Long-range Chromatin Domains in Yeast

Claudette Boa

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Declaration

I declare that the work presented in this thesis is my own and that the contribution of others has been clearly indicated.

Claudette Coert

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Abstract

The principal repeating subunit of chromatin is the nucleosome, which consists of a histone octamer complex, around which the DNA fibre coils. Adjacent nucleosomes are separated by short regions of linker DNA, within which a specialised 'linker' histone protein may bind to further compact the DNA. Chromatin organisation in *Saccharomyces cerevisiae* differs from that found in more complex organisms, primarily due to its relatively compact genome, which is made up of about 6000 genes. In yeast the average nucleosome repeat length is only 165bp compared to the 180-200bp linker length seen in higher eukaryotes. Therefore, the majority of the yeast genome does not have sufficient linker length between nucleosomes to enable the binding of 'linker' histones and only areas with increased repeat length will be able to bind these specialised proteins.

The *FLO1* gene locus includes a potentially gene-free 6kb upstream region that is mediated by SWI/SNF and Tup1-Ssn6. These complexes modulate nucleosome binding by altering the histone-DNA interactions and are pivotal to *FLO1* gene regulation (Fleming and Pennings, 2001). Interestingly, the nucleosome array in this region is most regularly spaced when the Tup1p co-repressor is present with an average nucleosome spacing of 180bp. Thus, it constitutes a region of chromatin in the yeast genome that could accommodate linker histones and / or the Tup1p repressor complex, leading to an area of higher-order chromatin compaction.

The causal relationship between Hho1p (yeast 'linker' histone) and Tup1p-Ssn6p binding was investigated in wildtype, *ΔHHO1*, *ΔSNF2*, *ΔSSN6* and *ΔTUP1* mutant

cells by chromatin immunoprecipitation in the *FLO1* upstream region. The 3D proximity of the two Tup1p peaks was investigated using chromatin conformation capture analyses, which showed that the Tup1p sites are closely aligned, except in *ΔSNF2* strains. The change in the conformation of DNA may be influenced by changes in the acetylation of the core histones and / or a chromatin structure which alters the fluidity of the chromatin fibre.

Chapter 1

Introduction

The term 'chromatin" was first coined by W.Flemming (~1880), for "that substance in the cell nucleus which is readily stained" (reviewed by Paweletz, N., 2001). Despite the the discovery of nucleic acids by Miescher (1871), followed shortly by the discovery of histones in 1884 (Kossel), its composition remained unknown for many decades until the breakthrough came in 1953 when Watson and Crick proposed the structure of the DNA double-helix. The discovery of the nucleosome structure, nevertheless, took a further 20 years to eludidate, starting in 1967 when histones were first fractionated by E.W. Johns (reviewed by Olins and Olins, 2003).

1.1 Chromatin

Chromatin is the term used to describe protein-packaged DNA in a eukaryote nucleus. The fundamental structural unit of condensed chromatin is the nucleosome (Oudet *et al.*, 1975). DNA is wrapped onto a histone octamer and can have an additional linker histone molecule associated with it, at its ends (reviewed by van Holde, 1988). Adjacent nucleosome complexes are connected by a length of linker DNA. This has been referred to as the beads-on-a-string conformation, or 10nm fibre, which was first visualised under low-ionic strength (Figure 1.1) by Olins and Olins (1974). The nucleosome crystal

structures were determined to 7.0Å by T. Richmond *et al* (1984) and subsequently to 2.8 Å by K. Luger *et al* (1997) (Figure 1.2).

The 10nm fibre is considered the first level of chromatin condensation (Figure 1.3). This relatively open form of chromatin can be increasingly compacted to form higher order chromatin structures. In the presence of low concentration monovalent ions, nucleosomal arrays appear in a zigzag conformation (Finch and Klug, 1976). Optical laser tweezer experiments suggest a model, where DNA folds in an irregular zigzag pattern with the linker DNA on the inside, forming a 30nm fibre (Dorigo *et al.*, 2004). This model is also supported by a recent study where a tetranucleosome structure was mapped to 9Å and a nucleosome and its associated linker DNA were mapped to 1.9 Å (Schalch *et al.*, 2005). This indicated that the tetramer adopts a zigzag architecture, with two nucleosomes and three segments of linker DNA passing between them (Woodcock, 2005).

At any one time, chromatin in the nucleus can take on two principal forms, either extended euchromatin or extensively condensed heterochromatin (Farkas *et al.*, 2000). Centromeres and telomeres are constitutively in the inactive heterochromatic state (Gilbert and Allan, 2000), while euchromatin is associated with transcriptionally active sequences. Euchromatin is more dynamic and can undergo dramatic changes in compaction.

Yeast chromatin was thought to exist in an constitutively open conformation as 40% of its genome is thought to be active at any one time. However, condensed chromosomes

have been visualised in yeast (Vas et al., 2007), and *in vivo* work by Gasser and colleagues (Bystricky *et al.*, 2004) which examined non-repetitive chromatin domains, suggested that yeast interphase chromatin exists in a compact higher-order conformation with a persistence length of 170-220 nm. This is equivalent to 7-10 nucleosomes per 11 nm within a 30 nm structure. This suggested that compact chromatin structures, such as the 30 nm fibre, occur at transcriptionally competent chromatin in living yeast cells. The authors postulated that this structure could form by default but could possibly be stabilised by a linker histone in yeast.

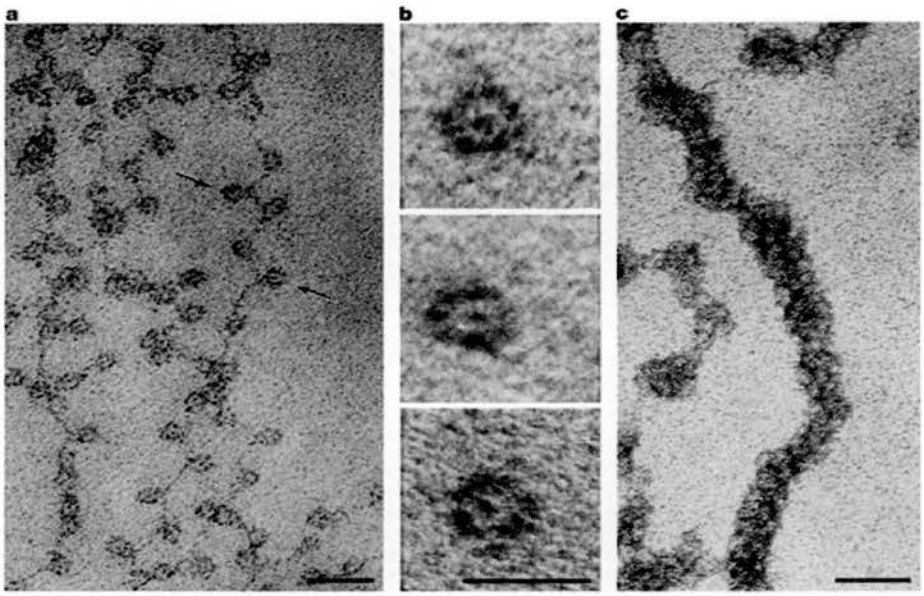

Figure 1.1 Electron micrographs of chromatin. A)Low ionic-strength chromatin: the 'beads on a string'. Size marker: 30 nm. b) Isolated mononucleosomes derived from nuclease-digested chromatin. Size marker: 10 nm. c) Chromatin spread at a moderate ionic strength to maintain the 30-nm higher-order fibre. Size marker: 50 nm. From Olins and Olins, 2003.

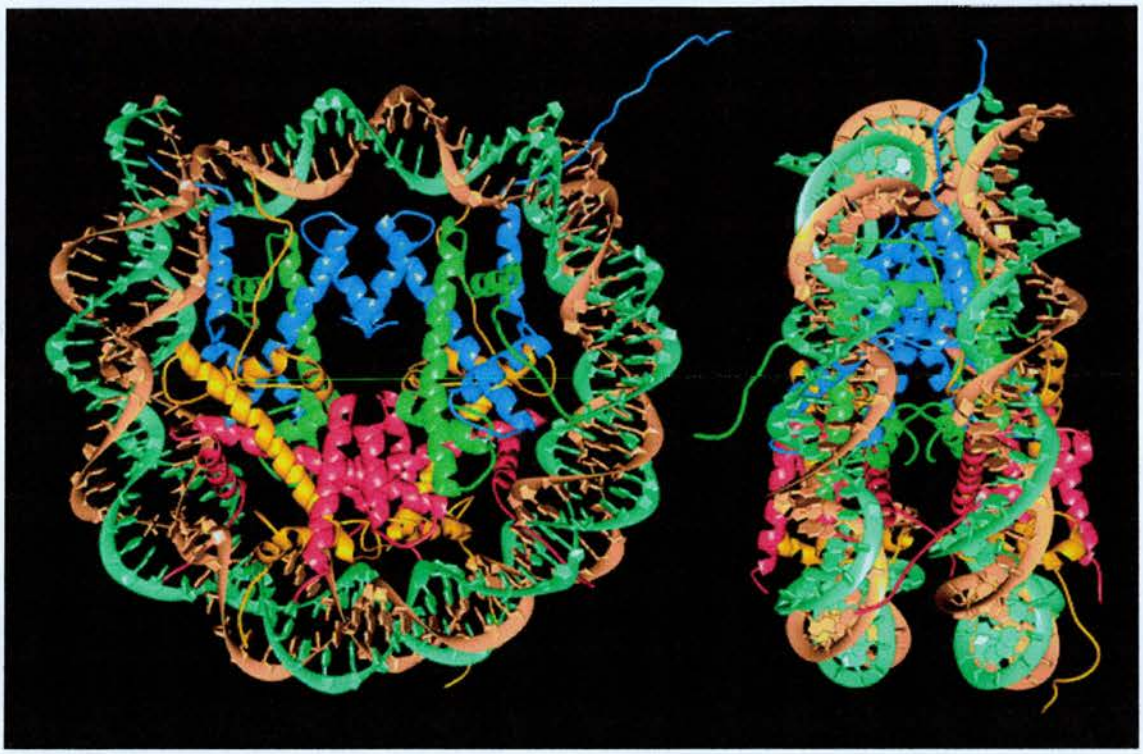

Figure 1.2: Nucleosome core particle: Ribbon traces for the 146-bp DNA phosphodiester backbones (brown and turquoise) and eight histone protein main chains (blue: H3; green: H4; yellow: H2A; red: H2B. From Luger *et al.*, 1997.

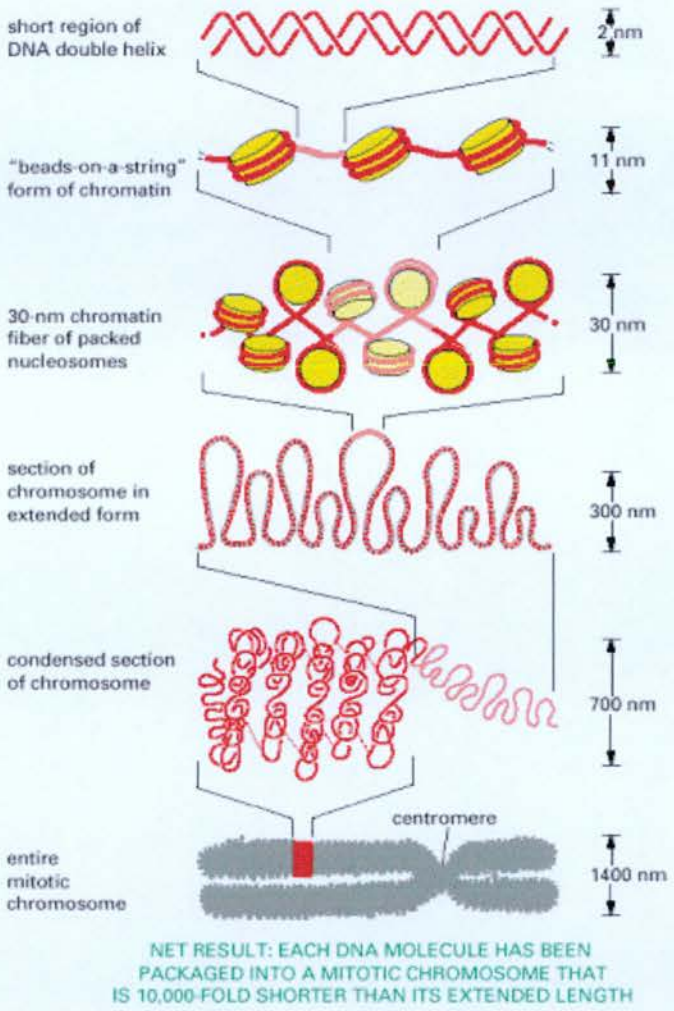

Figure 1.3. Chromatin folding.

This model illustrates the several levels of packaging of DNA which are postulated to give rise to the highly condensed mitotic chromosome. (Reproduced from Alberts *et al*).

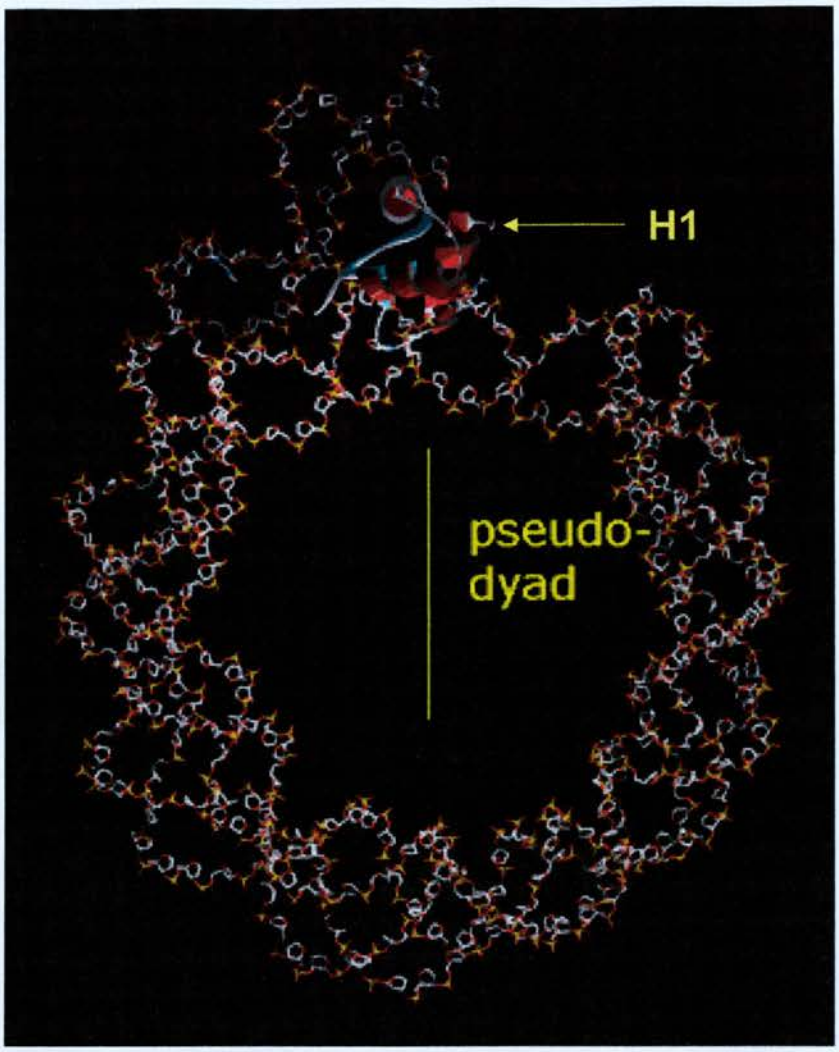

Figure 1.4 Binding of the globular domain of H1 to the nucleosome core.

Histone H1 binds to DNA at the terminal helical turn of the nucleosomal DNA, at a position close to the pseudo-dyad axis. Taken from Ramakrishnan *et al.*, 1993.

1.2 Histones

The histones are a family of basic proteins involved in organising the DNA in the nuclei of eukaryotic cells. There are 5 major classes of histones, the core histones H2A, H2B, H3, and H4 and the linker histone H1 (reviewed in Wolffe, 1999). The histone octamer consists of two molecules of each of the core histones. Two molecules of histones H3 and H4 associate as a tetramer, to which two H2A-H2B heterodimers bind (Figure 1.2). Histone H1 binds to DNA at the terminal helical turn of the nucleosomal DNA, at a position close to the pseudo-dyad axis (Figure 1.4) (Zhou *et al.*, 1998; reviewed by Kornberg and Lorch, 1999).

Not all eukaryotic cells utilise histones for packaging their DNA. Dinoflagellates utilise small basic proteins to package their DNA (Vernet *et al.*, 1990), while in most mammalian species, the DNA in spermatozoa is compacted by basic proteins known as protamines (Poccia, 1986) These arginine-rich proteins bind to DNA with high affinity (Ausio *et al.*, 1984), causing maximal compaction of the genome (reviewed in Eirín-López *et al.*, 2006). Evidence exists that vertebrate protamines have evolved from histone H1 (Lewis *et al.*, 2003). This change occurred rapidly when a frame-shift mutation appeared in a spermatozoa-specific histone H1 gene. It converted a lysine-rich H1 to arginine-rich protamine. In addition, a putative *Drosophila* protamine-like protein similar to histone H5, has also been identified in screens of transcripts expressed in the male germ line (Russell and Kaiser, 1996), supporting the theory that protamines were derived from linker histones.

1.2.1 The Core Histones

Core histones are highly conserved between species ranging from yeast to human (De Lange *et al.*, 1969). These small basic proteins contain high molar percentages of lysine and arginine (van Holde, 1988). In the histone octamer these residues lie on a ramp forming a left-handed protein superhelix matching that of the DNA in the core particle (Klug *et al.*, 1980). The $[H3\text{-}H4]_2$ tetramer lies at the centre of this superhelix, with an H2A-H2B dimer at either end of the path. Therefore, the nucleosome has evolved an optimal electrostatic charge alignment to facilitate DNA binding.

The core histones all assume a similar structure with a basic N-terminal tail, a globular domain organised as a histone fold, and a C-terminal tail. The central histone-fold domains of the core histones possess a high level of structural homology. The domain is formed through the interaction of three α-helices connected by two loops. The interaction of two histone folds generates a handshake motif between two different histone proteins, forming a hetero-dimer between H3 and H4, and also between H2A and H2B. It is the histone fold domains of the four core histones that mediate histone-histone and histone-DNA interactions (Luger *et al.*, 1997).

The unstructured amino termini of the histones, consisting of 15-30 residues, are referred to as the histone tails. The N-terminal tail regions of H3 and H2B have random coil segments that pass between the DNA gyres, while the N-terminal domain of H2A passes over the DNA along a minor groove. The amino acid base interaction within this groove may be important in modulating the nucleosomal association with particular DNA

sequences (Luger *et al.*, 1997). The H4 N-terminal tails appear to form divergent structures, though this might be a limitation of the crystallisation techniques used to visualise the histones.

The histone tails are subjected to various post-translational modifications (Figure 1.5; Grunstein, 1997), including methylation of lysine or arginine, phosphorylation of serine or threonine, acetylation of lysine, ubiquitination of lysine, sumoylation of lysine, ADP-ribosylation, biotinylation, glycosylation and carbonylation (reviewed in Margueron *et al.*, 2005). These may modulate chromatin structure and serve as signals for interactions with other proteins (Strahl and Allis, 2000), e.g. mitotic chromosome condensation is influenced by histone modification as H3S10 phosphorylation is needed for this to occur. In the interest of this study, only acetylation will be discussed in greater detail.

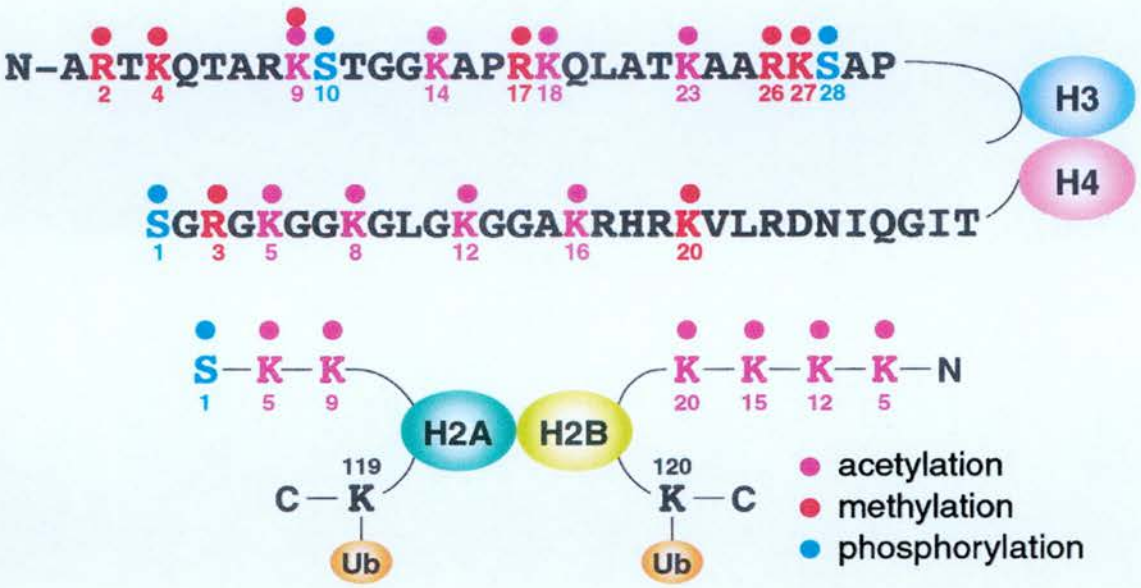

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Figure 1.5 Post-translational modifications on core histone tails. Arginine/Lysine methylation is indicated in red; Lysine acetylation is indicated in purple; serine phosphorylation is indicated in blue; lysine ubiquitination is indicated in orange.

1.3 Histone Acetylation

Lysine acetylation in the nucleosome plays a fundamental role in chromatin regulation (Figure 1.6; reviewed by Kurdistani and Grunstein, 2003). Acetylation can modulate histone deposition, transcription and DNA repair through the recruitment of proteins that bind acetyl lysine i.e. those with a bromodomain. Hyper-acetylation of the histone tail may also decrease the histone-DNA interactions (Hong *et al.*, 1993), and cause DNA to be more accessible to transcription factors.

A direct correlation between histone acetylation status and gene activity has been demonstrated (Hebbes *et al.*, 1988). The acetylation of the lysine residues of the core histone tails is associated with actively transcribing DNA. This may be caused by a more open chromatin structure, allowing transcription factors to access the regulatory regions of genes. Conversely, deacetylation can result in less accessibility for transcription factors due to a more compact chromatin structure. The enzymes that modulate the histone acetylation are known as histone acetyltransferases (HATs) and histone deacetylases (HDACs).

The histone acetyl transferase (HAT) domain acetylates lysines (reviewed in Roth *et al.*, 2001). HAT domains, which contain a central acetyl co-enzyme A binding site, are found in large complexes. An example is the Gcn5 HAT which is found in SAGA (reviewed in Marmorstein, 2001), and which is able to acetylate H3 and H2B *in vivo*. Sequential acetylation of a subset of lysines in histones H3 and H4 by Gcn5 HAT leads to the recruitment of bromodomain-containing transcription factors. This domain preferentially

recognises the acetylated lysine residues on N-terminal histone tails (Zeng and Zhou, 2002; Agalioti *et al.*, 2002). An example is the acetylation of H4K8 which causes TFIID to be recruited (Robert *et al*, 2004).

Although yeast contains on average 13 acetylated lysines per nucleosome, hinting at its very active genome (Waterborg, 2000), deacetylation is equally important in gene regulation. Lysine acetylation is reversed by histone deacetylases (HDACs) eg. Sir2p (Armstrong *et al.*, 2002), Hda1p and Rpd3p (Kurdistani *et al.*, 2002). HDAC activity has been shown to mediate gene silencing (Hassig *et al.*, 1997; Kadosh and Struhl, 1998). Moreover, hypoacetylation of a coding region can cause inhibition of transcription (Kristjuhan *et al.*, 2002). Conversely, *RPD3/HDA1* disruptions lead to increased acetylation at both the promoter and coding region of the *PHO5* acid phosphatase gene, leading to its activation (Vogelauer *et al.*, 2000). Similarly, in *rpd3* mutants, the acetylation of either H3 or H4 amino termini on genes which are normally repressed by Rpd3p, is sufficient for gene activation (Sabet *et al.*, 2004).

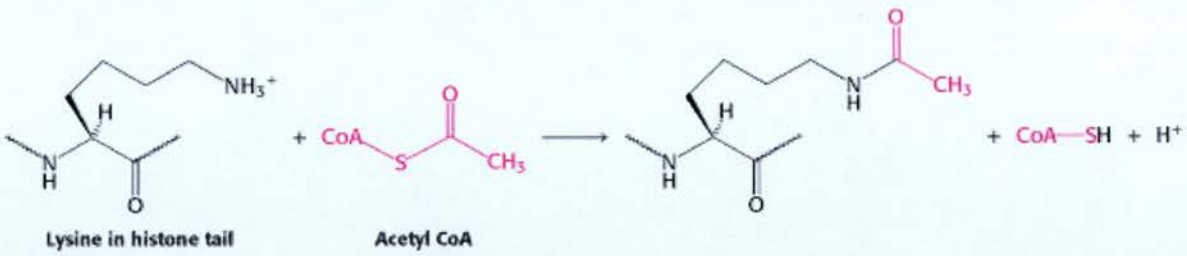

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Figure 1.6 Mechanism of acetylation of the lysine residues on core histone tails. Histone acetyltransferases (HATs) transfer an acetyl group from acetyl coA to the positively charged ammonium group on specific lysine residues on the amino-tail of histone H3 or H4, generating an uncharged amide group, which has less affinity for DNA.

1.4 Histone variants

In addition to histone modifications, core and linker histones can be substituted by variants (Figure 1.7), which alter the nucleosome structure or chromatin architecture (Reviewed in Sarma and Reinberg, 2005). Variant histones assemble at specific sites, mediated by specific chromatin complexes (reviewed in Henikoff *et al.*, 2004), allowing particular gene regions to associate with alternative proteins involved in gene regulation (Malik and Henikoff, 2003).

The H3 variant: centromeric protein A (CENP-A) occurs in mammalian centromeres (Palmer *et al.*, 1991), and is essential for centromere structure and function. It occurs in concert with H2A, H2B and H4 in centromeric nucleosomes (Reviewed by Ahmad and Henikoff, 2005). The N-terminal tail of CENP-A is different from canonical H3, and allows alternative histone modifications to occur, necessary for centromeric function. Another significant histone H3 variant, H3.3 is present at actively transcribing genes in *Drosophila* and accounts for approximately 25% of the total H3 present in a cell (McKittrick *et al.*, 2005).

Histone H2A also has many variants. For instance, H2A.Z modifies the accessible surface area of the nucleosome (Suto *et al.*, 2000) and regulates silencing of a subset of genes. In yeast, the Swr1p complex is needed for the exchange of H2A-H2B for H2A.Z-H2B (Meneghini *et al.*, 2003). Steric hindrance prevents H2A.Z and H2A co-existing on the same nucleosome (Suto *et al.*, 2000). Variant H2A.X has a C-terminal extension whose phosphorylation state is important for DNA repair by non-homologous end-joining

(Rogakou *et al.*, 1998). Notably, H2A.X is the major H2A variant in *S. cerevisiae*. It is phosphorylated after the formation of double strand breaks and facilitates the stable accumulation of repair proteins at damaged foci (Tsukuda *et al.*, 2005). The macro-H2A variant, which has a C-terminal 2kDa non-histone fold addition, is substituted in the inactive X-chromosome in female mammals (Okamoto *et al.*, 2004). Therefore, H2A variants are pivotal in such diverse events as gene silencing, DNA repair and chromosome segregation.

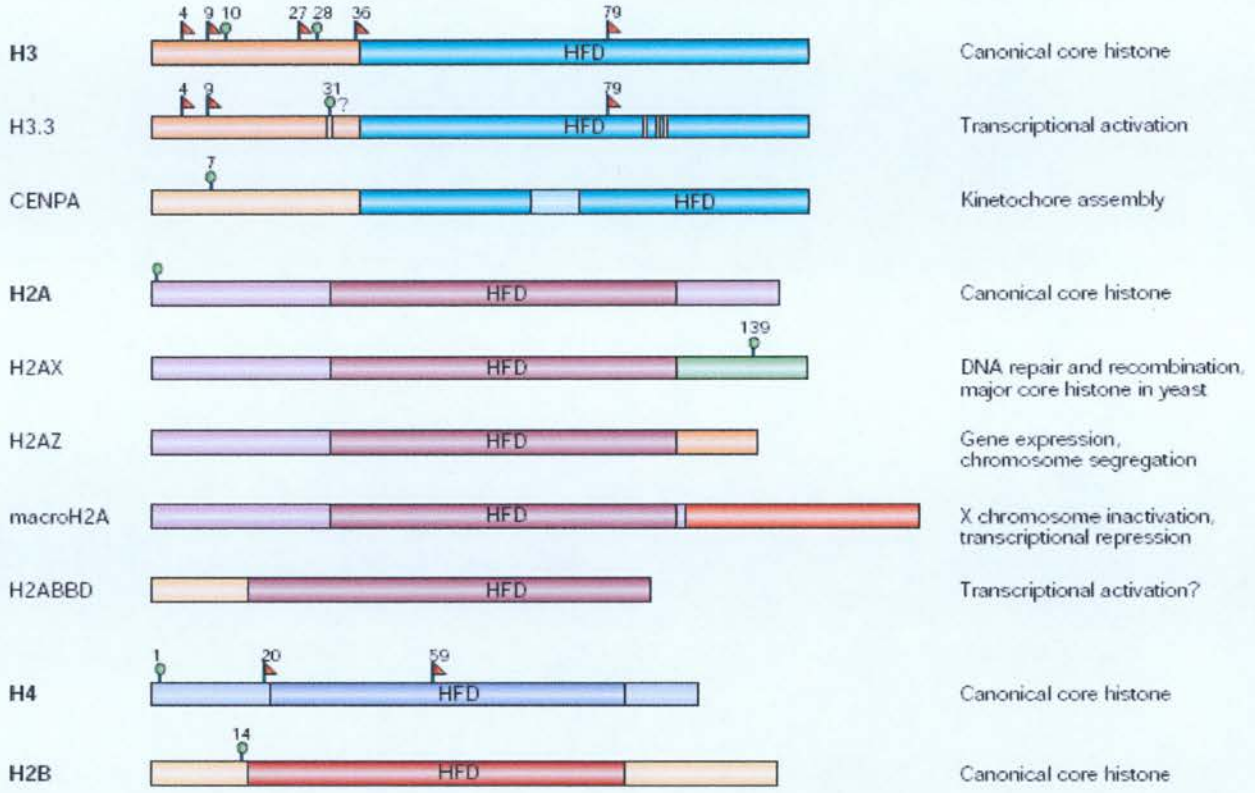

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Figure 1.7 Histone variants have diverse functions. Deposition of specific histone variants occur during specific cellular processes and have varying functions. Lysine methylation is denoted by the red flags, while serine phosphorylation is denoted by the green circles.

1.5 Linker Histones

Linker histones are the last component to be added in nucleosome assembly. They do not possess a histone-fold domain and are unrelated to core histones. They are less conserved when compared to core histones, and tissue-specific subtypes of H1 are present in many organisms. An example is the chicken erythrocyte-specific linker histone, H5, the object of many chromatin studies, which is related to H1o, the terminal differentiation variant.

Trypsin studies (reviewed by Bohm and Crane-Robinson, 1984) revealed that H1 has a tripartite structure, consisting of a central globular core and lysine rich N- and C-terminal domains (tails). The globular domain is made up of 3 α-helices terminating in a 3-stranded β-sheet (Ramakrishnan *et al.*, 1993). The globular domain appears to bind to one DNA strand as it enters or exits the nucleosome, as well as to the DNA near the dyad axis of symmetry of the nucleosome (Zhou *et al.*, 1998). These two proposed DNA-binding sites are required for the appearance of a chromatosome stop (with micrococcal nuclease digestion), which may indicate a requirement for correct positioning of the linker histone on the nucleosome (Goytisolo *et al.*, 1996). The highly basic linker histone tails interact with DNA between nucleosomes and partially neutralise negative charges on linker DNA (Clark and Kimura, 1990). The C-terminal domain has been shown to facilitate high affinity binding to chromatin fibres *in vivo* (Hendzel *et al.*, 2004). Furthermore, the C-terminal domain has also been found to mediate linker histone binding to nucleosomal arrays *in vitro* (Lu and Hansen, 2004).

Not all histones in the H1 family exhibit a tripartite structure. The ciliated protozoan *Tetrahymena* H1 lacks the central globular domain (Wu *et al.*, 1986), while the yeast *Saccharomyces cerevisiae* H1, contains two globular domains separated by a lysine-rich linker region (Ushinksy *et al.*, 1997).

Linker histones exhibit a binding preference for supercoiled, rather than relaxed DNA, as well as for AT-rich regions (Wolffe and Brown, 1987). It is generally accepted that H1 binds less tightly than other histones to DNA in chromatin (Wu *et al.*, 1986) and can readily exchange for other H1 proteins *in vivo* (Thomas and Rees, 1983). Exchange of histone H1 is rapid in both condensed and decondensed chromatin and occurs throughout the cell cycle (Lever *et al.*, 2000). This facet of H1 binding is thought to be an important step in modulating transcription (Shen and Gorovsky, 1996). Lever and colleagues (Lever *et al.*, 2000) have shown that H1-GFP is exchanged continuously within chromatin regions, with an average residence time of several minutes in both euchromatin and heterochromatin. Similarly, Misteli and colleagues (Misteli *et al.*, 2000) have shown using H1-GFP, that linker histones bind dynamically to chromatin in a human cell line. After hyperacetylation of core histones, the residence time of H1-GFP is reduced. This suggests a higher rate of exchange upon core histone modification, as a result of weaker binding. The dynamic nature of H1 binding is an essential feature of linker histones in relation to their function as regulators of chromatin remodelling and chromatin structure *in vivo*.

1.5.1 Linker Histone modifications

The major post-translational modification of histone H1 involves cdc2 kinase catalysed phosphorylation, targeted to serine (Ser) and threonine (Thr) residues on the N- and C-terminal domains (Spencer and Davie, 1999). In human cells, phosphorylation patterns differ according to the particular stage of the cell cycle, with the highest levels occurring during M-phase, when additional phosphorylation events take place at Thr residues. Furthermore, specific Ser/Thr kinases seem to be responsible for phosphorylation at different stages of the cell cycle (Sarg *et al.*, 2006).

The dynamic mobility of H1 is partly mediated by cyclin/cdk phosphorylation (Contreras *et* al., 2003). The C-terminal domain of H1 is able to bind to the hinge domain of HP1α, an important modulator of chromatin function. *CDK2* regulates the binding of these proteins by phosphorylation, which causes the proteins to disassociate (Hale *et al.,* 2006). Phosphorylation of H1 might therefore destabilise chromatin interactions, thereby relaxing chromatin.

The mechanism by which H1 phosphorylation affects transcription was investigated using *Tetrahymena* (Dou and Gorovsky, 2000). *Tetrahymena* strains with H1 mutations that mimick the charge of the phosphorylated region, without mimicking structure or hydrophillicity of the phosphorylated residues, were created. A charge patch, which interacts with DNA, is formed by residues dispersed throughout the H1 molecule, (Dou and Gorovsky, 2002). Phosphorylation of H1 acts by changing the overall charge of a small domain. This directly weakens the interaction of the basic tails of H1 with DNA,

thereby destabilising the chromatin fibre (Hill *et al.*, 1991). This transient chromatin decondensation allows DNA binding factors to bind (Thomas, 1999). These proteins may control DNA packaging and/or functional activities (Roth and Allis, 1992). Phosphorylation is cell-cycle dependent, with the highest level of phosphorylation occurring at metaphase during mitosis, which is when the chromosomes are most condensed. This has led to the argument that a causal relationship exists between H1 phosphorylation and chromatin compaction (Bradbury *et al.*, 1974; Bradbury, 1992).

There are currently two opposing models explaining the role of H1 phosphorylation in chromatin condensation (Figure 1.8). In the model proposed by Bradbury and colleagues (Bradbury, 1992), non-phosphorylated H1 is present in decondensed chromatin during interphase and is loosely bound to DNA through interactions involving amino- and carboxy-terminal tails, as well as the central globular domains. Upon phosphorylation of the H1 tails, the H1-DNA interactions are proposed to weaken, making way for H1-H1 interactions, which enable the formation of higher-order chromatin structures.

In the Roth and Allis model of 1992, the negative charge from linker DNA phosphates is shielded in condensed chromatin by the positively charged, non-phosphorylated tails of H1. Phosphorylation of the H1 tails increases the negative charge in the H1 molecule, thereby weakening H1-DNA interactions and causing a repulsion of adjacent fibres and subsequent decondensation of chromatin. This affirms the idea that chromatin folding *in vitro* is largely electrostatic in nature and is governed by repulsion between DNA regions that are reduced upon H1 binding. This decondensation might enable other proteins (e.g.

HMG proteins), involved in higher order DNA condensation, to interact with the DNA fibre, thereby facilitating mitotic chromatin condensation.

Interphase phosphorylation of histone H1 is correlated with the transcriptionally active states of chromatin (Roth and Allis, 1992; Lee and Archer, 1998). Several studies have shown an involvement of linker histone phosphorylation in gene transcriptional activation (reviewed in Spencer and Davie, 1999). H1 phosphorylation is essential for rapid gene activation of the mouse mammary tumour virus (MMTV) long terminal repeat promoter by the glucocorticoid receptor in response to hormone binding (Lee and Archer, 1998). In the absence of glucocorticoid, the MMTV promoter is incorporated into six regularly positioned nucleosomes (Richard-Foy and Hager, 1987). This closed chromatin structure prevents the binding of activators to the promoter, thus inhibiting transcription (Archer *et al.*, 1992). Glucocorticoid exposure rapidly disrupts the local chromatin structure, recruiting transcription factors and inducing activation of the gene (Lee and Archer, 1994). Phosphorylation of the H1 tails is a prerequisite for the partial H1 loss and nucleosome disruption at this promoter. The promoter is silenced by the dephosphorylation of histone H1.

