subunit and, like Cdc13p, is dispensable for telomerase activity *in vitro*. Loss of function of Est1p also leads to the gradual loss of telomeric repeats and cell senescence (Lingner *et al.,* 1997b). Est1p needs a free 3' terminus to bind to the single stranded telomeric DNA and binds to telomeres 500 times less efficiently than Cdc13p (Nungent *et al.,* 1996). Interactions between Est1p and Cdc13p improve the binding affinity of Est1p to telomeres (Qi and Zakian, 2000). Telomerase can be recruited to telomeres by fusing the DNA binding domain of Cdc13p to Est1p. Expression of the fusion protein in a $cdc13^{EST4}$ mutant causes elongation of telomeres as Cdc13p is no longer able to negatively regulate telomerase activity (Evans and Lundblad, 1999). Est1p would appear to be required solely for the recruitment of telomerase to telomeres (Virta-Pearlman *et al.,* 1996).

Cdc13p would appear to be important for the recruitment of telomerase, however it also recruits Stn1p, which is an inhibitor of telomerase. Stn1p and Cdc13p were found to physically interact by two hybrid analysis (Grandin *et al.*, 1997). Loss of Stn1p function leads to deregulation of telomerase recruitment and an increase in telomere length. Stn1p is proposed to function as a negative regulator of recruitment of telomerase by Cdc13p (Grandin *et al.*, 2000).

1.3.7 Cdc13p interacting proteins

Stn1p is primarily a protector of telomere ends that interacts with Cdc13p to negatively regulate the activity of telomerase and prevent degradation of the telomere (Grandin *et al.*, 1997, Chandara *et al.*, 2001). Deletion of *STN1* leads to the activation of a Rad9 cell cycle checkpoint in a similar manner to the deletion of *CDC13*. A *cdc13*Δ mutant can be partially rescued by a Stn1p fusion protein with the DNA binding activity of Cdc13p but still has gradual erosion of telomeric repeats as seen in a telomerase deficient or $cdc13^{EST4}$ strain. This phenotype can be overcome by introducing a second fusion protein comprising the DNA binding domain of cdc13 and a subunit of telomerase so that telomerase can be recruited to telomeres. A *cdc13*Δ mutant with both these fusion proteins has enhanced telomerase recruitment and thus has longer telomeres than a wild type strain (Pennock et al. 2001).

Ten1p was isolated as a weak suppressor of temperature sensitive *stn1* mutations and found to be essential for vegetative growth. Some alleles of *ten1* cause elongation of the telomeres and *ten1-16* mutants arrest at G2/M boundary in a DNA damage checkpoint dependent manner. It has been shown by two hybrid analysis that Ten1p binds to Stn1p and Cdc13p *in vivo*. A *stn1* mutant that has elongated telomeres is unable to interact with Ten1p but mutant alleles of *ten1* that confer deregulation of telomere length still interact with Stn1p and Cdc13p (Grandin *et al*, 2001).

Cdc13p interacts with the catalytic subunit of DNA polymerase α and it may be that this interaction recruits DNA polα to prime synthesis of the lagging strand (Qi and Zakian, 2000). The Polα and Polδ polymerases are essential for lagging strand

synthesis, mutations in either of these polymerases leads to difficulties in manufacturing the lagging strand after telomerase has synthesised the G-rich strand. Cells deficient for DNA polymerase α have elongated tracts of the 3' single stranded overhang and loss of telomeric silencing. Extended G-rich tails in a cell lacking Polα are only apparent when the cell is in S-phase (Martins *et al.*, 2000). It has been shown that G-rich strand and C-rich strand synthesis is coordinated in novel telomere addition (Fan and Price, 1997). Polα activity is necessary for the creation of novel duplex DNA binding sites for telomere binding proteins that regulate telomerase activity. Telomerase activity is therefore coupled to the lagging strand synthesis and failure to create the lagging strand leads to continued action of telomerase at telomeres (Deide and Gottschling, 1999).

1.3.8 Other proteins that affect telomeres in budding yeast

TEL1 and *MEC1* are homologous to ATM, the gene mutated in patients with ataxia telangiectasia. Budding yeast with mutations in either *tel1*$^-$ or *mec1*$^-$ have stably shorter telomeres but a *mec1*$^-$*tel1*$^-$ double mutants suffers from the progressive erosion of telomeres leading to cell senescence in a similar manner to that seen in cells lacking telomerase activity (Lustig and Petes, 1986, Ritchie *et al.*, 1999). Mec1p and Tel1p appear to be similar to protein kinases they but a *mec1*$^-$*tel1*$^-$*tlc1*$^-$ triple mutant that has no telomerase activity has a more pronounced phenotype than the *mec1*$^-$*tel1*$^-$ double mutant, suggesting that Tel1p and Mec1p do not act by altering telomerase activity. It is proposed that Mec1p and Tel1p may control the accessibility of the telomere repeats to telomerase (Ritchie *et al.*, 1999).

TEL2 is an essential gene that produces a low abundance protein. The *tel2-1* mutant causes shortening of telomeres with 100-150nt of telomeric repeats and alleviates silencing at telomeres but has no effect on silencing at *HMR* (Runge and Zakian, 1996). DNA binding studies show that Tel2p binds to telomeric DNA *in vitro*. (Kota and Runge, 1998). A *tel1tel2* double mutant has a similar telomere length to that of a *tel1* mutant alone suggesting that these genes act in the same pathway (Lustig and Petes 1986).

1.3.9 Factors involved in Non-homologous end joining and double strand break repair also have roles at telomeres

yKu70p and yKu80p are components of the NHEJ pathway that interact at the ends of double strand breaks. The role of yKu70p and yKu80p at telomeres in *S. cerevisiae* was detected in two screens; one for mutations that lowered the permissive temperature for growth of a *cdc13-1*^{ts} mutant and another for mutations that enhanced the senescence phenotype of an *est1*Δ mutant (Nugent *et al.*, 1998). Ku70p/Ku80p has been shown to bind telomeric DNA by crosslinking studies (Gravel *et al.*, 1998). Ku70p and Ku80p bind to telomeres as a heterodimer (Milne *et al.*, 1996, Boulton and Jackson, 1996) and are both required for telomere length maintenance with *Ku*Δ cells having stably shorter telomeres. KuΔ cells are also deficient for silencing at telomeres and are temperature sensitive for growth (Boulton and Jackson, 1996). *Ku70Δ/Ku80Δ* cells are deficient for telomeric clustering and localisation of telomeres to the nuclear periphery. Loss of Ku also leads to dispersal of Rap1p, Sir 3p and Sir4p (Laroche *et al.*, 1998). yKu70 has been shown to interact with Sir4p *in vivo* (Tsukamoto *et al.*, 1997). Cells lacking Ku70p/Ku80p maintain the normally S-phase specific G-rich single stranded overhang throughout the cell cycle (Polotnianka *et al.*, 1998). Ku is necessary to form an end structure at telomeres. *yKu80*$^{-}$*tel1*$^{-}$ double mutants have even shorter telomeres than either single mutation and thus yKu80p and Tel1p affect telomeres via different pathways (Gravel *et al.*, 1998).

The yKu70p/yKu80p heterodimer would appear to function as an end protector at telomeres. In a *cdc13Δ*^{EST4} background loss of yKu70p or yKu80p is lethal and double mutants between Ku proteins and *est1*, *est2* or *tlc1* senesce more quickly than their single mutant counterparts (Nugent *et al.*, 1998). In cells transcribing a mutant form of *cdc13* that leads to the formation of longer telomeres the phenotype is dependent on the presence of yKu70p and yKu80p (Grandin *et al.*, 2000). In the absence of yKu70p/yKu80p the Est1p dependent recruitment of telomerase by Cdc13p is compromised. yKu70p/yKu80p may have a telomerase loading function in

association with Cdc13p. A Cdc13p-yKu70p fusion protein has a similar telomere lengthening phenotype to that of a Cdc13p-Est1p fusion protein (Grandin *et al.*, 2000). yKu70 or yKu80 mutations impair growth in a *cdc13-1* background.

The action of the Ku70p/Ku80p heterodimer at telomeres therefore fulfils the criteria for a telomere terminus specific binding factor critical for TPE as predicted by experiments showing that additional internal tracts of telomeric DNA have no effect on TPE but that elongation of the telomere repeats reduces TPE. This was assumed to be due to telomere elongation moving the silencing complex/factor away from the site of the gene studied (Wiley and Zakian, 1995).

Heterogeneity of telomeres within a population and between chromosomes within a cell leads to difficulties for the direct examination of telomeres both *in vivo* and *in vitro*. An assay using an HO cleavage site in the genome next to 81bp of telomeric DNA provides a mechanism of making a unique chromosome end *in vivo*. Induction of the HO endonuclease generates a 81bp telomere with a 4bp G-rich 3' single stranded overhang (Deide and Gottschling, 1999). Using this system the yKu70p/yKu80p heterodimer is recruited to two types of HO induced breaks. One that is flanked by telomeric repeats and another that creates a double strand break in a random DNA sequence. In the former case a telomere is seeded and telomerase is recruited to form a novel telomere, in the other case NHEJ occurs. The telomeric sequence next to the double strand break must therefore have some kind of ability to determine which of the fates occurs at a double strand break and thus regulates the response generated by arrival of the yKu70p/yKu80p heterodimer. The same context probably applies at telomeres and yKu70p/yKu80p from initiating NHEJ at chromosome ends (Deide and Gottschling, 1999).

yKu70p/yKu80p are components of the NHEJ pathway. The Sir proteins also have a role at double strand breaks and are recruited to sites of DNA damage (Boulton and Jackson 1996). DNA damage mediated by bleomycin leads to reversible loss of silencing at telomeres and delocalisation of Sir3p from telomeres (McAinsh *et al.*, 1999). The release of Rap1p and the Sir complex from telomeres in response to DNA

damage is mediated by Rad9p, Mec1p and Ddc1p DNA damage checkpoint signalling proteins (Mills *et al.,* 1999). A single DNA double strand break mediated by an HO site on a plasmid cleaved by HO endonuclease is enough to cause the delocalisation of Sir3p from the telomeres. yKu70p/yKu80p is rapidly localised to the site of DNA damage, followed by low-level recruitment of SIR proteins near to the cleavage site (Martin *et al.,* 1999).

The Mre11/Rad50/Xrs2 complex from the NHEJ pathway is also required for mediating replication of the telomere via the telomerase pathway (Boulton and Jackson 1996, Nugent, *et al.,* 1998). *Mre11*-Δ and *rad50*-Δ strains have no alleviation of transcriptional repression at telomeres (Nugent, *et al.,* 1998).

1.3.10 *De novo* synthesis of telomeric repeats by the telomerase enzyme

Loss of telomerase activity would be expected to cause the gradual shortening of telomeres leading to a cumulative loss of telomere repeats. Screening for mutations that lead to an ever shortening telomere phenotype produced four Est mutants. Double mutants between est mutants have no additive effects indicating that all the est mutants act in the same telomere maintenance pathway (Lundblad and Szostak 1989, Lendvay *et al.,* 1996) The *est2* mutant produced from these screens was found to result from a mutation in the gene encoding the *S. cerevisiae* telomerase reverse transcriptase catalytic subunit. The gradual loss of telomeric repeats on each cell division in an *est2*$^{-}$ mutant leads to a decrease in cell viability and senescence (Lingner *et al.,* 1997). Telomerase activity levels in a cell does not always lead to an increase in telomere length suggesting that there is a method of controlling access of the enzyme to the telomere. Although telomerase activity is detectable in G_1 cells, *de novo* telomerase synthesis does not occur in G_1 phase, with active telomerase addition only happening in M-phase (Deide and Gottschling, 1999). This control of access to telomeres may be due to recruitment factors such as Cdc13p and Est1p not being at telomeres in G1 phase, the telomeric chromatin being in an inaccessible state, or a combination of such factors.

The RNA component of the telomerase holoenzyme is encoded by *TLC1* (Singer and Gottschling, 1994). Mutations in *TLC1* cause a similar phenotype to that of an *est2* mutant with the gradual erosion of telomeres leading to senescence and a high level of cell morbidity. Mutations in the telomerase RNA template that alter Rap1p binding sites in the telomeric repeats cause telomere elongation or erosion depending upon the site of the mutation. Over time, eroded wild type telomere repeats are replaced by the mutant form, which weakens Rap1p binding affinity for duplex telomeric DNA and destabilises length regulation. This causes a large increase in telomere length depending on the amount to which Rap1p is displaced, in a manner consistent with a loss of negative regulation of telomerase. Sequence changes that cause telomere shortening were also found suggesting that loss of negative regulation of telomerase activity caused by a loss of Rap1p binding is not enough to allow telomere addition. It may be the case that these mutations in the RNA template prohibit the binding of factors necessary for the recruitment of telomerase, such as Cdc13p or Est1p (Prescott and Blackburn, 2000).

EST1 and *EST3* encode additional subunits of the telomerase holoenzyme and *EST4* encodes the single strand overhang binding protein involved in recruitment of telomerase to the telomere, Cdc13p. Immunoprecipitation experiments have shown that Est3p interacts with telomerase RNA. *EST1*, *EST3* and *EST4-CDC13* are all dispensable for telomerase activity *in vitro* (Lingner *et al.*, 1997b) but interact with other components of the telomerase holoenzyme *in vivo* (Zhou *et al.*, 2000). Fusion of Est3p to Cdc13p greatly increases the access of telomerase to telomeres in a similar manner to the fusion between Est1p and Cdc13p (Hughes *et al.*, 2000).

1.3.11 Alternative lengthening of telomeres pathways in budding yeast

Telomerase is the major pathway of telomere elongation, however in the absence of telomerase a subset of cells in a population may escape senescence by adopting an Alternative Lengthening of Telomeres (ALT) pathway. There are two ALT pathways of telomere elongation in the absence of telomerase both of which are dependent on *Rad52* and one of either *Rad50* or *Rad51*. In the type I pathway amplification of the

Saccharomyces cerevisiae		
gene	**function at the telomere**	**reference**
CDC13	telomeric single stranded DNA binding protein	Lendvay *et al.*, 1996, Lingner *et al.*, 1997b
EST1	binds terminus of chromosome, interacts with Cdc13p and is involved in recruitment of telomerase to the telomere	Lundblad and Szostak, 1989
EST3	involved in recruitment of telomerase to the telomere	Lendvay *et al.*, 1996
HDF1	yKu70, NHEJ protein binds to telomere ends, involved in end protection	Boulton and Jackson,1996
HDF2	yKu80, NHEJ protein binds to telomere ends, involved in end protection	Boulton and Jackson,1996
MEC1	kinase with homology to TEL1 and human ATM1	Ritchie *et al.*, 1999
MRE11	DNA repair protein	Boulton and Jackson 1996, Nugent, *et al.*, 1998
POL1	DNA polymerase alpha	Qi and Zakian, 2000
RAD9	DNA repair protein	
RAD50	NHEJ protein necessary for ALT	Boulton and Jackson 1996, Nugent, *et al.*, 1998
RAD51	DNA repair and recombination protein, necessary for ALT	Lundblad and Blackburn 1993
RAD52	DNA repair and recombination protein, necessary for ALT	Lundblad and Blackburn 1993
RAP1	telomeric double stranded DNA binding protein, involved in telomere length regulation and recruiting silencing factors	Conrad *et al.*, 1990
RIF1	Rap1 interacting factor	Hardy *et al.*, 1992, Kyrion *et al.*, 1993
RIF2	Rap1 interacting factor	Wotton and Shore, 1997
SIR2	silent information regulator, histone deacetylase	Gottshling et al 1990, Imai *et al.*, 2000
SIR3	silent information regulator, recruited by Rap1 C-terminus and histone tails	Gottshling et al 1990
SIR4	silent information regulator, recruited by Rap1 C-terminus and histone tails	Gottshling et al 1990
STN1	suppressor of Cdc13p	Grandin *et al.*, 1997, Chandara *et al.*, 2001
TBF1	Telomere binding factor, binds to vertebrate	Brigati *et al.*, 1993

	telomere repeat, function in budding yeast unknown.	
TEL1	telomere length regulator protein	Lustig and Petes 1986
TEL2	telomere length regulator protein	Runge and Zakian, 1996
TERT	telomerase catalytic subunit	Lendvay *et al.*, 1996, Lingner *et al.*, 1997
TEN1	weak suppressor of STN1	Grandin *et al*, 2001
TLC1	telomerase RNA template	Singer and Gottschling 1994
XRS2	DNA repair protein	Nugent, *et al.*, 1998

Table 1.3 A summary of proteins involved in telomere structure and function in budding yeast and their apparent role at the telomere

subtelomeric Y' elements occurs and in type II sudden, bulk addition of long tracts of telomere repeats occurs, both of which are dependent upon RAD52 mediated mechanisms of recombination. Deletion of *RAD52* leads to an inability to generate survivors in the absence of telomerase activity, as does deletion of both *RAD50* and *RAD51* (Lundblad and Blackburn, 1993).

1.3.12 Telomere positioning in the *S. cerevisiae* nucleus

At interphase, yeast telomeres are clustered in six to seven foci near the nuclear periphery (Gotta *et al.*, 1996, Martin, *et al.*, 1999). Sequestration of DNA to the nuclear periphery facilitates transcriptional repression of genes in the surrounding region (Andrulis *et al.*, 1998). Telomeric localisation at the nuclear periphery is not sufficient for transcriptional repression, since telomeres are still located at the nuclear periphery in mutants deficient for TPE (Tham *et al.*, 2001). *Ku70Δ/Ku80Δ* cells, which are deficient for telomeric silencing are also deficient for telomeric clustering and localisation at the nuclear periphery (Laroche *et al.*, 1998). The functional significance of locating telomeres at the nuclear periphery is not known; however sequestering silencing proteins in a limited number of foci may protect the rest of the genome from transcriptional silencing (Tham and Zakian, 2000).

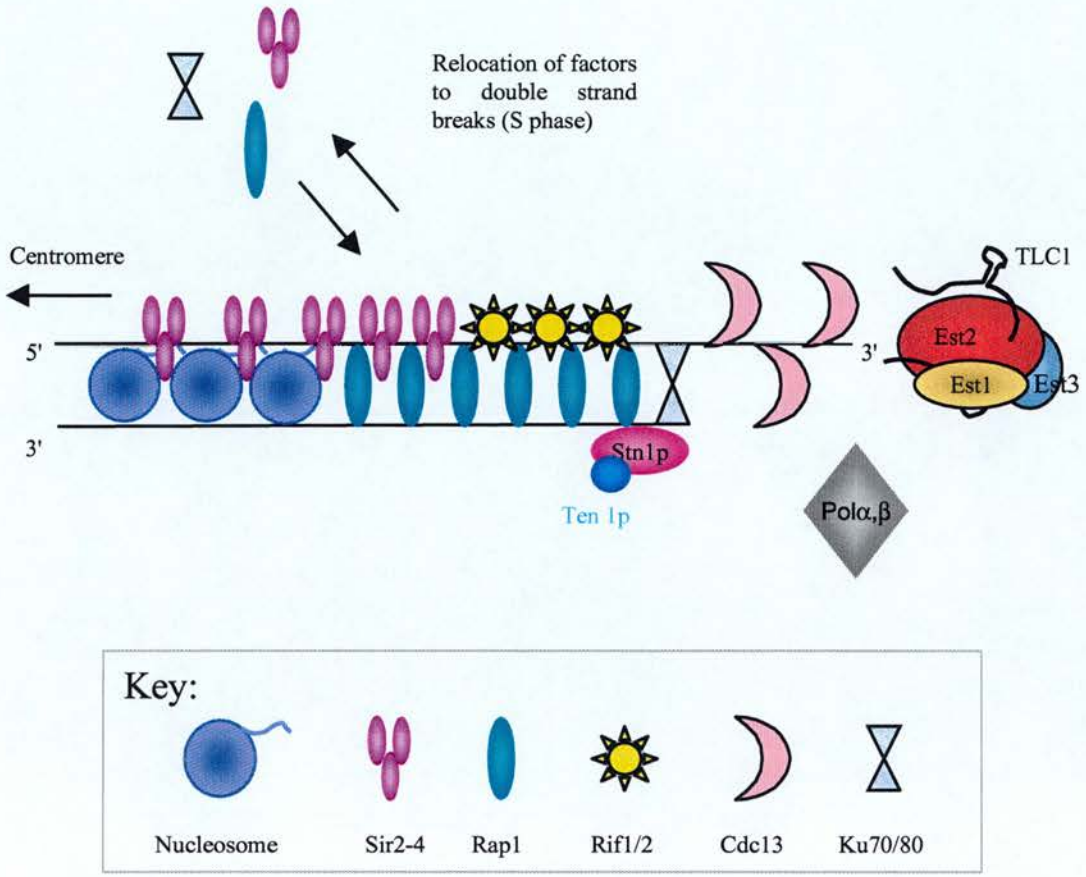

Figure 1.4 Diagrammatic representation of factors involved in telomere structure and function in budding yeast. Some factors involved in silencing at the telomere are also necessary for double strand break repair and are released from the telomere in response to DNA damage in a process that is dependent on the Rad9p, Mec1p and Ddc1p checkpoint proteins (Mills *et al.*, 1999).

Proteins with functions at the telomere in *S. cerevisiae* are summarised in Table 1.3. and a cartoon of their interactions at the telomere shown in Figure 1.4.

1.4 Telomeres in the fission yeast *S. pombe*

1.4.1 The fission yeast

Fission yeast *S. pombe* are about 330 to 420 million years divergent from budding yeast (reviewed Sipiczki, 2000). The fission yeast genome is around 13.9Mb in size and is divided amongst three chromosomes of 5.7Mb, 4.7Mb and 3.5Mb. These chromosomes are named chromosome I, II and III respectively and encode approximately 5-10,000 genes. Fission yeast is a genetically tractable organism that is equally divergent from budding yeast and vertebrates and therefore analysis of molecular genetics in both of the yeasts provides an excellent system with results that can be extrapolated to higher eukaryotes.

1.4.2 *S. pombe* chromosomes in meiotic and mitotic movement

In the fission yeast, telomeric clusters and centromeric clusters are positioned in the nucleus in a cell cycle-dependent manner, a summary of which is shown in Figure 1.5. In mitosis, telomeres are located at the nuclear periphery and centromeres are clustered near the spindle pole body (SPB), the *S. pombe* equivalent of a centrosome (Funabiki *et al.*, 1993). Upon entry into meiosis the nucleus undergoes reorganisation and the chromosomes adopt a characteristic formation often referred to as a 'bouquet' like arrangement (Fussel, 1987). The telomeres become delocalised from their previous positions and cluster at the nuclear periphery and the centromeres cluster away from the SPB (Chikashige *et al.*, 1997). The spindle pole body then leads the nucleus in rapid traverse of the cell called 'horsetail' movement in which the SPB oscillates between the cell poles dragging the chromosomes behind (Chikashige *et al.*, 1994). The bouquet arrangement of the chromosomes at meiotic prophase and subsequent 'horsetail' movement facilitates the alignment of and homologous recombination between sister chromatids (Niwa *et al.*, 2000).

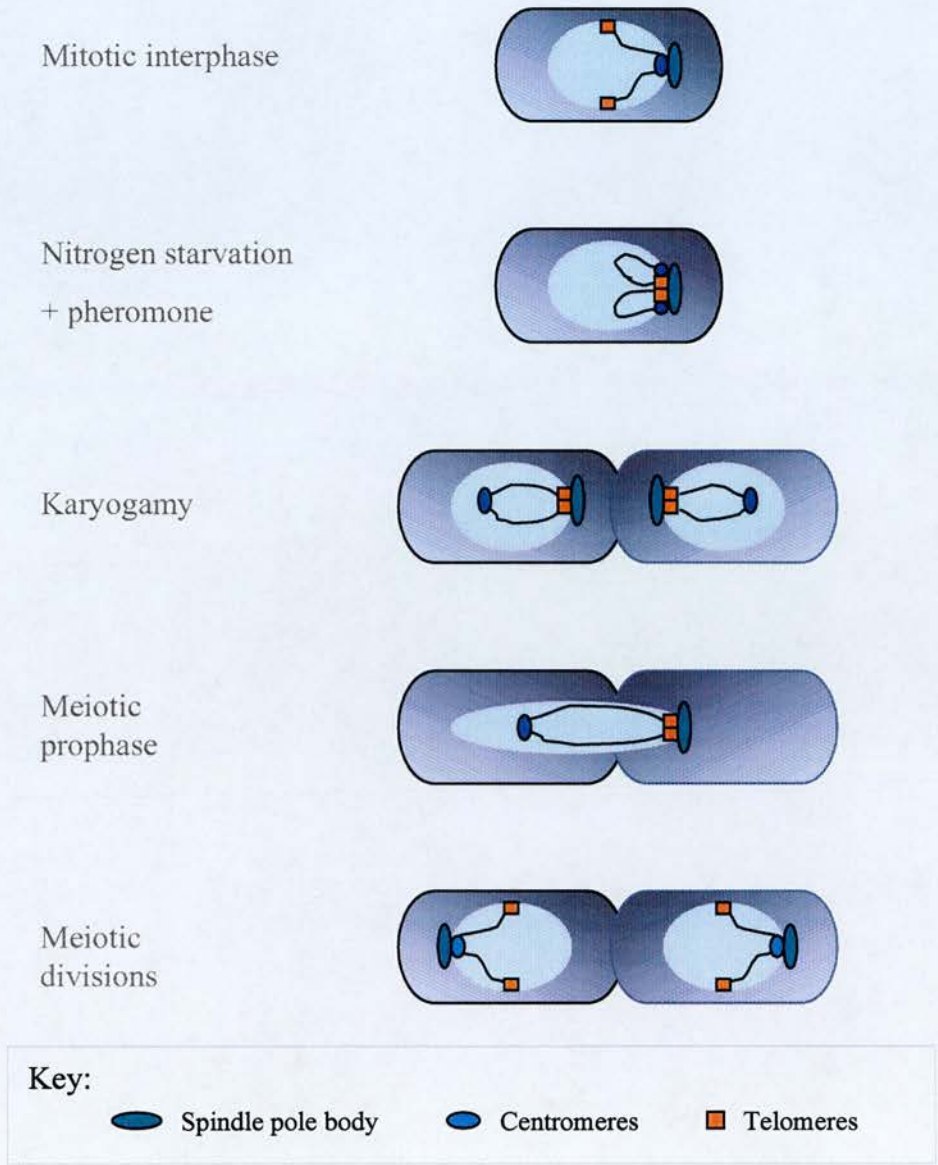

Figure 1.5 Diagram showing the position of the telomeres and centromeres at the nuclear periphery during the cell cycle in the fission yeast, *Schizosaccharomyces pombe*. During mitotic growth the centromeres are located at the spindle pole body (Funabiki *et* al., 1993). Upon entry into meiosis the telomeres and centromeres switch position so that the telomeres are at the leading edge of the cell during 'horsetail' movements (Chikashige *et al.*, 1997).

adapted from Yamamoto and Hiraoka (2001)

Mutations affecting dynein heavy chain formation affect the ability of the nucleus to make the 'horsetail' movements and are unable to pair homologous chromosomes efficiently (Yamamoto *et al.,* 1999). Cells with mutations in SPB, components Ndj1p and Kms1p are defective for recruitment of telomeres clustered at the nuclear periphery to the SPB, are impaired in karyogamy and have a reduced rate of recombination between homologous chromosomes (Trellis-Sticken *et al.,* Shimanuki *et al.,* 1997). Live analysis of strains carrying mutations in the telomeric genes *taz1* and *lot2* showed them to be defective for telomeric attachment at the SPB resulting in aberrant movement of the DNA during 'horsetail' phase. These mutants also have a reduced rate of recombination between homologous chromosomes (Nimmo *et al.,* 1998).

1.4.3 Silencing at heterochromatin-like regions in *S. pombe*

In the fission yeast chromatin is packaged into a transcriptionally repressed structure at centromeres, silent mating type loci and telomeres. When genes are inserted into centromeric regions they are subject to a reversible repression of gene expression similar to position effect variegation found at heterochromatic regions in *Drosophila* (Allshire *et al.,* 1994). Genes in the vicinity of telomeres in *S. pombe* are also subject to transcriptional repression, with telomere mediated chromosome breakage of minichromosomes resulting in the reversible silencing of genes adjacent to the break site (Nimmo *et al.,* 1994). The transcriptionally repressed state at all three regions of silent chromatin is disrupted by mutations in global silencing factors such as Swi6p, Rik1p and Clr4p. Mutations in *swi6* lead to alleviation of silencing at these transcriptionally repressed regions and mutation in either *rik1* or *clr4* disrupt the localisation of Swi6p resulting in a similar loss of silencing phenotype (Ekwall *et al.,* 1995). Alleviation of repression at centromeres and the silent mating type loci is associated with impaired function leading to chromosome missegregation, defective mating type switching and haploid meiosis (Ekwall *et al.,* 1996). Many proteins involved in higher order chromatin structures contain a signature motif called the chromodomain. The sequencing of the *S. pombe* genome has allowed for the identification of potentially homologous genes across species by searching for

sequence similarity or genes with a particular function by searching for characteristic protein motifs. Such searches allow for the identification of potentially interesting sequences in hypothetical open reading frames. A protein involved in centromeric silencing was identified as a hypothetical protein with an N–terminal chromodomain and named Chromodomain Protein 1, Chp1p (Doe *et al.,* 1998). A second chromodomain protein, Chp2p, was identified by sequence similarity to *swi6* and like Swi6p has a chromodomain and a shadow chromodomain. Deletion of *chp2* but not *chp1* leads to the alleviation of transcriptional repression at the telomere (Thon and Verhein-Hansen, 2000). As well as the global silencing factors, fission yeast also has silencing factors that affect specific regions of transcriptionally repressed chromatin. For example Taz1p and Rap1p specifically affect transcriptional repression at the telomere (Nimmo et al, 1998, Nimmo personal communication).

1.4.4 Telomeric DNA in the fission yeast

In fission yeast, a telomerase based system maintains telomeric repeats at an average length of 300nt (Hiraoka *et al.,* 1998), with the sequence of the telomeric repeats varying around a consensus sequence of $TTACAG_{1\text{-}8}$ (Hiraoka *et al.,* 1998). Telomere adjacent sequence repeats of up to 20kb are positioned internal to the telomeric repeats on chromosomes I and II, while on chromosome III the telomeric repeats directly abut tandem repeats of a 10.4kb rDNA sequence (Sugawara, 1989). A 3' G-rich single stranded overhang has been reported to be important for telomerase recruitment in *S. pombe* (Baumann and Cech, 2001).

1.4.5 Factors that bind to fission yeast telomeric DNA

The $taz1^+$ gene was identified by a one hybrid screen for factors that bound to *S. pombe* telomeric repeats (Cooper *et al.,* 1997) and isolated in a screen for mutants that specifically alleviated telomeric silencing (Nimmo *et al.,* 1998). The $taz1^+$gene encodes a protein with a telobox domain and a TRF domain similar to that found in TRF1 and TRF2 in human cells and which, like TRF1 and TRF2, binds to telomeric repeats in *S. pombe* as a preformed homodimer. In a *taz1*Δ cell telomeres are subject

to a dramatic increase in length and telomeric marker genes are no longer subject to transcriptional repression. *taz1*$^{-}$ cells also show a decreased association of telomeres with the SPB in meiotic prophase, resulting in defective 'horsetail' movement and reduced recombination between sister chromatids. Chromosome missegregation and aneuploidy in meiosis is more frequent, causing abnormal spore formation and low spore viability (Cooper *et al.*, 1998, Nimmo *et al.*, 1998). Although Taz1p is not essential for vegetative growth under stress-free conditions, *taz1*Δ cells exhibit lethal end-to-end telomere fusions in a Spku70p dependent manner when subjected to nitrogen starvation. This indicates that Taz1p has a role in telomere capping as well as length regulation and meiosis (Ferreira and Cooper, 2001).

Protein extract from fission yeast cells lacking Taz1p still displays significant telomere binding activity suggesting that this is not the only telomere binding factor in *S. pombe* (Vassetzky *et al.*, 1999, Spink *et al.*, 2000). It has been shown that at least two different proteins interact with telomere repeats (Duffy and Chambers, 1996). A second telobox protein was identified in *S. pombe* encoded by *teb1*, which binds with high affinity to vertebrate telomeres. However Teb1p only has low affinity for *S. pombe* telomere repeats. It could be that this protein has no major role at telomeric repeats but instead has another function in the cell (Vassetzky *et al.*, 1999).

Recently two independent routes have identified a *S. pombe* homologue of the budding yeast *RAP1* gene. A BLAST search using the *S. cerevisiae RAP1* sequence identified the similarities in an unidentified *S. pombe* open reading frame and a GFP fusion protein screen isolated a protein that specifically localised to the ends of telomeres (Kanoh and Ishikawa, 2001, Chikashige and Hiraoka, 2001). SpRap1p interacts with Taz1p by two hybrid analysis and colocalises with Taz1GFPp and the SPB. The *rap1*$^{+}$ gene encodes a protein with a telobox DNA binding domain, so it might be expected to be able to interact with duplex telomeric DNA, however in *taz1*Δ cells most of spRap1p is delocalised indicating that binding efficiency is reduced by loss of interaction with Taz1p. This is similar to the mediation of human Rap1 localisation through TRF2. A fusion protein consisting of Rap1 and the Taz1p

myb-like DNA binding domain dispenses with the need for an interaction between Taz1p and Rap1p for Rap1p localisation and function. The increased binding efficiency of Rap1 to telomeric DNA upon interaction with Taz1p is not reciprocal, as Taz1p remains localised in the absence of Rap1p (Chikashige and Hiraoka, 2001). Unlike Rap1p in budding yeast spRap1p does not appear to have other repressor activator functions essential for mitotic growth and, as found for the *taz1* disruption, deletion of fission yeast *rap1* has negligible effects on mitotic growth. Cells deleted for *rap1* have greatly elongated telomeres with genes placed in their vicinity no longer being subject to telomere position effect. *Rap1*Δ telomeres have meiotic defects including the declustering of telomeres at meiosis, deficient attachment to the spindle pole body and difficulties in performing 'horsetail' movement, which results in low spore viability. These are similar defects to those observed in a *lot2* mutant (Nimmo *et al.*, 1998), which was subsequently identified as a mutation in the *rap1* gene (Nimmo, personal communication).

A fission yeast protein that putatively binds to the single stranded overhang was isolated by homology to telomere binding proteins in ciliated protozoa. This protein was named Protection of telomeres 1, *pot1*$^{+}$ and shown to be able to bind to the *S. pombe* telomeric G-rich 3' overhang *in vitro* (Baumann and Cech, 2001). Deletion of *pot1*$^{+}$ in a *trt1*$^{-}$ background leads to an immediate rapid loss of telomeric DNA and telomere associated sequences and a high incidence of chromosome missegregation. When compared to the gradually senescing phenotype of a telomerase deletion this suggests that not only does Pot1p have a role in recruiting telomerase but it also functions to form a protective cap. *pot1*$^{-}$ colonies are deficient for telomere maintenance in a similar manner to a *trt1*Δ strain, and *pot1*$^{-}$ survivors are also generated by circularising chromosomes (Baumann and Cech, 2001).

1.4.6 Other proteins that affect telomeres in *S. pombe*

A protein with a function at telomeres has been identified by a BLAST search using the *S. cerevisiae RIF1* sequence. Cells deficient for spRif1p have an increase in telomere length of approximately 200bp and small increase in the frequency of

defects at meiosis. Like SpRap1p it would appear that Rif1p is localised to telomeres via interactions with Taz1p (Kanoh and Ishikawa, 2001).

Two genes with homology to human ATM have been identified in fission yeast; *tel1*$^+$ and *rad3*$^+$ (Naito *et al.*, 1998, Bentley *et al.*, 1996). *Tel1*$^+$ is homologous to the *S. cerevisiae TEL1* gene and *rad3*$^+$ is a fission yeast checkpoint gene homologous to *S. cerevisiae MEC1*. Neither gene is essential in *S. pombe* and mutation in either *tel1*$^+$ or *rad3*$^+$ leads to a small reduction in telomere length in the case of *tel1*Δ and a moderate reduction in telomere length in a *rad3* mutant. However, *tel1*Δ*/mec1*Δ double mutants have a huge increase in genomic instability. The cells grow poorly, with a high rate of aneuploidy observed, and the growth retardation increases during successive rounds of replication. Occasionally the population gives rise to survivor colonies that grow well during vegetative growth but are unable to pass through meiosis. Examination of the chromosomes of these survivors using pulsed field gel analysis and cytological methods found them to be circularised. This suggests that as telomeres shorten the frequency of end-to-end fusions increases. Fission yeast only has three chromosomes, so the probability of telomeres from the same chromosome fusing to each other is higher than in budding yeast.

DNA polII is necessary for lagging strand synthesis, and therefore is required to fill in the 5' strand following addition of repeats to the 3' overhang by telomerase. Strains that are deficient for DNA polII have elongated telomeres. This could be due to telomerase activity causing the accumulation of long single stranded overhangs but the lack of lagging strand synthesis meaning that there is no creation of extra duplex DNA containing Taz1p binding sites. As Taz1p is a negative regulator of telomerase activity telomerase is not down regulated following *de novo* repeat synthesis in the absence of DNApolII.

The *S. pombe* SUMO-1 homologue *pmt3*$^+$ is involved in post translational protein modification. *pmt3*$^+$ is required for telomere length maintenance with *pmt3*Δ cells displaying a large increase in telomere length of 500-900bp, intermediate between wild type and that found in a *taz1* or *rap1* mutant. Restoring pmt3p to the cell results

in gradual shortening of telomeres back to a wild type length. One of the foci observed by expression of a Pmt3GFP fusion protein is located at the SPB. This focus is formed in a cell cycle dependent manner, disappearing during prometaphase and metaphase (Tanaka *et al.*, 1999).

