Investigating the role of histones in fission yeast centromere function

Thesis

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INVESTIGATING THE ROLE OF HISTONES IN FISSION YEAST CENTROMERE FUNCTION

Barbara Mellone

Degree of Doctor of Philosophy
Open University
January 2002
PREFACE

This thesis was composed by myself and the research presented is my own unless otherwise stated.

Barbara Mellone

January 2002
A mamma e papa'
ABSTRACT

The centromere is the chromosomal region which is responsible for the accurate segregation of chromosomes during mitosis and meiosis. Failure to properly segregate replicated chromosomes causes aneuploidy and contributes to cancer progression.

Fission Yeast centromeres display several features in common with the centromeres of higher eukaryotes. They are assembled in a specialised silent heterochromatin composed of underacetylated histones and methylated histone H3.

To investigate the role of histone H3 and H4 N-tails in centromere structure and function, conserved lysines of histone N-termini were mutated to mimic hyperacetylated or non-methylated states. Since the fission yeast haploid genome contains three copies of both histones H3 and H4, initially a strain harbouring a single H3 and H4 gene was generated and analysed. This phenotypically wild type strain provided a genetic background in which to perform site-directed mutagenesis of the histone tails. These mutants showed that the H4 tail is not critical for silencing, while the H3 tail plays an essential role and is required for centromere function.

Centromeric nucleosomes contain an essential histone H3-like protein, CENP-A. Antibodies were raised against the fission yeast CENP-A homologue Cnp1 and were used to map Cnp1 association with the central domain of the centromere. Cnp1 appears to replace histone H3 in this domain and can coat a large fragment of non-centromeric DNA artificially inserted within this centromeric domain. Furthermore, strains expressing more histone H3 than H4 showed delocalisation of Cnp1, alleviation of centromeric silencing and missegregation of chromosomes in mitosis indicating that a fine balance between histone H3 and H4 is important for centromere function and that histone H3 can compete with Cnp1 in nucleosome assembly.
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<th>Abbreviation</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>ATP</td>
<td>adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin Immunoprecipitation</td>
</tr>
<tr>
<td>CENP</td>
<td>Centromere Protein</td>
</tr>
<tr>
<td>C-terminal</td>
<td>carboxy-terminal</td>
</tr>
<tr>
<td>DAPI</td>
<td>4,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>dH₂O</td>
<td>distilled water</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxynucleotide triphosphate</td>
</tr>
<tr>
<td>ECL</td>
<td>enhanced chemiluminescence</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene-diamine-tetraacetic acid</td>
</tr>
<tr>
<td>EGFP</td>
<td>Enhanced Green Fluorescent Protein</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>5-FOA</td>
<td>5-fluoro-oryotic acid</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>HA</td>
<td>haemagglutinin</td>
</tr>
<tr>
<td>HMTase</td>
<td>histone H3 methyltransferase</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IP</td>
<td>Immunoprecipitation</td>
</tr>
<tr>
<td>Kb</td>
<td>kilobase pairs</td>
</tr>
<tr>
<td>KDa</td>
<td>kilo daltons</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani broth</td>
</tr>
<tr>
<td>MNase</td>
<td>Micrococcal nuclease</td>
</tr>
<tr>
<td>NAD</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>nmt</td>
<td>no message in thiamine</td>
</tr>
<tr>
<td>N-terminal</td>
<td>amino-terminal</td>
</tr>
<tr>
<td>Abbr.</td>
<td>Term</td>
</tr>
<tr>
<td>-------</td>
<td>-------------------------------</td>
</tr>
<tr>
<td>ORF</td>
<td>Open Reading Frame</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PMG</td>
<td>Pombe Media Glutamate</td>
</tr>
<tr>
<td>PPTase</td>
<td>protein phosphatase</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SET</td>
<td>Suvar 3(9), Enhancer of zeste, Trithorax</td>
</tr>
<tr>
<td>TBE</td>
<td>tris-borate EDTA</td>
</tr>
<tr>
<td>TBZ</td>
<td>Thiabendazole</td>
</tr>
<tr>
<td>TSA</td>
<td>Trichostatin A</td>
</tr>
<tr>
<td>Tween</td>
<td>polyoxyethylenesorbitan monolaurate</td>
</tr>
<tr>
<td>YAC</td>
<td>Yeast Artificial Chromosome</td>
</tr>
<tr>
<td>YES</td>
<td>Yeast Extract Supplemented</td>
</tr>
</tbody>
</table>
CHAPTER 1
INTRODUCTION

During mitotic cell division the genome must be transported by an error-free mechanism from one cell generation to the next. Failure to correctly segregate replicated chromosomes leads to aneuploidy, the presence of extra copies or no copies of a chromosome in the cell. This aberrant set of chromosomes is an important cause of human birth defects (e.g., Down's syndrome) and an underlying cause of tumour progression. Cancer cells are known to be genetically unstable and mutations that lead to increased rates of chromosome missegregation in certain types of cancer may predispose cells to accelerate the process of tumorigenesis. The centromere and its monitoring components, which are essential for genome stability, must play an important role in providing some kind of prevention against aberrant chromosome segregation. However, despite the key role played by chromosome inheritance in cell viability our knowledge of the events that lead to chromosome segregation defects in these diseases remains minimal. By understanding the components and mechanisms involved in chromosome segregation, the molecular basis of this fundamental process can be uncovered.

Centromeres are the cis-acting regions of the chromosomes that specify the assembly of the protein components required for chromosome segregation in both meiotic and mitotic cell division. Centromeres were first identified more than a century ago, as a visible constriction in chromosomes and as a region of greatly reduced meiotic recombination [reviewed by B. Sullivan et al., 2001]. Electron microscopy studies [Cooke et al., 1990; Rieder et al., 1998; McEwen et al., 1998] have highlighted that this complex structure, called the kinetochore, is composed of three zones:

1) the centromeric chromatin comprising cis-acting DNA sequences specifying the chromosomal region where the active kinetochores are assembled, packaged in a specialised nucleosome structure containing the CENP-A histone H3-like protein.

2) a distinct chromatin interface on the chromosome surface, classically referred to as the inner kinetochore plate, composed of proteins involved in determining centromere identity, assembly and propagation;
Fig. 1. The DNA sequence of centromeres differs between species.

a) *Saccharomyces cerevisiae* centromere function depends on a small region containing the three conserved elements CDEI, II and III.

b) The minichromosome *Dp 1187*, the only defined *Drosophila melanogaster* centromere, consists of a core of 5bp satellites and transposons, flanked by other repetitive DNA (red).

c) Human centromeres consist of alpha-satellite DNA (red arrows) tandemly arranged into higher-order arrays (blue arrows), which extends over megabases.

d) *Caenorhabditis elegans* kinetochores (green circles) assemble along the length of each chromosome.

[from Sullivan B. et al., 2001].
3) an outer zone consisting of effector proteins such as microtubule-dependent motors responsible for the movement of chromosomes along the spindle microtubules and components of the spindle assembly checkpoint (SAC) responsible of monitoring kinetochore/microtubules attachment to centromeres and spindle orientation.

Centromeres are often surrounded or embedded in centric heterochromatin a repeat-rich gene-poor region of the genome that is transcriptionally silent and which mediates sister-chromatid cohesion at centromeres.

This Chapter will mainly focus on reviewing what is known about molecular components of the inner-kinetochore interface and their role in specifying centromeric chromatin.

CENTROMERES

Budding yeast centromeres: DNA and protein components.
The best characterised centromeres are those of *Saccharomyces cerevisiae* (Fig. 1-1a). The budding yeast minimal centromere is only 125bp in length and the same three functionally distinct elements (CDE I, II and III) are present in all 17 chromosomes. In native chromatin, centromeric DNA is contained within a 220-250bp DNA segment that is protected from nuclease cleavage and is flanked on both sides by hypersensitive cleavage sites and a highly ordered array of nucleosomes [Saunders et al., 1990; reviewed by Clarke, 1998]. Thus, in budding yeast the well-defined DNA of 125bp is sufficient to mediate spindle attachment, sister-chromatid cohesion, chromosome segregation and monitoring of these events by checkpoints.

Although small in size budding yeast centromeres do not lack complexity, a plethora of proteins is known to be associated with its centromeres, although only a subset of these are conserved in other eukaryotes [reviewed by Pidoux and Allshire, 2000; Clarke, 1998; Kitagawa and Hieter, 2001].

The DNA-inner kinetochore protein interface is composed of protein components interacting with the three centromeric DNA elements.

The CDEI 8-bp sequence is associated with the centromere-binding factor 1 (Cbf1/Cpf1 or Cep1) which contains a helix–loop–helix DNA-binding domain, and mediates both transcriptional regulation and chromosome segregation. Neither CDEI nor Cbf1 is essential for viability.
The 78–86-bp region of CDEII is composed of AT-rich DNA, and seems to act as a spacer between the conserved CDEI and CDEIII DNA elements. Mutations in the gene encoding Cse4, the centromere specific histone-H3 like protein, showed genetic interaction with mutations in the CDEII and CDEI DNA regions suggesting that this specialised centromere nucleosome containing Cse4 interacts with the DNA spanning these regions [Stoler et al., 1995; Keith and Fitzgerald-Hayes, 2000].

Only CDEIII (25bp) is essential, and point mutations within CDEIII abolish centromere function. The main CDEIII DNA-binding complex is CBF3, which is composed of the proteins Ndc10, Cep3, Ctf13 and Skp1. The CBF3 complex is activated by phosphorylation and this is thought to play a role in tying CBF3 assembly to S-phase duplication of centromeres [Kaplan et al., 1997]. The Sgt1 protein also associates with Skp1 and is required for regulation of CBF3 activity [Kitagawa et al., 1999].

Ctf19, Mcm21, and Okp1 were recently found to be centromere components and most likely form a protein complex that links CBF3 with the other components of the budding yeast centromere such as Cbf1, Mif2, and Cse4 [Ortiz et al., 1999]. The Mif2 protein shares some similarity with the conserved kinetochore component CENP-C and has been shown to bind centromeres by Chromatin Immunoprecipitation (ChIP) [Meluh and Koshland, 1997].

Bir1 is part of the Bir1/Cut17/Survivin family that is known to be involved in Aurora kinase function, it was isolated as a two-hybrid interactor of Ndc10 and appears to play a role in kinetochore function [Yoon and Carbon, 1999]. The Aurora kinase homologue of budding yeast, Ipl1, is responsible, in concert with the phosphatase Glc7, for regulating histone H3 phosphorylation at Ser10 during mitosis [Hsu et al., 2000]. Ipl1 was found to interact with Sli15, the budding yeast homologue of the INCENP passenger protein (see below), and to be stimulated by Sli15 [Kang et al., 2001]. These two proteins have been shown to bind to microtubules in vitro and to be associated with the centromeric DNA in vivo [Kang et al., 2001].

Recently, proteins that have been previously defined as part of the spindle pole body (SPB), namely Ndc80, Nuf2, Spc24 and Spc25, have been shown to also bind to centromeres in vivo and to function as kinetochore proteins [Wigge and Kilmartin, 2001]. Interestingly, homologues of all four proteins are conserved in fission yeast and Ndc80 and Nuf2 have human counterparts. Other candidate proteins that may provide a link between the kinetochore and
spindle microtubules are Slk19p and Mtw1p (the homologue of fission yeast Mis12) and Dam1, which is a microtubule binding protein that is physically and functionally associated with kinetochores [Jones et al., 2001].

The centromeres of all eukaryotic organisms mediate the same basic mechanism of coordinating the events responsible for chromosome segregation, but unlike budding yeast, their activity is not dependent on specific cis-acting DNA sequences.

There is no sequence conservation between centromeric DNA in different organisms, although they are often gene-poor, repetitive and AT-rich. Despite considerable efforts, the absolute sequence requirements for centromere function in many organisms remains elusive. Several studies have led to the conclusion that kinetochores may be assembled on an atypical DNA/chromatin region and subsequently propagated in an epigenetic manner [Karpen and Allshire, 1997]. Below are summarised the details of centromere structure and components in several model organisms with particular emphasis on fission yeast where kinetochore assembly is thought to rely on epigenetic regulation.

**Drosophila centromeres.**

The only molecularly and functionally defined *Drosophila* centromere is on a 1.3Mb X-derived minichromosome, *Dp1187* (Fig. 1-1b), progressive deletions of which have defined a 420kb region that genetically confers normal chromosome inheritance [Murphy and Karpen, 1995; Sun et al., 1997]. A 220kb essential core, composed predominantly of the complex island *Bora Bora*, is necessary to achieve good levels of stability, but full transmission also requires 200kb of the DNA flanking both sides [Murphy and Karpen, 1995]. Fine analysis of the DNA contained in this centromere revealed that the primary components are AATAT and AAGAG satellites, making up more than 85% (370kb) of this 420kb region whereas transposable elements comprise about 10% of it. These are found interspersed in the AATAT but not in the AAGAG satellite and also clustered in a terminal region of the centromere called *Maupiti* [Sun et al., 1997]. The current model proposes that *Bora Bora* includes the sites of kinetochore formation, based on the absolute requirement for this region, whereas the flanking satellite DNA is responsible for sister-chromatid cohesion and the assembly/stability of a fully functional kinetochore [Murphy and Karpen, 1995]. This model is supported by cytological studies which demonstrated that the domain containing the Cid (CENP-A) containing chromatin is flanked by, but does not overlap
with, chromatin that contains proteins involved in centric heterochromatin function, such as Su(var)2-5/HP1 and Prod (Proliferation disrupter) [reviewed by Sullivan B. et al., 2001]. In addition, also Mei-S332, a protein required for the maintenance of sister-chromatid cohesion in meiosis, is located near but not in the Cid-containing chromatin [Blower and Karpen, 2001]. Thus, Drosophila centromeres appear to be organised into two functionally distinct subdomains.

**C. elegans holocentric chromosomes.**

In most eukaryotes each chromosome contains one centromere (monocentric) whose location does not appear to change from one division to the next. However, organisms such as nematodes and crayfish, are holocentric (Fig. 1-1d), and the kinetochore is formed along the entire length of chromosomes [Pimpinelli and Goday, 1989]. Holocentric chromosomes bind to microtubules along their entire length and during mitosis, move broadside to the pole from the metaphase plate. These types of chromosomes are scattered throughout the plant and animal kingdom, and the most well known example is the holocentric nematode *C. elegans*. A number of proteins have now been implicated in centromere function or kinetochore structure in *C. elegans*, the majority by virtue of their homology to components already identified in monocentric organisms. In addition, the use of RNAi (RNA interference) screens have allowed the identification of novel protein components. An interesting question is what sequence determinants underlie the positioning of holocentric centromeres. In *C. elegans*, the HCP-3 (CENP-A homologue) antibody staining shows a punctate pattern within interphase nuclei and bands along mitotic chromosomes, indicating that not all genomic sequences are associated with this centromeric protein [reviewed by Dernburg 2001]. It will be interesting to uncover what DNA sequences are associated with HCP-3 to determine if they all have some feature in common. Neocentromere formation (the assembly and propagation of active centromeres at non-centromeric sequences), appears to be a relatively permissive event in *C. elegans* as any naked DNA microinjected in worms can assemble in extrachromosomal multimeric arrays containing HCP-3 [Howe et al., 2001]. Another interesting question is how do holocentric chromosomes accomplish bilateral attachment and avoid a single chromatid attaching to microtubules emanating from opposite poles. Interestingly, both homologues of the centromeric proteins CENP-A and CENP-F (see below) are only found on the poleward-facing side of the chromosomes.
**Mammalian centromeres.**

Studies of human centromeres over the last decade have led to controversy concerning the role of a candidate sequence, the centromere-associated alpha satellite, or alphoid DNA, in centromere function. The alpha-satellite is the only known human DNA that is exclusively localised to the centromeric regions of all normal human chromosomes and thus is a strong candidate for the centromere identifier sequence [reviewed by Murphy and Karpen, 1998].

Alphoid arrays are composed of tandemly arranged 171-bp monomers organised into higher-order arrays that extend from 100kb to several megabases [reviewed by Willard, 1998; Fig. 1-1c]. Chromatin immunoprecipitation has been used to isolate the sequences associated with the kinetochore protein CENP-A in vivo and this analysis resulted in a 10-20 fold enrichment of alpha-satellite compared to control genomic libraries [Vafa and Sullivan, 1997]. However, the entire alpha satellite array is unlikely to be involved in kinetochore assembly because antibodies to kinetochore proteins, such as CENP-A, localise to only a portion of the alpha satellite DNA [Warburton et al., 1997]. In addition, chromosomes that are naturally or artificially deleted for much of this array can still assemble a kinetochore and segregate normally [Wevrick et al., 1990; Yang et al., 2000]. Structural analyses are currently not detailed enough to exclude the importance of non-alphoid DNA. Alpha satellite arrays appear to be mostly homogeneous, but some interspersed sequences such as LINEs, Alu repeats and other satellites are also present; however, these sequences are also present in other parts of the genome that never associate with kinetochores, suggesting that they are not by themselves sufficient for centromere function [Lee et al., 1997].