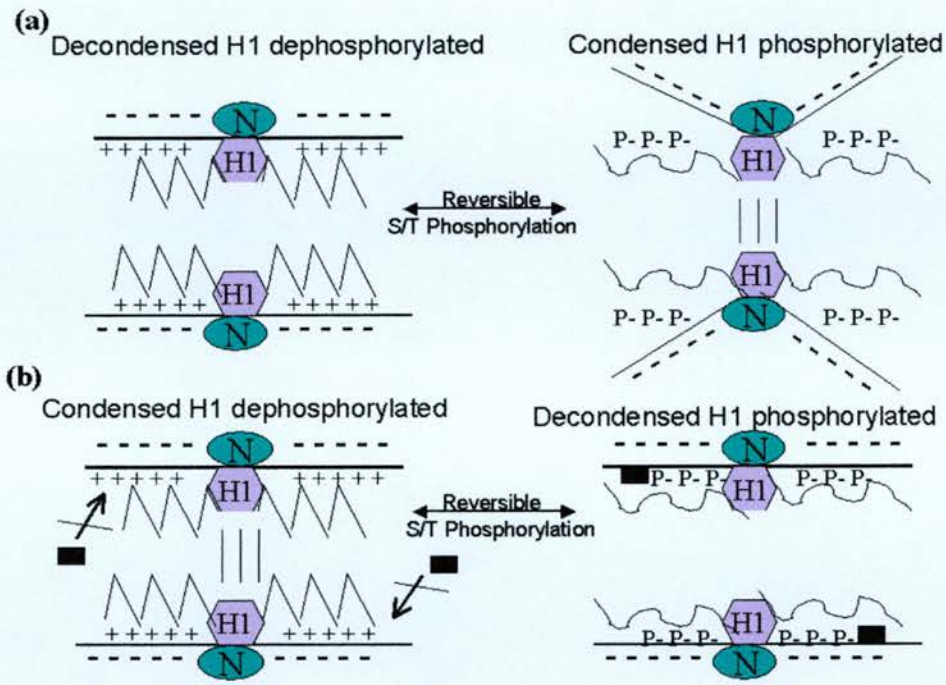

Figure 1.8 Models for the involvement of H1 phosphorylation in chromatin condensation. a) Bradbury model, 1974. Dephosphorylated H1 has a stronger interaction with DNA than with other H1s, favouring decondensation of chromatin. Once phosphorylated, H1 becomes less tightly bound to DNA, and favours H1-H1 interactions instead. Interactions between H1 molecules cause chromatin condensation. b) Roth/Allis model, 1992. Positive charge of the lysine rich tails of H1 enables chromatin condensation. Phosphorylation causes an increased negative charge of the H1 tails, and repulsion of the chromatin fibres, resulting in decondensation. Adapted from Roth and Allis, 1992.

H1 phosphorylation was shown to regulate specific gene expression *in vivo* by mimicking the partial removal of H1 (Dou *et al.,* 1999); Dou and Gorovsky, 2000). Phosphorylation of macronuclear H1 in *Tetrahymena* is nevertheless not essential for viability (Mizzen *et al.*, 1999). However, H1 phosphorylation was found to occur in response to starvation in *Tetrahymena thermophila*, thereby regulating the expression of specific genes (Dou *et al.*, 2005).

A lesser characterised modification of H1 is ubiquitination. $TAF_{II}250$ not only has acetyltransferase activity, but also has histone-ubiquitination activity (Pham and Sauer, 2000). *Drosophila* $TAF_{II}250$ can mediate the mono-ubiquitination of histone H1 *in vitro*. Since $TAF_{II}250$ is recruited to promoters, ubiquitination of histone H1 in eukaryotes may regulate chromosomal gene activity in a promoter specific manner (Wu and Grunstein, 2000).

1.5.2 Linker histone functions

Linker histones in various organisms function in very diverse ways (reviewed in Harvey and Downs, 2004). Histone H1 has long been thought to be a general repressor that ensured a strong and stable repression of tissue-specific genes (Weintraub, 1985). This was based on the premise that gene expression may be dominated by the higher-order structure of chromatin and that H1 was involved in the formation and maintenance of these structures. However, linker histones have been shown to contribute a large variety of functions.

In eukaryotes, transcriptional repression correlates, in general, with chromatin condensation. This can either be domain-wide or local, encompassing, in the latter case, just a few nucleosomes (Travers, 1999). For instance, histone H1 selectively represses the transcription of 5S rRNA genes (O'Neill *et al.*, 1995; Nightingale and Wolffe, 1995). During transcriptional repression, linker histones may also prevent access of transcription factors and chromatin remodelling complexes to DNA (Strahl and Allis, 2000). They have been found to specifically repress core histone acetylation, *in vitro*, possibly by hindering histone acetyltransferase binding (Herrera *et al.*, 2000).

A variety of structural roles have been attributed to linker histones, including locking the two DNA turns within a nucleosome, setting the inter-nucleosome spacing, and facilitation of folding of the 10nm nucleosome-containing fibre into higher order structures (Garrard, 1991; Zlatanova and Van Holde, 1992). Linker histones play a critical role in maintaining the structure of the 30nm fibre (Thoma *et al.*, 1979; Allan *et al.*, 1981). It has also been suggested that linker histones play a role in levels of chromatin structure beyond that found in the 30nm fibre (Weintraub, 1985). The globular domain of H1 and either the H1 or H3 tails are needed to stabilise the 3-D arrangement of nucleosomes (Zlatanova *et al.*, 1998). Linker histones have also been found to inhibit the mobility of positioned nucleosomes (Pennings *et al.*, 1994). However, the presence of H1 in a cell is not sufficient to condense the genome, in order for mitosis to occur (Ohsumi *et al.*, 1993).

Transcription factors that share structural features with histones or HMG proteins are able to replace H1. *HNF3*, which contains a winged-helix motif similar to that found in histone H1, can replace H1 in the chromatin of the mouse serum albumen enhancer (Cirillo *et al.*, 1998). Competition has also been shown to exist between H1 and HMGN proteins for chromatin sites (Catez *et al.*, 2002).

In yeast, linker histones seem to play a role in transcriptional regulation on a subset of genes rather than being responsible for global gene regulation (Hellauer *et al.*, 2001) as predicted by their abundance and biochemical properties. Early work on *Saccharomyces* chromatin, involving sea-urchin H1 under the influence of an inducible *GAL1* promoter, showed that transcriptional levels were affected on a global scale (Linder and Thoma, 1994) when H1 was over-expressed. However, this study was performed under the assumption that yeast did not possess a linker histone, and therefore adding too much H1 from another species, probably caused improper compaction of the genome, thereby affecting global transcription levels.

In stark contrast to core histones, linker histones are not necessary for viability in many systems (reviewed in Ausio, 2000). Unicellular eukaryotes generated with non-expressing linker histones are viable and show very few phenotypic changes e.g. *Tetrahymena* (Shen and Gorovsky, 1996), *Saccharomyces cerevisiae* (Patterton *et al.*, 1998), *Aspergillus nidulans* (Ramon *et al.*, 2000) and *Neurospora crassa* (Folco *et al.*, 2003). These strains were still able to undergo mitosis, transcribe essential genes and replicate their genomes successfully.

Tetrahymena lacking linker histones show no difference in vegetative growth, general transcription, protein synthesis and global nucleosome repeat length, but do show a small increase in nuclear volume size and less efficient meiosis (Shen *et al.*, 1995; Shen and Gorovsky, 1996; Karrer and VanNuland, 1999; Karrer and VanNuland, 2002).

H1-depleted A*scobolus immersus* show no differences in methylation-associated gene silencing, meiosis, germination or growth (Barra *et al.*, 2000). The authors did, however, notice a decrease in longevity, though this was probably due to mis-regulation of a subset of genes that happened to include longevity genes. *A. nidulans* with a deletion in one H1-encoding gene show no changes in gross nuclear morphology, growth, sexual reproduction, bulk nucleosomal repeat lengths or UV and DMSO resistance (Ramon *et al.*, 2000). *Neurospora crassa* linker histone, hH1, deletion shows no defects in morphology, DNA methylation, mutagen sensitivity, DNA repair, fertility, chromosome pairing or chromosome segregation (Folco *et al.*, 2003). However, expression of pyruvate decarboxylase gene expression was affected.

1.5.3 Linker histone variants

Linker histone levels can be important in development in multicellular animals and plants. In most multicellular organisms, a variety of H1 variants occur. The structures of these vary greatly during early stages in development concomitant with zygotic gene activation, and specific variants are therefore associated with functional changes. Redundancy might occur as in mice the deletion of single H1 subtypes causes no detectable phenotype (Fan *et al.*, 2001), whereas mice lacking three H1 subtypes (H1c,

H1d and H1e) are not viable and die by mid-gestation with a variety of phenotypic abnormalities, as well as a shortening of the spacing between nucleosomes (Fan *et al.*, 2003).

When these subtypes were deleted in mouse embryonic stem cells, dramatic changes in chromatin structure occurred (Fan *et al.*, 2005). The most marked change was a decrease in nucleosome repeat length from ~189 bp to ~174 bp. This was accompanied by differences in the levels of two key histone modifications in the linker histone-depleted nuclei: 4-fold reduction in H4K12 acetylation and a 2-fold reduction in H3K12 trimethylation. The reduced H4K12 acetylation and reduced nucleosome repeat length helps to compensate for H1 loss by increasing the neutralisation of DNA negative charges, thereby creating more compact chromatin. Microarray analyses showed only a small subset of differentially expressed genes. Interestingly, these genes were either imprinted or expressed on the X chromosome and their promoters contained CpG islands that were sensitive to DNA methylation. Therefore, linker histone variant depletion, though not altering the global DNA methylation pattern, does affect the DNA methylation pattern of specific loci, thereby altering their gene expression.

Further studies suggested that the preferential arrangement of linker histone subtypes on the chromatin fibre could play a role in contorting the fibre into a higher-order structure that altered gene expression (Alami *et al.*, 2003). In *Caenorabditis elegans*, a single H1 variant (H1.1), is essential for silencing genes important for the proliferation and differentiation of the hermaphrodite germ-line (Jedrusik and Schulze, 2003).

Interestingly, this variant is able to produce telomeric position-effect variegation when placed in *S. cerevisiae*, which suggests a relationship between germ-line and telomeric silencing.

In early *Xenopus* embryos maternally expressed histone B4 is the only linker histone found in eggs and is replaced by somatic histone H1 subtypes after the midblastula transition, when zygotic gene activation occurs (reviewed in Wolffe *et al.*, 1997). Linker histone B4 has been shown to allow chromatin remodelling to occur at specific loci, whereas somatic histone H1 prevents linker DNA from being accessed by chromatin remodelling factors at these regions (Saeki *et al.*, 2005).

It has been shown that human linker histones affect SWI-SNF ATP dependent remodelling (discussed in 1.8.2) *in vitro* (Hill and Imbalzano, 2000; Ramachandran *et al.*, 2003).Histone H1b cooperates with *MSX1* for transcription and myogenesis in embryonic eukaryotic cells. *MSX1*, a member of the Msx homeoprotein family of transcription factors, is responsible for differentiation of skeletal muscle in embryogenesis (Lee *et al.*, 2004). A role for linker histones in DNA double-strand break repair responses was also found in rodents (Konishi *et al.*, 2003). Here it was found that H1.2 was released into the cytosol, along with other linker histone variants, as a response to high doses of UV radiation, which caused cytochrome c to be released from mitochondria and ultimately caused cells to apoptose. Linker histones might therefore signal the state of genome integrity to cytosolic factors capable of inducing apoptosis.

Linker histones also have a role in replication. A lack of H1 in *Xenopus* embryos causes a lack of origin specificity, whereas an addition of H1 to *Xenopus* egg extract reduces the frequency of initiation events (Lu *et al.*, 1998). The findings that linker histones are implicated in ageing (Barra *et al.*, 2000) and DNA repair (Downs *et al.*, 2003) further suggest an important function in maintaining genomic integrity.

1.5.4 Linker histone in yeast

The existence of a linker histone in yeast had been controversial, as no histone H1 had been isolated from yeast; and it was considered unnecessary, as a large component of the yeast genome is transcriptionally active, compared to higher eukaryotes (Davie *et al.*, 1981). Early work by Linder and Thoma (1994), where sea urchin histone H1 was expressed in *S. cerevisiae*, showed that when this histone was bound to DNA, transcription, growth and viability were affected. This suggested that hypercondensation in yeast was undesirable, and that yeast H1 interacted with DNA in a different way. Since yeast possesses on average shorter nucleosomal spacing, 165bp compared to 200bp in higher eukaryotes, it was possible that an open chromatin structure was favoured, and that there was no requirement for neutralization of the linker DNA charge. However, partial purification of yeast chromatin showed the existence of a 30nm fibre higher order structure, suggesting that a protein fulfilling the role of a linker histone was present in yeast (Lowary and Widom, 1989; Bash and Lohr, 2001).

Yeast H1 was first detected by immunological techniques using anti-mouse H1 antibodies (Smith *et al* 1984; Srebreva *et al.,* 1987). The complete sequencing of the

yeast genome in 1996 (Goffeau *et al.*, 1996) ultimately revealed an open reading frame encoding a candidate H1, which was different to the normal tripartite H1, but had homology to the globular domain of known H1s in other organisms (Landsman, 1996; Ushinsky *et al.*, 1997). There is only one copy of this gene in the yeast genome (Ushinsky *et al.*, 1997), which was designated *HHO1*, after **h**istone **H** **o**ne.

HHO1 encodes a protein 258 amino acids in length with a predicted molecular weight of ~28 kDa. Hho1p differs structurally from canonical H1s in that it has two globular domains of about 80 residues, with a basic amino-terminal extension and a basic, lysine-rich linker region (38 residues), which connects the two globular domains (Figure 1.9; Figure 1.10) (Landsman, 1996). The linker region shows homology to the C-terminal tails of other histones, since it contains 12 lysines, 10 alanines and four prolines out of 42 residues. The first globular domain forms a winged-helix domain in 10 mM sodium phosphate pH 7, while the second globular domain was largely unstructured under these conditions (Ono *et al.*, 2003; Ali *et al.*, 2004). The second globular domain is able to form a winged-helix fold at high concentrations of large tetrahedral anions, such as phosphate, sulphate and perchlorate (Ali *et al.*, 2004). This might mimic the charge-screening effects of DNA phosphate groups, suggesting the possibility that the molecule folds only on contact with DNA. The second globular domain is also able to bind to DNA and 4-way junction DNA but, unlike the canonical linker histone globular domains, it does not produce a chromatosome stop (Ali and Thomas, 2004).

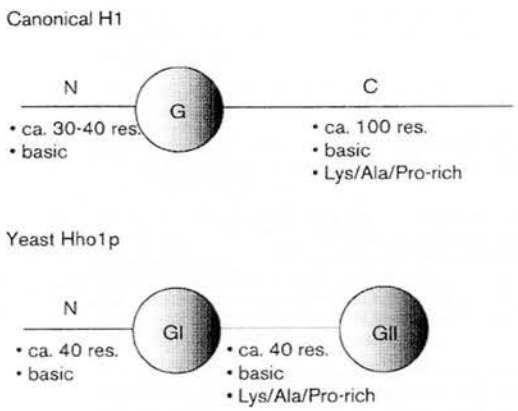

Figure 1.9 Comparison of the putative yeast H1 with the canonical H1.
Domain organisation. The two homologous domains (GI and GII) of Hho1 p, which are also homologous to the central globular domain (G) of a typical H1, are connected by a basic linker, which shows some similarity to the much longer carboxy-terminal basic domain (C) of H1. Both H1 and Hho1p have a basic amino-terminal domain (N). Reproduced from Thomas, 2000.

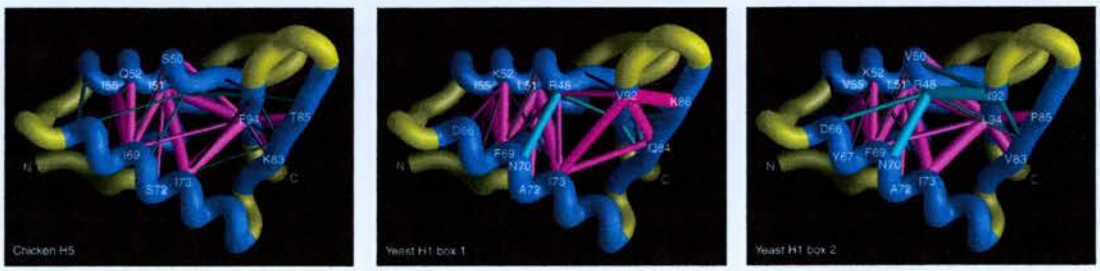

Fig. 1.10. Energy scaffolds for query sequences through the structure of chicken H1/H5. The alpha-carbon backbone of the protein is depicted as a curving "worm". Core segments of H5 are shown in blue, while the intervening loop regions are shown in yellow. Pairwise residue interaction energies between core residues are indicated by the thickness and coloring of the rods connecting alpha carbon positions on the protein backbone. Thick, magenta-colored cylinders indicate the most favourable interactions; thick, cyan-colored cylinders indicate the least favourable interactions. Residue numbering corresponds to the numbering in the multiple sequence alignment. Scaffolds were generated using the graphics program GRASP. *Left*, chicken H5; *Middle*, yeast H1 box 1; *Right*, yeast H1 box 2. Reproduced from Baxevanis and Landsman, 1998.

Using Hho1p-GFP fusion proteins, Hho1p has been shown to localize in the nucleus in close proximity to DNA (Ushinsky *et al.*, 1997). Recombinant Hho1p displayed electrophoretic and chromatographic properties similar to that of the linker histones and forms a stable ternary complex with a reconstituted dinucleosome core *in vitro* with molar rHho1p: core histone ratios of up to 1 (Patterton *et al.*, 1998). Deletion of the *HHO1* gene has little effect on telomeric silencing, basal transcriptional repression, or gene activation at a distance and does not affect nucleosome repeat length (Patterton *et al*, 1998; Escher and Schaffner, 1997). Indeed, although biochemical studies confirm its role as a linker histone in yeast, it is not clear whether the protein functions globally as a true H1, or whether it performs the role of H1 on a particular subset of genes in yeast. This view was supported by a microarray analysis of the *S. cerevisiae* genome, which demonstrated that in the absence of *HHO1,* only 27 genes had their expression altered by a factor of two or more (Hellauer *et al.*, 2001). The affected genes had reduced expression in the absence of *HHO1*, suggesting that yeast linker histone acts as an activator of transcription rather than a repressor. Hho1p was found to preferentially bind to rDNA sequences *in vivo* (Freidkin and Katcoff, 2001; Downs *et al.*, 2003). These studies also found that the stoichiometry of Hho1p to nucleosomes was less than that found in mammals: 1:37 in the Katcoff study and 1:4 in the Downs study, although the fact that Hho1p has two globular domains could mean that one Hho1p molecule could fulfill the role of two canonical H1s.

Recent studies have implicated *HHO1* in a DNA repair role. This is restricted to *RAD52*-mediated repair which involves repair by homologous recombination (Downs *et al.*,

2003). Moreover, the absence of Hho1p decreases the lifespan of yeast, an effect also reported for *A. immersus* (Ausio, 2000). It might therefore be possible that Hho1p binds to regions of the genome which are prone to double-strand DNA breaks, or indeed binds in order to maintain genome integrity.

Since linker histones have been found to inhibit SWI/SNF in human cells (Horn *et al.*, 2002), it will be interesting to see whether Hho1p affects the functions of ATP-dependent chromatin remodeling in yeast.

1.6 The "Histone Code"

The "histone code" was proposed in 2001 by Jenuwein and Allis and in a separate review by Turner (2000). This hypothesis proposes that co-ordinated histone modifications or combinations of histone modifications have specific impacts on transcriptional regulation (Figure 1.11). Modification of one residue can influence that of another, even when on different histones (Turner, 2002). Histone modifications occur in a particular order (reviewed by Imhof, 2003), and particular combinations of histone modifications result in different phenotypic outcomes by recruiting specific transcriptional regulators (Strahl and Allis, 2000). These mediate the functionality of the genome in response to upstream signaling pathways (reviewed by Fischle *et al.*, 2003) e.g. both acetylation and H3K4 methylation are associated with actively transcribing genes, though acetylation occurs at the beginning of genes, while methylation can occur throughout actively transcribing genes (Pokholok *et al.*, 2005).

Histone modifications existing on a genome-wide scale are also thought to reduce non-specific binding of silencing proteins (reviewed by van Leeuwen and Gottschling, 2002). This prevents these proteins from being titrated from their normal locations, thereby ensuring the stability at silent chromatin domains. In addition to providing activation and repression signals, certain histone "codes" are also able to mediate the multiple activities involved in DNA repair i.e. the phosphorylation of H2A.X (reviewed by Thiriet and Hayes, 2005).

1.7 Nucleosome Dynamics

It had been thought that nucleosomes were stationary, stagnant objects that were unable to move along the DNA (reviewed by Kornberg and Lorch, 1991). However, it has subsequently been demonstrated *in vitro* that nucleosomes are positioned in dynamic equilibrium along the DNA (Pennings *et* al., 1991; Meersseman *et* al., 1992). Work performed at gene promoters has also shown that nucleosomes are dynamic and that this can enable transcription factors to bind and therefore activate or repress genes. Chromatin remodellers exist that are able to move nucleosomes along the DNA (reviewed in Luger and Hansen, 2005) from the positions at which they are deposited very early in the chromatin assembly process, shortly after passage of the replication machinery (Lucchini *et al.*, 2001). Furthermore, histone acetylation is a very dynamic process that is closely connected to nucleosome remodeling (Reviewed by Gregory and Hörz, 1998).

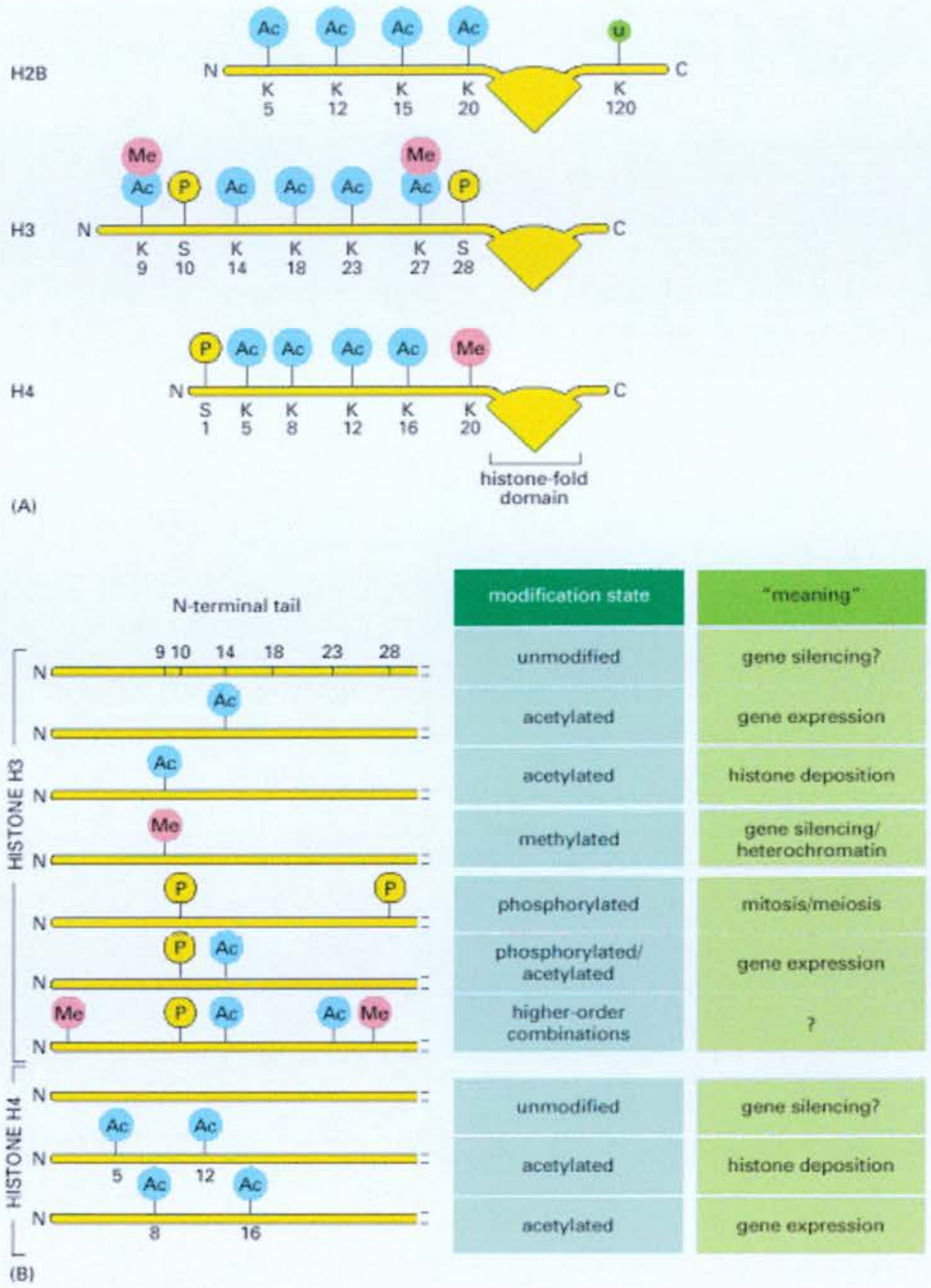

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Figure 1.11 The histone code. Single histone modifications or combinations thereof encode varying cellular processes.

1.8 Chromatin remodelling

Nucleosomes can sequester regulatory elements and compete with transcription factors for occupancy (Cairns, 2005). Since the positioning of nucleosomes is dynamic, nucleosomes can be repositioned at particular genes to cause repression or allow activation of genes. Chromatin remodellers are responsible for accelerating transcription factor binding to genomic DNA (Karpova *et al.*, 2004).

A nucleosome-free region flanked on both sides by a positioned nucleosome has been found approximately 200bp from the start codon at some RNA polymerase II-mediated promoters (Yuan *et al.*, 2005). These nucleosome-free areas coincide with transcription factor binding motifs, suggesting that nucleosome positioning is important for transcription factor access on a global scale. Low nucleosome density is a common feature at promoter regions; moreover, these regions have DNA sequences that do not favour nucleosome formation. This ensures that transcription factors bind preferentially to promoters and are not sequestered to non-relevant sites. In yeast cells at the *HIS3-PET56* gene, the promoter region is nucleosome-poor *in vivo*, as a direct result of its DNA sequence (Sekinger *et al.*, 2005). *In vitro*, this region associates poorly with histones. These recent findings are consistent with the early observations that nuclease hypersensitive sites occur at the *PHO5* promoter under repressive conditions (Almer and Hörz, 1986) and further work by this group which demonstrated that the chromatin structure of promoters is altered depending on the activity of the gene (Pavlović and Hörz, 1988).

The many protein factors that allow or limit accessibility of chromatin to transcription factors are highly conserved in eukaryotes (reviewed in Tsukiyama, 2002). There are two main classes: the first comprises those enzymes that covalently modify histones and the second comprises those that use the energy of ATP-hydrolysis to change the position or structure of nucleosomes (Figure 1.12; ATP-dependent remodelling factors).

There are a variety of chromatin remodeling mechanisms: firstly, nucleosome sliding, where the nucleosome position on the DNA changes. Secondly, remodeling where the DNA becomes more accessible, but histones remain bound. Thirdly, complete dissociation of DNA from the associated histones and fourthly, the replacement of histones with a histone variant (Mohrmann *et al*, 2004).

The complete removal of nucleosomes is exemplified by the *PHO5* promoter. Core histones at this promoter are first hyperacetylated and then lost completely from the *PHO5* promoter (Boeger *et al.*, 2003). Core histone and subsequent nucleosome loss from the promoter, takes place via the transient dissociation of all histones (Boeger *et al.*, 2004). Recent genome-wide studies in yeast suggest that nucleosome removal from promoter regions may occur generally in all cells (Bernstein *et al.*, 2004; Lee *et al.*, 2004), and the extent of nucleosome loss can be correlated with the number of binding sites for transcriptional activators occurring at the promoter. Disassembly of histones and nucleosomes has been found to be associated with the H3-H4 chaperone, Asf1p (Adkins *et al.*, 2004).

1.8.1 Nucleosome mobility

The nucleosome is rendered stable by clusters of weak interactions comprising hydrogen bonds and salt links which are formed as the DNA double helix winds around the nucleosome core particle (Luger and Richmond, 1998). Histone octamers have, however, been shown to display non-catalysed movement along the DNA, in *cis*, at physiological temperatures and low ionic strengths (Pennings *et al.*, 1991; Meersseman *et al.*, 1992). This is referred to as nucleosome mobility or sliding. Here all nucleosomal bonds are broken and reformed, although only a few histone-DNA contacts are able to be broken at any given time (Widom, 1999).

Chromatin remodeling enzymes can catalyse nucleosome mobility by coupling the disruption of histone DNA contacts to ATP-hydrolysis (Figure 1.12 - reviewed in Becker, 2002 and Cosgrove *et al.*, 2004). This imparts a dynamic nature to the nucleosomes, which enables them to slide over substantial distances in vitro (reviewed by Owen-Hughes, 2002).

An additional mechanism of nucleosome mobility is proposed in the loop propagation/recapture model, where DNA at the leading edge of the nucleosome comes off first. Distortion or thermal twisting of this DNA (Li *et al.*, 2005), such as bending it into a tight loop, will lead to the formation of equivalent non-identical histone-DNA interactions, which rapidly loops from the nucleosome (Schiessel *et al.*, 2001). This distortion then moves until it emerges on the other side, thereby translocating the DNA relative to the nucleosome and the size of the DNA loop. The associated proteins are able

to interact with neighbouring DNA, as the DNA loop reforms around a histone octamer (Brower-Toland *et al.*, 2005). The superhelical torsion is caused in an ATP-dependent manner (Havas *et al.,* 2000). This suggests that remodelling may cause rotation of DNA at the nucleosomal entry/exit sites causing over or under-winding and/or bulging on the octamer surface. However, removing torsional strain alone is not the main reason for remodeling, since Becker and colleagues (Becker and Langst, 2001) have shown that the presence of nicks in the nucleosomal DNA does not prevent sliding i.e. ISWI-dependent nucleosome mobility is facilitated by the introduction of specific DNA nicks at the site of the ISWI-nucleosome interaction. A further example of the loop recapture model is ACF, which is made up of two Acf1p and two ISWI molecules, which are capable of binding 4 DNA molecules at any given time (Strohner *et al.*, 2005). Acf1p binds symmetrically to the DNA entry site, causing increased nucleosome accessibility.

The alternative model for nucleosome mobility is the twist diffusion model, where DNA is rotated around its axis as it screws over the surface of the nucleosome (Widom, J., 2001). Thermal energy fluctuations are sufficient to twist the DNA helix at the edge of nucleosomes, which replaces histone-DNA interactions by interactions with neighbouring DNA base pairs.

All ATP-dependent chromatin remodelling factors are multi-subunit complexes that contain an ATPase subunit (reviewed by Cairns, 2005). Four different classes occur – CHD, INO80, ISWI and SWI/SNF (reviewed in Tsukiyama, 2002).

Mi-2, a member of the CHD family in higher eukaryotes, forms large complexes that contain HDAC subunits *in vivo* (Zhang *et al.*, 1998). These complexes also contain methylated DNA-binding proteins in mammalian cells and *Xenopus laevis* eggs, indicating an involvement in transcriptional repression that can be mediated by DNA methylation in higher eukaryotes (Zhang *et al.*, 1999).

The Ino80 complex contains actin and three actin-related proteins (Galarneau *et* al., 2000). Recruitment of Ino80 is negatively affected in cells which are unable to phosphorylate histone H2A. Furthermore, Ino80-mediated chromatin remodeling seems to be confined to regions affected by double-strand DNA breaks, where it appears to facilitate DNA repair (van Attikum *et al.*, 2004), and seems to require the binding of the Swr1 complex, which exchanges Htz1 for H2A (Downs *et al.*, 2004).

The ISWI complex is required for the formation of nuclease-insensitive chromatin structures at the promoter regions of genes not involved in meiosis, and has therefore been implicated in repression (reviewed by Varga-Weisz, 2001). ISWI is able to move nucleosomes bi-directionally and independently of DNA sequence, *in vitro* (Langst and Becker, 2001). It also has a role in replication, as evidence shows that ACF-ISWI is required for replication through highly condensed regions of chromosomes in mammalian cells (Collins *et al.*, 2002).

SWI/SNF will be described in greater detail.

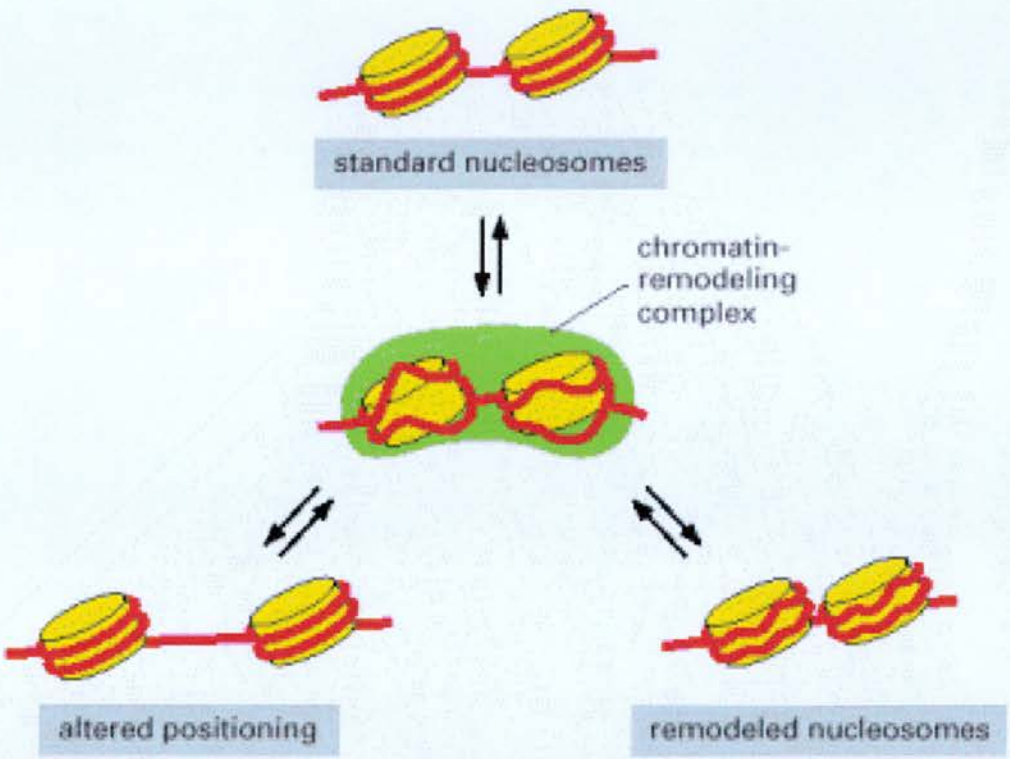

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Figure 1.12 ATP-dependent chromatin remodeling enzymes alter nucleosome position. Chromatin remodelling complexes use the energy of ATP to facilitate the movement of DNA. This either leads to the nucleosomes altering their position and moving along the DNA or allowing previously protected DNA access to transcription factors.

1.8.2 Yeast SWI/SNF

Subunits of SWI/SNF were identified using genetic screens looking for mating-type switching factors and those enabling sucrose to be used as a carbon source. The genes that were isolated during these screens were called SWI or **swi**tching (Stern *et al.*, 1984) and SNF or **s**ucrose **n**on-**f**ermenting (Neigeborn and Carlson, 1984). Biochemical evidence has shown that a 1.15 megaDalton complex containing Swi1/Adr6, Swi2/Snf2, Swi3, Snf5 and Snf6, can be isolated from yeast (Kwon *et al.*, 1994; Cairns *et* al., 1994; Smith *et al.*, 2003).

Apart from many putative histone binding motifs, SWI/SNF remodelling complexes contain a number of DNA-binding domains. Bromodomains within the catalytic units of SWI/SNF have been found to anchor these complexes to hyperacetylated nucleosomes on promoters (Hassan *et* al., 2002) and are important for the role of SWI/SNF in remodelling nucleosomes (Hassan, *et* al., 2007). Besides being recruited by hyperacetylated promoters, H3K56 acetylation has also been found to play an important role in recruiting SWI/SNF (Xu *et al.*, 2005). This modification is interesting in that it is located in the globular domain of H3, rather than at its histone tail. K56 acetylation is enriched preferentially at certain active genes, such as those encoding for histones. Recent evidence suggests that targeted histone acetylation by the SAGA complex predisposes promoter nucleosomes for displacement SWI/SNF complex (Chandy *et al.*, 2006). Moreover, at the *RNR3* gene, TFIID and RNA polymerase are required for SWI/SNF to be recruited to the promoter, suggesting that the general transcription machinery mediates the interaction of SWI/SNF with promoters.

The N-terminal domains of Snf5p and Swi1p make contact with gene activators, and the deletion of these N-terminal domains prevents activator binding (Prochasson *et al.*, 2003). SWI/SNF may be responsible for removing or rearranging the H2A/H2B dimer or altering the binding of the histone octamer (Coté *et al.*, 1994; Lorch *et al.*, 1998), though the removal of the histone octamer is not essential for SWI/SNF to act (Boyer *et al.*, 2000). Recently it has been shown that SWI/SNF remodelling occurs as a result of DNA being peeled from the edge of the nucleosome, effectively translocating the nucleosome along 50bp of the DNA strand. (Kassabov *et al.*, 2003). This may result in the exposure of DNA on the nucleosomal surface (Becker and Horz, 2002), the sliding of nucleosomes to new positions (Meersseman *et al.*, 1992), and the loss of nucleosomes from highly active genes (Boeger *et al.*, 2003; Narliker *et al.*, 2002; Reinke and Horz, 2003). Therefore SWI/SNF seems to play a strong role in destabilising nucleosomes.

SWI/SNF helps activate certain inducible genes e.g. the *SUC2* gene promoter adopts an open chromatin structure (Hirschhorn *et al.*, 1992) with the aid of SWI/SNF binding at both the *SUC2* TAT and UAS regions (Wu and Winston, 1997). In a DNA microarray, SWI/SNF mutant cells grown in rich media show more genes with increased gene expression, while reduced gene expression levels are seen in cells grown in minimal media. This shows that SWI/SNF is needed for inducible gene expression, and that SWI/SNF can activate and repress genes (Sudarsanam *et al.,* 2000). A further example of this occurs at the HTA1-HTB1 locus, where a component of the SWI/SNF complex is present at the promoter and involved in the negative regulation of the promoter. A direct interaction occurs between three SWI/SNF components and two locus-specific

repressors, which recruit SWI/SNF to the promoter. SWI/SNF might therefore cause a repressive chromatin structure by targeting regulatory proteins (Dimova *et al.*, 1999).

There are two possible mechanisms that might enable SWI/SNF to activate and repress genes. Firstly, SWI/SNF might affect nucleosome positioning, which will either increase or decrease the chance of transcription factors binding to promoters, thereby affecting transcription, or secondly, the SWI/SNF complex might always increase the accessibility of chromatin DNA and either activators or repressors might gain access to DNA, depending on the promoter (reviewed by Tsukiyama, 2002; Wade and Wolffe, 1999).

Apart from a local effect on specific genes (Kim and Clark, 2002), the SWI/SNF complex also functions globally in the regulation of chromatin structure, for example, SWI/SNF dependence is particularly evident during mitosis (Krebs *et al.*, 2000). Similarly, in a Gcn5 (catalytic subunit of histone acetyltransferase) mutant background, all genes display a SWI/SNF dependence (Sudarsanam *et al.*, 1999; Biggar and Crabtree, 1999).

These direct effects on chromatin indicate that either SWI/SNF has a role on higher-order chromatin structure (Horn *et al.*, 2002) or it has a highly catalytic action on nucleosomal arrays (Logie and Peterson, 1999). The latter effect has been shown in *S. cerevisiae*. Here SWI/SNF controls the chromatin structure of the *SER3* promoter, whose repression is directly controlled by the Snf2 component of SWI/SNF (Martens and Winston, 2002). Though SWI/SNF affects promoters to a greater degree, remodeling of the extended upstream region (Fleming and Pennings, 2001) or the entire gene (Kim and Clark, 2002)

has been seen *in vivo* i.e. a remodeled chromatin structure extends far beyond the promoter of *FLO1* or *HIS3*. In the latter case, SWI/SNF is recruited to the *HIS3* promoter by Gcn4, which therefore stimulates the mobilisation of nucleosomes over the gene (Kim *et al.*, 2006).