Disruption of the general silencing factors *swi6, clr4, rik1* and *chp2* also has an effect on silencing at the telomere (Ekwall *et al.*, 1996, Thon and Verhein-Hansen, 2000). Another mutation affecting telomeric silencing but not telomere length *rat1* has been found but the gene responsible has yet to be identified (Nimmo *et al.*, 1998).

1.4.7 DNA damage repair proteins also have a role at telomeres in fission yeast

A homologue of human Ku70 was found in *S. pombe* by DNA homology, which identified an ORF with 27% identity and 48% similarity to hKu70. SpKu70p is a part of the NHEJ pathway that binds and repairs broken DNA ends and is also bound to the telomeres. As in *S. cerevisiae,* cells with mutations in *SpKu70* have shorter telomeres by approximately 100bp, but no silencing defect is displayed (Manolis et al. 2001, Baumann and Cech, 2000). An increase in the rate of loss of linear mini-chromosomes but not circular minichromosomes in a *ku70Δ* background indicates that SpKu70p is necessary for the stability of chromosome ends (Manolis et al. 2001). SpKu70p is not located predominantly at the telomere, which is the case in budding yeast but instead is distributed throughout the nucleus. Nor does SpKu70p appear to be released from chromosome ends on induction of DNA damage (Manolis et al. 2001). Absence of SpKu70p in a $trt1^{+}$ cell has no noticeable effect of vegetative growth, however *Spku70Δtrt1Δ* cells senesce earlier than a strain deficient for *trt1* alone. They also display more difficulty in generating survivors, but do so by circularising chromosomes indicating that pKu70p is not necessary for this chromosome circularisation process. Chromosomes are also able to circularise in the absence of ligIV indicating that these chromosome fusion events are not mediated by components of the NHEJ pathway. Telomeres in a *Spku70* mutant undergo rearrangement of the telomeric sequences by homologous recombination between telomeres, suggesting that SpKu70p protects telomeres against recombinational

activities (Baumann and Cech 2000). It has been suggested that SpKu70p binds to the telomere as a broken DNA end but that its function is modified by the context of the other telomeric proteins with which it interacts, such as Taz1p. In a *taz1*Δ strain arrested in G1 end-to-end fusions occur in a pKu70p dependent manner. Taz1p would therefore appear to protect ends from fusion in a *Spku70* dependent manner performing a capping activity that distinguishes telomeres from broken DNA ends (Ferreira and Cooper, 2001).

1.4.8 Telomerase activity in *S. pombe*

The presence of telomerase in *S. pombe* was confirmed by primer extension assays that indicated that telomeres were extended by RNA dependent activity (Lue and Peng, 1997). The telomerase reverse transcriptase catalytic subunit encoded by $trt1^{+}$ was identified by degenerate PCR, deletion of which leads to the gradual erosion of telomere repeats and cells senesce and die after approximately 120 generations (Nakamura *et al.,* 1997). As the telomeres approach a critically short length there is an increased incidence of end-to-end fusions and chromosome breakage leading to genomic instability. A small number of cells survive by one of two methods; either by recombination between telomere ends in a similar manner to the RAD52 dependent alternative lengthening of telomere pathways studied in *S. cerevisiae* or by circularising their chromosomes (Haering *et al.,* 1998). Cells with circularised chromosomes become founders of a new population of cells, which are proficient for vegetative growth but unable to pass through meiosis. *trt1*Δ*/taz*Δ double mutants form circularised chromosomes by end-to-end fusions before the telomere has been eroded, suggesting that either telomerase reverse transcriptase or Taz1p may have a capping function at the telomere to prevent end-to-end fusions (Nakamura *et al.,* 1998, Ferreira and Cooper, 2001). The sequence encoding the telomerase RNA has not yet been identified in fission yeast.

Schizosaccharomyces pombe		
gene	**function at telomere**	**reference**
cdc17$^+$	DNA polymerase α, necessary for lagging strand synthesis	
chp1$^+$	general silencing factor	Thon and Verhein-Hansen, 2000
clr4$^+$	histone deacetylase, Swi6p localisation, silencing	Ekwall *et al.*, 1995
ku70$^+$	NHEJ protein, telomere end protection	Baumann and Cech, 2000, Manolis *et al.*, 2001
pot1$^+$	May bind to telomeric single stranded overhang, may recruit telomerase, role in end protection	Baumann and Cech, 2001
rad3$^+$	ATM homologue, telomere length maintenance	Bentley *et al.*, 1996
rap1$^+$	binds via interaction with Taz1p, telomere length regulator, transcriptional silencer, important for meiosis	Kanoh and Ishikawa, 2001, Chikashige and Hiraoka, 2001
rif1$^+$	Rap1p interacting protein, telomere length and silencing	Kanoh and Ishikawa, 2001
rik1$^+$	Swi6p localisation, silencing	Ekwall *et al.*, 1995
swi6$^+$	silencing factor	Ekwall *et al.*, 1995
taz1$^+$	telomere binding protein, myb-like DNA binding domain, telomere length regulator, transcriptional silencer, important for meiosis	Cooper *et al.*, 1997, Nimmo *et al.*, 1998
tel1$^+$	ATM homologue, telomere length maintenance	Naito *et al.*, 1998
trt1$^+$	telomerase catalytic subunit	Haering *et al.*, 2000, Nakamura *et al.*, 1997

Table 1.4 A summary of proteins involved in telomere structure and function in fission yeast and their apparent role at the telomere

1.4.9 Alternative Lengthening of Telomere pathways in *S. pombe*

In *trt1*Δ and *tel1*Δ/*mec* Δ mutant survivors arise at a rate of approximately 1 in 10,000 following complete deletion of the telomeric repeats (Naito *et al.*, 1998, Haering *et al.*, 2000). Examination of the chromosomes in these survivors found that in both cases the fission yeast was able to rescue the cell death phenotype by circularisation of the chromosomes. In the *trt1*Δ mutant a second pathway of

generating survivors was also noted in which the chromosomes remained linear and retained telomeric sequences in a similar manner to those maintained in RAD52 dependent ALT pathways in *S. cerevisiae* (Haering *et al.*, 2000).

Factors with an effect at the telomeres in *S. pombe* are summarised in Table 1.4 and their positions and interactions at the end of the chromosome shown in Figure 1.6.

1.5 Aims of this Thesis

It can be seen from the literature that there are many similarities between the mechanisms of telomere function and maintenance across both yeasts and vertebrates. Although some components appear to be functionally conserved across all three species there are others that have sequence similarity yet slightly different functions in the two yeasts and these can be compared to give insights into what may happen in higher eukaryotes. For instance in *S. cerevisiae,* Rap1p is vital for vegetative growth because as well as binding directly to the telomere it also acts as a repressor and activator elsewhere in the genome. In *S. pombe* however, *rap1* can be knocked out without detrimental effects in the mitotic cell cycle and localisation via a second telomere binding factor reflects that of human Rap1p. There are still several components at the telomere and aspects of telomere function observed in other organisms yet to be identified in fission yeast. In this thesis I set out to identify novel components with a role in telomere structure and function of *S. pombe*. Two genetic screens were performed using alleviation of repression of telomeric marker genes to isolate mutants of interest. In the first screen spontaneous mutagenesis and UV irradiation were used to create an extensive pool of mutants. In an attempt to overcome complementation difficulties arising from the nature of mutants isolated in the first screen a second screen was performed using insertional mutagenesis. The screens and analysis of mutants arising from them will be discussed.

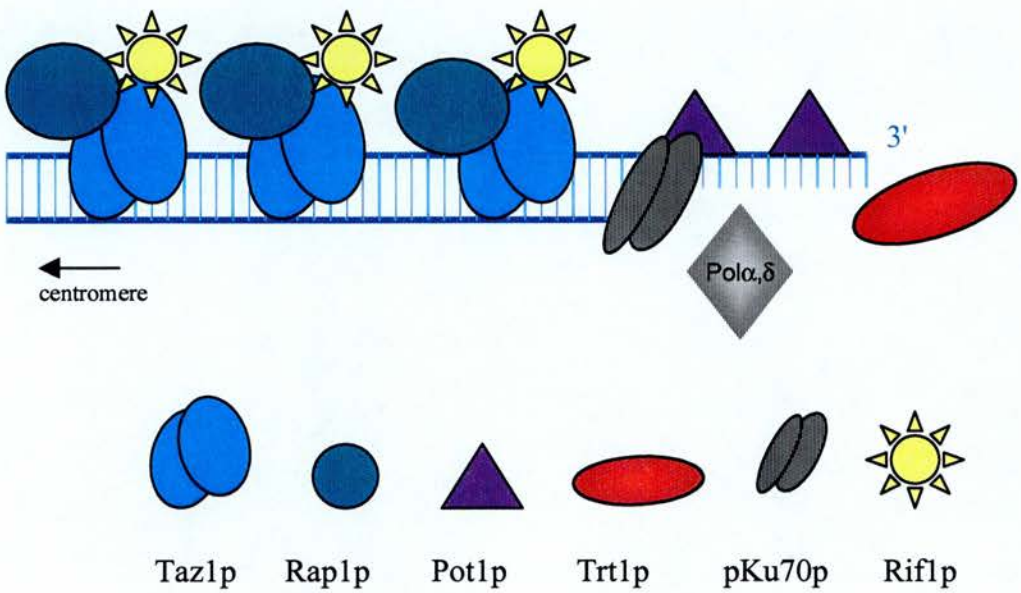

Figure 1.6 The telomeric localisation and relative interactions of proteins known to have a function at telomeres in *Schizosaccharomyces pombe*. Taz1p binds to double stranded telomeric DNA and is important for telomere length regulation. *S. pombe* Rap1p binds to the telomere via an interaction with Taz1p and in turn recruits spRif1p.

Chapter 2

Materials and Methods

2.1 General Chemicals and Solutions

GuHCl	4.5M Guanidine HCl, 99% grade (Bethesda Research Laboratories) 100mM EDTA 150mM NaCl (Fisher Scientific) 0.05% N-Lauoyl Sarcosine Sodium Salt (Sigma) dH_2O
SP1	1.2M D- Sorbitol (Sigma) 50mM Na Citrate (Fisher Scientific) 50mM $Na_2HPO_4.7H_2O$ (Riedel-deHaën) 40mM EDTA pH to 5.6 with orthophosphoric acid
20X SSC	175.3g NaCl (Fisher Scientific) 88.2g Tri-sodium citrate per litre dH_2O
20X TBE	216.0g Tris Base 110.0g Boric Acid 80.0ml 0.5 M EDTA (pH8.0) per litre
TE	10mM Tris-HCl pH8 1mM EDTA

2.2 Media and Strains

2.2.1 Yeast Protocols

Growth of fission yeast cells.

Cells for DNA preparations, RNA preparations and microscopy were grown in liquid culture in shaking incubators at 32°C unless otherwise stated.

Cell Stocks

A working stock of yeast cells were maintained on agar plates. Selection for marker genes was maintained where necessary by streaking strains on PMG agar lacking the relevant amino acid.

For permanent stocks cells were grown overnight in liquid culture and mixed in a 1:1 ratio with 100% glusulase. The cells were placed in a NUNC cryo tube, labelled and snap frozen. Permanent stocks were stored at –70°C.

2.2.2 Yeast Media

PMG liquid medium

20g/l D-Glucose anhydrous (Fisher Scientific)
3g/l phthallic acid (Sigma)
1.8g/l Na_2HPO_4 (Riedel-deHaën)
3.74g/l glutamic acid (Sigma)
1ml/l vitamins
0.1ml/l minerals
20ml/l salts
1X Amino acids, depending upon selection required

autoclave

Yeast Extract Suppliments (YES) liquid medium

30g/l D-Glucose anhydrous (Fisher Scientific)
5g/l yeast extract (DIFCO)
250mg/ l arginine (Sigma)
250mg/ l histidine (Sigma)
250mg/ l leucine (Sigma)

250mg/ l uracil (Sigma)

250mg/ l adenine (Sigma) autoclave

5-FOA liquid medium 2X YES liquid autoclaved

2g/l FOA (Melford Laboratories) filter sterilised

50:50 mix of YES and FOA

PMG solid medium 20g/l OXOID Bacteriological agar No. 1

20g/l D-Glucose anhydrous (Fisher Scientific)

3g/l phthallic acid (Sigma)

1.8g/l Na_2HPO_4 (Riedel-deHaën)

3.74g/l glutamic acid (Sigma)

1ml/l vitamins

0.1ml/l minerals

20ml/l salts

1X Amino acids, depending upon selection required

autoclave

Full PMG PMG medium plus 20 mls/l adenine, 10 mls/l arginine, 10 mls/l histidine, 10 mls/l leucine, 50 mls/l uracil

Minus histidine PMG medium plus 20 mls/l adenine, 10 mls/l arginine, 10 mls/l leucine, 50 mls/l uracil

Minus uracil PMG medium plus 20 mls/l adenine, 10 mls/l arginine, 10 mls/l histidine, 10 mls/l leucine

Yeast Extract Suppliments (YES) solid medium

20g/l OXOID Bacteriological agar No. 1

30g/l D-Glucose anhydrous (Fisher Scientific)

5g/l yeast extract (DIFCO)

250mg/ l arginine (Sigma)

250mg/ l histidine (Sigma)

250mg/ l leucine (Sigma)

250mg/ l uracil (Sigma)

250mg/ l adenine (Sigma)

autoclave

YES limiting adenine solid medium

As for YES solid medium but with only 10mg/l adenine (Sigma)

autoclave

Malt Extract (ME) plates

20g/l OXOID Bacteriological agar No. 1
30g/l Malt Extract (OXOID)
250g adenine (Sigma)
250g arginine (Sigma)
250g histidine (Sigma)
250g leucine (Sigma)
250g uracil (Sigma)

autoclave

FOA agar

Make agar as before, after autoclaving/melting cool to 65 °C and add 1g/l 5-FOA (Melford Laboratories)
Incubate at 65°C until powder has dissolved, shaking occasionally.

Pholoxin agar

As for YES agar add 2.5 mg/l Pholoxin B. Cells take up pigment from the pholoxin plate, dead cells are unable to excrete the pholoxin and so colonies containing cells that are unable to grow at a restrictive temperature are stained dark pink.

Amino acid Stocks In dH_2O autoclaved
50X adenine (Sigma) 0.5g/l
100X arginine (Sigma) 1g/l
100X histidine (Sigma) 1g/l
100X leucine (Sigma) 1g/l
20X uracil (Sigma) 0.2g/l might need to adjust to pH7.5 with NaOH pellets (Fisher Scientific)

1000X Vitamins	1g Inisotol (Sigma)	in 100mls
	1g Nicotinic acid (Sigma)	
	0.5g Panothenic acid (Sigma)	
	1mg Biotin (Sigma)	Filter Sterilise
10000X Minerals	1g H_3BO_3	in 200mls
	1g $MnSO_4.4H_2O$	
	0.8g $ZnSO_4.7H_2O$	
	0.4g $FeCl_3.6H_2O$	
	0.3g H_2MOO_4	
	80mg $CuSO_4.5H_2O$	
	2g Citric Acid	
	20mg KI	Filter Sterilise
Salts	53.3g $MgCl_2.6H_2O$	in 1 litre
	1g $CaCl_2.6H_2O$	
	50g KCl	
	2g Na_2SO_4	Autoclave

2.2.3 Mating and Random Spore analysis

In order to induce mating a small amount of cells from strains of opposite mating types (h^+/h^-) were mixed in a drop of sterile dH_2O and plated on ME agar, which is poor in nitrogen. The plates were incubated at 25°C for approximately 48 hours then the presence of spores was checked by light microscopy. Spores from each cross were scraped from the agar and placed into microfuge tubes containing diluted glusulase (NEN™) to digest the cell wall. The tubes were vortexed for one minute and incubated at 37°C for 24 hours to release the spores from the asci . The spores were concentrated by centrifugation and washed in dH_2O before being resuspended in 1ml dH_2O. 100-1000 spores were plated onto selective PMG media to select for the desired genotype.

Random Spore analysis

For random spore analysis mating was induced as above. Approximately a thousand spores from each cross were plated on non-selective YES media and incubated at 32°C for two days. The colonies that arose were replica plated to selective PMG media and a control plate of non-selective YES media and incubated for a further two days at 32°C. The number of colonies that arose on each plate were counted and the number growing on minus histidine divided by the total number growing on non-selective YES to calculate linkage between two loci.

Diluted Glusulase

1 in 100 dilution in dH_2O from glusulase stock (NEN™), filter sterilise.

Iodine staining of spores

H^{90} colonies were plated on ME medium to induce mating, incubated at 25 °C for approximately 48 hours and then exposed to iodine vapour. The presence of spores in a colony is indicated by black staining.

2.2.4 *S. pombe* transformation.

This method is based on electroporation and was used to transform both linear fragments of DNA and plasmids into fission yeast cells. Cells were grown to a concentration of $5x10^6$-$1x10^7$ cells/ml. The cells were harvested at 4°C for 2 minutes in a bench top centrifuge. The pellet was washed once with 20ml ice-cold 1.2M Sorbitol and twice with 10ml ice-cold 1.2M Sorbitol before being resuspended in ice-cold 1.2M Sorbitol to give a final concentration of $1x10^9$ cells/ml. Cells were divided into 200µl aliquots and mixed with approximately 50ng (plasmids) and 10µg (linear fragments) of DNA on ice. Immediately after mixing with DNA, cells were pulsed on an electroporation machine, using 2.25kvolts, 200Ω resistance and 25mF. 1ml of ice-cold 1.2M Sorbitol was added immediately after electroporation. Cells were pelleted by centrifugation at 4°C and resuspended in sterile cold dH_2O to remove the sorbitol. 100µl of cells was spread on selective medium using sterilised glass beads, and left at 25°C or 32°C. Transformants appeared after 2-5 days.

Linear fragments for electroporation were normally obtained by PCR amplification and purified prior to electroporation using a gel extraction kit (Quiagen). The fragments of DNA were dialysed against dH_2O on a Millipore 0.025μm dialysis disc for ten minutes prior to use.

2.2.5 Strains used in this study

ED 972 wild type
FY1554 h^+, *ade6-210, his3-D, leu1-32, ura4-D18* (from K Gould)
FY1555 h^-, *ade6-210, his3-D, leu1-32, ura4-D18* (from K Gould)
FY1645 h^+ *ade6-210, arg3-D4, his3-D1, leu1-32, ura4-D18*
FY1646 h^- *ade6-210, arg3-D4, his3-D1, leu1-32, ura4-D18*
FY1647 h^+ *ade6-216, arg3-D4, his3-D1, leu1-32, ura4-D18*
FY1648 h^- *ade6-216, arg3-D4, his3-D1, leu1-32, ura4-D18*
FY1862 h^{90} *ade6-210, his3-D, leu1-32, ura4-D18, ade6::SPH OTR,* TEL1L *-his3*$^+$ TEL2L *-ura*$^+$*4*
FY1863 h^{90} *ade6-210, his3-D, leu1-32, ura4-D18*, otr1RSph1::*ade6*$^+$, TEL1L*-his3*$^+$
FY1864 h^{90} *ade6-210, his3-D, leu1-32, ura4-D18,* otr1RSph1::*ade6*$^+$, TEL1L *-his3*$^+$
FY1867 h^{90} *ade6-210, his3-D, leu1-32, ura4-DS/E* otr1RSph1::*ade6*$^+$, TEL1L *-his3*$^+$ TEL2L- *ura*$^+$*4*
FY1868 h^{90} *ade6-210, his3-D, leu1-32, ura4-DS/E,* otr1RSph1::*ade6*$^+$, TEL1L *-his3*$^+$ TEL2L *-ura*$^+$*4*
FY1961 h^-, *ade6-210, his3-D, leu1-32, ura4-D18, taz1::ura4*$^+$, TEL1L *-his3*$^+$
FY1962 h^+, *ade6-210, his3-D, leu1-32, ura4-D18, taz1::ura4*$^+$, TEL1L *-his3*$^+$
FY2214 h^-, *nmt*81X-GFP-Swi6*::leu2 ade6-210, his3-D, leu1-32, ura4-D18*
FY3693 h^+, *ade6-210, his3-D, leu1-32, ura4-DS/E,* otr1RSph1::*ade6*$^+$, TEL1L *-his3*$^+$ TEL2L *-ura*$^+$*4*
FY3694 h^+, *ade6-210, his3-D, leu1-32, ura4-DS/E,* otr1RSph1::*ade6*$^+$, TEL1L *-his3*$^+$
FY3695 h^-, *ade6-210, his3-D, leu1-32, ura4-DS/E,* otr1RSph1::*ade6*$^+$, TEL1L *-his3*$^+$ TEL2L *-ura*$^+$*4*
FY3696 h^-, *ade6-210, his3-D, leu1-32, ura4-DS/E,* otr1RSph1::*ade6*$^+$, TEL1L *-his3*$^+$
FY3732 h^-, *ade6-210, his3-D, ura4-D18,* otr1RSph1::*ade6*$^+$, TEL1L *-his3*$^+$
FY3733 h^-, *ade6-210, his3-D, leu1-32, ura4-D18,* otr1RSph1::*ade6*$^+$, TEL1L *-his3*$^+$
FY3806 h^{90} *taz1GFP::ura4*$^+$, *ade6-210, his3-D, leu1-32, ura4-D18,* otr1RSph1::*ade6*$^+$, TEL1L *-his3*$^+$
FY3809 h^{90} *taz1GFP::ura4*$^+$, *swi6, ade6-210, his3-D, leu1-32, ura4-D18,* otr1RSph1::*ade6*$^+$, TEL1L *-his3*$^+$
FY3811 h^{90} h^{90} *taz1GFP::ura4*$^+$, *clr4, ade6-210, his3-D, leu1-32, ura4-D18,* otr1RSph1::*ade6*$^+$, TEL1L *-his3*$^+$
FY4585 h^+ *fat6, ade6-210, his3-D, leu1-32, ura4-D18,* TEL1L *-his3*$^+$
FY4586 h^+ *fat7, ade6-210, his3-D, leu1-32, ura4-D18,* TEL1L *-his3*$^+$
FY4945 h^+ *rat2-j543, ade6-210, his3-D, leu1-32, ura4-DS/E,* TEL1L *-his3*$^+$ TEL2L- *ura*$^+$*4*
FY4946 h^- *rat2-j543, ade6-210, his3-D, leu1-32, ura4-DS/E,* TEL1L *-his3*$^+$ TEL2L- *ura*$^+$*4*

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FY4947 h$^+$ *rat2-j543, ade6-210, his3-D, leu1-32, ura4-DS/E,* TEL1L -*his3*$^+$
FY4948 h$^-$ *rat2-j543, ade6-210, his3-D, leu1-32, ura4-DS/E,* TEL1L -*his3*$^+$
FY4949 h$^+$ *rat2-j556, ade6-210, his3-D, leu1-32, ura4-DS/E,* TEL1L -*his3*$^+$ TEL2L- *ura*$^+$*4*
FY4950 h$^-$ *rat2-j556, ade6-210, his3-D, leu1-32, ura4-DS/E,* TEL1L -*his3*$^+$ TEL2L- *ura*$^+$*4*
FY4951 h$^+$ *rat2-j556, ade6-210, his3-D, leu1-32, ura4-DS/E,* TEL1L -*his3*$^+$
FY4952 h$^-$ *rat2-j556, ade6-210, his3-D, leu1-32, ura4-DS/E,* TEL1L -*his3*$^+$
FY4953 h$^+$ *rat4-j575, ade6-210, his3-D, leu1-32, ura4-DS/E,* TEL1L -*his3*$^+$ TEL2L- *ura*$^+$*4*
FY4954 h$^-$ *rat4-j575, ade6-210, his3-D, leu1-32, ura4-DS/E,* TEL1L -*his3*$^+$ TEL2L- *ura*$^+$*4*
FY4955 h$^+$ *rat4-j575, ade6-210, his3-D, leu1-32, ura4-DS/E,* TEL1L -*his3*$^+$
FY4956 h$^-$ *rat4-j575, ade6-210, his3-D, leu1-32, ura4-DS/E,* TEL1L -*his3*$^+$
FY4957 h$^+$ *rat2-j589, ade6-210, his3-D, leu1-32, ura4-DS/E,* TEL1L -*his3*$^+$ TEL2L- *ura*$^+$*4*
FY4958 h$^-$ *rat2-j589, ade6-210, his3-D, leu1-32, ura4-DS/E,* TEL1L -*his3*$^+$ TEL2L- *ura*$^+$*4*
FY4959 h$^+$ *rat2-j589, ade6-210, his3-D, leu1-32, ura4-DS/E,* TEL1L -*his3*$^+$
FY4960 h$^-$ *rat2-j589, ade6-210, his3-D, leu1-32, ura4-DS/E,* TEL1L -*his3*$^+$
FY4961 h$^+$ *rat2-k596, ade6-210, his3-D, leu1-32, ura4-DS/E,* TEL1L -*his3*$^+$ TEL2L- *ura*$^+$*4*
FY4962 h$^-$ *rat2-k596, ade6-210, his3-D, leu1-32, ura4-DS/E,* TEL1L -*his3*$^+$ TEL2L- *ura*$^+$*4*
FY4963 h$^+$ *rat2-k596, ade6-210, his3-D, leu1-32, ura4-DS/E,* TEL1L -*his3*$^+$
FY4964 h$^-$ *rat2-k596, ade6-210, his3-D, leu1-32, ura4-DS/E,* TEL1L -*his3*$^+$
FY4965 h$^+$ *rat4-k601, ade6-210, his3-D, leu1-32, ura4-DS/E,* TEL1L -*his3*$^+$ TEL2L- *ura*$^+$*4*
FY4966 h$^-$ *rat4-k601, ade6-210, his3-D, leu1-32, ura4-DS/E,* TEL1L -*his3*$^+$ TEL2L- *ura*$^+$*4*
FY4967 h$^+$ *rat4-k601, ade6-210, his3-D, leu1-32, ura4-DS/E,* TEL1L -*his3*$^+$
FY4968 h$^-$ *rat4-k601, ade6-210, his3-D, leu1-32, ura4-DS/E,* TEL1L -*his3*$^+$
FY4969 h$^+$ *rat3-k620, ade6-210, his3-D, leu1-32, ura4-DS/E,* TEL1L -*his3*$^+$ TEL2L- *ura*$^+$*4*
FY4970 h$^-$ *rat3-k620, ade6-210, his3-D, leu1-32, ura4-DS/E,* TEL1L -*his3*$^+$ TEL2L- *ura*$^+$*4*
FY4971 h$^+$ *rat3-k620, ade6-210, his3-D, leu1-32, ura4-DS/E,* TEL1L -*his3*$^+$
FY4972 h$^-$ *rat3-k620, ade6-210, his3-D, leu1-32, ura4-DS/E,* TEL1L -*his3*$^+$
FY4973 h$^+$ *rat7-k639, ade6-210, his3-D, leu1-32, ura4-DS/E,* TEL1L -*his3*$^+$ TEL2L- *ura*$^+$*4*
FY4974 h$^-$ *rat7-k639, ade6-210, his3-D, leu1-32, ura4-DS/E,* TEL1L -*his3*$^+$ TEL2L- *ura*$^+$*4*
FY4975 h$^+$ *rat7-k639, ade6-210, his3-D, leu1-32, ura4-DS/E,* TEL1L -*his3*$^+$
FY4976 h$^-$ *rat7-k639, ade6-210, his3-D, leu1-32, ura4-DS/E,* TEL1L -*his3*$^+$
FY4977 h$^+$ *rat3-l646, ade6-210, his3-D, leu1-32, ura4-DS/E,* TEL1L -*his3*$^+$ TEL2L- *ura*$^+$*4*
FY4978 h$^-$ *rat3-l646, ade6-210, his3-D, leu1-32, ura4-DS/E,* TEL1L -*his3*$^+$

TEL2L- *ura*$^{+}$*4*

FY4979 h^{+} *rat3-l646, ade6-210, his3-D, leu1-32, ura4-DS/E,* TEL1L *-his3*$^{+}$

FY4980 h^{-} *rat3-l646, ade6-210, his3-D, leu1-32, ura4-DS/E,* TEL1L *-his3*$^{+}$

FY4981 h^{+} *rat5-l655, ade6-210, his3-D, leu1-32, ura4-DS/E,* TEL1L *-his3*$^{+}$
TEL2L- *ura*$^{+}$*4*

FY4982 h^{-} *rat5-l655, ade6-210, his3-D, leu1-32, ura4-DS/E,* TEL1L *-his3*$^{+}$
TEL2L- *ura*$^{+}$*4*

FY4983 h^{+} *rat5-l655, ade6-210, his3-D, leu1-32, ura4-DS/E,* TEL1L *-his3*$^{+}$

FY4984 h^{-} *rat5-l655, ade6-210, his3-D, leu1-32, ura4-DS/E,* TEL1L *-his3*$^{+}$

FY4985 h^{+} *rat6-l675, ade6-210, his3-D, leu1-32, ura4-DS/E,* TEL1L *-his3*$^{+}$
TEL2L- *ura*$^{+}$*4*

FY4986 h^{-} *rat6-l675, ade6-210, his3-D, leu1-32, ura4-DS/E,* TEL1L *-his3*$^{+}$
TEL2L- *ura*$^{+}$*4*

FY4987 h^{+} *rat6-l675, ade6-210, his3-D, leu1-32, ura4-DS/E,* TEL1L *-his3*$^{+}$

FY4989 h^{-} *rat6-l675, ade6-210, his3-D, leu1-32, ura4-DS/E,* TEL1L *-his3*$^{+}$

FY4990 h^{+} *rat2-l678, ade6-210, his3-D, leu1-32, ura4-DS/E,* TEL1L *-his3*$^{+}$
TEL2L- *ura*$^{+}$*4*

FY4991 h^{-} *rat2-l678, ade6-210, his3-D, leu1-32, ura4-DS/E,* TEL1L *-his3*$^{+}$
TEL2L- *ura*$^{+}$*4*

FY4992 h^{+} *rat2-l678, ade6-210, his3-D, leu1-32, ura4-DS/E,* TEL1L *-his3*$^{+}$

FY4993 h^{-} *rat2-l678, ade6-210, his3-D, leu1-32, ura4-DS/E,* TEL1L *-his3*$^{+}$

FY4994 h^{90} *rat2-j543, ade6-210, his3-D, leu1-32, ura4-D18,* TEL1L *-his3*$^{+}$

FY4995 h^{90} *rat2-j556, ade6-210, his3-D, leu1-32, ura4-D18,* TEL1L *-his3*$^{+}$

FY4996 h^{90} *rat3-j575, ade6-210, his3-D, leu1-32, ura4-D18,* TEL1L *-his3*$^{+}$

FY4997 h^{90} *rat2-j589, ade6-210, his3-D, leu1-32, ura4-D18,* TEL1L *-his3*$^{+}$

FY4998 h^{90} *rat2-k596, ade6-210, his3-D, leu1-32, ura4-D18,* TEL1L *-his3*$^{+}$

FY4999 h^{90} *rat4-k601, ade6-210, his3-D, leu1-32, ura4-D18,* TEL1L *-his3*$^{+}$

FY5000 h^{90} *rat4-k620, ade6-210, his3-D, leu1-32, ura4-D18,* TEL1L *-his3*$^{+}$

FY5001 h^{90} *rat7-k639, ade6-210, his3-D, leu1-32, ura4-D18,* TEL1L *-his3*$^{+}$

FY5002 h^{90} *rat3-l646, ade6-210, his3-D, leu1-32, ura4-D18,* TEL1L *-his3*$^{+}$

FT5003 h^{90} *rat5-l655, ade6-210, his3-D, leu1-32, ura4-D18,* TEL1L *-his3*$^{+}$

FY5004 h^{90} *rat6-l675, ade6-210, his3-D, leu1-32, ura4-D18,* TEL1L *-his3*$^{+}$

FY5005 h^{90} *rat2-l678, ade6-210, his3-D, leu1-32, ura4-D18,* TEL1L *-his3*$^{+}$

FY5158 h$^{-/+}$ *rat4-k601 taz1GFP::ura4*$^{+}$*, ade6-210, his3-D, leu1-32, ura4-D18,* otr1RSph1::*ade6*$^{+}$, TEL1L *-his3*$^{+}$

FY5159 h$^{-/+}$ *rat3-k620 taz1GFP::ura4*$^{+}$*, ade6-210, his3-D, leu1-32, ura4-D18,* otr1RSph1::*ade6*$^{+}$, TEL1L *-his3*$^{+}$

FY5163 h$^{-/+}$ *rat6-l675 taz1GFP::ura4*$^{+}$*, ade6-210, his3-D, leu1-32, ura4-D18,* otr1RSph1::*ade6*$^{+}$, TEL1L *-his3*$^{+}$

2.3 DNA techniques

2.3.1. Basic manipulations

DNA digestion

Restriction enzymes were all purchased from Roche apart from Not1 which was purchased from NEB. Following digestion enzymes were denatured by heat inactivation unless otherwise stated.

Ligation

DNA fragments were dialysed against dH_20 on Millipore dialysis discs prior to ligation reactions. 1 unit of DNA ligase IV (Roche) was used per 10µl reaction and ligation reactions were incubated for at least four hours at 20°C.

Agarose gels 1% agarose gel melt 1g agarose (Biogene) per 100ml in 1XTBE, cool to 60°C before pouring

Ficoll loading buffer 0.25% Bromophenol Blue
30% Ficoll

2.3.2 Polymerase Chain Reaction (PCR) (Saiki et al, 1988)

reaction mix:

1X Buffer (Roche)
2.5Mm $MgCl_2$ (Roche)
0.25µM dNTPs (ABGene)
0.5mM Oligonucleotide 1
0.5mM Oligonucleotide 2
0.5 units taq polymerase (Roche)
dH_2O to 20µl
approximately 20 ng DNA (Plasmid)
or 20-100ng DNA (genomic)

When necessary optimal PCR conditions were obtained by titration of the $MgCl_2$ concentration in the reaction.

Template for PCR was obtained from plasmids, Genomic DNA or SPZ treated cells

Inverse PCR

DNA was isolated from the mutant strains and digested for two hours at 37°C with CfoI, followed by heat inactivation of the enzyme at 80°C for 15 minutes. To circularise the fragments and form a template for the PCR amplification step the digested DNA was dialysed on Millipore discs for one hour, then incubated overnight at 25°C with DNA ligase IV. In order to optimise the amount of DNA fragments in each reaction the ligation mixes for the digested DNA were set up in a series of ten-fold dilutions of 800ng, 80ng, 8ng and 0.8ng DNA in a 100µl reaction mix. Ligation reactions were performed in a large volume of liquid to encourage intramolecular rather than intermolecular interactions. A diagram describing the steps of the reaction is show in Figure 2.1.

Ten percent of each ligation mixture was used as a template in a 200µl PCR reaction using primers complementary to regions inside each end of the *ura4*$^+$ fragment priming towards the flanking sequence. Two different Oligonucleotide pairs were used; W679 and W680 with the 5' primer positioned 115 bp downstream of the ATG and the 3' primer located 220bp downstream of the *ura4* stop codon, and U456 and U457 with the 5' primer positioned 100 bp downstream of the ATG and the 3' primer located 100bp upstream of the *ura4* stop codon in case the 3'UTR of *ura4*$^+$ was lost on insertion. PCR programs with 35 cycles of amplification were used with 4 minutes of primer extension time at 72 °C and annealing temperatures of 58°C and 65°C for primer pairs W679 and W680 and U456 and U457 respectively.

After amplification the PCR product could be sequenced then searched against the *S. pombe* genome at the Sanger centre using BLAST programs to identify regions of homology and therefore the likely site of the *ura4*$^+$ insertion.

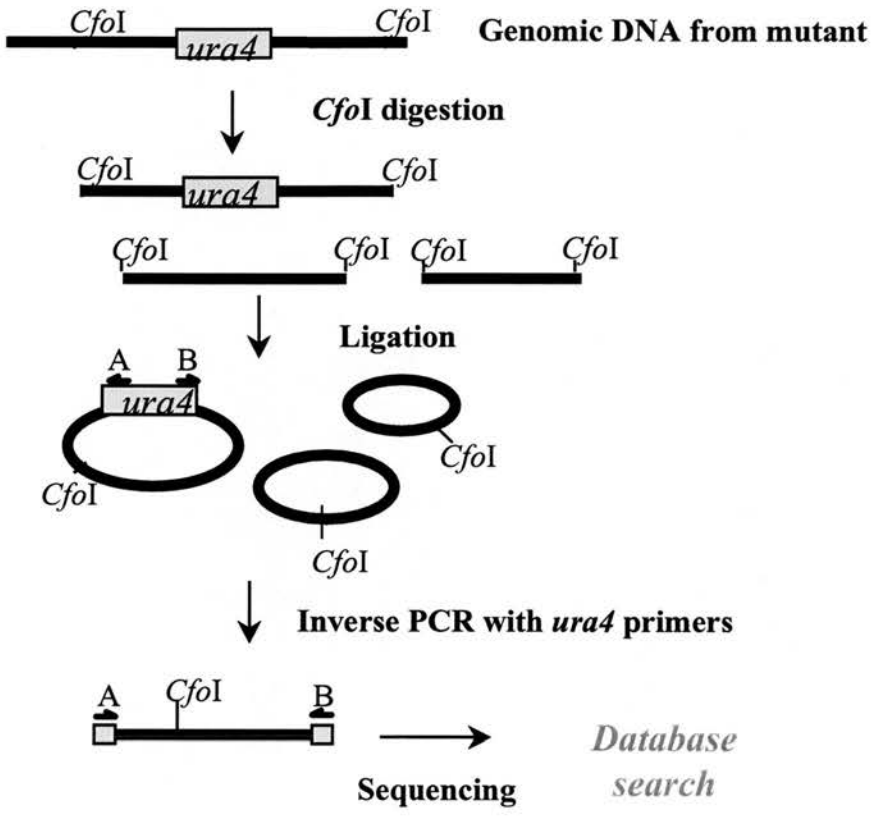


Figure 2.1 Genomic DNA was digested with CfoI restriction enzyme that does not cut within the *ura4* sequence. The DNA fragments were then circularised and inverse PCR from the ends of the *ura4* sequence across the sequence surrounding the insertion site attempted.