If alpha satellite specifies the site of kinetochore formation, then it should be possible to create functional centromeres *de novo* from cloned alphoid DNA. Synthetic alpha satellite arrays of 1Mb introduced into cells along with telomeric and total genomic DNA resulted in the recovery of clones containing significantly larger alphoid arrays (6-10Mb) and among these some contained *de novo* centromeres [Harrington et al., 1997]. In a different approach, a YAC (Yeast Artificial Chromosome) containing 100kb of alpha satellite was introduced into human cells. Some of the recovered clones contained centromere activity and were composed of about 30 copies of the original YAC. FISH analysis suggested that the minichromosomes had not acquired large amounts of host sequences [Ikeno et al., 1998]. The creation of these MACs
(mammalian artificial chromosomes) demonstrates that transfection of naked alpha satellite DNA can result in de novo centromeres that bind the active kinetochore proteins CENP-C and CENP-E (see below). However, other fates were observed for the transfected alpha satellite DNA in the majority of selected transformants, primarily integration at or near endogenous centromeres or telomeres. In addition, the stable MACs were substantially larger than the input DNA and the nature of the events that led to these rearrangements, including the possibility of uptake of non-alphoid DNA from the host, are not known. This suggests that additional epigenetic processes during transfection, and amplification/rearrangement of these sequences are necessary to permit centromere formation and mitotic propagation.

Identical alphoid sequences can differ in their ability to bind active kinetochore proteins. Dicentric chromosomes, which are naturally occurring abnormal chromosomes that contain two regions of centromeric DNA, are unstable because they frequently attach to opposite spindle poles and produce chromosome bridges in anaphase [Sullivan and Willard, 1998]. However, numerous stable dicentric chromosomes have been described in which one of the two alpha satellite blocks is inactivated and no longer binds critical kinetochore proteins [Sullivan and Schwartz, 1995]. Finally, centromeres can be assembled in the absence of alphoid DNA (discussed below).

**Neocentromeres.**

Several different human marker chromosomes have been described that lack detectable amounts of alphoid DNA by FISH [reviewed by Murphy and Karpen, 1998]. These chromosomes harbour functionally active centromeres assembled on regions of the genome that do not contain centromeric DNA. Patients with such neocentromere-containing chromosomes are heterozygous for the chromosome aberration. The mechanism that led to the formation of these neocentromeres is unclear and may have occurred to compensate for loss of the normal centromere. The best characterised example is the neocentromere formation associated with an 80kb region of chromosome 10 euchromatin [du Sart et al., 1997]. Restriction analysis of this 80kb neocentromere (mardei(10)) showed that this domain has identical genomic organisation to the corresponding normal chromosomal region [du Sart et al., 1997]. Recently, chromatin immunoprecipitation experiments combined with DNA array analyses have identified a CENP-A binding region within the mardel(10) neocentromere that is
Fig. 1-2. Fission yeast centromeres organisation.
S. pombe genome is organised into three chromosomes 1, 2 and 3. The largest chromosome (Ch1) contains the smallest centromere (35kb) whereas the smallest chromosome (Ch3) contains the largest centromere (110kb). Centromere 2 occupies 60kb. The outer repeats (otr) are composed of dg (grey arrow) and dh (cross hatched arrow). cen3 contains 9 additional copies represented by x3 on the left and x8 on the right side. The central core region (cc) of cen1 and cen3 share an element, TM (checked rectangle). Inner repeats at all three centromeres have a distinct sequence represented by the differently shaped arrowheads, imr1 (white) imr2 (stippled) imr3 (striped). The multiple tRNA genes are denoted by the sharp vertical arrowheads (from Allshire, 2001)
significantly A+T rich, gene poor and contains repeated tandem repeats, features that are in common with centromeric sequences [Lo et al., 2001]. Thus, it is possible that in these rare events of neocentromere formation, kinetochores are assembled in preferred regions of the genome that display certain features resembling the centromere.

Neocentromere formation has also been described in *Drosophila* [Murphy and Karpen, 1995]. Structurally acentric mini-chromosomes that display efficient mitotic and meiotic transmission were isolated after irradiation of a *Drosophila melanogaster* mini-chromosome despite their small size (under 300 kb) and lack of centromeric DNA [Murphy and Karpen, 1995; Williams et al., 1998]. These acentric mini-chromosomes were transmitted by a microtubule-based mechanism as they were associated with the centromere-specific protein ZW10 and associate with the spindle poles in anaphase [Williams et al., 1998].

The acquisition of neocentromere activity may initially require the proximity to an endogenous centromere and spreading *in trans* of centromere components. Alternatively, neocentromere activity could arise once an inhibitory element, such as the presence of a functional centromere on the same chromosome, is removed by a rearrangement. The ability to form neocentromeres indicates that a certain degree of plasticity is involved in kinetochore assembly. Remarkably, once established, these rare neocentromeres are faithfully propagated suggesting that once acquired, centromere activity is retained and transmitted through DNA replication in the absence of any centromeric *cis*-acting sequences. However, in normal cells this ability to form neocentromeres must be restrained to prevent the formation of dicentric chromosomes.

**FISSION YEAST CENTROMERES.**

**Centromeric sequences.**

The unicellular fission yeast, *Schizosaccharomyces pombe*, whose genome size is similar to that of budding yeast, requires significantly more DNA for centromere function. The three centromeres have a very similar overall organisation of DNA elements, but they differ in size and in the number and arrangement of the flanking repetitive sequences [reviewed by Clarke, 1998]. The simplest and better characterised centromere in fission yeast is *cen1* occupying a region of approximately 38kb, whereas *cen2* is approximately 60kb and *cen3* is about 110kb in size (Fig. 1-2). Each centromere contains a 4-7kb single-copy central core and 20-100kb of
flanking repetitive sequences, arranged as long inverted structures. The central core of centromere 2 (cc2) lacks a 3.3 kb sequence shared by centromeres 1 and 3 (99% identity) and the flanking elements present within a few kilobases on both sides of the core (the innermost repeats imrL and imrR) are unique to each centromere. The central core and the innermost repeats constitute the central domain, a region that is functionally distinct from the outer repeats [Partridge et al., 2000]. While the outer repeat sequences share a high degree of conservation among centromeres, they are organised differently in different centromeres. The outer repeats are composed of two elements dg and dh (also called K and L). There are two copies of the dg/dh repeats surrounding the central domain of cen1, however, the number of dg/dh repeats varies from between two and four at cen2 to 11-13 at cen3.

An interesting feature of S. pombe centromeres is that approximately one third of the total number of tRNA genes present in the haploid genome is contained within the centromeric regions (vertical bars in Fig. 1-3). These tRNA genes appear to be intact and they are found within (imr) or adjacent to the centromeres [Takahashi et al., 1991]. The presence of these tRNA genes in the imr spatially defines the boundary between the central domain and the outer repeats which is also a functional transition point [Takahashi et al., 1992; Partridge et al., 2000]. tRNA genes are also found at the left and right extremities of each centromere except for cen1, where they are absent from the right hand side. It is not known whether these genes are actively transcribed or whether they play a specific role at centromeres.

Reconstructed minimal centromeres of 12-15kb that retain partial function, as measured by mitotic and meiotic transmission fidelity, all contain at least a portion of the central region and some flanking dg sequences. Deletion of the entire central core region or of all dg from these constructs results in high mitotic instability and loss of functional meiotic segregation [Baum et al., 1994; Clarke et al., 1993]. In brief, a minimum sequence of 25Kb, which contains the non-repetitive central core, inner repeats and a portion of the outer repeats, is an absolute requirement for centromere function and for stable chromosome transmission [Baum et al., 1994; Hahnenberger et al., 1991].

Circular minichromosomes based on cen3 containing a 2.1Kb fragment of dg (K), dh (L), all of the left arm innermost repeats (imrL), the cc3 and a small portion of the innermost repeats from the right arm (imrR), showed the ability to acquire two possible states one unstable and one
stable without any change or rearrangement of the structure of the constructs themselves [Steiner and Clarke, 1994]. This indicated that constructs that were not expected to retain the ability to be stably transmitted through mitosis, on the basis of previous studies [Baum et al., 1994], occasionally switched to a stable state in an epigenetic manner which may reflect the ability of the centromere to adopt a particular conformation. Once this conformation is established, it can be propagated for several generations. Thus, in contrast to budding yeast centromeres, fission yeast centromeres do not appear to rely entirely on defined cis-acting DNA sequences necessary and sufficient for centromere function, but are regulated in part by an epigenetic mechanism that perhaps contributes to the formation of some higher order structure.

The specialised chromatin of the central domain.
Like the rest of nuclear DNA, centromeres are packaged into nucleosomes. While most of the centromere-specific repeated DNA sequences are packaged into nucleosomes typical of bulk chromatin, the central core and the innermost repeats sequences from the centromere regions show an altered chromatin structure, with little or no evidence of regular nucleosomal packaging [Polizzi and Clarke, 1991; Takahashi et al., 1992]. Nucleosomal arrays can be detected by limited digestion of chromatin with Micrococcal Nuclease (MNase) followed by Southern analysis of the resulting DNA with probes specific for different centromeric regions. Such analyses result in a normal nucleosomal ladder when probing with genomic probes or outer repeat probes, whereas a smeared pattern is detected with central core or imr probes. Interestingly, these same DNA sequences are packaged into nucleosomes typical of bulk chromatin when present in a non-functional environment on a minichromosome in the budding yeast Saccharomyces cerevisiae [Polizzi and Clarke, 1991]. Moreover, the epigenetic switch to a stable conformation of minichromosomes carrying abbreviated centromeric DNA constructions was accompanied by a change from the normal to the unusual nucleosome packaging [Steiner and Clarke, 1994]. Thus, this specialised chromatin structure appears to correlate with the assembly of a functional centromere, and is disrupted in cells mutated in essential central domain interacting factors such as Mis6, Mis12 and Cnp1 [Saitoh et al., 1997; Goshima et al., 1999; Takahashi et al., 2000].
CENTROMERE AND KINETOCHORE COMPONENTS.
Centromere/kinetochore proteins can be grouped into at least two classes. The first can be observed constitutively at the kinetochore throughout the cell cycle and form the interphase pre-kinetochore, the second is only associated transiently with kinetochores.

Constitutive centromere components.
Constitutive centromere/kinetochore proteins are found either in the underlying heterochromatin or in the inner kinetochore plate and are thought to play a fundamental role in centromere identity and propagation. They were originally identified as autoantigens recognised by sera of certain patients with scleroderma spectrum disease [Moroi et al., 1980; Earnshaw et al., 1985]. These sera are often referred to as CREST sera, referring to the spectrum of clinical signs displayed by a subclass of scleroderma spectrum patients (calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, and telangiectasia). Three constitutive centromeric components are recognised by these anti-centromere antibodies: CENP-A (see below) [Sullivan et al., 1994], CENP-B [Earnshaw et al., 1987] and CENP-C [Saitoh et al., 1992].

CENP-B is a DNA-binding protein that specifically binds to a DNA motif, the CENP-B box, which is found interspersed in the alphoid DNA. The consequences and relevance of this binding are unknown. Interestingly, African green monkey cells, which contain extensive alpha-satellite arrays at centromeres, appear to lack the CENP-B box although they express a functional CENP-B homologue [Goldberg et al., 1996]. CENP-B boxes are also absent from the Y chromosome and no CENP-B is detected at the Y centromere [He et al., 1998]. Three independent studies have shown that mice lacking CENP-B are viable and display normal mitotic and meiotic cell division [Hudson et al., 1998; Perez-Castro et al., 1998; Kapoor et al., 1998]. In addition, CENP-B is present at both active and inactive centromeres of stable dicentric autosomes [Earnshaw et al., 1989]. The function of this non-essential centromere component remains unknown.

CENP-C is an essential protein both in mouse [Kalitsis et al., 1998] and in chicken cells [Fukagawa et al., 1997] and is only found at active centromeres of stable dicentrics [Earnshaw et al., 1989]. Immunoelectron microscopy has established that CENP-C is a component of the inner kinetochore plate [Saitoh et al., 1992]. DNA binding activity has been shown for CENP-C,
Indicating a role in the formation of centromeric chromatin [Sugimoto et al., 1994]. Anti-CENP-C antibody injection experiments have demonstrated the importance of the levels of CENP-C at the centromere for kinetochore assembly and establishment and/or maintenance of proper kinetochore size [Tomkiel et al., 1994]. Antibody microinjection during interphase, caused metaphase arrest by reducing the amount of CENP-C at centromeres and electron microscopy of kinetochores from these metaphase-arrested cells revealed a reduced number of normal trilaminar structures, in which the diameter was significantly reduced [Tomkiel et al., 1994]. Proper targeting of CENP-C appears to be dependent upon the presence of the H3-like protein CENP-A at centromeres. Mouse cells lacking the essential component CENP-A showed severe mitotic defects and absence of discrete CENP-C signal on centromeres [Howman et al., 2000]. RNA interference experiments that reduced the expression of HCP-3/CENP-A in the holocentric worm *C. elegans* caused HCP-4/CENP-C to fail to localise at centromeres [Moore and Roth, 2001].

CENP-G has been identified more recently, using anti-centromere antibodies from the serum of a patient with gastric antral vascular ectasia disease [He et al., 1998]. This protein was detected at centromeric regions throughout the cell cycle and in mitosis it appeared restricted to the kinetochore inner plate. It may preferentially associate with a subtype of alphoid DNA [He et al., 1998], however, it was also found associated with the Y chromosome and with a neocentromere both lacking this alphoid subtype indicating that other DNA sequences can associate with CENP-G [Gimelli et al., 2000].

CENP-H is another novel component constitutively associated with the inner kinetochore plate [Sugata et al., 1999]. CENP-H colocalised with CENP-A and CENP-C and was detected at neocentromeres, but not at the inactive centromere of stable dicentric chromosomes [Sugata et al., 2000]. A conditional loss-of-function mutant in the chicken DT40 cell line showed that in the absence of CENP-H, cells arrest at metaphase [Fukagawa et al., 2001] consistent with an essential role for this component. In addition, CENP-H deficient cells demonstrated that CENP-H is necessary for CENP-C localisation at the centromere [Fukagawa et al., 2001].

**CENP-A and the propagation of centromere identity.**

As mentioned above, human CENP-A was originally identified along with CENP-B and C using serum from CREST patients [Earnshaw et al., 1985]. Further analysis and partial sequencing
Fig. 1-3. Protein sequence alignment of members of the CENP-A like family.
Amino acid sequences of CENP-A homologues obtained from the Sanger Centre were analysed with ClustalX. Highlighted in red are identical residues whereas in gray are highlighted similar residues. From the top line down are shown: S. pombe Cnp1, S. cerevisiae Cse4, human CENP-A, mouse Cenpa, C. elegans HCP-3, Drosophila CID. These proteins share high conservation amongst their histone-fold domains and high divergence amongst their N-termini.
revealed that the protein contained some regions with homology with histone H3 and some regions unrelated to histone H3 [Palmer et al., 1991]. The budding yeast CENP-A homologue, Cse4, was identified as a temperature sensitive mutant that showed genetic interaction with mutations in CDEI and II and caused cells to arrest in metaphase at elevated temperature [Stoler et al., 1995]. Both CENP-A and Cse4 have been shown to be components of active centromeres and are localised at the inner kinetochore plate of animal cells [Warburton et al., 1997] and associated with centromeric yeast DNA in vivo [Meluh et al., 1998].

CENP-A homologues have now been identified in several species, demonstrating that a highly conserved histone H3-like protein is a characteristic component of centromeres of highly divergent eukaryotes. Proteins from this H3-like family display the same basic organisation as histone H3 consisting of an amino-terminal domain and a carboxy-terminal histone-fold domain. The histone fold domain shares the highest homology and is required for human CENP-A targeting to the centromere [Sullivan et al., 1994], whereas the amino-terminal region is highly divergent, Fig. 1-3. The amino terminal tail of Cse4 tail has been shown to display an essential function distinct from that of histone H3 [Chen et al., 2000].

All the CENP-A homologues identified to date have been shown to be essential for viability. RNA mediated interference (RNAi) aimed to reduce the expression of the C. elegans homologue, HCP-3, resulted in 100% embryonic lethality and caused missegregation of chromosomes from the first mitotic division [Buchwitz et al., 1999]. Mice null for Cenpa failed to survive beyond 6.5 days post-conception with cells displaying severe mitotic problems [Howman et al., 2000]. Similarly, antibody injection and RNAi were used to deplete the Drosophila melanogaster CENP-A (CiD) and caused chromosome segregation defects and disruption of kinetochore assembly [Blower and Karpen, 2001]. In addition, CENP-A is only present at active centromeres of dicentric chromosomes and is associated with neocentromeres, thus the presence of this protein correlates tightly with kinetochore activity [Warburton et al., 1997]. All these data indicate that CENP-A is an essential component of active centromeres.

What is the function of CENP-A? Biochemical data showed that on extraction from the nucleus, CENP-A copurifies with histones and nucleosome particles indicating that this centromere protein behaves as a histone-like component of chromatin [Palmer et al., 1987; Palmer et al.,
Genetic interactions have been demonstrated between Cse4 and histone H4 through their histone fold [Smith et al., 1996; Glowczewski et al., 2000]. In contrast, an antagonistic effect has been demonstrated between Cse4 and histone H3, as overexpression of the latter is synthetically lethal with cse4 alleles [Glowczewski et al., 2000]. Human CENP-A has been shown to form homodimers [Shelby et al., 1997]. In addition, nucleosome reconstitution showed that CENP-A can replace histone H3 in vitro [Yoda et al., 2000]. These data support a model in which CENP-A replaces histone H3 in centromeric nucleosomes.

As discussed previously, CENP-A is required for CENP-C localisation at the centromere in mouse and in C. elegans. Thus, CENP-A could act as a nucleation signal for the assembly of the centromere, connecting the underlying centromeric DNA with the kinetochore.

How is CENP-A recruited to centromeric sites?