The RSC group of chromatin remodellers (**r**emodels **s**tructure of **c**hromatin) is also a member of the SWI/SNF class, though it differs from *SWI2/SNF2* in that its Sth1 catalytic subunit is essential for viability (Laurent *et al.*, 1993). Both RSC and Sth1 are DNA translocases (Saha *et al.*, 2002), which are able to twist and remodel nucleosomes. The RSC complex contains eight of the fifteen bromodomains in yeast, suggesting that acetyl-lysine recognition is important for RSC. Rsc4 interacts with H3 K14 (Kasten *et* al., 2004). Mutations in RSC lead to altered gene expression, especially in those genes involved in ribosomal function (Angus-Hill, 2001). Furthermore, the localization of RSC changes when the cell is exposed to various stresses (Damelin *et al.*, 2002).

The functional diversity of SWI/SNF complexes allows distinct biological roles to be fulfilled in the context of chromatin. These include an involvement in the expression, maintenance and duplication of the genome (Mohrmann and Verrijzer, 2005). When SWI/-SNF is targeted to the nuclear infrastructure of particular genes, it facilitates activation or repression of genes by interacting with other proteins (Wade and Wolffe, 1999).

1.9 Transcriptional Co-Activator Complexes

The central components of RNA polymerase transcriptional machinery are the same in bacteria and eukaryotic cells (reviewed by Kornberg, 2005). In eukaryotes, gene-specific activator proteins stimulate transcription by recruiting general transcription factors to promoters (Ptashne and Gann, 1997). The mediator complex has been identified as a requirement for activator-dependent stimulation of RNA polymerase II (RNA pol II) transcription (Kelleher *et al.*, 1990; Flanagan *et al.*, 1991) by means of its Srb4p subunit, without which transcription will not be initiated (Takagi and Kornberg, 2006). Here mediator functions as a bridge between regulatory proteins and the basal RNA pol II transcriptional machinery. This takes place in eukaryotic species ranging from yeast to humans.

The yeast mediator complex (reviewed by Bjorklund and Gustafsson, 2005) has 21 subunits which occur both in the free form as well as in a holoenzyme with RNA pol II (Kim *et al.*, 1994). Mediator adopts an elongated conformation in the presence of RNA pol II, forming a head, middle and tail region. Direct contacts occur between RNA pol II and the mediator head and middle region. The C-terminal domain (CTD) in RNA pol II is important for mediator function (Myers and Kornberg, 2000). The CTD is made up of Tyr-Ser-Pro-Thr-Ser-Pro-Ser repeats and truncations of these in yeast cause problems with gene activation *in vivo* (Scafe *et al.*, 1990). RNA pol II movement through the transcriptional cell cycle is regulated by CTD phosphorylation. In the unphosphorylated form of the CTD, the initiation complex is bound to the promoter. Upon phosphorylation, active elongation takes place (Cadena and Dahmus, 1987; Payne *et al.*, 1989; O'Brien *et*

al., 1994). The mediator complex directly binds the unphosphorylated CTD (Myers *et al.*, 1998). Mediator is unable to bind hyperphosphorylated RNA pol II and is found to dissociate when transcriptional elongation begins (Svejstrup *et al.*, 1997).

Separate recruitment of mediator and the general transcriptional machinery has been demonstrated at the *HO* promoter (Cosma *et al.*, 2001). Inactivation of *cdk1* kinase leads to activation of *HO*. The Swi5 transcription factor is translocated from the cytoplasm which stimulates chromatin remodelling at the *HO* promoter. Remodelling allows the transcriptional activator SBF (Swi4-6 cell-cycle box factor) to bind. Mediator is recruited but no recruitment of RNA pol II or general transcription factors (GTF) takes place. Activation of the *HO* promoter only takes place in the G1 phase of the cell cycle when Cdk1 is activated by binding to the G1 cyclins (Bhoite *et al.*, 2001). After recruitment to the promoter, mediator might form a scaffold at the promoter, which allows a functional transcription complex to assemble, thereby allowing multiple rounds of transcription to take place.

1.9.1 Mediator in transcriptional repression

Mediator is a co-activator complex but can also function in regulated transcriptional repression. The Tup1-Ssn6 co-repressor complex does not bind directly to DNA, but rather is recruited by DNA –binding proteins to target specific promoters. Tup1p recruits mediator that contains a Srb8-11 module by direct interactions with the Srb10 subunit (Zaman *et al.*, 2001). Genes encoding the components for Srb 8-11 were identified in genetic screens for Tup1-mediated repression (Lee *et al.*, 2000). Repressors might

therefore recruit mediator to promoters in a form in which interactions with repressive Srb 8-11 module becomes stabilized (Mo *et al.*, 2004).

1.10 Co-repressor complexes

Though chromatin remodelling factors have been shown to remodel nucleosomes in order to allow access to DNA-binding proteins (Coté *et al.*, 1994), the nucleosomal arrays also makes use of co-regulators in order to mediate gene activity. These co-regulator proteins recruit multi-protein subunits that associate with the chromatin structure, thereby modulating transcription (reviewed by Burke and Baniahmad, 2000).

Co-repressors are responsible for gene silencing and actively repress transcription but do not bind DNA directly. They are recruited by transcription factors bound to regulatory regions of target genes, and either aid gene silencing or inhibit gene activators. Co-repressors bind to a wide range of targets, though specific interactions by certain co-repressors with silencers can in turn recruit repressive protein complexes, thus increasing the complexity of the silencing. Examples of these include the yeast Sir proteins, heterochromatin-forming Polycomb proteins, NuRD complex, and the Tup1-related transcriptional repressors. In the interest of this study, Tup1p repression will be discussed in greatest detail.

1.10.1 HDAC Co-repressor function

HDAC 1 and 2 exist as core components of the SIN3 and Mi-2/NuRD complexes, which both function as deacetylases in eukaryotes (Knoepfler and Eisenman, 1999). Mi-2 is in

addition a chromatin remodeller. The ability of the NuRD complex to remodel nucleosomes aids the deacetylation process by making the DNA more accessible to the HDACs (Tong *et al.*, 1998).

1.10.2 Tup1-Ssn6 Co-repressor Complex

Active repression of genes occurs when a gene is repressed even though the activators are present in the cell. Eukaryotic cells can actively repress genes in several connected ways: Firstly, they may modulate the local histone acetylation state; secondly, they may build up special chromatin structures; thirdly, they may interfere with activators and fourthly, they may interfere with the transcriptional machinery. Tup1p has been implicated in each of these modes of action.

Tup1p belongs to the evolutionarily conserved Tup1/GROUCHO (Figure 1.13) protein family, and is made up of WD repeats which are comprised of a 44-60 amino acid sequence containing a GH di-peptide 11-24 residues from the N-terminus and a WD dipeptide at the C-terminus.

The WD repeat motif adopts a B-propeller fold (reviewed by Smith *et al.*, 1999). This symmetrical structure creates a stable platform for forming multi-protein complexes and allowing simultaneous interactions between multiple proteins, though the mechanisms of repression are likely to vary between genes. Crystallography of the C-terminal domain shows a 43 kDa fragment containing seven copies of WD motifs (Figure 1.14; Sprague *et al.*, 2000). Interestingly, this portion of the protein can partially substitute for full-length

Tup1p and is able to cause partial repression, though the first WD motif is most important for oligomerisation and binding to Ssn6 (Zhang *et al.*, 2002). The Tup1-Ssn6 complex is composed of four Tup1p molecules and one Ssn6p molecule and adopts an elongated conformation (Varanasi *et al.*, 1996). In most cases Ssn6p associates with a DNA-binding molecule and might therefore play the role of an adapter molecule (Tzamarias and Struhl, 1995). A specific region of the Ssn6p TPR (tetratricopeptide repeat) domain associates directly with Tup1p (Gounalaki, *et al.*, 2000). Distinct TPR motifs of Ssn6p are required for the repression of certain pathway-specific genes. The Tup1-Ssn6 complex is a very efficient repressor complex (>1000 fold for some genes) which acts on 3% of *S. cerevisiae* genes (Smith and Johnson, 2000). Deletion of either gene is not lethal but the phenotypes include flocculation, loss of mating in α strains, poor sporulation and loss of some aspects of glucose repression. These phenotypes are caused by improper expression of certain genes affected by Tup1p or Ssn6p repression (Keleher *et al.*, 1992). Tup1-Ssn6 itself has no DNA-binding capabilities but rather represses genes by interaction with specific DNA binding proteins (Keleher *et al.*, 1992).

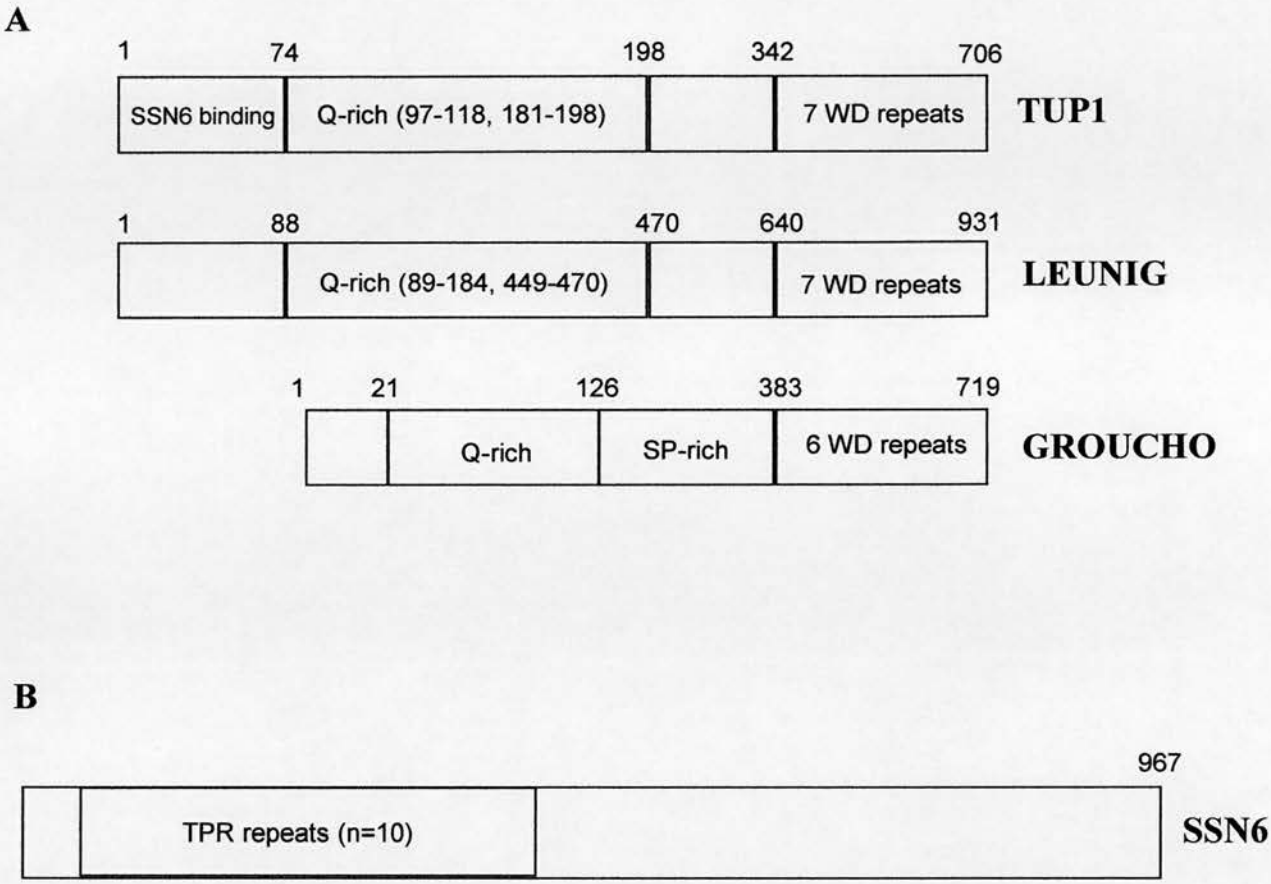


Figure 1.13 Structural domains of GROUCHO/*TUP1* family of transcriptional repressors and *SSN6*. (A) shows a schematic comparison of domains from *TUP1* homologues. Numbers correspond to amino acids. The Q-rich domain corresponds to a glutamine-rich region and SP-domain correspond to regions rich in serine and proline. (B) shows a schematic of the domains present in the *SSN6* co-repressor protein. TPR corresponds to tetratricopeptide repeats, which are 34 amino acid repeats. TPR1 to TPR3 are associated with Tup1p binding (Gounalaki et al., 2000).

Reproduced from Smith *et al.*, 1999.

Figure 1.14. The WD repeat of the G_B subunit of a heterotrimeric G-protein seen from the top and side. The α-carbon backbone is shown in grey, while the red and yellow ribbons shown the N- and C-termini respectively. Each blade (blue) is made up of four anti-parallel B-sheets, combining to form a propeller structure.

For repression to occur, a sequence-specific DNA binding protein that recognizes a sequence in the promoter region of the target gene recruits the Tup1-Ssn6 complex (Treitel *et al.*, 1995; Tzamarias and Struhl, 1994). Examples of these proteins are Mig1p (glucose repression), Crt1p (DNA damage) and Rox1p (hypoxia). At the *HO* gene promoter Tup1-Ssn6 is recruited by α1- α2 proteins (Mathias *et al.*, 2004).

Tup1-Ssn6 repression can be divided into three classes (Figure 1.15): firstly, it can repress by the direct interference with a gene activator. In the case of the *GAL4* gene, the UAS is occupied by the activator but Tup1-Ssn6 is able to repress the gene (Redd *et al.*, 1997). The contact with Tup1-Ssn6 affects the ability of the activator to allow transcription, by inhibiting TATA-binding protein from associating with DNA (Kuras and Struhl, 1999).

Secondly, it can repress genes by altering the local chromatin structure around the genes it regulates. An example of this is that positioned nucleosomes are found upstream of both α2 and *FLO1* promoters (Shimizu *et al.*, 1991; Fleming and Pennings, 2001). The SWI/SNF complex has been proposed to antagonise Tup1-Ssn6 by controlling remodeling activity (Gavin and Simpson, 1997). Interestingly, *ISW2* is required for nucleosome positioning to occur at the Tup1-repressed *RNR3* locus (Zhang and Reese, 2004i). Deletion of *ISW2*, *HDA1* or mediator subunit genes lead to enhanced transcription of *RNR3* and *HUG1* (Zhang and Reese, 2004), suggesting that Tup1p utilizes multiple redundant mechanisms to repress transcription.

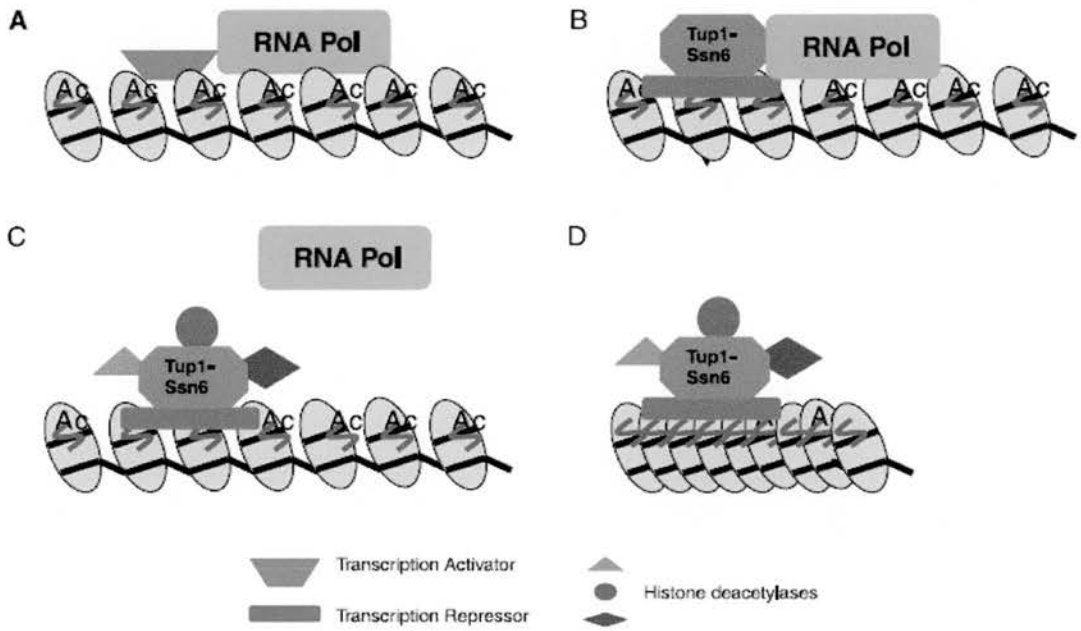


Figure 1.15 Model of step-wise Tup1-Ssn6 repression.

A) Tup1 is recruited to the active promoter by a sequence-specific repressor.

B) The corepressor interacts with Mediator complex prohibiting transcription.

C) Tup1–Ssn6 recruits HDACs

D) Histones are deacetylated, chromatin is compacted, genes are repressed.

Taken from Malavé and Dent, 2006.

Tup1p interacts with H3 and H4 tails *in vivo* (Edmondson *et* al., 1996) and deletion of these can partially relieve Tup1-Ssn6 repression (Edmondson *et al.*, 1998). Tup1-Ssn6 has been found to interact with multiple histone deacetylases *in vivo* (Davie *et al.*, 2003), including Rpd3p, Hos2p and Hos1p, which are class I HDACs. Interactions have also been seen with the class II HDAC, Hda1p. A disruption in either Tup1p or Hda1p at the stress-response promoter, *ENA1*, induces hyperacetylation of H3/H2B (Wu *et al.*, 2001). Alternate repressive structures might therefore be created on different classes of repressed genes. Stable decreases in histone acetylation levels may be directed by the Tup1-Ssn6 complex (Bone and Roth, 2001), as a decrease in acetylation of H3 co-localises with Tup1p (Davie *et al.*, 2002). Histone tail mutations and histone deacetylase mutations may prevent Tup1 from associating with target loci. The Tup1-Ssn6 repressor complex might therefore alter histone modification states to allow its own histone interactions to occur, which maintain a stable repressive state.

Thirdly, Tup1-Ssn6 may interact with the general transcription machinery. In this case, Tup1-Ssn6 binds at the promoter region and when it comes into contact with the transcription machinery, it prevents the transcription machinery from moving along the DNA backbone, causing a modest amount of repression. This was discovered using a non-nucleosomal template where Tup1-Ssn6 activity was seen on naked DNA *in vitro* (Redd *et al.*, 1997). Tup1p has also been seen to compete with an activator (Med 6p) for binding to a subunit of RNA polymerase II (Srb7), which further facilitates repression (Gromoller and Lehming, 2000). As discussed before, Tup1p may in addition interfere with Mediator function.

Tup1-Ssn6 repression can be lifted relatively quickly in response to the relevant cellular signals. For example, some genes required to repair DNA damage are repressed by Tup1-Ssn6. Upon DNA damage, Crt1p is hyperphosphorylated and is then unable to remain bound to DNA (Huang *et al.*, 1998, Li and Reese, 2001). Thus, the DNA-damage signal causes Crt1p and Tup1-Ssn6 to be released from the *RNR* promoters. This de-repression leads to co-activator recruitment and the *RNR* gene activation. Crt1p therefore plays a crucial role in the switch between repression and activation. Crt1p has two repression domains and a region required for gene activation (Zhang and Reese, 2005). The N-terminal domain of Crt1p is the major repression domain which is dependent on HDACs and Tup-Ssn6, while the C-terminal repression domain is independent of HDACs and Tup1-Ssn6. TFIID and SWI/SNF are found to bind to distinct but overlapping regions of the C-terminal domain, and might therefore have dual repressor/activator functions. A similar case is presented at the *SUC2* gene where Snf1 kinase phosphorylates Mig1p, and so abolishes its interaction with Tup1-Ssn6 (Papamichos-Chronakis *et al.*, 2004).

There is conflicting evidence as to whether Tup1p itself spreads along the region it represses e.g. like SIR proteins, since ChIP analyses at the *STE6* gene showed a high density of Tup1p over the whole locus (Ducker and Simpson, 2000). However, other studies showed Tup1p localisation is limited to the α2 binding site (Wu *et al.*, 2001).

1.11 Aims

Yeast flocculation is a calcium-dependent aggregation of yeast cells caused by the expression of flocculation (FLO) genes. *FLO1,* the dominant flocculating gene, is regulated by the Tup1-Ssn6 co-repressor complex (Treitel and Carlson, 1995). Past work by Fleming and Pennings (2001) demonstrated that antagonistic remodelling by the SWI/SNF and Tup1-Ssn6 chromatin remodelling factors rearranges nucleosomal arrays up to 5Kb from the *FLO1* transcription start site, thereby modulating *FLO1* promoter activity. When the nucleosome positions in this upstream domain were mapped (Fleming and Pennings, 2001), it was noted that regions of the 32-nucleosome array showed a nucleosomal spacing of 180bp, compared to the 160bp spacing generally seen in yeast. Therefore, the yeast linker histone, Hho1p may be able to bind in these regions of increased linker length.

The aim of this study therefore, was to investigate the hypothesis that a dynamic relationship exists between chromatin remodelling and co-repressor complexes and Hho1p in a long-range chromatin domain at the *FLO1* upstream locus, localising various proteins involved in *FLO1* regulation in a variety of mutant strains. Earlier work had demonstrated regions of increased nucleosomal spacing, suggesting a possible role for the yeast linker histone. Linker histones have been shown to have an inhibitory effect on SWI/SNF binding (Horn *et al.*, 2002) while Tup1p is known to interact with H3/H4 tails as well as HDACs. Therefore, this work focuses on the possible interplay between Hho1p and Tup1p, and their influence on gene activation, specifically with regard to their effects (if any) on histone acetylation.

Chapter 2

Materials and Methods

Most of the general molecular biology protocols and standard solutions were derived from Current Protocols in Molecular Biology (edited by Ausubel *et al.*, 2004).

2.1 Reagents and Stock Solutions

All solutions were made using deionised water (Elgar option 4Y). Chemicals were from BDH (AnalaR grade) unless otherwise mentioned.

Agarose Gel Loading Buffer at 5X concentration consisted of 0.208% orange G (Sigma), 12.5% Ficoll-400 (Amersham), and 100mM EDTA.

Bead beater lysis buffer contained 50 mM Hepes-KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100 and 0.1% sodium deoxycholate (Sigma).

Buffered Phenol was prepared as follows: 250g of solid phenol (Fluka) were dissolved in 127ml of 2M Tris-HCl pH 7.5, and the phases left to settle. The aqueous phase was removed and discarded. To the organic phase, 55ml 2M Tris-HCl pH8, 13.75ml m-cresol, 550µl β-mercaptoethanol and 275mg 8-hydroxyquinoline were added. The solution was mixed well and left to settle. The phenol layer was retained, aliquoted, and stored at -20°C.

ChIP Elution buffer contained 50 mM Tris/HCl pH 8.0, 10mM EDTA and 1% SDS.

ChIP gel loading buffer *5X* consisted of 15% Ficoll-400 and 0.05% bromophenol blue.

ChIP Wash buffer 1 consisted of 50 mM Hepes-KOH, pH 7.5, 500 mM NaCl, 1 mM EDTA, 1% Triton X-100 and 0.1% sodium deoxycholate.

ChIP Wash buffer 2 consisted of 10 mM Tris/HCl pH 8.0, 0.25 M NaCl, 0.5% Triton X-100, 0.5% sodium deoxycholate and 1 mM EDTA.

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

Diethyl pyrocarbonate (DEPC)-water was prepared by diluting diethyl pyrocarbonate (Sigma) to 0.1% in distilled water, followed by incubation at 37°C for 1 hour/room temperature (RT) overnight. The solution was then autoclaved to deactivate the DEPC.

Dithiothreitol (DTT) was prepared by dissolving solid dithiothreitol to 1M in distilled water, and was stored at 4°C.

Ethylene diamine-tetraacetic acid (EDTA) (disodium salt) was dissolved in distilled water and adjusted to pH 8.0 with NaOH. The volume was adjusted to give a final concentration of 0.5M.

Ethidium bromide stock solution was prepared by dissolving ethidium bromide to 10mg/ml in distilled water, and stored at RT in a light-proof bottle.

MOPS solution was prepared as a 10X solution by dissolving 20.93g MOPS and 2.05g sodium acetate in DEPC-water. 25ml of 0.1M DEPC-EDTA pH 8 was added. The volume was adjusted to 250ml, to give final concentrations of 0.4M MOPS, 0.1M sodium acetate, 0.01M EDTA.

Phenol:Chloroform:IAA consisted of a 25:24:1 ratio of buffered phenol, chloroform, and iso-amyl alcohol.

Phosphate buffered saline (PBS) contained 140 mM NaCl, 2.5mM KCl, 8.1 mM Na_2HPO_4 and 1.5mM KH_2PO_4, pH 7.5.

RNaseA (Sigma) was dissolved in water to 1mg/ml and boiled for 30 minutes to an hour to inactivate DNase. Aliquots were stored at -20°C.

Salmon sperm DNA (Sigma) at 2, or 10mg/ml in TE (pH 8) was dissolved by stirring slowly overnight at 4°C. Aliquots were stored at -20°C, and denatured before use by boiling for 5 minutes.

SDS sample buffer at 2X concentration contained 25ml 4X Tris-Cl/SDS pH6.8 (0.5M Tris-Cl pH6.8, 0.4% SDS), 20ml glycerol, 4g SDS, 1mg bromo-phenol-blue and 55ml water. This was stored at 4°C, and 20µl β-mercaptoethanol was added per ml prior to use.

SDS electrophoresis buffer was prepared as a 5X stock by dissolving 15.1g Tris, 72g glycine and 5g SDS in 1l of water.

Sodium acetate was dissolved in water, and the pH adjusted to 5.2 with glacial acetic acid. The volume was adjusted to give a final concentration of 3M.

Sodium dodecyl-sulphate (SDS) stock was prepared at 10% (w/v) in distilled water.

SSC was prepared as a 20X stock by dissolving 175g (3 moles) NaCl, and 88g (0.3 moles) tri-sodium citrate in 1l of distilled water.

TBE (Tris-borate-EDTA) buffer for agarose gel electrophoresis was prepared as a 10X stock by dissolving 108g Tris (0.89 moles) and 55g (0.89 moles) boric acid in 960ml distilled water, and adding 40ml of 0.5M EDTA pH8.

TBS-T buffer comprises 20mM Tris-Cl pH7.5, 150mM NaCl and 0.05% [v/v] Tween 20.

Transfer Buffer (TB) comprises 25mM Tris, 192mM glycine, and 20% methanol. SDS was added to 0.1% after mixing, to prevent bubbles.

Tris(hydroxymethyl)aminomethane (Tris)-HCl) was dissolved in distilled water and adjusted to the appropriate pH with concentrated HCl. The volume was adjusted to give a final concentration of 1M.

Tris/EDTA (TE) buffer is 10mM Tris-HCl pH 8, 1mM EDTA pH8.

2.2 Culture and Manipulation of *Saccharomyces cerevisiae*

All phenotypic characterisations were performed using the W303 background strain which was HHO1-Myc tagged (kind gift from Dr J Downs, Cambridge University) and its corresponding knockout strains, unless indicated otherwise.

2.2.1 Yeast strains, media and growth conditions

S. cerevisiae strains that were used in this study were derived from W303 [MAT**a**, *leu 2-3, 112ura 3-1, trp 1-1, his 2-11, 15 ade 2-1, can 1-100, GALSUC1 mal0;* Rothstein, ,1983]. Yeast media were made as described previously (Adams et al., 1998). All media were made using distilled water, and were autoclaved at 15lb/in^2 for 15 minutes prior to addition of supplements. The rich medium (YPD) contained 1% (w/v) Bacto-yeast extract, 2% (w/v) Bacto-peptone (Difco) and 2% (w/v) glucose. For plates, 2% (w/v) Bacto-agar was added before autoclaving. Where geneticin selection was used, geneticin G418 (250μg/μl) was added to plates prior to pouring. Yeast cultures were incubated at 30°C in plate incubators or with agitation in an orbital shakers. In the case of Δ*SNF2*-selection, recombinants were checked for growth on YP raffinose, containing 2% raffinose and 1 μg/ml antimycin A.

Where glycerol stocks were needed, 1ml of a saturated culture was added to an equal volume of sterile glycerol solution (65% glycerol, 0.1M $MgSO_4$, and 0.025M Tris.HCl pH8), mixed well by vortexing and stored at -70°C.

2.2.2 Construction of knockout strains

DNA for transformations was amplified from the kanMX plasmid (Wach et al, 1994), which enables transformants to be selected using geneticin (G418) resistance as a selection marker. Yeast cells were transformed with a DNA fragment amplified by PCR and spread onto YPD plates containing 250 μg/ml geneticin G418.

2.2.3 High Efficiency Transformation of Saccharomyces cerevisiae

Yeast were transformed by the lithium acetate/single-stranded carrier DNA/polyethylene glycol (LiAc/ss-DNA/PEG) protocol (Gietz *et al*, 1995). Briefly, the cell density of an overnight culture was determined by counting cells using a haemocytometer. An appropriate volume was used to inoculate 50ml of fresh media to a density of 5×10^6 cells/ml. This culture was shaken at 200rpm at 30°C until a density of 2×10^7 cells/ml had been reached. Yeast cells were harvested by centrifugation at 5000 rpm for 5 minutes at room temperature, washed in sterile water and re-centrifuged as before. They were resuspended in 1ml of 100mM lithium acetate, then harvested in a microcentrifuge and resuspended in 400μl of 100mM lithium acetate. This was divided into 50μl aliquots of cells and the lithium acetate was removed. The following was carefully layered over the cells in a eppendorf tube (to prevent the cells from being damaged by the hugh concentration of lithium acetate): 240μl 50% polyethylene glycol, 36μl 1M lithium acetate, 50μl 2mg/ml denatured salmon sperm DNA and the appropriate DNA in a volume of 36μl sterile water. The solution was vortexed for 1 minute to mix the cells and transformation ingredients, incubated at 30°C for 30 minutes, and then heat shocked 30 minutes at 42°C. Cells were gently harvested after a spin at 6000 rpm for 1 minute, and

resuspended in 20ml YPD. Out-growth was allowed at 30°C for 2-3 hours, with shaking at 100rpm. Cells were harvested by centrifugation at 5000 rpm for 5 minutes at room temperature, washed in sterile water and recentrifuged as before. Cells were resuspended in 1ml sterile water. Appropriate volumes were spread onto selection plates, and incubated at 30°C.

2.3 Cloning and Manipulation of DNA

2.3.1 Polymerase Chain Reaction (PCR)

For amplification of DNA fragments to be used for cloning or as probes, PCR reactions were normally performed in a volume of 50µl as follows: 10-500ng template DNA; 1X Vent polymerase buffer (Promega); 1.5mM $MgSO_4$; 0.2mM deoxy-nucleotide triphosphates (dNTPs); two primers at 0.5µM; 1U Vent polymerase (Promega). In some instances Taq polymerase (Promega) was used. In this case, 1X Taq buffer was used, and 1.5mM $MgCl_2$ was substituted for 1.5mM $MgSO_4$. A standard PCR program comprised 5 minutes at 95°C, followed by 30 cycles of: denaturation at 95°C for 1 minute; annealing at an appropriate temperature for 1 minute; extension at 72°C for 60 seconds (approximately 1 minute per kilobase of DNA to be amplified), followed by a final elongation at 72°C for 10 minutes to ensure complete extension of the fragments. Reaction products were separated in an agarose gel and purified as described.

In the case of Chromatin Immunoprecipitation, the PCR program was adjusted (see 2.8.1.3) on account of the small PCR fragments that needed to be amplified. Titanium

Taq (Clontech) and its corresponding buffer was used to allow the reaction to undergo a hot-start and a higher yield.

2.3.2 Phenol/Chloroform Extraction and Ethanol Precipitation

Phenol/chloroform/IAA was added to an equal volume of aqueous DNA solution and mixed well by vortexing for 30-60s. The aqueous and solvent phases were separated by microcentrifugation at 13000 rpm for 2-10 minutes. The aqueous phase was removed to a clean tube, and the extraction was repeated until the interphase was clear. An additional extraction was performed with an equal volume of chloroform/IAA to remove the residual phenol from the sample.

DNA was precipitated at -20°C for 30 minutes, to 16 hours by 1/10 volume 3M sodium acetate (pH 5.2) or 4M LiCl, and 2.5 volumes 100% ethanol. DNA was collected by centrifugation at 13000 rpm for 10 minutes. The pellet was washed in 0.5ml of 70% ethanol (at -20°C) to remove any residual salt. The DNA pellet was air-dried and resuspended in an appropriate volume of sterile water.

2.3.3 Agarose Gel Electrophoresis

DNA fragments were separated according to size by agarose gel electrophoresis. Depending on the size of fragments to be resolved, gels between 1 and 1.5% agarose were used. DNA samples were loaded in 1X agarose gel loading buffer. Gels were run in 1X TBE, at 80 to 110 volts for an appropriate time. Gels were then stained in 3μg/ml

ethidium bromide solution for 10 minutes, to visualize DNA. De-staining was achieved by washing twice for 10 minutes in distilled water. Images of stained gels were obtained by scanning in a Fujifilm FLA-2000 in fluorescent mode.

2.3.4 Gel Extraction

DNA fragments were resolved on an agarose gel and stained with ethidium bromide. The band of interest excised with a razor blade. The DNA was eluted using Perfectprep Gel Cleanup kit (Eppendorf) and eluted in 30 µl sterile water. For chromatin conformation capture experiments, QIAEX II Gel Extraction Kit (QIAGEN) was used instead.

2.3.5 DNA Concentration

DNA concentration was determined spectrophotometrically by measuring the absorbance of dilutions at 260nm, and using the conversion:

1 A_{260} absorbance unit = 50µg DNA/ml.

2.3.6 Restriction Enzyme Digestion

DNA was digested with restriction enzymes as specified by the relevant manufacturer. Digestion products were resolved by agarose gel electrophoresis. Generally, restriction enzyme digestions were carried out in 100 µl as follows.

DNA	_ µl
10X Buffer	10µl
Restriction Enzyme	1 U/µg DNA
Water	up to 100µl

2.3.7 DNA Ligation

Ligation reactions were carried out overnight at 16°C in 50µl. DNA was incubated in the presence of 400U T4 DNA ligase and buffer containing ATP (NEB).

2.3.7 DNA Sequencing

DNA sequencing was performed using the comfort read sequencing service from MWG-Biotech. Samples of plasmid (>5µg) or fragment DNA (20ng/100bp) were air-dried and sent to the company to be processed.

2.4 Radio-labelling of DNA fragments

2.4.1 Marker DNA

300ng of 1kb ladder (Promega) was labelled on the 5'-end by incubating with 1X polynucleotide kinase buffer, 5U polynucleotide kinase (NEB) and 4 picomoles of [γ-^{32}P]ATP at 37°C for 1 hour. The enzyme was deactivated by heating to 68°C for 20 minutes. Unincorporated label was removed by passing the sample through a MicroSpin^{TM} G-25 Column (Amersham Biosciences).

2.4.2 Probe DNA

DNA fragments to be used as probes were labelled by random priming. 1-2µg of DNA in a volume of 9µl was combined with 5µg hexanucleotide mix (in a volume of 5µl) in a volume of 14µl, boiled for 5 minutes to denature the DNA and then put on ice. The following was then added: 1X Klenow polymerase buffer (2.5µl) ; 0.5mM 3dNTP's (-

dCTP); 12.5U Klenow polymerase (NEB; 1µl); and 8 picomoles [α-^{32}P]dCTP (5µl_. The reaction was allowed to proceed for four hours at RT, and was then stopped by adding 1µl 0.5M EDTA. 75µl of TE were added, and the sample passed through a MicroSpin^{TM} G-25 Column (Amersham Biosciences) to remove unincorporated label. The probe was denatured before use by boiling for 5 minutes. The denatured probed was immediately added to a hybridisation bottle containing a prepared nitocellulose membrane.

2.5 Preparations from *S. cerevisiae*

2.5.1 Genomic DNA

Yeast from a 10ml overnight culture were harvested at 5000rpm for 5 minutes and washed in 0.5ml sterile water. Cells were resuspended in 200µl of breaking buffer (2% Triton X-100, 1% SDS, 100mM NaCl, 10mM Tris.HCl pH8, 1mM EDTA pH8). 200µl glass beads and 200µl phenol/chloroform/IAA were added, and the cells lysed by vortexing for 1 minute. Samples were cooled on ice before another minute of vortexing. 200µl of TE buffer was added, and the sample briefly vortexed again before microcentrifugation at 13000 rpm for 10 minutes. 1ml of ethanol was added to the aqueous phase to precipitate the DNA. DNA was harvested by microcentrifugation as before, and resuspended in 400µl of TE buffer and 30µl of RNaseA (1mg/ml). This was incubated at 37ºC for 30 minutes. DNA was precipitated at RT with 10µl of 4M ammonium acetate and 1ml ethanol. DNA was harvested by microcentrifugation and resuspended in TE. This DNA was further purified by repeated phenol/chloroform/IAA

extractions, and ethanol precipitated again once the interface was clean. This procedure yields approximately 20µg of DNA.

2.5.2 Total RNA

For preparation and manipulation of RNA, all glass and plasticware was soaked for 20 minutes in 3% hydrogen peroxide, and then rinsed with DEPC-water. Solutions were either DEPC treated themselves, or made using DEPC-water. Phenol was adjusted to pH 4 and equilibrated with AE buffer.

10ml yeast cultures were grown to 4 x 10^7 cells/ml in YPD. Cells were then harvested by centrifugation at 5000rpm for 5 minutes. Cells were resuspended in 400µl AE buffer (50mM sodium acetate pH5.3, 10mM EDTA pH8) and transferred to a 1.5µl Eppendorf tube. 40µl 10% SDS was added and the sample vortexed. 440µl of AE equilibrated buffered phenol (pH 4) was added, and the mixture vortexed for 60s before heating at 65°C for 5 minutes. Tubes were then transferred to dry ice for 15 minutes to precipitate protein and DNA. Care had to be taken during this process to ensure that the protein-DNA mixture was not completely frozen, as this caused reduced yields of RNA. Phases were separated by microcentrifugation at 13000rpm for 15 minutes. The aqueous phase was re-extracted with phenol (pH 4)/chloroform/IAA and ethanol precipitated. Pellets were resuspended in 50µl DEPC-water, and stored at -70°C. This method was adapted from Schmitt *et al.*, 1990.

Anecdotal evidence suggests that the pH of the phenol is irrelevant in this preparation with RNA being extracted at both pH 4 and pH 7, however, in our hands the RNA was contaminated with DNA, when phenol pH7 was used.