Vectorette PCR

Two vectorettes were designed; one with a BamHI compatible end and a blunt end and a second with EcoRI and HindIII overlaps at either end. 2µg of DNA from *fat6* and *fat7* were digested for 4 hours with; BamHI, BglII, Sau3A, EcoRI or HindIII. before the digestion was halted by heat inactivation of the enzyme and the DNA fragments dialysed against dH_2O on Millipore 0.025µm dialysis discs for one hour. 2pg of vectorette, 20 units of DNA VI ligase (Roche), ligation buffer and dH_2O were added to a final volume of 100µl and the reaction incubated at 16°C for 12-24 hours. 400µl dH2O was added to the sample and 3µl of DNA used as template in a 50µl PCR reaction.

To form vectorette

The vectorette was constructed by heating the two Oligonucleotides to 95°C followed by cooling slowly to room temperature to allow the two strands to anneal in the homologous regions. The vectorette was made to a final concentration of 1pg/µl.

The PCR program for vectorette PCR was:
94 °C 5 minutes, (94 °C 20 sec, 58 °C 20 sec, 72 °C 90 seconds) x 35 cycles, 72 °C 5 minutes.

PCR programs

A touchdown PCR program was used for all PCR reactions in this thesis unless otherwise stated. The primer extension time is altered depending on the expected product size. For products up to 1kb one minute extension time was allowed, products up to 2kb had two minutes extension time, products up to 3kb had three minutes extension time and products up to 5kb had 5 minutes extension time. The touchdown program for a 1 minute touchdown PCR is shown;

Touchdown: 94°C 4min; (94°C 30sec; 65°C 30sec; 72°C 1min) 3X; (94°C 30sec; 62°C 30sec; 72°C1min)3x; (94°C 30 sec; 59°C 30sec; 72°C 1min)3X; (94°C 30 sec; 56°C 30sec; 72°C 1min)3X; (94°C 30 sec; 53°C 30sec; 72°C 1min)3X; (94°C 30 sec;

50°C 30sec; 72°C 1min)3X; (94°C 30 sec; 47°C 30sec; 72°C 1min)5X; (94°C 30 sec; 45°C 30sec; 72°C 1min)10X; 72°C 5min.

TOUCH1: 1 minute touchdown program
TOUCH2: 2 minute touchdown program
TOUCH3: 3 minute touchdown program
TOUCH4: 4 minute touchdown program
TOUCH5: 5 minute touchdown program

SPZ PCR

Approximately $1x10^5$ to $3x\ 10^5$ cells were picked from colonies to be tested using a toothpick and the cells resuspended in 10µl SPZ containing $2.5mgml^{-1}$ zymolyase. The cells were incubated at 37°C for 10 minutes to permeablise the cell wall, then 1µl of the mixture was used as template for a PCR reaction using primers R568 and R569 and a two minute touchdown PCR program.

SPZ Buffer
1.2M D-Sorbitol (Sigma)
100mM Sodium Phosphate buffer pH 7.4
2.5mg/ml zymolyase (ICN Biochemicals Inc.)

Radioactive competitive PCR.

This technique was used to quantify PCR products after RT-PCR. 1µl of [α–^{32}P]dCTP was added per 200µl of PCR mix. The URA25 PCR program was used, after the PCR was completed, samples were loaded onto a 4% acrylamide gel made in 1X TBE and run on a minigel apparatus (Hoefer) at 100V for 40 min. The gel was then transferred to 3mm paper and dried on a vacuum dryer prior to exposure on a phoshorimager cassette. Data were visualised on a Storm phosphorimager and quantified using ImageQuant software.

URA25 PCR program: 94 °C 5 minutes, (94 °C 20 sec, 55 °C 20 sec, 72 °C 90 seconds) x 25 cycles, 72 °C .

2.3.3 DNA preparation from fission yeast

A 20ml culture was grown overnight to a density of approximately $1x10^7$ cells ml^{-1}. The cells were pelleted by centrifugation and resuspended in 1ml SP1. The cells were transferred to a microfuge tube and zymolyase (ICN Biochemicals Ltd.) added to a final concentration of 50µg/ml. The cells were then incubated at 37°C for 1 hour prior to being spun down and resuspended in 300µl 4.5M GuHCl (prepared as described in 2.1). They were then incubated for 10 minutes at 65 °C before being cooled to room temperature prior to addition of 300µls cold 100% ETOH. The cells were centrifuged for 5 minutes at 10,000 rpm, the supernatant removed and the pellet resuspended in 600µl 10X TE. RNAse was added to a final concentration of 0.1mg/ml and the mixture was incubated for 1 hour at 37°C. Proteinase K (Roche) was then added to a final concentration of 0.2mg/ml and incubated for 1 hour at 65°C. DNA was extracted twice with 1 volume Phenol Chloroform at pH 8 (Sigma) followed by a single extraction with Chloroform (Fisher Scientific). The DNA was precipitated by adding $1/10^{th}$ volume 3M NaOAc and 2-3 volumes 100% cold ETOH. The contents of the tubes was mixed by inversion and the DNA precipitated at -20°C for 10 minutes. The tubes were centrifuged at 10,000rpm for 10 minutes and the supernatant removed. The pellet was washed with 70% ETOH then air-dried and resuspended in 30-50µl TE or dH_2O if it was to be used for salt sensitive applications. A small amount of each sample was run on a 1% agarose gel in 1X TBE with DNA markers to quantify their concentration.

2.3.4 Southern Blotting

Preparation of a filter for Southern Blotting

DNA fragments were separated to the required distance on a 0.9% agarose gel made in 1XTBE containing Ethidium Bromide. The gel was exposed on a UV light source and a photograph taken with a ruler alongside. The gel was covered with denaturing solution and incubated at room temperature with rotation for 15 minutes before the denaturing solution was replaced and the step repeated once. The denaturing solution

was poured off and the gel covered and incubated in 1X PO_4 solution for 15 minutes, PO_4 was replaced and the gel incubated for a further 15 minutes. The gel was then placed onto a raised platform in a dish containing approximately 500mls 1X PO_4. The platform was covered by two wide strips of 3mm chromatography paper pre-soaked in PO_4 and with their ends dipping in the PO_4 solution to act as a wick. Genescreen membrane (NEN™ Life Science Products) was cut to the size of the gel and pre-soaked in dH_2O before being carefully place on top of the gel. Two pieces of 7mm chromatography paper were cut to the same size and place on top, these were then covered with a stack of paper towels approximately 5cm deep. The sides of the stack and dish were wrapped in cling film to ensure that evaporation only occurred through the stack and not around the edges. A light weight was placed on top to apply a small amount of pressure and the stack left overnight at room temperature for the DNA to transfer to the membrane. The next morning the stack was disassembled and the membrane rinsed in 2XSSC before being blotted dry in two sheets of 3mm paper. The membrane was exposed to 150mj UV light in a Strata linker and baked for one hour at 80°C to fix the DNA to the membrane.

Radioactive labelling of DNA probes with [α-^{32}P]CTP

The membrane was pre wet in 2XSSC and incubated with 10-20mls Church and Gilbert buffer in a hybaid tube in a rotating oven set at 65°C for at least 2 hours prior to the addition of probe.

20-50ng of double stranded DNA was made up to a volume of 13µl with dH_20 in a microfuge tube and denatured by incubating at 96°C for 5 minutes. The tube was then placed on ice for two minutes before adding 30µCi [α-^{32}P]CTP and 4µl of a High Prime (Roche) random priming kit. The mixture was incubated at 37°C for 10 minutes to prime the random synthesis of copies of the DNA incorporating [α-^{32}P]CTP into the synthesised strand. The reaction was passed through a Nic column (Pharmacia Amersham) in order to separate incorporated from unincorporated nucleotides. The High Prime reaction was placed on top of column and washed once with 350µl TE, the flow through was collected and discarded before washing for a

second time with 450µl TE. The flow through that contained the labelled probe was collected in a microfuge tube, whilst unincorporated nucleotides remained in the Nic column. The labelled probe was sealed in the tube and boiled in a water bath for 10 minutes to dissociate dsDNA then added immediately to the Hybaid tubes containing the filters in a 65°C rotating incubator. The filters and probe were incubated overnight and washed the next day 3x for 30 minutes at 65°C with 0.1%SDS, 0.1X SSC solution. The blot was rinsed in the wash solution and monitored with a Geiger counter before being patted dry in 3mm chromatography paper, wrapped in Clingfilm and exposed on phosphorimager cassette overnight. The phosphoimager cassette was read on a phosphorimaging machine and analysed using Imagequant Software.

The telomere repeat sequence probe was prepared by cutting plasmid pEN42 with Apa1 (Roche) to release a 1.9 kb fragment containing telomere repeat sequence and a kanamycin maker which was purified by gel extraction. (A gift from Elaine Nimmo)

For *ura4* probe the template was prepare by amplifying the gene sequence by PCR
For cosmid probe 20-30ng of the relevant cosmid was used per reaction

Denaturing Solution 1.5M NaCl
0.5M NaOH

40X PO_4 105g $NaH_2PO_4.2H_20$ Disodiumhydrogen phosphate dihydrate, (Riedel-deHaën)
44g Na_2HPO_4 Sodiumdihydrogen Orthophosphate 1.hydrate(BDH Laboratory Supplies)
in 1litre of dH_20.

Church and Gilbert Buffer
1X PO_4
7% Sodium Diodoecyl Sulphate (Fisher Scientific)

2X PO_4 for C+G Buffer

0.5M Disodiumhydrogen phosphate dihydrate, Na_2HPO_4
(Riedel-deHaën)
titrate to pH 7.5 with Orthophosphoric Acid

2.3.5 Pulsed Field Gels

Preparation of *S. pombe* chromosomes in agarose plugs for PFG analysis

Cells were cultured to a density of approximately 1-4x10^7 cells ml^{-1}, pelleted by centrifugation and washed three times in 50mM EDTA pH 8.0. The final pellet was resuspended in SP1 at a density of 2x10^9 cells ml^{-1} and Zymolyase added to a final concentration of 0.5mg/ml. The cells were incubated at 37°C for 2 hours before adding an equal volume of 2% Low Melting point agarose (GIBCO Brl) at a temperature of 42°C (agarose made in 125mM EDTA pH8). The cell/agarose mixture was aliquoted into 100µl moulds and allow to set at room temperature. Once set, the plugs were removed from the moulds and placed in NDS buffer. Proteinase K was added to a final concentration of 0.5mg/ml and the plugs were incubated at 50°C for 24 hours. The NDS buffer was removed and replaced and Proteinase K added again to a final concentration of 0.5mg/ml, the plugs were then incubated at 50°C for a further 24-48 hours. The plugs were then washed repeatedly in TE until they became translucent and were stored at 4°C in TE.

The plugs were digested overnight with NotI (NEB) at 37°C or SfiI (Roche) at 50°C, which cut at known sites in the genome. Double digests were performed by digesting first with Sfi1I then adjusting the Salt concentration of the buffer to a total of 50mM Tris, 100mM NaCl and digesting overnight at 37°C with NotI. The plugs were placed in a microfuge tube with 3µl enzyme, 25µl buffer and 142µl dH2O to a final volume of 250µl.

The plugs were loaded onto a 0.9% gel made in 0.25X TBE and run on a Rotophor Pulsed Field Gel apparatus (Biometra) for 24 hours at a maximum temperature of

13°C. The Rotorphor apparatus was set at 150-140volts descending in a logarithmic scale, with the angle of the electrodes decreasing from 120° to 110° on a linear scale and the switching time descending from 100 to 10 seconds on a linear scale. The gel was removed from the apparatus and soaked in dH_20 containing 1µg/ml ETBR before being exposed on a UV light box and a photograph taken. The gel was exposed on the UV light box for a further minute to break the DNA and aid the transfer of the DNA to a membrane for Southern blotting. ETBR was washed out by incubating the gel with water on a rotating platform.

NDS Buffer 0.5M EDTA
10mM Tris pH7.5
autoclave then add 1% N-lauryl sarcosine (Sigma)

2.3.6 DNA Sequencing

The gene to be sequence was amplified by PCR. The reaction was split into ten different tubes and the amplification products pooled to ensure that errors by taq polymerase did not produce a false mutation site. The amplification product was cleaned using an ExoSapIT enzyme mix (USB), containing exonuclease I and Shrimp Alkaline Phosphatase. 2µl of the enzyme mix was used per 5µl amplification product. The mixture was incubated at 37°C for fifteen minutes followed by fifteen minutes at 80°C to heat inactivate the reaction. The sequencing reaction was performed using a Big Dye Sequencing Kit (Perkin Elmer); 4µl Big Dye sequencing mix was mixed with 100ng amplification product, 20ng oligonucleotide, 4µl 2mM $MgCl_2$ (Roche) and dH_20 added to a total volume of total volume 20µl. The amplification program consisted of an initial denaturing step at 96°C for 5 minutes followed by 25 cycles of; 96°C for 30 seconds, 55°C for 15 seconds and 60°C for 4 minutes. The products of the sequencing reactions were mixed with 50µl absolute alcohol and 2µl 3M Sodium Acetate and precipitated for one hour at room temperature. The DNA was precipitated by centrifugation for twenty minutes at 10,000 rpm. The supernatant was removed and the pellet rinsed in 70% ETOH and air-dried before being given to technical services to run on a sequencing machine.

2.4 RNA techniques

2.4.1 *S. pombe* Total RNA prep

Strains were grown at 32°C in YES broth or PMG broth lacking uracil to a density of approximately $1x10^7$ cells ml^{-1}. Cultures were pelleted by centrifugation, washed in TE and transferred to microfuge tubes prior to being resuspended in 300µl RNA extraction buffer. 300µl of glass beads (Sigma) were added followed by 300µl phenol/chloroform pH 4.7 (Sigma). Cells were lysed by shaking at high speed on a multi-head vortexer for 30 minutes at 4°C before centrifuging at 10,000 rpm for 5 minutes and removing the supernatant. The supernatant was extracted twice with phenol chloroform, and then once with chloroform. The RNA was precipitated with 3 volumes cold 100% ETOH and centrifuged at 10,000rpm for 15 minutes at 4°C. The supernatant was removed and the pellet air-dried before being resuspended in 25µl dH_2O. The concentration of each sample of RNA was determined by measuring the optical density of a 1/500 dilution in a spectrophotometer set at a wavelength of 280λ. Samples were then diluted to a concentration of 1µg/µl with dH_2O.

2.4.2 RT-PCR

For rtPCR 2.5 µg of each sample of RNA was aliquoted into a microfuge tube. DNAse buffer, 1µl DNAse and dH_2O were added to a final volume of 10µl and the mix was incubated at 25°C for 1 hour. After this time 1µl 25mM EDTA was added and the reactions incubated at 65 °C for 10 minutes to denature the DNAse. 1µl of the reaction was used as template for a PCR reaction to check that all of the DNA had been digested. Once it had been established that there was no DNA remaining as a contaminant in the sample 1µg oligonucleotide dT_{12-18} and dH_2O were added to a final volume of 25µl. The samples were incubated for 10 minutes at 70 °C, then placed on ice. The samples were centrifuged briefly at 4°C and returned to ice before adding 4µl 10X PCR buffer (Roche), 4µl $MgCl_2$ (Roche), 4µl DTT and 2µl 2.5mM dNTPs. The samples were shaken gently then split into two 19µl aliquots and incubated at 42 °C for 5 minutes. 1µl reverse transcriptase (Superscript GIBCO) was

added to one of each pair of tubes only. The incubation at 42 °C was continued for another 50 minutes then the samples were incubated at 70 °C for 15 minutes to stop the reaction and placed on ice. 1µl of each sample was used as template in a 20µl PCR reaction to determine the amount of transcript.

RNA Extraction Buffer

50mM Tris-HCl pH7.5
10mM EDTA
100mM NaCl
1% SDS autoclaved

4% polyacrylamide gel for RT-PCRs

25ml dH_2O
4ml 30% acrylamide
1.5ml 20X TBE
300µl Ammonium Persulphate (10%)
60µl TEMED

2.5 Cytological Methods

2.5.1 Live Imaging

GFPswi6 is transcribed from a NMT promoter and inserted along with a *leu2* marker gene at the *ars1* locus. In a wild type background the GFPSwi6 fusion protein localises to the centromere, silent mating type locus and telomeres, which are all competent for silencing. The desired strains were selected from genetic crosses by plating the spores on minus histidine minus leucine plates to select for the telomere silencing mutant phenotype and *GFPSwi6* respectively.

Taz1GFP::ura4$^+$ is at the endogenous *taz1* locus. TazGFPp was crossed into *rat* mutant strains that did not have the *ura4*$^+$ telomeric marker. The desired strains were selected from genetic crosses by plating the spores on minus histidine minus uracil

plates to select for the telomere silencing mutant phenotype and *Taz1GFP::ura4*$^+$ respectively.

Cells derived from strains with the GFPswi6p or Taz1GFPp fusion proteins were grown to log phase in 2ml of thiamine-free medium (PMG) supplemented with all the necessary amino acids. Cells were concentrated 100 fold by centrifugation and embedded in in 1% low melting point agarose (GIBCO BRL) in PMG broth, melted and cooled to 37°C. The cells and agarose were spread onto sides and a glass cover slip was applied on top and pushed down firmly. Cells were visualised under a fluorescence microscope.

2.5.2 Swi6 Immuno Localisation

Cells were grown in YES broth at 32°C to mid logarithmic phase then the temperature was shifted to 18°C for 2 hours. Sorbitol was added to a final concentration of 1.2M and the cells incubated for a further 5-10 minutes at 18°C before adding paraformaldehyde to a final concentration of 3.7% and incubating for 30 minutes at room temperature. The cells were pelleted by centrifugation and washed thoroughly in PEMS. They were then resuspended at a final concentration of $1x10^8$ cells ml^{-1} in PEMS containing 1mg/ml zymolyase and incubated at 37 °C for 90 minutes in order to remove the cell wall and enable antibodies to enter the cell. The cells were pelleted by centrifugation and washed in PEMS, resuspended in 1ml PEMS containing 1% Triton-X100, incubated for 1 minute at room temperature, washed twice in PEMS followed by one wash in PEM. Cells were then resuspended in 0.5mls PEMBAL and incubated for 1 hour with rotation at room temperature. From this pool of cells an aliquot containing approximately $0.5x10^6$ cells was removed and pelleted by centrifugation then resuspended in 100µl PEMBAL containing a 1:30 dilution of affinity purified anti-swi6 antibody. Cells were incubated with the primary antibody overnight at 4°C with rotation, then washed 3x for 20 minutes in PEMBAL to remove excess primary antibody before adding 100µl of PEMBAL containing a 1:100 dilution of FITC conjugated anti-rabbit secondary antibody. This was incubated with rotation, in the dark, for 4 hours at room

temperature. The cells were washed once in PEMBAL, then incubated for 5 minutes in PEM and 0.1% azide and 25µg/mlDAPI to stain the nucleus. Excess DAPI was removed by washing the cells in PEMazide and the cells resuspended in a small amount of PEMazide to make a cloudy suspension. 5µl of the cell suspension was spread onto a polylysine-coated cover slip and allowed to dry before mounting onto slides and sealing with nail varnish. Cells were then viewed on a fluorescence microscope.

Swi6 Cnp1 double Immuno localisation

Cells were prepared as above.Secondary antibodies specific to either sheep or rabbit are conjugated to fluorochromes that fluoresce in response to different wavelengths of light enabling the relative position of the foci of staining for each antibody to be seen. Where the protein localisations coincide the antibody signals merge to give a single focus indicating the position of the centromere in the cell.

PEM	0.1M PIPES (Sigma) pH7 1mM $MgSO_4$ 1mM EGTA
PEMS	PEM + 1.2M Sorbitol (Sigma)
PEMBAL	PEM + BSA (Sigma) + 0.1% Azide + 100mM Lysine hydrochloride (BDH)
PEMAZIDE	PEM + 0.1% Azide

2.5.3 Propidium Iodide Staining of Spores

Mating was induced in the mutants by plating h^{90} strains on ME plates, a low nitrogen medium, and incubating at 25°C. After two days the spores were washed off

the plates with distilled water and concentrated by centrifugation. Fixation was done by resuspending the spores in 70% ETOH at a concentration of $1x10^6$ asci ml^{-1} and incubated overnight at 4°C. These were pelleted and washed in 50mM NaCitrate, resuspended at a concentration of $1x10^7$ cells ml^{-1} in 50mM NaCitrate with 250μgml^{-1} RNAse and incubated at 37°C for 1 hour. The cells were diluted two fold with NaCitrate and Propidium Iodide was added to a final concentration of 250μgml^{-1}. Asci were spread onto cover slips coated with 1x polylysine and left to dry before being mounted onto slides with Vectashield (Vector Laboratories Inc.) and sealed with nail varnish.

2.6 Constructs and Oligonucleotides used in this study

2.6.1 Cosmids

SPBc1778, SPBc405, SPBc4c5 and SPBc609 were a gift from Rhian Gwilliam at the Sanger centre

SPBc1778: *S.pombe* chromosome II, database size 19011bp
genes and hypothetical open reading frames;
actin binding; actin polymerisation complex; Calponin; fimbrin; LTR; rad35/dfp1- like; TBC domain; zuotin-like.

SPBc4c3: *S.pombe* chromosome II , database size 29767bp
genes and hypothetical open reading frames;
ade1; dna-directed rna polymerase i 190 kd subunit; fork head domain; GHMP_kinases; homoserine kinase; nuc1; phosphoribosylamine-glycine ligase; proteasome component precursor; protein kinase phosphorylase; sep1.

SPBc405: *S.pombe* chromosome II, database size 14425
genes and hypothetical open reading frames;
60s ribosomal protein l36; ade1; dna-directed rna polymerase ii largest subunit; dnaj related protein; phosphoribosylamine--glycine ligase; ras-related protein; rpl36-2.

SPBc609: *S.pombe* chromosome II, database size 15301
genes and hypothetical open reading frames;
5S rRNA gene; guanine nucleotide binding protein beta subunit;

major facilitator superfamily protein; possible chromatin-associated HMG protein; protein phosphatase; protein-tyrosine phosphatase; SSRP; structure specific regognition protein; trp-asp repeat; WD domain.

2.6.2 Oligonucleotides used in this study

Oligonucleotides used in this study were manufactured by Genosys or MGW. Stocks were made at a concentration of 100 mM in 1X TE and stored at –20°C unless otherwise stated. Working stocks were made from these by diluting in dH_2O to a concentration of 5 mM

Oligonucleotides for PCR and Sequencing reactions:

Gene	Oligonucleotide name	Position relative to gene ATG	Size and direction relative to gene
his3	R568	590-609	20mer, 5'-3'
	R569	1196-1178	19mer, 3'-5'

Gene	Oligonucleotide name	Position relative to gene ATG	Size and direction relative to gene
rap1	D1364	342-363	21mer, 5'-3'
	D1365	1245-1266	21mer, 5'-3'
	D1366	1864-1841	21mer, 3'-5'
	D1367	2694-2673	21mer, 3'-5'
	D1632	874-854	20mer, 3'-5'
	D1633	2394-2414	20mer, 5'-3'
	D1667	-146--126	20mer, 5'-3'
	D1668	3454-3432	22mer, 3'-5'
	E1880	774-794	20mer, 5'-3'
	E1881	1762-1782	20mer, 5'-3'
	E1882	2464-2444	20mer, 3'-5'

Gene	Oligonucleotide name	Position relative to gene ATG	Size and direction relative to gene
taz1	L829	0-29	29mer, 5'-3'
	L830	1990-1960	29mer, 3'-5'
	N147	297-317	20mer, 5'-3'
	N148	1074-1094	20mer, 5'-3'
	N149	1442-1462	20mer, 5'-3'
	N150	691-711	20mer, 5'-3'
	N151	1714-1734	20mer, 5'-3'
	N218	391-371	20mer, 3'-5'
	N219	857-837	20mer, 3'-5'
	N220	1470-1450	20mer, 3'-5'
	W883	2157-2137	20mer, 3'-5'
	Y802	-200--180	22mer, 5'-3'

Gene	Oligonucleotide name	Position relative to gene ATG	Size and direction relative to gene
ura4	C1221	469-490	22mer, 5'-3'
	C1222	1420-1401	20mer, 3'-5'
	R180	0-20 + "tail"	31mer, 5'-3'
	R181	-1-19 + "tail"	32mer, 5'-3'
	R182	-2-18 + "tail"	33mer, 5'-3'
	R183	1181-1160 + "tail"	32mer, 3'-5'
	R893	0-20 + "tail"	41mer, 5'-3'
	R894	1181-1160 + "tail"	42mer, 3'-5'
	U457	101-76	24mer, 5'-3'
	W679	114-94	20mer, 3'-5'
	W680	1000-1022	22mer, 5'-3'
	X961	-537- -515	22mer, 5'-3'
	Y255	-438- -462	24mer, 3'-5'
	Y256	1225-1206+ "tail"	42mer, 3'-5'

Oligonucleotide sequences:

C1221: 5' primer for amplification across the region deleted in DSE *ura4*.

5'- CCG TAT TGT CCT ACC AAG AAC C -3'

C1222: 3' primer for amplification across the region deleted in DSE *ura4*.

5'- GCC TTC TGA CAT AAA ACG CC -3'

D1362: primer for sequencing *S. pombe rap1* homologue.

5'- GTT GAG GAT ACT AGG AAA CC -3'

D1363: primer for sequencing *S. pombe rap1* homologue.

5'- GAA CAA CAA TTC CTT GAA GC -3'

D1364: primer for sequencing *S. pombe rap1* homologue.

5'- GGG GAT TCA AAA ATG CAA TTG -3'

D1365: primer for sequencing *S. pombe rap1* homologue.

5'- CGA CAT ACA TTT TCC GAA TGG -3'

D1366: primer for sequencing *S. pombe rap1* homologue.

5'- CTA TCA CTA TAG TCA CTC AGC -3'

D1367: primer for sequencing *S. pombe rap1* homologue.

5'- GTT GAT TTA CTC TGC TAC ATC -3'

D1632: primer for sequencing *S. pombe rap1* homologue.

5'- GTT GAG GAT ACT AGG AAA CC -3'

D1633: primer for sequencing *S. pombe rap1* homologue.

5'- GAA CAA CAA TTC CTT GAA GC -3'

D1667: 5' primer for amplification and sequencing of *S. pombe rap1* homologue.

5'- CCA GTA GAG GCG ATG TTG AT -3'

D1668: 3' primer for amplification and sequencing of *S. pombe rap1* homologue.

5'- GCC ATT GAC ATT ACT GGC ATC G -3'

E1880: 5' primer for amplification and sequencing of *S. pombe rap1* homologue.

5'-GCC AGA AAG GTA TGA TCT TG - 3'

E1881: 5' primer for amplification and sequencing of *S. pombe rap1* homologue.

5'- GAT CGC TTG ATG ACA TGA GT –3'

E1882: 3' primer for amplification and sequencing of *S. pombe rap1* homologue.

5'- GAA AGT AGT TTA GCAATG GC –3'

F1023: primer at 3' end of c4c3 c1778 region of overlap, priming in 3'-5' direction

5'- CAC ACT GGC TGT TAA CCC TC -3'

L829: 5' primer for amplifying *taz1* ORF.

5'- CGC GGA TCC ATG ATA AGC GTG CAA AGT AC -3'

L830: 3' primer for amplifying *taz1* ORF.

5'- CGC GGA TCC TTA AGA TTG ATA ATT AAC AA -3'

N147: primer for sequencing *taz1*.

5'- GAA AAC GAA GTC TCG ACT GC -3'

N148: primer for sequencing *taz1*.

5'- TCG CTT AGA AAG AAA GGA CC -3'

N149: primer for sequencing *taz1*.

5'- AAA CCG GAG TAA GCT GAT TG -3'

N150: primer for sequencing *taz1*.

5'- CAG GCT TGA TTG ATC TCC TA -3'

N151: primer for sequencing *taz1*.

5'- ATG ATT TCT CAG CAT GGC TG -3'

N218: reverse primer for sequencing *taz1*.

5'- CCC ATA TCG ATC GAA CTA TC -3'

N219: reverse primer for sequencing *taz1*.

5'- GAA GGA TTT GAA TCG GTA CC -3'

N220: reverse primer for sequencing *taz1*.

5'- CCT CTT CGC AAT CAG CTT AC -3'

R180: 5' primer for amplification of *ura4* without ATG with built on BamHI site, frame1.

5'-GAC TGG ATC CGA TGC TAG AGT ATT TCA AAG C-3'

R181: 5' primer for amplification of *ura4* without ATG with built on BamHI site frame2.

5'GAC TGG ATC CAG ATG CTA GAG TAT TTC AAA GC-3'

R182: 5' primer for amplification of *ura4* without ATG with built on BamHI site frame3.

5'-GAC TGG ATC CAT GAT GCT AGA GTA T TT CAA AGC-3'

R183: 3' primer for amplification of *ura4* without ATG with built on BamHI site.

5'-GCT AGG ATC CTC CAA CAC CAA TGT TTA TAA CC-3'

R568: 5' primer for amplification of an internal region of *his3*.

5'- CTT CTT GAT GCC AAT GAA TG -3'

R569: 3' primer for amplification of an internal region of *his3*.

5'- ACC GAA TTC CTG CTA GAC C -3'

R893: 5' primer with tails for amplification of *ura4* without ATG for insertional mutagenesis.

5'-NNN NNN NNN NNN NNN NNN NNG ATG CTA GAG TAT TTC AAA GC-3'

R894: 3' primer with tails for amplification of *ura4* for insertional mutagenesis.

5'-NNN NNN NNN NNN NNN NNN NNT CCA ACA CCA ATG TTT ATA ACC-3'

U456: Inverse primer at 5' end of *ura4* sequence, to PCR into sequence surrounding insertion site after insertional mutagenesis.

5'-CAA GTT GCT TTG CTT TTC TTC CAT C-3'

U457: Inverse primer at 3' end of *ura4* sequence before stop codon, to PCR into sequence surrounding insertion site after insertional mutagenesis.

5'-CGG TAG CGA TAT CAT CAT TGT TGG-3'

W679: Inverse primer at 5' end of *ura4* sequence, to PCR into sequence surrounding insertion site after insertional mutagenesis.

5'-GAC CGC GAC TGA CAA GTT GC-3'

W680: Inverse primer at 3' end of *ura4* sequence after stop codon, to PCR into sequence surrounding insertion site after insertional mutagenesis.

5'-CAT GCT CCT ACA ACA TTA CCA C-3'

W883: 3' primer downstream of *taz1*.

5'- AAA GGA TGA TTA AGG CCG TG-3'

X961: 5' primer for amplification of the whole *ura4* HindIII fragment for insertional mutagenesis.

5'-GCT TAG CTA CAA ATC CCA CTG G-3'

Y255: Inverse primer at 5' of *ura4* ATG to PCR into sequence surrounding insertion site after insertional mutagenesis.

5'-CCT ATT TAG AGA AAG AAT GCT GAG-3'

Y256: 5' primer with tails for amplification of whole *ura4* HindIII fragment for insertional mutagenesis.

5'-NNN NNN NNN NNN NNN NNN NNG CTT AGC TAC AAA TCC CAC TGG-3'

Y802: 5' primer 200bp upstream of *taz1* ATG

5'-CCG ACA GTT CCT TTC TTT TCG C-3'

2.6.4 Vectorette linker

Oligonucleotides to make Vectorette

B1764 V1 57mer 1st strand of vectorette construct 1 with BamHI and blunt ends.

5'-GAT CCA AGG AGA GGA CGC TGT CTG TCG AAG GTA AGG AAC GGA CGA GAG AAG GGA GAG- 3'

B1766: V2 53mer 2nd strand of vectorette construct 1 with BamHI and blunt ends.

5'-CTC TCC CTT CTC GAA TCG TAA CCG TTC GTA CGA GAA TCG CTG TCC TCT CCT TG-3'

D1161: 58mer 1st strand of vectorette construct 2 with EcoRI overhang.

5'-AAT TCA AGG AGA GGA CGC TGT CTG TCG AAG GTA AGG AAC GGA CGA GAG AAG GGA GAG A- 3'

D1162: 58mer 2nd strand of vectorette construct 2 with HindIII overhang.

5'-AGC TTC TCT CCC TTC TCG AAT CGT AAC CGT TCG TAC GAG AAT CGC TGT CCT CTC CTT G-3'

Oligonucleotides to PCR or sequence from Vectorette

B1768: primer for PCR from vectorette sequencing towards the BamHI or EcoRI overhang.

5'- CGA ATC GTA ACC GTT CGT ACG AGA ATC GCT -3'

D1163: primer for PCR from vectorette sequencing towards blunt end or HindIII overhang.

5'- GCT GTC TGT CGA AGG TAA GGA ACG GAC GAG -3'

Chapter 3

A genetic screen to identify mutations in genes that act at telomeres

3.1 Introduction

In *S. pombe* the terminal telomere repeat arrays are maintained at an average length of 300bp by a telomerase-based system. The fission yeast telomere repeat unit is more complicated than in many other eukaryotes, with a consensus sequence of $TTACAG_{1-8}$ (Hiraoka *et al.,* 1998), compared to that of TTAGGG in mammals and TG_{1-3} in *Saccharomyces cerevisae* (Blackburn and Greider, 1995). Genes placed adjacent to the telomeric repeats are subject to transcriptional repression. Several mutations affecting telomere structure and function in *S. pombe* have already been identified. The *taz1* and *lot2* mutations have an increase in the number of telomere repeat sequences and alleviate silencing of telomeric marker genes. Other mutations, such as those affecting the catalytic subunit of telomerase reverse transcriptase, *trt1,* cause the progressive shortening of telomeres resulting in telomere loss and cell senescence and death within approximately 80 generations (Nakamura *et al.,* 1998). Mutations affecting general silencing factors can lead to transcription of telomeric marker genes without changing the telomere length, for example mutations in the general silencing factors *swi6, clr4* and *rik1* (Ekwall *et al.,* 1996). Homologues of some factors acting at telomeres in other organisms have not yet been identified in fission yeast, for example *CDC13* and *EST3*, which are involved in telomerase recruitment and regulation in *S. cerevisiae* (Lendvay *et al.,* 1996). The *S. pombe* telomerase RNA template has not been identified but the structure of the telomere repeats and comparison with telomere maintenance pathways in other organisms indicates that it must exist. Given the complexity of telomere length regulation and structure in other organisms there are probably more factors involved in telomere structure and function still to be discovered in the fission yeast. Such factors may share regions of similarity with telomeric genes identified in other organisms, indicating that they are conserved in evolution, or they may prove to be quite different, thus offering alternative pathways for telomere maintenance. Either

outcome will be useful in helping to identify the components of telomere structure and function in higher eukaryotes.

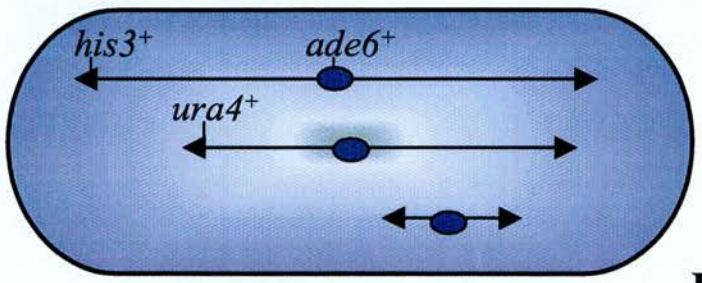
An initial screen for loss of telomeric silencing generated three different mutants: *rat1, lot2* and *lot3* (Nimmo *et al.,* 1998). Whilst *rat1* maintained wild type telomere length, *lot2* and *lot3* mutants displayed a large increase in the number of telomeric repeats. During meiosis telomeric attachment to the spindle pole body was defective, resulting in aberrant movement of the DNA in 'horsetail' phase. Such defects in meiosis lead to reduced rates of homologous recombination and irregular spore formation. It was found that the *lot3* phenotype was caused by a mutation in the myb domain of *taz1* (Cooper, *et al.* 1997, Nimmo *et al.,* 1998) and that the *lot2* strain contained a mutation in the gene encoding *S. pombe* Rap1p (Nimmo, personal communication). However this screen was not saturated and only a handful of mutants were examined. Due to this a further screen was devised to isolate and analyse a larger collection of independent mutants. The details and results of this screen are presented.

3.2 Results

3.2.1 Screening for spontaneous and UV generated mutations in telomere genes

Genes inserted into regions of silent chromatin at centromeres, telomeres and the silent mating type locus are transcriptionally repressed in a wild type background. A screen for telomere specific mutations in fission yeast was carried out using a strain containing selectable marker genes in the centromere and telomeres, which may become expressed when genes affecting the structure or function of these regions are disrupted. These markers consist of a *his3*$^+$ gene and an *ura4*$^+$ gene at the left hand telomeres of chromosome one and two respectively and an *ade6*$^+$ gene at centromere one, as shown in Figure. 3.1.