One favoured hypothesis was that coupling CENP-A expression with centromeric DNA replication, both occurring in late S phase, could be a requirement for correct loading, as expression of human CENP-A under the human histone H3 promoter, which is on early in S phase, fails to drive proper loading at centromeres [Shelby et al., 1997]. However, human CENP-A associated chromatin has been recently shown to replicate asynchronously in mid- to late S phase, simultaneously to non-centromeric DNA [Sullivan and Karpen, 2001]. Thus, human centromeres replicate at time points when new CENP-A has not yet been synthesised. In addition, CENP-A loading can take place in the absence of DNA replication since ectopic expression of CENP-A in the presence of a replication inhibitor resulted in proper localisation [Shelby et al., 2000].

Are specific centromeric sequences required for CENP-A targeting?

The fact that CENP-A like proteins are highly conserved among species whereas centromeric sequences are highly divergent suggests that centromeric DNA may not be essential for targeting of CENP-A. In addition, the infrequent events that allow neocentromere formation supports the idea that CENP-A can associate with regions not associated with centromeric sequences. Conversely, the presence of canonical centromeric sequences does not necessarily drive the assembly of a functional centromere. This is indicated by the fact that in dicentric chromosomes, CENP-A is only found at the active centromere [Warburton et al., 1997]. CENP-A proteins from human, worm and budding yeast, when expressed in Drosophila cells were
found to preferentially localise at *Drosophila* pericentric heterochromatin, suggesting that CENP-A homologues perhaps share some intrinsic property that enables them to be targeted similarly to the endogenous CID [Henikoff et al., 2000]. These studies point toward a sequence-independent CENP-A recruitment at centromeres. How therefore is CENP-A deposited specifically at active centromeres? It has been previously discussed how centromeres of organisms other than *S. cerevisiae* may rely on epigenetic mechanisms rather than on primary DNA sequence for continued centromere propagation. The propagation of centromere identity could be simply based on a cyclical mechanism of depositing CENP-A in regions of the genome that previously contained CENP-A. During DNA replication of CENP-A chromatin, individual CENP-A nucleosomes are distributed to both daughter strands, as demonstrated in HeLa cells [Shelby et al., 2000]. At this stage chromatin could exist in two possible states 1) no new nucleosomes have been deposited on the newly replicated centromere or 2) histone H3 is momentarily deposited as a “placeholder” for CENP-A nucleosomes [reviewed by Sullivan, 2001]. In both cases this CENP-A hemichromatin could act as a template for further deposition of CENP-A, in addition the second model would imply that histone H3 was actively removed from centromeric chromatin. To date no candidates responsible for CENP-A deposition or histone H3 dissociation have been identified.

Such a cyclical epigenetic mechanism could account for the faithful propagation of both monocentric and holocentric centromeres. However, this model could not be applicable to the small centromeres of budding yeast. As these are thought to contain a single Cse4 nucleosome replication will produce daughter strands of which only one sister is likely to contain the Cse4 nucleosome. A *cis*-acting based mechanism is therefore necessary to deposit one Cse4 nucleosomes at all centromeres.

**Transient kinetochore proteins.**

The second class of centromere proteins is found at the kinetochore only during specific stages of mitosis. Some of these components have been termed chromosomal passengers and were originally defined by their change in distribution during mitosis. They are concentrated at centromeres during metaphase, transfer to the spindle during early anaphase and shortly after to the cell cortex at the future cleavage furrow and finally concentrate at the midbody during cytokinesis [Earnshaw and Bernat, 1991]. INCENP is a passenger protein and its role appears
to be strictly linked with Aurora B kinase function. *Drosophila* INCENP is required for normal localisation of Aurora B in mitosis and for its function as a histone H3 kinase suggesting a role for INCENP in recruiting Aurora B to the centromeres and the spindle [Adams et al., 2001; Greaves, 2001].

Two others passenger proteins are high molecular weight components that are involved in microtubule capture and chromosome movements along the spindle microtubules. CENP-E is a kinesin-like motor protein that localises to the outer kinetochore plate and is involved in the congression of chromosomes to the metaphase plate [Cooke et al., 1997; Wood et al., 1997]. The distribution of CENP-F on the outer kinetochore and its dynamics during mitosis closely parallels that of CENP-E and it is proposed to be involved in eliciting the mitotic checkpoint (like Zw10). These components are homogeneously distributed throughout the nucleus of HeLa cells in G2. Upon prophase, these proteins localise to the kinetochores and this association is maintained throughout metaphase, but at the onset of anaphase they are found at the spindle mid-zone. By telophase, they are concentrated into a narrow band on either side of the midbody [Rattner et al., 1993].

CLIP-170 is a microtubule-binding protein, MCAK a kinesin-related protein; dynamitin, Arp1 and p150 Glued are components of the dynactin complex [Faulkner et al., 1998; reviewed by Craig et al., 1999]. These proteins are likely to be associated with the kinetochore rather than the centromeric chromatin, because they are all present at active, but not inactive, centromeres on stable dicentric chromosomes [Faulkner et al., 1998], the function of these proteins is not known.

The Drosophila protein Zw10 is another component which transiently associates with the kinetochore and is required for chromosome segregation [Williams et al., 1992]. Its human homologue is present only at active centromeres in dicentrics [Faulkner et al., 1998]. Zw10 plays a role in a tension-sensing checkpoint at the anaphase onset [Williams et al., 1996], and in targeting the dynactin/dynein complex to the kinetochore [Starr et al., 1998].

The Drosophila protein *rough deal* (ROD) has been shown to be in a complex with Zw10. In flies, the two proteins always colocalise and require each other for their recruitment to the mitotic apparatus [Scaerou et al., 2001]. Drosophila cells lacking either ROD or Zw10 exhibit a phenotype that is similar to that of Bub1 mutants: they do not arrest in metaphase in response
to spindle damage, but instead separate sister chromatids, degrade cyclin B and exit mitosis [Basto et al., 2000; Chan et al., 2000]. Interestingly, these are the first spindle checkpoint components to be identified that do not have obvious homologues in budding yeast.

Finally, some transiently kinetochore-associated components are involved in the spindle assembly checkpoint (such as hMad2, hBub1 and hBubR1) and disappear from the kinetochore upon proper attachment to the spindle [Li and Benezra, 1996].

**Fission yeast protein components associated with the central domain.**
The central domain of fission yeast centromeres is composed of the central core (cc) flanked by central-core associated repeats or innermost repeats (imr). This domain is clearly distinct spatially and functionally from the flanking outer repeats (otr). ChIP analysis across centromere 1 defined the boundaries between these two distinct domains which reside in the region across two tRNA genes located at the two ends of imrR and imrL [Partridge et al., 2000]. The Mis6 protein [Saitoh et al., 1997] associates with sequences located internally to the two tRNA genes, whereas the Swi6 and Chp1 proteins associate with the flanking elements [Partridge et al., 2000].

A screen for temperature-sensitive (ts) fission yeast mutants that display high loss rates of minichromosomes at permissive or semi-permissive temperature was used to isolate 12 genetic loci. Two of these mutants, Mis6 and Mis12, exhibit unequal segregation of chromosomes at mitosis Takahashi et al., 1994]. The mis6 gene is essential for viability: its function is required at the onset of S phase, and it shares weak homology with a novel mammalian protein [Saitoh et al., 1997] and with a budding yeast protein, Ctf3 [Measday et al, 2002]. Mis6-HA is localised at the centromeres and it was mapped to the central domain by ChIP. Cells lacking Mis6 display a high rate of unequal segregation, but no lagging chromosomes on the mitotic spindle [Saitoh et al., 1997]. These data, led to a model where it was proposed that Mis6 is not required for the capture of the kinetochore by the spindle microtubules, but instead it is required to mediate the biorientation of sister kinetochores during metaphase [Saitoh et al., 1997]. Mis12 is another essential protein which has homologues in budding yeast and filamentous fungi. Like Mis6, Mis12 is associated with the central domain and is required for the presence of the specialised nucleosomal structure [Goshima et al., 1999].
In fission yeast three proteins with overlapping functions have been isolated which are homologous of mammalian CENP-B, they are Abp1/Cbp1, Cbh1 and Cbh2. As with their mammalian counterpart, none of these genes are essential and only combinations of gene-disruptions caused chromosome segregation defects [Irelan et al., 2001]. Abp1/Cbp1 was originally isolated in gel mobility-shift assays with oligonucleotides containing the ARS (Autonomously Replicating Sequence) consensus sequence [Murakami et al., 1996]. Abp1 is able to bind in vitro to the dg/K type repeat DNA [Lee et al., 1997] and cc2 [Halverson et al., 1997; Baum and Clarke, 2000]. In vivo, Cbh1 associates with the dg/K repeats but also to several non-centromeric locations [Baum and Clarke, 2000] whereas Cbh2 appears to be specifically associated with the central domain [Irelan et al., 2001].

Homologues of Ndc80p, Nuf2p, and Spc24p have been found in *S. pombe* and GFP tagged proteins localised at the centromere [Wigge and Kilmartin, 2001]. In addition, preliminary ChIP data suggests that these factors are associated with the central domain of the centromere [J. Abbott and A. Pidoux, unpublished observations].

A candidate for a CENP-A homologue was uncovered by the *S. pombe* sequencing project and in parallel, was isolated by degenerate PCR [Takahashi et al., 2000]. Cnp1 was found to be essential and spore germination experiments showed that lack of Cnp1 caused a high frequency of chromosome missegregation and disruption of the specialised chromatin structure of the central domain [Takahashi et al., 2000]. Epitope tagged Cnp1 expressed from the endogenous promoter was found to localise at centromeres by immunofluorescence. ChIP analysis showed that Cnp1 was specifically associated with the central core and *imr* region, but not with the outer repeats. Interestingly, *cnp1* transcription is cell cycle regulated and takes place early in S phase just prior to the expression of histone H3 [Takahashi et al., 2000] and during the same time window of centromere replication [Kim and Huberman, 2001]. Intriguingly, Cnp1-GFP localisation was lost in the absence of a functional Mis6 protein, but was independent of Mis12 [Takahashi et al., 2000]. On the basis of this evidence the authors proposed a role for Mis6 in loading Cnp1 into chromatin. However, the Mis6 homologue in budding yeast, Ctf3p, is not required for Cse4 localisation and the human Mis6 homologue has been localised to the outer kinetochore plate [Measday et al., 2002 and references therein].
HETEROCROMATIN

Heterochromatin of complex eukaryotes such as fruit flies and mammals was first identified cytologically. Unlike euchromatin, heterochromatin stains darkly and stays condensed throughout the cell cycle. Heterochromatin is often found in close association with the nuclear periphery, replicates mainly in late S phase, has a low density of RNA polymerase II transcription units, and has a reduced meiotic recombination [Karpen and Allshire, 1997]. The fact that heterochromatin is less accessible to transcription and recombination factors does not however imply that it lacks cellular functions. Heterochromatin is responsible for a number of essential biological functions like centromere and telomere activity, ribosomal RNA gene regulation, pairing of sister chromosomes in metaphase, and nuclear organisation. Heterochromatin function relies on a specialised chromatin that is epigenetically regulated and a number of factors that specifically associate with it.

Histone modifications and the "histone code" hypothesis.

Chromatin, the physiological template of all eukaryotic genetic information, is subject to a diverse array of post-translational modifications that largely involve histone amino termini, thereby regulating the accessibility of the underlying DNA. Distinct modifications can act synergistically or antagonistically to mediate recruitment of chromatin associated proteins, which ultimately dictate dynamic transitions between transcriptionally active and silent states (histone code hypothesis).

Histones are small basic proteins consisting of a globular domain and a more flexible and charged NH₂-terminus (histone "tail") that protrudes from the nucleosomes. In each nucleosome, approximately two superhelical turns of DNA wrap around an octamer of core histone proteins formed by an H3-H4 tetramer and two H2A-H2B dimers [Luger et al., 1997]. Nucleosomal arrays containing histone H1 are further twisted and folded through an as yet unknown mechanism forming a more compacted higher structure. Although histone proteins come in generic or specialised forms, further complexity is added by the number of different covalent modifications (such as acetylation, phosphorylation and methylation) of the histone tail domains.
Fig. 1-4. Histone modifications and chromatin states.
Models for euchromatic or heterochromatic histone tail modifications.
A) Schematic representation of euchromatin and heterochromatin as accessible or condensed nucleosome fibers containing acetylated (Ac), phosphorylated (P), and methylated (Me) histone NH2-termini.
B) General model for antagonistic E(var) and Su(var) gene function in mediating euchromatic (EU) or heterochromatic (MET) modification marks onto nucleosomes. Su(var)s also function in removing euchromatic signals and E(var)s can destabilise heterochromatin.
C) Examples of combinatorial modifications in histone N-termini that are likely to act like “imprints” for active or inactive chromatin. The presence of acetylated H4 K12 at heterochromatic sites is subject of debate (see text).
D) Proposed synergistic (connected arrowheads) or antagonistic (blocked oval line) modifications in histone H3 N-terminus. The arrow with the scissors indicates possible proteolytic cleavage of the H3 N-terminus [from Jenuwein and Allis, 2001].
Acetylation of lysine residues within the N-terminal tails is associated with the transcriptionally active regions within the genome and plays a causal role during the gene activation process [Tazi and Bird, 1990; Wade et al., 1997; Kuo and Allis, 1998; Mizzen and Allis, 1998; Turner et al., 1992]. Conversely, the removal of acetyl groups from the histones has a negative effect on gene expression in many model organisms [Zhang et al., 1997; Zhang et al., 1999; Jones et al., 1998; Wade et al., 1999]. In addition, H3 and H4 N-tails are consistently underacetylated in the heterochromatic regions of diverse organisms ranging from budding yeast [Ekwali et al., 1997; Grunstein, 1998] to mammals [Jeppesen and Turner, 1993; Taddei et al., 2001]. Centromeres, that tend to be embedded in pericentric heterochromatin, are characterised by a depletion of acetylated histone H4 lysines, however acetylation of histone H4 K12 has been reported to be a hallmark property of heterochromatin in organisms ranging from yeast to fly [Turner et al., 1992; Turner et al., 1993; Turner, 2000]. Nevertheless, a recent report in which highly-specific anti-acetylated lysines are employed argued that budding yeast heterochromatin is depleted also for histone H4 K12 acetylation [Suka et al., 2001].

Reversible disruption of the hypoacetylated state of mammalian pericentric heterochromatin by inhibiting HDAC activity with trichostatin A caused defective mitosis and transient loss of localisation of the Heterochromatin Protein 1 (HP1) [Taddei et al., 2001]. Thus, the hypoacetylated state of histone N-tails appears to be required for heterochromatin formation and function.

Phosphorylation of histone H3 at S10 has been shown to play a dual role in different contexts: transcriptional activation of genes and chromosome condensation in mitosis [reviewed by Strahl and Allis, 2000]. Finally, methylation of histone H3 at K9 has recently been shown to be a key event for heterochromatin formation [Rea et al., 2000; Nakayama et al., 2001].

The enzymes mediating histone tail modifications are highly specific for particular amino acid positions [Strahl and Allis, 2000], thereby extending the information contained in the genome. Accordingly, the "histone code" hypothesis predicts that: 1) modifications of the histone N-tails would determine the affinity for specific chromatin-associated proteins; 2) modifications on the same or different histone tails may be interdependent and generate various combinations on any one nucleosome and 3) distinct features of higher order chromatin, such as euchromatic or
heterochromatic domains, are largely dependent on the local concentration and combination of
differentially modified nucleosomes [Jenuwein and Allis, 2001], Fig. 1-4A.

It is now widely recognised that heritable, but reversible, changes in gene expression can occur
without altering the DNA sequence. Early studies on radiation-induced chromosomal
translocations [Muller, 1932] provided some of the earliest findings that epigenetic "on" and "off"
transcriptional states are largely dependent on the position of a gene within an accessible
(euchromatic) or an inaccessible (heterochromatic) chromatin environment. This phenomenon
known as position-effect variegation (PEV), allowed the development of genetic screens in
have identified ~30 loci involved in modifying PEV. Similar to PEV mating type switching in
budding [Haber, 1998] and fission yeast [Grewal and Klar, 1996] represent another example for
a variegating mechanism where the location of a gene within a distinct chromatin environment,
the mat region, mediates the establishment of an active or a silent transcriptional state.

Among the modifier genes identified in Drosophila, one subclass suppresses variegation, the
Su(var) group, and comprises gene products such as histone deacetylases (HDACs) and
protein phosphatases (PPTases) as well as chromatin-associated components such as
HP1/Su(var)2-5 [Jenuwein and Allis, 2001]. The antagonising class of PEV modifiers, the E(var)
group, enhances variegation, and counteracts the Su(var)-induced silent state, Fig. 1-4B.
Several E(var) components are part of ATP-dependent nucleosome remodeling machines, such
as the SWI/SNF and brahma complexes [Tsukiyama and Wu, 1997; Kal et al., 2000] which by
increasing nucleosome mobility increase accessibility of underlying genes to the transcription
machinery. Both Su(var) and E(var) contain protein modules such as the bromo-, chromo- and
SET-domains that are also found in two other classes of antagonistic components: the
Polycomb (Pc-G) and Trithorax (trx-G) groups. The Pc-G and trx-G genes are important for
maintaining the expression boundaries of the homeotic genes and several other key
developmental genes in Drosophila [Paro et al., 1998].
The bromodomain [Dhalluin et al., 1999] is found in SNF2, TAFII250 and mammalian trithorax
(HRX/MII); the chromodomain [Paro and Hogness, 1991; Jones et al., 2000] is shared between
Polycomb and HP1; and the SET domain [Tschiersch et al., 1994] is found in Su(var)3-9, in the
Pc-G member E(z) and in trithorax. These modules have been widely used during evolution to generate a remarkable functional diversity among proteins specialised in modulating chromatin structure. The SET domains of the Su(var)3-9 homologues, human SUVAR39H1 and fission yeast Clr4, have been shown to have histone H3 methyltransferase activity (HMTase) specific for K9 [Rea et al., 2000; Nakayama et al., 2001]. However, enzymatic HMTase activity has not yet been demonstrated for Pc-G and trx-G SET domains.