2.5.3 RNA Concentration

RNA concentration was determined spectrophotometrically by measuring the absorbance of dilutions at 260nm, and using the conversion:

1 A_{260} absorbance unit = 40μg RNA/ml

2.5.4 Nuclei

One litre yeast cultures were grown to 2 x 10^7 cells/ml. Cells were harvested by centrifugation at 4000rpm for 5 minutes at 4°C. The weight of the cell pellet was determined and designated as 1 volume (1g = 1ml). Cells were washed in 3 volumes of water and then harvested in a JA14 rotor in a Beckman centrifuge, at 5000rpm for 5 minutes at 4°C. The supernatant was decanted and cells resuspended in 1 volume zymolyase buffer (50mM Tris-Cl pH7.5, 10mM $MgCl_2$, 1M sorbitol, 14mM β-mercaptoethanol) containing 30mM DTT. The suspension was incubated at RT for 15 minutes to break disulphide bonds, and then cells harvested at 5000rpm for 5 minutes at 4°C. Cells were resuspended in 3 volumes zymolyase buffer containing 1mM DTT. 100mg of yeast lytic enzyme were added per 5g of cells. Cells were incubated for 30min-1 hour at 30°C with gentle agitation to form spheroplasts. Spheroplast formation was mintored by periodically checking the cells under the microscope. Spheroplasts were harvested by centrifuging as before, and washed 3 times in 2 volumes ice-cold zymolyase

buffer containing 1mM DTT. All subsequent steps were performed at 4°C. Spheroplasts were lysed by stirring gently for 20 minutes in 15 volumes ficoll buffer (18% Ficoll-400, 10mM Tris-HCl pH7.5, 20mM KCl, 5mM $MgCl_2$, 1mM EDTA, 3mM DTT, 1mM PMSF). The suspension was centrifuged at 5000 rpm for 5 minutes to pellet cell debris and unlysed spheroplasts. This step was repeated once more, and the supernatant removed and centrifuged at in a JA20 rotor at 13000rpm for 20 minutes. The pellet volume (~2g) was estimated and resuspended in an equal volume of storage buffer (20mM Tris-HCl pH7.5, 0.1mM EDTA pH8, 10% glycerol, 100mM KCl, 1mM DTT, 1mM PMSF, 14mM β-mercaptoethanol). Aliquots were stored at -70°C.

2.5.5 Spheroplast preparation for direct MNase analysis

Spheroplasts were produced by following a protocol developed by Kent and Mellor (1995). Briefly, 100ml yeast cultures were grown to mid-log phase (2×10^7 cells/ml) and their cell count was determined using a haemocytometer. The cells were harvested, and the pellets were washed in sterile water, resuspended in 950µl Yeast Lytic Enzyme Buffer [10mg/ml Yeast Lytic Enzyme, 20 000 units/g (ICN), 1M sorbitol, 5mM B-mercaptoethanol], and incubated for 15min at room temperature with gentle shaking, to allow spheroplast formation. The spheroplasts were harvested at 5000rpm at 4°C and resuspended in.1M sorbitol. This wash step was repeated. The sphaeroplasts were resuspended in 1.2ml spheroplast digestion buffer [1M sorbitol, 50mM NaCl, 10mM Tris-Cl pH 7.5, 5mM $MgCl_2$, 1mM B-mercaptoethanol, and 0.075% Triton]. Spheroplasts were stored at -70°C.

2.6 DNA Analysis

2.6.1 Southern Blotting

Prior to Southern blotting (Southern, 1975), DNA fragments resulting from a restriction digest of approximately 10µg genomic DNA were separated in an agarose gel. 50 counts of radiolabelled 1kb marker were also included on the gel. DNA was denatured in-situ by washing for 2 x 20 minutes in 1.5M NaCl, 0.5M NaOH. The gel was neutralized for 2 x 25 minutes in 1mM ammonium acetate, 20mM NaOH. DNA was transferred overnight to nitrocellulose membrane (Zeta-Probe GT, BIO-RAD), by upward capillary transfer in 20X SSC.

The membrane was washed in 2X SSC and air-dried for 20 minutes. The DNA was immobilized by baking on a vacuum dryer at 80°C for 1 hour. The membrane was then incubated at 65°C in pre-hybridization buffer (3X SSC, 10mM EDTA pH8, 0.2% PVP, 0.2% Ficoll-400, 0.2% BSA, 0.1% SDS, 0.1mg/ml denatured salmon sperm DNA, 0.5mg/ml heparin), for 2-3 hours in a rotating oven. The denatured probe was added to 25ml of hybridization buffer (pre-hybridization buffer supplemented with 2.25g of dextran sulphate) at 65°C. Hybridization was performed overnight at 65°C. The membrane was washed at 65°C for 4 x 15 minutes in 2X SSC/0.1% SDS, and for 2 x 20 minutes in 0.1X SSC/0.1% SDS. Finally, the membrane was rinsed in 2X SSC at RT, and exposed to a phosphorescent screen (Fuji).

2.7 RNA Analysis

2.7.1 Agarose Gel Electrophoresis

RNA fragments were size separated in a denaturing gel containing 1.5% agarose, 1X MOPS and 7.2% formaldehyde. 10-20µg samples of RNA were prepared in 15µl of MMF solution (500µl formamide, 162µl 40% formaldehyde, 100µl 10X MOPS). Ethidium bromide was added to a final concentration of 0.1mg/ml, and samples were heated at 60°C for 15 minutes. Samples were loaded in 1X loading buffer (1mM EDTA, 0.25% bromo-phenol-blue, 0.25% xylene-cycol, 50% glycerol), and gels were run in 1X MOPS. Images of stained gels were obtained by scanning in a Fujifilm FLA-2000 in fluorescent mode.

2.7.2 Northern Blotting

Prior to Northern blotting, RNA was separated in a denaturing agarose gel. Gels were washed for 2 x 20 minutes in DEPC-water to remove formaldehyde. RNA was then transferred to nitrocellulose membrane, and hybridized to a cDNA probe as for a Southern blot.

2.8 Chromatin Analysis

2.8.1 Chromatin Immunoprecipitation (ChIP)

The chromatin immunoprecipitation protocol was adapted from Hecht *et al* (1999).

2.8.1.1 Growth, *in vivo* crosslinking, harvest and lysis of yeast cells

Two falcon tubes, each containing 20 ml YPD, were inoculated with an appropriate amount of overnight culture of the strain of interest, and grown to the desired cell density (~3 x 10^7 cells/ml). A 550µl amount of 37% formaldehyde (final concentration 1%) was added to each tube in order to crosslink protein-DNA complexes. The tubes were incubated for 15 minutes at room temperature, with occasional mixing on a rotating platform, to allow the crosslinking reaction to take place. The reaction was quenched with the addition of 1ml 2.5M glycine (final concentration 125mM) and incubated for 5 minutes at room temperature, again on the rotating platform. The cells were harvested by centrifugation at 3000 rpm at 4°C for 5 minutes. The supernatant was discarded and the cell pellets were resuspended in 10ml ice-cold PBS and pelleted again. This step was repeated. The supernatant was discarded and 250 µl ice-cold bead beater lysis buffer and 10 µl protease inhibitor mix for yeast and fungi (Sigma) was used to resuspend the pellet, by pipetting up and down several times. The cell suspension was transferred to a 1.5 ml screw-cap eppendorf tube. An equal volume of silica beads was added to the cell suspension. The tubes were subjected to bead-beating at 4°C for 30s-1 minute in order to lyse the cells. The eppendorf tube was punctured top and bottom using a red-hot 0.6 mm (25G) needle. The tube was placed on top of another and centrifuged at 13000 rpm for 5

seconds. This allowed the crude cell lysate to be captured in the bottom eppendorf tube. The cell lysate was placed on ice. The chromatin was sheared by sonication with 3 pulses of 10 seconds each with a 20 second rest interval while cooling samples on ice. *The optimal sonication time was determined by doing a sonication time course.* The lysates were then centrifuged at 13000 rpm for 20 minutes at 4°C. The supernatant was transferred to a fresh eppendorf tube. An aliquot (20 µl) of this crude cellular extract was set aside as INPUT material and stored at 4°C until further processing.

2.8.1.2 Immunoprecipitation and DNA Isolation

A 200 µl aliquot of whole cell extract (WCE) was transferred to a fresh eppendorf tube and an empirically determined amount of antibody (2.5-10 µl and 30µl of the corresponding protein sepharose suspension was added. The samples were incubated on a nutator for 2 hrs - overnight at 4°C and then centrifuged for 5 seconds at 1000 rpm at 4°C. The supernatant was discarded. One ml of bead-beater lysis buffer was added to the protein sepharose beads and incubated for 5 minutes on a nutator and then centrifuged to pellet the beads. This step was repeated. ChIP wash buffer 1 (1ml) was added to the beads and incubated for 5 minutes at 4°C on a nutator and the the beads were then centrifuged to pellet the beads. ChIP Wash buffer 2 (1ml) was added to the beads and incubated for 5 minutes at 4°C on a nutator. The beads were then centrifuged and the supernatant was discarded. ChIP Elution buffer (60 µl) was used to resuspend the beads and they were then incubated at 65°C for 10 minutes. The beads were centrifuged for 2 minutes at 13000 rpm and the supernatant was transferred to a fresh eppendorf tube. This was designated the PRECIPITATE. A 20 µl amount of precipitate and 10 µl of the input

samples were transferred to a fresh eppendorf tube and 100 µl TE/1%SDS was added. The tubes were incubated at 65°C in a waterbath overnight to reverse the DNA-protein crosslinks and the remainder of the precipitate and input samples were retained at 4 °C for later analyses.

The next day samples were removed from the waterbath and allowed to cool. TE (120 µl), 2 µg glycogen and 100 µg proteinase K was added the tubes and incubated at 37°C for 1 hour. LiCl (25 µl) and 250 µl Phenol/ Chloroform solution was added to the samples. The tubes were vortexed vigorously for 10 seconds. The aqueous and organic phases were then separated by centrifugation for 5 minutes at 13000 rpm. The upper (aqueous) layer was transferred to a fresh tube and 750 µl absolute ethanol was added. The tube was mixed carefully and the nucleic acid was pelleted by centrifugation at 13000 rpm for 20 minutes. The supernatant was discarded and the pellet was washed with 500 µl 70% ethanol. This was repeated. The 70% ethanol was discarded and the pellet was allowed to air-dry for 15 minutes. The DNA from INPUT samples was resuspended in 50 µl TE and DNA from the PRECIPITATE sample was resuspended in 70 µl TE.

2.8.1.3 Polymerase Chain Reaction for Chromatin Immunoprecipitation

PCR reactions were carried out in 25 µl volumes with 25 pmol of each primer, 0.2 mM dNTPs and 0.25 units Titanium Taq Polymerase (Clontech) and 1X PCR buffer. DNA was amplified on a Biometra PCR machine using the following program: 2 min initial denaturation at 96°C, followed by an 26-35 cycles with 30 seconds at 96°C, 30 seconds at 54°C, 60 seconds at 72°C and a final extension step of 2 minutes at 72°C.

2.8.1.4 Polyacrylamide Gel Electrophoresis

DNA fragments were separated according to size by polyacrylamide gel electrophoresis. Gels contained 6% polyacrylamide and were run in 1X TBE. DNA samples were loaded in 1X ChIP gel loading buffer. Gels were run in 1X TBE, at 100 volts for 45 minutes. Gels were then stained in 3μg/ml ethidium bromide solution for 10 minutes, to visualize DNA. De-staining was achieved by washing twice for 10 minutes in distilled water. Images of stained gels were obtained by scanning in a Fujifilm FLA-2000 in fluorescent mode. Bands were then quantitated using the AIDA software.

2.8.2 Chromosome Conformation Capture (3C)

The chromosome conformation capture assay was performed using the technique developed by Kleckner and colleagues (2002). Briefly, purified nuclei (~1 X 10^8) in 50μl were crosslinked with 1% formaldehyde for 2 minutes at room temperature. The reaction was quenched by the addition of glycine to 0.125M. SDS was added to a final concentration of 0.1% and the reaction was incubated at 37°C for 10 minutes in order to remove any non-crosslinked proteins from the DNA. To sequester SDS and allow subsequent restriction digestion, Triton X-100 was added to a final concentration of 1%. The DNA was digested with a restriction enzyme at 37°C in a final volume of 500 μl. The restriction enzyme was inactivated by the addition of 1.6% SDS and incubation at 65°C for 20 minutes. Triton X-100 was added to 1% and DNA was ligated overnight at 16°C using T4 ligase. The crosslinks were reversed by overnight incubation at 65°C in the presence of 5μg/ml proteinase K. Finally the DNA is cleaned by running the decrosslinked solution through a PCR purification column (QIAGEN) and eluted in 30 μl

TE. Two sets of control DNA were produced by (i) eliminating the formaldehyde crosslinking step in one set, and (ii) eliminating both formaldehyde cross-linking and ligation in another set. The DNA was subjected to PCR amplification and the products were run on 6% polyacrylamide gel, stained in 0.75 μg/ml ethidium bromide and quantified using the AIDA software.

2.8.3 Digestion of Nuclei and Spheroplasts with Micrococcal Nuclease (MNase)

Nuclei or spheroplasts were washed 3 times in 1ml of micrococcal nuclease digestion buffer (1M sorbitol, 15mM Tris-HCl pH8, 1mM $MgCl_2$, 50mM NaCl, 0.5mM PMSF) and harvested by microcentrifugation at 11000rpm for 2 minutes. Nuclei were resuspended in 400μl digestion buffer and pre-incubated for 2 minutes at 37°C. 1-3U of micrococcal nuclease was added, and the reaction initiated by addition of 5μl $CaCl_2$ (100mM). Digestion proceeded at 37°C. A 90μl aliquot was removed to 10μl of termination solution (250mM EDTA pH8, 5% SDS, 50mM Tris-Cl pH8) at 30 seconds, 1 minute, 2 minutes and 4 minutes.

For spheroplast digestions, 100U MNase (Worthingtons) was added to 1.2ml spheroplasts resuspended in spheroplast digestion buffer and the reaction was incubated at 37°C. At 30 seconds, 1 minute, 2 minutes and 4 minutes, 200 μl aliquots were removed and added to fresh eppendorf tubes containing 20μl termination solution.

Protein was digested by 5μl of 20mg/ml proteinase K at 50°C for 45 minutes. DNA was extracted with phenol/chloroform/IAA, and residual phenol removed with

chloroform/IAA. After ethanol precipitation the samples were resuspended in 90µl 1mg/ml RNaseA and incubated at 37°C for 45 minutes. DNA was extracted from the RNaseA, and precipitated as above. DNA pellets were resuspended in 20µl TE and loaded onto a 1% agarose gel in 1 X TBE, and electrophoresed for 1hr at 120V. The gels were stained in 3µg/ml ethidium bromide for 20 minutes and destained in water for 20 minutes. The DNA was visualised using a phosphorimager.

2.9 Protein Analysis

2.9.1 Protein Extraction from Yeast Cells

Total crude protein was extracted from cells as previously described (Methods in Yeast Genetics, 1997). Briefly, an overnight culture of yeast cells (10ml; OD_{600} 0.7) was pelleted by centrifugation in a benchtop centrifuge (13000 × g, 25°C, 1min). The cells were washed in water and resuspended in 100 µℓ sample application buffer (0.06 M Tris-Cl (pH 6.8), 10% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 0.0025% (w/v) bromophenol blue). The samples were heated at 95°C for 5 minutes, cooled to room temperature, and loaded onto a 15% SDS-PAGE gel.

2.9.2 Protein co-immunoprecipitations

For co-immunoprecipitations 200µl whole cell extract was added to 2.5µl Flag antibody (Sigma) and 30µl protein sepharose A beads (Pharmacia) and rotated for 2 hours at 4C. The protein was recovered with 10µl SDS sample loading buffer.

2.9.3 Western blots

Gels were electrophoresed at 150 V for 90 min in 1X SDS-PAGE buffer. Following electrophoresis, gels were stained with Coomassie Blue stain solution for 1 h (0.25% (w/v) Coomassie Brilliant Blue in methanol), and destained overnight in destain solution (7% acetic acid, 25% ethanol). Unstained protein was transferred using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad Laboratories, Hercules, CA, USA) following the manufacturer's instructions. Briefly, after electrophoresis, the gel was equilibrated in transfer buffer (25 mM Tris, 192 mM glycine, 20 % (v/v) methanol, pH 8.3) for 20 minutes, before being assembled in the transfer cell. The protein was transferred to a 0.45 μm Trans-Blot Transfer Medium nitrocellulose membrane (Bio-Rad), at 100V, 350 mA over 2 hours. The membrane was treated with blocking solution (0.1 % [v/v] Tween 20, 10 % [w/v] dry milk powder in PBS) for two hours at room temperature, followed by three washes with 0.1 % [v/v] Tween, 1× TBS (20 mM Tris-Cl (pH 7.6), 137 mM NaCl). Thereafter, the nitrocellulose membrane was incubated with primary antibody (1:1000) overnight at 4°C. After washing 6 times in 0.1 % Tween, 1× TBS, a second incubation with phosphatase-labelled secondary antibody (1:20000; Sigma), was performed over 2 hours at room temperature. The membrane was washed 6 times in 0.1 % Tween, 1× TBS and antibody-labelled proteins were visualised with LumiPhos WB (Pierce, Rockford, Illinois, USA). Briefly, 5 ml LumiPhos was incubated with the nitrocellulose membrane for three minutes. The membrane was wrapped in Saran Wrap and exposed to Kodak Scientific Film X-OMAT AR (Eastman Kodak Company, Rochester, New York, USA). The x-ray film was developed using a Konica SRX-101A X-ray processor (Konica Corporation, Tokyo, Japan).

Chapter 3

Generation and Characterisation of Mutant Yeast Strains

3.1 Introduction

The *FLO1* gene is the predominant member of the *FLO* group of genes encoding lectin-like proteins (Teunissen *et al.*, 1995). Flo1p promotes cell adhesion by causing flocculation (Miki *et al.*, 1982), a phenomenon whereby yeast cells aggregate in clumps and drop out of solution in a liquid medium.

The *FLO1* gene locus comprises of an upstream regulatory region over 7kb long. This region is relatively gene-free and is found to be under the influence of the Tup1-Ssn6 co-repressor complex, which has been shown to bind in discrete foci (Tsukihashi and Pennings, in preparation). Nucleosomal mapping over a 32 nucleosome array in this region in various yeast mutants (Fleming and Pennings, 2001) demonstrated that antagonistic remodelling by Tup1-Ssn6 and SWI/SNF formed the background for *FLO1* gene regulation.

Interestingly, the nucleosomal spacing over the upstream region shows an average nucleosomal spacing of 180bp, which is significantly longer than the average nulceosomal spacing of 165bp in yeast. The nucleosomal array is at its most regular in the presence of the Tup1-Ssn6 co-repressor, which might be indicative of a form of higher-order folding. Because the increased nucleosomal spacing leaves sufficient

linker DNA between nucleosomes, we speculated that Hho1p might be a possible factor involved in the chromatin organisation of the upstream region or even the regulation of the *FLO1* gene.

At the promoter of the *SUC2* gene in yeast, Tup1p is evenly distributed over four nucleosomes and has been shown to de redistributed upon derepression of the gene, possibly as a result of role-reversal from repressor to activator complex (Boukaba *et al.*, 2004). Furthermore, Tup1-Ssn6 is continuously associated with the promoters it represses, whether in the active or repressed state (Papamichos-Chronakis *et al.*, 2002). Therefore the mechanism by which Tup1-Ssn6 repression is alleviated is not its removal; instead an activator complex alters the chromatin environment in such a way, that the Tup1-Ssn6 co-repressor complex is no longer able to perform its repressive functions. The protein that recruits Tup1-Ssn6 to the *FLO1* promoter is unknown.

The aim therefore was to localise various candidate proteins involved in the regulation of the *FLO1* gene in various mutant strains. Previous work had been performed in a By447 (Mat**a**) strain where Tup1p had been Flag-tagged, but Tup1p and Ssn6p antibodies had since become available. In order to investigate the localisation of specific proteins at the *FLO1* upstream region, the W303 yeast strain with a Myc-tagged-Hho1 protein was selected as the parental strain, as no Hho1p antibodies are available commercially and previous attempts to raise an Hho1p antibody generated a non-specific antibody (Coert and Patterton, unpublished data). Further attempts to tag the protein at the C-terminal end, with both Flag and HA tags, though successful, were either not detectable via western blot (HA-plasmid) or produced non-specific

binding, in the case of the Flag-tag (refer to appendix II). The Myc-tagged Hho1p W303 strain is a kind gift from Dr J. Downs (Downs *et al.*, 2003). This strain was used as the background strain from which *ΔSNF2, ΔSSN6* and *ΔTUP1* were constructed, and was tested for its specificity to the Myc antibody by western blot (Appendix II). The *ΔHHO1* mutant strain was a kind gift from Dr H.G. Patterton (Patterton *et al.*, 1998).

3.2 Methodology used in this Chapter

Gene deletion strains were generated using PCR-mediated gene disruptions (Baudin *et al,* 1993). This allows the replacement of a gene at its normal chromosomal location with a mutant allele of that gene or a selectable marker gene, produced in vitro, such that the only difference between the initial and final strain is that particular allele. A series of plasmids and strains have been created in order to facilitate this technique (Brachmann *et al.*, 1998). The plasmids contain common yeast selectable marker genes cloned into a conserved site. These selectable marker genes could code for auxotrophic marker genes which have been deleted from the yeast genome, as well as antibiotics like geneticin (G418). The technique is based on the premise that homologous recombination in yeast is very efficient with DNA fragments, and that only 40bp of homology is required for efficient recombination. The selectable marker gene is amplified by PCR with 40+ bp of homologous sequence on either side. This is achieved by designing primers to the selectable marker which have 40bp of sequence homologous to the gene to be disrupted, flanking the primer sequence of the selectable marker (Figure 3.1). The gene disruption fragment is then transformed into the original strain and the strain with the disrupted gene is selected for by growth on the appropriate media.

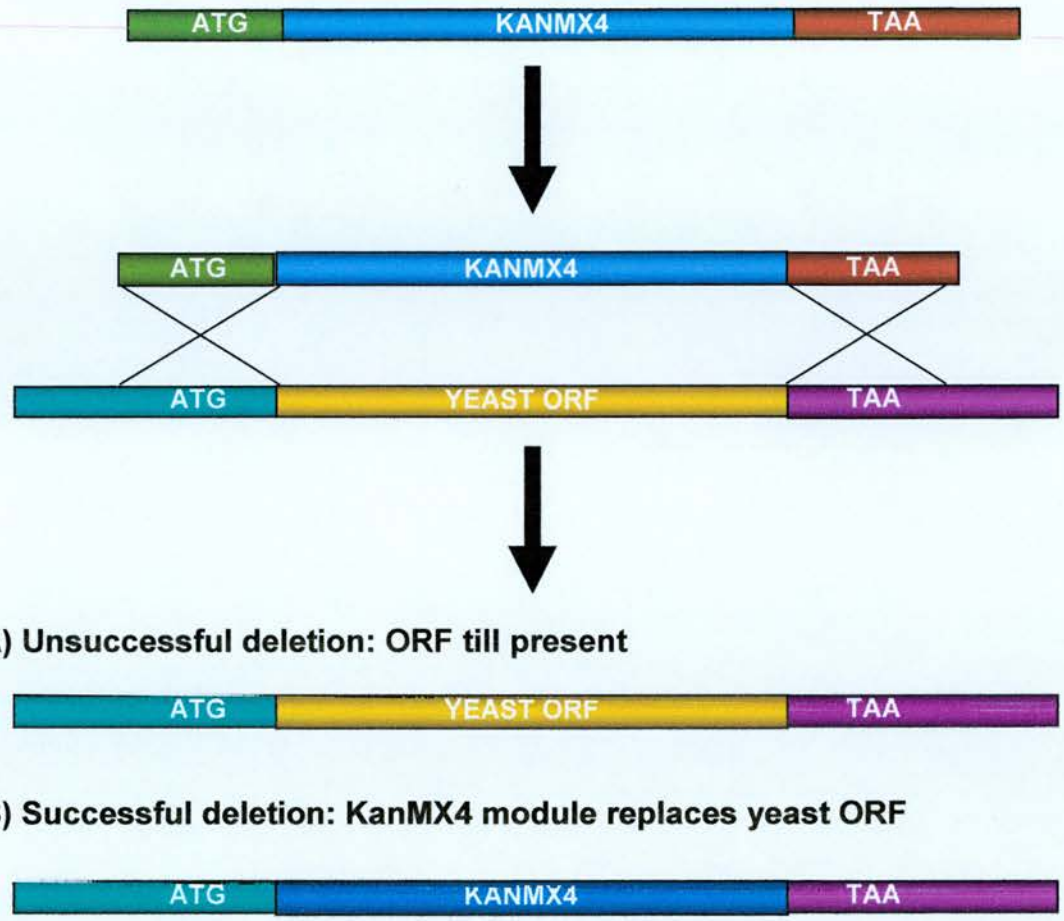


Figure 3.1 Strategy for PCR-mediated gene disruption in yeast resulting in chromosomal integration by homologous recombination.

A fragment for transformation is produced by amplifying the kanMX integration fragment with 40bp of homology to the each end of the gene to be disrupted. After transformation into the yeast cell, the flanking ends are recognised by the yeast genome and might lead to homologous recombination. If successful the ORF will be disrupted by the integration of the kanMX fragment into the yeast genome. If unsuccessful, the yeast ORF will still be present. Adapted from the Saccharomyces Genome Database.

3.3 Generation and Analysis of the Knockout strains

In order to further investigate the interaction between the *FLO1* upstream region and the co-repressor/co-activator system, *TUP1*, *SSN6* and *SNF2* were deleted in the following way:

Ethidium Bromide gel stains had not been kept and therefore could not be included. Preliminary PCR tests should have been performed to verify that the genes had been deleted.

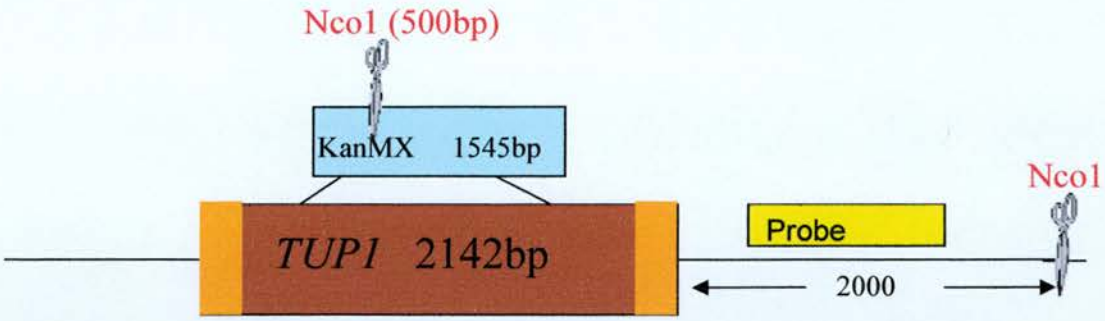
3.3.1 Generation and Analysis of Δ*TUP1*

The Δ*TUP1* deletion strain was generated using primers homologous to the kanamycin kanMX gene (Wach *et al.*, 1994) containing flanking regions homologous to the *TUP1* gene. These were designed to replace the *TUP1* gene from the ATG to downstream of the stop codons by PCR-mediated disruption, with a geneticin selectable marker. The kanamycin gene was amplified from plasmid kanMX with primers Tup1kanMX_1 and Tup1kanMX_2. The disruption fragment was transformed into yeast strain JD397 (W303-*HHO1*-Myc), and following growth at 30°C for 2-7 days on YPD plates supplemented with 200μg/ml geneticin, positive clones were selected. A flocculation test was performed to test for the ability for the strain to flocculate. Flocculation is dependent upon Ca 2+ ions and can therefore be distinguished from other cellular processes which may cause clump formation, by resuspending cell pellets in 250mM EDTA. Therefore, after allowing cells to flocculate after overnight growth, EDTA was added to ensure that they could be dispersed when calcium ions were chelated. The Δ*TUP1* strain was a good flocculator (Figure 3.5). Cells were generally found in small clumps in liquid media and cultures

took approximately two days to reach saturation, due to fermentation of the flocculating clumps and possible contact inhibition.

Genomic DNA (10µg) was prepared from a saturated culture of wildtype cells, as well as from the putative Δ*TUP1* strain. The DNA was then subjected to an overnight restriction enzyme digestion with 1U *Nco1* (NEB)/µg DNA at 37°C. The reaction was stopped by incubating the digest at 65°C to denature the remaining enzyme, before subjecting it to a phenol/chloroform wash and ethanol precipitation. The DNA was dissolved in 20µl of sterile water. The digested DNA was electrophoresed on a 1% agarose gel (1X TBE) gel for 2.5 hours using a radiolabelled 1kb ladder as a size marker.

A Southern blot (see materials and methods) was performed on genomic DNA digests, and hybridised with a probe which confirmed complete deletion of the *TUP1*. The probe DNA had previously been amplified by PCR from genomic DNA (Invitrogen), using oligonucleotides Tup1kanSB1 and Tup1kanSB2. This reaction yielded a PCR fragment of 347 bp. Nco1 cuts 500bp into the kanMX insert and again at 2000bp downstream of the *TUP1* gene (Figure 3.2). A band 3045bp in length is expected in the Δ*TUP1* mutant strain.



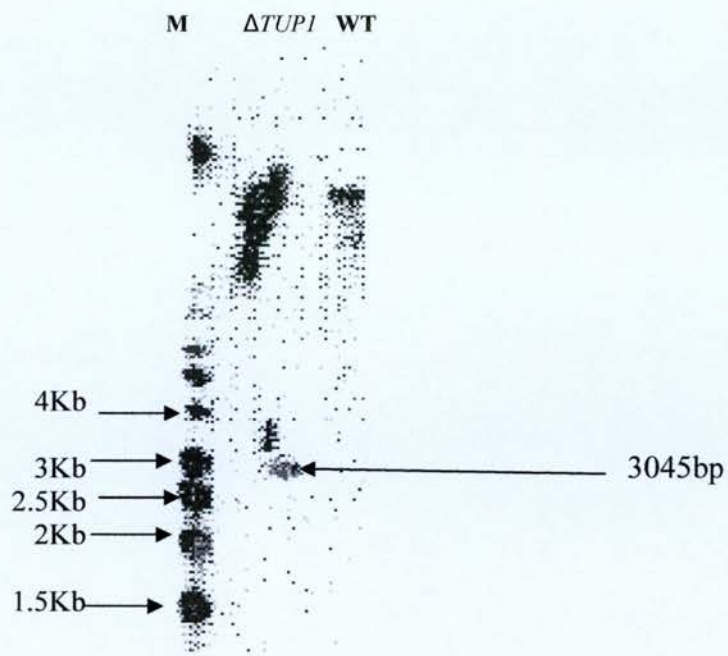


Figure 3. 2 Strategy and Southern blot of *TUP1* knockout.

The *TUP1* gene in its entirety including the start and stop codons was disrupted. The disruption fragment was amplified by PCR from plasmid kanMX using primers Tup1kanA and Tup1kanB. A probe amplified from primers Tup1kanSB1 and Tup1kanSB2 was used to visualise bands indicating the differences in restriction fragment sizes. The yellow box denotes the probe. The orange boxes denote the 40bp of homology which enables homologous recombination. M denotes the 1kb ladder used as a marker. The blot shows a band of 3045bp in the Δ*TUP1* lane confirming that *TUP1* has been successfully deleted.

3.3.2 Generation and Analysis of Δ*SSN6*

The *SSN6* strain was generated by PCR-mediated disruption, by replacing the *SSN6* gene with a geneticin selectable marker from the ATG and downstream of the stop codons. The kanamycin gene was PCR amplified from plasmid kanMX with primers Ssn6knAand Ssn6knB, containing flanking sequences homologous to the *SSN6* gene. The disruption fragment was transformed onto yeast strain JD397, and following growth at 30°C for 2-7 days supplemented with 200μg/ml geneticin, positive clones were selected on YPD plates. A flocculation test was performed to verify the strain's ability to flocculate.

The Δ*SSN6* mutant strain displayed a very strong flocculation phenotype. In all experiments the ssn6 mutant strain produced a "round ball of cells" which was unable to break up. This meant that the cells in the middle of the "ball" were less well-aerated and subsequently the culture always took upwards of two days to reach saturation, compared to overnight incubation for wildtype cells. The clumps were, however, able to disperse in the presence of 250mM EDTA, which is able to chelate the Ca+ ions responsible for flocculation. A composite picture of all flocculating strains is seen in Figure 3.5). The Δ*SSN6* mutant strain is seen to drop completely out of solution; however, the Δ*TUP1* strain which also flocculates does not have such a strong flocculating phenotype.

Genomic DNA (10μg) was prepared from saturated cultures of wildtype and putative Δ*SSN6* mutant cells and digested with *Dra1* (NEB; 1U/μg DNA) overnight at 37°C. After denaturing the remaining enzyme at 65°C, the digested DNA was cleaned using

phenol/chloroform extraction and ethanol precipitation, before being dissolved in 20µl sterile water. The DNA was electrophoresed as before and visualised using an ethidium bromide stain. A radiolabelled 1kb ladder was run alongside the digested DNA as a size marker. After a denaturing step, the DNA was transferred to a nitocellulose membrane by overnight Southern blot in 20X SSC.

The oligonucleotides Ssn6kanSB1 and Ssn6kanSB2 yielded a 521bp DNA fragment which was used as a radiolabelled probe for hybridisation to the nitrocellulose membrane overnight at 65°C. The excess probe was removed in a washing step and the membrane was exposed to a phosphorescent screen overnight.

The blot shows a band of 1836bp in the Δ*SSN6* lane confirming that *SSN6* has been successfully deleted, while the wildtype shows a band of 4191bp (Figure 4). The fragments are produced as a result of *Dra1* cutting the wildtype strain 461bp upstream of the SSN6 gene and again 751bp downstream of the SSN6 gene, while in the Δ*SSN6* mutant strain the kanMX insert is cut 541bp from the start.

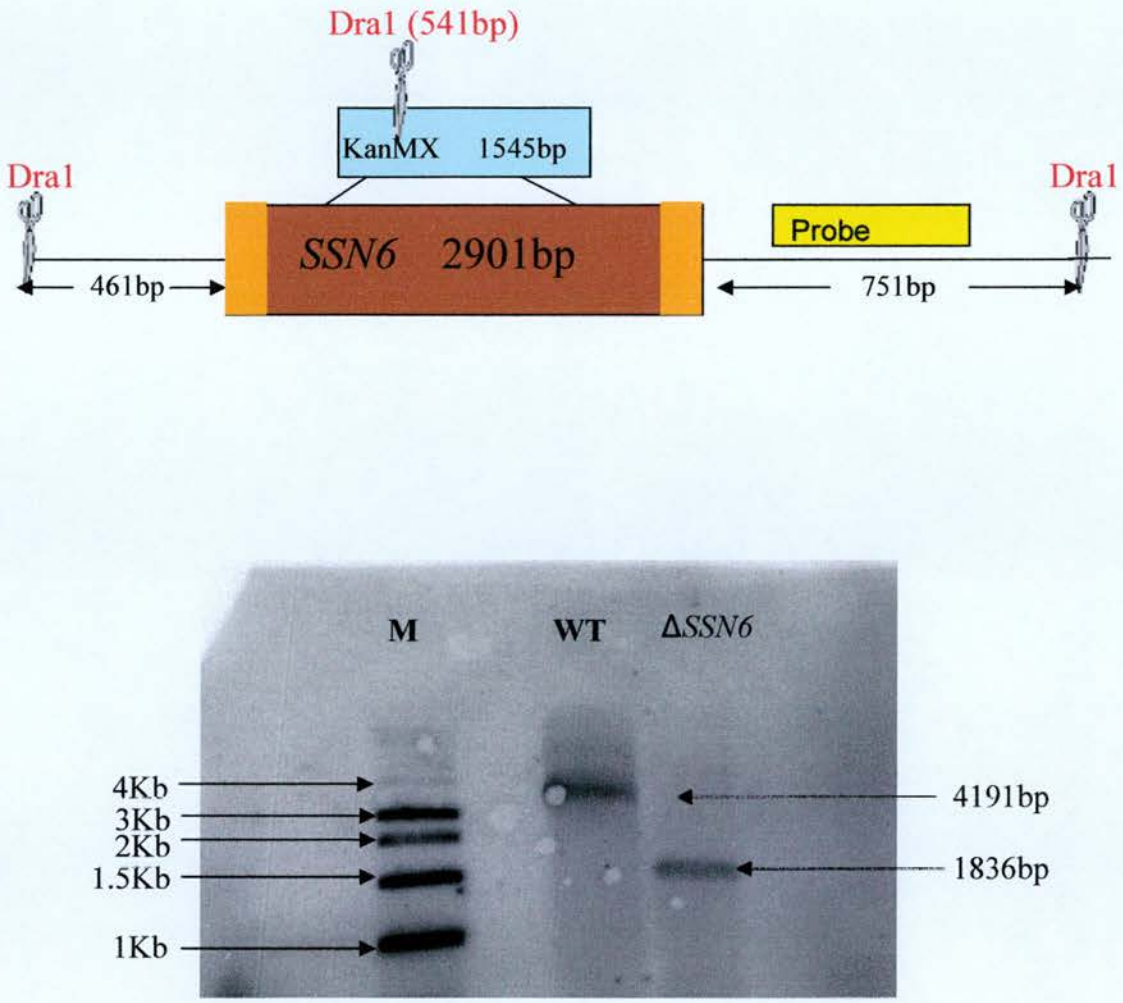


Figure 3. 3 Strategy and Southern blot of *SSN6* knockout.

The *SSN6* gene in its entirety including the start and stop codons was disrupted. The disruption fragment was amplified by PCR from plasmid kanMX using primers Ssn6knA and Ssn6knB. A probe amplified from primers Ssn6kanSB1 and Ssn6kanSB2 was used to visualise bands indicating the differences in restriction fragment sizes. The yellow box denotes the probe. The orange boxes denote the 40bp of homology which enables homologous recombination. M denotes the 1kb ladder used as a marker. The blot shows a band of 1836bp in the Δ*SSN6* lane confirming that *SSN6* has been successfully deleted.

3.3.3 Generation and Analysis of Δ*SNF2*

The *SNF2* strain was generated by PCR-mediated disruption, by replacing the *SNF2* gene with a geneticin selectable marker from the ATG to downstream of the stop codons. The kanamycin gene was PCR amplified from plasmid kanMX with primers Snf2kanMXback and Snf2kanMXforward, containing flanking sequences homologous to the *SNF2* gene. The disruption fragment was transformed onto yeast strain JD397, and following growth at 30°C for 2-7 days on YPD plates supplemented with 200μg/ml geneticin, positive clones were selected. A southern blot was performed on genomic DNA digested with *DraI*, and probed using probe Snf2KanMXSB (148bp) which confirmed the complete deletion of the *SNF2* gene.

As expected, the Δ*SNF2* mutant strain did not display a flocculation phenotype (Figure 3.5) but was extremely slow growing in liquid culture. Cultures took approximately three days to reach saturation (compared to overnight for wildtype cultures) and could only tolerate speeds of no more than 110 rpm in the orbital shaker.