The original screen was repeated with a few small adjustments in the hope of achieving saturation and identifying more factors involved in telomeric structure and function. The starting strain was cultured for three to four days at 32°C on rich



FY1862

ade6-210, his3D-1, leu1-32, ura4-D18

Figure 3.1: The strain used in the mutagenesis screen showing the location of the *his3*$^{+}$ and *ura4* $^{+}$ marker genes at the left hand telomeres of chromosomes one and two respectively and the *ade6*$^{+}$ gene at the centromere of chromosome one (strain FY1862). Expression of the nutritional markers inserted in the heterochromatin like regions is repressed in a wild type background.

medium agar containing 0.1% fluro-orotic acid (5-FOA), a chemical toxic to cells expressing the *ura4*$^+$ gene. This ensures that silencing is maintained at the telomeres and thus selects against the presence of a pre-mutation of the starting strain, which could lead to the over representation of a single mutation in the screen. Twelve colonies were selected from these plates and the screen carried out using the method described in Figure 3.2. Only cells with alleviation of silencing at telomeres allow expression of telomeric *his3*$^+$ and *ura4*$^+$ genes and grow on the selective plates. Selecting for loss of silencing at two telomeres guards against growth on selective plates of colonies that result solely from chromosomal rearrangements removing the marker genes from the influence of a neighbouring telomere. Those that appeared within five days were classed as early mutants and slower growing colonies were classified as late. Subdividing the pools of cells in this manner created a possible 48 final pools of independently arising mutants.

3.2.2 Selection of telomere specific mutants

Mutations affecting silencing at telomeres may be telomere specific or the result of a more general disruption in the structure of silent chromatin. Colonies that displayed alleviation of silencing at the telomere were streaked onto limiting adenine plates, to examine them for alleviation of repression of the *ade6*$^+$ marker at the centromere. Cells unable to transcribe *ade6*$^+$ are blocked at a point in the adenine biosynthesis pathway and accumulate a metabolite that produces a red pigment. The presence of limiting supplementary adenine enables colonies to develop, but as they become deprived of adenine colonies turn red in colour. Loss of centromeric silencing allows transcription of *ade6*$^+$ and thus the formation of white colonies. A summary of the mutants generated by this screen is shown in Table 3.1. The mutants generated from each pool of cells in the screen were split into groups according to whether they were: telomere specific or more general silencing factors, based on colony colour; mutants that arose spontaneously or generated following exposure to UV light; and colonies that arose early or late. Red colonies that grew on minus histidine and minus uracil were presumed to have mutations in genes that specifically affect silencing at telomeres and were selected for further analysis.

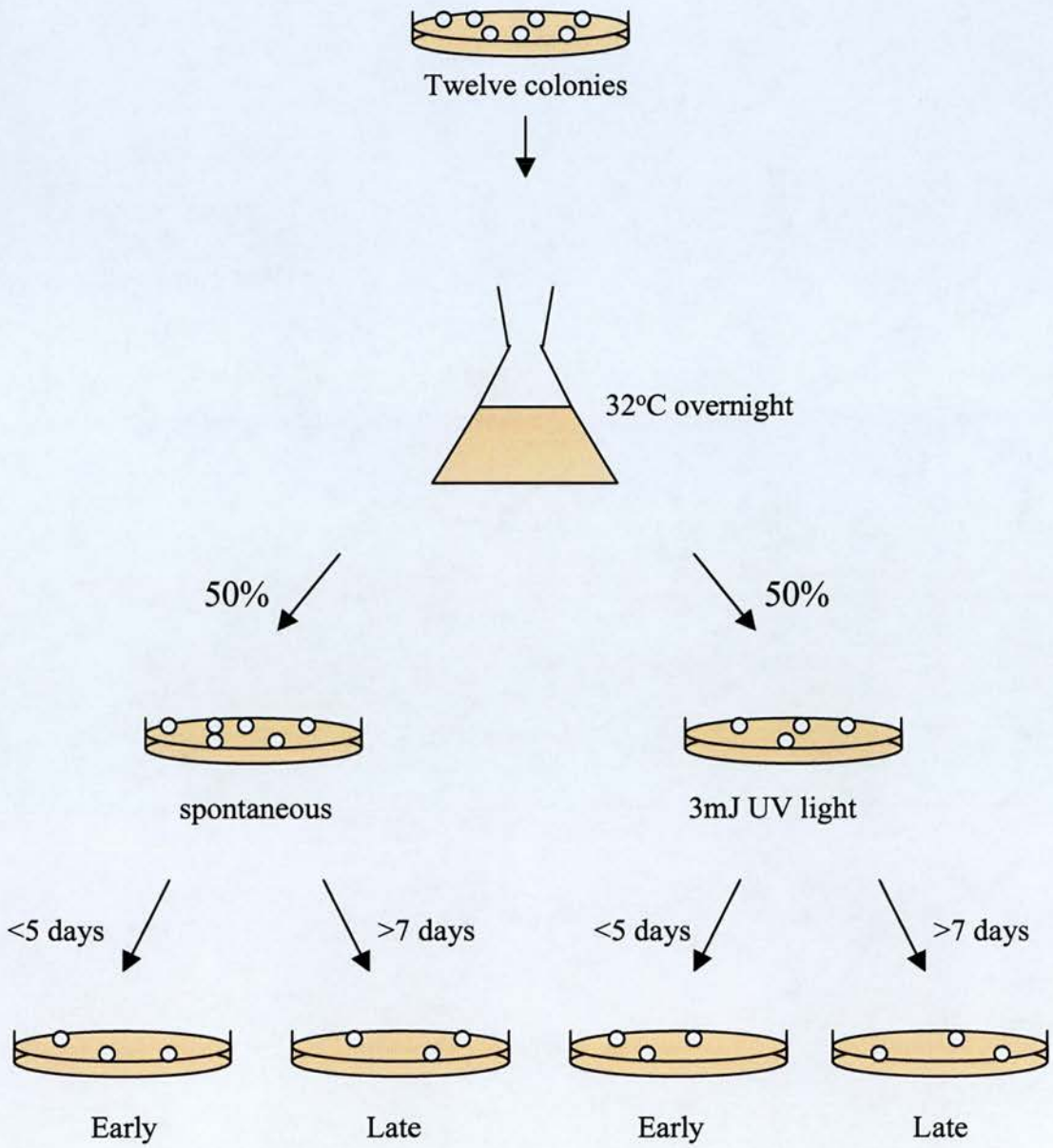


Figure 3.2: Mutagenesis screen for factors involved in silencing at telomeres; twelve separate colonies were picked from the starting plate and grown to log phase at 32°C in YES containing 5-FOA. The concentration of cells in each culture was determined and $1x10^7$ cells from each pool were plated onto two plates minus both histidine and uracil. After plating, half of each pool of cells was left untreated to screen for spontaneous mutations, while the other half was subjected to 3mJ UV light, which resulted in killing of 50% of cells. The plates were incubated at 25 °C and colonies picked as they became visible. Colonies were subdivided into fast and slow growing colonies depending on whether they appeared early (<5days) or late (>7days).

The strain utilised for mutagenesis had a wild type mating type so that mating type switching could occur. This allowed defective silencing at *mat2* and *mat3* to be analysed. Mating in an h^{90} colony normally results in asci containing four spores that contain starch and stain black on exposure to iodine vapour. Defective silencing at the mating type locus results in inefficient mating type switching and thus less spore formation. Upon exposure to iodine vapour these colonies stain grey or white compared to the black of colonies competent for mating type switching. The selected mutants were therefore screened for defects in mating type silencing. Colonies that stained black after growth on ME plates, as in the starting strain, were selected as isolates bearing a telomere silencing specific mutation and preserved for further study. On completion of this analysis the screen generated a total of 777 telomere specific mutants derived from 46 of the 48 original groups.

A temperature sensitive mutant would provide a convenient means with which to identify the affected gene. Colonies were tested for temperature sensitivity by streaking cells onto several YES Pholoxin (Vital stain) plates, which were incubated at 25°C, 32°C and 36°C. No temperature sensitive mutations were found amongst the mutants obtained in this screen. This may suggest that mutations in genes affecting telomere structure and function are not immediately lethal to the cell or that the affected genes are not essential. This may reflect the fact that telomere maintenance machinery is not continuously required for vegetative growth. For instance, it is known that for some genes that affect telomere structure and function, such as $trt1^{+}$ in *S. pombe* and the *EST1* and *EST2* in *S. cerevisiae,* mutation or loss of function eventually leads to cell death but only after a number of generations (Nakamura *et al.,* 1998, Lundblad and Szostak, 1989, Lendvay *et al.,* 1996). This is due to the erosion of the telomeres which leads to cell senescence and crisis.

3.2.3 Dividing the *rat* mutants into complementation groups

To reduce the number of strains to a manageable number a subset of the mutants generated was selected for further analysis and sorted into complementation groups.

Pool:		A		B		C		D		E		F	
		red	white	red	white	red	white	red	white	red	white	red	white
spontaneous	early	13	--	0	24	17	1	8	5	3	15	6	7
	late	20	--	22	12	38	4	21	7	4	14	21	20
3mj UV	early	11	--	0	22	21	1	13	5	7	5	7	7
	late	8	--	7	27	33	6	35	6	20	11	24	15

Pool		G		H		I		J		K		L	
		red	white	red	white	red	white	red	white	red	white	red	white
spontaneous	early	4	15	0	34	12	3	2	18	9	12	1	21
	late	37	2	29	1	13	14	21	12	35	3	35	7
3mj UV	early	3	16	5	15	11	4	6	10	10	10	17	1
	late	36	4	35	4	35	3	24	18	31	10	7	0

Table 3.1 A summary of the number of mutants generated in each class; spontaneous or UV generated, fast or slow growing and telomere specific or a general silencing factor (red or white respectively), for each pool of cells used to perform the screen.

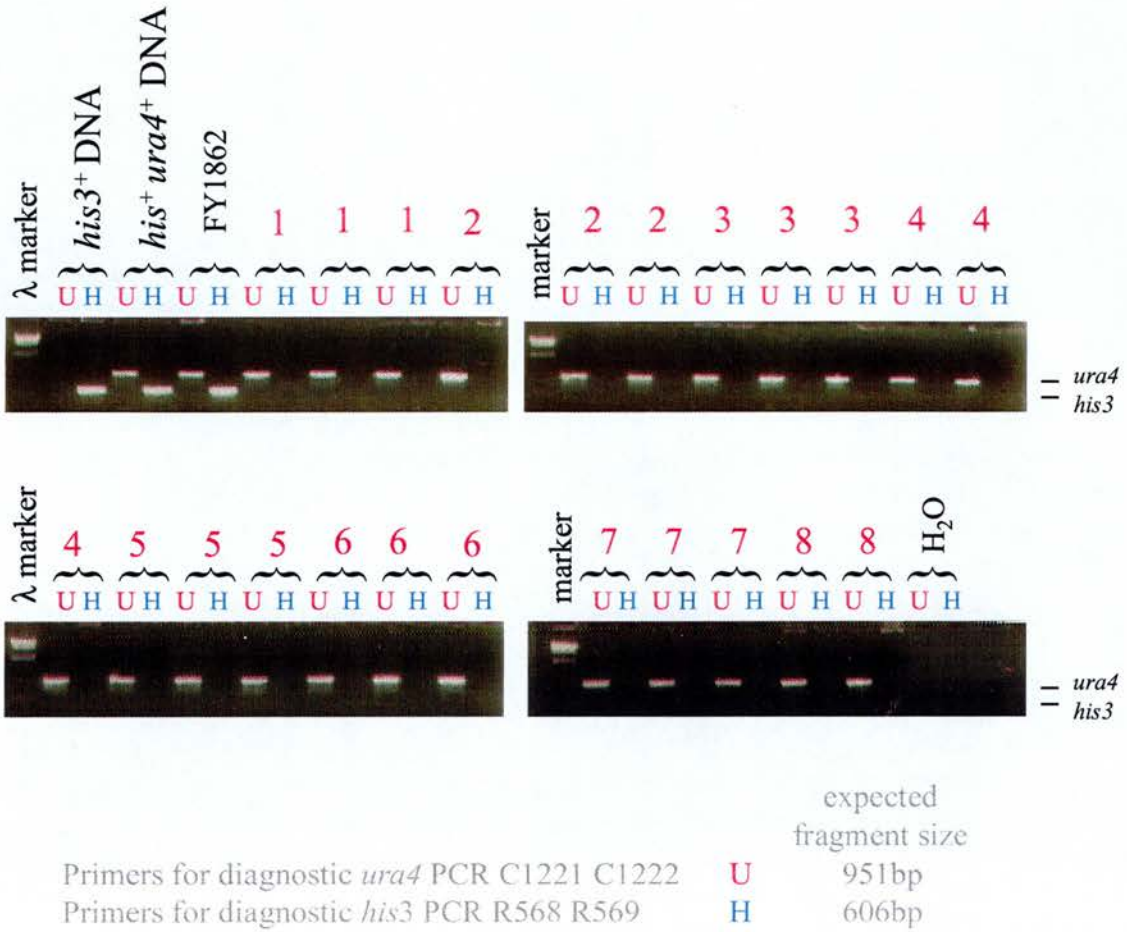
The subset to be analysed were chosen by taking one early and one late growing mutant from both the UV generated and spontaneously mutated sets of each of three of the different starting pools. This ensured that the mutants arose independently from one another and still represented a cross section of the subgroups generated by the screen. These twelve mutant strains were then backcrossed to h^+ and h^- strains to ensure that a single mutation was responsible for the phenotype and that self-mating would not occur. The strains were crossed to one another and approximately 1000 of the resulting spores from each cross were plated on non-selective YES plates. The resulting colonies were replica plated to minus histidine plates. The number of colonies that grew on minus histidine plates and therefore those expressing the telomeric *his3*$^+$ marker from each cross was counted and compared to the total number of colonies growing on the non-selective YES media to determine whether alleles at the same locus had been isolated. At least 700 progeny from each cross were counted. These data were used to place the twelve mutants in complementation groups. The proportion of *his3*$^+$ colonies produced for each genetic cross is shown in Table 3.2.

The results in Table 3.2 show that rarely are all of the offspring able to grow on minus histidine plates. The *his3*$^+$ marker at the telomere is occasionally lost when the cells pass through meiosis, an event that is likely to be due to recombination at telomeres. A small proportion of *his3*$^-$ colonies in the progeny of a cross could be misinterpreted as linkage of two mutant loci rather than alleleism. When a small number of progeny were found to be unable to grow on plates lacking histidine they were streaked onto minus uracil to see if the *ura4*$^+$ marker gene was still expressed. Amplification of the telomeric markers by PCR was attempted in order to clarify whether the *his3*$^-$ phenotype was due to loss of the telomeric marker or linkage. This allows for confirmation that the telomeric marker has been lost during crossing. Colonies that gave no amplification product were found to be *his3*$^-$ due to loss of the telomeric marker and not because of re-imposition of silencing at the telomere. If the *his3*$^+$ marker in the *his3*$^-$colonies could be amplified by PCR then the mutations in the two parental strains must be closely linked but perhaps in two different

mutant allele		h^+											
		j543	*j556*	*j575*	*j589*	*k596*	*k601*	*k620*	*k639*	*l646*	*l655*	*l675*	*l678*
h^-	*j543*	98[1]	96[2]										96[3]
	j556	96	-	72	83	100						77	99[4]
	j575		72	-		85	99[6]			87			
	j589		83		-	100							
	k596		100	85	100	97[8]		61	67	75			99[5]
	k601	81		99			-						
	k620	72		78		61		-	77	97[7]			
	k639					67		70	-		76	67	
	l646			87		75		97		-			77
	l655	72		76	72				78		99	74	78
	l675								73		74	99	79
	l678	97	99			99				76	78	79	100

Table 3.2. The proportion of progeny from each cross that express the *his3* gene inserted at the telomere. If two mutations in the same gene are causing the alleviation of repression phenotype in the parental strains then all the offspring will inherit a mutant gene and express the *his3* marker gene at the telomere. If two different genes are causing the loss of silencing phenotype approximately 25% of the offspring will be wild type with silencing maintained at the telomere rendering them unable to grow on PMG media lacking histidine. The crosses which had a high proportion of $his3^+$ progeny, marked with a red number, were PCR checked for loss of the telomeric $his3^+$ marker gene

genes. Colonies that grew on minus uracil and in which no amplification product was produced were presumed to be his^- due to loss of the telomeric marker gene and not transcriptional repression. In Figure 3.3 it can be seen that, for the crosses marked with a number in Table 3.2, no $his3^+$ amplification product was observed. We conclude from the genetic data and PCR analysis that the inability of progeny to grow on media lacking histidine was due to loss of the $his3^+$ marker gene and not re-imposition of silencing at the telomere. The same was also true for crosses between *k620* and *l646* and between *j575* and *k601*.



		expected fragment size
Primers for diagnostic *ura4* PCR C1221 C1222	U	951bp
Primers for diagnostic *his3* PCR R568 R569	H	606bp

Figure 3.3. Occasionally the *his3*$^{+}$ marker gene at the telomere is lost as the cells pass through meiosis. The presence of the telomeric marker gene in colonies that are unable to grow on plates lacking histidine was checked for by PCR. Each colony was resuspended in SPZ, as detailed in 2.3.2, and two PCR reactions were carried out, one for a diagnostic *his3*$^{+}$ fragment (H) and one for a diagnostic *ura4*$^{+}$ fragment (U). The letter above each lane indicates which of the two PCR reactions the product stems from and the numbers above each pair of these corresponds to the crosses from which the his3^{-} colony originated, as indicated in table 3.2. Those that show no product are *his3*$^{-}$ due to loss of the *his3*$^{+}$ gene and not re imposition of silencing at telomeres.

From this data the twelve mutants fall into six complementation groups as shown in Table 3.3. The complementation groups are named *rat* for repression alleviated at telomere. *Rat2* contains five alleles, the *rat3* and *rat4* groups each contain two alleles, while the other three complementation groups all consist of one allele each.

	Complementation group					
	rat2	***rat3***	***rat4***	***rat5***	***rat6***	***rat7***
allele	*j543*	*k620*	*j575*	*l655*	*l675*	*k639*
	j556	*l646*	*k601*			
	j589					
	k596					
	l678					

Table 3.3. The complementation groups into which the twelve *rat* mutants were divided following genetic crossing.

3.2.4. Identification of genes responsible for the *rat* mutant phenotypes

The level of variegation of expression of the *ura4*$^+$ marker gene at the telomere in the *rat* mutants, discussed in chapter 5, is too high for complementation of the phenotype by use of plasmid genomic libraries. Thus the affected genes cannot be identified by this approach. One possibility is that some of the *rat* mutants are alleles of genes already identified as affecting telomeric silencing. Strains carrying point mutations in one of *rap1, rat1, taz1, swi6, rik1* or *clr4* were crossed to the *rat* mutants and the effect on a *his3*$^+$ gene at the telomere monitored. The spores from the crosses were plated on YES and grown for two days at 32°C, then replica plated onto minus histidine and YES. The relative number of colonies formed on the plates was then counted for each cross and the mutants identified as alleles of known genes or novel genes.

		Complementation group					
		rat2	*rat3*	*rat4*	*rat5*	*rat6*	*rat7*
Candidate gene	*taz1*	96	88	76	78	73	67
	rap1	69	76	92	63	81	85
	rat1	77	67	82	-	-	80
	swi6	68	78	75	69	73	73
	clr4	75	85	78	81	73	80
	rik1	79	69	74	82	73	69

Table 3.4. The groups of *rat* mutants were crossed to candidate genes that affect telomeric silencing and the percentage of his3$^+$ progeny arising from each genetic cross counted.

A summary of the proportion of progeny expressing the *his3*$^+$ gene in all the crosses carried out is displayed in Table 3.4. The number of *his3*$^+$ colonies produced from crosses between individual alleles of a complementation group and a candidate gene were divided by the total number of colonies from those crosses to give the final Figure shown in the Table. It can be seen from this data that the alleles of the *rat2* complementation group are either allelic to *taz1*$^+$ or closely linked to the *taz1*$^+$ locus. PCR analysis of the telomeric *his3*$^+$ marker gene showed that the small proportion of colonies unable to grow on minus histidine had lost the telomeric *his3*$^+$ marker. Thus the *rat2* mutants are likely to be caused by mutations in the *taz1* gene. None of the other *rat* mutants show alleleism with any of the candidate genes tested. It was noted that the progeny of a cross between the *rat5-1655* mutant and *rap1*Δ grew very poorly.

3.2.5 Sequencing the *taz1* gene in the *rat2* mutants

Data obtained from the genetic crosses show that the alleles of the *rat2* complementation group are very closely linked to the *taz1*$^+$ gene. In order to confirm that the *rat2* mutants were indeed alleles of *taz1* they were sequenced to identify the site of the mutation. The oligonucleotides used to prime the sequencing reactions and their positions relative to the *taz1* gene open reading frame are shown in Figure 3.5.

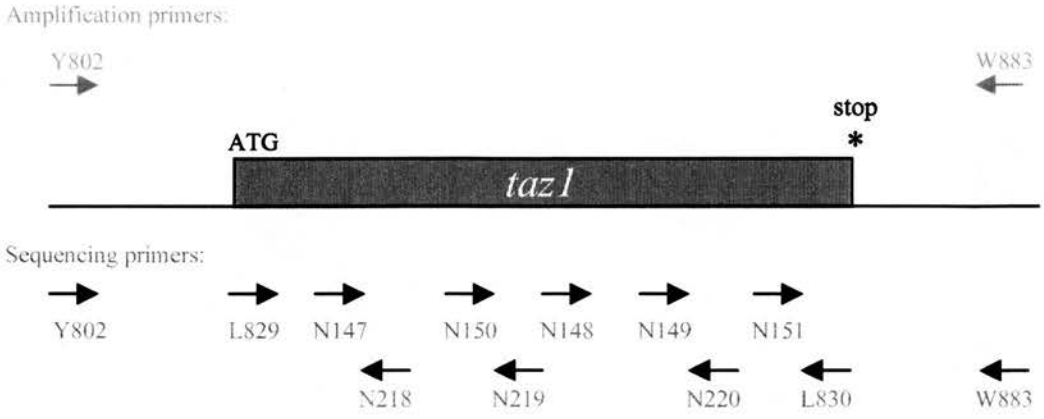


Figure 3.6 The oligonucleotides used to amplify and sequence the *taz1* gene in the *rat2* mutant strains.The exact sequences and positions of the oligonucleotides relative to the *taz1* ATG are detailed in 2.6.2.

Sequence was obtained for *rat2-k596* and *rat2-j556* and was searched against the wild type *taz1* gene sequence using the *S. pombe* BLAST server at the Sanger Centre to identify potential changes in the sequence. The *rat2-k596* allele has an adenine to cytosine change at A840, relative to the *taz1* ATG, making the codon change from ACC to CCC and thus resulting in a change in the amino acid sequence from threonine to proline. Sequencing the *rat2-j556* mutant revealed that 1114t is deleted in this strain, resulting in a frame shift followed by premature truncation of the protein seven amino acids after the mutation site. It is possible to amplify the *taz1* open reading frame in *rat2-j589* only as far as the N219 primer, 1825bps downstream of the start codon. This indicates that part of the 3' end of the gene containing the myb-like DNA binding domain is deleted in this strain. The positions of the mutations observed in *rat2-k596* and *rat2-j556* are shown in Figure 3.6.

3.3 Discussion

In this chapter I have described an extensive screen for mutations affecting silencing at fission yeast telomeres but not at centromeres or the silent mating type loci. A total of 777 mutants matching all criteria were generated, which were isolated from twelve independent pools of the starting strain. One early spontaneous mutant, one late spontaneous mutant, one early UV generated mutant and one late UV generated mutant were selected from three of these twelve pools. Genetic analysis revealed that these mutants represent six different complementation groups. At the outset of this project there were three loci known to specifically affect silencing at the telomere in fission yeast; *taz1, lot2 and rat1* and an additional three silencing factors that generally affect areas of silent chromatin including the telomere; *swi6, clr4* and *rik1*. Members of each complementation group were tested in genetic crosses to known mutants affecting telomere silencing. Random spore analysis suggested that *rat2* and *taz1* are allelic. Sequencing analysis identified mutations in the *taz1* open reading frame of *rat2-k596* and *rat2-j556*, a third *rat2* mutant, *rat2-j589* has a suspected

Starting strain: MISVQSTETIQKVLENEGDQQFDKEVVQNSDSNIETGQISDSLTKAVEERAETESSSNLSNTSESESSKPAYCFNSHSQNMAEGSISIPVISHSMNVENEVSAE
rat2-k596 : MISVQSTETIQKVLENEGDQQFDKEVVQNSDSNIETGQISDSLTKAVEERAETESSSNLSNTSESESSKPAYCFNSHSQNMAEGSISIPVISHSMNVENEVSAE
rat2-j556 : MISVQSTETIQKVLENEGDQQFDKEVVQNSDSNIETGQISDSLTKAVEERAETESSSNLSNTSESESSKPAYCFNSHSQNMAEGSISIPVISHSMNVENEVSAE

Starting strain: GQDSRTGESENDQNAMIVRSIWDIEKASLLVNDCQNIANMAEQKVMMVSAIFSESSKDIVNPESFSERLGKETVKDLYEFNEQLTTKYGLEFRTIFFSYIRKYD
rat2-k596 : GQDSRTGESENDQNAMIVRSIWDIEKASLLVNDCQNIANMAEQKVMMVSAIFSESSKDIVNPESFSERLGKETVKDLYEFNEQLTTKYGLEFRTIFFSYIRKYD
rat2-j556 : GQDSRTGESENDQNAMIVRSIWDIEKASLLVNDCQNIANMAEQKVMMVSAIFSESSKDIVNPESFSERLGKETVKDLYEFNEQLTTKYGLEFRTIFFSYIRKYD

Starting strain: AYWCLFEDLEKPLKSIQFFTGLIDLLDNTNKHLTLRSIVLDALLSADEEDSFYGDALVLFEELVIRYFGTDSNPSIDASEFILSCLPYTSLDALNVVCGQVWKSQK
rat2-k596 : AYWCLFEDLEKPLKSIQFFTGLIDLLDNTNKHLTLRSIVLDALLSADEEDSFYGDALVLFEELVIRYFGPDSNPSIDASEFILSCLPYTSLDALNVVCGQVWKSQK
rat2-j556 : AYWCLFEDLEKPLKSIQFFTGLIDLLDNTNKHLTLRSIVLDALLSADEEDSFYGDALVLFEELVIRYFGTDSNPSIDASEFILSCLPYTSLDALNVVCGQVWKSQK

Starting strain: ICDFLKSTIGNTSNSPLQLRASFPAFVNAVIHFLLEFKNVRRLERKDLSVKGMLYDSDSQQILNRLRERVSGSTAQSADEASGHESDASEDTFSERTLGLNSIDNT
rat2-k596 : ICDFLKSTIGNTSNSPLQLRASFPAFVNAVIHFLLEFKNVRRLERKDLSVKGMLYDSDSQQILNRLRERVSGSTAQSADEASGHESDASEDTFSERTLGLNSIDNT
rat2-j556 : ICDFLKSTIGNTSNSPLQLRASFPAFVNAVIHFLLEFKNVRRLERKDLSVKGMLSTLIANRS*

Starting strain: EISEVVSLGLVSSALDKITGLLSADNLSETVSQARDFSHTLSKSLKSRAKSLSQKEAANRSKLIAKRGDNLRREASLSSEQDDLSEDFPPVRESDEQESRSGGRSS
rat2-k596 EISEVVSLGLVSSALDKITGLLSADNLSETVSQARDFSHTLSKSLKSRAKSLSQKEAANRSKLIAKRGDNLRREASLSSEQDDLSEDFPPVRESDEQESRSGGRSS
rat2-j556

Starting strain: AMRVSIERSAARSGTRRSQGNPYEGYRTRRKWTDEEENELYEMISQHGCCWSKIIHIQKLENGPLKTFGPTQIKDKARLIKARFMKQNRLQELYSKSLNWKN
rat2-k596: AMRVSIERSAARSGTRRSQGNPYEGYRTRRKWTDEEENELYEMISQHGCCWSKIIHIQKLENGPLKTFGPTQIKDKARLIKARFMKQNRLQELYSKSLNWKN
rat2-j556

Starting strain: VTVGQAYCELHKIPYIEATPPLLREELVNYQS
rat2-k596 VTVGQAYCELHKIPYIEATPPLLREELVNYQS
rat2-j556

Figure 3.6 The amino acid sequence of *taz1* with the myb domain highlighted in purple and the amino acid changes resulting from mutations identified in the open reading frames of *rat2-k596* and *rat2-j556* highlighted in red. The amino acid changes and early truncation of the protein in *rat2-j556* are highlighted in orange. Mutations identified in the *taz1* open reading frame in the *rat2* mutants confirms that they are alleles of *taz1*.

truncation as PCR amplification of the 3' end of the gene was impossible. The *rat2-k596* mutant has alleviation of repression at the telomere but a wild type telomere length, thus separating the silencing and telomere length functions of *taz1*. The mutation found in *rat2-k596* may identify a domain of the protein that has an important role in forming the silencing complex at the telomere.

The phenotypes of the *rat* mutants will be further analysed in chapters 4 and 5. For clarity alleles in the *rat2* complementation group will continue to be called *rat2* to distinguish them from previously isolated *taz1* alleles.

Chapter 4

Analysis of the *rat* mutants

4.1 Introduction

There are already several known mutants affecting the telomere in *S. pombe*. Phenotypic analysis of loci that affect telomeric silencing revealed that two of these genes, *taz1* and *rap1*, have roles in telomeric length regulation, transcriptional repression and meiosis (Cooper *et al.*, 1998, Nimmo *et al.*, 1998, Chikashige and Hiraoka, 2001, Kanoh and Ishikawa, 2001). Mutation at third telomere specific locus, *rat1*, or deletion of one of the general silencing factors *clr4*, *rik1* or *swi6* has no effect on telomere length (Nimmo *et al.* 1998, Ekwall *et al.*, 1996), however the *clr4-lot1* mutation results in a small increase in telomere length (Nimmo personal communication). In budding yeast mutations in yKu70 or yKu80 alleviate telomeric silencing and shorten the telomere repeat tract. Fission yeast lacking *pku70* also have a shorter telomeric repeat but no effect on alleviation of transcriptional repression at the telomere is seen (Maniolis *et al.*, 2001). Alleviation of silencing at the telomere has not been reported in *trt1*Δ or *tel1*Δ*rad3*Δ strains (Nakamura *et al.*, 1998, Naito *et al.*, 1998). Thus alleviation of silencing at the telomeres does not always signify that a change in telomere length has occurred, nor do structural changes at the telomere always give rise to alleviation of silencing.

The *rat* alleles in each complementation group were analysed for their phenotypes. All alleles in each group were analysed as mutations affecting different regions of the same gene may have different phenotypes, as for *cdc13* alleles studied in *S. cerevisiae* (Grandin *et al.*, 2000, Pennock *et al.*, 2001).

4.2 Results

4.2.1. Analysis of telomere length in *rat* mutants

In a wild type population of *S. pombe,* the telomere repeats are maintained at an average length of 300bp. Mutations that affect silencing at telomeres may also cause imbalances in mechanisms of telomere maintenance and lead to changes in the average telomere repeat size of a population. This is demonstrated by the increase in telomere length seen in *taz1* and *rap1* mutants (Cooper *et al.,* 1997, Nimmo *et al.,* 1998). The mutations causing alleviation of silencing at telomeres in the *rat* mutants may also have an effect on the length of the telomere repeats in these strains. Digesting DNA from the starting strain and the mutant strains derived from it with a BamHI restriction enzyme cuts within the $his3^{+}$ telomeric marker gene on chromosome I, thus releasing a fragment containing telomere repeats (Nimmo *et al.,* 1998). The released telomere fragment appears as a smear on a Southern blot when probed with telomere repeat sequences. In the starting strain this telomeric smear extends between 600nt and 800nt in size.

DNA was extracted from the *rat* mutant strains, digested with BamHI and a Southern blot prepared using telomeric sequence as a probe, as described in 2.3.4. The resulting Southern blot can be seen in Figure 4.1.

rat7-k639, rat4-j575, rat2-j543 and *rat2-k596* display telomere smears of a similar size to that of the starting strain. A slight increase in the telomere lengths of the *rat3-k620* and *rat6-l675* mutants is observed, with the telomeres approximately 100bp longer than wild type at between 700bp -900bp. A slightly larger increase resulting in a telomere length of between 800bp and 1200bp was observed in *rat2-l678.* In the *rat5-l655* strain a very faint smear of telomere repeats is detected. This smear has a larger distribution than that of a wild type strain and begins at a shorter size extending between 500bp and 800bp, and is similar to that seen in a *ku70* mutant. No telomere repeat smear was detected in the lanes for *rat3-l646, rat2-j556, rat2-j589* and *rat4-k601.*

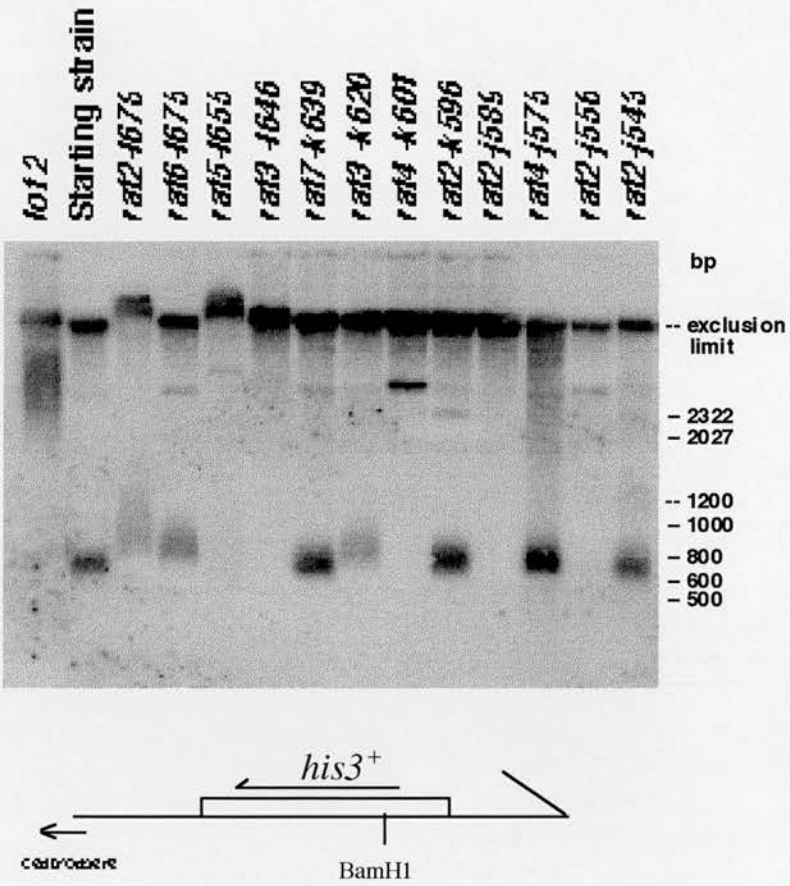


Figure 4.1. Southern Analysis of BamHI digested DNA from the *rat* mutants, starting strain and a *lot2* mutant. Digesting the genomic DNA of these strains cuts within the *his3*$^{+}$ marker gene and releases a 600-800bp from the marked telomere, as shown in the cartoon beneath the blot. Some of the *rat* mutants display altered telomere length. Not all of the alleles in any one complementation group share the same telomere phenotype.

Mutants in any one of the complementation groups did not share a common telomeric phenotype. For instance; of the five *rat2* alleles two had a wild type telomere length, one displayed a small increase in telomere length and no telomere repeat smear was detecTable in the fourth and fifth alleles.

In the *rat4-k601* allele no telomere repeat smear is detected, but a strong band of approximately 4kb becomes prominent. This could be due to a telomere fusion event occurring.

Thus in addition to affecting telomeric silencing some of the *rat* mutants are also defective in telomere length regulation. A change in the number of telomere repeat units indicates that the process of measuring telomere length or the balance of erosion and addition of telomere repeats is disrupted. The observation that some of the *rat* mutants have altered telomere length also validates the use of disruption of silencing as a tool for identifying mutations affecting telomere structure and function. The differences in telomere length between alleles within each of the complementation groups may reflect a different effect of each mutation on the protein function. Separation of function alleles have been found previously for other telomeric genes, including Cdc13p and Est1p in *S. cerevisiae,* and have proved useful in dissecting the function of this protein at telomeres (Grandin *et al.,* 2000, Pennock *et al.,* 2001).

4.2.2 Pulsed field gel analysis of *rat* mutant chromosome fragments

In the southern blot for telomere length four of the *rat* mutants, *rat2-j556, rat2-j589, rat3-l646* and *rat4-k601* were observed to have lost the telomeric repeats. These strains are proficient for vegetative growth on plates and in culture at 32°C and 36°C. It is possible that, like the survivor colonies that can arise from *trt1*Δ or *rad3*Δ*mec1*Δ populations of cells, these strains are able to overcome a loss of telomeric repeats by have circularising their chromosomes. To investigate this possibility two pulsed field gels were prepared.