The histone code hypothesis predicts that the histone modifications provide a mark which can be recognised as a binding site for effector proteins. The bromodomain, present in many transcriptional regulators (e.g., Gcn5, PCAF, TAFII250) with histone acetylase activity (HAT), has been shown to selectively interact with a covalent mark, acetylated lysine, in the histone N-terminus [Jacobson et al., 2000].

The chromodomain of HP1 on the other hand has been shown to bind histone H3 methylated at K9 [Lachner et al., 2001; Bannister et al., 2001]. However, it seems likely that not all chromodomains behave similarly, for example Polycomb does not bind methylated H3 [Lachner et al., 2001] and the chromodomain of Mof has been proposed to bind RNA [Akhtar et al., 2000]. Interestingly, Su(var)3-9 HMTase family members also contain a chromodomain, which is critical for silencing in vivo [Nakayama et al., 2001; Ivanova et al., 1998]. A hallmark property of all HP1 proteins is the presence of a chromo-shadow domain which is separated by a hinge from the chromodomain. The role of this domain is not entirely understood but it has been shown to dimerise [Cowieson et al., 2000; Brasher et al., 2000].

Studies of histone methylation have shown that H3 can be methylated at K4 or K9. Whereas as previously discussed methylation of H3 K9 is linked to gene silencing and assembly of heterochromatin, H3 K4 has been correlated with active gene expression [Strahl et al., 1999; Noma et al., 2001; Boggs et al., 2001]. Thus, histone N-terminus modifications such as acetylation, phosphorylation and methylation play dual roles in both active and inactive chromatin (Fig. 1-4C), raising the question of how combinatorial specificity is used to define an imprint for euchromatin or heterochromatin. Recent studies are progressively highlighting a complex crosstalk between different modifications taking place between histone N-tails. For example histone H3 S10 phosphorylation and K9 methylation appear to be antagonistic modifications [Rea et al., 2000] whereas S10 phosphorylation and K14 acetylation work in
concert to activate transcription [Lo et al., 2000]. In addition, deacetylation of H3 K14 by the histone deacetylase Cir3 is required to facilitate methylation of K9 in fission yeast heterochromatin by Cir4 [Nakayama et al., 2001].

How stable are these histone modifications?

It has been proposed that, on the basis of chemical principles alone, the methyl groups may have a considerably lower turnover than acetyl or phosphoryl groups [Jenuwein and Allis, 2001]. Acetylation can be removed by HDAC activity, and phosphoryl groups are removed by protein phosphatases (Fig. 1-4D). To date, a demethylase activity has not been reported, however the methyl groups could in principle be removed by proteolytic cleavage of the histone N-tail. Alternatively, methylation of histones could be progressively diluted by semiconservative DNA replication at each mitotic division. Perhaps proteolytic cleavage is used by cells to remove marks of the so called "facultative" heterochromatin (composed of genes that are temporarily "off") whereas the constitutive heterochromatin can only be erased by dilution after many cell divisions. If this were the case, then histone H3 methylation would be a very stable and inheritable imprint comparable to that of DNA methylation.

**Budding yeast heterochromatin.**

Budding yeast has provided a genetic and molecular model system for the study of heterochromatin. Yeast heterochromatin appears to be condensed since it prevents access to DNA-altering enzymes (such as the DAM methylase), is late replicating in S phase and is associated with the nuclear periphery [Wright et al., 1992; Gotta and Gasser, 1996]. Yeast heterochromatin also contains hypoacetylated histone H4 at lysines K5, K8 and K16, but not at K12 [Lowell and Pilius, 1998]. In budding yeast such heterochromatic regions are found at the telomeres and at the silent (HM) mating loci [Grunstein, 1998]. Yeast heterochromatin has been shown to silence transcription of marker genes placed in the vicinity. *URA3* or *ADE2* inserted near a telomere are repressed and the mechanism clearly involves epigenetic regulation [Gottschling et al., 1990]. This telomere position effect (TPE), similar to PEV, spreads into adjacent DNA up to approximately 3kb from the telomeric end but it can be extended up to 16kb upon overexpression of SIR3 [Renaud et al., 1993]. The ends of yeast chromosomes appear to lack nucleosomes and to be instead associated with Rap1 [Wright et al., 1992]. Genetic and biochemical data have now shown that Rap1 participates in a large macromolecular complex
Fig. 1-5. Budding yeast heterochromatin.
A) A Model for the formation of telomeric heterochromatin. RAP1 (green) interacts with DNA at the telosome. The telosome is proposed to fold back onto subtelomeric chromatin allowing RAP-SIR, SIR-SIR, and SIR-histone interactions. Nucleosomes are shown as blue circles with extended H3/H4 N termini that interact with SIR3 and SIR4.
B) The heterochromatin-specific hypoacetylation state of H4 may promote interaction of SIR3 and the SIR complex with histone H4 at residues 16–29. It is speculated that in euchromatin, acetylation of K16 prevents H4-SIR3 binding, targeting SIR3 and the SIR complex to regions of heterochromatin [from Grunstein, 1998].
that includes Sir2 (NAD-dependent HDAC), Sir3, Sir4 and histones H3 and H4, which is essential for telomere and HM silencing, Fig. 1-5A [Grunstein, 1998]. In vitro, both Sir3 and Sir4 interact directly with the histone H3 tail (residues 4-20) and the histone H4 tail (16-29) [Hecht et al., 1995]. In vivo, this interaction is dependent upon non-acetylated K16 of histone H4, as amino acid substitutions of this residue to mimic acetylation (to G or Q) results in silencing defects, Fig. 1-5B [Johnson et al., 1990]. Rap1p interacts directly with DNA and with Sir3p, this interaction is dependent upon histone H4 as a substitution of the acetylatable lysine K16 to Q, which prevents its interaction with Sir3p in cell extracts, prevents the coimmunoprecipitation of Sir3p with Rap1p [Hecht et al., 1996; Hecht et al., 1995]. Silencing in budding yeast relies mainly on the histone H4 tail and specifically, on K16. Other amino acid changes, even in combination, of the other acetylatable lysines in H3 and H4 did not affect repression [Johnson et al., 1992]. The removal of the entire histone H3 tail alleviated silencing of the telomeric URA3 marker ten fold less than the single amino acid substitution (K16 to Q) of histone H4 [Thompson et al., 1994]. In conclusion, budding yeast heterochromatin is characterised by the presence of a silencing complex composed of Rap1 and the Sir proteins which is recruited via underacetylated histone tails, in particular via histone H4.

Since histones play a central role in mediating silencing one might expect that proper deposition of histones on newly replicated DNA also plays a role to establish and maintain silenced domains. CAF-1, the chromatin assembly factor involved in histone H3 and H4 deposition at the replication fork, is not essential for viability in S. cerevisiae [Enomoto et al. 1997; Kaufman et al. 1997]. Mutations in CAF-1 impair transcriptional silencing of reporter genes integrated next to telomeres and, to a lesser extent, silencing of the mating type loci [Enomoto et al. 1997; Kaufman et al. 1997; Monson et al. 1997; Enomoto and Berman 1998]. At the HML locus, CAF-1 is required for the maintenance, but not the establishment of transcriptional silencing [Enomoto and Berman 1998]. In addition, deletion of HIR genes, which regulate histone gene expression, synergistically reduced gene silencing at telomeres and at the HM loci in CAF mutants, although hir mutants had no silencing defects when CAF-1 was intact [Kaufman et al., 1998]. These studies suggest that, CAF-1 plays a role in the maintenance of heterochromatin at telomeres and the silent mating type loci [reviewed by Verreault, 2000].
Transcriptional silencing in fission yeast.
A phenomenon similar to budding yeast mating type and telomeric silencing and to Drosophila PEV has been described for all three fission yeast heterochromatic sites: telomeres, mating type and centromeres [reviewed by Ailshire, 1995].

The transcription of the ura4' and the ade6' genes became repressed when they were inserted within the central core of cen1, 2 and 3 [Ailshire et al., 1994]. The ura4' and ade6' gene activity in these locations fluctuated between repressed and expressed states and, similarly to Drosophila PEV, was influenced by temperature, expression being increased at higher temperatures. Interestingly, once incorporated within the central core of cen1 the ura4' gene acquired the unusual chromatin structure typical of the central domain of fission yeast centromeres as detected by Mnase digestion and Southern analysis. Noticeably, the stronger resemblance with the endogenous smeared pattern was observed toward the 5' and the 3' end of the ura4' gene, whereas the middle of ura4' showed a more nucleosomal pattern [Ailshire et al., 1994]. This phenomenon suggests that this specialised chromatin organisation could "spread" over artificially inserted DNA fragments in order to maintain the function of the centromere intact.

Variegated expression of the ura4' gene occurs at multiple sites within cen1 [Ailshire et al., 1995]. Expression of the ura4' gene was measured in strains harbouring the ura4' gene inserted at 13 centromeric locations: 7 across the central domain, 4 across the outer repeats and two immediately outside of cen1. Both plating assay and Northern analysis of the ura4' transcripts showed that the ura4' insertions across cen1 are transcriptionally silent, with the central domain less strongly silenced than the outer repeats. In contrast, insertions just adjacent to cen1 were fully expressed. Mutations in cir4, rik1 [Ekwall and Ruusala, 1994; Ivanova et al., 1998] and swi6 genes [Lorentz et al., 1994], affected transcriptional silencing of insertions across the outer repeats, but not across the central domain, in particular the alleviation of silencing caused by mutations in cir4 and rik1 was more pronounced than in swi6 mutants [Ailshire et al., 1995]. More recently, it was shown that the mis6-302 mutation [Takahashi et al., 1994; Saitoh et al., 1997] caused alleviation of transcriptional silencing of the ura4' gene inserted within the central core of centromere 1 [Partridge et al., 2000]. These studies demonstrate that two distinct types of silencing are present at fission yeast centromeres: the
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Strong outer repeat silencing which is alleviated by mutated clr4, rik1 and swi6, and the weaker, more variable silencing of the central domain which relies on an intact mis6 product. As more information becomes available about the role of Clr4 and Swi6 at the outer repeats (see below) it is clear that transcriptional silencing is a useful tool for the identification of mutations affecting the two functionally distinct regions of fission yeast centromeres. Screens for factors affecting outer repeats or central core silencing successfully allowed the identification of novel centromere components [Ekwali et al., 1999; Pidoux and Ailshire, manuscript in preparation].

Centromeric outer repeat silencing also depends on the histone H3 and H4 acetylation status. Treatment of cells with the histone deacetylase inhibitor trichostatin A (TSA) caused hyperacetylation of the histone H3 and H4 tails and, consequently, alleviation of the ade6" gene inserted within otrR [Ekwali et al., 1997]. Interestingly, the loss of transcriptional silencing was accompanied by remarkable alterations of normal centromeric functions such as chromosome segregation and disperse localisation of the Swi6 protein from its normal heterochromatic foci [Ekwali et al., 1995]. After removal of TSA from culture medium, cell lineages were isolated which maintained the altered centromeric acetylation state for several generations although bulk levels of acetylation dropped back to normal [Ekwali et al., 1997]. This suggests that a change in transcriptional silencing can be inherited in an epigenetic manner, implicating the histone acetylation state in the propagation of either the silenced or the expressed state.

Three HDACs have been identified in fission yeast Clr3, Clr6 and Hda1 [Grewal et al., 1998; Olsson et al., 1998]. Clr6 is essential and shares homology with known HDACs from other organisms. In addition mutations in clr6 alleviated silencing at the mating type locus and at the centromeric outer repeats [Grewal et al., 1998].

Thus, transcriptional silencing is a useful indicator of the state of the centromeric chromatin. In the silenced state, centromeric chromatin is underacetylated, inaccessible to the transcription machinery and is competent for all centromeric functions whereas in the expressed state, this chromatin becomes hyperacetylated, open and accessible to transcription and no longer able to recruit centromeric components and direct chromosome segregation.

Components of fission yeast heterochromatin.

clr4, rik1 and swi6 were originally identified as mutations which alleviate repression of the silent mating type loci mat2 and mat3 [Lorentz et al., 1992; Thon and Klar, 1992; Ekwali and Ruusala,
1994]. Mutation at these loci were found to also affect silencing at the outer repeats and, to some extent at telomeres [Allshire et al., 1995].

The Swi6 protein contains a chromodomain, a motif first identified as a region of similarity between HP1 and Polycomb [Paro and Hogness, 1991], that is 46% identical to the chromodomain of Drosophila HP1 [Lorentz et al., 1994]. Like HP1, Swi6 also contains a shadow domain, which was recently crystallised revealing a dimeric structure forming a putative protein-protein interaction pit [Cowieson et al., 2000]. Interestingly, the pit formed at the dimer interface is commensurate with the binding of an extended pentapeptide motif (PxVxL) that has been identified as the consensus sequence for interaction with HP1 chromo-shadow domain [Cowieson et al., 2000; Smothers and Henikoff, 2000]. Swi6 not only affects silencing at the heterochromatic loci, but also localises at centromeres, mating type locus and telomeres as shown by immunostaining and FISH [Ekwali et al., 1995] forming 2-5 discrete spots per haploid nucleus. Moreover, cells lacking the Swi6 protein showed increased frequency of lagging chromosomes in late mitotic cells and of minichromosome loss [Ekwali et al., 1995]. Swi6 localisation is dependent upon the presence of intact \textit{cir4} and \textit{rik1} gene products [Ekwali et al., 1996] as the absence of either of the two components resulted in Swi6 delocalisation to fill the nucleus, in particular the nucleolus [Ekwali et al., 1996]. Mutated \textit{cir4}, \textit{rik1} and \textit{swi6} showed cold sensitivity at 18°C and an increased frequency of lagging chromosomes. In addition, \textit{swi6Δ}, \textit{cir4} and \textit{rik1} were sensitive to the microtubule poison TBZ [Ekwali et al., 1996]. Mutated \textit{cir4}, \textit{rik1} and \textit{swi6} also showed a genetic interaction with β-tubulin (\textit{nda3}) indicating that these three factors may play a role in the spindle/kinetochore interactions [Ekwali et al., 1996].

Clr4 is the fission yeast homologue of \textit{Drosophila} Su(var)3-9, human SUV39H1 and murine Suv39h1. It contains a chromodomain and a SET domain, both of which are essential for silencing [Ivanova et al., 1998]. The SET domain of Cir4, ike human SUV39H1 and murine Suv39h1, also displays histone H3 K9-specific methyltransferase activity in vitro (HMTase) [Nakayama et al., 2001]. Methylated histone H3 K9 provides a binding site for Swi6 [Bannister et al., 2001] with a similar mechanism to that occurring at mammalian heterochromatin [Lachner et al., 2001]. ChIP experiments, using a specific antibody against H3 methylated at K9, showed that this modification is present across the outer repeats and mating type locus and that it is lost in \textit{cir4Δ} cells [Nakayama et al., 2001]. In addition, cells mutated in the histone deacetylase Cir3
showed slightly reduced methylated K9 at centromeric repeats, which decreased even further in cells mutated in both Clr3 and Clr4 [Nakayama et al., 2001]. Thus, the underacetylated state of histone H3 could be important to allow histone H3 tail methylation by Clr4.

Little is known about the Rik1 protein. Computational analyses revealed that Rik1 contains β-propeller domains typically found in UV-damaged DNA-binding protein and in related proteins [Neuwald and Poleksic, 2000]. Thus, one possibility is that Rik1 functions by binding DNA and recruiting the HMTase Clr4. This possibility is supported by the fact that cells lacking Rik1 did not contain methylated histone H3 at K9 at the centromeric outer repeats and at the mating type chromatin [Nakayama et al., 2001] indicating that Rik1 may act upstream of Clr4. In retrospect these data explain the delocalisation of the Swi6 protein from heterochromatic foci in both the rik1Δ and clr4Δ backgrounds [Ekwall et al., 1996] where the lack of methylated H3 failed to mediate recruitment of Swi6.

Another component of fission yeast centromeric outer repeats heterochromatin is Chp1, a protein that also contains a chromodomain. Cells lacking Chp1 also display chromosomes lagging on the spindle during anaphase and a high rate of chromosome loss [Doe et al., 1998]. In addition, Chp1 is required for outer repeat transcriptional silencing [Partridge et al., 2000; Thon and Verhein-Hansen, 2000] and shows genetic interaction with α-tubulin (nda2) [Doe et al., 1998]. A GFP-Chp1 fusion showed, like Swi6, discrete foci within the nucleus, but unlike Swi6, Chp1 was not required to repress the silent mating type [Doe et al., 1998].

Role of outer repeats components.
Which of these factors have been shown to associate with centromeres in vivo?