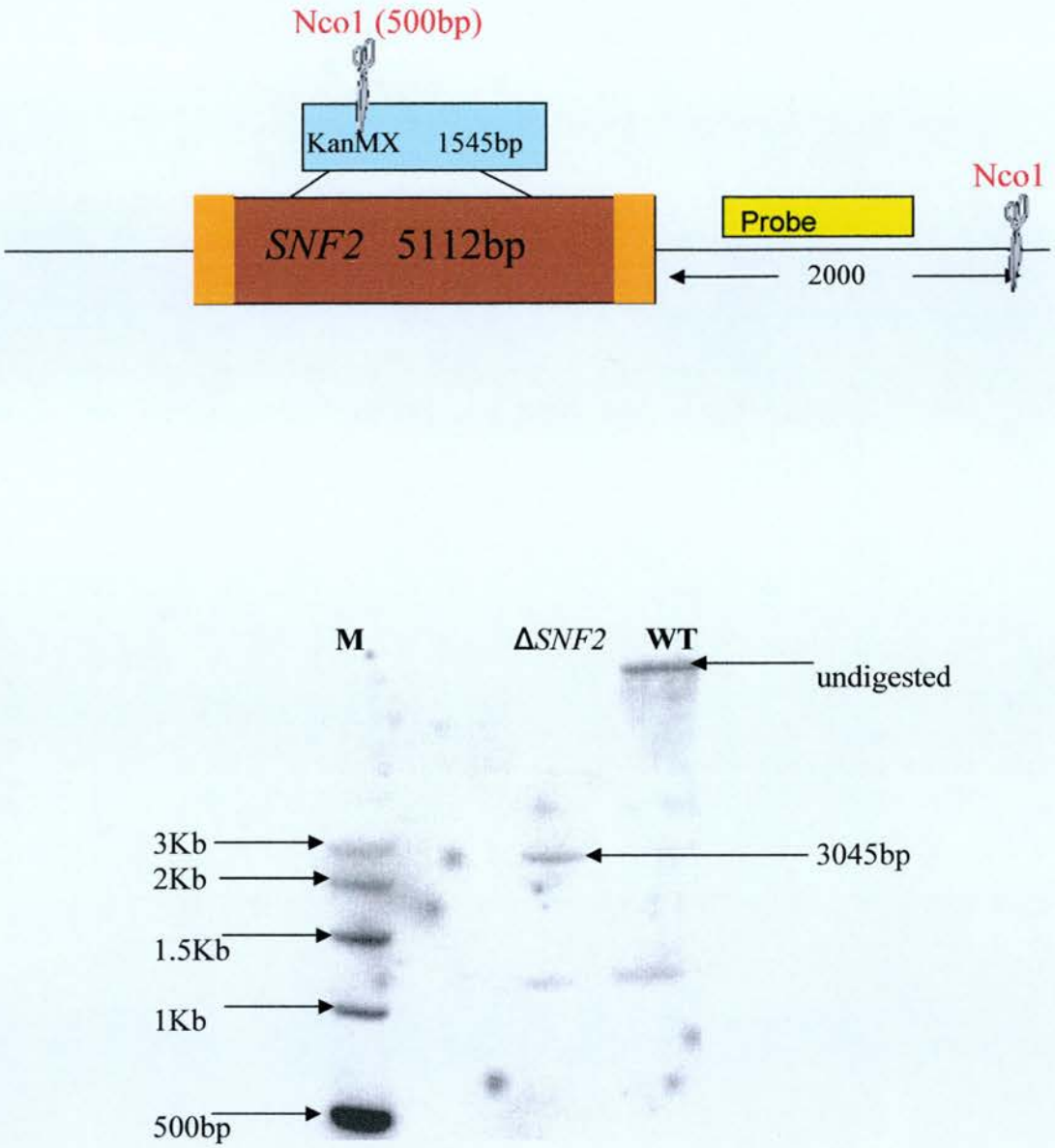


Figure 3. 4 Strategy and Southern blot of *SNF2* knockout.

The *SNF2* gene in its entirety including the start and stop codons was disrupted. The disruption fragment was amplified by PCR from plasmid kanMX using primers Snf2knA and Snf2knB. A probe amplified from primers Snf2kanMXback and Snf2kanMXforward was used to visualise bands indicating the differences in restriction fragment sizes. The yellow box denotes the probe. The orange boxes denote the 40bp of homology which enables homologous recombination. M denotes the 1kb ladder used as a marker. The blot shows a band of 3045bp in the Δ*SNF2* lane confirming that *SNF2* has been successfully deleted.

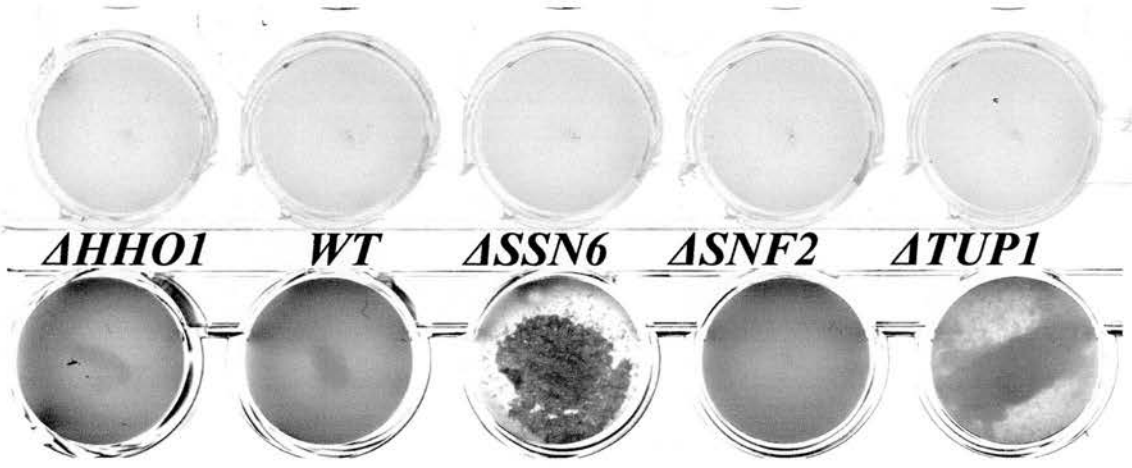


Figure 3.5 Flocculation assay. Yeast strains were inoculated into 5ml YPD and allowed to grow till saturated. 1ml of inoculum of each strain was pipetted into a one 16-well plate well and allowed to settle for one hour. The plates were then scanned using a UMAX Powerlook 1000. It is evident that *ΔSSN6* has a severe flocculation phenotype, while *ΔTUP1* has a weaker flocculation phenotype. The wells in the top row were treated with 250 mM EDTA, while the bottom row was allowed to settle.

As a further confirmation of a successful *SNF2* deletion, the positive clones were streaked onto raffinose plates, supplemented with antimycin A, an electron transport inhibitor. The *SNF2* mutation abolishes the cell's primary ATPase activity and also disrupts the integrity of the SWI/SNF complex (Peterson *et al.*, 1994). The *SUC2* gene encodes invertase which enables the cell to use raffinose and sucrose as a carbon source. The activation of *SUC2* is dependent on *SWI/SNF* remodelling at the *SUC2* promoter (Hirschhorn et al., 1992). *SNF2* mutants are therefore unable to utilise either sucrose or raffinose as they are unable to remodel the *SUC2* promoter (Santisteban *et al.*, 1997). The ability for cells to grow on raffinose is a sensitive assay for invertase activity, as raffinose is a poorer substrate for invertase than sucrose (Neigeborn and Carlson, 1984). Putative mutants were therefore spotted onto plates containing raffinose, supplemented with antimycin A. This prevents the mitochondria from functioning, which in turn forces the cell to switch to fermentative growth. If the cell is unable to utilize its carbon source, it will therefore be unable to grow. The putative *ΔSNF2* colonies were therefore restreaked onto YEP plates supplemented with 2% raffinose and 1 μg of antimycin A/ml.

The raffinose sensitivity experiment showed that colony 1 and 2 were positive *ΔSNF2* transformants, while colony 13 was a false positive. Both wild type and colony 13 grew on the raffinose plates, though the colonies were smaller in size than on glucose plates.

Mutant 1 produced colonies of equal size on solid medium, while mutant 2 produced unevenly sized colonies. Mutant 1 was therefore selected for further analyses.

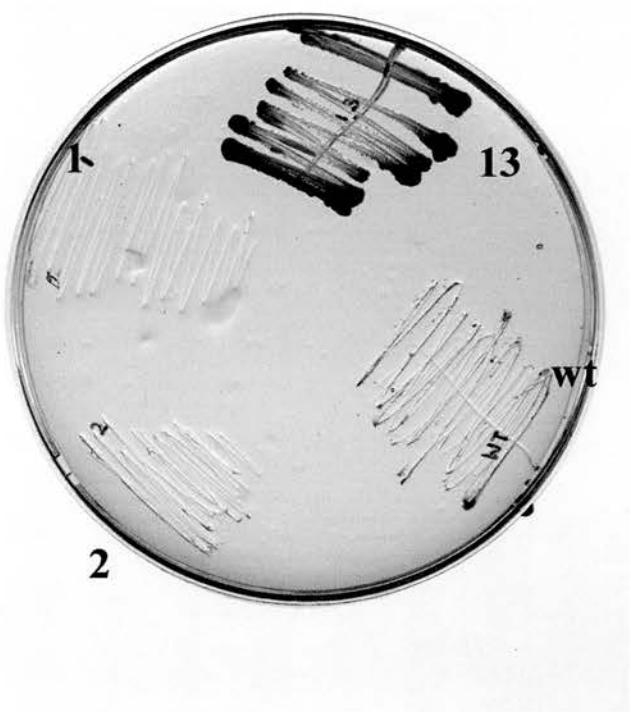


Figure 3.6 Raffinose sensitivity assay. The sensitivity of the putative *ΔSNF2* mutant was tested by restreaking those colonies that had successfully grown on kanamycin plates, onto YEP-raffinose plates supplemented with 1 μg of antimycin /ml. Colonies one and two were therefore confirmed as successful transformants.

3.3.4 Northern blot analyses of Mutant strains

The *ΔTUP1* and *ΔSSN6* mutants displayed a flocculation phenotype. Northern blots were therefore performed on all strains used in the study in order to determine the activation status of the *FLO1* gene.

In Figure 3.7 *ACT1* probe hybridisation was used as a loading control. It is evident that the *FLO1* gene is derepressed in the *ΔSSN6* and *ΔTUP1* strains (Figure 3.8). Interestingly, the gene is also partially derepressed in the wildtype strain. However, this partial derepression is not easily visible with the naked eye in terms of flocculation, as is evident in Figure 3.5. Unfortunately this northern blot was not repeated and we can therefore not confirm if this result is real or if the partial depression seen is due to the wildtype strain being contaminated with a flocculating strain.

In order to ensure that repression or derepression of the *FLO1* gene was not caused by intergenic transcription of any upstream sequences (Martens *et al.,* 2004), probes were generated against five 1Kb upstream regions. No mRNA transcripts were detected, however, these experiments did not include a positive control on each blot, and so no definitive observation can be made by these experiments, since the signal might have been washed off during the stringent wash conditions. If the wildtype strain does indeed have a slight flocculation phenotype then one might expect that the chromatin over the *FLO1* upstream region might be in an already remodelled state. As a control, a chromatin IP (with Tup1p antibodies) on the 6Kb *FLO1* upstream region in wildtupe cells will be performed. The chromatin IP should recreate results generated by Tsukihashi and Pennings (manuscript in preparation).

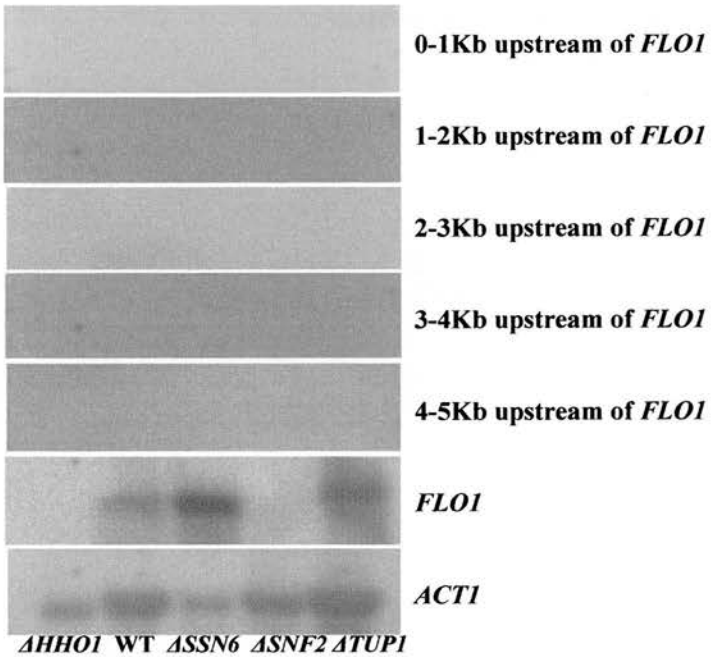


Figure 3.7 Northern blot analysis on the mutant strains.

RNA generated from all the strains used in this study were subjected to northern blot anlysis. The RNA was probed with *FLO1* to detect mRNA from the *FLO1* gene, and *ACT1* to detect the *ACT1* gene which was used as a loading control. The RNA was also probed with 1Kb probes across the 5Kb *FLO1* upstream region, in order to detect mRNA transcripts being transcribed in this region. None were detected.

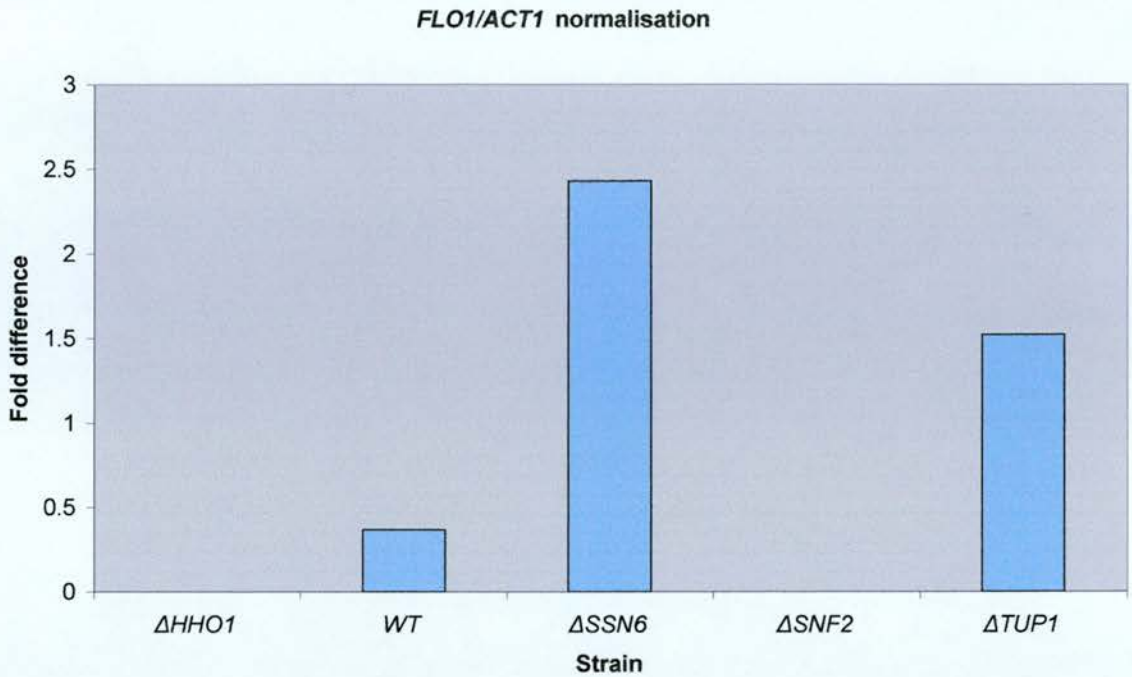


Figure 3.8 Upregulation of the *FLO1* gene in mutant strains.

Bands from the *FLO1* and *ACT1* expression were quantitated. *FLO1* expression is plotted after being normalised to ACT1. WT shows slight upregulation which was not confirmed by a second northern blot. *ΔSSN6* and *ΔTUP1* show upregulaton of *FLO1*.

3.4 Summary

The results from the Southern blots show that *SNF2, SSN6* and *TUP1* have been successfully knocked out in their respective strains. This is confirmed by additional phenotypic evidence.

Chapter 4

Chromatin Immunoprecipitation over the *FLO1* upstream domain

4.1 Introduction

Regulation of the *FLO1* gene is mediated by an antagonistic relationship between a chromatin remodelling complex and a chromatin co-repressor, SWI/SNF and Tup1-Ssn6, which are able to rearrange the nucleosomes in the 32-nucleosome array (Figure 4.1) across the *FLO1* upstream domain (Fleming and Pennings, 2001). This may regulate access of certain DNA binding proteins to the DNA and either activate or repress the gene.

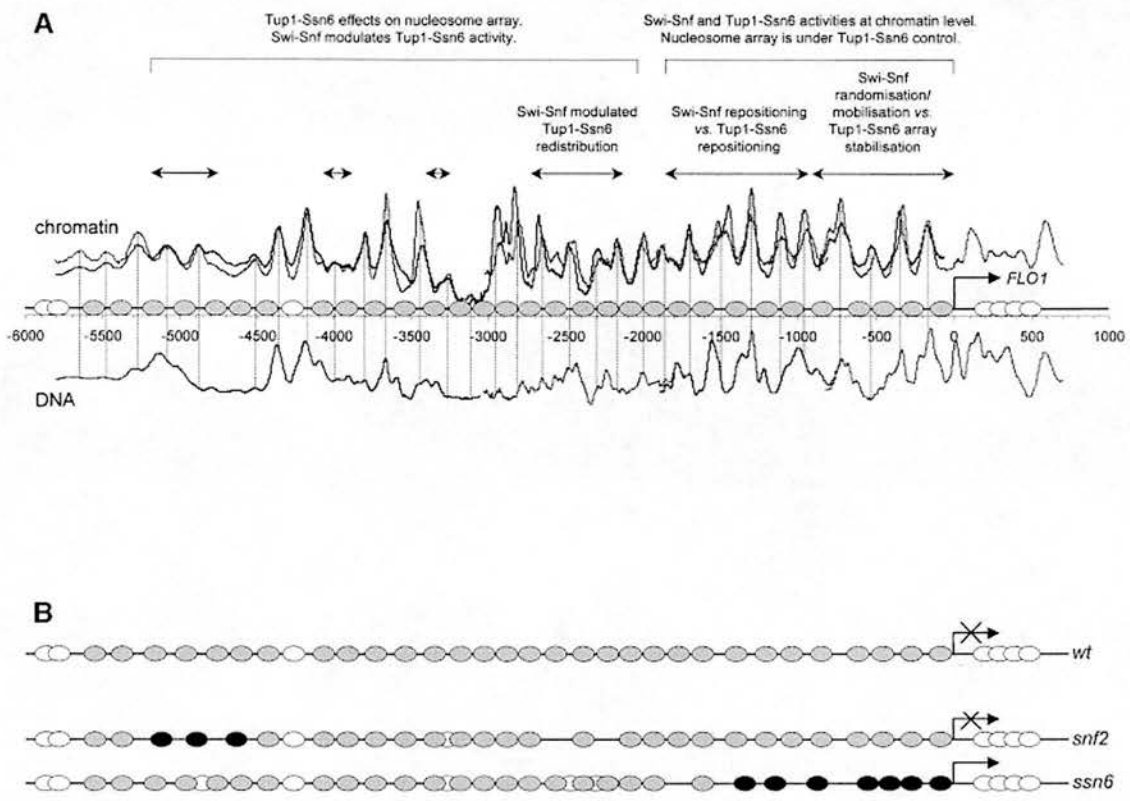
The presence of Tup1-Ssn6 at the promoter causes *FLO1* to be repressed (Fleming and Pennings, 2001). Tup1-Ssn6 binds to deacetylated histone H3 and H4 tails but not to acetylated histones (Edmondson *et al*, 1996). In general, repression by Tup1-Ssn6 is associated with reduced acetylation of histone H3 and H4 at promoters *in vivo* (Bone and Roth, 2001). Active promoters, on the other hand, are found in the context of acetylated histone H3/H4. Therefore repression of *FLO1* by Tup1-Ssn6 may be modulated by changes in histone acetylation.

Intriguingly, the 32-nucleosome array in the *FLO1* upstream domain contain regions of increased nucleosomal spacing of approximately 180bp, rather than the normal 160bp spacing (Fleming and Pennings, 2001; Figure 4.1). We reasoned that it would be interesting to see whether this increased nucleosomal spacing can incorporate a yeast linker histone as it is more similar to the linker length seen in higher eukaryotes.

Our first aim was therefore to determine the localisation of Tup1p across the *FLO1* upstream region up to 6Kb upstream from the transcription start site, in various yeast strains, including those where *SSN6* and *SNF2* have been deleted. This might show how the binding pattern alters as the gene is repressed or expressed.

Secondly we aimed to localise Hho1p across the domain in order to elucidate whether it binds in areas of increased nucleosomal spacing, and how it is related to the chromatin remodelling complex and chromatin co-repressor mediating the regulation of the *FLO1* gene.

Finally, we aimed to determine the H4 acetylation pattern to firstly, investigate the possibility that the acetylation pattern changes in association with the activity of the gene, and secondly, whether the absence of the co-repressor complex or co-activator (remodelling) complex influences the acetylation pattern over the domain.



Reproduced from Fleming and Pennings, 2001.

Figure 4.1 Nucleosome positioning at the *FLO1* upstream region in an array of mutant strains. Indirect end-labelling gels were scanned and intensity plots linearised to determine where the nucleosomes (oval discs – black/white/grey) were positioned (**A**) over a 6Kb region upstream of the *FLO1* gene. This was repeated with *snf2* and *ssn6* strains (**B**). Increased linker lengths on the DNA (black line) are visible.

4.2 Methodology used in this chapter

Chromatin immunoprecipitation is a technique used to determine whether a given protein is localised to a specific DNA sequence *in vivo* (Hecht and Grunstein, 1999; Figure 4.2). Briefly, chromatin-associated proteins are crosslinked to DNA by formaldehyde *in vivo*. The chromatin is isolated from the cells and then sheared to an appropriate fragment size of 500-1000bp (Figure 4.3). The fragmented chromatin is subsequently incubated with an antibody specific to the protein of interest, and then selectively pelleted with Sepharose beads binding to the antibody in order to retrieve the protein bound DNA complex. The crosslinks are then reversed to release the DNA, which is further purified. Finally, PCR amplification of specific DNA sequences is performed to see whether they were co-precipitated with the antibody (Figure 4.2).

Chromatin immunoprecipitation is a very sensitive technique and considerable optimisation had to be undertaken before the procedure could be reliably performed. Sonication was optimised by performing a 0-50s timecourse (Figure 4.3). Antibody-binding of the immunoprecipitation procedure was performed at 4°C overnight. The sepharose bead retrieval also needed to be optimised. Initially the beads were washed 4 times in Lysis buffer, however this was too stringent. Instead we opted for washing the beads twice in Lysis buffer and then once in 500mM NaCl (ChIP Wash Buffer 1) and once in 250mM NaCl (ChIP Wash Buffer 2). For most experiments protein Sepharose A was employed, which uses protein A with very high affinity to IgG molecules, however for goat antibodies (Santa Cruz), protein Sepharose G was used instead.

The amount of input DNA had to be optimised in order to assure that the PCR was in the logarithmic scale and was not saturated. Input DNA was therefore diluted in a 5-fold dilution series and amplified by PCR with a primer set (Figure 4.4A). ChIP primers

were all designed to have a Tm of 56-58°C and were between 20-22bp long. Finally, a PCR was performed on input DNA to determine the optimal cycle number in order to ensure that the PCR was in the logarithmic stage of the reaction. A PCR reaction timecourse was therefore performed with cycle numbers ranging from 0-40 cycles (Figure 4.4 B). 33 cycles were determined to be the cycle optima for subsequent experiments (Figure 4.4 C).

For the experiments presented, two separate yeast cultures were inoculated and grown for each strain and processed in parallel, to yield duplicate datasets. The graphs shown group experiments performed in parallel. All PCR fragments in this chapter were run on 6% polyacrylamide gels in 1X TBE, stained in Ethidium Bromide and destained under fixed conditions, and visualised on a phosphorimager (Fuji FLA-2000) under the fluorescent setting.

The intensity of each band was determined as follows: For the precipitate signal, a rectangle was drawn around the bands in the 2D Mode using Aida 2.0 Software (Raytek). An equally-sized rectangle was drawn in an empty gel area adjacent to the band. The blank signal was subtracted from the precipitate signal. The same was done for the input signal. The precipitate was then divided by the input for each band and plotted on a graph. The graph shows the average between two separate experiments.

The chromatin immunoprecipitation methodology used in this study yields semi-quantitative results. Since a real-time PCR machine was not available at the start of this study, the above methodology was chosen in order to add to an existing dataset which had been performed by Tsukihashi and Pennings (manuscript in preparation), and which

had used the semi-quantitative method. Additional controls (not repeated in this study) such as radioactive PCR quantitation and the use of the RNR2/STE6 genes as an internal reference, had further established the conditions subsequently used in this study, along with gels showing single band amplification with a dynamic intensity range (not shown).

In order to make the results more robust, more replicates of the chromatin immunoprecipitations should be performed. In addition, more PCR probes could be incorporated in the experiments, especially over areas which have sparse representation in the ChIP experiments.

Finally, repeating the whole dataset with Real-time PCR would make the technique quantitative.

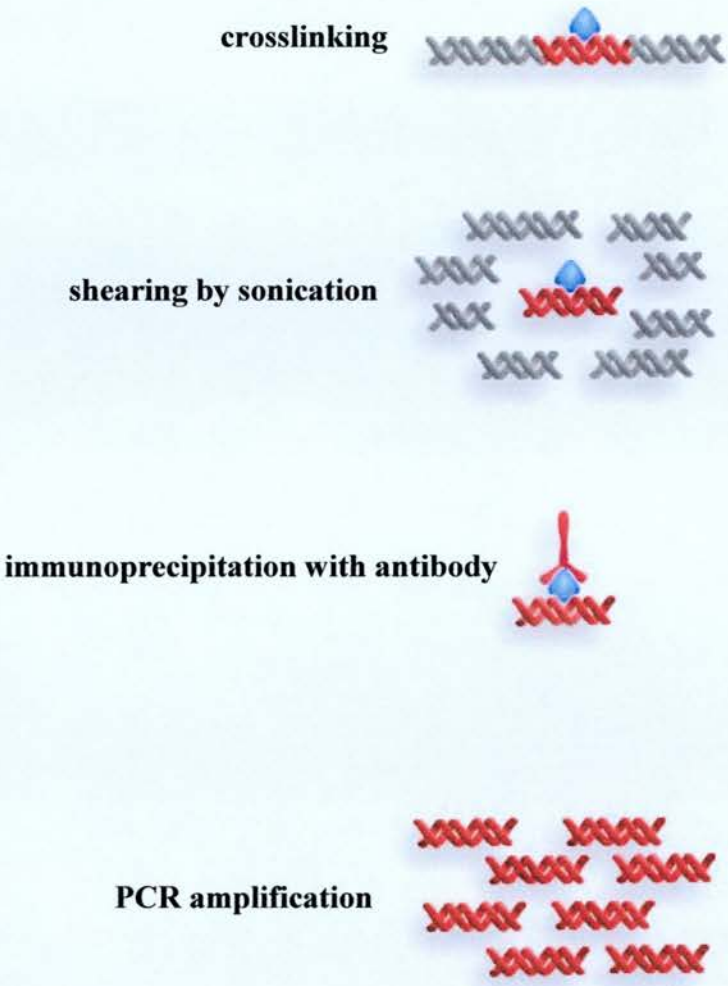


Figure taken from Molecular Biology of the Cell. Alberts *et al.*

Figure 4.2 Chromatin immunoprecipitation protocol.

Protein (blue) is crosslinked to a DNA sequence of interest (red) using a crosslinking agent, such as formaldehyde. DNA is sheared by sonication yielding a desired fragment length. The protein-DNA complex is immunoprecipitated from the whole cell extract using an antibody specific to the protein of interest, by means of Sepharose beads (not pictured). The DNA-protein complex is decrosslinked and the fragment of interest that was previously bound to protein is amplified by PCR using primers specific to the DNA sequence.

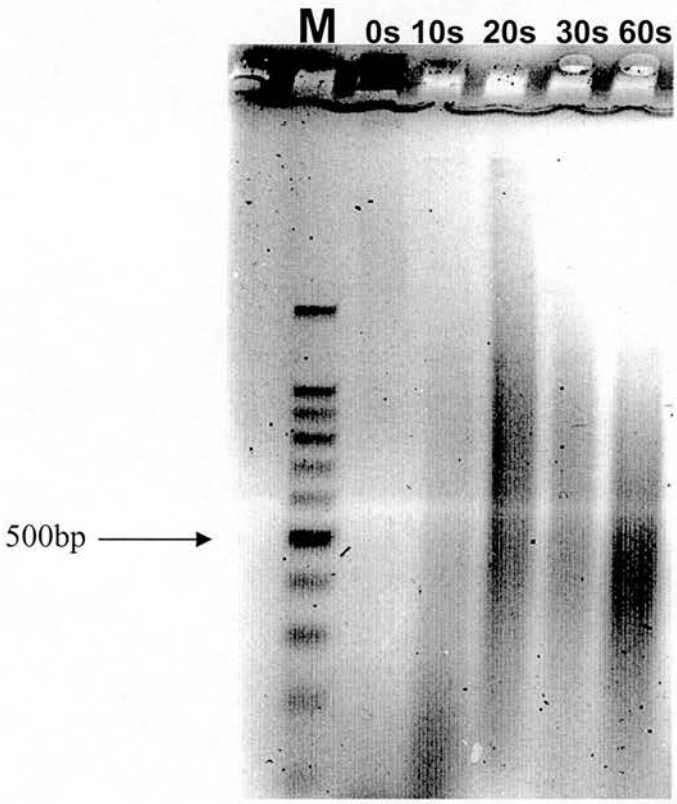


Figure 4.3 Sonication timecourse.

Crosslinked yeast chromatin was sheared by sonication in a timecourse ranging from 0-50s. DNA was purified and electrophoresed on a 1% agarose gel.

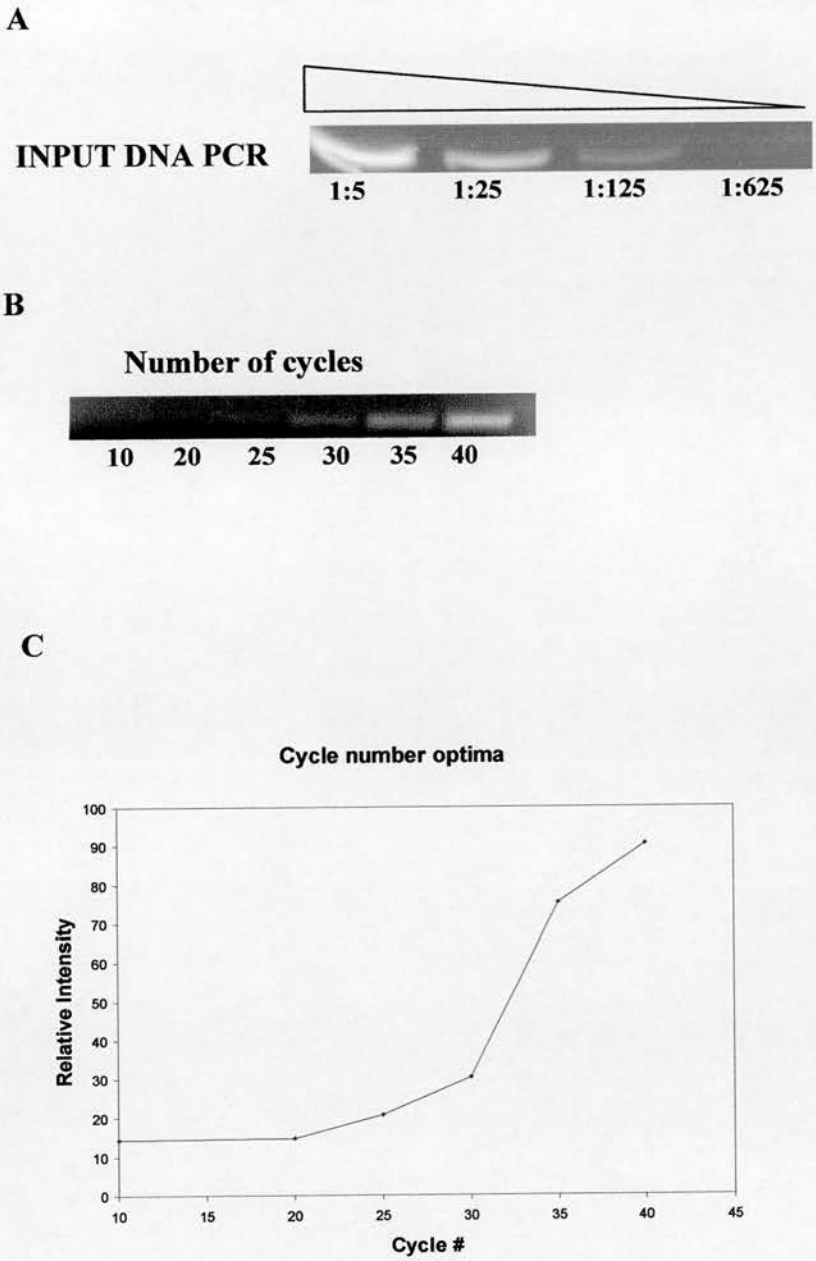


Figure 4.4 Chromatin immunoprecipitation control experiments.

In **A**, input DNA is diluted and then amplified over 30 cycles to determine the optimal DNA concentration (1:125). In **B**, PCR reactions of input DNA at a ratio of 1:125 is amplified for increasing amounts of cycles ranging from 10-40 cycles to determine the optimal number of cycles. 33 cycles were optimal, visible in the adjacent graph **C**.

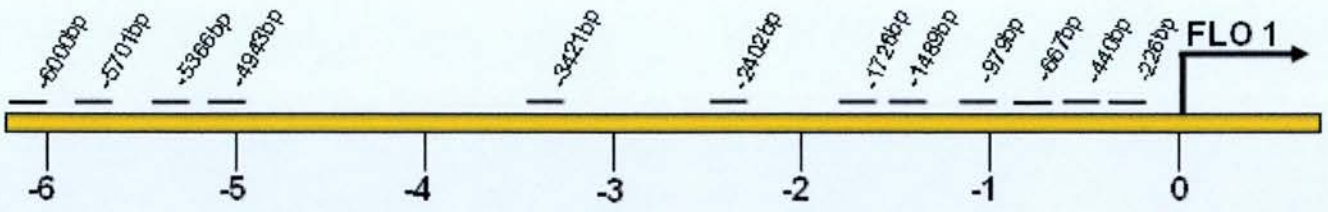


Figure 4.5 Localisation of chromatin immunoprecipitaton PCR fragments.

Primers, of uniform length and T_m were designed across a 6Kb domain upstream of the *FLO1* gene. These were used to amplify DNA prepared by chromatin immunoprecipitation.

4.3 Results

4.3.1 Localisation of Tup1p over the *FLO1* upstream region

The relative distribution of Tup1p over the promoter and extended upstream region of the *FLO1* gene was determined using the chromatin immunoprecipitation (ChIP) assay, an *in vivo* technique that combines antibody detection with PCR-based mapping, which has been used for mapping chromatin-associated proteins and histone modifications along the DNA sequence (Hecht and Grunstein, 1999).

To detect Tup1p, an antibody generated against the N-terminal of the Tup1p protein (Santa Cruz Biotechnology) was used to probe the *FLO1* upstream region in four strains, in order to determine the differences in Tup1p localisation. In three of these strains, the coding regions of specific DNA packaging or chromatin remodelling genes (*SSN6, HHO1, SNF2*), had been replaced with the kanMX coding region as a selective marker.

Tup1p localisation has been studied by other groups at the *STE6, STE2, RNR2, SUC2* promoters (Ducker and Simpson, 2000; Davie *et al.*, 2002; Boukaba *et al.*, 2004). These studies showed that Tup1p was localised at the promoter regions of these genes. Here, the localisation of Tup1p was analysed over a 6Kb chromatin region upstream of the *FLO1* coding sequence. The diagram in Figure 4.5 indicates the DNA fragments that were amplified from either input chromatin or the immunoprecipitated fraction, using 12 sets of primers under conditions of semi-quantitative amplification (Figure 4.5).

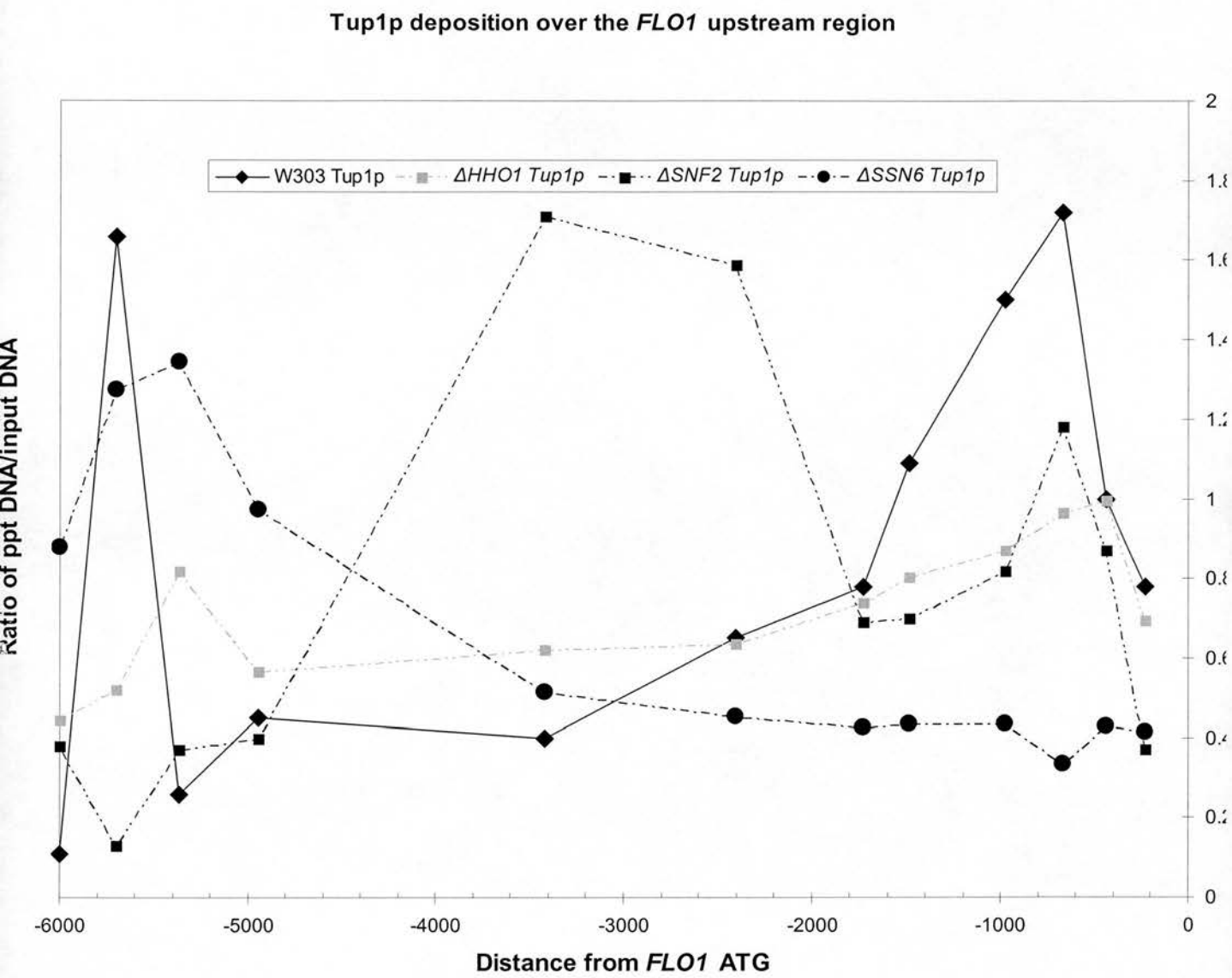


Figure 4.6 Tup1p deposition over the *FLO1* upstream region.