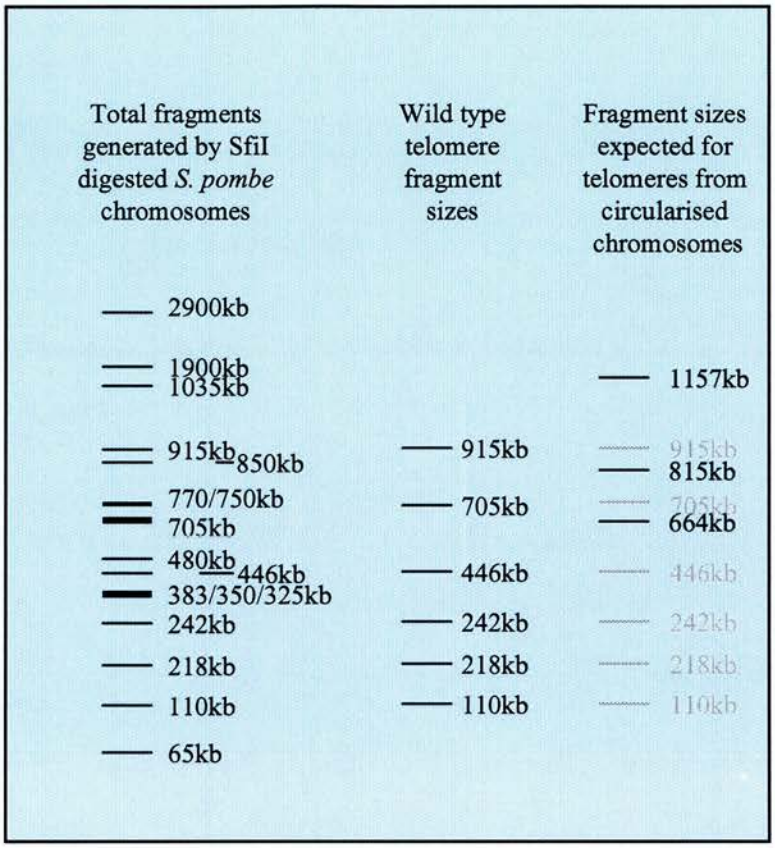


Figure 4.2. to demonstrate the fragment sizes generated by Pulsed Field gel analysis of wild type *S. pombe* chromosomes digested with Sfi1, which of these fragments contain telomeres and the size of fragments expected should circularisation of the chromosomes occur, leading to fused telomere ends.

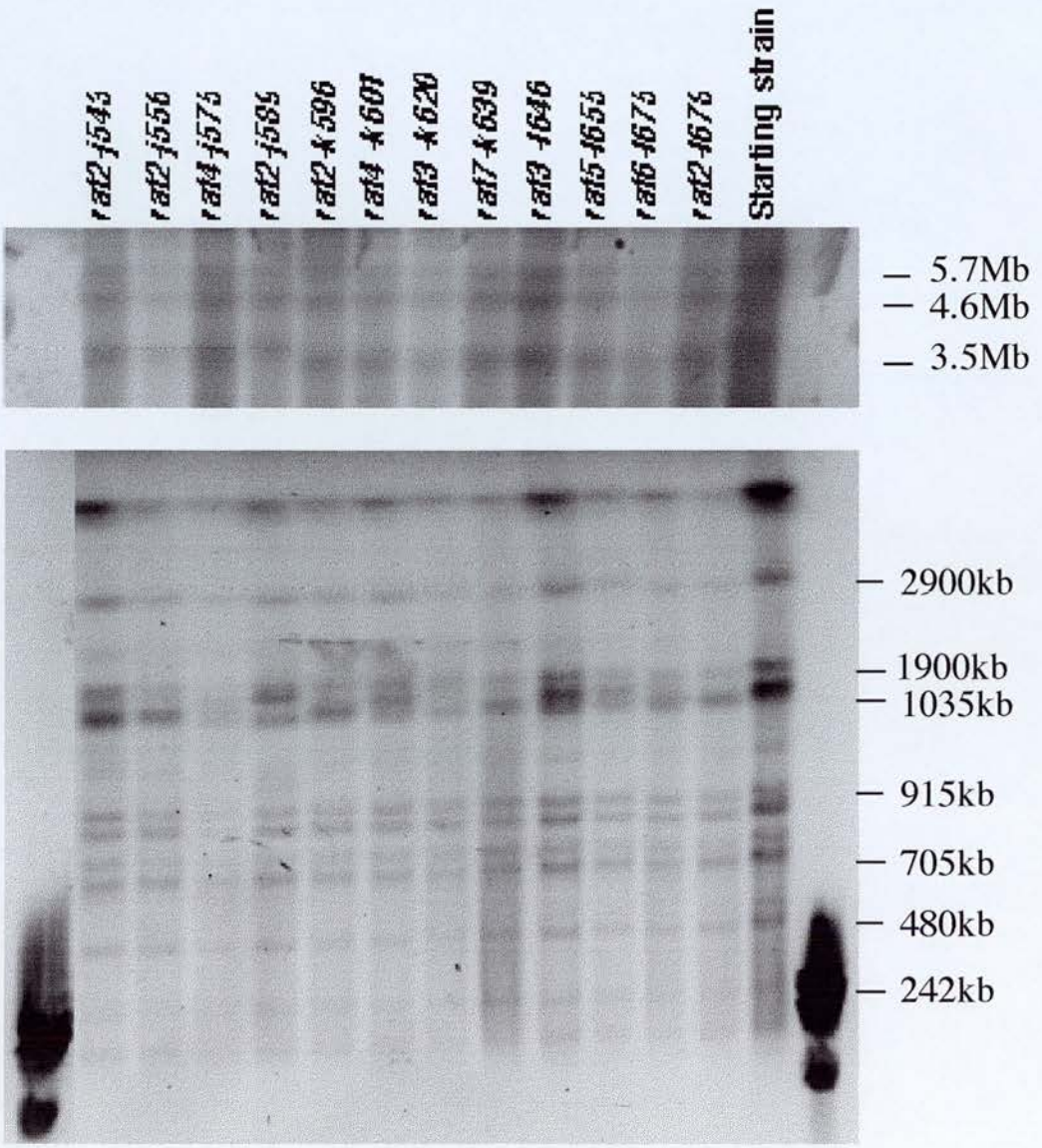


Figure 4.3. Pulsed Field gel analysis of high molecular weight DNA from the *rat* mutants. DNA from each strain was isolated in agarose plugs. In the upper panel the DNA is undigested and whole chromosomes are separated by size. In the lower panel the DNA is digested with SfiI. In all of the mutants it is possible to separate linear chromosomes buy pulsed field gelelectrophoresis. In the digested DNA it can be seen for *rat2-j589, rat4-k601, rat5-l655* and *rat3-l646* that an extra fragment of over 705kb is formed in these strains.

Circular chromosomes are unable to migrate into a pulsed field gel using the parameters for linear DNA molecules (Fan *et al.,* 1992). A pulsed field gel was prepared as detailed in 2.3.5. For the first pulsed field gel the plugs were not digested and the gel was run over 120 hours to separate whole chromosomes. A second pulsed field gel was prepared using the same batch of plugs to ensure that the concentration of DNA was equal in corresponding lanes on the two gels. The plugs of DNA used for the second gel were digested with Sfi1, an enzyme whose restriction site occurs 16 times in the pombe genome, giving rise to nineteen fragments of a known size as shown in figure 4.2. Six of these fragments end in a telomere and the sizes of these fragments are 110kb, 218kb, 242kb, 446kb 705kb and 915kb (Fan *et al.,* 1991). If circularisation of the chromosomes has occurred in the *rat* mutants then one would expect to observe two changes relative to a wild type strain; the migration of whole chromosomes into the gel should be retarded, giving rise to fainter bands than expected, and extra bands equivalent to the sum of two wild type telomeric bands should appear in the Sfi1 digested gel. These bands are expected to be around 664, 815 and 1157kb in size, as shown in figure 4.2.

In Figure 4.3 an extra band of approximately 1157kb is visible in *rat2-j589*, *rat5-l655, rat4-k601* and *rat3-l646*. This is potentially consistent with a telomeric fusion on chromosome three forming a ring chromosome. However for all the *rat* mutants the migration of whole chromosomes into the gel appears to be unperturbed. The extra fragments in the Sfi1 digested DNA were suspected to be the result of telomere fusion events, but may also be due to chromosomal translocations.

4.2.3. Defective spore formation in the *rat* mutants.

The attachment of telomeres to the spindle pole body is important for the alignment of homologous chromosomes and recombination in meiosis in the fission yeast (Funabiki *et al.* 1993). Following meiosis in wild type *S. pombe,* asci are formed that contain four evenly sized spores. Defects in meiosis lead to reduced rates of homologous recombination and an increased proportion of cells with chromosome

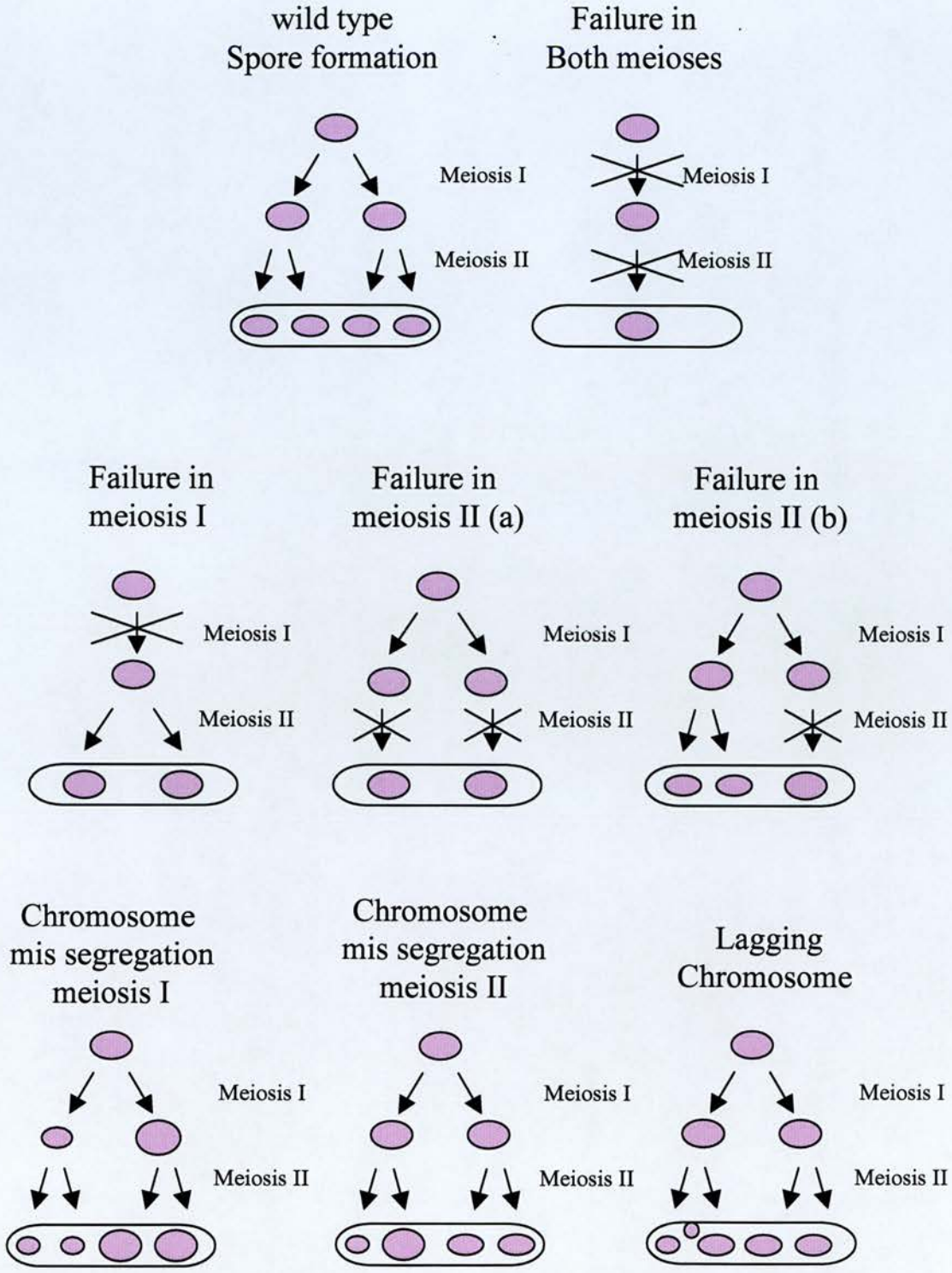


Figure 4.4. Cartoons showing the predicted spore pattern in an ascus following a wild type meiosis and the potential contents of asci following difficulties in meiosis I or meiosis II.

mis-segregation defects, which can result in aberrant spore formation. The *lot2* and *taz1-lot3* mutants are both defective in meiosis and have a high percentage of misshapen asci containing an odd number of spores or spores of different sizes to one another. 74% and 53% of the asci produced by *lot2* and *taz1-lot3* strains respectively contain abnormal spores (Nimmo *et al.*, 1998). Aberrant spores may form following a number of different difficulties arising in meiosis; these are demonstrated in Figure 4.4. Failure to separate chromosomes in meiosis I or II may generate asci containing one or two large spores. Mutations that result in aneuploidy may result in uneven spore size and occasionally lagging chromosomes or fragments of chromosomes created by a chromosome breakage event may result in DNA remaining external to the spores or give rise to microspores within the ascus. The asci of the *rat* mutants

Strain	Appearance of Ascus				% Abnormal	Increase relative to wild type
	Wild type	Abnormal				
		Fewer spores	Uneven spores	Extra Spores		
Wild type	112	1	1	-	1.8	-
rat2-j543	85	15	2	-	16.6	9.5X
rat2-j556	105	2	3	1	5.4	3.1X
rat2-j589	90	1	3	3	7.2	4.1X
rat2-k596	64	2	2	-	5.8	3.3X
rat2-l678	104	4	1	1	4.6	2.6X
rat3-k620	69	1	-	2	1.4	0.8X
rat3-l646	113	-	-	-	0	0X
rat4-j575	102	6	-	3	8.1	4.6X
rat4-k601	79	3	-	1	4.8	2.7X
rat5-l655	95	1	-	-	1.1	0.6X
rat6-l675	100	-	1	-	1.0	0.6X
rat7-k639	103	2	-	-	3.7	2.1X

Table 4.1. Analysis of the spores of the *rat* mutants. The asci were fixed in 70% ethanol and stained with Propidium Iodide. Approximately one hundred asci were counted for each strain and the number and type of abnormal asci observed in each strain are displayed here.

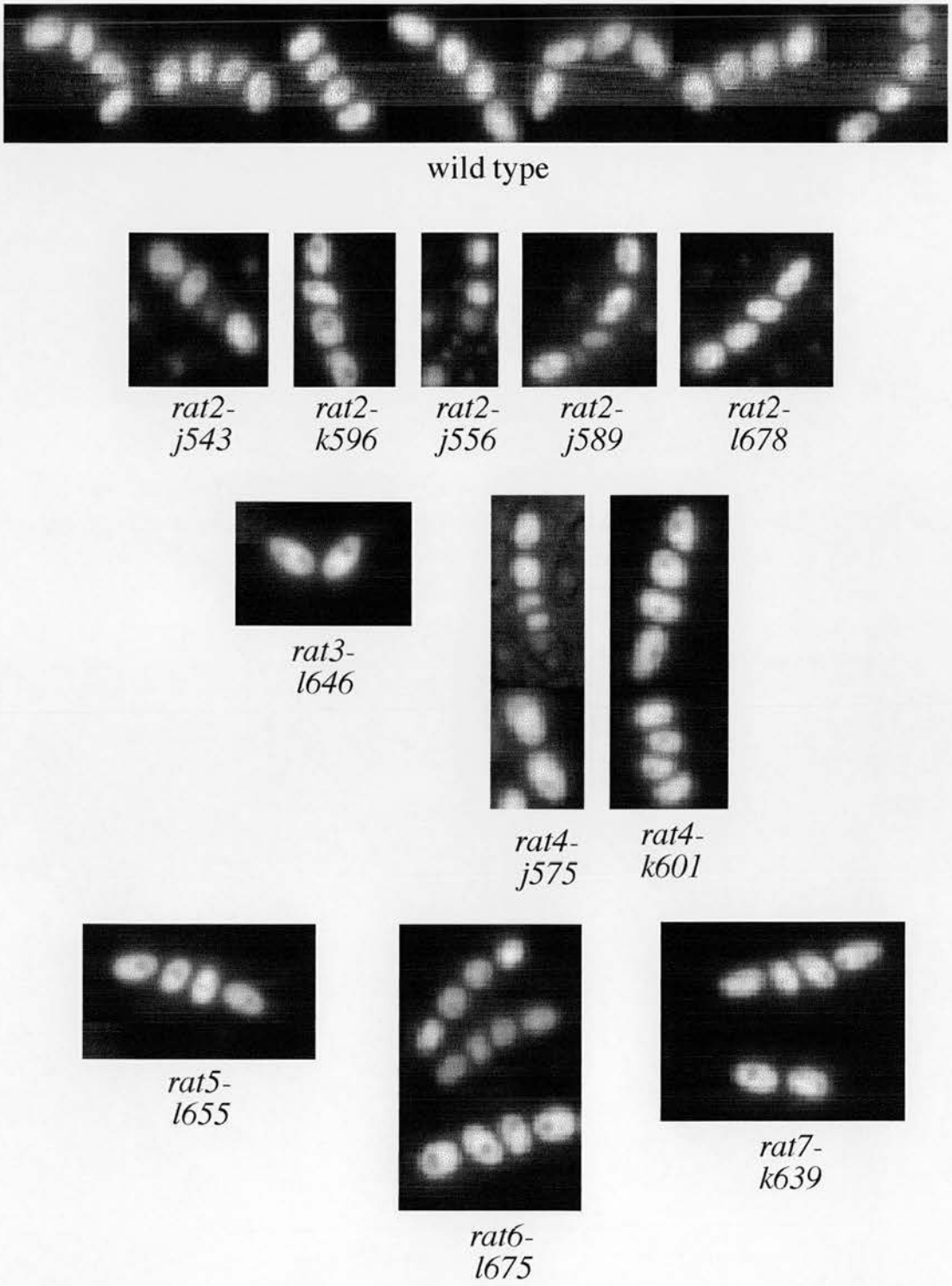


Figure 4.5. Mating was induced in the *rat* mutants and a wild type strain. Spores were fixed in 70% ethanol, stained with Propidium Iodide and examined under a fluorescence microscope at 100X magnification. Wild type asci contain four evenly sized spores. The *rat* mutants display a number of different types of malformed asci. In *rat2* three of the alleles show an increase in the number of asci with microspores and all have an increase of asci containing two large spores. The *rat4* mutants display an increase in the number of asci with an abnormal number of spores. *Rats 5, 6* and *7* all have little or no increase in the amount of malformed asci relative to the starting strain.

were fixed in ethanol, stained with Propidium Iodide and examined for defective spores, as described in 2.5.3. The number of asci counted and type of spores found for each strain is recorded in Table 4.1 and examples of the abnormalities found in the *rat* mutants are shown in Figure 4.5.

The proportion of malformed asci found in the starting strain used for this screen was 1.8%. In all of the *rat2* alleles there was an increased but variable level of abnormality in the asci compared to the wild type strain. The allele with the highest level of malformed spores was *rat2-j543* with 16% of asci containing fewer than four spores. A large increase in the number of asci with fewer spores than in a wild type might indicate that this mutant is having difficulties in chromosome separation at meiosis. The other four *rat2* alleles all have a less pronounced increase in the number of abnormal spores with mutants *rat2-j556, rat2-j589 and rat2-l678* all display asci with extra areas of DNA staining outside of the normal spores. This might indicate that chromosomes or chromosomal fragments are forming microspores as seen in the *lot2* mutant and may be indicative of chromosome lagging or breakage (Nimmo *et al.*, 1998). An increase in the levels of asci with the wrong number of spores was observed in *rat4-j575*, suggesting that this mutant may be having problems separating sister chromatids. *rat4-k601* also displays similar types of malformations to those in *rat4-j575*, but less frequently. The asci of the *rat3, rat5, rat6 and rat7* mutants all have levels of abnormalities similar to those seen in the wild type strain and therefore display no indication of meiotic defects.

The levels of abnormalities observed in the *rat* mutants are not as high as those observed in *taz1-lot3* where 53% of asci are malformed (Nimmo *et al.*, 1998). The defects seen in the *rat2* and *rat4* mutants indicate that these strains are all suffering from chromosome mis-segregation problems. The allele with the highest rate of abnormality within the *rat2* complementation group has wild type telomere length indicating that the increase in meiotic defects observed in *taz1-lot3* and *lot2* mutants

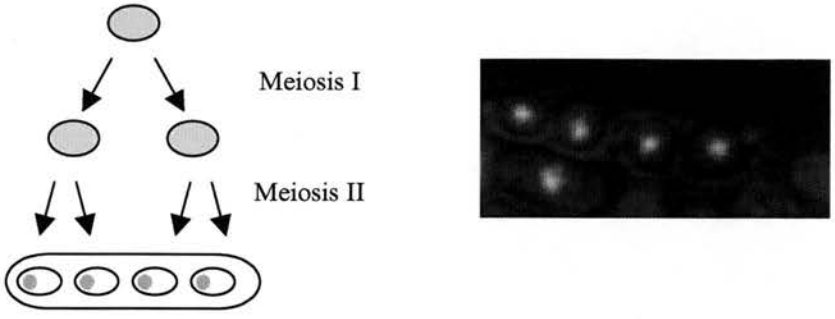


Figure 4.6. A proportion of *rat3-l646 and rat3-k620* asci displayed an abnormal pattern of Propidium Iodide staining. The outline of the spores within the nucleus and by light microscopy indicated that the asci had passed through wild type meiosis. It would appear that the Propidium Iodide has only stained the DNA in the spore nucleus, this may be due to the degradation of RNA in the rest of the spore by RNAse used in the preparation of the cells for microscopy. This may indicate that the *rat3* mutants have a spore wall defect. Asci of this appearance are referred to as Dark Zygotes.

are not solely the result of the increase in length of their telomeres (Nimmo *et al.*, 1998).

A proportion of the asci in the *rat3-l646* and *rat3-k620* mutants displayed an abnormal staining pattern. Propidium Iodide stains both DNA and RNA in the spores giving a bright staining pattern of four equal spores in a wild type ascus. Although RNAse is used in the preparation of the spores for microscopy there is no permeabliseation step in the protocol to allow the RNAse to cross the spore wall. In *rat3-l646* and *rat3-k620* 7% and 5.5% asci respectively contained two or more spores in which only the nucleus was stained by Propidium Iodide, indicating that there was an absence of RNA in the spore. An example of the abnormal staining pattern is shown in Figure 4.6. In the starting strain 0.9% of asci displayed a similar same staining pattern. In the *rat2* mutants 0-1.4% of asci were dark and in *rat5-l655* 3.1% of asci have a similar staining pattern. It may be that the *rat3* and *rat5* mutants have a spore wall defect that allows RNAse to enter some of the spores during preparation for microscopy, although there is no reason why this should be associated with the alleviation of telomeric silencing for which they were selected.

4.2.4. Immunolocalisation of Swi6p in the *rat* mutants

Swi6p is a general binding factor found at all regions of silent chromatin in *S. pombe* (Ekwall *et al.*, 1995). Delocalisation of Swi6p leads to the alleviation of silencing in these regions. Mutations of *clr4* or *rik1* delocalise Swi6p from centromeres telomeres and the silent mating type loci (Ekwall *et al.*, 1996). All the *rat* mutants generated by the screen described in chapter 3 maintain silencing at *mat2, mat3* and the centromere but not at the telomere. It may be that these mutants are unable to recruit Swi6p to the telomere while it remains associated with centromeres, *mat2* and *mat3*. Cells from cultures of *rat* mutants were stained with an α-Swi6p antibody, as described in 2.5.2. The protein localisation pattern in the *rat* mutants is shown in Figures 4.7. and 4.8.

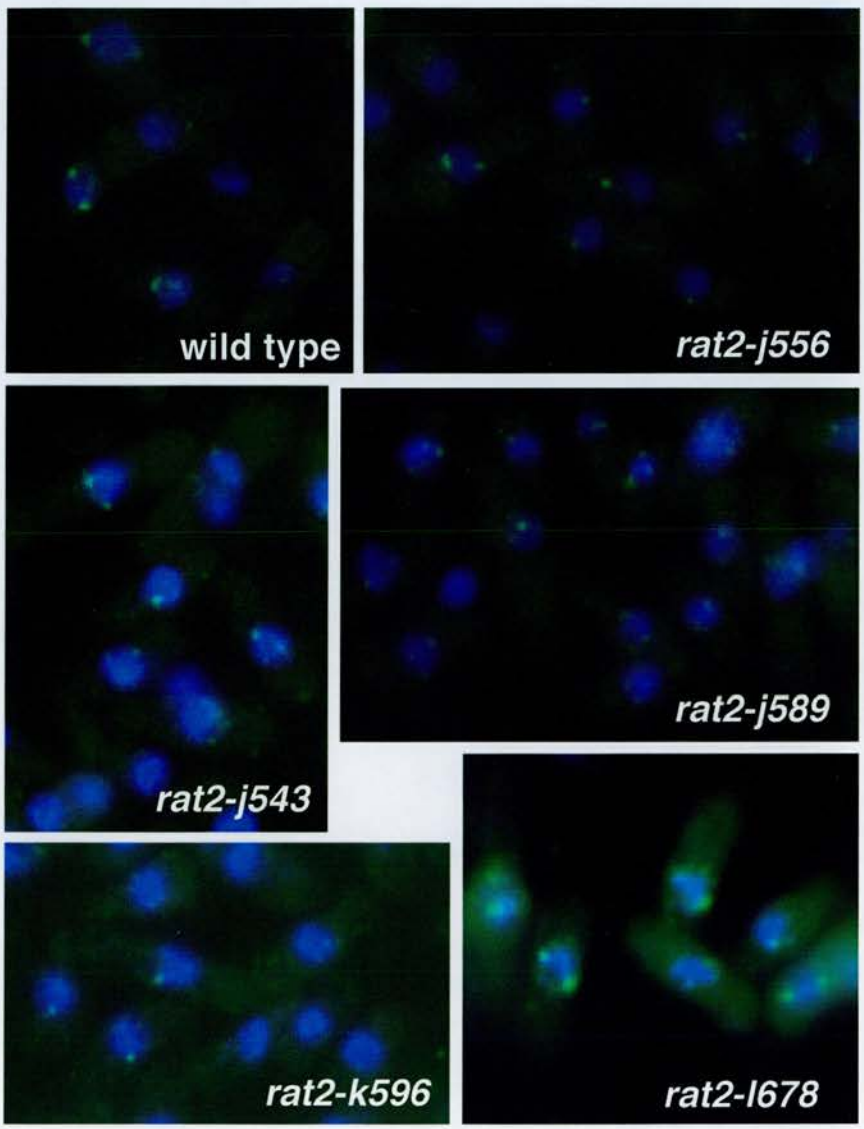


Figure 4.7. Immuno localisation of Swi6p in the *rat2* mutants. Swi6p (green) forms a punctate staining pattern in the nucleus (blue) of all the *rat2* mutants in a similar pattern to that of the wild type strain.

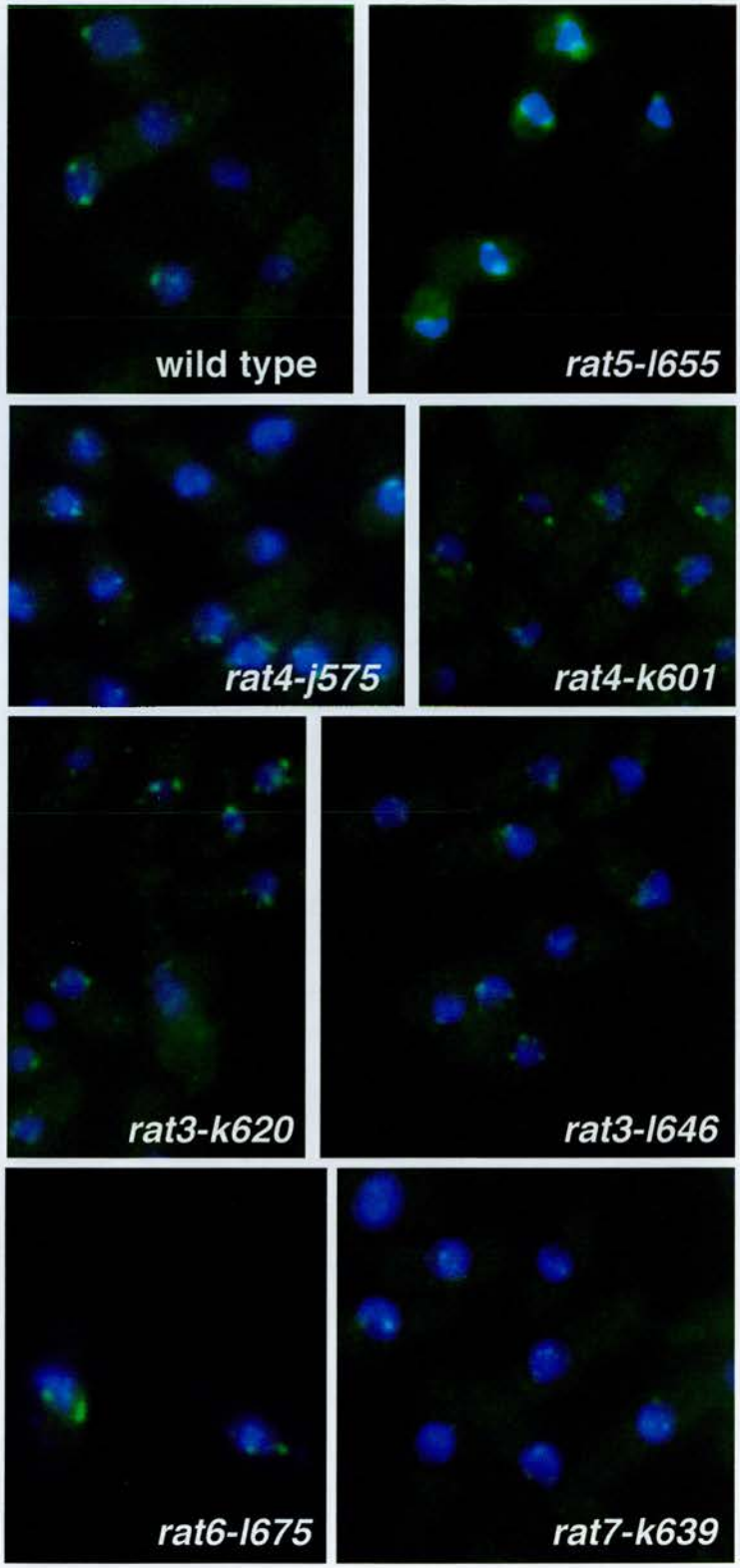


Figure 4.8. Immuno localisation of Swi6p in *rat3, rat4, rat6, rat7*. Swi6p (green) forms a punctate staining pattern in the nucleus (blue) of all of the *rat* mutants shown, in a similar pattern to that of the wild type strain.

Two to three foci of Swi6p are visible in the nucleus of each cell in the wild type strain. In Figures 4.7. and 4.8. it can be seen that Swi6p is localised at more than one spot of fluorescence in all of the *rat* mutants investigated. In all these strains the Swi6p localisation pattern is similar to that of the wild type strain, suggesting that Swi6p is recruited to the telomere.

4.2.5. Immuno localisation with anti-Swi6p and anti-Cnp1p antibodies

When using an antibody against Swi6p for immunofluorescence it is not possible to determine which of the foci seen is centromeric and which are at telomeres unless the cells are dividing, when the centromeric focus is at the leading edge of the chromosome movement (Funabiki *et al.,* 1993). Alternatively antibodies can be used to determine which of the Swi6p foci are telomeric or centromeric. This confirms that Swi6p forms foci in the cell at sites other than those at centromeres, and that multiple foci in the nucleus are due to a wild type localisation pattern of Swi6p at telomeres and not declustering centromeres. Staining the cells with an antibody raised against Cnp1p, a histone H3 variant specifically located in the central core of centromeres, labels the three clustered centromeres in interphase nuclei (Barbara Mellone, personal communication). Using primary antibodies to Swi6p raised in rabbit and Cnp1p raised in sheep it is possible to produce distinct signals for both proteins in the same cell. Co-localisation of the Swi6p and Cnp1p signals identifies which of the Swi6p foci is at the cluster of centromeres in the cell and therefore, by exclusion, which of the foci are at telomeres. Chromosomal DNA was stained with DAPI.

Simultaneous localisation of Swi6p and Cnp1p in various *rat* mutants is shown in Figure 4.9. In the wild type strain centromeres are distinguished by a yellow-green signal resulting from the localisation of Cnp1p. The red foci represent Swi6p localisation at telomeres and perhaps the silent mating type loci. In all the strains analysed a similar pattern of Cnp1p and Swi6p localisation to that of the wild type strain is observed.

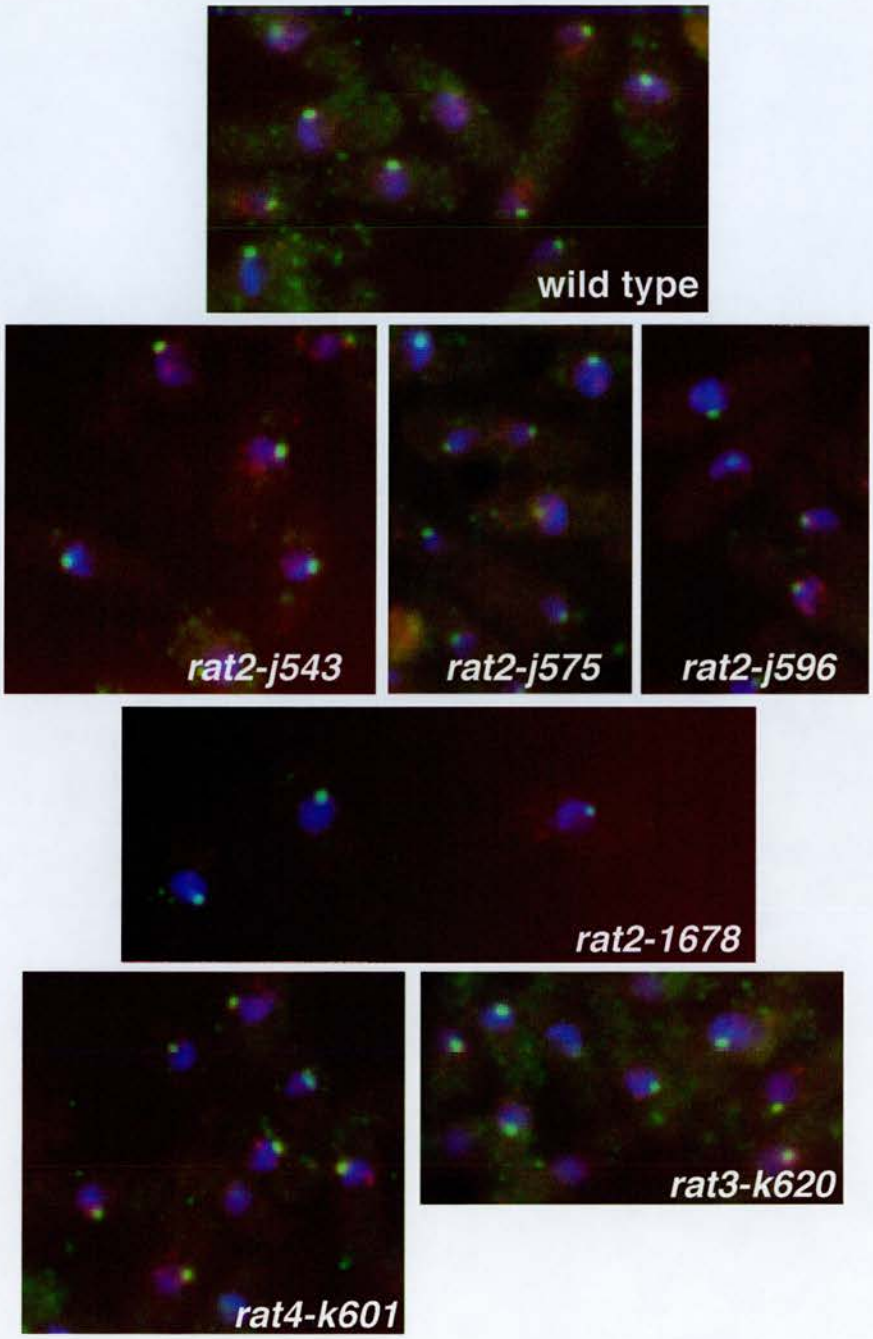


Figure 4.9. Immuno localisation of Swi6p and Cnp1p in the *rat* mutants. Cnp1p (green) is localised at the centromere, thus distinguishing which of the Swi6p (red) foci is centromeric and therefore by default which are telomeric. Swi6p (red) displays a punctate staining pattern in the nucleus (blue) in *rat2*, *rat3* and *rat4* mutant strains, in a similar pattern to that of the wild type strain, suggesting that Swi6p is localised at the telomeres in the *rat* mutants.

In all strains tested, antibodies against Swi6p detect more than one region of localisation of Swi6 protein in the nucleus. This indicates that Swi6p is recruited to the telomere in all of the *rat* mutants. Due to variability of staining with α-swi6 it is difficult to discern small changes in the localisation pattern. Investigation of Swi6p localisation was also attempted in live cells with a GFPSwi6 fusion protein expressed from the *ars1* locus, however the fusion protein gave inconsistent results. This may have been due to endogenous Swi6p being present in the same cell. Complete delocalisation of GFPSwi6p was observed in a high proportion of the *rat* mutants. However, immunolocalisation of endogenous Swi6p in the *rat* mutants shows that Swi6p does form foci of fluorescence in these cells. Although GFPSwi6p has been a useful tool for investigating the localisation of Swi6p in previous experiments it may not be suitable for analysis of mutations like those represented by the *rat* mutants.