As previously introduced, extensive chromatin immunoprecipitation analyses (ChIP) have been used to study the association of centromeric components with the centromere in vivo [Partridge et al., 2000]. The ura4Δ insertions within cen1 were used to measure association of three components with this marker gene inserted at different locations. The centromeric chromatin was composed of two defined regions: a central domain (central core and central core-associated repeats or imr) to which the Mis6-HA associates surrounded by flanking outer repeats, coated with Swi6 and Chp1. Interestingly, extended ura4Δ insertions within the outer repeats, where ura4Δ is flanked by extra non-centromeric DNA, can be coated by Swi6,
indicating that this heterochromatic protein is able to spread over non-centromeric sequences [Partridge et al., 2000]. Thus, outer repeat elements contain underacetylated histone H3 and H4 N-tails, histone H3 methylated at K9 by Clr4 (perhaps recruited by Rik1), Swi6 binding to H3 and Chp1.

Recently an important link between heterochromatin and centromere cohesion has been demonstrated. Sister-chromatids cohesion is mediated by the cohesin complex which is distributed along the entire chromosome with an enrichment at the centromeres. Anaphase is triggered by the cleavage of the Scc1/Rad21 subunit of cohesin, allowing sister-chromatid separation. In metazoans, arm cohesion is released early in mitosis and the sister-chromatids remain associated through the centromeric pool of cohesion [reviewed by Cohen-Fix, 2001]. It is not known what distinguishes arm cohesion from centromere cohesion. In fission yeast Swi6 was found to be required for recruitment of Rad21 indicating that centromeric heterochromatin is important for centromere cohesion [Bernard et al., 2001]. Importantly, cells lacking intact Swi6 showed loss of centromeric cohesion but they maintained arm cohesion. The high frequency of lagging chromosomes observed in mutants lacking Clr4, Rik1 and Swi6 can now be explained as being due to depletion of centromeric cohesion [Bernard et al., 2001].

Genetic evidence has implicated Clr4, Rik1, Chp1 and Swi6 in the kinetochore/spindle interaction pathway [Ekwali et al., 1996; Doe et al. 1998], suggesting that the outer repeats might be the region of the centromere mediating the kinetochore attachment to microtubules. However, the recent study of two microtubule-binding proteins, Dis1 and Aip14, suggests that both regions, outer repeats and central core might be involved in this process. Dis1 and Aip14 bind microtubules in vitro, are localised along interphase cytoplasmic microtubules and are mobilised onto the spindle upon mitotic commitment [Nakaseko et al., 2001]. GFP-Dis1 fusion protein behaved as a kinetochore protein during the progression from metaphase to anaphase and ChIP experiments showed association of Dis1 mainly with the central core DNA and to a lower degree with the inner repeats (imr) [Nakaseko et al., 2001]. Aip14 is a component of the Mad2-dependent spindle checkpoint pathway and appears to be associated with both central domain (imr) and with the outer repeats, but not with the central core [Garcia et al., 2001].
Mutations affecting outer repeat silencing were isolated and identified 12 loci of novel silencing components 10 of which specifically affected centromeric outer repeat silencing [Ekwail et al., 1999], however the proteins encoded by these loci have not yet been identified.
AIM OF THE PROJECT.
This project was focused on understanding the role of histones H3-H4 and histone variants in the structure and function of fission yeast centromere/kinetochore.

At the beginning of this work it was clear that the underacetylated state of histone H3 and H4 N-termini plays an important role in centromere function [Ekwall et al., 1997]. However, it was not known whether one or more specific N-terminal residues of histone H3 and H4 had to be in this underacetylated state to mediate recruitment of heterochromatin factors such as Swi6.

To study the role of histone H3 and H4 in centromere function, one strategy was to perform site-directed mutagenesis of residues likely to play such a role. As fission yeast contains multiple histone H3-H4 gene sets, the first step was to establish whether a fission yeast strain could be generated which contained only one histone H3-H4 pair. Once this strain was generated, it was important to design a strategy to perform single amino acid changes and to rapidly assess the integrity of centromeric silencing. By systematically changing conserved histone N-terminal lysines with residues mimicking an hyperacetylated state, it was expected to identify residues responsible for mediating centromeric silencing.

CENP-A is a histone H3-like protein, essential for centromere activity in all eukaryotes. Several observations have led to the idea that CENP-A is a specialised histone assembled exclusively at active centromeres, perhaps replacing normal histone H3. The presence of a CENP-A homologue in fission yeast had been postulated. One way to identify such a homologue was to frequently search the S. pombe database. The identification of fission yeast CENP-A would allow the investigation of a number of interesting questions. One was to identify which region of the centromere it is associated with and to test whether it replaces histone H3 in this region. Another interesting aspect was to test whether overexpression of histone H3 could interfere with normal CENP-A/Cnp1 association. The requirement for cis-acting DNA sequences for CENP-A assembly is a major task of centromere research. As several studies point toward an epigenetic mechanism for CENP-A loading/centromere propagation, it was interesting to investigate whether fission yeast Cnp1, like its human counterpart, can be incorporated onto DNA which does not contain classic centromeric sequences.
CHAPTER 2
MATERIALS AND METHODS

2.1. GENERAL SOLUTIONS

PBS per litre:
- 10g NaCl
- 0.25g KCl
- 1.43g Na,HPO\textsubscript{4}
- 0.25g KH\textsubscript{2}PO\textsubscript{4}
- Autoclaved.

TE:
- 1mM EDTA
- 10mM Tris-HCl pH8
- Autoclaved.

20xTBE per litre:
- Tris Base 216.0g
- Boric Acid 110.0g
- 0.5 M EDTA (pH8.0) 80.0ml

2.2. YEAST PROTOCOLS

Growth of fission yeast cells.
Cells were grown in liquid cultures of PMG (selective medium) supplemented with the desired supplements (adenine, leucine, uracil, histidine, arginine), or YES (rich medium) at 32°C or 25°C (temperature-sensitive strains) in incubators with agitation. Alternatively, cells were plated on PMG-agar supplemented with the above supplements and/or with the drugs 5-FOA (to select for ura\textsuperscript{-} cells) and TBZ (thiabendazole, a microtubule polymerisation poison), or YES-agar supplemented with 20ml of 50X adenine (full adenine plates) or with 2ml of 50X adenine (1/10 adenine plates) per litre of medium.

Yeast Media.
All of the following media were autoclaved unless otherwise stated.

Per litre

PMG Agar in 900ml:
- Phthallic acid 3.0g
- Di-sodium orthophosphate 2.2g
- Glutamic acid 3.75g
D-Glucose anhydrous 20.0g (Fisher Scientific)
Vitamins 1000X 1.0ml
Minerals 10,000X 0.1ml
50X Salts 20.0ml
Agar 20.0g (OXOID)

PMG in 900ml:
Phthallic acid 3.0g
Di-sodium orthophosphate 2.2g
D-Glucose anhydrous 20.0g (Fisher Scientific)
Glutamic acid 3.75g
Vitamins 1000X 1.0ml
Minerals 10,000X 0.1ml
50X Salts 20.0ml

YES (no ade) agar:
Yeast Extract 5.0g (DIFCO)
D-Glucose anhydrous 30.0g (Fisher Scientific)
Arginine 0.2g (Sigma)
Histidine 0.2g (Sigma)
Leucine 0.2g (Sigma)
Lysine 0.2g (Sigma)
Uracil 0.2g (Sigma)
Agar 20.0g (OXOID)

YES liquid:
Yeast Extract 5.0g (DIFCO)
D-Glucose anhydrous 30.0g (Fisher Scientific)
Adenine 0.2g (Sigma)
Arginine 0.2g (Sigma)
Histidine 0.2g (Sigma)
Leucine 0.2g (Sigma)
Lysine 0.2g (Sigma)
Uracil 0.2g (Sigma)

50X Salts:
Magnesium Chloride 6H2O 53.5g
Calcium Chloride 6H2O 1.0g
Potassium Chloride 50.0g
Di Sodium Sulphate 2.0g

1000X Vitamins (100ml):
Pantothenic acid 0.5g
Nicotinic acid 1g
Inositol 1g
CHAPTER 2 MATERIALS AND METHODS

Biotin 1mg
(Filter sterilised)

10,000X Minerals: Boric acid 5g
MnSO₄ 4g
ZnSO₄ 4g
FeCl₃ 6H₂O 2g
Molybdcic acid 1.6g
KI 1g
CuSO₄ 5H₂O 0.4g
Citric acid 10g
(Filter sterilised)

Supplements Stocks: 50X adenine (Sigma) 5g/l
100X arginine (Sigma) 10g/l
100X histidine (Sigma) 10g/l
100X leucine (Sigma) 10g/l
100X uracil (Sigma) 10g/l (dissolved by adding NaOH)

Malt Extract plates (ME): Agar 20g/l (OXOID)
Malt Extract 30g/l (OXOID)
adenine 250g/l (Sigma)
arginine 250g/l (Sigma)
histidine 250g/l (Sigma)
leucine 250g/l (Sigma)
uracil 250g/l (Sigma)

5-FOA plates: PMG or YES-agar
1g/l 5-FOA (Meiford laboratories)
(added to melted agar when this is under 60°C)

TBZ plates: YES-agar
TBZ (stock 10mg/ml in DMSO) to 10μg/ml or 15μg/ml
(added to melted agar when this is under 60°C)

Mating and random spore analysis.
A small amount of cells from two strains of opposite mating type (h⁺/h⁻) were mixed in a droplet of sterile dH₂O on a nitrogen-free plate (Malt extract or ME plates), and incubated for 1-2 days at 25°C. After this time, the presence of spore-containing ascii was checked by light microscopy.
A loopfull of cells was resuspended in 400μl of sterile dH₂O containing 4μl of Glusulase (NEN) to digest the cell wall and incubated at 25°C or 32°C. Liberated spores were plated on the required selective medium at a density of approximately 500 spores per plate and incubated at 32°C or 25°C until colonies appeared.

**Tetrad dissection analysis.**
Cells from two strains with opposite mating type were crossed on ME plates. When ascii were formed, a loopfull of them were spread until forming a one-cell layer on one side of a thin YES-agar plate supplemented with adenine. Single ascii (18 per plate) were then picked with a micromanipulator (Singer) and positioned on the plate following a matrix and incubated at 18°C overnight. The next day, ascii were broken with the tip of a needle and single spores were isolated following the matrix, and incubated at 25°C. When colonies appeared, the plate was replica-plated on various media to determine the genotype of cells.

**Colony-colour assay.**
Colonies derived from cells containing the ade6 gene inserted at silent sites accumulate a red pigment when grown on media containing limiting concentration of adenine. When transcription of ade6 is activated, colonies are white. To assess the transcriptional state of the silent ade6, cells from various backgrounds were streaked or serially diluted on YES plates supplemented with 10mg/l of adenine.

**Minichromosome loss assay.**
Chromosome loss rates were measured using the linear minichromosome Ch16 [Niwa et al., 1989]. This 530-kb is a derivative of chromosome 3 and is lost in 0.2% of all cells. Since it carries a suppressor of ade6 mutations, when it is lost from cells, they form red rather than white colonies on limiting adenine plates. If loss occurs at the first division of a single cell on such a plate, then half of the resulting colony will retain the minichromosome and form a white sector, whereas the other half will form a red sector. By scoring the frequency of half-sectored colonies, an accurate rate of chromosome loss per division can be calculated. Cells containing Ch16, obtained by crossing with FY537, were plated on YES-agar plates supplemented with 10mg/l of adenine at 500-1000 cells/plate and incubated for 5 days at 25°C. The number of
colonies with a red sector covering at least half of the colony was counted and the rate of chromosome loss was calculated by the formula:

\[ \frac{n^o \text{ of half-sectored colonies}}{n^o \text{ of white} + n^o \text{ of half-sectored colonies}}. \]

Approximately 3000 colonies per strain were counted.

**Serial dilution assay.**
To assess the growth of various mutant strains on different media or at different temperatures, cells from a fresh plate were resuspended in 0.5ml of liquid YES and counted with a coulter counter, followed by serial dilutions in YES in a sterile microtiter plate. 5μl of cells were spotted at the following concentrations per plate: 5x10^5, 5x10^6, 1x10^7, 200, 40, 8. Plates were then incubated at the desired temperature until colonies appeared.

**Estimation of yeast cell number.**
Cell number was estimated using a coulter counter. A 100μl sample of cell culture was mixed in 10ml FacsFlow solution, sonicated for 5 sec at setting 26 on a Sonicator (MSE Soniprep) and counted.

**S. pombe transformation.**
This methods is based on electroporation and was used to transform fission yeast cells with both plasmids and linear fragments of DNA.
Cells were grown to a concentration of 5x10^5-1x10^7 cells/ml. The cells were harvested at RT for 3 min at maximum speed in a benchtop centrifuge. The pellet was washed once with 20ml and twice with 10ml of ice-cold 1.2M Sorbitol then it was resuspended in ice-cold 1.2M Sorbitol to give a concentration of 1x10^6 cells/ml. Cells were divided into 200μl aliquots and mixed with between 50ng (plasmids) and 10μg (linear fragments) of DNA. Linear fragments were often obtained by PCR amplification, agarose electrophoresis and gel purification with the quiagen gel extraction kit. Immediately after mixing with DNA, cells were pulsed, using settings of 2.25kv, 200Ω and 25μF. After the pulse, 1ml of ice-cold 1.2M Sorbitol was added. 100μl of cells was spread on selective medium using sterilised glass beads, and left to incubate at 25°C or 32°C. Transformants appeared after 2-5 days. When transforming with DNA fragments designed to replace the *ura4* gene, cells were co-transformed with a *LEU2* plasmid, grown overnight in
CHAPTER 2 MATERIALS AND METHODS

liquid medium lacking leucine at RT and plated on medium containing 5-FOA and lacking leucine.

Immunostaining.
20-25ml of a cell culture was grown to a concentration of 5x10^6 cells/ml. Cultures were shifted down to 18°C for 30 min prior to fixation. Cells were fixed by adding 3.8% paraformaideyde dissolved in culture medium (a 10X stock was dissolved at 65°C and cooled-down to RT) and shaking the culture at RT or 18°C for 30 min. Cells were spun in a refrigerated benchtop centrifuge at 18°C, washed once with 15ml PEM, transferred to a 1.5ml tube and washed twice with PEMS. Cells were then incubated at 36°C for 90 min in PEMS containing 1mg/ml zymolyase 100T (ICN) at a concentration of less than 10^5 cells/ml. After this time cells were observed on a light microscope to check for digestion of the cell wall (cells become dark). After being washed with 1m of PEMS, cells were resuspended in 200µl of PEMS containing 1% Triton-X100 and incubated on the bench for 5 min. Cells were then washed 3 times with 500µl of PEM, resuspended in 500 µl of PEMBAL and incubated on a rotating wheel for 30 min at RT. Aliquots of cells were taken to be incubated with the appropriate dilution of primary antibodies in 100µl of PEMBAL overnight at 4°C on a rotating wheel.
The antibodies and the dilutions used in this thesis were:
anti-αtubulin (TAT-1), monoclonal, 1/15;
anti-HA (12CA5), monoclonal, 1/30;
anti-phosphoS10 H3, polyclonal (rabbit), affinity purified, 1/5;
anti-Swi6, polyclonal (rabbit), affinity purified, 1/30;
anti-Cnp1; polyclonal (sheep); crude serum, 1/500.
After incubation with primary antibodies, cells were washed three times with 500µl of PEMBAL incubating for 5 min with rotation at each wash. The required secondary antibodies (anti-mouse, anti-rabbit or anti-sheep) conjugated with the desired fluorescent conjugate (Texas red or FITC) were added at the concentration of 1/100 in 100 µl of PEMBAL. Tubes were wrapped in foil and incubated at RT with rotation for 4hrs followed by one 5 min wash with 500µl of PEM, one 5 min wash with PBS containing 0.1% Sodium Azide and one 5 min wash with PBS-0.1% Sodium Azide containing 1µl /ml DAPI (stock 500X stored at -20°C). Cells were finally spun and
resuspended in 20μl of PBS-0.1% Sodium Azide. 5μl of cells were spread in a thin layer on a glass coverslip previously coated with 1mg/ml Poly-L-lysine hydrobromide (Sigma) and allowed to dry. Coverslips were then placed face-down avoiding formation of air bubbles on a glass slide where 100μl of Vectashield were placed. The coverslips were sealed with transparent nail polish and observed at a fluorescence microscope. Images were acquired using the IPLab software.

PEM: 100mM Pipes, pH6.9; 1mM EDTA; 1mM MgSO₄
PEMS: PEM containing 1.2M Sorbitol
PEMBAL: PEM containing 1% BSA (Sigma), 0.1% Na Azide; 100mM Lysine hydrochloride (BDH).

Live analysis of GFP-Swi6 expressing cells.
Cells derived from the nm81X-GFP-Swi6 (FY2214) were grown to log phase in 2ml of thiamine-free medium (PMG) supplemented with all amino acids. 1% low melting point agarose (Gibco) in culture medium was boiled and left at 37°C ready for use. Cells were pelleted, resuspended in 4μl of culture medium and put on a microscope glass slide. 6μl of agarose were added to the cells and mixed well. A glass coverslip was applied on top and pushed down firmly. Slides were observed at the fluorescence microscope.

2.3. DNA AND RNA TECHNIQUES

Agarose gel electrophoresis.
Agarose gel electrophoresis was utilised to separate DNA fragments of different size. Routinely, agarose (BG) was melted in 1XTBE at the desired concentration, ranging from 0.8% to 1.2%. 0.25μg/ml Ethidium Bromide was added to the gel before pouring. DNA was loaded onto the gel in 1X loading buffer and electrophoresed in 1XTBE in a gel apparatus (Flowgel) at 50-120mV. A ladder DNA was also loaded to assess DNA size (100bp ladder, 1Kb ladder or λDNA/HindIII). Gels were photographed on a UV transilluminator.