ChIP experiments were performed in duplicate using the Tup1p antibody, and the average of the ratio of precipitate DNA/input DNA was plotted. WT-black diamond; *ΔHHO1*-grey square; *ΔSNF2*-black square; *ΔSSN6*-black circle.

.The results (Figure 4.6; 4.9) show that in the WT strain, W303 *HHO1*::Myc, Tup1p is highly concentrated between -226bp and -1726bp, a finding which was also demonstrated by Tsukihashi and Pennings (unpublished data) in the BY4733a strain with a FLAG-His epitope tag. In addition, a sharper area of Tup1p binding is seen further upstream between -5366bp and -6000bp. However, to solidify this observation, it would be useful to probe this area with more primer sets in order to localise Tup1p more exactly.

To test the contribution of Hho1p (Figure 4.6; 4.10) to the localisation of the Tup1-Ssn6 complex, the ChIP assay was repeated on an *HHO1* deletion mutant. However, this strain did not show any significant peaks in Tup1p localisation.

In the *ΔSNF2* strain (Figure 4.6; 4.11), Tup1p is localised in a broad region from -1726bp and -4943bp, though Tup1p localisation can also be seen to a lesser extent between -226bp and -979bp. This result suggests interplay between Tup1-Ssn6 and SWI/SNF, which correlates with the findings of Fleming and Pennings, which found that Tup1-Ssn6 and SWI/SNF work in antagonistic manner at the *FLO1* upstream locus.

In the *ΔSSN6* strain (Figure 4.6; 4.12), Tup1p localisation between the -226bp and -1726bp locus is completely absent. This dependence of Tup1p on the presence of Ssn6p suggests that at this locus, Tup1p is recruited as a complex with Ssn6p, confirming observations at the *RNR2* and *STE6* genes (Davie *et al.*, 2002). Tup1p localisation at the site distal to the promoter remains intact, however Tup1p localisation at this site, occurs over a broader region i.e. -4943bp to -6000bp. This suggests that Tup1p is recruited by a factor other than Ssn6p at this site.

4.3.2 Localisation of Hho1p over the *FLO1* upstream region

Published papers show varied reports of the stoichiometry of linker histones in yeast (Freidkin and Katcoff, 2001; Downs *et al.*, 2004), with values ranging from 1 linker histone per 37 nucleosomes in the former, to 1 linker histone per 4 nucleosomes in the latter. The authors of Downs *et* al suggested that the reason for Freidkin and Katcoff estimating such a low stoichiometry of linker histone to nucleosomes was that they did not measure the abundance of core histones and could therefore have underestimated the ratio of Hho1p to nucleosomes. In any case, it suggests that the 1:1 ratio of linker histone to nucleosomes seen in higher eukaryotes does not exist in yeast. This could imply that linker histone binding occurs at specific regions in the genome, and might therefore have a specific function in yeast. Indeed, work by Downs and colleagues (Downs *et al.*, 2003) implied a specialised function for Hho1p in homologous recombination. It is worth noting that the yeast linker histone consists of two globular domains instead of one, which also might explain the decreased stoichiometry of linker histones to nucleosomes in yeast.

Fleming and Pennings (2001) demonstrated that the average nucleosomal spacing over the *FLO1* upstream region was increased from approximately 165bp (average for yeast) to 180bp. This region of increased linker length might therefore be better able to accommodate a yeast linker histone. No suitable antibodies to native or tagged Hho1p (both commercial or generated in the Patterton and Pennings laboratories) were found. We therefore performed ChIP analysis of strains in a W303 background where Hho1p had been Myc-tagged. This strain was a kind gift from Dr J Downs at Cambridge University,

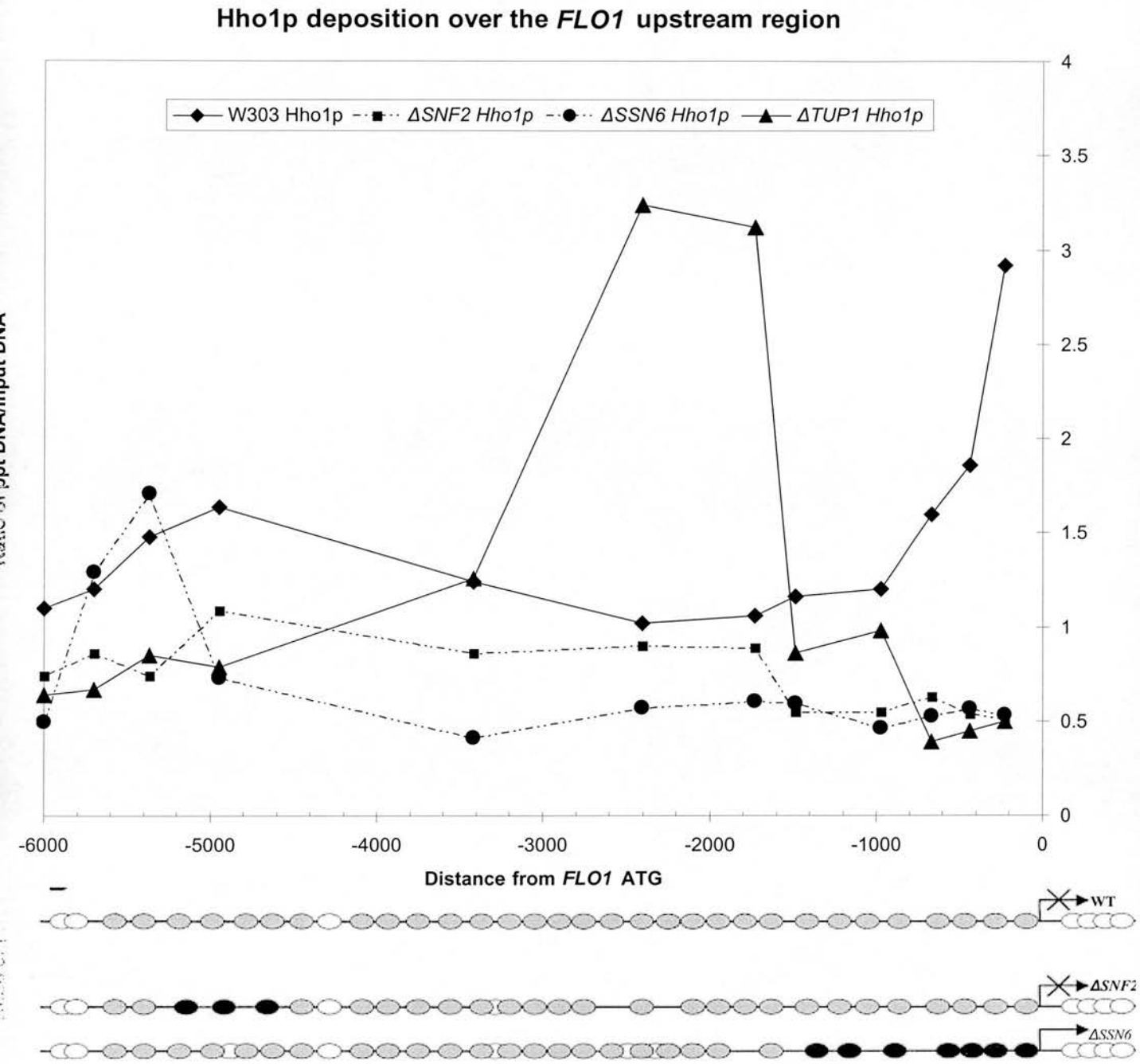


Figure 4.7 Hho1p deposition over the *FLO1* upstream region.

ChIP experiments were performed in duplicate using the Tup1p antibody, and the average of the ratio of precipitate DNA/input DNA was plotted. WT-black diamond; *ΔSNF2*-black square; *ΔSSN6*-black circle; *ΔTUP1*-black triangle. Nucleosome positioning plots lie below.

and was used as a background strain for a number of subsequent gene deletions. A Myc-antibody (Upstate) was used for chromatin immunoprecipitation experiments for Hho1p. The caveats associated with using this strain are discussed in Appendix II.

In wildtype strains (Figure 4.7; 4.9), Hho1p deposition occurs between -226bp and –979bp upstream of the *FLO1* gene.

In the *ΔSNF2* strain (Figure 4.7; 4.11), the Hho1p peaks seen in the wildtype strain disappear, suggesting that Hho1p is mostly absent in a *ΔSNF2* mutant strain. Hho1p was completely absent in the *ΔSSN6* mutant strain (Figure 4.7; Figure 4.12) but this could be due to the active state of the *FLO1* gene. Nucleosomes may be disrupted at the promoter, allowing transcription factors to bind, which subsequently switch on the *FLO1* gene, and thus causes flocculation.

However, the most dramatic change in Hho1p deposition occurs in the *ΔTUP1* strain (Figure 4.7; 4.13), where the Hho1p peak seen in the wildtype strain, makes way for a broad peak of Hho1p between –1489bp to –3421bp upstream of the *FLO1* gene.

The hypothesis that Hho1p would bind in areas of increased nucleosomal spacing was tested by aligning the ChIP graphs with the nucleosomal spacing determined by Fleming and Pennings (Figure 4.7), though this would not be conclusive, especially since their study did not include *ΔHHO1* and *ΔTUP1* strains. However, the correlation between increased nucleosomal spacing and Hho1p deposition was not convincing. In the wildtype strain the area of Hho1p localisation (-226bp to -979bp) overlaps loosely with a region of increased nucleosomal spacing (0 to -1400bp). However, the

nucleosomal spacing over this area is similar in the *ΔSNF2* strain and Hho1p deposition is not visible in this strain. In addition, Hho1p is not deposited in an area of increased nucleosomal spacing between -500bp and -1800bp in the *ΔSSN6* strain (although this might be due to DNAse I hypersensitivity, Tsukihashi and Pennings). These results do not support the hypothesis that Hho1p binds in areas of increased nucleosomal spacing over the *FLO1* upstream region.

4.3.3 Histone H4 lysine acetylation over the *FLO1* upstream region

Acetylation of promoters plays an important role in the activity of genes. HDAC and HAT recruitment to promoters is associated with repression or activation of genes, respectively. The *PHO5* promoter is heavily acetylated when the gene is in the active state (Svaren and Hörz, 1997; Vogelauer *et al.*, 2000). Moreover, acetylation of the repressed *ADH2* promoter allows the TATA-box to become destabilised, allowing the recruitment of transcriptional activators (Verdone *et al.*, 2002).

Work by Boukaba *et al* (2004) at the *SUC2* promoter, a gene whose activity is also modulated by the Tup1-Ssn6 co-repressor complex, shows that an increase in acetylation occurs when the gene is derepressed. Tup1-Ssn6 is associated with low levels of histone acetylation (Bone and Roth, 2001) and has been shown to interact with HDACs (Davie *et al,* 2003).

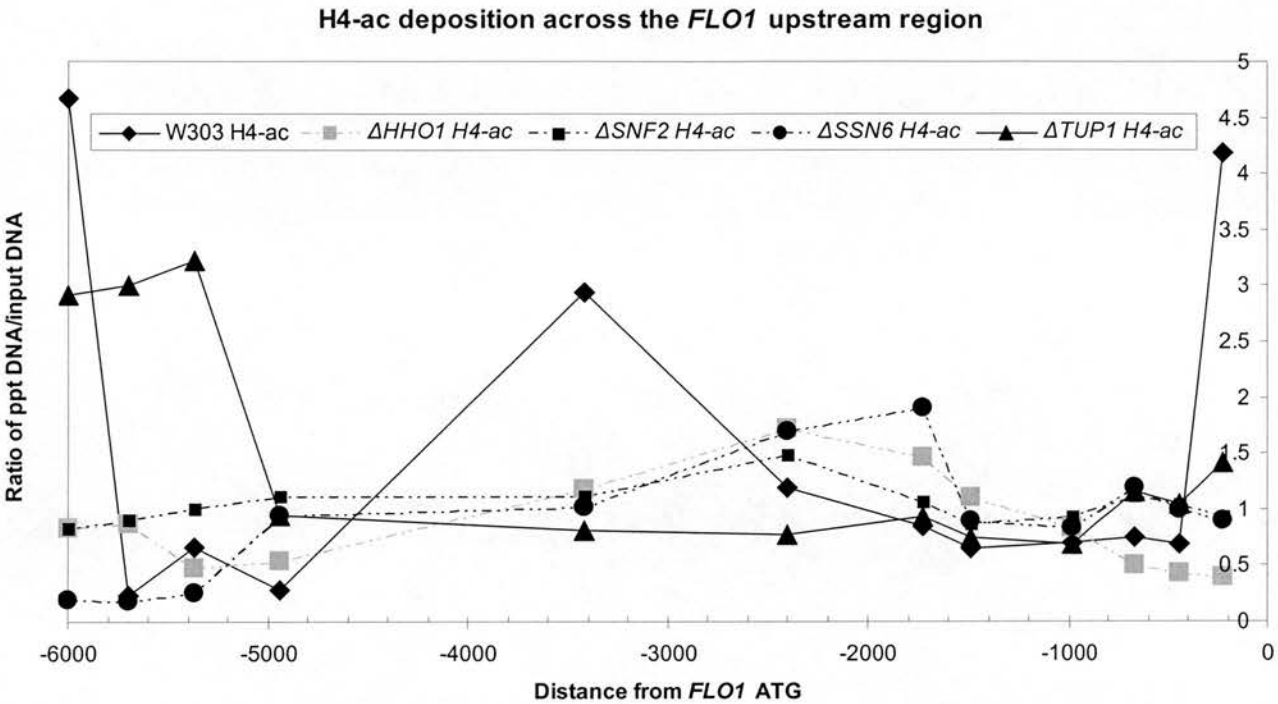


Figure 4.8 H4-ac deposition over the *FLO1* upstream region.

ChIP experiments were performed in duplicate using the Tup1p antibody, and the average of the ratio of precipitate DNA/input DNA was plotted. WT-black diamond; *ΔHHO1*-grey square; *ΔSNF2*-black square; *ΔSSN6*-black circle; *ΔTUP1*-black triangle.

We therefore hypothesised that in wildtype strains, the histone tails associating with Tup1-Ssn6 would be hypoacetylated. Chromatin immunoprecipitation was therefore performed over the *FLO1* upstream locus with a pan acetyl-H4 antibody (Upstate; lysines 5, 8, 12 and 16) in order to determine the H4-acetylation pattern over the region.

In wildtype strains acetylated H4 (Figure 4.8; 4.9) was localised between -1726 and -4245 . In addition, high levels of acetylated H4 were found at -226bp and -6000bp, though the high result was restricted to a single point, in both these cases. In order to verify this result, it would be useful to incorporate more primer pairs close to these regions, and perform more replicates.

Similar patterns of acetylation were seen in the *ΔHHO1, ΔSNF2* and *ΔSSN6* strains (Figures 4.8; 4.10; 4.11; 4.12), though in this case acetylated H4 was localised from -1489 to -3421.

The *ΔTUP1* strain (Figure 4.8; 4.13) shows a markedly altered pattern of acetylated H4 localisation compared to the other strains, with acetylated H4 localised between -4943 and -6000.

Previous figures shown grouped the data according to antigen. The following figures group the same data per experiment and show the localisation of different markers within the same strain. These graphs used the same input chromatin and all reactions were done in parallel, bearing in mind that relative intensities are partly determined by antibody affinity.

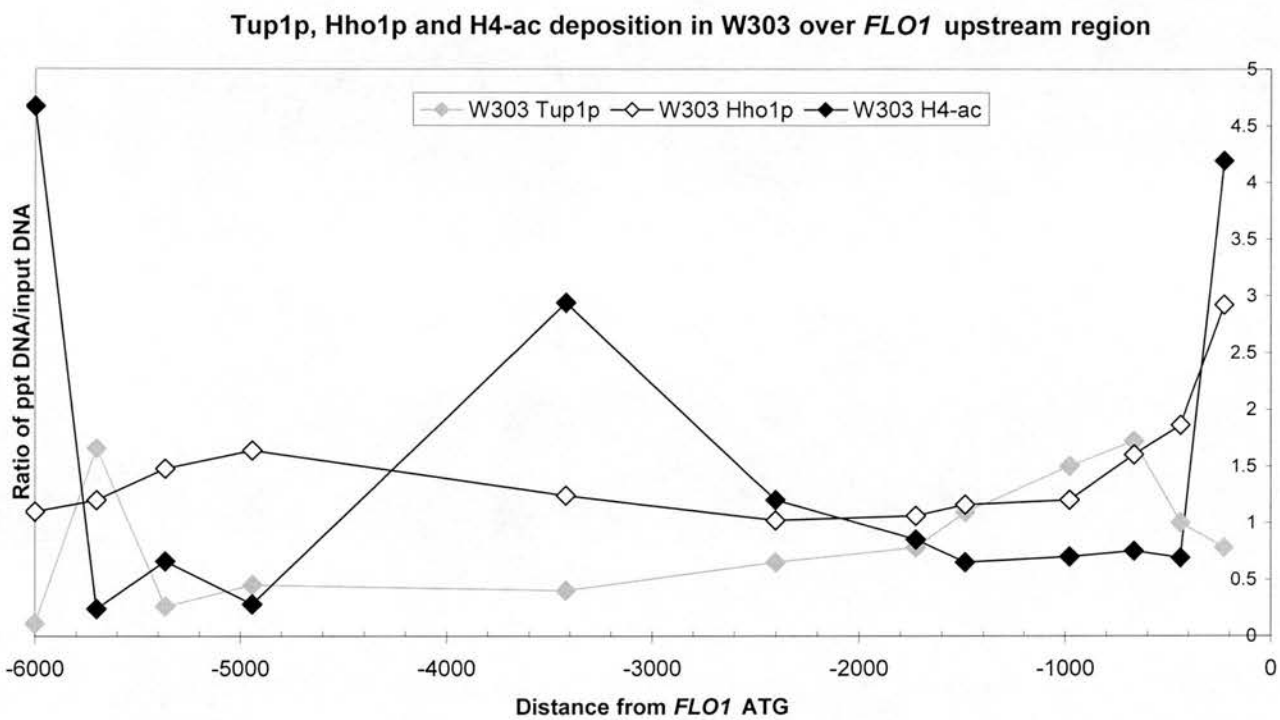


Figure 4.9 Localisation of Hho1p,Tup1p and H4-ac inW303 at the *FLO1* upstream domain. Crosslinked chromatin fragments from wildtype (W303) were immunoprecipitated with antibodies to Tup1 (grey), Myc (Hho1 - white) and acetyl H4 (black) and the DNA content was analysed by PCR. Amplified fragments were gel separated and visualised with ethidium bromide on a phosphorimager. The graph shows an average of two independent experiments.

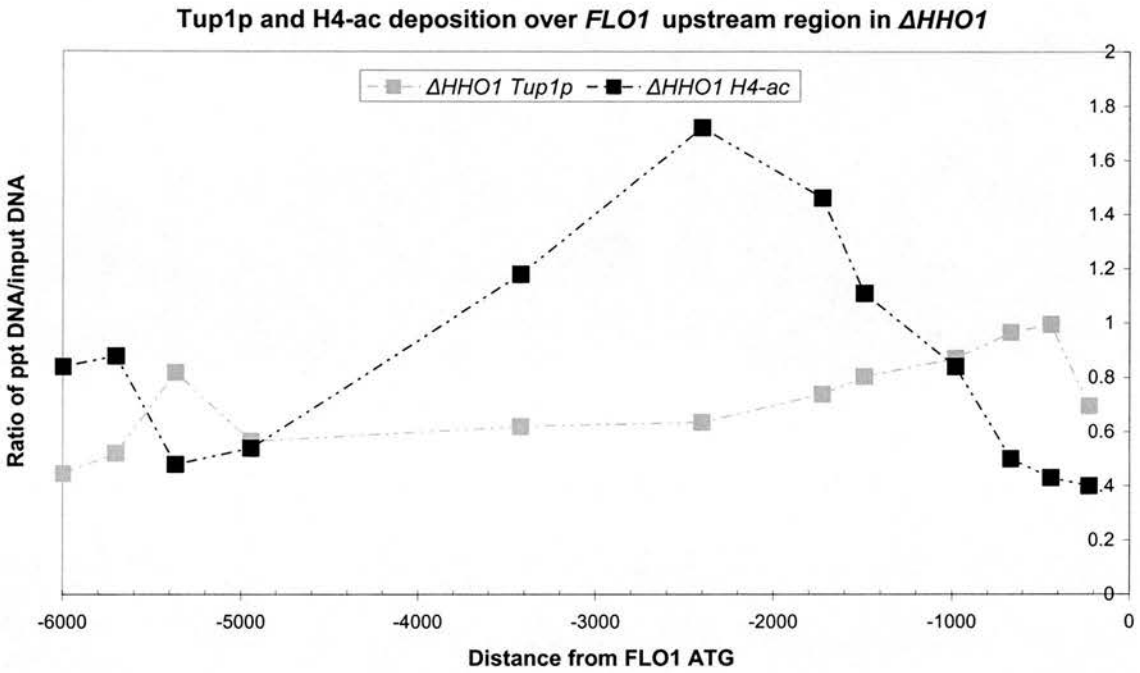


Figure 4.10 Localisation of Tup1p and acetyl-H4 in *ΔHHO1* at the *FLO1* upstream domain. The analysis was the same as in Figure 4.9, except here the ChIP experiments were carried out on a *ΔHHO1* strain immunoprecipitated with Tup1p (grey) and acetyl H4 (black).

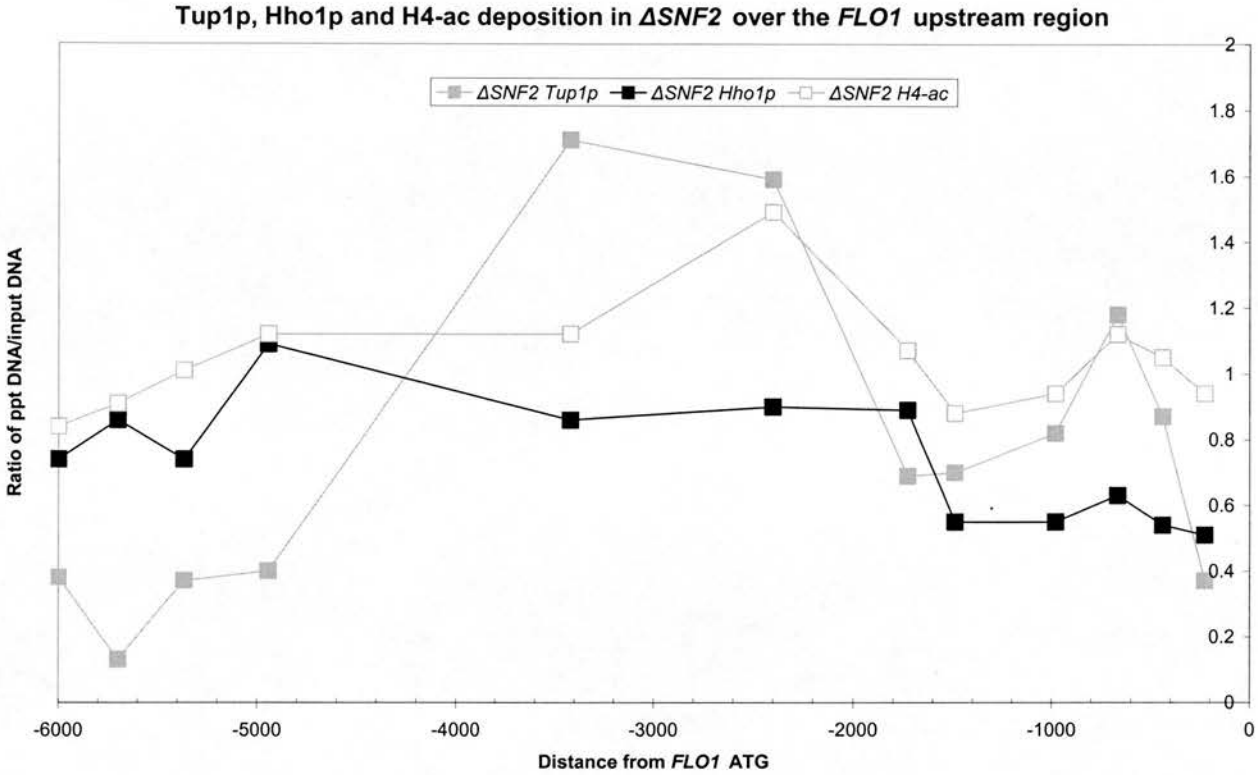


Figure 4.11 Localisation of Hho1p,Tup1p and acetyl-H4 in *ΔSNF2* at the *FLO1* upstream domain. The analysis was the same as in Figure 4.9, except here the ChIP experiments were carried out on a Δ*SNF2* strain immunoprecipitated with Tup1p (grey), Myc (Hho1p- white) and acetyl-H4 (black).

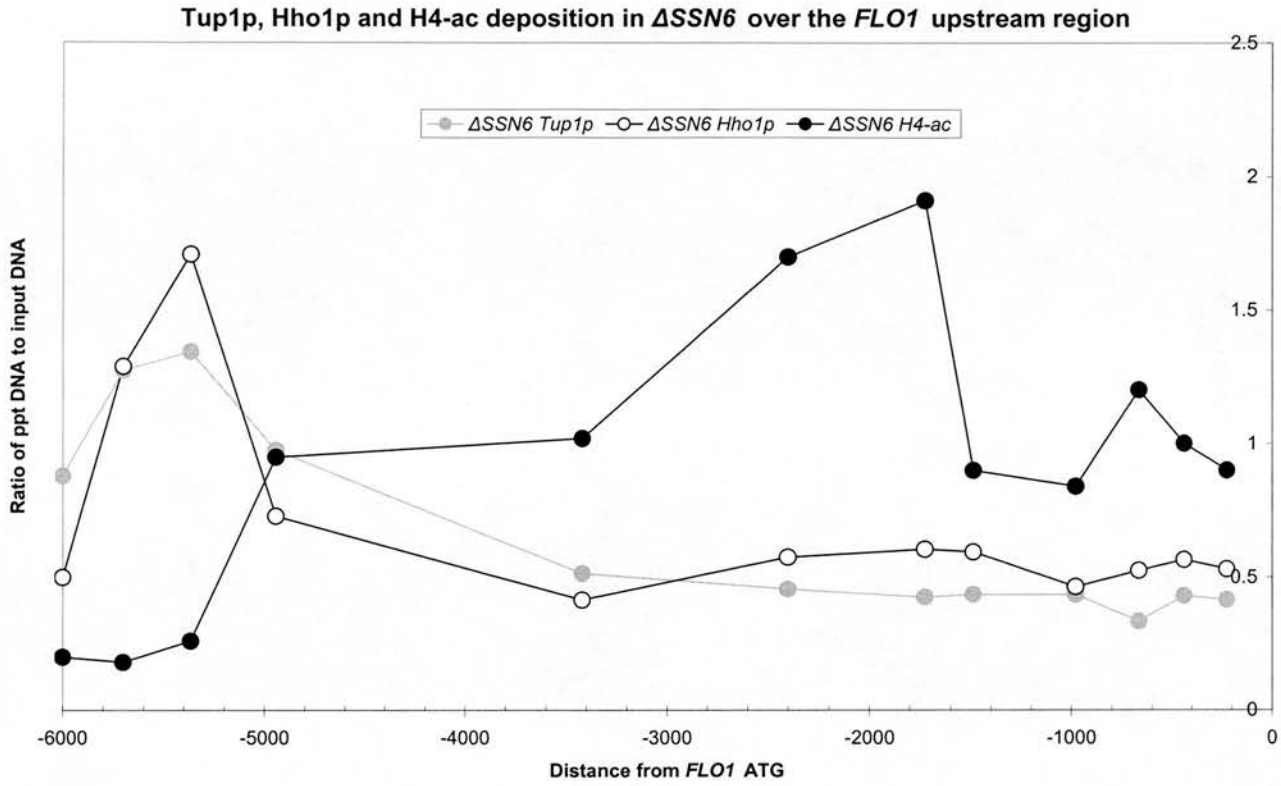


Figure 4.12 Localisation of Hho1p, Tup1p and acetyl-H4 in Δ*SSN6* at the *FLO1* upstream domain. The analysis was the same as in Figure 4.9, except here the ChIP experiments were carried out on a Δ*SSN6* strain immunoprecipitated withTup1p (grey), Myc (Hho1p- white) and acetyl H4 (black)

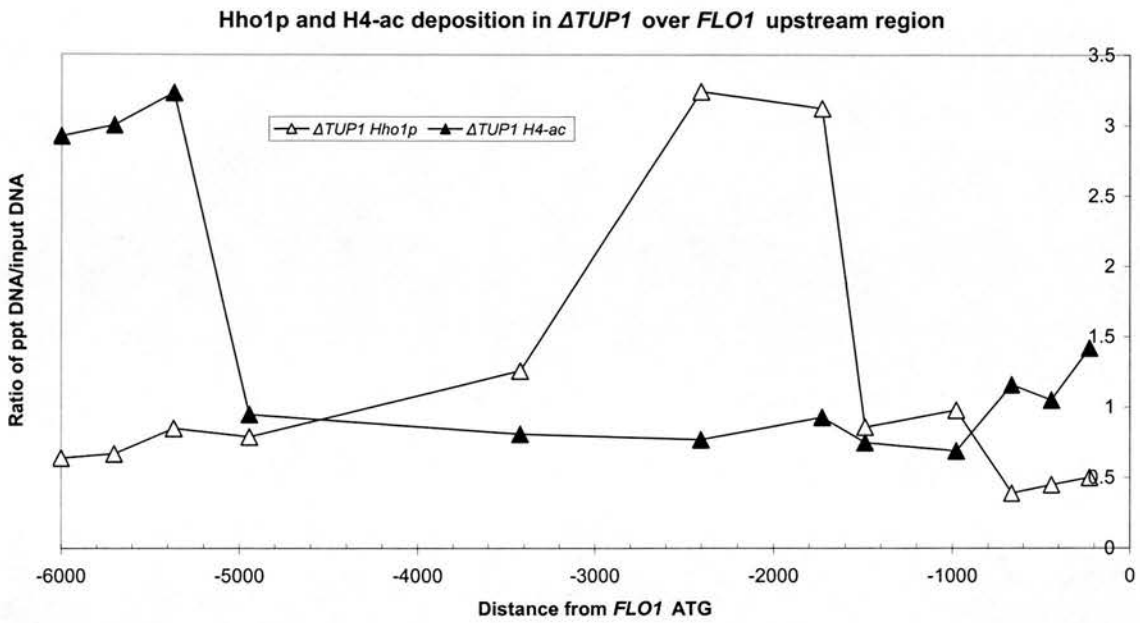


Figure 4.13 Localisation of Hho1p and H4-ac in *ΔTUP1* at the *FLO1* upstream domain. The analysis was the same as in Figure 4.9, except here the ChIP experiments were carried out on a *ΔTUP1* strain immunoprecipitated with Myc (Hho1- white) and acetyl-H4 (black).

4.4 Summary

The localisation of Tup1p over the *FLO1* upstream region in wildtype cells was determined by replicating the experiments of Tsukihashi and Pennings (unpublished). These showed that Tup1p deposition is highly concentrated between -226bp and -1726bp and also further upstream between -5366bp and -6000bp. In the *ΔSNF2* strain, Tup1p is localised in a broad region from -1726bp and -4943bp, though Tup1p localisation can also be seen to a lesser extent between -226bp and -979bp, suggesting an interplay between Tup1-Ssn6 and SWI/SNF.

The *ΔTUP1* strain displayed markedly altered Hho1p and acetylated H4 binding patterns compared to wildtype.

The Hho1p deposition results do not seem to support the hypothesis that Hho1p binds in areas of increased nucleosomal spacing over the *FLO1* upstream region.

	WT	*ΔHHO1*	*ΔSNF2*	*ΔSSN6*	*ΔTUP1*
Tup1p antibody	-226→-1726 -5366→-6000	Not significant.	-1726→-4943	-4943→-6000	Not tested
Hho1p-Myc antibody	-226→-979	Not tested	Not significant	Not significant	-1489→-3421
H4-acetyl antibody	-226 -6000 -1726→-4245	-1489→-3421	-1489→-3421	-1489→-3421	-4943→-6000

Chapter 5

Chromosomal Conformational Analyses

5.1 Introduction

The complexity of the tertiary arrangement of chromatin extends beyond the 30nm fibre, since DNA arranged in this conformation attains the ability to organise itself into active and inactive regions i.e. euchromatin and heterochromatin, and is highly dynamic (Heun *et al.*, 2001).

Chromosomes are known to occupy specific territories within the nucleus (Cremer *et al.*, 2000) and in some cases euchromatic regions are seen to extend out from the body of the chromosome in defined loops containing active genes. These loops are found to extend towards regions of the nucleus which contain RNA polymerase II (Osborne *et al.*, 2004). This suggests that the nucleus operates efficiently by confining RNA polymerase to specific regions in order to prevent improper transcription, for instance to prevent constitutively inactive genes from being switched on. The latter is further ensured by the binding of proteins that confer an inactive conformation, which prevents activators from binding.

Chromosome looping allows distal genes, separated by tens of kilobases (in higher eukaryotes) to colocalise to discrete foci (Osborne *et al*, 2004). Active genes are therefore shepherded into these foci when the need arises for them to be switched on, and conversely transcription is switched off when they leave these foci. Active genes are therefore shuttled to pre-assembled transcription sites, rather than transcription complexes being assembled every time a gene needs to be activated (Osborne *et al.*,

2004). In this way several active genes may occupy the same transcription factory (Jackson *et al.*, 1998), which might enable the nucleus to co-ordinate the timing of the activation of distal genes involved in the same pathway. The migration of genes to these nuclear subcompartments might therefore play an important role in gene expression. The higher order chromatin structure, as well as histone modifications may affect the binding capacity of a particular site, thereby affecting its residence time in the transcription factory.

Chromosomal looping also adds a level of control over transcriptional elements in gene clusters separated by large distances, by bringing them into close proximity (Tolhuis *et al.*, 2002). In the human beta globin gene locus, the chromosome conformation enables the locus control region to interact with one gene of the cluster and its flanking hypersensitive sites at a time, and in so doing forms an active chromatin hub. In this case, chromosomal looping enables distal regulatory regions of select genes to come into juxtaposition to control expression. The locus control region's proximity to the gene is imperative for proper functioning of the active chromatin hub (Patrinos *et al.*, 2004). Interestingly, the gene is still active when the promoter is deleted and the locus control region is intact and proximal to the gene, however, when additional specific hypersensitive sites in the locus control region are introduced, the active control hub is no longer maintained. The locus control region therefore adopts a specific conformation and in so doing ensures the activity of the beta globin gene, by forming multiple interactions with the gene.

In yeast, a further role for chromosome looping was proposed after studies performed on the *Saccharomyces cerevisiae FMP27* and *SEN1* genes (O'Sullivan *et al.*, 2004). The average size of yeast genes is 1.6 kb, and at 7887 and 6696, respectively, these genes are relatively long. RNA polymerase II is localised at both the promoters and terminators of these genes, perhaps as a result of the transcriptional elongation (Figure 5.1). The chromosome is seen to loop, thereby juxtaposing the initiator and terminator, possibly defining the start and end sites of the transcription unit in this fashion. Gene loops might therefore play an important role during the early phases of transcriptional activation.

5.2 Rationale

The *FLO1* upstream regulatory region is under the influence of the Tup1/Ssn6 co-repressor complex. Interestingly, Tup1p deposition is enriched at two sites, and the aim therefore, was to determine if these sites were proximal *in vivo*. This could be of functional relevance to *FLO1* regulation and/or its chromatin environment. Tup1p has been reported to associate with longer stretches of chromatin in some instances, and has been shown to form folded chromatin structures (Ducker and Simpson, 2000).

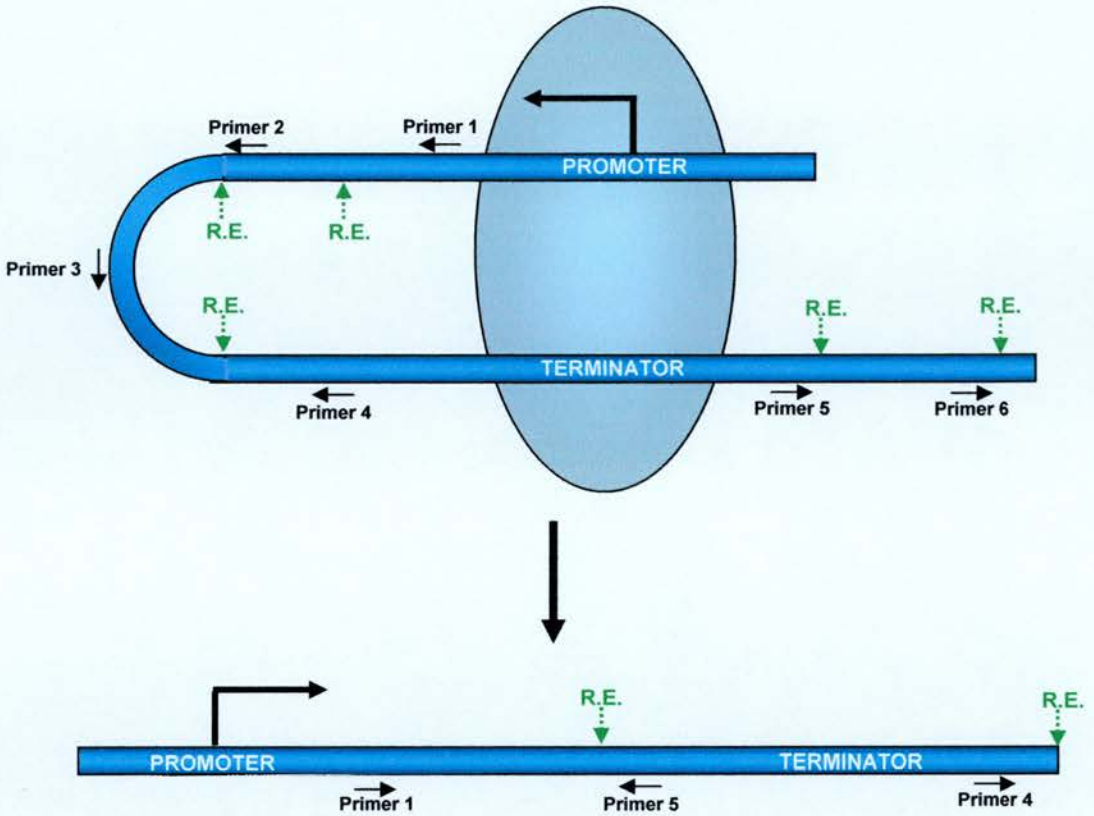


Figure 5.1 Model of a Transcription Gene Loop.