4.2.7. Monitoring telomere clustering using a TazGFP fusion protein

Since it can be difficult to discern whether the telomeres are clustering correctly by the immunolocalisation pattern of Swi6p in Figures 4.7, 4.8 and 4.9 the clustering of telomeres was also investigated using a Taz1GFP fusion protein. As Taz1p binds directly to the double stranded telomere repeats it is unlikely that other mutations affecting the telomere would delocalise Taz1p. Band shifting assays using Taz1p alone suggest that Taz1p is not modified by or bound to another factor prior to binding the DNA (Spink *et al.*, 2000). The Taz1GFP fusion protein is transcribed from the endogenous locus for *taz1* so can only be used to monitor the telomere in strains whose phenotypes are not caused by a mutation in the *taz1* gene. In Figure 4.10 it can be seen that in a wild type cell, Taz1GFPp forms two foci per nucleus. In *rat3-k620, rat4-k601* and *rat6-l675* backgrounds the same pattern is observed, indicating that Taz1p is still recruited to the telomeres in these mutants and that the telomeres do not decluster. This is also true for the pattern of taz1p localisation in cells disrupted for the general silencing factors *clr4Δ*, *rik1Δ* and *swi6Δ*.

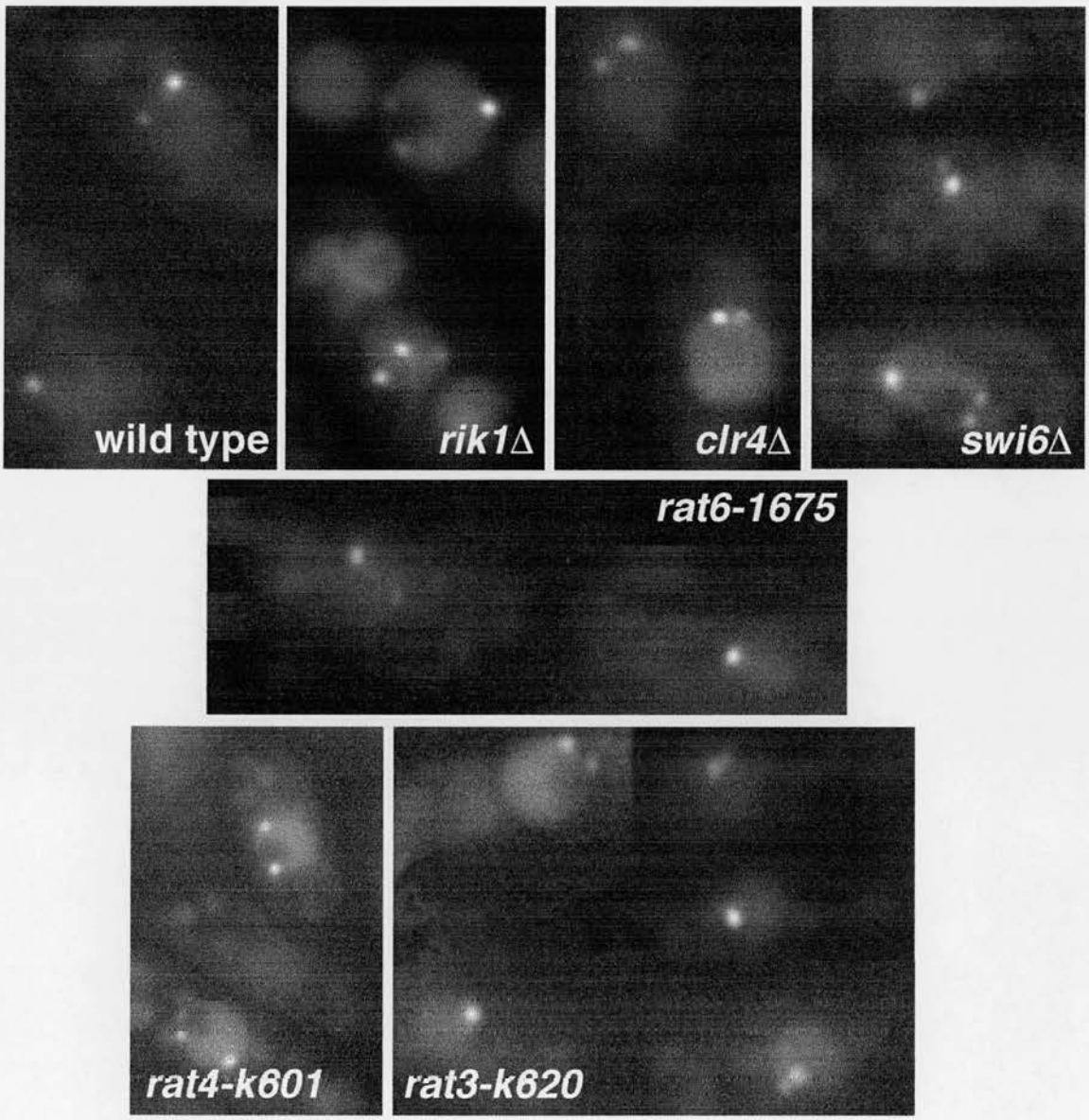


Figure 4.10. In a wild type cell there are two to three spots of Taz1GFPp localisation per cell. This is also the case for *rik1*Δ, *swi6*Δ and *clr4*Δ and in *rat3*, *rat4* and *rat6* mutants indicating that in these mutants the telomeres remain clustered

4.4. Discussion

Some of the rat mutants selected for alleviation of silencing were also found to have altered telomere length. This validates the use of alleviation of silencing at the telomere to screen for factors involved in telomere length maintenance. The differences in telomere length between alleles within each of the complementation groups may reflect a different effect of each mutation on the protein function. This screen identified five alleles of *taz1* with different telomere length phenotypes; *rat2-j543* and *rat2-k596* had wild type telomere lengths, *rat2-l678* had a small increase in telomere length of approximately 200bp and no telomere repeat sequence was discernable for two others; *rat2-j556,* and *rat2-j589*. Separation of function alleles have been found previously for telomeric proteins, such as Cdc13p and Est1p in *S. cerevisiae,* and have proved useful in dissecting their functions (Grandin *et al.,* 2000, Pennock *et al.,* 2001). As mutants of *taz1* with wild type telomere length have been isolated this will allow for dissection of function of Taz1p in the same way as Cdc13p and Est1p in budding yeast.

In the *rat4-k601* allele no telomere repeat smear is detected, but a strong band of approximately 4kb becomes prominent. This could be due to a telomere fusion event occurring. In *trt1*Δ and *rad3*Δ*tel1*Δ mutants telomeres shorten over successive cell divisions until the majority of cells senesce and die (Nakamura *et al.,* 1998, Naito *et al.,* 1998). Occasionally colonies arise that can overcome the ever-shortening telomere phenotype, and analysis of these cells has shown that they contain circularised chromosomes. Telomeric repeats are no longer detectable in the survivors, and deletion of the telomere associated sequences removing up to 4kb from each chromosome end has been found to occur prior to circularisation in these mutants. If the 4kb band is caused by fusion of telomeric sequences in a circularised chromosome then the chromosome must have circularised before the telomeric repeats were lost. This may indicate that the protective telomeric cap that prevents end-to-end fusions has been disrupted in this strain. PFG analysis confirmed that

changes had occurred in the chromosomal banding pattern that might be indicative of end-to-end fusions occurring in mutants *rat2-j589*, *rat4-k620* and *rat3-l646*.

There is a slight increase in the number of malformed asci in all strains in the *rat2* and *rat4* complementation groups, although not as high as that observed in a *taz1* or *lot2* strain (Nimmo *et al.*, 1998). The *rat2-j543* mutant has the highest increase in the number of malformed asci indicating that meiotic defects are occurring at a rate of approximately 9.4% in this strain. This allele of *taz1* has a wild type telomere length suggesting that increased telomere length of a *taz1* null strain is not the sole cause of the meiotic defect seen in that strain.

In all the *rat* mutants examined multiple foci of Swi6p were seen in the nuclei, indicating that the alleviation of silencing at the telomere is not a result of telomere specific delocalisation of this general silencing factor. It could be that Swi6p is nucleated at the telomere associated sequences in these mutants but not recruited to the terminal telomeric repeats, thus alleviating silencing while appearing to remain localised. This question could be resolved using chromatin immunoprecipitation (CHiP) experiments to investigate the presence of Swi6p at the telomeric markers, however they were not carried out due to time constraints.

The double immunostaining of cells with anti-Swi6p and anti-Cnp1 antibodies confirmed that Swi6p was localised at both the telomeres and centromeres in the *rat* mutants. As this failed to resolve the question of whether or not the telomeres were declustering in these cells a Taz1GFP fusion protein was used to investigate telomeric clustering. In all of the *rat* mutants examined the Taz1GFPp pattern was similar to that seen in a wild type strain indicating that the telomeres remain clustered and Taz1p remains localised in these strains.

Chapter 5

Variegation of telomeric marker expression in the *rat* mutants

5.1 Introduction

Identification of the affected genes in various *rat* mutants requires the complementation of defective silencing. All *rat* mutants express the telomeric *ura4*$^+$ gene, therefore complementation with a genomic library containing the wild type *rat* gene would reimpose silencing at the telomere, thus allowing growth on FOA. Previous studies have shown, however, that there is variegation in the levels of expression of the *ura4*$^+$ marker gene in *taz1-lot3* and *rap1-lot2* mutants resulting in a proportion of cells retaining the ability to grow on FOA plates (Nimmo, personal communication). Even a low level of background growth of mutants on FOA plates would hamper complementation. Before attempting complementation analysis of the *rat* mutants, cells from each mutant were tested for growth on minus uracil, FOA and non-selective plates. Only those mutants displaying little or no growth on FOA would be amenable to complementation.

5.2 Results

5.2.1. Comparison of growth of the *rat* mutants under different selective pressures

Serial dilutions of cells from non-selective or minus uracil cultures were plated on non-selective, minus uracil, minus histidine and FOA plates, which were incubated at 25°C, 32°C and 36°C. The growth on selective plates following non-selective growth in culture reflects the proportion of cells in the culture that are expressing the *ura4*$^+$ gene at the telomere. In Figure 5.1 colonies pregrown in non-selective YES and grown for 2 days at 32°C are shown. Cells from the strain used to perform this screen, FY1862, grow well on FOA and very poorly on minus uracil at all temperatures tested. ED972, a wild type strain competent for all nutritional markers, grew well on minus uracil and very poorly on media containing FOA.

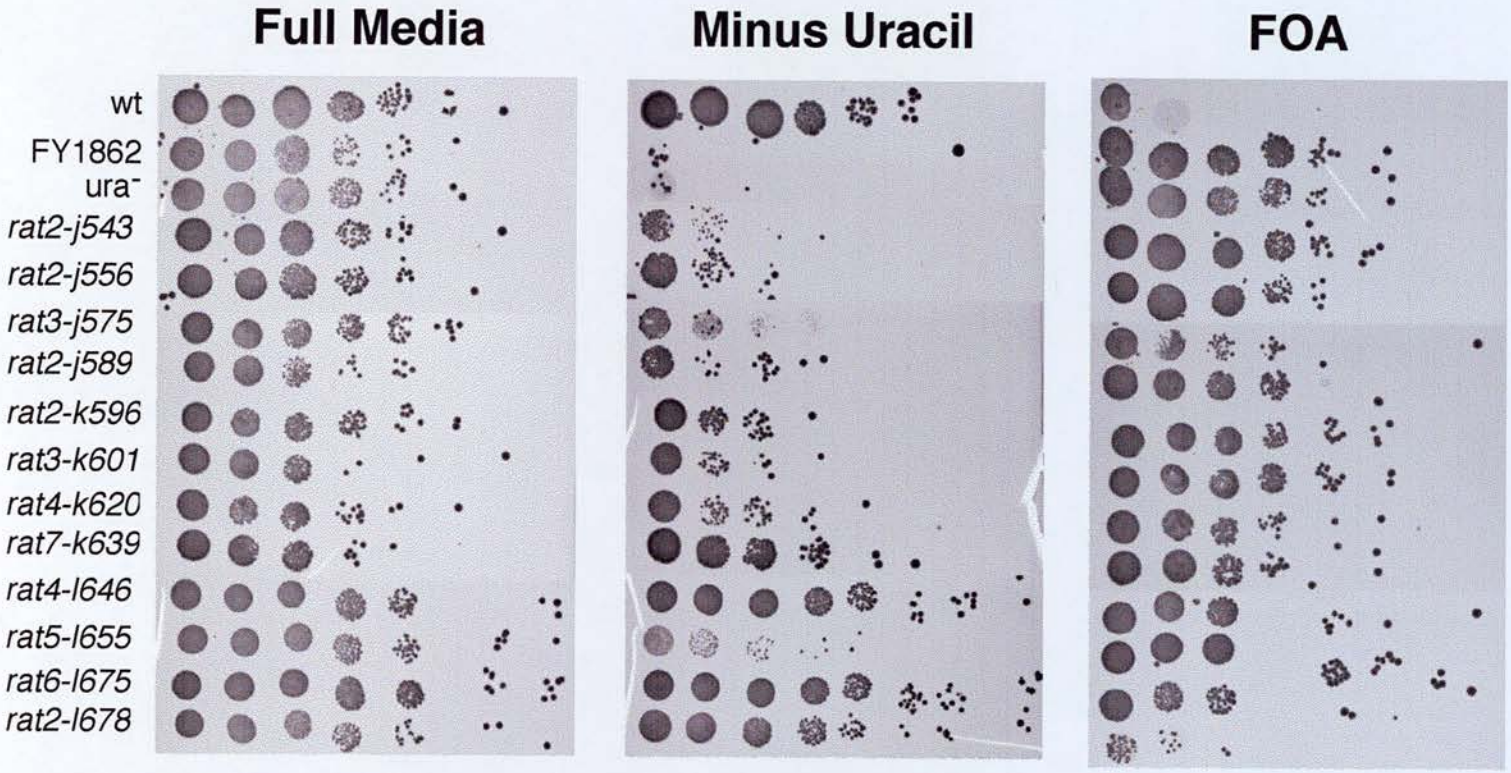


Figure 5.1 Serial dilution of the *rat2* mutants on non-selective, minus uracil and FOA plates. Cells from all of the *rat* strains are able to produce colonies on all types of plates, suggesting that these strains may be able to change the silencing status of genes in the telomeric regions

In general, cells plated on media of a similar type to that used for pre-growth form evenly sized colonies. When the culture medium imposes a different type of selection to that used in the serial dilution assay the colonies sizes vary, with a mixture of large and small colonies in the same dilution spot. An ability to adapt the transcriptional status of the telomeric region containing the *ura4*$^+$ marker gene inserted at the telomere could explain why cells are able to grow on media containing FOA following pre-culture in minus uracil in which only cells expressing *ura4*$^+$ are able to thrive. Alternatively a proportion of the cells in the population may not transcribe *ura4*$^+$ and therefore are growth arrested or grow slowly in minus uracil but are able to resume growth and form colonies following plating on FOA.

As found in previous experiments when investigating the phenotypes of different alleles of the *rat* mutants, the growth phenotype of the different alleles within any one group is not the same. All of the *rat* mutants produced colonies on both FOA plates and minus uracil, a variation of the expression of the telomeric marker gene in the cell populations not demonstrated by the starting strain or the wild type strain. A variety of growth patterns were observed in the *rat* mutants; *rat2-l678, rat7-l675* and *rat3-l646* all grow better on minus uracil than on FOA, *rat6-k639* grows equally well on either type of selective plate, *rat2j-556, rat2-j589, rat2k-596, rat4-k601, rat3-k620* all display a slight preference for growth on FOA over minus uracil and *rat2-j543*, *rat5-l655,* and *rat4-j575* all form many more colonies on FOA.

In Figure 5.2 the starting strain, pregrown in non-selective YES, and *rat2-j543,* precultured in either FOA or minus uracil, have been serially diluted onto similar sets of media and grown for two days at 25 °C, 32 °C or 36°C. It can be seen that the *rat2-j543* strain grows poorly on FOA and well on PMG minus uracil when pregrown in minus uracil while the opposite pattern of growth can be seen for the same strain precultured in FOA. The ability of the *rat2-j543* cultures to grow on the counter selective media is reduced at 25 °C compared with the higher temperatures tested. Overall this demonstrates the ability of cells from a *rat2-j543* derived population to form colonies on counter selective media following culture in either minus uracil or FOA. This could be due to an ability to adapt the transcriptional status of the telomeric region containing the *ura4*$^+$ marker gene, FOA not being toxic to cells with a low level of *ura4*$^+$ transcription or cells arrested for growth in one type of selective

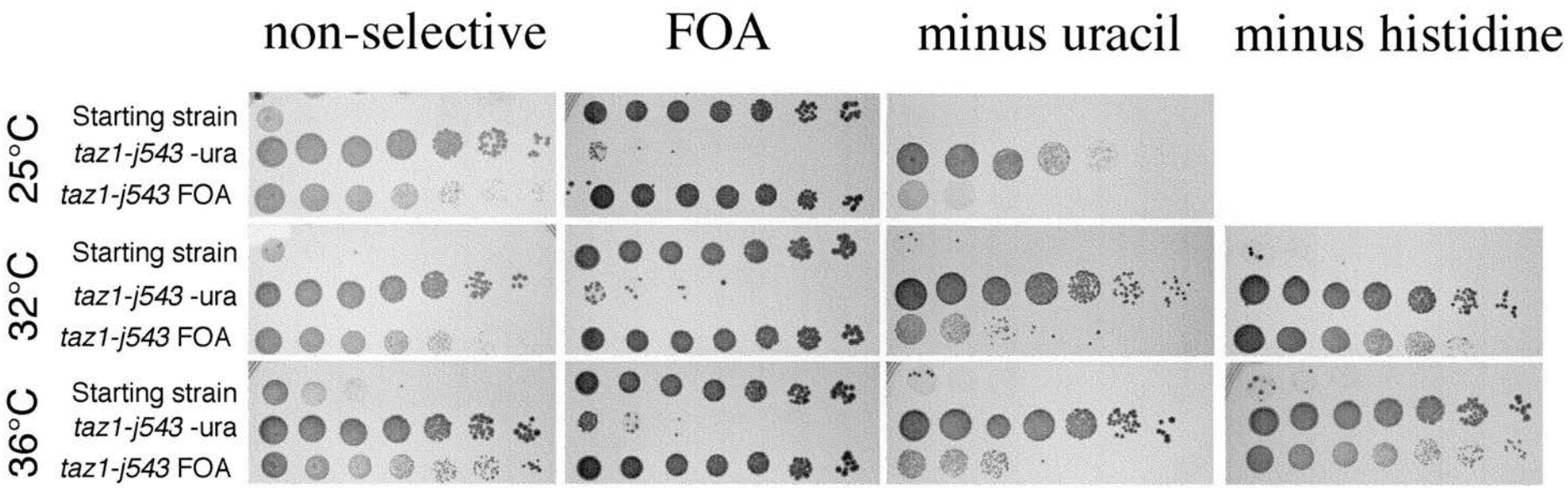


Figure 5.2 serial dilution of *rat2-j543 on* non-selective, minus uracil, minus histidine and FOA plates following culture in either FOA or minus uracil. Cells from either type of preselection are able to grow on all types of plates, suggesting that the transcriptional status at the telomere may be changeable.

media resuming growth when placed in counterselection. Interestingly *rat2-j543* grows equally well on PMG media lacking histidine under selection both for and against the expression of the *ura4*$^+$ marker gene. This may indicate that the silencing status of the *his3*$^+$ marker gene on chromosome I is unaffected by the silencing status imposed upon the *ura4*$^+$ marker gene on chromosome II. Alternatively, it could be that growth on PMG lacking histidine is possible with varying levels of *his3*$^+$ expression.

5.2.2 Comparison of growth of *rat* mutants on PMG minus uracil and PMG containing FOA

The *rat* mutants were grown to log phase in YES and the density of each culture measured using a haemocytometer. From each culture approximately 1000 cells were plated onto non-selective YES, FOA and minus uracil media. After incubation for two days at 32°C the number of colonies growing on each plate were counted. The relative amount of colonies present on each type of plate can be seen in the graph in Figure 5.3. This shows that even the strains such as *rat2-l678, rat3-l646* and *rat7-l675,* which appear to grow less well on FOA than minus uracil in the serial dilution assay, have a large proportion of colonies that are able to grow on media containing FOA. In this assay only one strain, *rat4-k601,* had less than 100% growth on media containing FOA. In all of the *rat* strains more colonies grew on FOA plates than minus uracil. The high level of growth on media containing FOA means that it will not be possible to clone the genes responsible for the *rat* mutants by complementation of the phenotype using plasmid libraries.

5.2.3 Quantification of the silencing defect in the *rat* mutants by RT-PCR

To quantify the level of expression of the telomeric *ura4*$^+$ marker in the *rat* mutants, RT-PCR analysis was performed. In addition to *ura4*$^+$ at the left telomere on chromosome II each strain also contains a truncated *ura4* mini-gene (*ura4DS/E)* at the endogenous *ura4*$^+$ locus. This *ura4DS/E* mini-gene is deleted in the centre of the gene and is transcribed from the endogenous *ura4* promoter to produce a nonfunctional RNA with identical sequence at each end to the full length gene, as shown in Figure

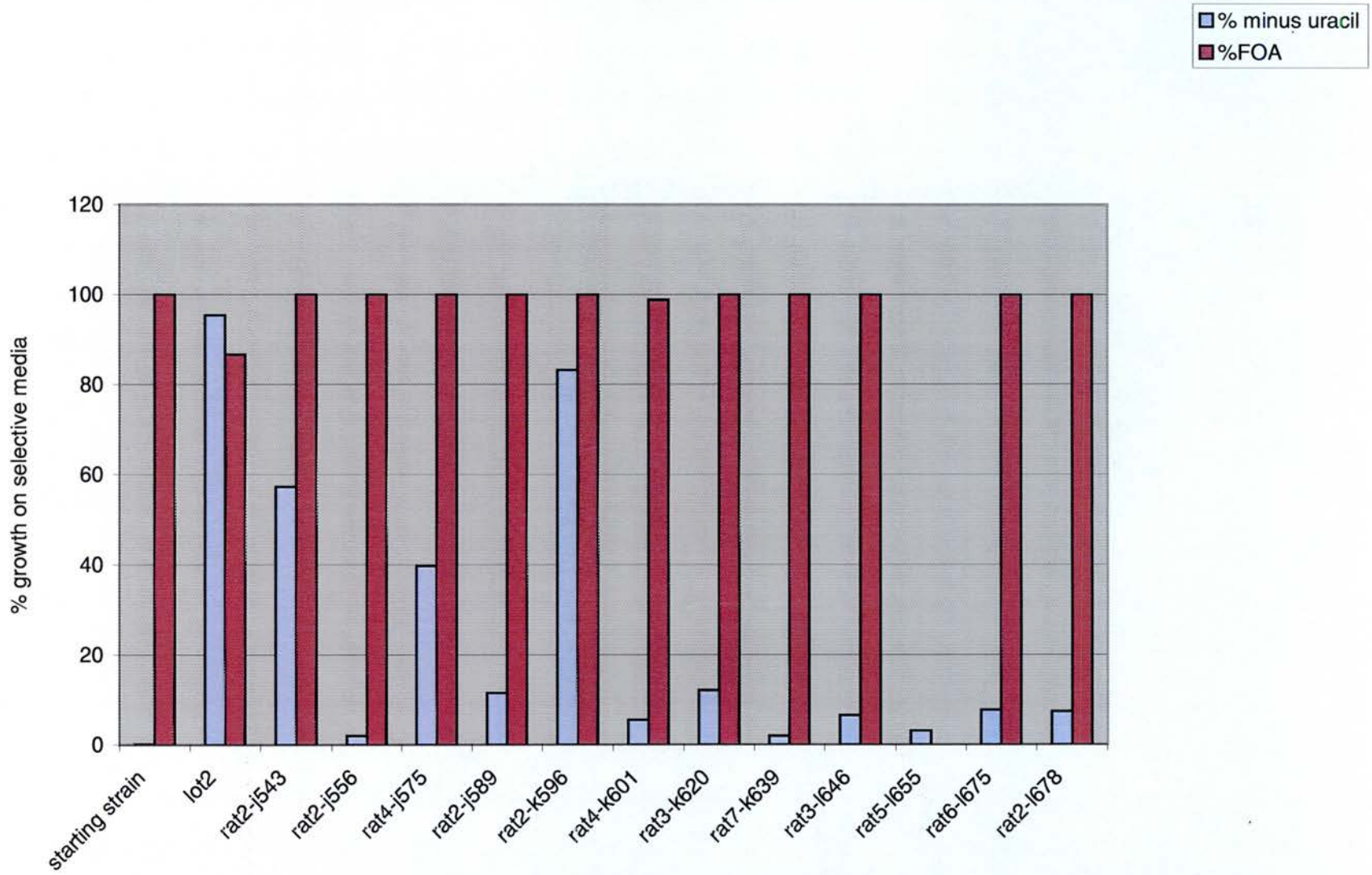


Figure 5.3 Random spore analysis of growth on the rat mutants on FOA and minus uracil. In all strains tested more colonies grew on FOA plates than on minus uracil

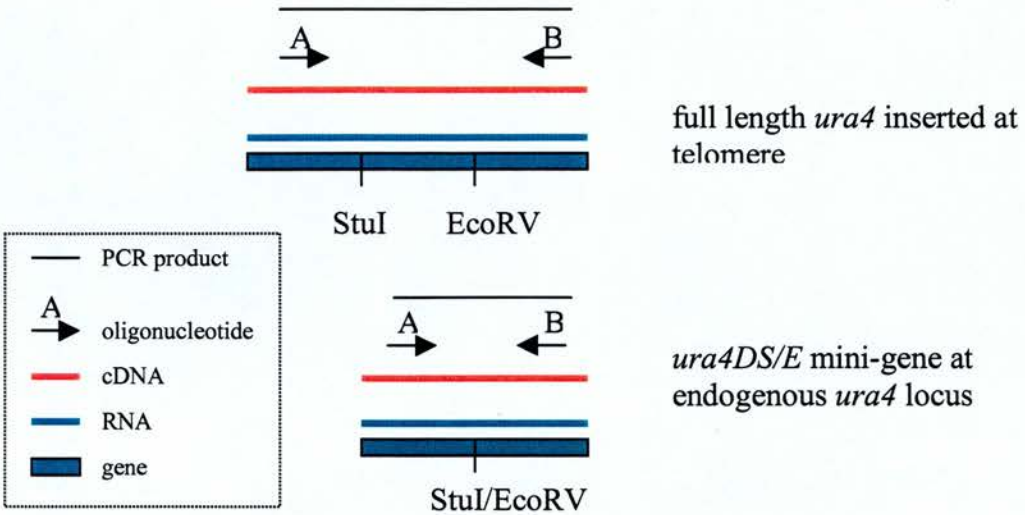


Figure 5.4. A *ura4* mini-gene deleted internally between a StuI and an EcoRV site, deleting approximately 280bp, is inserted at the endogenous *ura4* locus in *rat* strains that also contain a full length and functional *ura4*$^+$ gene at the telomere. The ends of the full length gene and the mini-gene are identical so that a single pair of oligonucleotides can be used to PCR amplify from the cDNA template and thus the amount of transcript produced from each of these loci can be compared using RT-PCR techniques.

5.4. Measuring the amount of transcript of the full sized *ura4*$^+$ telomeric marker gene relative to the amount transcript of *ura4DS/E* in the same population enables the relative level of transcription at the telomere to be measured and therefore the overall alleviation of silencing in that mutant strain to be quantified.

Total RNA was prepared from the strains grown at 32°C to logarithmic phase in YES and PMG minus uracil. A cDNA copy of the RNA was prepared using a Superscript kit (GIBCO BRL). Quantitative PCR was carried out using a single set of oligonucleotide to amplify both the telomeric *ura4*$^+$ marker gene product and that of *ura4DS/E* at the endogenous *ura4*$^+$ locus using the cDNA as template, as shown in Figure 5.4. To measure the amount of PCR amplification products and allow for comparison of expression levels between the telomeric *ura4*$^+$ marker and *ura4DS/E* at the endogenous ura4 locus, the PCR reaction was radioactively labelled with 1µCi [α-^{32}P]CTP per 20µl reaction. The reaction was loaded onto a 4% acrylamide gel to separate the amplification products. The gel was dried using a gel dryer and exposed

overnight on a phosphorimager screen. It was then possible to measure the amount of signal from each amplification product using Imagequant software. The amount of cDNA available as template for the PCR reaction reflects the amount of RNA in the cell. This makes it possible to use levels of PCR product to measure the relative levels of transcription from the telomeric *ura4*$^+$ marker gene and the *ura4DS/E* mini-gene, the latter of which is unaffected by the silencing status of the telomere. If the telomeric gene is silenced, as in a wild type background, little product should be detected for the full sized transcript. If silencing at the telomere is disrupted the amount of full length *ura4*$^+$ transcript increases relative to that from the *ura4DS/E* locus.

The control strains for this experiment were chosen to allow for comparison of the level of transcription from the telomere in the *rat* mutants to that of telomeric mutants known to have a level of background growth on FOA that is too high to allow for identification of the mutant gene by complementation. *taz1-lot3* and *rap1-lot2* have point mutations that affect telomeric silencing. *clr4-lot1* is an allele of the *clr4*$^+$ gene with a point mutation that also affects telomeric silencing and has a slight increase in telomere length of between 500 and 1000bp. These allele was isolated in the initial screen for telomere specific mutants (Nimmo *et al.*, 1998, Nimmo personal communication).

It can be seen in Figure 5.5 that there is a very low level of expression of the telomeric *ura4* marker gene in the original strain used to generate the mutants. The ratio of *ura4*:*ura4DS/E* transcript was calculated for the *rat* mutants and the *clr4-lot1* *rap1-lot2* and *taz1-lot3* controls.

None of the *rat* mutants have as high a level of transcription at the telomere as the control strains. The *rat2-j543* and *rat2-k596* strains display the highest levels of telomeric *ura4*$^+$ transcription ratios of 25% and 24% respectively when grown in non-selective YES. *rat2-j556* also produces *ura4*$^+$ transcript in non selective media but all of the other *rat* mutants analysed produce very low levels of transcript from the telomeres when grown non-selectively.

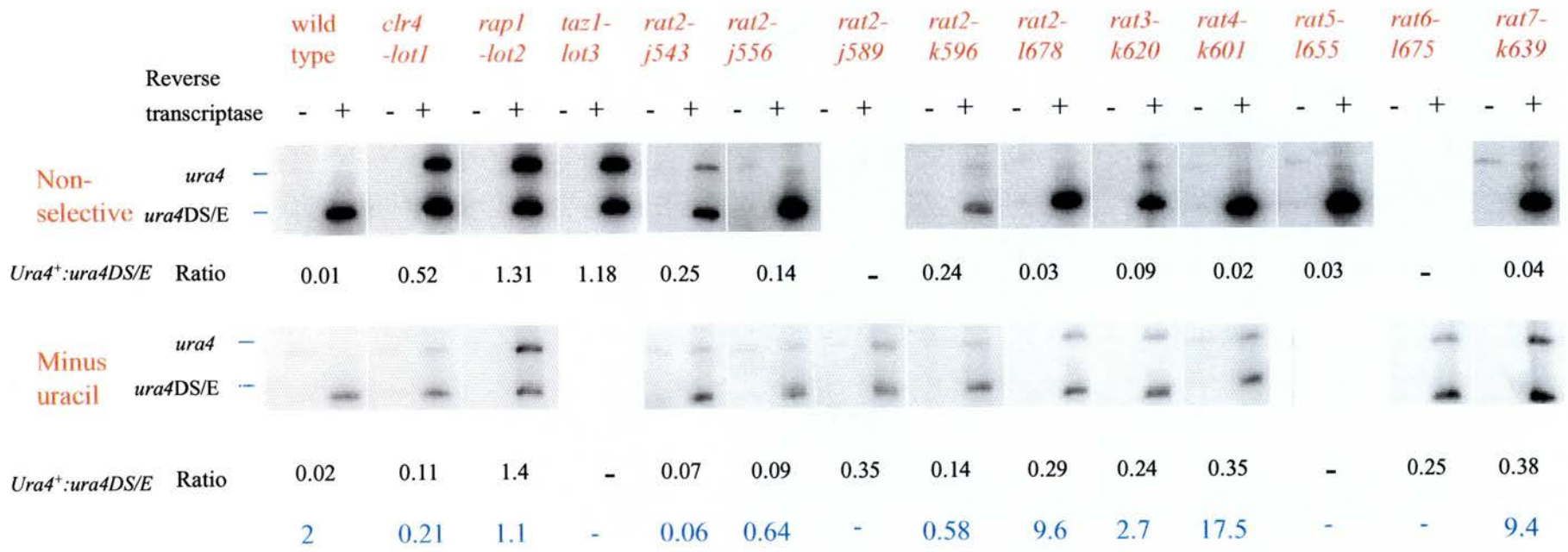


Figure 5.5. RT-PCR analysis was used to measure the level of transcription at the telomere in the *rat* mutants. The first column of each set of PCRs is a control reaction on RNA without reverse transcription. In each case the upper band represents the amount of transcript produced from the *ura4* gene inserted at the telomere and the lower band represents the amount of transcript produced from the DS/E *ura4* mini-gene at the endogenous *ura4* locus in the same reaction. The ratio of *ura4*$^{+}$*:ura4DS/E* transcript is shown in black underneath the relevant column and the relative amount of transcription at the telomere for cells grown in PMG compared to cells grown in YES is shown in blue.

Strain	Ratio of the amount of telomeric *ura4* transcript to truncated *ura4* control product	
	non-selective YES	PMG minus uracil
Starting strain	0.01	0.02
clr4-lot1	0.52	0.11
lot2	1.31	1.4
rat2-lot3	1.18	-
rat2-j543	0.25	0.07
rat2-j556	0.14	0.09
rat2-j589	-	0.35
rat2-k596	0.24	0.14
rat2-l678	0.03	0.29
rat3-k620	0.09	0.24
rat4-k601	0.02	0.35
rat5-l655	0.03	-
rat6-l675	-	0.25
rat7-k639	0.04	0.38

Table 5.1 Summary of the data showing the relative amounts of transcript produced from the *ura4* marker gene inserted at the telomere and the truncated *ura4D/SE* gene at the endogenous locus for each strain, normalised to a random integrant strain. The strains were cultured at 32°C in both non-selective YES broth and PMG broth lacking uracil and the ratio of the amount of transcript produced from the telomeric *ura4* gene to that of the mini-gene calculated in each case.

There is a decrease in the ratio of transcript produced from the telomeric *ura4*$^+$ marker compared to *ura4DS/E* in the *clr4-lot1, lot2* and *lot3-taz1* controls when the strains are grown in minus uracil when compared to culture in YES. The same unexpected reduction in transcription levels is seen in *rat2-j543, rat2-j556* and *rat2-k596* when they are cultured in minus uracil. For all other *rat* mutants tested the relative level of transcription at the telomere increased in minus uracil. *rat3-k620* has a very small increase in the level of telomeric transcription when grown in minus uracil. For *rat2-l678, rat4-k601* and *rat7-k639* a large increase in the level of transcription is detected when the cells are grown in minus uracil. In no case is there

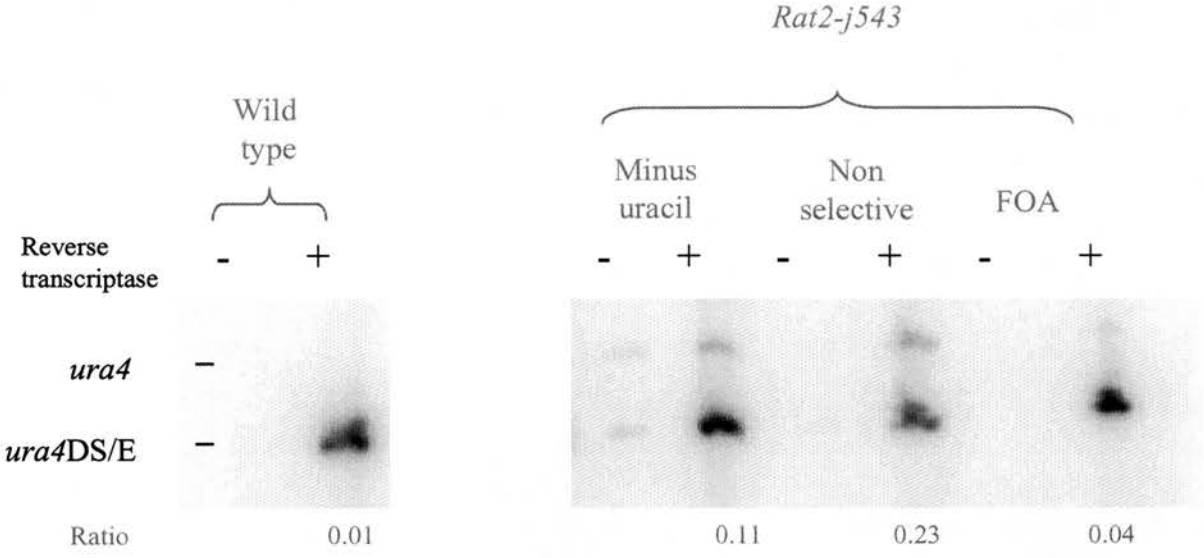


Figure 5.6. RT-PCR analysis of the effect of different selective pressures on the amount of transcript produced from the telomeric *ura4* marker gene in the *rat2-j543* strain. This figure shows that the amount of transcript is decreased when the strain is grown in YES broth containing 5-FOA compared to growth in non-selective media. Unexpectedly the amount of *ura4* transcript also appears to decrease when the strain is grown in PMG lacking uracil.

total alleviation of silencing at the telomere. When grown in uracil minus media none of the *taz1-rat2* alleles have as high a level of transcription at the telomere as *taz1-lot3*, which is a null allele of *taz1*. The apparent decrease in transcription observed for *rap1-lot2, taz1-lot3, rat2-j543, rat2-j556* and *rat2-k596* when the cells are grown in minus uracil may be an artefact caused by up regulation of the *ura4DS/E* promoter in selective media.