Loading buffer (6X): 0.25% Bromophenol blue or Orange G, 30% glycerol in water.
PCR.

All the PCR performed in this thesis were based on the touchdown program. The only variable was the extension time (1 min; 2 min) which depended on the size of the desired PCR product. The 2 min cycles were used to obtain PCR products larger than 1.5kb (all the gene replacements with ura4'). The template DNAs were: SPZ treated cells for PCR checks of genotypes after crosses or transformations; genomic DNA obtained with the small scale method (1\mu l of a 1:100 dilution in 20\mu l of PCR mix); plasmid DNA (1\mu l of a 1:100 dilution in 20\mu l of PCR mix).

The standard PCR mix contained: 50ng/\mu l of primer, 0.5\mu l of ampiltaq polymerase (Roche-Applied biosystems), 250\mu M dNTPs (AB gene), 10x PCR buffer (Roche), 1.5mM MgCl₂ (Roche). Optimal PCR conditions were obtained by titration of the MgCl₂ concentration.

Touchdown: 94°C 4min; (94°C 30sec; 65°C 30sec; 72°C 1min or 2min)3X; (94°C 30sec; 62°C 30sec; 72°C 1min)3X; (94°C 30 sec; 59°C 30sec; 72°C 1min)3X; (94°C 30 sec; 56°C 30sec; 72°C 1min)3X; (94°C 30 sec; 53°C 30sec; 72°C 1min)3X; (94°C 30 sec; 50°C 30sec; 72°C 1min)3X; (94°C 30 sec; 47°C 30sec; 72°C 1min)5X; (94°C 30 sec; 45°C 30sec; 72°C 1min)10X; 72°C 5min.

'ura4' program: 94°C 4min; (94°C 30sec; 55°C 30sec; 72°C 1min) 30 times; 72°C 5 min.

PCR performed in 0.2ml thin walled tubes in a MJR thermal cycler.

Radioactive competitive PCR.

This technique was used to quantify PCR products after RT-PCR and after ChIP. The conventional 'ura4' PCR program was used on PCR mix containing 1\mu l of α²P-dCTP for every 200\mu l of mix. This method was used to PCR both ade6/ade6-D/NN RT-PCR and ura4/ura4-D/SE ChIP. After the PCR was completed, samples were electrophoresed on 4% acrylamide gel in TBE1X with a minigel apparatus (Hoefer) at 100V for 40 min, followed by vacuum drying of the gel and exposure to a phoshorimager cassette. Data were collected using the Storm phosphorimager and quantified with the imageQuant software.

Small scale S. pombe DNA preparations.

5ml of stationary phase cell culture was pelleted at maximum speed in a benchtop centrifuge. The pellet was resuspended in 250\mu l SP1 containing 0.4mg/ml Zymolyase 100T (ICN) and incubated for 60 min at 37°C. Spheroplasted cells were pelleted at 8,000 rpm in an eppendorf
centrifuge for 15 sec. The pellet was resuspended in 0.5ml TE, 50μl of 10% SDS was added, followed by vortexing and addition of 165μl 5M K acetate. Samples were stored on ice for 30 min. The supernatant was added to 0.75ml isopropanol, placed on dry ice for 5 min and centrifuged for 10 min. The pellet was resuspended in 0.3ml TE and RNase added to 10μl/ml. After 1hr 30min at 37°C, the sample was extracted with phenol/chloroform and precipitated with Ethanol. Genomic DNA was resuspended in 20μl TE.

SP1: 1.2M Sorbitol, 50mM Sodium Citrate; 50mM Sodium Phosphate; 40mM EDTA. pH to 5.6.

**Rapid S. pombe genomic DNA preparation for PCR (Ling et al., 1995).**
A small amount of cells was picked with a sterilised cocktail stick from a fresh patch of cells and placed in a sterile microfuge tube containing 15μl of the SPZ buffer. After mixing the suspension was incubated at RT for 10-15 min and 1μl of it was used as DNA template for a 20-50μl PCR reaction.

SPZ buffer: 1.2 Sorbitol; 100mM Sodium phosphate, pH 7.4; 2.5mg/ml Zymolyase 100-T. Stored at –20°C.

**Total S. pombe RNA preparation.**
Cells were grown at 32°C in YES medium to a density of approximately 1x10⁷ cells/ml. The cultures were pelleted by centrifugation, washed in TE and transferred to microfuge tubes prior to being resuspended in 300μl RNA extraction buffer. 300μl of glass beads (Sigma) were added followed by 300μl phenol/chloroform pH 4.7 (Sigma). The microfuge tubes were shaken at high speed on a multi-head vortexer for 30 minutes at 4°C to lyse the cells, followed by centrifugation at 10,000 rpm for 5 minutes and removal of the supernatant. The supernatant was extracted twice with phenol chloroform, and then once with chloroform. The RNA obtained was precipitated with 3 volumes cold 100% ETOH and centrifuged at 10,000rpm for 15 minutes at 4°C. The pellet was air-dried and resuspended in 25μl dH₂O. The concentration of each sample of RNA was determined by measuring the optical density of a 1/500 dilution in a spectrophotometer set at a wavelength of 260nm. The samples were then diluted to a concentration of 1μg/μl with dH₂O.
CHAPTER 2 MATERIALS AND METHODS

RNA Extraction Buffer: 50mM Tris-HCl pH7.5, 10mM EDTA, 100mM NaCl, 1% SDS.

RT-PCR.
For RT-PCR 2.5 µg of each sample of RNA was aliquoted into a microfuge tube. DNAse buffer, 1µl DNAse and dH₂O were added to a final volume of 10µl and the mix was incubated at 25°C for 1 hour. After this time 1µl 25mM EDTA was added and the reactions incubated at 65°C for 10 minutes to denature the DNAse. 1µl of the reaction was used as template for a PCR reaction to check that all of the DNA had been digested. Once it had been established that there was no DNA remaining as a contaminant in the sample 1µg oligo dT₁₂₋₁₈ and dH₂O were added to a final volume of 25µl. The samples were incubated for 10 minutes at 70°C, then placed on ice. The samples were centrifuged briefly at 4°C and returned to ice before adding 4µl 10X PCR buffer (Roche), 4µl MgCl₂ (Roche), 4µl DTT and 2µl 2.5mM dNTPs. The samples were mixed then split into two 19µl aliquots and incubated at 42°C for 5 minutes. 1µl reverse transcriptase (Superscript GIBCO) was added to one of each pair of tubes only. The incubation at 42°C was continued for another 50 minutes then the samples were incubated at 70°C for 15 minutes to stop the reaction and the samples returned to ice. 1µl of each sample was used as template in a 20µl PCR reaction to determine the amount of transcript.

Mnase digestion of chromatin.
300ml of cell cultures were grown until a concentration of 5x10⁵ cells/ml. Cultures were spun down at maximum speed in a benchtop centrifuge for 2min. Cells were resuspended in 20ml of SP1 buffer containing 40µl of β-Mercaptoethanol and incubated for 10min at RT. After centrifugation, cells were resuspended at the concentration of 2x10⁵ cells/ml in SP2 buffer containing 5mg/ml Zymolase 100T and incubated at 36°C for 15 min with vigorous shaking. Spheroplasts were pelleted and washed three times with 10-30ml of SP3 buffer (by resuspending gently at each wash) and resuspended in 10ml of NDB buffer. Micrococcal nuclease (50,000 units/ml- Worthington) was diluted in 1ml of NDB-BS containing 0.15% NP-40 at the following concentrations: 0, 25, 50, 100, 150, 200, 300 and 500 units/ml. Spheroplasts were pelleted, resuspended in 1.6ml of NDB-BS and divided in 200µl aliquots. Enzyme and spheroplasts were pre-warmed at 36°C. 200µl of the 0units/ml Mnase dilutions were added to the first series of spheroplasts aliquots. After 1min the 25units/ml Mnase dilution was added to
the second series of spheroplasts and so on. After 5 min 40 µl of warmed 250 mM EDTA with 5% SDS (stop solution) was added to the tubes containing the 0 units/ml Mnase dilution. After 1 min the stop solution was added to the tubes containing 25 units/ml Mnase dilution and so on. 3 µl of 10 mg/ml Proteinase K were added to all samples and tubes were then incubated at 50°C overnight. 200 µl of dH₂O were added and samples were extracted once with phenol/chloroform and once with chloroform. 1/10th of the volume of 3 M NaOAc and 3 volumes of ethanol were added, samples were mixed thoroughly and precipitated for 10 min on dry ice. Samples were centrifuged at 13,000 rpm in a eppendorf centrifuge for 10 min at 4°C. Pellets were washed with 70% Ethanol, air-dried and resuspended in 30 µl of TE containing 100 µg/ml RNase. Samples were incubated at 37°C for 1 hr, orange G dye was added and half of the volume was loaded on a 1.2% agarose gel. DNA was subject to Southern analysis.

SP1: 20 mM citrate/phosphate, pH 5.6; 40 mM EDTA, pH 8
SP2: 50 mM citrate/phosphate; 1.2 M Sorbitol, pH 5.6
SP3: 10 mM Tris-HCl pH 7.6; 1.2 M Sorbitol
NDB: 1.2 M Sorbitol. 10 mM Tris-HCl, pH 7.5; 50 mM NaCl; 5 mM MgCl₂; 1 mM CaCl₂
NDB-BS (for four strains): 15 ml NDB; 1 µl b-Mercaptoethanol; 7.5 µl 1 M Spermidine (free base).

Southern transfer.
Following run, the ethidium bromide stained agarose gel was photographed next to a fluorescent ruler. The gel was soaked in depurinating solution with gentle rocking for 10 min only. After rinsing twice with distilled water the gel was immersed in denaturing solution twice for 15 min with gentle rocking. In a tray containing denaturing solution the following stack was made: a large gel tray upside-down, 3 large pieces of 3MM paper soaking in the denaturing solution, 3 gel-size pieces of 3MM, the gel upside-down, Genescreen membrane previously soaked in distilled water followed by denaturing solution, large stack of paper towels, a glass plate and a weight of approximately 500 g on top. Care was taken while stacking papers and gel that no air bubbles were formed. The exposed parts of the stack were wrapped in cling film to prevent evaporation, the transfer was left on overnight.
After disassembly, the location of the wells was marked on the membrane. The membrane was floated on 50mM phosphate pH 7.2 twice for 10 min, quickly dried on a piece of 3MM paper and crosslinked with Stratalinker program C3 (damp membrane). The membrane was stored at room temperature until required.

Depurinating Solution: 25ml of concentrated HCl in 500ml dH2O

Denaturing Solution: 0.5M NaOH, 1.5M NaCl.

1M Sodium Phosphate (per litre): 89g of Na2HPO4, pH to 7.2 with orthophosphoric acid.

**Probe manufacture.**

25ng of the fragment of DNA to be used as probe were diluted to 13μl with dH2O, boiled for 10min and immediately put on ice. The following were mixed: 13μl of denatured DNA, 3μl (30 μCi) of α32P-dCTP, 4μl of High Prime (Roche) solution. The mix was incubated for 30 min at 37°C, followed by addition of 80μl of 25mM EDTA to stop the reaction. The probe was kept on ice and boiled for 5 min before use.

**Pre-hybridisation and hybridisation.**

10-15ml of Church buffer were added to the hybridisation bottle containing the membrane with the DNA side in and incubated in the roller oven for 1hr at 65°C. The probe was boiled for 5 min and added immediately to the bottle. The membrane was incubated at 65°C overnight. The hybridisation solution was poured off and the membrane was washed three times with 100ml Wash buffer for 15 min at 65°C. The membrane was then dried on 3mm paper and exposed into a phosphorimager cassette. The data were collected in the Storm phosphorimager.

Church buffer: 0.5M Na phosphate pH7.2; 7% SDS; 1mM EDTA; 1% BSA.

Wash buffer: 40mM Na phosphate pH7.2; 1mM EDTA; 1% SDS.

**Sequencing.**

The DNA to be sequenced was prepared by PCR followed by gel purification with gel-extraction kit (Qiagen). For each reaction the following were mixed: template DNA (100-400ng), 8μl ABI Dye terminator mix (Perkin Elmer), 3.2pmol primer, dH2O to 20μl. The SEQ PCR program was used in a thermal cycler (MJ Research) PCR machine. After PCR 2μl 3M NaAc pH5.5 and 50μl
of 95% ethanol were added. After vigorous mixing, the DNA was precipitated for 30 min at RT, followed by 20 min of centrifugation at RT in an eppendorf centrifuge at maximum speed. The supernatant was discarded and the pellet was washed once with 250 μl of 70% ethanol, followed by air-drying. The sequencing mix was run by the MRC sequencing facility.

SEQ program: 96°C 5 min, (96°C 30 sec, 55°C 15 sec, 60°C 4 min)25X.

2.3. PROTEIN TECHNIQUES

Chromatin immunoprecipitations (ChiP).
This protocol was adapted from Ekwali and Partridge, 1998.

50 μl of exponentially growing cells (5x10⁶ cells/ml) were shifted from the growing temperature (25°C or 32°C) at 18°C for 30 min. The same number of cells were utilised in all samples. Cells were fixed for 30 min at 18°C with 3% paraformaldehyde dissolved in culture medium. Fixation was stopped by adding 2.5M glycine (20X) to cultures for 5 min at RT with agitation. Cells were then washed twice with 20 μl of ice-cold PBS, resuspended in 1 ml of PBS and transferred to a 2 ml screw-cap tube. Cells were spun in microfuge for 30 sec at 4°C at maximum speed and resuspended in 400 μl of ice-cold lysis buffer containing protease inhibitors cocktail (100X Sigma) and 2 mM PMSF. Glass beads (acid-washed, Sigma) were added until a thin layer of liquid is left in the tube (~1 ml). Cells were lysed in a bead beater at top speed for 2 min on ice. The cell lysate was recovered by piercing the bottom of 2 ml screw-cap tube, placing on top of a fresh 1.5 ml tube without the lid and by placing both tubes in a 15 ml snap-cap tube. Tubes were spun for 3 min at 4°C on a benchtop centrifuge and the recovered lysate was resuspended gently using a blue-tip micropipette. Lysates were sonicated to shear the chromatin for 10 sec at #22 on ice. This should result in shearing the chromatin to approximately 500-1000 bp. The lysate was spun at maximum speed in a microfuge for 5 min at 4°C to eliminate debris. The supernatant was transferred to a fresh tube and spun again for 15 min. The chromatin obtained was pre-cleared by adding 1/6 of the volume of beads (Roche) protein G agarose (for anti-Cnp1 ChiP) or protein A agarose (for anti-HA, anti histone H3N/H3C, anti-AcKH3 and anti-ACKH4 ChiP) in a 1:1 v/v suspension in lysis buffer and incubating with gentle rocking for 1-2 hs at 4°C. Beads were spun at 8000 rpm for 3 minutes at 4°C and the supernatant was transferred to a fresh tube. 1/10 of this pre-cleared lysate was frozen and constituted the total input DNA.
sample. The appropriate amount of antibodies was then added to the remaining lysate at the following concentrations:

- anti-Cnp1: 10μl in 300μl lysate (1:30).
- anti H3 N-terminus, anti-Ac H3 and H4 lysines (M. Grunstein): 2μl in 20μl lysate (1:10).
- anti H3 C-terminus (A. Verreault): 20μl in 400μl lysate (1:20).
- anti methylated K9 H3 (upstate): 6μl in 120μl (1:20).

Tubes were incubated at 4°C with gentle rocking for 1hr. A volume of beads resuspended 1:1 in lysis buffer equivalent to 1/6 of the volume of lysate was added and tubes were incubated overnight at 4°C with gentle rocking. Beads were spun at 8000rpm, washed for 5min at RT with gentle rocking with 1ml of each of the following buffers: Lysis buffer, Lysis buffer 0.5M salt, Wash buffer, TE pH8. After the washes 50μl of TES were added to the beads and tubes were incubated at 65°C for 10min. The beads were spun and the supernatant transferred to a fresh tube. Beads were washed again with 200μl of TES, spun and the two supernatants were pooled. TES was added to the total input DNA samples (T) to obtain the same final volume. Tubes were incubated overnight at 65°C to reverse the crosslinking. After cooling down samples, 250μl of TE and 25μl of 10mg/ml Proteinase K (Roche) were added and tubes were incubated at 37°C for 2hrs. Samples were phenol/chloroform extracted to eliminate proteins and the DNA samples were transferred into 2ml tubes. To the DNA samples were added 1/10 of the volume of 3M NaOAc, 2.5 volumes of ice cold ethanol 1.5μl of 10mg/ml Glycogen. Samples were mixed thoroughly by vortexing and incubated in dry ice for 1hr. DNA was recovered by centrifuging the samples at 4°C for 30min at maximum speed. The pellet was dried under a fume hood. ChIP DNA (IP) was resuspended in 30μl and total Input DNA (T) in 300μl of TE. 2μl of DNA were used for 20μl PCR reactions containing 3mM MgCl₂ with primer pair F1944/F1945 for ura4' PCRs and primers Q947/Q949, P75/P76, E615/E616 for multiplex PCR. The 'ura4' program was used for all the PCR of ChIP DNA described in this thesis: 94°C 4min; (94°C 30sec; 55°C 30sec; 72°C 1 min) 30 times; 72°C 5 min.

Lysis buffer: 50mM Hepes-KOH, pH7.5; 140mM NaCl; 1mM EDTA; 1% (v/v) triton X-100; 0.1% (w/v) sodium deoxycholate.
Lysis buffer 0.5M NaCl: Lysis buffer containing 500mM NaCl.