Promoter and terminator sequences are in close proximity at the yeast *SEN1* gene locus. The numbers depict oligonucleotide primers (1-6), and the green arrows depict *Eco RI* restriction enzyme cut sites (R.E.). RNA polymerase (light blue oval) draws the promoter and terminator into close proximity. Following crosslinking, digestion and ligation, the DNA is decrosslinked and amplified. A possible PCR fragment resulting from an amplification event with Primer 1 and 5 is shown. Adapted from O'Sullivan *et al.*, 2004.

5.3 Methodology used in this chapter:

Chromosome Conformation Capture Analysis (or 3C Analysis) is a technique used for determining the spatial arrangement of one part of a chromosome in relation to another (Figure 5.2). The technique was developed by Kleckner and colleagues (2002), and initially was used on chromosome III in yeast to show that its telomeric ends are in proximity. Chromosome III is the shortest yeast chromosome and can circularise as a ring structure (Dekker *et al.,* 2002) and has also been found to be proximal to chromosome VI, as determined by Gasser and colleagues (Bystricky *et al.,* 2005) using high resolution microscopy. This study showed that the nucleus is an ordered structure and that rather than moving freely in solution, the chromosomal telomeric ends are tethered to the nuclear membrane, and that certain chromosomes showed a preferential proximity to others.

The 3C Analysis technique (Figure 5.2 generates a population average of juxtaposition between any two genomic loci, thus providing information on their relative proximity in the nucleus (Dekker *et al.*, 2002). Formaldehyde is used to fix cells, which forms DNA-protein and protein-protein cross-links between regions of the genome in proximity (Figure 5.2). Subsequent restriction enzyme digestion and intra-molecular ligation produces novel junctions between restriction fragments in proximity in the nucleus. Novel ligation products can be detected by PCR. The 3C assay can also be used to reveal proximity between active genes and distal genomic elements (Tolhuis *et al.*, 2002).

During interpretation of 3C data, it is important to understand that not all pairs of restriction fragments that provide a positive result, by generating a positive PCR product, are necessarily engaged in a functional interaction in the nucleus. Distal fragments can be cross-linked by formaldehyde simply because they are occasionally near each other in the nucleus, therefore fixation conditions are critical in the 3C assay since increased fixation leads to greater probability of cross-linking resulting in the detection of chromatin fragments that may be in relative proximity in the nucleus but not necessarily engaged in a specific intermolecular interaction with implied function.

In our experiments, the yeast nuclei or cell culture is crosslinked for 2 minutes. The reaction is quenched with glycine and SDS is added to sequester the uncrosslinked protein. The DNA is digested with a suitable enzyme, in this case *BclI*. The nuclei are diluted 25X (determined empirically – see Figure 5.3), to prevent random, intermolecular interactions, followed by DNA ligation overnight. Ligation covalently joins DNA fragments that are crosslinked in the same complex (intramolecular ligation). These ligations reflect crosslinking between otherwise separate restriction fragments which are tethered into close proximity by protein-DNA interactions. The nuclei are then decrosslinked overnight at 65°C to reverse the protein-DNA crosslinks, and the DNA cleaned. The ligated DNA is then subjected to PCR with appropriate oligonucleotide primers and electrophoresed on a polyacrylamide gel, which is stained in Ethidium Bromide, visualised by phosphorimaging and quantitated using AIDA 2.1 software (Raytek). As is expected, a vast array of possible ligation products could form, yielding an assortment of PCR products (Figure 5.4). It is therefore essential that the necessary controls are performed.

5.4 Control experiments

As a positive control, and also to confirm that the technique was reproducible in my hands, we analysed the *SEN1* gene, which formed a loop structure in a previously published study (O'Sullivan *et al.*, 2004; Ansari and Hampsey, 2006). This gene has an extensive open reading frame, with 6.7 kb between the promoter and terminator region. The latter were found to be held in close spatial proximity by a protein-DNA complex at the proposed gene loop (Figure 5.1). In order to test that the PCR fragments only formed under experimental conditions, we included two further reaction conditions with all yeast strains used in this study. These strains were wildtype W303::Myc (Lane 1, 6 & 11), *ΔHHO1* (Lanes 2, 7 & 12), *ΔSNF2* (Lanes 3, 8 &13), *ΔSSN6* (Lanes 4, 9 & 14) and *ΔTUP1* (Lanes 5, 10 & 15). Under the first condition, the chromatin was not subjected to either restriction enzyme digestion or intramolecular ligation, while in the second, ligation was omitted, but restriction enzyme digestion proceeded as normal. These controls were necessary to confirm that the DNA fragments produced by PCR could only be amplified in the experiments where the DNA in the initial experiments had been both restriction enzyme digested and ligated, and were therefore not produced by an experimental artefact.

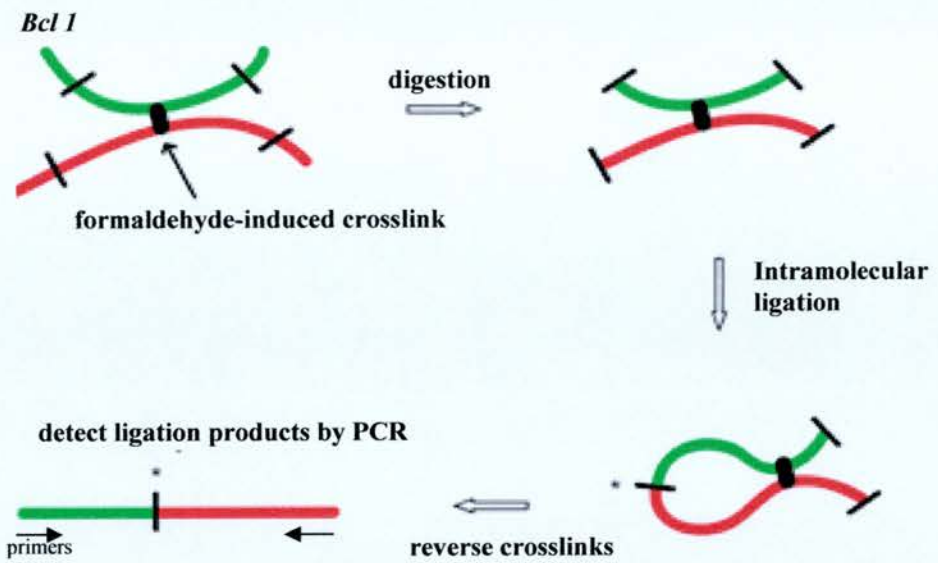


Figure 5.2 An overview of the 3C technique. Fixation with formaldehyde is followed by *Bcl 1* digestion and intra-molecular ligation. Cross-links are reversed and novel ligation products are detected by PCR (adapted from Dekker *et al.*, 2002).

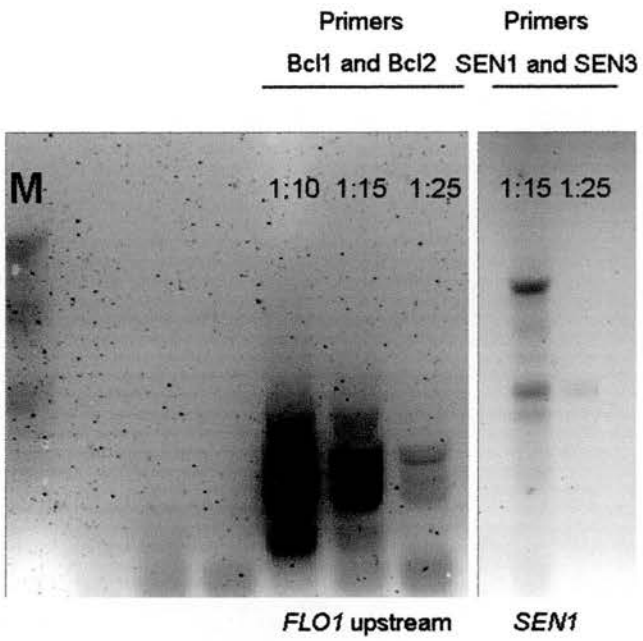


Figure 5.3 Determination of nuclei dilution factors.

The 3C technique was performed by diluting the nuclei 1:10, 1:15 and 1:25 before ligation in order to determine the optimal dilution factor. By carrying out the technique to completion with all the dilutions and then amplifying the resultant DNA with primers Bcl1 and Bcl2, as well as primers SEN1 and SEN3, the optimal nuclei dilution factor of 1:25 was determined. DNA was electrophoresed on a 1X TBE/1% agarose gel, stained using ethidium bromide and visualised using a phosphorimager in the fluorescent mode.

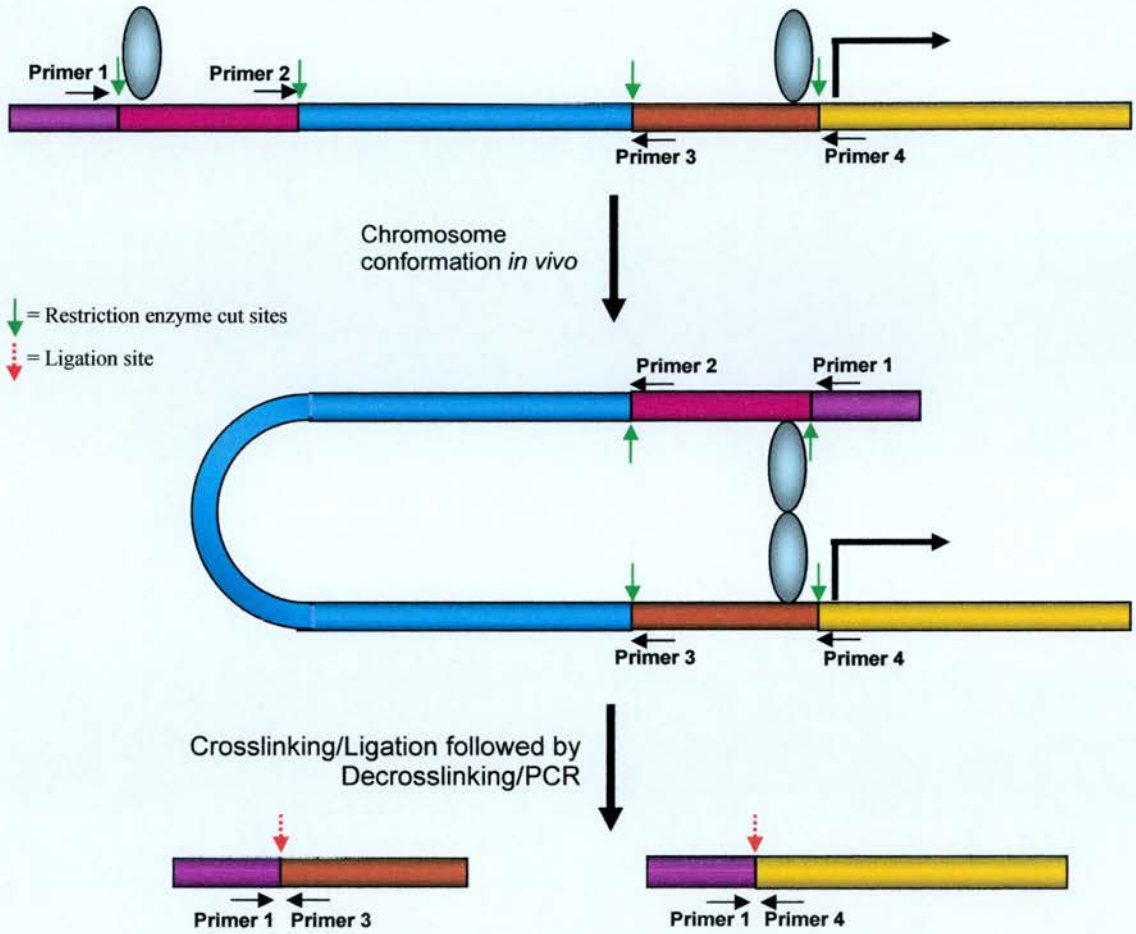


Figure 5.4 Many possibilities of ligation products and PCR fragments.

DNA is denoted by the coloured ribbons. Protein is denoted by the grey discs. The green arrows depict restriction enzyme cut sites. For any given ligation there are a host of ligation events and subsequent PCR products. Control experiments are therefore essential.

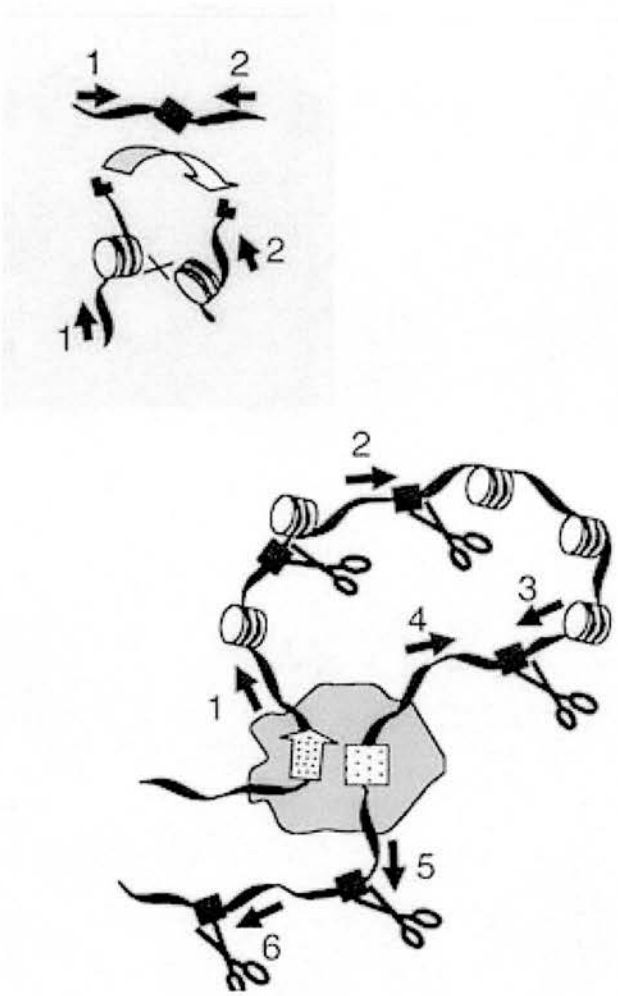


Figure 5.5 3C Analyses at the *SEN1* gene locus using *EcoR1*

In order to determine whether the restriction enzyme digested DNA ligated with fragments in close proximity, 3C Analysis was performed on the *SEN1* gene locus. The DNA is depicted by the ribbon and the nucleosomes are depicted by spools (not drawn to scale). The scissors depict restriction enzyme cut sites and the arrows depict oligonucleotide primer sets. In our case, PCR was performed between primers 1 and 2 and also between primers 1 and 5. Reproduced from O'Sullivan *et al.*, 2004.

In the control experiment at the *SEN1* locus, *Eco R1* is used to digest the crosslinked DNA (Figure 5.5). In Figure 5.6 lanes 1-5 show the undigested and unligated control, while lanes 6-10 show the digested/unligated control. Lanes 11-15 show the PCR products from a complete experiment where both restriction enzyme digestion and ligation have taken place. The PCR reactions performed on the DNA produced from the control experiments (Lanes 1-10) yielded no fragments in all strains, which confirmed that the bands produced in lanes 11-15 could only produced where both restriction digestion and ligation had proceeded.

The absence of PCR product with primers SEN1 and SEN4 (Figure 5.6B) in all strains indicated that these nonadjacent *Bcl 1* fragments did not ligate and consequently were not in close spatial proximity. By contrast, primers SEN1 and SEN5 (Figure 5.6A) produced products indicative of intramolecular ligation. These data replicate the findings of the previous studies and show that the promoter and terminator regions are in close spatial proximity and that the 3C technique is working in our hands. The oligonucleotide primer sequences were the same as in the O'Sullivan paper, however, in later experiments looking at the *FLO1* locus, new *SEN1* primers were designed as *Bcl 1*, rather than *EcoRI* was used. This was necessary because the *FLO1* upstream domain has only two, inconveniently situated *EcoR1* sites (Figure 5.7A), while the *Bcl 1* sites are more suitable. The new *SEN1* primers were designed approximately 250bp from the cut site. This generated a PCR fragment of 500bp.

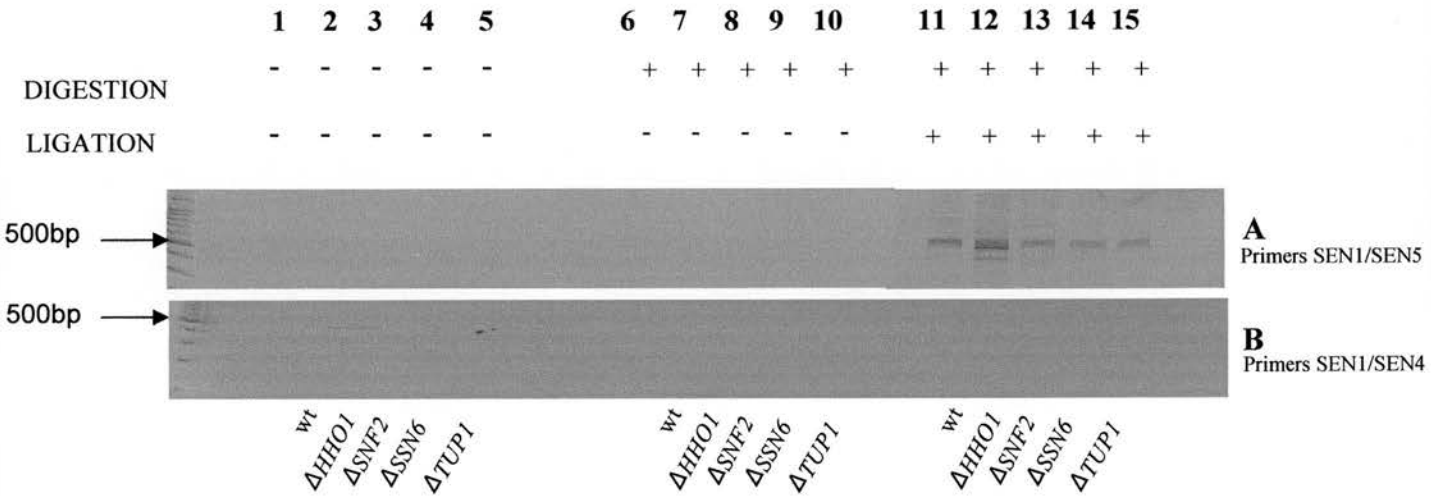


Figure 5.6 Chromosome conformation capture at the *SEN1* locus.

Lanes 1-10 show the negative control lanes which contain either undigested/unligated or digested/unligated samples. Lanes 11-15 show the PCR products digested/ligated samples. Row A shows the PCR products of primers SEN1 and SEN5, while Row B shows the PCR products of SEN1 and SEN4. Lane 12 shows a few non-specific bands. These strains were wildtype W303::Myc (Lane 1, 6 & 11), *ΔHHO1* (Lanes 2, 7 & 12), *ΔSNF2* (Lanes 3, 8 &13), *ΔSSN6* (Lanes 4, 9 & 14) and *ΔTUP1* (Lanes 5, 10 & 15).

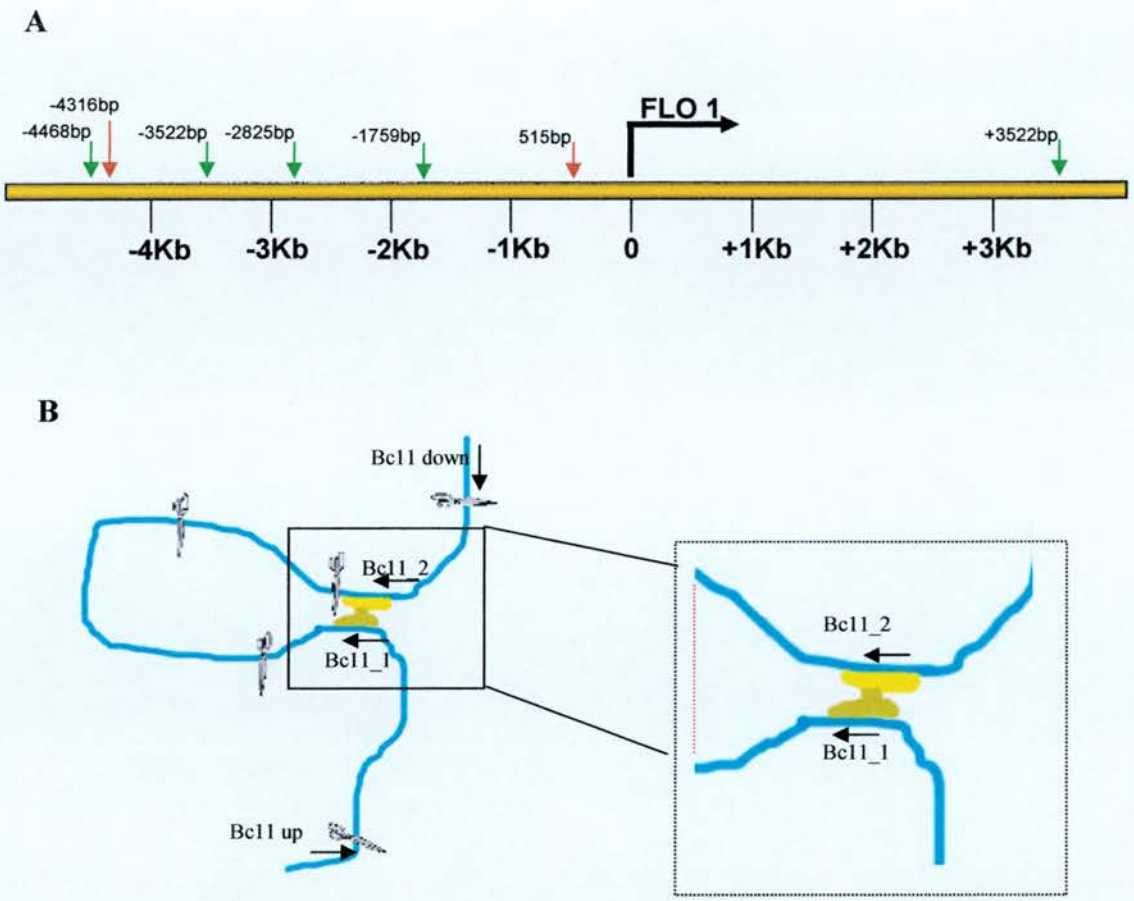


Figure 5.7 3C Analyses at the FLO1 gene locus using *Bcl1*

A shows the restriction enzyme cut sites at the *FLO1* locus. *Bcl1* sites are depicted by the green arrows, while *EcoR I* sites are shown by red arrows. In order to determine whether the restriction enzyme digested DNA ligated with fragments in close proximity, 3C Analysis was performed on the *FLO1* gene locus (**B**). The DNA is depicted by the blue line (not drawn to scale). The scissors depict restriction enzyme cut sites (*Bcl 1*) and the arrows depict oligonucleotide primer sets. In our case PCR was performed between primers Bcl1_1 and 1_2 and also between primers Bcl1 upstream and Bcl 1 downstream. The inset shows that a ligation event (red dotted line) between two fragments.

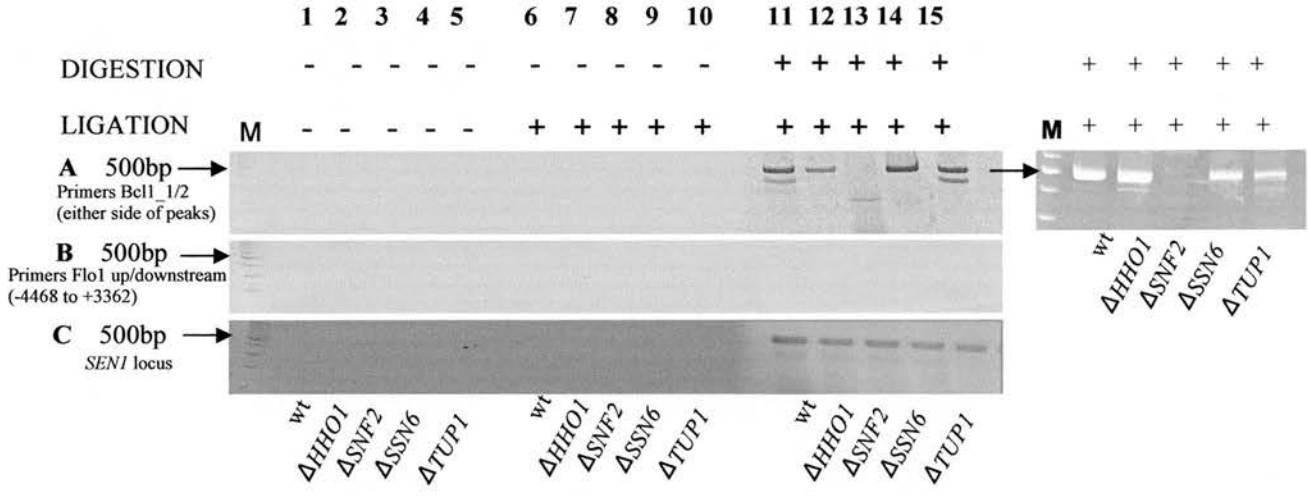


Figure 5.8 Chromosome conformation capture at the FLO1 locus.

These strains were wildtype W303::Myc (Lane 1, 6 & 11), *ΔHHO1* (Lanes 2, 7 & 12), *ΔSNF2* (Lanes 3, 8 &13), *ΔSSN6* (Lanes 4, 9 & 14) and*ΔTUP1* (Lanes 5, 10 & 15). Row A shows the PCR products of Primer Bcl1_1 and Bcl1_2. These are two separate experiments. Positive signals occur in lanes 11, 12, 14 and 15. A non-specific band is present in *ΔSNF2*. Row B shows the PCR products of Primer Bcl1 upstream and Bcl1 downstream. Row C shows the PCR product at the *SEN1* locus. The 500bp marker band is denoted by the black arrow.

5.5 Chromosome conformation at the *FLO1* upstream locus

The hypothesis that the *FLO1* upstream region exists as a gene loop structure can be investigated by means of chromosome conformation capture. 3C analysis was carried out on a region of DNA surrounding the *FLO1* gene in wildtype W303::Myc, *ΔHHO1*, *ΔSNF2*, *ΔSSN6* and *ΔTUP1* yeast strains (Figure 5.7B). These strains were wildtype W303::Myc Lane 1, 6 & 11), *ΔHHO1* (Lanes 2, 7 & 12), *ΔSNF2* (Lanes 3, 8 &13), *ΔSSN6* (Lanes 4, 9 & 14) and *ΔTUP1* (Lanes 5, 10 & 15). Control experiments were included so as to ensure that a positive result occurred as a result of both restriction enzyme digestion and ligation of DNA fragments.

In Figure 5.8 lanes 1-5 show the undigested, unligated control, while lanes 6-10 show the restriction enzyme digested/unligated controls. Lanes 11-15 show the PCR products of DNA produced by the full 3C procedure, which have been amplified by oligonucleotides specifically designed for these experiments. Oligonucleotides were designed 250bp up-or downstream of a *Bcl1* restriction enzyme cut site.

Lanes 1-10 all gave negative results suggesting that a positive result could only occur in cases where both restriction enzyme digestion and ligation had taken place. The absence of PCR product with primers Bcl 1 upstream and Bcl 1downstream in all strains indicated that these nonadjacent *Bcl1* fragments did not ligate and consequently were not in close spatial proximity.

By contrast, primers Bcl1_1 and Bcl1_2 produced a DNA fragment of 500bp indicative of intramolecular ligation (Figure 5.8). This fragment was produced in all strains apart from in the *ΔSNF2* strain (Lane 13 – A). This experiment was repeated to show that the result was reproducible. These data provide evidence that the two Tup1p-bound domains are in close spatial proximity in the wildtype strain. suggesting that a loop structure between the 1kb and 5kb Tup1-Ssn6 localised regions in the *FLO1* upstream domain exists under wildtype conditions, however the loop structure also occurs in *ΔHHO1*, *ΔSSN6* and *ΔTUP1*. This suggests that Tup1p is not responsible for tethering the chromatin in such a way, as to form the loop.

The results in Figure 5.8, are encouraging, however, primers Bcl_1 1_1 and Bcl_1 1_2, in addition to producing the expected PCR fragment size, also produced additional non-specific bands, which were present in lanes 11-15. The experiment was repeated, but non-specific bands were seen there too. In order to verify the data, it is therefore necessary to sequence all PCR fragments, and also repeat the 3C experiment with another restriction enzyme. The latter would allow one to rule out the possibility that a *Bcl 1* restriction cut site is being obscured by formaldehyde crosslinking.

The failure for the *ΔSNF2* chromatin to produce a loop seems to suggest a different chromosome conformation and might occur as a by-product of the changes in the nucleosome positioning, which alters the accessibility of the DNA to DNA-binding proteins. As seen in the chromatin immunoprecipitation experiments (Chapter 4), the acetylation pattern as well as the Tup1p pattern in this strain differs from that seen in wildtype DNA. In the *ΔSNF2* strain, the acetyl H4 in the region between 1kb and 5kb in the wildtype strain, is replaced withTup1p. This suggests that the deposition of Tup1p in a region which is flexible under wildtype conditions, causes the chromatin to revert to a less flexible state, which prevents a loop from forming. In addition, the nucleosome positioning in the *ΔSNF2* strain appears to be altered in this region (Fleming and Pennings, 2002), suggesting that an altered nucleosome positioning could also affect the flexibility of chromatin. As a means of testing this hypothesis, we could repeat the 3C analyses in a *TUP1/SNF2* double knockout strain to investigate the possibility that Tup1p-binding is responsible for the loss of flexibility in the *FLO1* upstream region. This would also further investigate the interplay between Tup1p and Snf2p suggested by Fleming and Pennings (2002).

5.6 Summary

The aim of this chapter was to determine whether two Tup1p-bound domains were in close spatial proximity, using the chromosome conformation capture technique (Dekker *et al.*, 2002).

Our initial experiments reproduced data from a previous report in order to confirm that this procedure could work in our hands. We found that at the *SEN1* gene locus, the promoter and terminator sequences were in close spatial proximity (Figure 5.6), which confirmed the work by O'Sullivan *et al* (2004). In this paper, they attributed the conformation to the action of RNA pol II.

In our own experiments at the *FLO1* gene locus, we found that the areas of Tup1p localisation in the wildtype strain (at 1kb and 5kb upstream of *FLO1*), were in close proximity (Figure 5.8) in all strains except the *ΔSNF2* strain (Figure 5.8 – lane 13). This may occur due to the altered Tup1p binding pattern found in this yeast strain.

However, these results need to be verified due to the occurrence of non-specific bands, the identity of which needs to be determined. Moreover, the identity of the bands corresponding to the expected product sizes for Primer Bcl 1_1 and Bcl1_2 need to be confirmed by sequencing.

Chapter 6

General Discussion

6.1 Introduction

The *FLO1* gene is an example of a gene controlled by its chromatin environment. Chromatin remodelling events are observed far upstream from its promoter sequence (Fleming and Pennings, 2001) in an extended gene-free region of 6Kb where a 30-nucleosome array was mapped (Figure 4.1). Apart from remodelling in the upstream region, additional remodelling events occur at the proximal promoter sequence. The chromatin remodelling over the extended *FLO1* upstream region, has been attributed to the SWI/SNF co-activator and Tup1-Ssn6 co-repressor complexes, which function antagonistically.

Tup1p is known to interact with deacetylated histone H3/H4 tails (Edmondson *et al.*, 1996), whereas promoter sequences are associated with acetylated histone H3/H4 tails (Bone and Roth, 2001). Therefore *FLO1* gene activity may be influenced by the acetylation state of the promoter, which we hypothesized would be connected with the presence and location of Tup1p.

A number of mutants for Tup1-Ssn6 and SWI/SNF components were analysed in the Fleming and Pennings (2001) study and some of these showed an increase of 20bp in DNA linker length at various locations in the 32-nucleosome array (Figure 4.1). This increase may have been caused by the actions of chromatin remodellers altering the chromatin structure. The increased linker length might allow transcription factors to

bind, but it might also enable linker histones to bind in these regions. Yeast linker histone contains two globular domains, rather than just the one seen in higher eukaryotes (Landsman, 1996), which might in part account for the decreased stoichiometry of linker histone to nucleosome seen in yeast (Freidkin and Katcoff, 2001; Downs *et al.*, 2004). Yeast linker histones, nevertheless, do not bind to every nucleosome, and therefore we hypothesized that Hho1p would bind in regions of increased nucleosomal spacing.

6.2 Does Hho1p bind in regions of increased nucleosomal spacing?

Linker histones are thought to compact the chromatin and prevent spurious binding of transcription factors, by competition for their binding sites (Kermekchiev *et al.*, 1997). They also have an important role to play in the activation state of genes, as was shown by Bhattacharjee *et al* (2001).

In the wildtype strain Hho1p was localised between -226bp and -979. This binding pattern was not seen in any of the other strains examined. In the *ΔSSN6* and *ΔTUP1* strains, where the *FLO1* gene is active, Hho1p is removed from the proximal promoter site, perhaps as a result of the promoter occupancy associated with gene activity. This is consistent with findings at the MMTV promoter which demonstrated that linker histones are not present at active promoters in that system (Bresnick *et al.*, 1992).

It is surprising that Hho1p was not localised in the inactive *FLO1* upstream region in *ΔSNF2*. In humans, linker histones have been implicated in the modulation and inhibition of SWI/SNF-induced chromatin remodelling (Hill and Imbalzano, 2000;

Ramachandran *et al.*, 2003). Therefore, one might have expected an increase in Hho1p deposition in *ΔSNF2*. Conversely, the presence of SWI/SNF in the absence of Hho1p might have a greater effect on chromatin remodelling, and this could be investigated by evaluating the nucleosome positioning array over the *FLO1* upstream region in *ΔHHO1*. However, this experiment remains to be completed. It is worth noting that the *FLO1* gene is not switched on by *HHO1* depletion.

Finally, in *ΔTUP1*, Hho1p deposition is altered greatly. Unfortunately, the nucleosome positioning array over the region of interest has yet to be determined. However, one may speculate that at the *FLO1* gene, Tup1-Ssn6 might alter the chromatin into a more repressive structure. This process could be aided by the binding of Hho1p, which further compacts the DNA into a less flexible state, which in so doing, it might block the binding of transcriptional activators.

The initial aim was to compare the Hho1p deposition pattern with existing data generated by Fleming and Pennings (2001), which had shown that regions of nucleosome repeat-length of 180bp existed in the *FLO1* upstream region. However, the results could not be correlated, partly due to the ChIP probes not overlapping the regions of increased DNA linker length. In order to test this hypothesis more stringently, ChIP primer pairs could be designed to specifically amplify regions of increased linker length.

The absence of Hho1p at the inactive promoter of the *ΔSNF2* strain, with the same nucleosomal spacing as wildtype, is at face value, not consistent with the possibility that Hho1p might bind opportunistically in regions of increased nucleosomal spacing

(180bp linker length). This needs to be tested more fully, for example in *ΔSNF1 ΔTUP1* double mutants, as the possibility emerging from this study is that the locations of Hho1p, Tup1p and Snf2p may be interdependent.

6.3 Is Tup1p deposition altered?

Tup1p and Ssn6p both have an array of DNA-specific binding partners, and therefore, the complexes could function in different ways, depending on the proximity of certain proteins. Three mechanisms have been proposed for how Tup1-Ssn6 interacts with chromatin. Firstly, Tup1-Ssn6 is thought to alter the chromatin structure into a repressive state; secondly, it is thought to inhibit RNA polymerase from initiating transcription; and thirdly, it is thought to block transcriptional activators (Smith and Johnson, 2000). Tup1-Ssn6 repression may also differ at specific genes (Tzamarias and Struhl, 1995). Alternatively, because Tup1p is known to associate with HDACs (Watson *et al.*, 2000), it may change the acetylation patterns in the vicinity of where it is bound.

Tup1p has been localised at the promoters of a number of genes studied: *STE6, STE2, RNR2, SNF2* promoters (Ducker and Simpson, 2000; Davie *et al.*, 2002; Boukaba *et al.*, 2004). Two alternate modes of Tup1-Ssn6 binding have been proposed. Tup1-Ssn6 is thought to either continuously polymerise along the chromatin fibre (Ducker and Simpson, 2000) or localise at distinct foci (Wu *et al.*, 2001). To test these hypotheses, we mapped the 6Kb *FLO1* upstream sequence for Tup1p, using the chromatin immunoprecipitation technique in the wildtype strain and various mutant strains.

In the wildtype Tup1p was localised at two foci i.e. -226 to -1726 and -5366 to -6000. At face value, it appears that Tup1p does not polymerise across the chromatin but rather binds at discrete foci. However our results are not definitive. Tup1-Ssn6 may polymerise across the chromatin but our crosslinking might not allow us to see this as Tup1p lies too far away from the chromatin. Nevertheless, more extensive crosslinking with DMA did not confirm this in wildtype strains (Tsukihashi and Pennings, in preparation).

In *ΔSSN6* the Tup1 deposition at the binding site proximal to the *FLO1* start site disappears. Possibly it is displaced by activator complexes, since the gene is active in this strain, while another explanation is that Ssn6p is required to recruit Tup1p.

Interestingly, though still being localised at two foci, the Tup1p localisation differs greatly in the *ΔSNF2* strain i.e. -226 to -979 and -1726 to -4943. This hints at the antagonistic relationship existing between Tup1-Ssn6 and SWI/SNF (Fleming and Pennings, 2001), whereby SWI/SNF may antagonize Tup1-Ssn6 by controlling chromatin remodelling activity (Gavin and Simpson, 1997). The altered Tup1p deposition pattern may be a by-product of the absence of SWI/SNF chromatin remodelling over this region, disrupting this balance.

6.4 Is Tup1-Ssn6 binding influenced by changes in acetylation?

Tup1-Ssn6 has been found to be associated with Hda1p deacetylase activity at the *ENA1* and *STE6* promoters (Wu *et al.*, 2001; Davie *et al.*, 2002). We observed a Tup1-Ssn6 dependent H4 deacetylation across the *FLO1* upstream region. In the wildtype strain, H4 acetylation is seen from -1726bp to -4245bp upstream of the

FLO1 start site, and also at sharp peaks at -226bp and at -6000bp. Tup1p is known to interact with deacetylated histone tails, and in correlation with this, acetylation seems to taper close to regions where Tup1p is present, and then increase where Tup1p is absent.

The acetylation state of the *FLO1* upstream region was similar in *ΔHHO1*, *ΔSNF2*, and *ΔSSN6* mutants which showed H4 acetylation from -1489bp to -3421bp.

H4 acetylation pattern are greatly altered in *ΔTUP1* i.e. -4943 to -6000. Upon activation of the *FLO1* gene, the histone tails at the promoter sequence are acetylated and might therefore lose their affinity for Tup1-Ssn6. This correlates with work performed on the *PHO5* promoter, which demonstrated that the promoter is heavily acetylated when the gene is active (Svaren and Hörz, 1997; Vogelauer *et al.,* 2000). It is surprising to note that the altered acetylation pattern extends up to 6Kb from the promoter sequence. This, however, is consistent with the result of Fleming and Pennings (2001), which showed that SWI/SNF could remodel nucleosomes up to 5Kb upstream in *ΔSSN6.*

6.5 Do the two Tup1p peaks interact with each other?

Chromosome looping allows distal genes to colocalise in discrete foci (Osborne *et al.*, 2004), and also controls transcriptional elements separated by large distances, by bringing them into close proximity (Tolhuis *et al.*, 2002). This is evident at the human beta globin gene locus, where chromatin looping brings about a distinct conformation that allows the gene to be active (Patrinos *et al.*, 2004). At certain yeast genes e.g.