The ratios of transcription of telomeric *ura4*$^+$: *ura4DS/E* for each strain is summerised in Table 5.1 and the relative amount of transcription of the telomeric *ura4*$^+$ gene is indicated in blue underneath the RT-PCR bands in Figure 5.5.

5.2.4 Selective pressure in the growth medium affects the level of expression of the telomere marker gene in a population.

The levels of expression of the telomeric marker in *rat2-j543* were investigated under different selective pressures. The cells were grown at 32°C to logarithmic phase in three types of growth media; FOA, which selects against the expression of the telomeric marker gene, YES and minus uracil, to select for expression of the telomere marker gene. RNA was isolated from these cultures and RT-PCR carried out using the methods described previously. The levels of expression of the telomeric *ura4*$^+$ gene resulting from the different selective pressures is shown in Figure 5.6. This Figure shows that, as predicted, the amount of transcript is decreased when the strain is grown in FOA compared to growth in non-selective media. Unexpectedly the amount of *ura4* transcript also appears to decrease when the strain is grown in minus uracil. This demonstrates that for *rat2-j543* the silencing complex at the telomere is adaptable, allowing the strain to grow under different selective pressures.

5.3 Discussion

All of the *rat* mutant strains are able to form colonies on both minus uracil and FOA. Quantitative PCR demonstrates that in non-selective media many of the *rat* mutants do not transcribe the *ura4*$^+$ telomeric marker gene at a greater rate than the starting strain, however when in grown in uracil$^-$ medium the level of transcription in *rat2-l678, rat4-k601* and *rat7-k639* is increased. Thus increased growth on minus uracil is

accompanied by a change in the amount of *ura4*$^{+}$ transcript from the telomeric gene. In *taz1-lot3*, *rap1-lot2*, *rat2-j543* and *rat2-j556* the amount of transcription from the telomere appears to decrease in minus uracil. This could be an artefact due to up regulation of the *ura4*$^{+}$ promoter at the endogenous locus causing more *ura4DS/E* transcript to be produced.

In all the strains analysed it was found that variegation of expression of the telomeric *ura4*$^{+}$ gene was too high to allow the genes responsible for the *rat* mutant phenotypes to be identified by complementing the telomere silencing phenotype with a genomic library.

Chapter 6

Insertional mutagenesis screen

6.1 Introduction

In Chapter 5 it was found that all of the *rat* mutants retain a relatively high level of telomere repression, resulting in a high incidence of FOA resistant colonies and rendering identification of the affected gene by normal complementation strategies extremely difficult or impossible. Therefore, in order to identify genes involved in telomeric silencing and telomere function, an insertional mutagenesis strategy was decided upon. The approach used involved screening for random insertion of a traceable fragment of DNA into the genome, which disrupted silencing at a marked telomere but not at centromeres. Insertion of *ura4*$^{+}$ into the genome is selected for, then cells are screened for loss of silencing at the telomere and the insertion fragment acts as a marker for the mutation site. The sequence of the surrounding DNA can be obtained and can then be searched against an *S. pombe* sequence database to identify the gene that has been disrupted and is therefore presumed to cause the mutant phenotype.

Insertional mutagenesis has rarely been used in fission yeast. In most organisms transposons or retroviruses have been used as an insertional mutagen, such as P-elements in *Drosophila melanogaster* and retroviral provirus in Zebrafish (Ballinger and Benzer 1989, Allende *et al.*, 1996). In mammalian systems "gene trapping" has been developed in which a lacZ or similar reporter gene is inserted into the genome at random. The reporter gene lacks the initial ATG codon so must insert into an open reading frame and form a functional fusion protein to be expressed. Total RNA is isolated from the cells and 5' rapid amplification of cDNA ends (RACE PCR) used to identify the gene into which lacZ reporter gene has inserted (Sutherland *et al.*, 2001).

Large screens for genes affecting various cellular functions are now possible using insertional mutagenesis methods. Such an approach has been used to identify genes

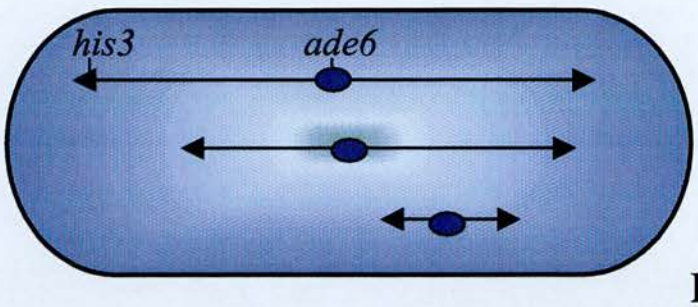
affecting salt tolerance in budding yeast. A transposon was used to generate 25,000 insertions which were screened for salt sensitivity. This identified 61 separate insertions affecting 31 genes of which only 12 were previously identified as necessary for salt tolerance (de Jesus Ferreira et al 2001). A suitable transposition system for insertional mutagenesis is not currently available for use in *S. pombe*. The Tf1 transposon (Levin *et al.*, 1990) has been found to insert preferentially at intragenic regions so is clearly not suitable for use as an an insertional mutagen (Behrens *et al.*, 2000).

In fission yeast insertional mutagenesis screens have been developed based on linear cassettes generated by PCR amplification. This approach depends upon illegitimate recombination between the ends of the insertion fragment and genomic DNA to randomly integrate the insertion fragment into the DNA and potentially cause a mutation. Mutants generated in this manner allow identification of the gene of interest by retrieving the sequence surrounding the insertion site and thus removing the need for cloning by functional complementation. The sequence surrounding the site of insertion can be searched against the *S. pombe* sequencing database (www.sanger.ac.uk) to rapidly identify the insertion site. The marker gene *ura4*$^+$ is a good choice for the mutagenic fragment, as its presence can be selected for on media lacking uracil and counter selected with media containing 5′-Fluoro-Orotic Acid (FOA), which is toxic to cells expressing *ura4*$^+$.

6.2 Results

6.2.1 Setting up insertional mutagenesis in *S. pombe*

During the course of setting up this system another group demonstrated its worth (Chua *et al.*, 2000). We have adapted this method by using a promoterless *ura4*$^+$ insertional fragment and a full length *ura4*$^+$ insertional fragment and attempted to reconfirm its validity as a screening method. A strain was used in which *ade6*$^+$ and *his3*$^+$ marker genes had been inserted into regions of silent heterochromatin in the centromere and the telomere respectively on chromosome I, as shown in Figure 6.1. These nutritional marker genes are not expressed in a wild type background, but may



FY1863

Figure 6.1: Strain used for insertional mutagenesis screen. Expression of the nutritional markers inserted at the centromere and telomere on chromosome one is repressed in a wild type background.

become so if the structure or function of the region in which they are located is perturbed. The open reading frame and approximately 500bp of the 3' untranslated region of *S. pombe ura4*$^+$ was amplified from a plasmid using PCR, as described in Figure 6.2 and Table 6.1. After PCR amplification the *ura4*$^+$ product was gel purified to ensure that the plasmid template was removed. Between 500ng and 1μg DNA per 10^8 cells was introduced by electroporation into the marked tester strain, FY1863. Whilst increasing the amount of DNA in each electroporation event increases the number of ura4$^+$ transformants obtained it also increases the risk of multiple insertions of *ura4*$^+$ into any one cell.

In initial experiments a "genetrap" approach was followed in which the gene promoter and start site were excluded in the amplification of *ura4*$^+$, so that the gene would only be expressed if inserted in the correct frame downstream of a promoter for another gene (Nos.1-3, Figure 6.2). In another approach twenty random nucleotides were included at the ends of the primers used to amplify the *ura4*$^+$ (Nos.4 and 6, Figure 6.2). It has been shown that at junctions between the insertion fragment and the genome micro deletions occur and a small buffer region of DNA flanking the marker gene prevents degradation of its ends during insertion (Chau *et al.*, 2000). It was also hoped that these randomised tails might promote homologous recombination between the ends of the PCR product and random sites in the pombe genome, thus aiding insertion events. This approach generated a low yield of ura4$^+$

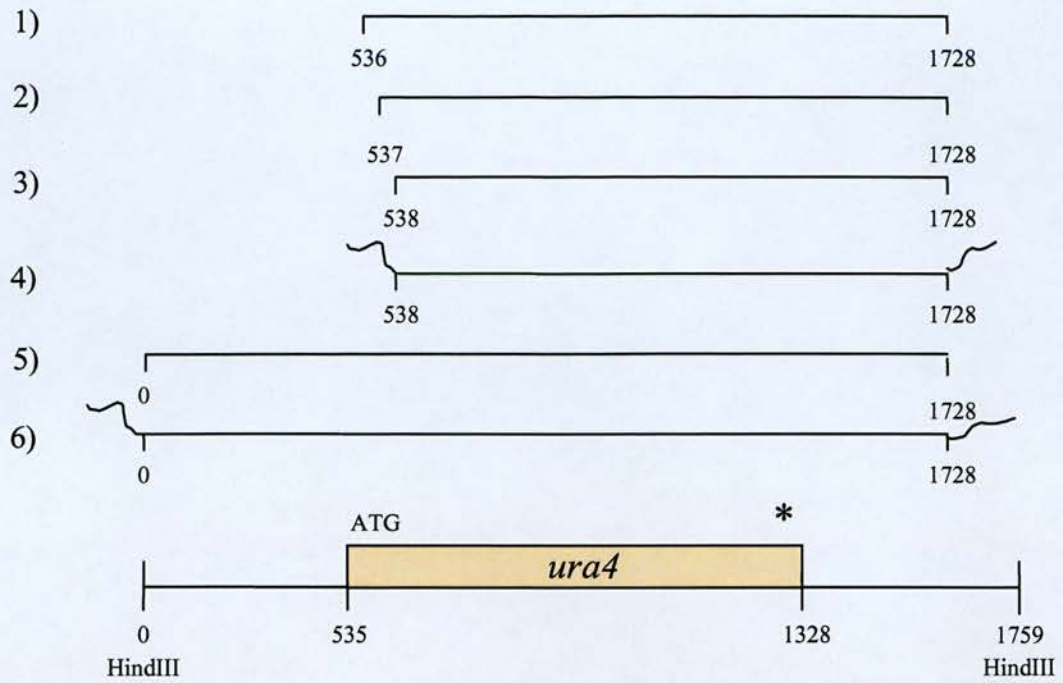


Figure 6.2 Diagram of the different *ura4*$^+$ PCR products used for transformation into *S. pombe* in the insertional mutagenesis screen. In initial experiments, using a genetrap approach, the *ura4* gene was amplified without the ATG so that the fragment had to insert downstream of another promoter and form a functional fusion protein to be expressed. This was changed to a less stringent screen in which the *ura4*$^+$ fragment included the gene's own promoter to increase the numbers of transformants obtained. Primers for products 4 and 6 had 20 random nucleotides incorporated onto the ends in the hope that this would buffer the ends of *ura4*$^+$ against micro deletions and promote homologous recombination and aid insertion into the genome.

	Product	5' Primer	3' Primer
1	*ura4* -ATG frame 1	R180	R183
2	*ura4* -ATG frame 2	R181	R183
3	*ura4* -ATG frame 3	R182	R183
4	*ura4* –ATG with random ends	R893	R894
5	*ura4* + 5' UTR	X961	R183
6	*ura4* + 5' UTR with random ends	Y256	R894

Table 6.1 Primers used to amplify each of the PCR products in figure 6.2 Sequences for the primers are in materials and methods.

transformants and so a less stringent approach was devised where the mutagenic PCR fragment included the promoter region of the *ura4*$^+$ gene. The full length *ura4*$^+$ PCR fragment (Nos. 5 and 6, Figure 6.2) can insert within or next to a gene and cause disruption while being transcribed from its own promoter and does not require insertion in frame with an endogenous ORF to be expressed. Reducing the constraints on the system increased the number of positive colonies generated on minus uracil. Alternative methods of obtaining ura4$^+$ colonies were used so that the production of random insertions was maximised, as described in Table 6.2. Initially transformed cells were plated on media lacking uracil following a very short recovery time after electroporation. In an attempt to reduce the number of unstable, episomal ura4$^+$ transformants and favour random integration events transformed cells were grown in non-selective media for 12-24 hours prior to spreading on selective plates. Approaches involving non-selective outgrowth can lead to a high occurrence of siblings in a culture. Therefore to lower the number of identical mutants, pre-growth was also performed on non-selective YES plates before imposing selection by replica plating to minus uracil or minus uracil/minus histidine plates. Cell division during non-selective growth on solid media results in cells remaining in one position on the plate and thus the number of sibling mutants is greatly reduced.

6.2.2 Insertional mutagenesis affecting telomeric silencing

Using these approaches a total of 10,180 ura4$^+$ colonies were obtained, as shown in Table 6.2. All of these colonies were subsequently streaked onto minus histidine plates to screen for mutations affecting silencing at telomeres. Of the ura4$^+$ colonies isolated only twenty-one also expressed the telomeric *his3*$^+$ marker gene. These were named fat for factor affecting telomeric silencing, and numbered fat1 to 21 in order of occurrence.

Since transformation techniques such as electroporation can be mutagenic it is possible that the inserted *ura4*$^+$ is not responsible for the loss of telomeric silencing resulting in the his3$^+$ phenotype. To test this the 21 *fat* mutants were backcrossed to a

Insertion fragment	Selection	Number of colonies
ura4$^+$ - ATG	immediate selection on uracil minus plates	1014
	o/n outgrowth in rich medium, selection on uracil minus plates	378
ura4$^+$ - ATG , + random tails	immediate selection on uracil minus plates	1304
	o/n outgrowth in rich medium, selection on uracil minus plates	677
HindIII *ura4*$^+$ fragment + random tails	immediate selection on uracil minus plates	3276
	o/n outgrowth in liquid rich medium, selection on uracil minus plates	3531

Table 6.2 The numbers of ura4$^+$ cells obtained using various selection techniques after introduction of the *ura4* fragments into *S. pombe* cells by electroporation.

strain similar to the marked tester strain and only those where the *ura4*$^+$ insertion co segregated with expression of telomeric *his3*$^+$ were selected for further study.

As shown in Table 6.3, eleven of the 21 *fat* mutants lost all *ura4*$^+$ expression when crossed to a wild type strain suggesting that although the *ura4*$^+$ gene had been expressed these were unstable insertion events or episomal forms of *ura4*$^+$. *fat16* and *fat5* had very low numbers of progeny which retained *ura4*$^+$ expression, this may have been due to incomplete loss of episomal *ura4*$^+$. Seven *fat* mutants displayed a stable *ura4*$^+$ insertion, but in only two of these, *fat6* and *fat7,* does *ura4*$^+$ appear to co segregate with the expression of the telomeric *his3*$^+$.

Only *fat6* and *fat7* were selected for further investigation since the apparent co segregation of *ura4*$^+$ and *his3*$^+$ in these strains is consistent with the *ura4*$^+$ insertions

	ura4+ his3+	ura4+ his3$^-$	ura4$^-$ his3+	ura4$^-$ his3$^-$
fat1	0	0	1021	51
fat2	0	0	53	2027
fat3	0	0	130	1062
fat4	0	0	53	1234
fat5	3	50	18	874
fat6	107	13	4	151
fat7	378	17	0	591
fat8	0	0	14	165
fat9	183	136	240	135
fat10	350	87	289	237
fat11	0	0	775	920

	ura4+ his3+	ura4+ his3$^-$	ura4$^-$ his3+	ura4$^-$ his3$^-$
fat12	0	0	60	19
fat13	0	0	543	764
fat14	0	0	60	19
fat15	0	0	543	764
fat16	5	7	127	543
fat17	0	0	213	968
fat18	29	0	749	369
fat19	36	25	46	82
fat20	199	82	243	
fat21	78	41	50	

Table 6.3. Table of progeny phenotypes following the crossing of *fat1– fat21* to a wild type strain with a telomeric *his3*$^+$ marker.

disrupting genes whose products have a role at the telomere. However, a proportion of *ura4*$^+$ progeny from the backcross of *fat6* and *fat7* did not display expression of telomeric *his3*$^+$. As discussed in chapter 3, section 2.3, the telomeric marker is occasionally lost in meiosis due to recombination between telomeres. Therefore colonies unable to grow on minus histidine were checked for the presence of the *his3*$^+$ marker gene by PCR. No product was obtained in these his3$^-$ colonies. This indicates that the telomeric *his3*$^+$ marker had been lost from these progeny. In the backcross of progeny from *fat6*, four colonies were isolated in which the telomeric marker was expressed in the absence of the *ura4*$^+$ insertion. Thus it is possible to separate the inserted *ura4*$^+$ from the mutation at a low frequency. This suggests that the inserted *ura4*$^+$ insertion event in *fat6* may be linked to the mutation but is not the cause of it.

fat6 and *fat7* were plated on limiting adenine to investigate the silencing status at the centromere. Wild type strains with silent centromeric *ade6*$^+$ form red colonies whereas *ade6*$^+$ expressing strains form white colonies. The *fat6* and *fat7* colonies are the same shade of red as the starting strain, indicating that silencing at the centromere is maintained and that these mutants affect telomere specific silencing factors (data not shown).

6.2.3 Telomere length of *fat6* and *fat7*

Mutations that affect silencing at telomeres may also cause imbalances in mechanisms of telomere maintenance and lead to changes in the average telomere repeat size of a population. In order to investigate telomere length in the *fat6* and *fat7* mutants total genomic DNA was prepared from these strains, a wild type starter strain (FY1863) and the *rap1-lot2* mutant and Southern analysis was performed, as described in 2.3.4. Digesting genomic DNA from these strains with BamHI cuts within the telomeric *his3*$^+$ marker releasing a fragment of between 500 and 800bp in a wild type strain. The released telomere fragment appears as a smear on a Southern blot when probed with telomere repeat sequences.

It can be seen from the Southern blot shown in Figure 6.3 that telomere length in *fat6* and *fat7* ranges between 2kb and 10kb, and is much longer than that of a wild type strain, which displays a 500bp–800bp smear. The increase in telomere length of the *fat6* and *fat7* mutants is similar to that seen in a *rap1-lot2* mutant.

6.2.4 Localisation of GFPswi6 fusion protein in *fat6* and *fat7*

Swi6p is a general silencing factor present at centromeres, telomeres and the silent mating type locus in *S. pombe* (Ekwall *et al.*, 1995). Localisation of Swi6p is dependent upon Clr4p and Rik1p and delocalisation of Swi6p due to mutation in *swi6*, *clr4* or *rik1* alleviates silencing in these regions (Ekwall *et al.*, 1996). GFPswi6p is a functional fusion protein that localises to the same regions as wild

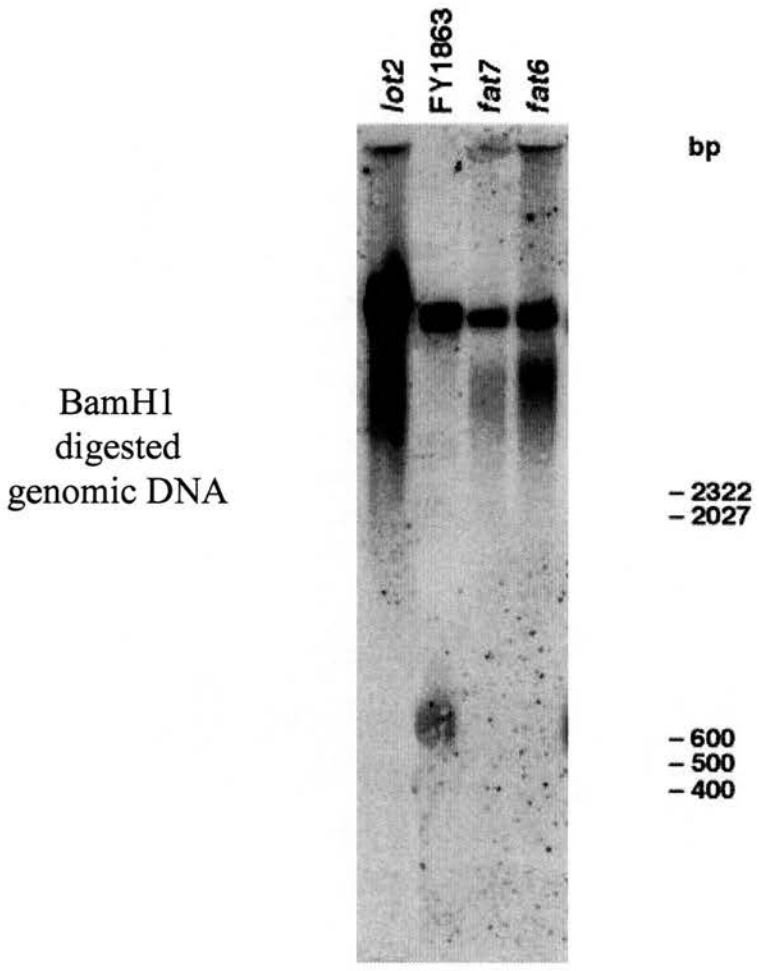


Figure 6.3. Southern analysis of telomere length in *fat6, fat7, rap1-lot2* and wild type strains. Compared to a wild type strain the *fat6* and *fat7* mutants both have an increased length of telomere repeat sequence, which is similar to that found in a *rap1-lot2* mutant.

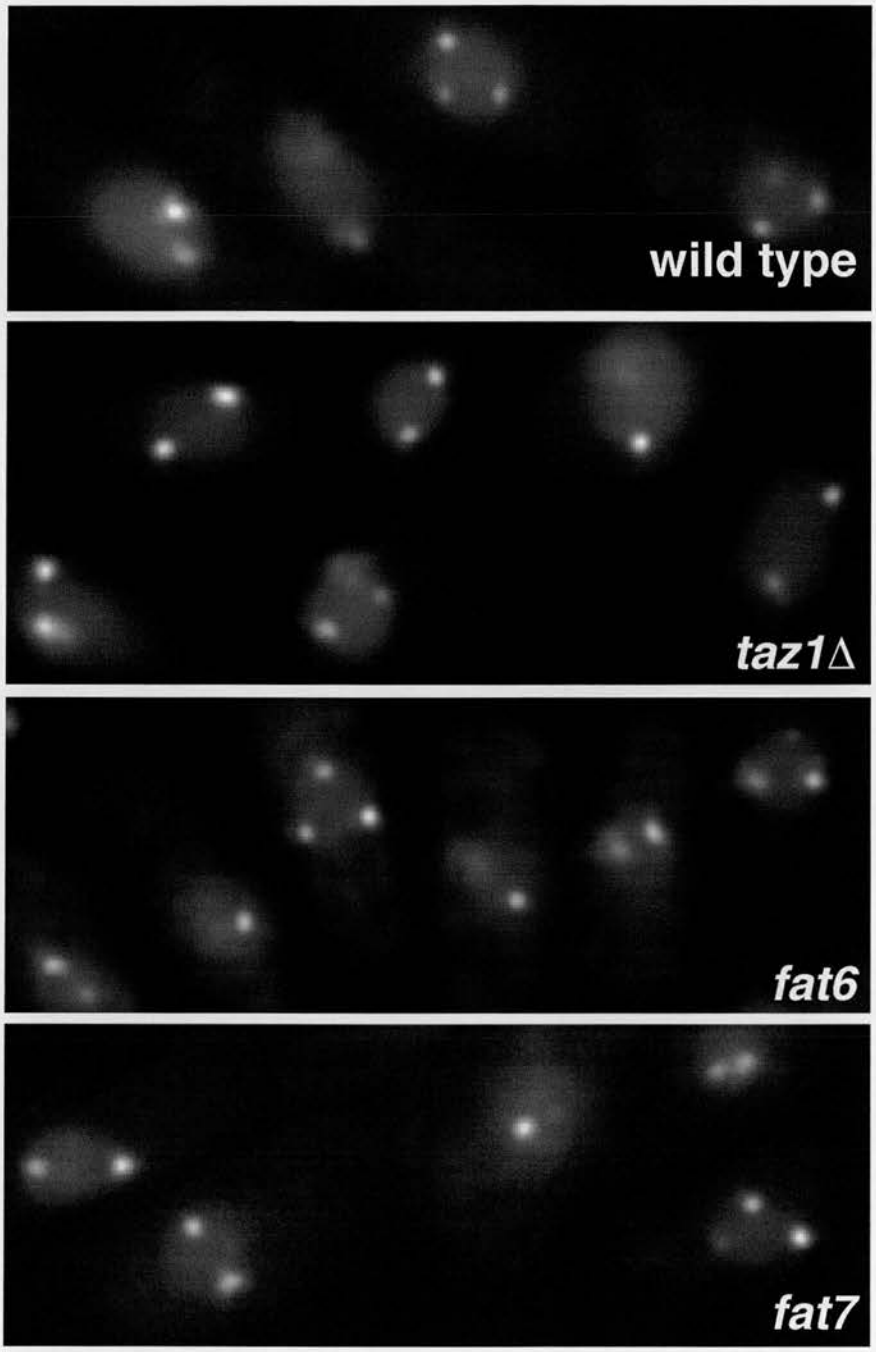


Figure 6.4. Swi6GFP localisation in both the *fat6* and *fat7* mutants displays a similar pattern of foci to *taz1*Δ and wild type strains. This suggests that alleviation of silencing in these mutants is not attributable to the delocalisation of swi6p

type Swi6p. It was possible that the localisation of Swi6p to the telomeres was disrupted in *fat6* and *fat7* mutants resulting in the loss of telomeric silencing.

To test this, a construct expressing GFPswi6p from the *nmt1* promoter integrated at *ars1* (FY2214) was crossed into the *fat6* and *fat7* mutant backgrounds. Cells were prepared for live microscopy as described in 2.5.1. In wild type strains two to three bright foci are visible in cells expressing GFPSwi6p. These foci correspond to clustered centromeres, aggregated telomeres and the silent mating type locus. In *fat6* and *fat7* cells the number of foci remains the same as in a wild type cell, suggesting that Swi6p is recruited to the telomeres in these mutants, shown in Figure 6.4. Although GFPswi6p remains localised at centromeres and telomeres in *fat6* and *fat7*cells this is not sufficient to silence the telomeric *his3*$^{+}$ marker. It is possible that Swi6p alone is insufficient for complete telomeric silencing and that the *fat6* and *fat7* mutations affect another telomeric silencing factor Alternatively although GFPswi6p forms foci at the telomeres in *fat6* and *fat7* cells its interactions with telomeric chromatin may be altered resulting in alleviation of silencing. The interaction of Swi6p and the telomeric marker gene could be further investigated by Chromatin immunoprecipitation analysis.

6.2.5. Analysis of spore formation in *fat6* and *fat7*

Following meiosis in wild type *S. pombe,* asci are formed that contain four evenly sized spores. Defects in meiosis lead to reduced rates of homologous recombination and an increased proportion of cells with chromosome mis-segregation defects, which can result in aberrant spore formation (Funabiki *et al.* 1993). The *lot2* and *taz1-lot3* mutants exhibit defects in meiosis, which result from failure in attachment of the telomeres to the SPB, and have a high percentage of misshapen asci containing an odd number of spores or spores of different sizes to one another (Nimmo *et al.*, 1998).

To test if *fat6* and *fat7* mutants affect meiosis and spore formation mating was induced in h^{90} derivatives. Asci from *fat6*x*fat6* and *fat7*x*fat7* crosses display a variety

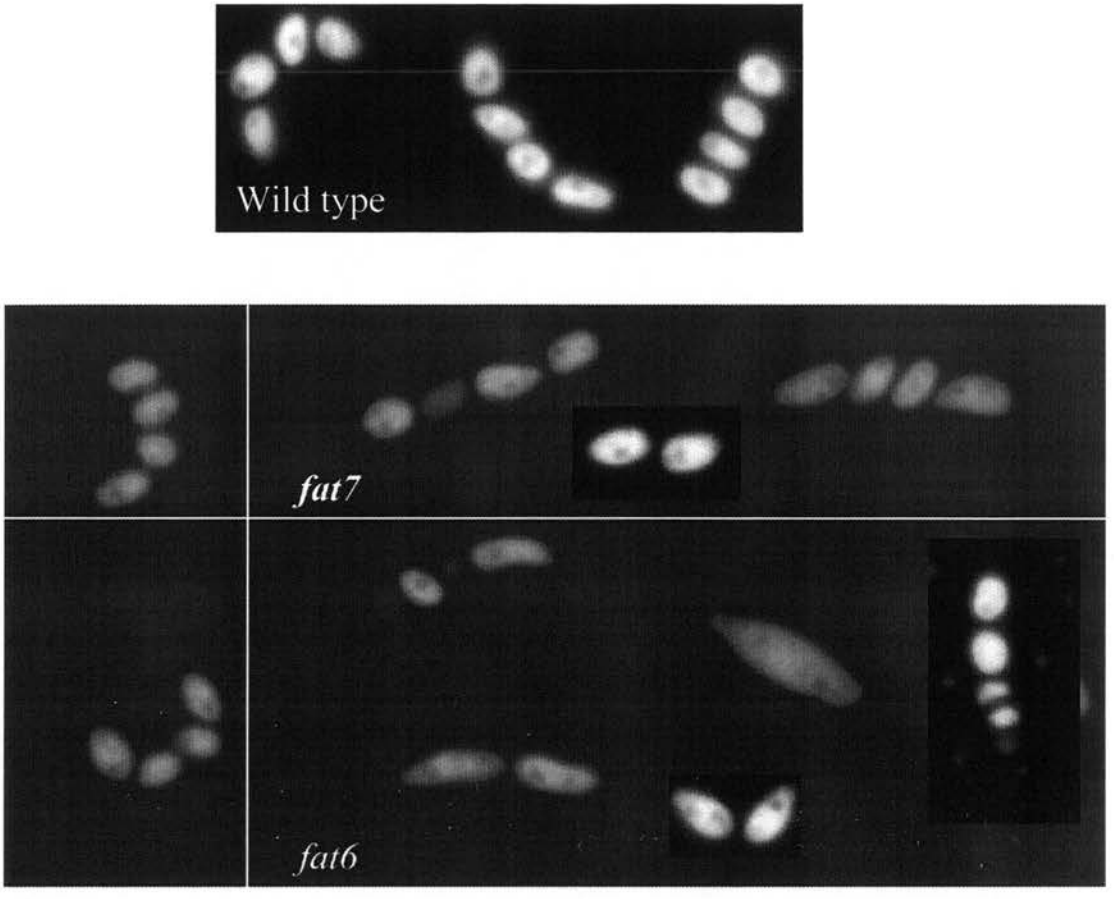


Figure 6.5. Spores from the *fat6* and *fat7* mutants display a variety of defects compared to a wild type strain. The asci from wild type colonies contain 4 evenly sized spores. In the lower panels asci from the *fat6* and *fat7*mutants are displayed. Not all of the asci in the fat mutants are malformed. 70-80% are wild type , such as those on the left hand side of these pictures. Other asci contain an abnormal number of spores, misshapen spores or both.

of defects compared to a wild type strain. The asci shown in the upper panel of Figure 6.5 are from wild type colonies and contain four evenly sized spores. In the lower panel asci from the *fat6* and *fat7* mutants are displayed. Not all asci from *fat6*x*fat6* and *fat7*x*fat7* crosses are malformed, 70-80% are similar to wild type. However a proportion of asci (32% *fat6*, 20% *fat7*) contain either an abnormal number of spores, misshapen spores or both. Clearly defects in the *fat6* and *fat7* strains are leading to aberrant spore formation, which are likely to result from failure to complete meiosis I or meiosis II.

6.2.6. Identifying the affected gene in *fat6* and *fat7*

In order to identify the *lot2* mutation a strain in which a random *ura4*$^+$ gene insertion linked to the *rap1-lot2* mutation was created. In this strain the *ura4*$^+$ inserted in a gene on chromosome II with homology to budding yeast *pob3* (Elaine Nimmo, personal communication). The *fat6* and *fat7* mutants have a similar phenotype to that seen in the *rap1-lot2* mutant. Cosmids from the region surrounding *pob3* were ordered for further analysis from the Sanger centre. Because of similar phenotypes displayed by *rap1-lot2, fat6* and *fat7,* crosses between all three mutants were performed to determine if *fat6* or *fat7* were *rap1-lot2* alleles. By genetic crossing and following expression of the *ura4* gene, the insertion sites in *fat6* and *fat7* were mapped relative to one another and to *pob3*::*ura4*. The sites of *ura4* insertion in *fat6* and *fat7* appeared to be very close but not identical to one another, since 96.4% of progeny were ura4$^+$. In *fat6* and *fat7* the *ura4*$^+$ insertion sites were found to be linked by 6.2cM and 8cM to the *pob3* disruption. *fat6* and *fat7* were also crossed to a disruption of a a nearby ORF(SPBC1778.02) with homology to *S. cerevisae Rap1*. *fat6* and *fat7* were also clearly linked to *rap1*::*ura4*. The linkage data is displayed in Table 6.4 and Figure 6.6. From these data it was concluded that the *fat6* and *fat7* mutations lie between the *pob3* and *rap1* loci.

The genetic mutation responsible for *lot2* was previously unmarked, *fat6* was therefore crossed to a *lot2* mutant strain and expression of telomeric *his3*$^+$ was followed in the progeny. Of 1060 colonies examined only ten did not express the

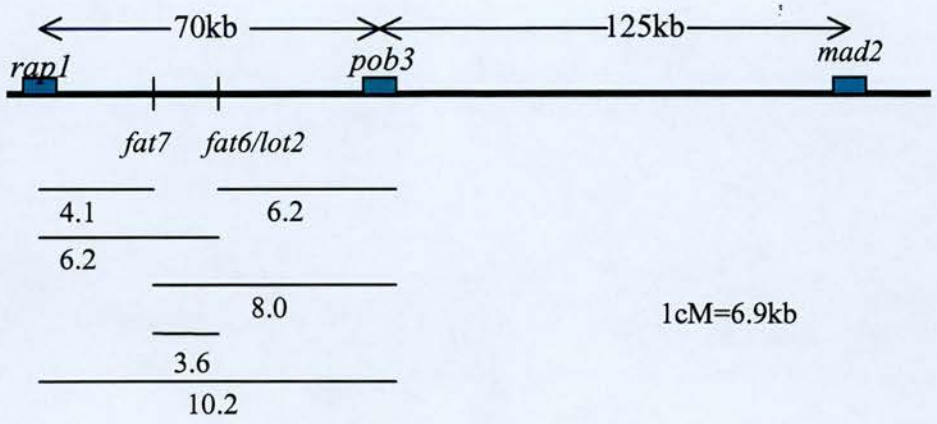


Figure 6.6. linkage map of the region containing *fat6*, *fat7*, *rap1-lot2* and *pob3*. Genetic crosses place the *fat6* and *fat7* mutations 6.2 and 4.1 centiMorgans from the *rap1* gene respectively. They are also linked to the *pob3* gene by a distance of 6.2 and 8cM respectively, thus would appear to lie between these two genes. Genetic crosses also indicate that *fat6* and *rap1-lot2* are caused by mutations in the same gene.

Strains mated	Marker gene followed	Total number of progeny	% Progeny positive for marker	Linkage in centiMorgans
fat6 x fat7	*ura4*	1329	96.4	3.6cM
fat6 x pob3	*ura4*	1238	93.8	6.2cM
fat7 x pob3	*ura4*	2020	92.0	8cM
fat6 xrap1	*ura4*	1949	93.8	6.2cM
fat7 xrap1	*ura4*	2619	95.9	4.1cM
fat6 xlot2	*his3*	1060	99.1	0.9cM
lot2 x taz1	*his3*	736	78.3	unlinked

Table 6.4 Linkage between *fat6*, *fat, pob3 and rap1* was calculated following the segregation of the *ura4* genes inserted into the fat mutants with *ura4* disrupted genes. Linkage to *rap1-lot2* was calculated by following the expression of the *his3* marker gene at the telomere. The *fat* mutants and *rap1-lot2* are all linked to the two genes in this region.

marker gene at the telomere. Subsequent investigation of these progeny by PCR demonstrated that they had lost the telomeric *his3*$^{+}$ marker, thus all putative recombinants were actually due to loss of the telomeric *his3*$^{+}$. This indicates that the *fat6* and *lot2* mutations are tightly linked and are likely to be caused by mutations in the same gene.

6.2.7 Analysis of DNA in the *fat* region

An insertion event in the chromosomal region between *pob3* and *rap1* where the *fat6* and *fat7* mutations map should result in a detectible change on a Southern analysis of DNA from *fat6* and *fat7* when compared to that of the starting strain. Four cosmids span this region: SPBC1778, SPBC405, SPBC4c3 and SPBC609; these cosmids were labelled with [α-^{32}P]CTP and used as probes. The results are presented in Figure 6.7.