Wash buffer: 10mM Tris-HCl, pH8; 0.25M LiCl; 0.5% NP-40; 0.5% (w/v) sodium deoxycholate; 1mM EDTA.

TE: 10mM Tris-HCl pH8; 1mM EDTA.

TES: 50mM Tris-HCl pH8; 10mM EDTA; 1%SDS.

**Total protein extraction from S. pombe cells.**

10ml of cells were grown to 5x10^6 cells/ml in YES or PMG selective medium. The same number of cells was used for each strain. Cells were spun in a benchtop centrifuge at maximum speed for 2 min. Pellet was resuspended in 1ml dH_2O, transferred to microfuge tube, and centrifuged for further 30 sec followed by removal of the supernatant. Cell pellet was resuspended to obtain 5x10^7 cells in 100μl of 2XSB containing PMSF (50μl of 100mM stock per ml of SB) and transferred to a screw-cap tube. An equal amount of glass beads (Sigma) was added and cells were lysed in a bead beater for 2min at maximum speed, followed by boiling for 5-10min. The extract was transferred to a fresh microfuge tube using a duck-bill tip to avoid glass beads. The extract was then spun and transferred to a fresh tube avoiding cell debris. 10-20μl were used in SDS PAGE.

2X SB (Sample buffer) for 10ml: 2.5ml o.5M Tris-HCl, pH6.8; 2ml Glycerol; 4ml 10% SDS; 0.5ml 0.1% (w/v) Bromophenol blue; 0.5ml 2-mercaptoethanol; dH_2O. Stored at -20°C.

**Rapid S. pombe histones preparations (adapted from Ekwali et al., 1997).**

Cultures were grown in YES approximately to a concentration of 5x10^6 cells/ml. For each preparation 1x10^8 cells (20 ml of culture) were washed in 10 ml of ice cold NiB buffer and resuspended in 500μl of NiB buffer with proteins inhibitors and PMSF. 500 μl of glass beads (acid washed, 400-600nm SIGMA) were added. Cells were lysate using the beadbeater for two minutes, keeping samples on ice all the time, good percentage (>70%) of lysis was checked at the microscope. The lysate was recovered by punctuating the bottom of the beadbeater tube and placing it on top of a new 1.5ml tube. Both tubes were then placed in a “snap top” 15ml tube and centrifugated for 1 min at max speed in a benchtop centrifuge at 4°C. The lysate was spun in a fresh 1.5ml tube for 10 min at max speed at 4°C. The supernatant was discarded. The pellet
was resuspended in 0.5ml of 0.4M sulfuric acid (Aldrich) and incubated for 1hr on ice to extract basic proteins. The extract was then centrifuged at max speed for 5 min at 4°C and the supernatant was collected to a fresh microfuge tube. The acid extraction is repeated once, without further incubation on ice. The pooled supernatants (1ml total) were precipitated overnight in glass Corex tubes at -20°C with 12 volumes of ice-cold acetone. The precipitate was collected by centrifugation at 7,000 rpm for 15min at 4°C (Sorvall SS-34). The pellet was air dried the pellet under a fume hood and resuspended in 4M urea.

The protein concentration was determined using Biorad protein assay kit at 595 nm following the manufacturer's instructions. This method yielded 50-100 µg of protein. 3-5 up to 10 µg were used to visualise histone bands in Coomassie-stained gels and in Western blots. 2XSB was added, samples were boiled and electrophoresed on a 16% acrylamide (Prosieve) gel; pre-stained protein marker (Benchmark or Prosieve) were used to run the gel long enough (10KDa band almost to the bottom of the gel) to separate well the histone bands.

NIB buffer: 0.25M Sucrose, 60mM KCl, 15mM NaCl, 5mM MgCl2, 1mM CaCl2, 15mM Pipes pH 6.8, 0.8% Triton X-100.

2X SB (Sample buffer) for 10ml: 2.5ml 0.5M Tris-HCl, pH6.8; 2ml Glycerol; 4ml 10% SDS; 0.5ml 0.1% (w/v) Bromophenol blue; 0.5ml 2-mercaptoethanol; dH2O. Stored at -20°C

SDS-PAGE (Laemmli, 1970).

Proteins were separated on 1mm thick discontinuous SDS-PAGE (Sodium dodecyl sulphate-polyacrylamide gel electrophoresis) with the Hoefer minigel apparatus. The Prosieve 50 gel solution (FMC) was used to improve the separation of small molecular weight proteins, such as histones and histone variants. Two concentrations of resolving gel (14% and 16%) were used which should separate optimally proteins within the size range of 5-50 KDa.

Resolving gel (for 10ml):

14%: 4.5ml dH2O, 2.8ml Prosieve 50; 2.5ml 1.5M Tris-HCl, pH8.8; 0.1ml 10% SDS; 0.1ml 10% APS; 4µl TEMED.

16%: 4.1ml dH2O, 3.2ml Prosieve 50; 2.5ml 1.5M Tris-HCl, pH8.8; 0.1ml 10% SDS; 0.1ml 10% APS; 4µl TEMED.
CHAPTER 2 MATERIALS AND METHODS

Stacking gel (5%) for 5ml: 3.75ml dH₂O, 0.5ml Prosieve 50; 0.65ml 1.5M Tris-HCl, pH6.8; 0.05ml 10% SDS; 0.05ml 10% APS; 5µl TEMED.

Resolving gel was poured first, followed by stacking gel. Gels were run in 1X Tris/Tricine/SDS buffer at 130V (constant voltage) for approximately 30min.

10X Tris/Tricine/SDS (1 litre): 121g Tris Base; 179g Tricine; 100ml 10% SDS, dH₂O.

Western blot analysis.
Proteins were transferred on Protran nitrocellulose (Schleicher & Schuell) using a Hoefer semi-dry electrophoretic transfer apparatus. The membrane was floated on dH₂O then soaked on blotting buffer, then placed on top of 6 pieces of 3MM paper of the gel size, also previously soaked in blotting buffer. The SDS gel was placed on top of the membrane followed by 6 more pieces of 3MM paper soaked in blotting buffer. As each layer was added, bubbles were rolled out using a plastic pipette. Transfer was done at the constant amperage of 65mA for 30min-1hr. The membrane was washed in dH₂O, followed by staining with Ponceau solution (Biorad) to verify good protein transfer. The membrane was rinsed in PBS, followed by incubation in blocking buffer for 1hr at RT with agitation. After a brief wash with PBS-0.2% Tween, the membrane was placed in a sealed plastic bag and incubated with the primary antibody of interest in PBS-0.2% Tween overnight at 4°C with agitation. The membrane was washed three times for 15 min with PBS-0.2% Tween at RT with agitation followed by incubation with the appropriate HRP (Horseradish peroxidase) conjugated antibody diluted 1:2,500 in blocking buffer for 1hr with agitation. Membrane was washed again as described and washed twice for 10min in PBS. Excess liquid was removed from the membrane on 3MM paper, and proteins detected using the Enhanced Chemi-Luminescence kit (Amersham) following the manufacturer's instructions. The blot was exposed to kodak film for 10min-1hr.

Blotting buffer (for 100ml): 10ml 10X Tris/Tricine/SDS; 70ml dH₂O; 20ml MeOH.
Blocking buffer: 5% Marvel dried milk; 0.2% Tween in PBS.

Western blot to detect histone H3 and H4.
A modified western blot procedure was applied to detect various acetylated histone H3 and H4.
The membrane was blocked in freshly prepared PBS 3% non-fat milk (PBS-MLK) for 20min at
RT with constant agitation, followed by incubation with a 1:2,000 dilution of anti-acetylated H3/H4; 1:3,000 of anti-Nter H3/H4 (gift from M. Grunstein) or 1:2,000 anti-H3C (A. Verreault) in PBS-MLK overnight at 4°C with agitation. The membrane was washed twice with water and incubated with secondary antibody HRP conjugated anti-rabbit 1:2500 in PBS-MLK for 1.5 hrs at RT with agitation. The blot was washed twice with water, followed by one wash with PBS-0.05% Tween for 5 min, and by one wash with water for 5 min. The histones were revealed using the ECL kit as previously described.

**Immunoprécipitations.**

100 ml of cell cultures were grown to the concentration of 5×10⁶ cells/ml. Cells were spun in a benchtop centrifuge for 2 min at top speed and washed once with dH₂O. Cells were transferred to a 2 ml screw-cap tube, centrifuged and the pellets frozen in liquid N₂. Pellets were resuspended in 500 μl of ice-cold Lysis Buffer containing protease inhibitors (100X Sigma) and 1 mM PMSF (100 mM stock in methanol). Approximately 500 μl of glass beads (acid washed-Sigma) was added. Cells were lysate in bead-beater for 2 min at maximum speed on ice, spun briefly to pellet beads/debris and transferred to a new tube. DTT was added to 0.5 mM and lysate was spun 3 times for 5 minutes, transferring the supernatant to a fresh tube. The lysate was pre-cleared at 4°C with rotation with 25 μl of PtA or PtG agarose (Roche) as appropriate that has been washed twice in Lysis buffer for 10 min-1 hr. The pre-cleared lysate was transferred to a fresh tube and 20 μl were kept as whole cell extract (WCE) and stored at -20°C. Volume of extract was made up to 500 μl with Lysis buffer and the desired antibody was added (10 μl of anti-Cnp1; 30 μl of anti-HA). Samples were mixed and incubated with gentle rotation at 4°C for 1 hr. 25 μl of the appropriate PtA or PtG agarose (Roche) (1:1 in Lysis buffer). Immunoprécipitations were incubated with gentle rotation at 4°C overnight. Beads were washed 3 times with 500 μl of ice-cold Lysis buffer and at the last wash transferred to a new tube. Beads were further washed twice with PBS and the supernatant was removed using duckbill tips. 20 μl of 2X sample buffer were added to IP and WCE samples and tubes were heated at 65°C for 10 min. 5-10 μl of material was loaded on a 14% SDS-PAGE (Prokieve) and followed by Western blot analysis.
CHAPTER 2 MATERIALS AND METHODS

Lysis buffer: 50mM Hepes, pH7.6; 75mM KCl; 1mM MgCl₂; 1mM EGTA; 0.1% TritonX-100.

2.3. STRAINS GENERATED IN THIS THESIS.

Generation of strains with reduced histone h3-h4 gene sets.

Strains with replaced histone gene sets were generated by Leslie Bali (Honours Project, 1998) using primers containing 58bp of homology with the histone set of interest fused to 22bp of the ura4* gene. PCR products obtained were gel purified and transformed into yeast FY1180. Selection was made on plates lacking uracil. Transformants were PCR checked with the following primer pairs: P681/T952 (for the deletion of h3.1/h4.1), P683/T952 (for the deletion of h3.2/h4.2), and P684/T952 (for the deletion of h3.3/h4.3). The histone deleted strains were backcrossed three times with the wild type. Double-deleted strains were obtained by mating of single followed by selection of the progeny on medium lacking uracil and PCR check. The presence of the otriR::ade6* insertion was checked using primers L72/ade6B.

To generate a strain containing only the h3.2/h4.2 pair to be used to generate H3 and H4 N-tails mutants, single ura4* histone deletions were replaced with different marker genes. The h3.1/h4.1Δ::ura4* was replaced with the his3* gene using primers P720/P724. Whereas the h3.3/h4.3Δ::ura4* was replaced with the arg3* gene using primers P719/P723. After backcrossing of the two strains obtained, a strain containing both these replacements was generated by cross and used to place the ura4* tag downstream of h3.2 and of h4.2. This ura4* tag was obtained amplifying the ura4* gene from plasmid pUR19 (lab stock) with two primers containing 62bp homologous to the 3' end of the histone gene (just after the stop codon) and 18bp of ura4* (U690/U691 for h3.2 and V985/V986 for h4.2).

Disruption of the h4.2 gene was obtained by replacement again with the ura4* gene using primers amplifying histone h3.2 flanking sequences fused to ura4* (P674/P671) and transformation of the wild type FY1645. Replacement of h3.2 was obtained in the same way using the primer pair R635/Q164.

Generation of histone H3 and H4 N-tail mutations.

Mutated h4.2 or h3.2 genes were generated by a two-step PCR strategy. Two overlapping primers containing the mutated codons were designed to point in opposite directions. Each primer was used in a PCR reaction in combination with an appropriate primer annealing
upstream or downstream of the gene. The template DNA used was genomic DNA from a wild type strain (FY106). These PCR resulted in two DNA fragments, the 5' and the 3' of the histone gene that overlapped in the region where they were mutated. The PCR products were gel purified using the gel-extraction kit (Quiagen), following the manufacturer’s instructions. The two DNA fragments were then mixed together and used as DNA template in a PCR where the two external primers were used to amplify the entire h4.2 or h3.2 locus. This PCR generated a mutated h4.2 or h3.2 gene that was introduced in the genome of the strains described in Chapter 4.

The two mutagenic fragments were generated using the following primer pairs:

3. H4K8AK16G: same primers used for K8A, using as template DNA the K16G genomic. Followed by amplification with B2/B1. And:

\textit{cnp1} replacement with \textit{ura4}. The \textit{ura4} gene was amplified by PCR from plasmid pUR19 using primers containing 58bp of the \textit{cnp1} flanking sequences fused to 22bp of \textit{ura4} (X26/X27). The DNA fragment obtained was gel purified using the quiagen kit and introduced by transformation in the genome of the diploid strain FY1963, as it was predicted that \textit{cnp1} might be essential. Cells were plated on PMG lacking uracil and adenine and colonies appeared after 2 days.

\textbf{2.4 BACTERIAL METHODS.} Bacterial cells DH5α were used for all the cloning performed in this thesis. Cells were grown at 37°C in LB medium, solid or liquid, supplemented with 30μg/ml ampicillin for plasmid selection.

\textbf{Bacterial media.} LB (per litre): 10g Bacto-peptone; 5g Yeast extract; 10g NaCl. Autoclaved.
Preparation of competent cells.
A single colony of DH5α cells was grown overnight in 5ml LB. This was diluted into 100ml LB and grown for about 2 hrs until the OD₅₅₀ reached 0.48. Cells were chilled on ice then pelleted at maximum speed for 5 min in a eppendorf centrifuge at 4°C. The supernatant was discarded and the pellet resuspended in a minimal volume of LB. 40ml ice-cold TFB-I was added and the cells were gently mixed with a pipette. Cells were incubated on ice for 15-45 min, then centrifuged for 10 min at maximum speed at 4°C. The pellet was gently resuspended in 8ml ice-cold TFB-II and incubated on ice for further 30 min. Cells were aliquoted into pre-chilled tubes and frozen on dry ice. Competent cells were stored at -70°C until use.

TFB-I: 30mM K acetate, 0.1M RbCl₂, 10mM CaCl₂ 2H₂O; 50mM MnCl₂; 15% glycerol; pH to 5.8 with acetic acid. Filter sterilised.

TFB-II: 10mM PIPES; 75mM CaCl₂ 2H₂O; 10mM RbCl₂; 15% glycerol; pH to 6.5 with KOH. Filter sterilised.

Transformation of competent cells.
An aliquot of frozen competent bacteria was thawed on ice. 100µl of cells was added to a 10 µl ligation mixture or to 50ng plasmid DNA and incubated on ice for 30 min. Cells were heat-shocked for 1 min at 42°C followed by 2 min on ice. 1ml of LB was added and cells were incubated at 37°C for 30-45min. Cells were spread on LB-agar+Amp plates.

Plasmid constructions.
Restriction enzymes were obtained from Boehringer-Mannheim and were used in the reaction buffer supplied by the manufacturer. All digests were carried out at 37°C for 2-3hs. Digested DNA fragments were gel-purified using the gel extraction kit (Qiagen), following the instructions supplied by the manufacturer. Ligations were carried out using T4 DNA-ligase (Roche) in the supplied buffer, in 20µl final volume, overnight at 16°C. A typical ligation contained large excess of insert DNA fragment (15-10:1) with respect to the vector.

Plasmid minipreps.
2-3 ml of LB+Amp cultures of bacteria were grown overnight. The plasmids were isolated using a miniprep kit (Qiagen) following the instructions provided by the manufacturer.
Plasmids constructed in this thesis.

<table>
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<tr>
<th>Plasmid</th>
<th>Description</th>
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<tr>
<td>pREP42EGFP-cnp1†</td>
<td>N-terminal fusion of cnp1† (cloned Ndel/BamHI) with EGFP under medium-strength nmt promoter. Marker for selection: ura4†</td>
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<tr>
<td>pREP41HA-cnp1†</td>
<td>N-terminal fusion of cnp1† (cloned Ndel/BamHI) with HA under medium-strenght nmt promoter. Marker for selection: LEU2†</td>
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<td>pREP41X-h3.2</td>
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<td>histone h4.2 (cloned Xhol/BamHI) under strong nmt promoter. Marker for selection: LEU2†</td>
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### Primers used in this Thesis.

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**CHAPTER 2 MATERIALS AND METHODS**

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CHAPTER 2 MATERIALS AND METHODS

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INTRODUCTION

The histone proteins are among the most invariant proteins known. These five small, highly conserved basic proteins serve to organise the DNA of eukaryotes into its fundamental unit, the nucleosome. Each nucleosome contains a core of histones around which 146 bp of DNA are wrapped. Recently, a detailed view of the histone organisation within the nucleosome has been provided by crystallographic studies [Luger et al., 1997]. Each nucleosome is an octamer composed of a histone (H3)2(H4)2 tetramer at the centre, and two H2A-H2B heterodimers at the ends of the DNA path. All histone proteins exhibit a similar C-terminal polypeptide chain fold, called the histone fold composed of three α-helices, with 15-30 unstructured residues at the N-terminus that extend outward from the nucleosome, commonly referred to as the histone tails. Histone H1 acts as a linker connecting two nearby nucleosome particles together and promoting coiling or folding of the chromatin fibre.