SEN 1, the initiator and terminator sequences are brought into proximity by RNA polymerase II (O' Sullivan *et al.*, 2004), possibly defining a transcriptional unit.

In the wildtype strain, Tup1p is localised in two distinct regions. We hypothesized that if Tup1p was not continuously "polymerised" across the region, then perhaps these two peaks might be proximal, to allow the chromatin to take on a long-range repressive higher order structure.

Chromatin conformation capture was performed on wildtype, *ΔHHO1, ΔSNF2, ΔSSN6 and ΔTUP1*. The results suggest that the chromosome adopts a looped structure in all strains except in the Δ*SNF2* mutant. However, we can not attribute the chromosomal looping to the Tup1p peaks as looping takes place in *ΔSSN6* which does not exhibit any Tup1p binding at the proximal binding site. Chromosomal looping also takes place in Δ*TUP1*, therefore the looping must be caused by some hitherto unknown protein, or as a consequence of the acetylation pattern over the region, or may instead require the absence of Tup1p across the intervening upstream region.

No 3C positive signal was obtained for the *ΔSNF2* mutant using primer *Bc1*1_1 and *Bcl* 1_2. Unique to the *ΔSNF2* mutant, Tup1p invades the -2000bp to -4000bp upstream region. It is tempting to conclude that the Tup1-Ssn6 repressive structure is preventing the chromosome from looping. The *ΔSNF2* mutant might also adopt a different chromosome conformation due to changes in the nucleosomal positioning array, which is most regular in this mutant (Fleming and Pennings, 2001).

Histone acetylation levels are known to modulate ATP-dependent nucleosome positioning (Krajewski, 2002). Changes in the acetylation status of histones are known to influence the folding of the nucleosomal fibre (reviewed by Eberharter *et al.*, 2005), suggesting that the inability of the *ΔSNF2* mutant to loop occurs as a function of its acetylation state, it Tup1p deposition status and the nucleosome array profile.

6.6 Future work

6.6.1 Chromatin immunoprecipitation

I would scan the *FLO1* upstream region for total histone H4, so as to infer the histone H4 baseline. It would also be interesting to compare the H4 acetylation levels seen in our study, with an acetyl H3 antibody, to elucidate whether all core histones are affected in the same way, as these acetylation pattern reflect to different extents the activated and poised states.

The number of replicates for experiments would be increased, primer pairs would be redesigned to cover the areas which were not probed in our experiments, and quantitative methods (real-time PCR) would be added to further substantiate our results.

6.6.2 Chromatin conformation capture

My current hypothesis is that the *FLO1* upstream region loops as a function of its H4 acetylation state and its Tup1p deposition profile. Firstly, I would verify my findings with quantitative PCR. Secondly, I would repeat the experiment using another

restriction enzyme to ensure that the experiment can be replicated with another enzyme. The experiment will also need to be repeated, so that the PCR fragments can be subcloned into a p-GEMT-easy vector and sequenced, so as to verify that the PCR fragment is made up of the correct ligation event. The dependence of looping on the Tup1p deposition profile would be assessed in *ΔSNF1 ΔTUP1* double mutants

6.6.3 Indirect end-labelling and nucleosome positioning

Throughout this study, it was clear that it was necessary to perform nucleosome positioning assays on *ΔTUP1* and Δ*HHO1* strains as well as *ΔSNF1 ΔTUP1* mutants. This work has been started (Appendix III). This might link the changes in protein binding demonstrated in the chromatin immunoprecipitation studies to nucleosome linker length.

6.7 Conclusions

I have investigated the chromatin environment of a long-range DNA domain extending over 6Kb upstream upstream of the *FLO1* gene in yeast. This gene is under the influence of antagonistic relationship between the Tup1-Ssn6 co-repressor complex and the SWI/SNF co-activator complex (Fleming and Pennings, 2001). This relationship seems to be acted out at the level of histone acetylation, a dynamic histone modification that allows for a quick switch between the active and inactive states and also may influence the flexibility of the chromatin fibre, either indirectly or directly.

Appendix I

Oligonucleotide primers

3C Analyses:

Bcl1 1_1	5' –ATG GTC GTT TAA GGC CTG AAG A -3'
Bcl1 1_2	5' –AAC ATA AGG TGA GCA ACG TCT -3'
Bcl1 upstream	5' –GTC ACA TTC AAC AAT GGA ATC T- 3'
Bcl1 downstream	5' –TAC ATG GGT GCA ATT CCT TGT G- 3'
SEN1	5' –CCA ACA ACT CAA GAA ACA GCT C- 3'
SEN2	5' –GCA TCT TCA ATC AAA TCT CTC CA -3'
SEN3	5' –AGC GCG GAT GAA GAT TAC AA -3'
SEN4	5' –AAA TTT AAT AAT GCT GTG ACT AA -3'
SEN5	5' – GCT TCA TCT ATA ATG ACC GTA -3'

Knockout Primers:

Ssn6knA 5' -GCA GCA GTT CCT CAG CAG CCA CTC GAC CCA
TTA ACA CAA TCA GCT GAA GCT TCG TAC GC -3'

Ssn6knB 5'–AAC AGA AGC TGC TTT GGT AGC TTC TTC AGC
AGG ACT AGC TGC ATA GGC CAC TAG TGG ATC TG -3'

Tup1kanMXA '5 –AGC AGG GGA AGA AAG AAA TCA GCT TTC CAT
CCA AAC CAA TCA GCT GAA GCT TCG TAC GC -3'

Tup1kanMXB 5' -GCC GGA TTT CTT ATC CCA AAA CAG GAC ACC
ACG ATC TTT GGC ATA GGC CAC TAG TGG ATC TG-3'

Snf2kanMXback 5' -ATG AAC ATA CCA CAG CGT CAA TTT AGC AAC
GAA GAG GTC CAG CTG AAG CTT CGT ACG C-3'

Snf2kanMXforward 5'CTA TAC ACT CGC TTC TGT CAT GCT CGA GTC
CGC TTC ATC TGG CAT AGG CCA CTA GTG GATCTG3'

Southern blot Primers for testing Knockout Strains

Snf2_For	5'-GGC TAT TCT GAG TGA ACA TAA GG-3'
Snf2_Rev	5'-CGA AAG TCG TGA AAA TAG CAG-3'
Tup1KanSB1	5'-TTA CAT TAT CGC TAC CGA CGG-3'
Tup1KanSB2	5'-TGG TTT GGA TGG AAA GCT GA-3'
Ssn6KanSB1	5'-GCT TAA TAC GGA ACC AGA GTC A-3'
Ssn6KanSB2	5' -CCA CAACAT AAT GAA TGA ATT G -3'

Northern blot primers for probes checking FLO1 upstream region expression

NB1000_1:	5'-ACG TAA TTG GTA ACG ATG AGG G-3'
NB1000_2:	5'-GAG CTT ACA TCA ACG AGC AAG A-3'
NB2000_1:	5'-GTG TAT TGT CTG CAA CAT CTG A-3'
NB2000_2:	5'-TCG CGT ATT ATG CTA GGT TGT G-3'
NB3000_1:	5'-ATG CTG TAT GAT GTT GAG CGG-3'
NB3000_2:	5'-CAA TTC ACC TCG GTG CAT TA-3'
NB4000_1:	5'-GAA TGG TTC GTT CAA GAG TCC A-3'
NB4000_2:	5'-AAA CCA GGT ATG GCC TAG AGT T-3'
NB5000_1:	5'-TGG TTC TCG GCT TCT TGT TCT-3'
NB5000_2:	5'-GAT ACT GAA GAC ATT TCA TTC G-3'

Additional probes used in chapter 3:

FLO1Pr	Full-length *FLO1* gene, isolated from plasmid YY10 after Eco RV digest
ACTPr	PCR amplified, contains *ACT1* ORF sequences between +411 and +1422

Chromatin immunoprecipitation oligonucleotide primers

YT1/IPFLO1A	5'-TCTCTGGTAAAGAGCTCTGC-3'
YT2/IPFLO1B	5'-GGATGTTCTGTTTACTGGTG-3'
YT3/IPFLO2A	5'-CAGAGGTATTGTGGAACCTTC-3'
YT4/IPFLO2B	5'-GCCGTTAATGCTGATTGTTG-3'
YT5/IPFLO3A	5'-AGCTTTTGGCTTCCAGTATG-3'
YT6/IPFLO3B	5'-AATGAGCAGAGGAAGGCTAG-3'
YT7/IPFLO4A	5'-GGTAAGTCTCATTACCTAAAC-3'
YT8/IPFLO4B	5'-CTAGTCGAATGTTCTCTTGC-3'
YT9/IPFLO5A	5'-CTCGCTAATCGTTAGTGGGA-3'
YT10/IPFLO5B	5'-ACAGGATCGGGGAAAGATAC-3'
YT11/IPFLO6A	5'-CTAAGAAGAGCCACTAAAC-3'
YT12/IPFLO6B	5'-GTAGAGGATGTTCCTACAAG-3'
YT13/IPFLO7A	5'-ATTTGCCTTCATGACCCAC-3'
YT14/IPFLO7B	5'-AGCGGAATTGTTGCAAGAGG-3'
YT15/IPFLO8A	5'-CTATGTAACGTTCACTCTTAC-3'
YT16/IPFLO8B	5'-CTAAACCAGGTATGGCCTAG-3'

YT17/IPFLO9A	5'-GTAACACAAAGCTCCACTGG-3'
YT18/IPFLO9B	5'-TTTTTGGTGGGCAGAAGTGC-3'
FLO9.1A	5'-ATCAAAACTCATTAGCTTCGG-3'
FLO9.1B	5'-ATCGCTGGGAATGAGCAATA
FLO9.2A	5'-TTCAGTCCCACATGATTCACCT-3'
FLO9.2B	5'-TCTCCTCATCATGGTTTCACCA-3'
YT19/IPFLO10A	5'-AAACAGAAAGGCCGTTACAG-3'
YT20/IPFLO10B	5'-TCGTTTCATCTCTTTGACTG-3'

Appendix II

Construction of *HHO1*-Tagged strains

AII.1 HHO1-Flag tag construction

A 1446bp region, incorporating the *Candida-URA3* gene flanked by Flag-His tags, and 40bp of the C-terminal end of the *HHO1* gene on each side, was amplified from pSP-C-Flag-Ca-Ura (Pennings *et al.*, in preparation) with Vent polymerase (NEB) and template mismatched primers, to introduce *HHO1* homologous sites at the fragment ends (Figure AII.1).

The *Candida albicans URA 3* gene can be used as a selectable marker in *Saccharomyces cerevisiae*. It encodes for uracil and cells containing this marker are therefore able to grow in synthetic complete media which does not contain uracil. The recovered PCR fragment was transformed into the wildtype strain, FY2, using the Lithium acetate transformation method (Schiestl *et al.,* 1995). Yeast cells are able to recognise the incoming fragment by its homology to the genome, and are able to insert the new sequence by homologous recombination. The original fragment contains two Flag-His6 sequences, but since these are also homologous; they can recombine to leave only one Flag-His6 sequence followed by a stop codon. The recombination of the Flag-His6 tag into the 3' end of the *HHO1* in the recovered uracil phototrophs was confirmed in two clones by polymerase chain reaction (Figure AII.2). Following growth on uracil, the colonies were replica-plated onto 5-flouro-orotic acid plates (5-FOA), so as to remove the *URA3* moiety by counterselection, due to toxicity of 5-FOA metabolite. This strain was denoted y*HHO1*-Flag.

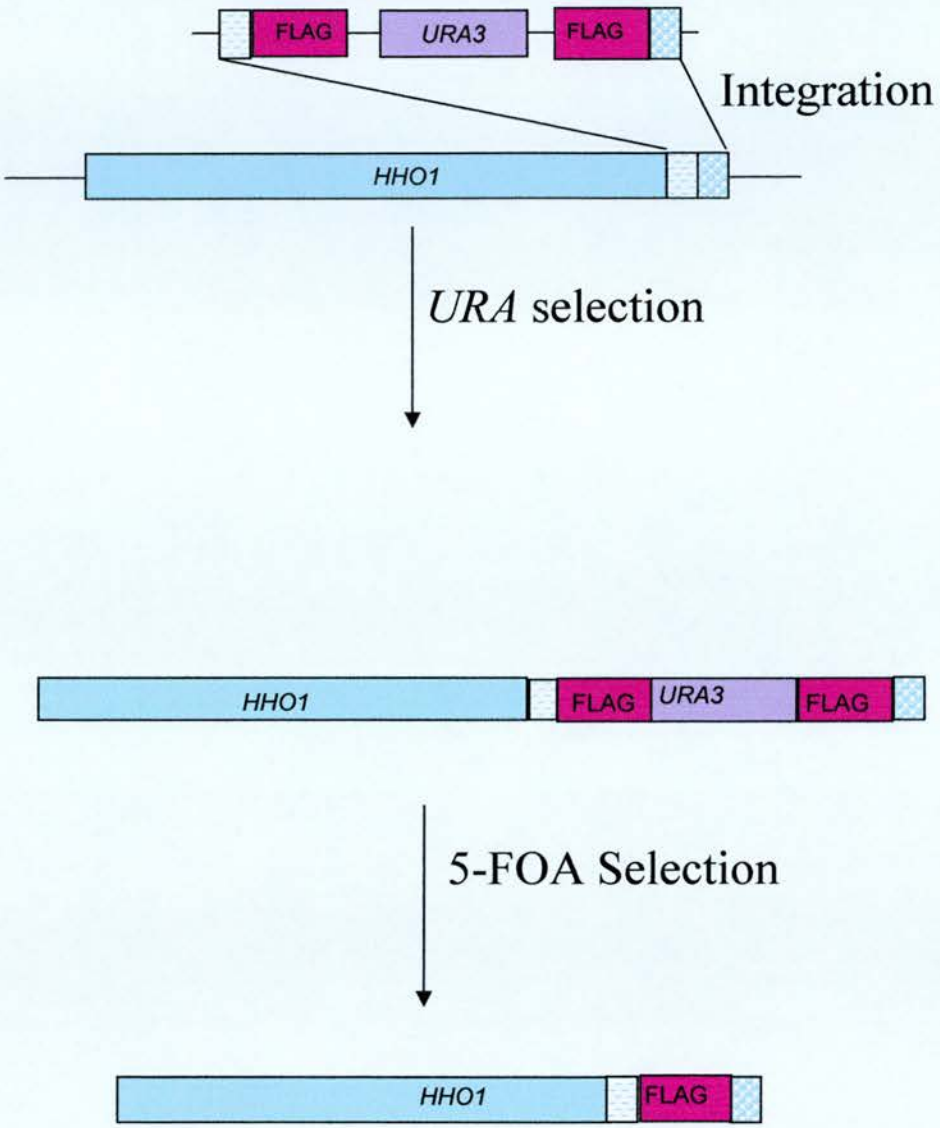


Figure AII.1 Cloning strategy for attaching the Flag-tag to *HHO1*.

The *Candida URA3* gene, flanked by the dual Flag-His6 tag and regions of *HHO1* homologous to the C-terminal end of the gene are transformed into wildtype cells. The transformants are retrieved by *URA* selection. *URA3* is lost by 5-FOA counter-selection.

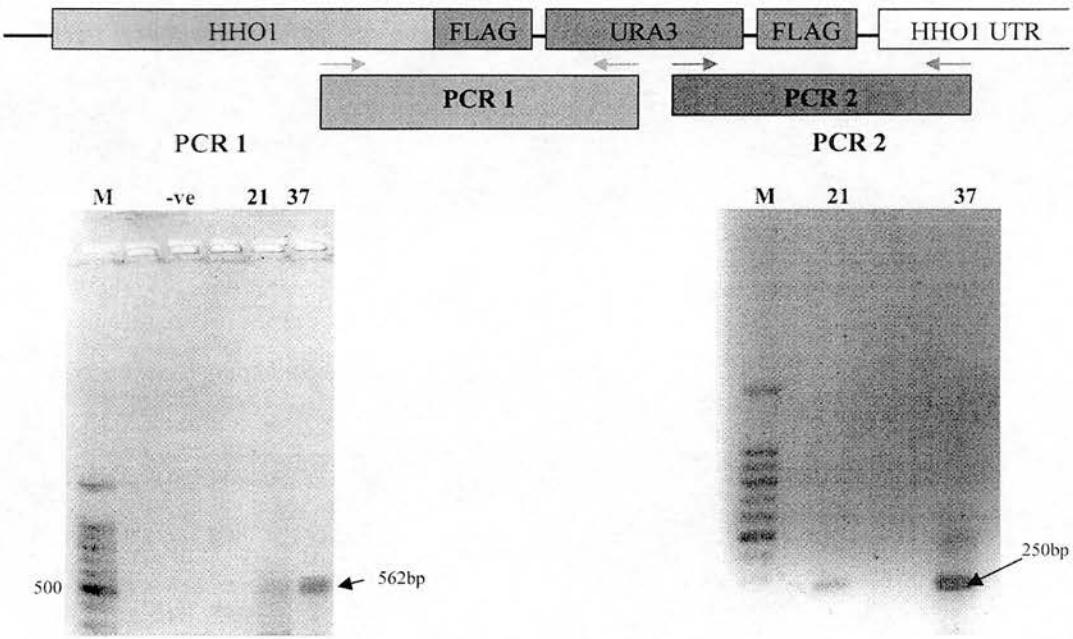


Figure AII.2 PCR screening strategy for selecting positive clones.

Two PCR tests were performed on genomic DNA extracted from clones, in order to detect whether the *HHO1*-Flag fragment had been incorporated into the *HHO1* gene. The expected fragment sizes are indicated by arrows.

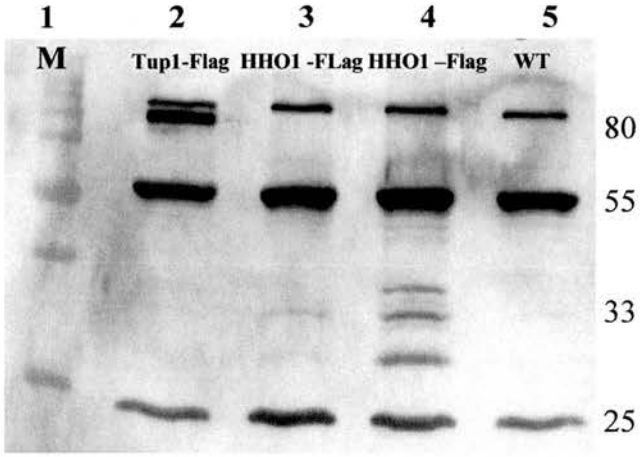


Figure AII.3 Western blot for detecting the HHO1-Flag.

Lane1 contains the protein size marker (M), Lane 2 contains the immunoprecipitated Tup1-Flag (20µl). The Tup1 band is visible at 80kDa. Lane 3 contains 10µl HHO1-Flag immunoprecipitate, while Lane 4 contains 20µl of HHO1-Flag immunoprecipitate. Bands recognised by the Flag antibody appear around 33kDa. Lane 5 contains 20µl wildtype whole cell extract immunoprecipitate. The bands at 55 and 25 kDA correspond to light and heavy chains of IgG.

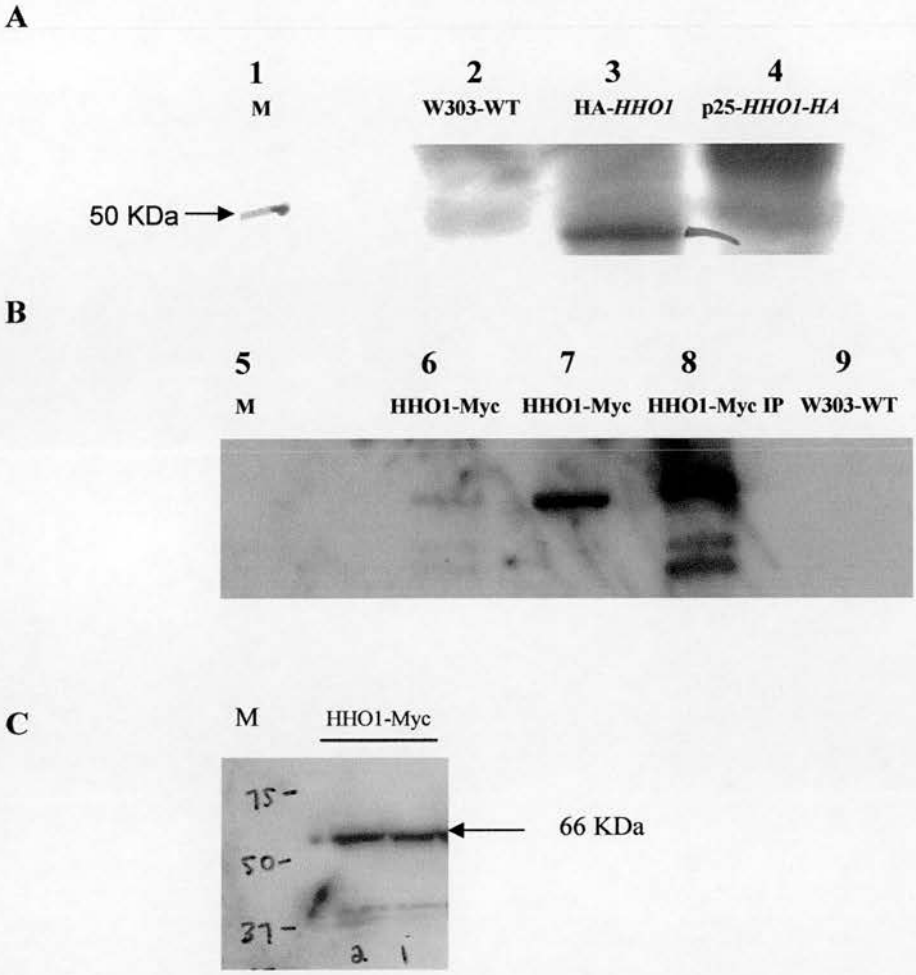


Figure AII.4 Western blot for detecting the HHO1 in tagged strains.

In **A:** Lane1 contains the protein size marker (M), Lane 2 contains the wildtype w303 negative control (20µl). Lane 3 contains 20µl *HHO1*-HA (commercial) whole cell extract, while Lane 4 contain 20µl of p25-*HHO1*-HA (Freidkin and Katcoff, 2001) whole cell extract. Bands recognised by the HA antibody appear around 50kDa.In **B:** Lane 5 contains the protein size marker (however the marker had not been drawn on the X-ray film prior to developing therefore part C of this figure has been included to show the marker.) Lane 6 contains 10µl *HHO1*-Myc whole cell extract. Lane 7 contains 20µl of *HHO1*-Myc (Downs *et* al., 2003) whole cell extract and Lane 8 contains *HHO1*-Myc immunoprecipitate. Lane 9 contains the WT-W303 whole cell extract negative control. In C: Lane 10 contains the protein size marker (KDa), determined by overlaying the X-ray onto the western blot with a prestained broad range marker (BIORAD), and Lane 11 and 12 contain 60µl and 70µl of *HHO1*-Myc respectively. The *HHO1*-Myc is found around 66KDa. The bands around 37 KDa were also seen in Downs et al, 2003 but the authors did not comment on their presence.

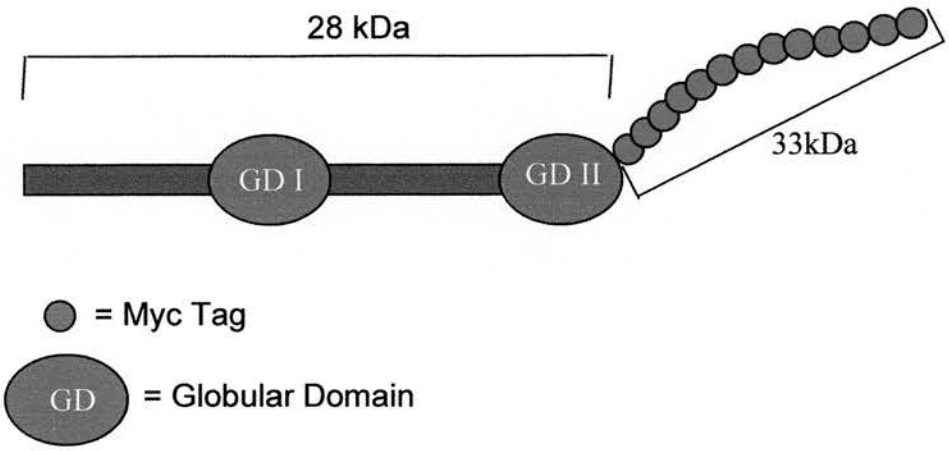


Figure AII.5. Schematic of Myc-tagged Hho1p.

The 33kDa Myc tag is made up of 13 Myc tags attached to the 28kDa Hho1p. The protein runs as 66kDa protein due to the increased electrophoretic mobility of histones. Hho1p is made up of two globular domains separated by a basic linker region.

Unfortunately this strain could not be used in subsequent studies as the western blot showed two additional bands, both larger than Hho1p. (Figure AII.3). The western blot was repeated numerous times with various antibody concentrations, to rule out the possibility that these bands were non-specific, however the bands appeared in all subsequent western blots. The fact that the bands are larger than 33kDa suggests that these are not breakdown products of the protein of interest, however, they could be phosphorylated forms of the linker histone. This could be tested by cutting out the bands and sequencing them, or subjecting them to mass spectroscopy.

A previous study with *HHO1*-2HA tagged plasmid transformed into yeast (p25-*HHO1*-HA; Freidkin and Katcoff, 2001), showed that Western blots with an HA antibody only proved successful when the histones were isolated from nuclei. However, this C-terminally tagged-*HHO1* plasmid when transformed into FY2 did not produce any bands in a western blot (Figure AII.4; Lane 4). When the procedure was repeated on a commercial N-terminally HA tagged *HHO1* (Open Biosystems; Figure AII.4 Lane 3), the western blot was successful, and the Hho1p was detected. However, it could not be detected in ChIP experiments (not shown).

Finally, after the publication of a paper by Downs *et al* (2003) where a *HHO1*-Myc W303 strain was used in chromatin immunoprecipitation experiments, I requested the strain from the authors and all subsequent experiments were performed on this strain. This strain was designated wildtype. Western blot experiments demonstrated that the Myc-tag was visible in both whole cell extract (Figure AII.4; Lane 6, 7, 11 and 12), as well as in immunoprecipitated samples (Figure AII.4; Lane 8).

The Myc-tag comprises 13 Myc tags, each 10 amino acids long (Figure AII.5). Therefore, the Myc tag used in this study is actually larger than the Hho1p itself. Previous groups have found that the second globular domain can fold in 250nM sodium phosphate. It is not certain whether the addition of a Myc-tag to Hho1p will allow the second globular domain to fold correctly, since the tag is larger than the actual protein. However, the binding mechanism of Hho1p on yeast chromatin is also uncertain, and it is therefore not clear whether the binding ability of Hho1p-Myc is altered when compared to the wildtype protein.

The presence of the Myc tag on Hho1p might not necessarily affect the function of Hho1p in the nucleus at all; however, other possibilities need to be discussed. Firstly,the presence of the Myc tag might cause the chromatin fibre to have irregularities where the linker histone is bound, secondly, inappropriate binding of Hho1p might cause altered gene expression. This could be tested by performing microarray experiments on wildtype and *HHO1*-myc strains, as a way of testing whether gene expression is altered when the Myc tag is present. A third possibility, is that the Myc tag does alter the folding of the protein, but the second globular domain might not have a significant role, and therefore the altered protein folding does not impact the gene expression in the nucleus.

The paper from which the Hho1-Myc strain was derived states that the Hho1-Myc strain has no visible defects in phenotype compared to a wildtype W303 strain (Downs *et al.*, 2003). However, it is important to note that their experiments focussed on recombination effects and did not look at flocculation. It remains a possibility that if the folding of the second globular domain was altered when the Myc tag is present,

the subsequent function of Hho1p could be altered. For example, the addition of a tag the same size as the protein could form a "bulge" wherever Hho1p is incorporated, and this might alter the folding of the chromatin. Hho1p has not been directly implicated in any functions other than in homologous recombination to date. It is important, therefore to bear in mind that though using this tag has provided a useful tool for visualising the deposition of the protein by chromatin immunoprecipitation, the results might be skewed by the incorporation of a large tag. It is worth noting that early work on Hho1p using an even larger GFP tag at the C-terminal end did not report any changes in protein folding or function (Ushinsky *et al.*, 1997). Neither did work on human H1 tagged with GFP at the C-terminal end demonstrate any changes in the dynamics of the protein (Misteli *et al.*, 2000).

The findings in this thesis are therefore based on the premise that the *HHO1*-Myc tagged strain does not have an improperly-folded protein compared to wildtype and the function of Hho1p is not altered in this strain.

Appendix III

MNase digests: nuclei vs spheroplasts

Micrococcal nuclease (MNase) test digests were performed on W303 nuclei, which had been prepared by differential centrifugation (Ausubel *et al.*, 2004) and as a comparison, also on W303 spheroplasts prepared according to Kent and Mellor (1995). This was deemed necessary because the differential centrifugation technique is inefficient and the yield of nuclei is very low. I had initially hoped to perform DNase I digests, but time constraints intervened.

The spheroplast preparation was performed as prescribed by Kent and Mellor (1995). Briefly, yeast cells (1 $X10^8$) were harvested and then allowed to sheroplast in a sorbitol solution containing yeast lytic enzyme for 2.5 minutes. The cells are then washed before being transferred to MNase I digestion buffer which contains a detergent which makes the cells permeable to the MNase I. Initially, we used Triton X-100, which is very similar to the Nonidet NP-40. However, we found that the MNase I (1U/ml) digests (refer to materials and methods) were not very efficient, and after consulting the corresponding author of the paper we decided to used Nonidet NP-40 after all (which is no longer manufactured).

Subsequent investigations showed that Triton X-100 and Nonidet NP-40 are very similar molecules which differ in the number of polycarbon chains they possess. Triton X-100 has approximately ten polycarbon chains, while Nonidet NP-40 has about 8 polycarbon chains. This might enable to Nonidet NP-40 to have a slight

advantage over Triton X-100 in disrupting the yeast membrane. However, in other techniques, Nonidet NP-40 and Triton X-100 can be used interchangeably.

Both nuclei and spheroplasts were digested with 1U/ml MNase (Worthingtons) over a timecourse of 0 to 4 minutes. After digestion the material was treated with proteinase K and RNAse A, and ethanol precipitated. The DNA was resuspended in 20µl TE and electrophoresed on a 1% agarose gel (1X TAE) at 100V for 3 hours. The DNA was visualised by staining the gel in ethidium bromide and photographing the gel on a phosphorimager in the fluorescent mode.

Figure AIII.1 shows that nuclei and spheroplasts (**A** and **B**) both digest well with MNase 1, however spheroplasts that are made permeable with Triton X-100 (**C**) as a detergent, digest less well than those made permeable with Nonidet NP-40, suggesting that Nonidet NP-40 is more efficient at permeabilising the cell membrane in yeast. Figure AIII.2 demonstrates an MNase digestion timecourse on spheroplasts produced from wildtype, *ΔSNF2*, *ΔSSN6*, and *ΔTUP1*,.

The MNase digests were conducted as part of a pilot study which was designed to investigate the nucleosome positioning and DNase 1 hypersensitivity in the yeast strains produced during this study. Unfortunately, due to time constraints, this work could not be completed.

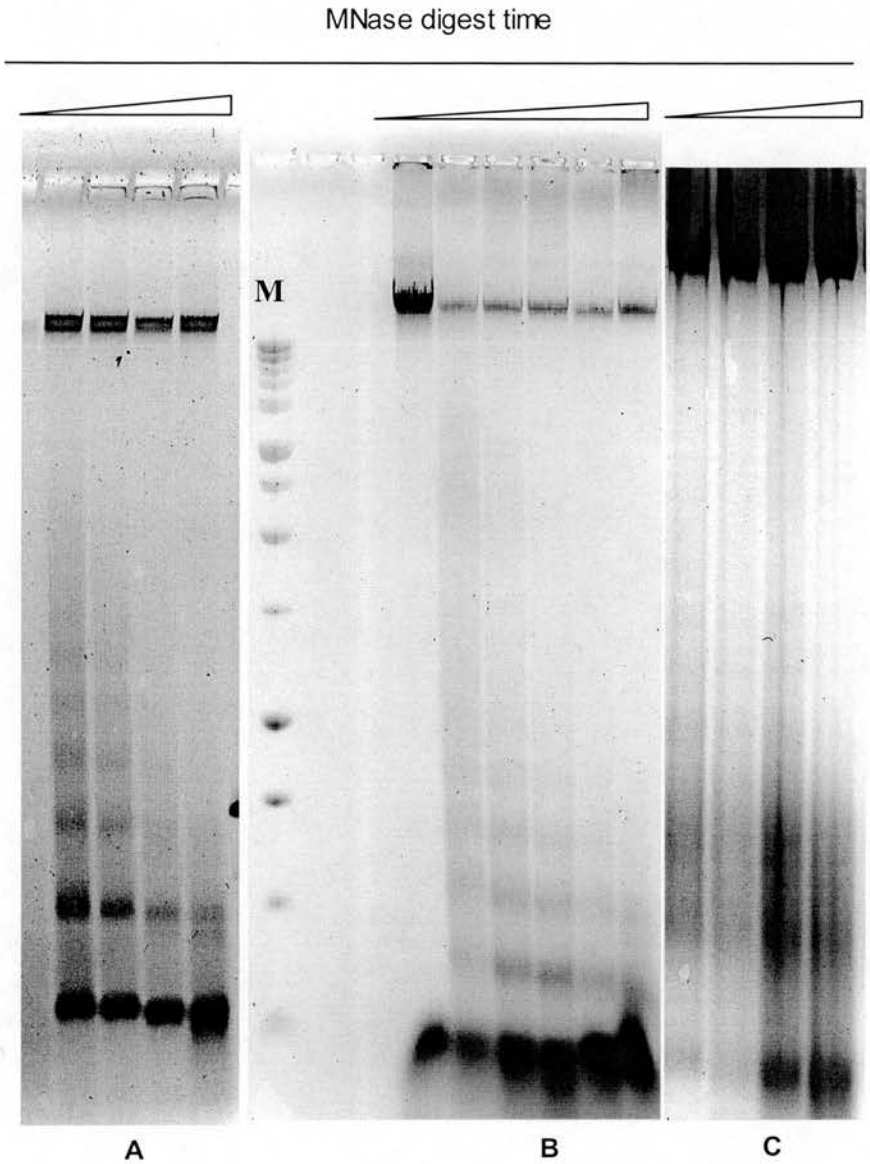


Figure AIII.1 MNase digestion on nuclei and spheroplasts.

Wildtype W303 nuclei (A) were digested with 1U/ml MNase over 0.5, 1, 2 and 4 minutes at 37°C. Digestion **A** shows nuclei digested with MNase, while Digestion **B** shows a spheroplast digestion using Nonidet NP-40 as a detergent. Digestion **C** shows a spheroplast digestion using Triton X-100 as a detergent. Digestions **A** and **B** show similar digestion patterns, while digestion **C** shows a large proportion of undigested material.

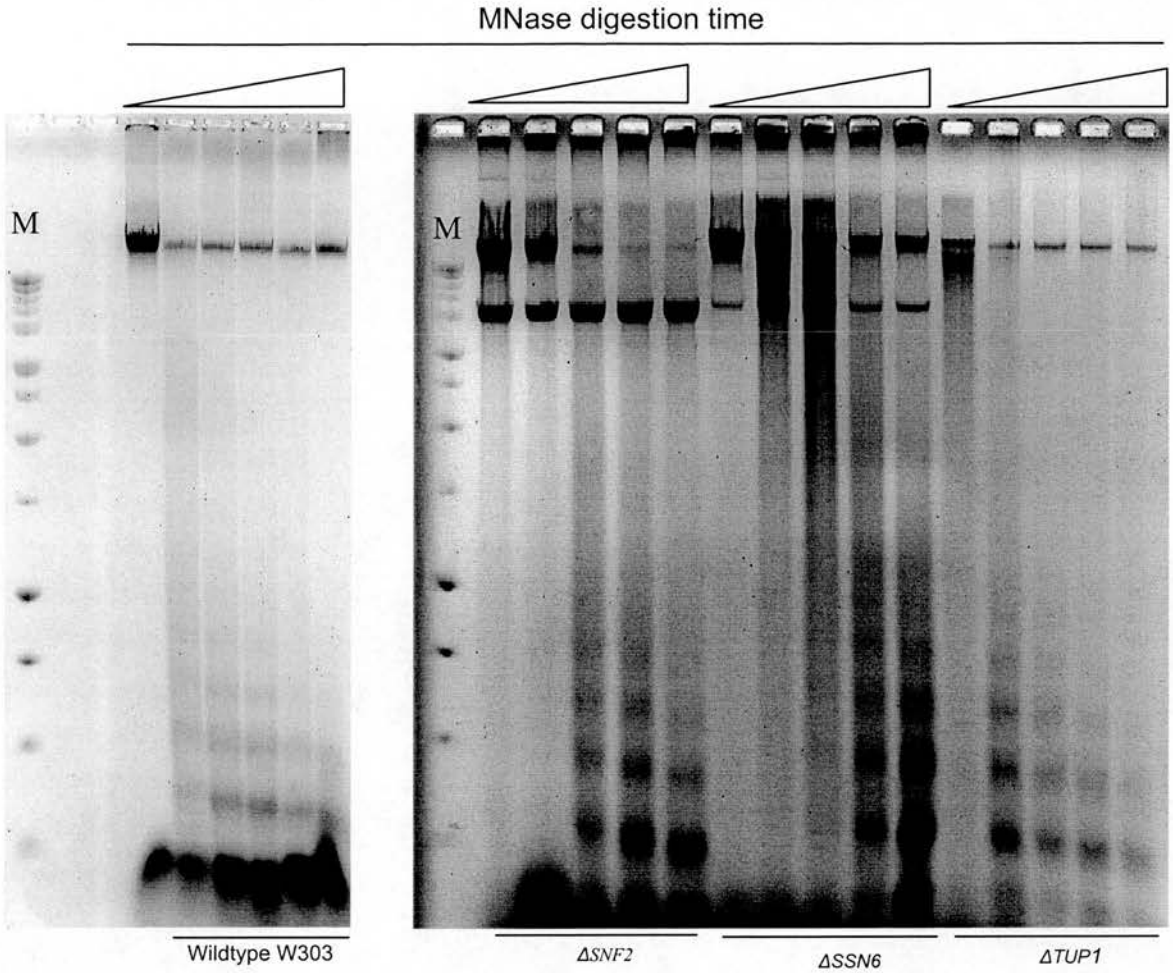


Figure AIII.2 MNase digestion on spheroplasts.

Spheroplasts prepared with Nonidet NP-40 (as a detergent) were digested with 1U/ml MNase over 0, 0.5, 1, 2 and 4 minutes at 37°C. Digests were carried out over a 0 – 4min timecourse on wildtype, *ΔSNF2*, *ΔSSN6* and *ΔTUP1*. DNA was purified by phenol extraction and ethanol precipitation and dissolved in TE, before being loaded and electrophoresed on a 1% agarose gel in 1X TAE.

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