Several hybridising bands detected in wild type are missing in *fat6* and *fat7*. DNA changes are detectable with more than one cosmid probe: with SPBC405 or SPBC609 *fat6* and *fat7* share a missing band of approximately 2.4kb. In addition the wild type 3.6kb, 1.2kb and 1.05kb bands are not detected in *fat6* and *fat7* after probing with SPBC609. A band of 1.45kb is also absent in *fat6* and *fat7* DNA probed with cosmids SPBC1778 and SPBC4c3. Although several bands appeared to be absent in *fat6* and *fat7* DNA no new bands indicative of the presence of an insertion event or loss of a restriction site, were detected. The loss of restriction fragments could be explained by a deletion induced by the insertional mutagenesis in *fat6* and *fat7* in the region covered by a particular cosmid. However the fact that deletions occur in regions corresponding to more than one cosmid suggests that a complete rearrangement may have occurred. Similar bands affected in adjacent cosmids are probably due to overlapping sequences. A map of EcoR1 restriction sites across the region was calculated using the GCG sequencing program at HGMP and used to calculate the expected fragments from digestion of the region between *rap1* and *pob3* by enzymes. The predicted bands were then matched to fragments found to be absent by Southern analysis of *fat6* and *fat7* DNA and used to determine the affected

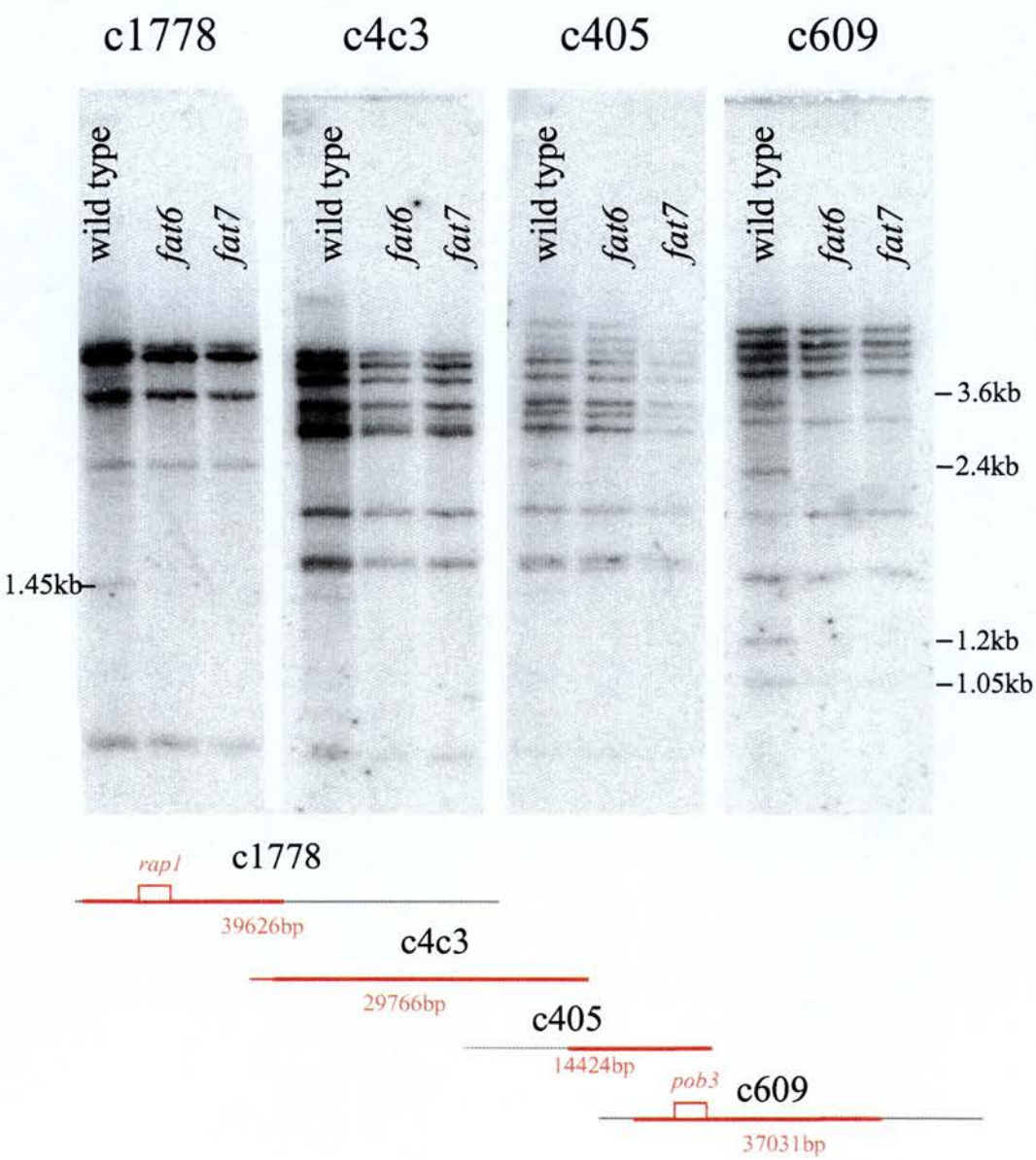


Figure 6.7 Southern analysis of EcoR1 digested DNA from wild type, *fat6* and *fat7* strains probed with cosmids spanning the suspected region of the *ura4* insertions in the *fat6* and *fat7* strains. The position of the cosmids relative to one another is indicated in the diagram. The size of bands visible in the wild type strain that appear to be missing in *fat6* and *fat7* strains are indicated.

regions. It was concluded that none of the fragments missing by Southern analysis are from neighbouring regions in the contiguous sequence. This means that the missing fragments do not represent a single large deletion event. It may be that a rearrangement accompanied by deletion of sequence either end has occurred. Such a deletion/rearrangement event is not necessarily the result of a *ura4*$^+$ insertion event into the region.

Interestingly, the cosmid SPACc1778 was found to complement the *lot2* mutation (Elaine Nimmo, personal communication). This cosmid carries a gene encoding an ORF with homology to *S. cerevisiae Rap1*. Sequence analysis showed that *lot2* cells carry a mutation in the *rap1* gene. Primers spanning the *rap1* ORF and including approximately 500bp of 5' and 3' flanking sequence were used to amplify the *rap1* gene from *fat6* and *fat7* DNA. No difference in the size of this PCR product was detected relative to wild type, as demonstrated in Figure 6.8. Thus the mutagenic *ura4*$^+$ insertion did not disrupt the *rap1* gene in *fat6* and *fat7*.

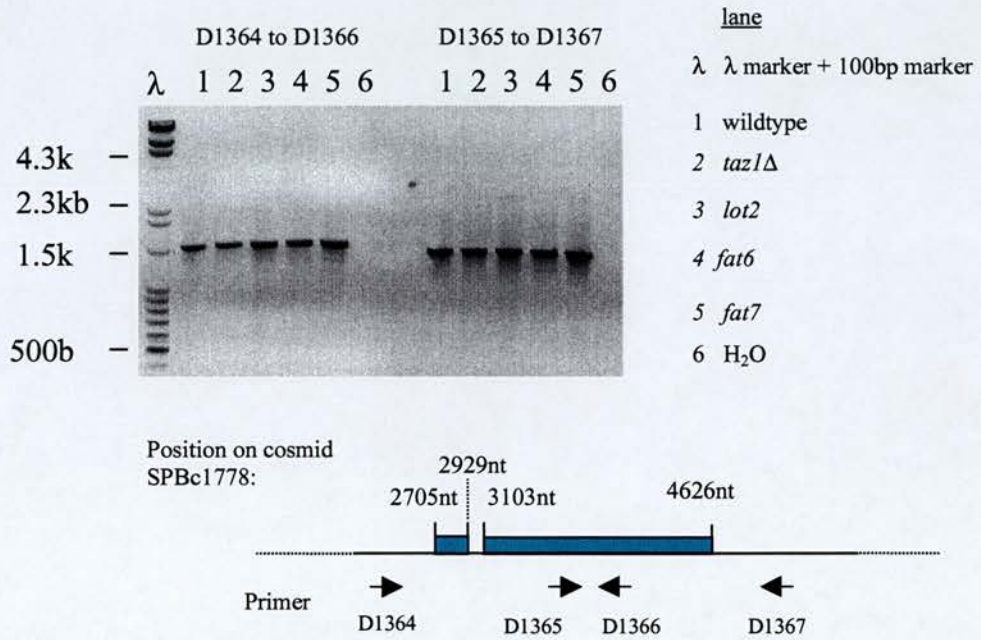


Figure 6.8. the predicted open reading frame of the *rap1* gene cosmid SPBC1778. PCR amplification of the region in wild type, *rap1-lot2*, *fat6* and *fat7* displayed no differences in the size of the amplification product indicating that there has been no insertion of *ura4*$^+$ into this gene in *fat6* and *fat7*.

6.2.8 Identification of the site of insertion of the *ura4* gene.

To identify the site of insertion of a *ura4*$^+$ gene causing mutations it is necessary to sequence the region immediately adjacent to *ura4*$^+$. Since the entire *S. pombe* genome has been sequenced, less that 100bp of sequence is required to perform a BLAST search against the *S. pombe* genome at the Sanger Centre and identify the site of insertion.

A method for recovery of unknown DNA flanking an insertion and previously used in *Drosophila* to identify P-element insertion sites is Vectorette or "bubble PCR" (Eggert *et al.*, 1998). In this method a specialised linker, comprising of a section of non-homologous sequence in the centre flanked by two short regions of homology and termed a vectorette, is ligated onto the ends of all fragments in a digest of genomic DNA (Riley *et al.*, 1990). The non-homologous regions in the centre of the construct remain single stranded forming a "bubble". The end of the vectorette can be designed with 5' overhangs to be compatible with restriction enzyme sites of choice. To amplify the sequence between the site of *ura4*$^+$ insertion and the ligated vectorette linker two primers are used; one is complementary to the *ura4*$^+$ insertion sequence, priming out from the *ura4*$^+$ to the flanking sequence, and another, which is identical in sequence to one side of the vectorette "bubble" and priming towards the *ura4*$^+$ gene. Only when the single stranded region has been used as a template by passage of taq polymerase is a complimentary strand created for annealing of the vectorette primer. This only occurs if *ura4*$^+$ has been ligated nearby and therefore provides specificity to the PCR reaction as described in Figure 6.9.

This method produced strong reproducible PCR bands from EcoRI digested DNA for both of the *fat* mutant strains. The amplification products were of an identical size in *fat6* to those produced in the *fat7* mutant. These were sequenced and the sequence obtained was used to search for homology against the *S. pombe* genome. Using this method it was found that the first 640nt of the amplified sequence were homologous to a region of cosmid SPCC330, which contains the wild type *ura4*$^+$ locus. At the

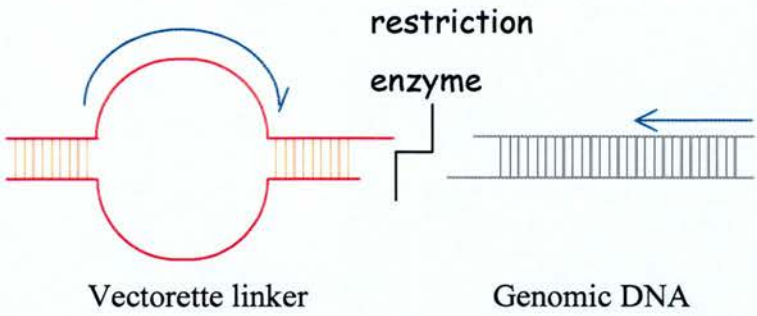


Figure 6.9a. The Vectorette linker consists of a central region of non-homologous sequence flanked by short regions of homology. When annealed the oligonucleotides form a linker with a bubble of single stranded DNA in the centre. The vectorette can be designed with ends compatible with restriction enzymes of choice (Riley *et al.*, 1990)

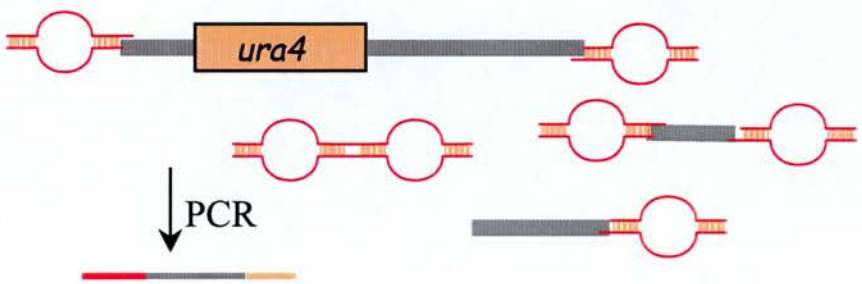


Figure 6.9b. Genomic DNA is digested and vectorettes are ligated onto the ends of the DNA fragments. PCR from the sequence of the insertion fragment, in this case *ura4,* to the vectorette initiates rounds of PCR amplification across the region next to the insertion site. The amplification product can then be sequenced and searched against genome sequence data with blast servers to identify the site of insertion in the genome.

TNTNTTCACANAGTGCNNACNTTNTCATGAAAAAGAACCATTTTAATTTANAGCAAGGGCA
TAT ATTCACAAAGTGCAAACATTATCATGAAAAAGAACCATTTTAATTTAAAGCAAGGGCA

TTAAGGCTTATTTACAGAATNTCTTACTTTTGTAAAGATTATAAGGCTGATTATCTTTTTCA
TTAAGGCTTATTTACAGAATTTCTTACTTTTGTAAAGATTATAAGGCTGATTATCTTTTTCA

CCATGCCAAAAATTACACAAGATAGAATGGGATGTTTGAAATTAAACGTGAGTATACAAAC
CCATGCCAAAAATTACACAAGATAGAATGG - ATGTTTGAAATTAAACGTGAGTATACAAAC

AAATACACTAGGTAAATCGAAACATTTTTTTCTCCATTAAGTAACAAATTCCTATTTAGAGA
AAATACACTAGGTAAATCGAAACATTTTTTTCTCCATTAAGTAACAAATTCCTATTTAGAGA

AAGAATGCTGAGTAGATTAAATAATCTATACAAACTTTTTTAACACAAATGCATACATATAG
AAGAATGCTGAGTAGATTAAATAATCTATACAAACTTTTTTAACACAAATGCATACATATAG

CCAGTGGGATTTGTAGCTAAGCTCAATAGTGGGGGCATTGTATTTGTGAAAAAAAACTTAA
CCAGTGGGATTTGTAGCTAAGCTCAATAGTGGGGGCATTGTATTTGTGAAAAAAAACTTAA

GTAAATTTATTTTTTTATGAATACCGGATTTCATTTCTATTTCTTTATTCA -CTTACCGC -CT
GTAAATTTATTTTTTTATGAATACCGT ATTTCATTTCTATTTCTTTATTCAACTTACCGCACT

TCCTAAATTCCCTTTATTCTATATTGGTACTCCGTTNCCCAATTAATTATGACTGGGTG
TCCTAAATTCCCTTTATTCTATATTGTTACTCCGTTACCCAATTAATTATGACTG AGTG

TCCTAA	sequencing from *fat6* Vectorette fragment	
CACAAG	SPCC330 [Full Sequence] S. pombe chromosome 3	13267 - 13697
CTTTAT	pNSU28 [Full Sequence] S. pombe telomere	166-325

Figure 6.10 The sequence data from the PCR amplified fragment between vectorette and the site of *ura4*$^+$ insertion was identical in the *fat6* and *fat7* mutants. The sequence of the amplified product is indicated in black. The portion that matches *ura4* sequence is indicated in orange and the portion that matches telomeric DNA is indicated in blue. The sequencing data indicates that the *ura4*$^+$ fragment has inserted into telomere associated sequence transcribed away from the centromere as shown in the cartoon.

end of the *ura4*$^+$ sequence this fragment contains sequence adjacent to the *ura4*$^+$ insertion site, as shown in Figure 6.10. The sequence obtained from the site of *ura4*$^+$ insertion in the *fat6* and *fat7* displays most homology to sequence on plasmid pNSU28, displayed in Figure 6.11, which contains telomere associated sequences. The sequence data shows that the *ura4*$^+$ gene has inserted in an orientation where it is transcribed towards the telomere repeats. However all the telomeric cosmids contain highly similar stretches of sequence and as these cosmids have yet to be to be joined to the chromosome contigs it is not possible to distinguish which telomere the *ura4*$^+$ fragment is associated with from this data.

Sequences producing High-scoring Segment Pairs:		High Score	Smallest Sum Probability P(N)	N
SPCC330	S. pombe chromosome 3	2006	1.8e-85	1
pNSU28	S. pombe telomere	742	1.7e-29	1
pNSU64	S. pombe telomere	747	1.2e-28	1
pNSU70	S. pombe telomere	747	1.2e-28	1
pNSU21	S. pombe telomere	747	1.2e-28	1
pNSU65	S. pombe telomere	747	1.2e-28	1
pNSU77	S. pombe telomere	747	1.3e-28	1
pNSU71	S. pombe telomere	747	1.3e-28	1
pNSU31	S. pombe telomere	476	2.3e-17	1

Figure 6.11 The vectorette PCR products from the 5' end of the *ura4*$^+$ insertion site in *fat6* and *fat7* were sequenced and found to be identical. The sequence was searched against the Sanger Centre S. pombe genome database. The sequence following the *ura4*$^+$ insertion is largely similar to sequence found in telomere cosmids. These cosmids contain regions of homology to one another and are unassigned to particular telomeres making it impossible to tell which telomere the *ura4*$^+$ fragment is inserted into in the *fat6* and *fat7* mutants from this data.

The sequence surrounding the *ura4*$^+$ insertion sites in *fat6* and *fat7* were found to be identical; these strains therefore must represent the same mutation isolated as siblings of a progenitor during the selection process. The apparent difference seen in crosses between the strains can be explained by the occasional loss of the *ura4* gene from the telomere during meiosis. Further sequence analysis was performed on previously constructed strains which carry a *ura4*$^+$ inserted adjacent to the telomere. This showed that *fat6* and *fat7* strains are not descended from these strains created in the lab.

To confirm that *fat6* and *fat7* cells contained only a single insertion of *ura4*$^+$ Southern analysis was performed on high molecular weight DNA prepared and digested with SfiI and NotI in agarose plugs. This allows identification of the telomere into which the *ura4*$^+$ fragment had inserted.

The resulting filter was hybridised with a *ura4*$^+$ probe and a single band was detected in *fat6* and *fat7* DNA digested with NotI or SfiI, as shown in Figure 6.12. This indicates that there has been a single *ura4*$^+$ insertion event in these cells. In SfiI digested DNA this *ura4*$^+$ positive band corresponds to a fragment of approximately 915kb. There is a faint band corresponding to a much larger fragment of over 3Mb in lanes with NotI digested DNA, which has not transferred to the membrane as efficiently as the smaller SfiI fragment. After NotI SfiI double digestion the fragment remains the same size indicating that the SfiI fragment does not contain a NotI restriction site. The size of this fragment is consistent with the insertion of *ura4*$^+$ into a SfiI fragment of approximately 915kb at the right telomere of chromosome III.

6.2.9. Sequencing the *rap1* gene in the *fat* mutants

Since the only site of *ura4*$^+$ insertion in the *fat6* and *fat7* mutants appears to be at a telomere the phenotypes must be due to a non insertional mutation elsewhere in the genome. The mapping data in Figure 6.6 indicated that *fat6* is allelic to *rap1-lot2* and that *fat6* and *fat7* are sib mutations. It is therefore likely that all these mutations affect the same gene. SPBC1778 alone complements the *rap1-lot2* phenotype and disruption of the *rap1* ORF causes increased telomere length and loss of telomeric silencing similar to that seen in a *rap1-lot2* mutant Finally sequencing of *rap1* indicated that it is mutated in *rap1-lot2* mutant (Elaine Nimmo, personal communication).

The *rap1* gene and approximately 500bp flanking the coding region was amplified from the *fat6* and *fat7 DNA* by PCR and sequenced, as shown in Figure 6.13. A T to G change was identified 635bp downstream of the *rap1* start codon in both the *fat6*

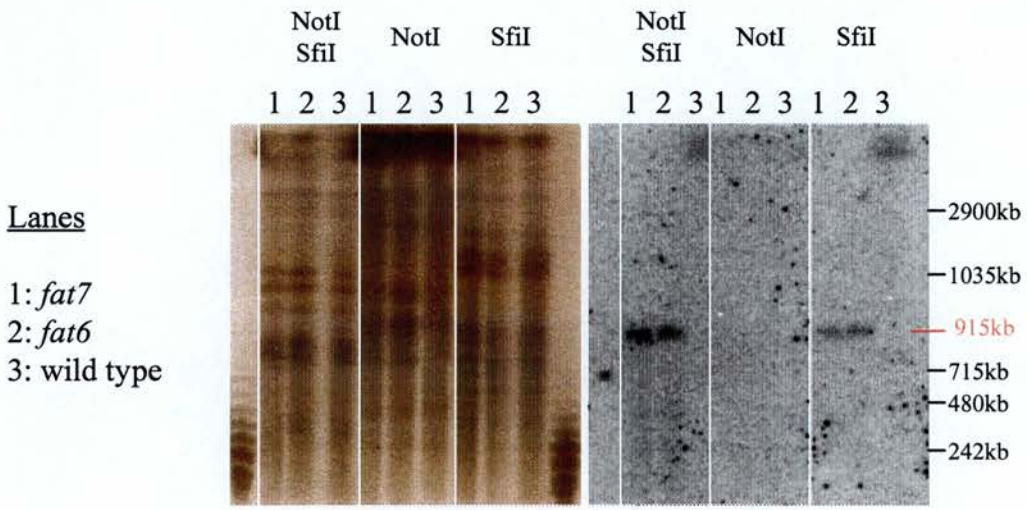


Figure 6.12. Southern analysis of high molecular weight DNA probing with *ura4*$^{+}$. DNA from *fat6* and *fat7 was* digested with Not1, Sfi1 or both. Digesting whole chromosome preparations with these enzymes gives fragments of a known size. These gels were blotted to Genescreen membrane and probed with a ^{32}P labelled *ura4* PCR product to show into which fragment the *ura4* gene was inserted. This shows that there is only one *ura4*$^{+}$ fragment inserted into each of the *fat6* and *fat7* strains.

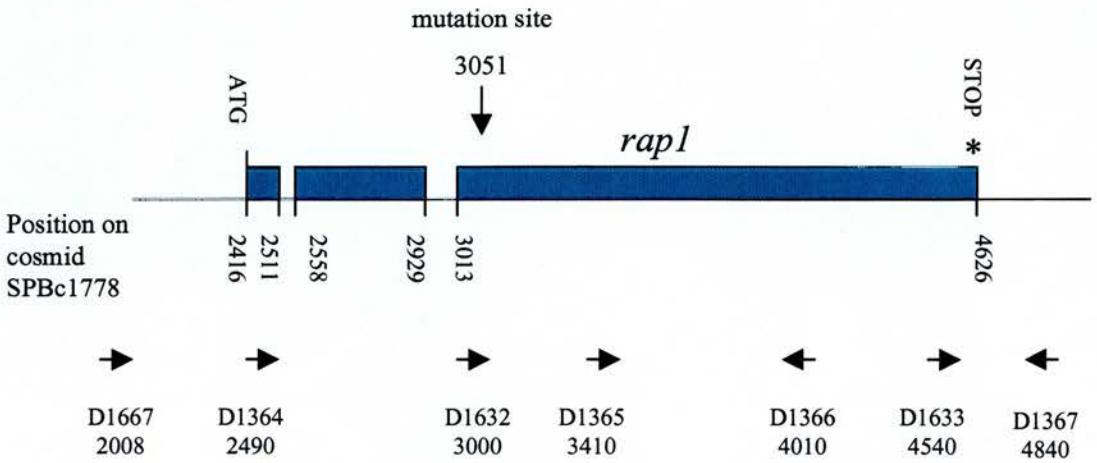
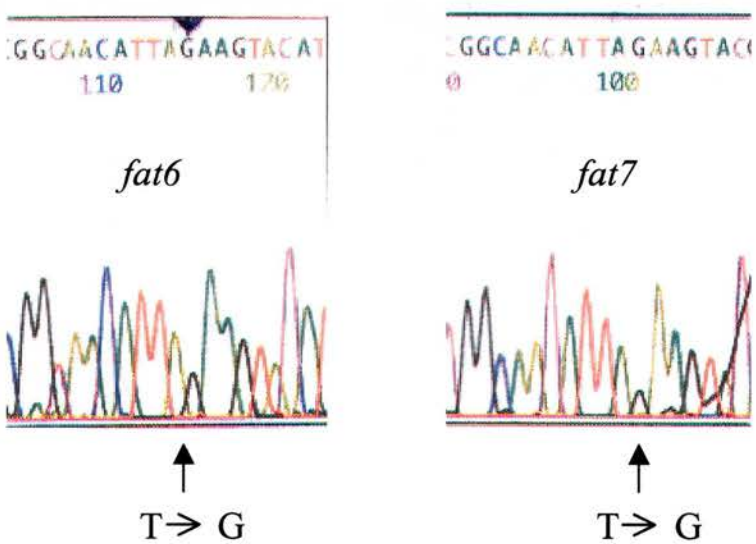


Figure 6.13. The structure of the open reading frame of *rap1* showing the position of the gene and the mutation site relative to cosmid SPBc1778 and the primers used to sequence *rap1* in the *fat6* and *fat7* mutants. Below are the sequence analysis of the mutation site in the *rap1 gene* in *fat6* and *fat7*. On the left is the trace from *fat6* sequencing and on the right the sequence of the identical region of *rap1* in fat7.



and *fat7* strains. This alters a TAT to a TAG codon, resulting in a stop codon in place of a tyrosine in Rap1p. The stop codon in *fat6* and *fat7* is introduced within a region of homology between the *S. pombe* and *S. cerevisae rap1* sequences, in the C-terminal part of region homologous to the myb-like DNA binding domain and therefore is likely to cause a truncated product.

6.3 Discussion

A total of 10,180 ura4$^+$ colonies were isolated and investigated for defective telomeric silencing using the insertional mutagenesis method. Of those selected for further study 67% were unstable *ura4*$^+$ insertions. An increase in the number of unstable insertions from 20% to 55% was found when the whole *ura4*$^+$ gene with its own promoter was used to generate insertions. All of the colonies that were ura4$^+$ were examined for expression of a normally silent telomeric *his3*$^+$ marker gene. Of these, 21 were alleviated for silencing at the telomere enabling them to form colonies on minus histidine media. After these strains were backcrossed only seven of these mutants displayed a stable integration of the *ura4*$^+$ gene, and in only two did the *ura4*$^+$ insertion fragment and telomeric *his3*$^+$ marker gene appear to co segregate, giving the appearance that the insertion events had caused mutations that had an effect on the telomeric silencing. Genetic crosses between *fat6* and *fat7* indicated that the strains were linked.

Vectorette linkers were used to successfully identify the sequence surrounding the *ura4*$^+$ insertion. It was discovered that *fat6* and *fat7* had identical sites of *ura4* insertion and that the *ura4*$^+$ gene had inserted at the telomere. Southern analysis with a probe for the *ura4* sequence found that the insertion had occurred at the right telomere on chromosome II.

The screen was unsuccessful in identifying novel telomere genes by disrupting their open reading frames with a marker gene, however a similar method has been used successfully for generating mutations in other laboratories (Chau *et al*., 2000).

Studying the phenotypes of the *fat6* and *fat7* strains showed them to have an increase in telomere length of up to 10kb, defects in meiosis and loss of silencing at the telomere, a similar phenotype to that seen in a *lot2* mutant. Genetic crossing between the *fat6* and *lot2* strains demonstrated that the mutations were allelic, and since the *lot2* mutation could be complemented by cosmid SPBC1778 that contains the pombe *rap1* gene the *rap1* gene was sequenced in the *fat6* and *fat7* strains. It was found that there was a mutation causing a stop codon to be generated 635bp downstream of the *rap1* start codon, so truncating the protein in *fat6* and *fat7* cells. This is identical to the mutation found in a *rap2-lot2* mutant. The duplication of the same mutation site in the *fat* and *lot2* mutants may be due a mutation hotspot existing at this point in the gene or the deletion of a large part of the gene being necessary for a telomere silencing defect to occur.

Genetic crosses between a *rap1::ura4*$^+$ strain and the *ura4* insertion in the *fat* mutants had indicated that the inserted *ura4*$^+$ was linked to but not at the *rap1* locus. It is expected that if the two marked loci are unlinked only 75% of the progeny of a cross should be ura4$^+$ and the other 25% will be wild type. The high numbers of ura4$^+$ progeny resulting from this cross may have been due to the genetic cross being performed between two strains with mutations in *rap1,* which are defective in meiosis. If karyogamy and meiosis is defective then there may have still been parental cells existing amongst the spores after mating, therefore increasing the number of *ura4*$^+$ colonies and giving rise to these aberrant numbers.

Chapter 7

Concluding remarks

In this thesis I performed two screens based on alleviation of TPE to identify novel factors with a role in telomere structure and function. The first screen used 3Mj UV irradiation and spontaneous mutagenesis to create a large pool of mutants, a cross section of which were selected for further analysis. These twelve mutants formed six complementation groups by genetic crossing, one of which was allelic to *taz1*. Subsequent investigation of telomere length in these strains showed that two of these alleles of *taz1*, *rat2-j543* and *rat2-k596* had wild type telomere length but were alleviated for silencing, thus separating the telomere length regulation and silencing roles of *taz1*. Further investigations using these alleles of *taz1* could be undertaken to dissect the role of Taz1p at the telomere and identify proteins that interact with Taz1p.

The mutation in *taz1* in the *rat2-j543* mutant is outside of the DNA binding domain and does not appear to affect the ability of the protein to bind to telomeric DNA, as such a defect would also lead to delocalisation of the telomere length regulation complex and lead to increased telomere length. Since failure to recruit Rap1p to the telomere leads to a large increase in the length of telomeres and loss of Rif1p function leads to a smaller increase in telomere length it might be assumed that in the *rat2-j543* and *rat2-k596* mutants Taz1p still localises these proteins. The *rat2-j543* mutant has an increased number of malformed asci relative to a wild type strain, which might suggest that Taz1p is unable to interact correctly with factors involved in attachment of the telomeres to the SPB in this strain. Both the *rat2-j543* and *rat2-k596* mutants are defective for telomeric silencing indicating that the mutations in these strains may affect a domain whose function is to recruit silencing factors to the telomere. Unlike in *S. cerevisiae* a *S. pombe ku*Δ strain is not alleviated for silencing at the telomere and immunolocalisation of Swi6p in the *rat2* strains found that there was no disruption of Swi6p at the telomere. This suggests that there may be other unidentified factors involved in silencing at the telomere recruited by Taz1p.

Five of the complementation groups identified in the first screen did not display linkage to any of the genes tested that affect telomere silencing. This suggests that there are still several genes affecting telomeric silencing that have not yet been identified. Analysis of the expression of the telomeric marker genes in some of the *rat* mutants suggested that they would not be amenable to complementation with a genomic library. The difficulties in isolating the mutations responsible for alleviation of telomeric silencing suggests that the traditional random mutagenesis methods, although capable of generating large collections of mutants as demonstrated in chapter 3, are of limited use for identifying genes involved in regulating telomere position effect.

Recently *rif1*, *rap1* and *pot1* (Kanoh and Ishikawa, 2001: Chikashige and Hiraoka, 2001: Baumann and Cech, 2001) have been identified as genes involved in telomere structure and function in *S. pombe* through homology to telomeric factors in other organisms. The overall homology of each of these genes to the corresponding genes in other organisms is not particularly high, with 27% identity between *S. pombe rap1* and *S. cerevisiae RAP1*. Regions of greater homology occur within these genes, however, such as the myb-like domain which has 32% identity between the two species (Kanoh and Ishikawa, 2001). This indicates that it may be more fruitful to search for sequence homology with small conserved domains than with the whole gene. *S. pombe* and *S. cerevisiae* are equally divergent from each other and vertebrates by between 330 and 420 million years (reviewed Sipiczki, 2000). The sequencing and public availability of whole genome sequences facilitates searching for genes by homology and the manipulation of sequence data. There are many more genes known to be involved in telomere structure and function in *S. cerevisiae* and vertebrate systems than are currently identified in *S. pombe* (see tables 1.1, 1.2 and 1.3). Although more might be identified in *S. pombe* by searching for regions of homology other factors acting at *S. pombe* telomeres might be too divergent or unrelated to those in other organisms for this type of approach to be possible. Although homology will be a useful tool for identifying some components of the *S. pombe* telomere it will not discover novel components that are not identified elsewhere.

Analysis of the method by which Rap1p is tethered to the telomere in *S. pombe* suggests that the localisation of Rap1p to the telomere is mediated by Taz1p (Chikashige and Hiraoka, 2001), in a similar manner to the interaction between human RAP1 and TRF1 (Bibo, *et al.*, 2000). Comparing this method of recruitment to the direct interaction of *S. cerevisiae* Rap1p with the terminal telomeric repeats demonstrates that although these proteins have regions of structural similarity their modes of action at the telomere differ. hRap1 was identified by homology to *S. cerevisiae RAP1* but subsequent identification and analysis of *S. pombe* Rap1p suggests that in this case the human and fission yeast proteins share more similarity in their function. Thus studying telomeres in different systems can help to guide the analysis of telomere function in complex eukaryotes. This demonstrates the value of studying telomere structure and function in more than one model organism.

Alleviation of silencing at the telomere is a valid tool for identifying mutations that result in structural changes, as discussed in chapter 4, however this phenotype is not suitable for complementation of the mutation by genomic libraries. Therefore in order to identify novel components of the telomere in fission yeast it is desirable to develop methods of mutagenesis that mark the site of mutation. Insertional mutagenesis with a mutagenic fragment consisting of the *ura4*$^+$ gene was attempted in a strain with a *his3*$^+$ marker gene inserted at the telomere. The telomeric *his3*$^+$ marker gene is silent in a wild type background. Screening 10,180 insertional mutants for alleviation of silencing at the telomere identified 21 candidate strains of which two appeared to co-segregate with the *ura4*$^+$ insertion event and expression of the telomeric marker and therefore were of interest. Subsequent analysis demonstrated that the *ura4*$^+$ insertion had occurred at the telomere in these strains and that a second unconnected mutation was responsible for the telomeric silencing defect. A high level of unstable insertions or episomal expression of *ura4*$^+$ was discovered in the insertional mutants produced by this screen. A similar screen has been used effectively to identify mutations in genes involved in down regulation of growth following nitrogen starvation (Chua *et al.*, 2000). In *S. cerevisiae* insertional mutagenesis has been has used to efficiently identify genes involved in tolerance of

salt stress (de Jesus Ferriera *et al.*, 2001). Improvements to the insertional mutagen may be beneficial to the use of this system in fission yeast. A mutagenic fragment with a growth phenotype that is not found in a wild type *S. pombe*, such as kanamycin resistance may be desirable to reduce the chance of accidental contamination. Other methods of transformation, such as the lithium method used by Chau *et al.*, 2000, may be less mutagenic than the electroporation technique used in the insertional mutagenesis screen detailed in Chapter 6.

A fission yeast transposon has been identified, Tyl, but this inserts preferentially at intergenic regions and so is not suitable for use as an insertional mutagen (Devine and Bockle, 1996, Nelson *et al.*, 1997). It may be that a transposon system from another organism such as Ac/Ds in maize, which has been shown to successfully reproduce in *S. cerevisiae,* could be adapted for use as an insertional mutagen in *S. pombe* (Weil and Kunze, 2000).

Insertional mutagenesis generates loss of function mutations. It has been shown that deletion of telomeric factors is not immediately lethal to the cell during vegetative growth. Spontaneous mutagenesis causes point mutations, which generate mutations with different phenotypes, allowing the functions of the gene to be separated and identifying functional domains of known proteins. As more factors affecting telomere structure and function are identified in fission yeast it will be interesting to study the interactions between different components of the telomere. Factors involved in telomere structure and function would appear not to be constitutively required for vegetative growth in *S. pombe*, as demonstrated by the cumulative senescence of *trt1*Δand *tel1*Δ*rad3*Δ mutants and the failure of the screen described in Chapter 3 to produce temperature sensitive mutants. It is relatively easy to perform genetic manipulations and create double mutant strains that can be analysed from an early stage in model organisms such as *S. pombe*, which allows for the investigation of gene function in a genetically homogeneous background. These are therefore suitable for studying synthetic lethality between different components of the telomere maintenance complex. *S. pombe taz1*$^{-}$ cells have no mitotic phenotype under wild type growth conditions. Upon nitrogen starvation, however the telomeres

form end-to-end fusions in a pKu70p dependent manner (Ferreira and Cooper 2001). It might be possible that creating double mutants between the *rat* strains and another factor such as pKu70 would result in a less variable telomeric phenotype, thus enabling the genes involved to be cloned by conventional methods. Double mutants may have a stronger phenotype than single mutants, and it may be possible to identify temperature sensitive mutants that are more amenable to complementation with genomic libraries than the mutants produced in the screen in Chapter 3.

Insertional mutagenesis may also be used as a method of identifying the *rat* mutants, which are not amenable to complementation with plasmid libraries. A diploid strain containing the *rap1-lot2* mutant was subjected to insertional mutagenesis and colonies selected that had regained the *rap1*-lot2 phenotype. One of the insertion sites generated in this way was shown to be linked to the mutation and cosmids in the area used to try to complement the mutation. One of these cosmids was found to reimpose silencing at the telomere. Open reading frames represented on the cosmid were then knocked out in a strain with a $his3^{+}$ telomeric marker and investigated for alleviation of silencing at the telomere. One of the open reading frames was deemed to be a likely candidate and the gene sequenced revealing the mutation in the *lot2* mutant to be in the *rap1* open reading frame (Nimmo, personal communication)

Five of the six *rat* complementation groups isolated in the *rat* complementation groups and lack of a telomerase RNA template indicate that there are still several genes involved in telomere structure and function in *S. pombe* that have yet to be identified.

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