In many organisms, histone genes are organised into tandemly repeated, highly conserved quintets; each quintet contains five genes, encoding the five major histone protein types (H1, H2A, H2B, H3, H4). These quintets of genes are highly reiterated [reviewed by Hentschel and Birnstiel, 1981; Old and Woodland, 1984] with 10 histone gene copies in the chicken genome, 300 in the sea urchin and 100 in Drosophila melanogaster. Mammals have fewer histone genes, with on the order of 10- to 40- fold reiteration. Lower eukaryotes retain even less histone genes.

The S. cerevisiae haploid genome contains two loci, each displaying one divergently transcribed H3 and H4 genes [Smith and Andresson, 1983] and two loci encoding a single divergently transcribed histone H2A and H2B [Hereford et al., 1979]. A homology search in the yeast S. cerevisiae genome using the globular region of histone H1 from Pisum sativum, identified a single open reading frame with good homology, which is thought to be the yeast histone H1 homologue [Landsman, 1996]. In Neurospora crassa the number of genes is even lower, with only one copy of the histone H3 and H4 genes present in the haploid genome [Woudt et al., 1983].
Fig. 3-1. Organisation and sequence of fission yeast core histones.
A) Diagram of the organization of histone genes in S. pombe [based on Lind et. al, 1994].
B) Protein sequence alignments showing that the different histone genes encode almost identical proteins. Only one amino acid change is found between the three histone H3 proteins (position 45) and three amino acid changes amongst the two histone H2A proteins (positions 121, 124, 125).
The genome of *S. pombe* contains an H2A-H2B pair (H2A.1-H2B.1 or H2A-H2Balpha) on chromosome 3, a single isolated H2A gene (H2A.2 or H2Abeta) on chromosome 3, and three H3-H4 pairs, one (H3.1/H4.1) on chromosome 1, and two (H3.2/H4.2 and H3.3/H4.3) on chromosome 2. In total, there are three H3, three H4, two H2A and one H2B genes organised in five distinct loci in the *S. pombe* haploid genome [Matsumoto and Yanagida, 1985] see diagram in Fig. 3-1A. A common upstream sequence of 17bp, which could be involved in histone gene regulation, is present in the flanking regions of all the five histone loci. The reiterated *S. pombe* histone genes encode for almost identical proteins. Histone H2A.1 and H2A.2 contain 131 and 130 residues respectively, and differ at three positions near the carboxy end with one deletion (position 125) and two replacements (T121 to Q, R124 to K). Histone H3.3 differs from histone H3.1 and H3.2 by one residue at position 45 (G to P). The three H4 genes encode for identical proteins (see Fig. 3-1B). No linker histones have yet been isolated in *S. pombe*.

The evolutionary significance of the high reiteration of histone genes is not entirely understood. Histone genes clusters could have originated by duplication events and sequence variants might have been generated by accumulation of mutations during evolution. Organisms displaying a high reiteration of histone genes may have a selection advantage because of the importance of histone genes for chromosome function. In multicellular organisms, the degree of reiteration could also be necessary to respond to the requirement for large amounts of histone mRNA during proliferation or oogenesis. It has been proposed that the cluster organisation of histone genes may facilitate the synthesis of large quantities of histones for the packaging of rapidly replicating DNA, for example in the rapidly proliferating Drosophila embryo. It may also permit the accumulation of a large store of histone mRNA during the extended period of oogenesis of amphibia [Old and Woodland, 1984]. In contrast, mammalian cells do not undergo such high rates of cell division and therefore may not need such large numbers of histone genes. Finally, differential expression of stage-specific subsets of histone genes has been observed during the development of diverse organisms. For instance, in the sea urchin, development is accompanied by selective transcription of sequence variant histone genes, with a concomitant disappearance of histone mRNA of the earlier variant types [Newrock et al., 1978]. Thus, in multicellular organisms different histone gene clusters are utilised at different developmental stages.
While these examples suggest that different histone genes perform specific functions, there is as yet no direct experimental evidence that these repeated histone gene copies are required for normal function, or that they play distinct roles in the cell. Some insight into the role of histone gene dosage has come from studies carried out in budding yeast and in chicken cell lines. In *S. cerevisiae*, a constant steady state level of histone H2A-H2B transcripts is maintained by regulation of their mRNA degradation. The presence of an extra H2A-H2B gene pair, inserted by transformation, did not increase the H2A-H2B mRNA level. Instead the steady-state levels of H2A-H2B mRNA were maintained at a constant level by increasing the turnover of the histone transcripts [Osley and Hereford, 1981]. Deletion of approximately half of the histone genes in chicken DT40 cell lines caused transcriptional upregulation of the remaining histone genes, resulting in constant steady state levels of histone mRNA [Takami et al, 1997]. These studies suggest that maintenance of the constant ratio between histone mRNA levels by altered transcription or transcript turnover may be a general mechanism to allow the cell to retain a constant amount of histone proteins, thus the multiple histone gene copies could simply be redundant in these systems.

There are however some instances that suggest that transcriptional or post-transcriptional alteration may not suffice to compensate for the effect of altered gene dosage. In *S. cerevisiae*, strains deleted for either one of the two H3-H4 pairs were viable and progressed normally through the cell cycle [Smith and Stirling, 1988]. However, deletion of one of the two loci caused a dramatic increase of minichromosome loss, suggesting that the two H3-H4 gene pairs are not equally important and that one may be expressed at higher levels. An early study highlighted a role for histone gene dosage in position-effect variegation (PEV) in *Drosophila melanogaster* [Moore et al, 1979]. When cells bear a chromosomal rearrangement, which juxtaposes an euchromatic gene near to heterochromatin, a fraction of the cells exhibit expression of that gene and the resultant organism is a mosaic for activity of the rearranged gene. This process is generally referred as PEV. Reduction in the number of genes encoding histone proteins altered the degree of mosaicism, by causing a greater proportion of cells to express the variegating genes. As discussed previously, the cell regulates the level of histone proteins through mRNA turnover, maintaining a constant level of histone proteins. However it is possible that the compensation for the reduced histone coding capacity is in some case incomplete and that the
level of cellular histone proteins is partially reduced during the time when the transcriptional fate of the variegating genes is determined. Similarly, the subtle effect caused by reduction of the histone H3-H4 gene dosage in the *S. cerevisiae* system could be revealed only during chromosome segregation.

The relatively simple arrangement of histone genes and the genetic tractability of *S. pombe* provide a good experimental system in which to test the roles of the multiple copies of histone genes *in vivo*. In addition, the role of the multiple histone genes in heterochromatic gene silencing and proper chromosome segregation can be tested using the well-defined fission yeast centromeres.

To date, there has been no genetic dissection of the role of multiple histone genes in *S. pombe*. In particular, it would be of interest to understand the function of the three different histone h3-h4 pairs. The reagents and the literature available for histone H3 and H4 are well developed and accessible and the construction of a strain harbouring one h3-h4 gene pair would provide a genetic tool in which to generate site specific histone mutants. In this chapter the function of the three different h3-h4 pairs will be dissected, with particular emphasis on their role in gene silencing and proper chromosome segregation. The data presented in this chapter suggest that the three different histone h3-h4 gene pairs differentially contribute to these functions and to cell viability. The results obtained provide a robust framework for the generation and analysis of mutated H3 and H4 N-termini, presented in Chapter 5, and for the study of the effect of altered H3 versus H4 gene ratio on proper centromere function, presented in Chapter 7.

**RESULTS**

3.1. Replacement of the histone H3 and H4 genes with the *ura4* marker gene (Leslie Ball, honours project 1998).

To study the effects of reduced histone gene dosage, histone h3.x and h4.x gene pairs were replaced with the *ura4* marker gene to generate the *S. pombe* strains h3.x/h4.xΔ::ura4*. To construct these strains, cells were transformed with a PCR generated DNA fragment containing the *ura4* gene flanked by 58bp of homology with the histone locus. The PCR primers were designed to entirely replace the histone h3/h4 pair, as described in chapter 2.
Fig. 3-2. Characterisation of histone h3-h4 deleted strains.

A) PCR check of the histone h3-h4 deleted strains.
Integration of the ura4⁺ or his3⁺ marker genes at the histone h3-h4 loci was verified by PCR of yeast genomic DNA using one primer flanking the histone loci and the other internal to the marker. The primers used were:
forward primer within ura4⁺ T592 or R568 for his3⁺.
reverse primers downstream of histone h3.x P681 (h3.1); P683 (h3.2); P684 (h3.3)

B) Serial dilution assay to assess viability of histone h3-h4 deleted strains.
To test viability at different temperatures, serial dilutions of cells were plated on YE plates at 25°C and at 36°C. To test sensitivity to the microtubule poison, TBZ cells were also plated at 25°C on YE supplemented with 10μg/ml TBZ and growth compared with the TBZ sensitive mutant swi6Δ (FY 1200). Strains deleted for only one gene pair showed normal growth in all conditions. Amongst strains retaining only one h3-h4 gene set strain .1/.2Δ showed temperature sensitivity at 36°C and strain .2/.3Δ was sensitive to TBZ. The strain .1/.3Δ grew well in all conditions. A wild type strain (FY 1180) was plated as control.
The following mutants were generated: \( h3.1/h4.1\Delta::ura4^* \), \( h3.2/h4.2\Delta::ura4^* \) and \( h3.3/h4.3\Delta::ura4^* \); these are henceforth abbreviated to \( h3.x/h4.x\Delta \) throughout this thesis (\( x\Delta \) in the figures).

### 3.2. Generation of strains retaining only one histone gene set.

After being backcrossed three times with a wild type strain, crosses were carried out between histone \( h3-h4 \) deleted strains in all combinations to obtain strains retaining only one histone \( h3.x/h4.x \) pair. Progeny were selected on medium lacking uracil and \( \text{ura}^+ \) progeny were checked by PCR for double deletion of two histone \( h3/h4 \) pairs, abbreviated to \( h3.x/h4.x\Delta::h3.y/h4.y\Delta \) (\( x\Delta/y\Delta \) in the figures). The result of the PCR check is shown in Fig. 3-2A. To increase the likelihood of obtaining the strain \( h3.1/h4.1\Delta::h3.2/h4.2\Delta \) the \( \text{ura}^4^* \) gene was replaced by the \( \text{his}3^+ \) gene in the \( h3.1/h4.1\Delta::\text{ura}4^* \) strain. Strain \( h3.1/h4.1\Delta::\text{his}3^+, h3.2/h4.2\Delta::\text{ura}4^* \) was then generated by crossing and selection of the progeny on medium lacking uracil and histidine. The morphology of cells retaining reduced copies of histones H3 and H4 was normal (not shown). These results show that \( S. \text{pombe} \) cells are viable when retaining only one histone \( h3-h4 \) gene pair. The frequency of double-deleted recombinants is being assessed.

### 3.3. Viability.

To uncover mutant phenotypes associated with the histone \( h3-h4 \) gene deletions, the histone deletion strains were tested for growth under different conditions. In the laboratory the normal temperature for growth of wild type fission yeast cells is 32°C. At 36°C the growth of fission yeast temperature-sensitive mutants is impaired. Serially diluted cells from each histone \( h3-h4 \) deleted strain were therefore plated on YE medium at three different temperatures (32°C-not shown; 25°C, and 36°C) and on YE medium containing 10μg/ml of thiabendazole (TBZ, a microtubule poison). Thiabendazole is a compound that is able to interfere with microtubule polymerisation, affecting this process also during mitosis. The centromeres of strains bearing mutations of proteins involved in centromere function, such as the \( \text{swi6}^\Delta \) mutant, are often impaired in their ability to interact with microtubules. The presence of TBZ exacerbates this defect, reducing cell viability. The results are shown in Fig. 3-2B. All of the single histone \( h3-h4 \) pair deleted strains grew well under all conditions. The strain \( h3.1/h4.1\Delta::h3.2/h4.2\Delta \) was temperature sensitive at 36°C. As predicted the \( \text{swi6}^\Delta \) mutant was sensitive to TBZ whereas the
Fig. 3-3. Monitoring outer repeat silencing of h3-h4 deleted strains by colony-colour.

A) Colony-colour assay for silencing at the outer repeats of the centromeres. Histone h3-h4 deleted strains containing the ade6" marker gene inserted at the outer repeats of centromere 1 were plated on YE medium containing 1/10 of the normal supplement of adenine (limiting adenine see Chapter 2) to reveal their colour. This is indicative of the transcriptional status of the ade6" gene. Red colour indicates repression of the ade6" (wt), whereas white colour indicates transcription (swi6A). Red/white and pink colonies indicate metastable silencing (1A and 1/.3A).

B) and C) Verification of h3-h4 deleted strains in the ade6-D/NN/ade6" mat3 and tel1 background by PCR. Progeny obtained from the of all combination histone h3-h4 and the two strains mat3-M(RV):ade6", ade6-D/NN (FY 3249) and (Ch16LEU2 ade6+-tel1), ade6-D/NN (FY 3180) were selected on medium lacking uracil and medium lacking uracil and leucine, respectively. DNA from these progeny was then checked by PCR. The same primers used in Fig. 3-2A, were employed to check the presence of the disrupted h3-h4 gene loci. One set of primers (T904/T905) encompassing the ade6" and the ade6-D/NN deletion was used to amplify both alleles, resulting in two PCR products of 490 and 336bp, respectively.
wild type control was very mildly affected. The strain h3.2/h4.2Δh3.3/h4.3Δ was sensitive to thiabendazole at 25°C, while the strain h3.1/h4.1Δh3.2/h4.2Δ was only slightly sensitive. These results suggest that the histone h3.2/h4.2 gene pair is the most important as loss of this set in combination with any other pair causes temperature sensitivity or sensitivity to TBZ.

3.4. Monitoring silencing defects.

The centromeres, telomeres and mating type loci of fission yeast display features of heterochromatin. The complex of proteins occupying these regions blocks access of the RNA polymerases to underlying DNA sequences resulting in transcriptional repression of marker genes artificially inserted within these regions [Allshire et al., 1995]. At the centromere this silent chromatin is thought to reflect the formation of a functional centromere/kinetochore. Experiments were carried out to determine whether mutants with a reduced number of histone h3-h4 genes showed defective silencing at these transcriptionally repressed sites.

3.4.1. Colony colour silencing assay.

The ability of the histone h3-h4 deleted strains to repress the ade6 gene marker gene inserted at silent regions was assessed by colony colour assay [Allshire et al., 1994]. Due to their heterochromatic nature, genes artificially inserted within heterochromatic regions, such as the outer repeats of the centromere, mating type loci and telomeres, are normally repressed. Repression of the ade6 gene renders cells ade6. This leads to the accumulation of a substrate of adenine metabolism that when exposed to oxygen confers a red colour to colonies grown on limiting adenine plates. When heterochromatin is disrupted, such as in a swi6Δ background, the ade6 gene is expressed and colonies are white. Variegation of silencing results in a mixture of red, pink and white colonies that arise when the transcription of the marker gene is flipping between an on and an off state.
A

Full Adenine | 1/10 Adenine | $mat3::ade6^+$

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B

Full Adenine | 1/10 Adenine | ade6' - tel1

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Fig. 3-4. Colony-colour assay for silencing at the mating type locus and at telomere.
A) Histone deleted strains containing the ade6" marker gene inserted at mat3 were plated on YE medium containing limiting adenine. Silencing was unaffected at 25°C, but was slightly affected at 32°C in the strain .3Δ (h3.3/h4.3Δ) and in all of the double gene pair deleted strains.
B) Histone deleted strains, containing the ade6" marker gene adjacent to tel1 carried on minichromosome Ch16, were plated on YE limiting adenine medium. At 25°C the ade6" marker was repressed in all of the strains. At 32°C the wild type strain showed variegation of silencing as did the single h3-h4 gene pair deleted strains. One strain .1/.3Δ (h3.1/h4.1Δ h3.3/h4.3Δ) showed increased alleviation of silencing and two strains .1/.2Δ (h3.1/h4.1Δ h3.2/h4.2Δ) and .2/.3Δ (h3.2/h4.2Δ h3.3/h4.3Δ) showed increased repression of silencing, compared to the wild type strain.
Silencing at the outer repeats of the centromere.

Two distinct domains are present within fission yeast centromeres: the outer repeats and the central domain. To assay centromeric silencing at the outer repeats of the centromere, histone deleted strains were crossed into a background in which the wild type ade6** gene is inserted into the Sph1 site at the otr1limr junction of centromere 1 (FY1180: ade6-210, leu1-32, ura4-D18, otr1R::ade6t) and the endogenous ade6 gene is mutated (ade6-210). The results are illustrated in Fig. 3-3A. Strains h3.1/h4.1Δ (.1Δ) and h3.1/h4.1Δh3.3/h4.3Δ (.1/3Δ) showed variegated colony colour indicative of metastable silencing. However, cells from strains harbouring other histone h3-h4 deletion combinations formed red colonies on limiting adenine medium, indicating that centromeric silencing was not affected. This result suggests that the individual histone h3-h4 pairs contribute differentially to the formation of silent chromatin at the outer repeats of the centromere.

Silencing at mating type locus and at telomeres.

To test mating type silencing, strains were crossed with a tester strain in which the wild type ade6** gene is located adjacent to the mat3 locus, and is transcriptionally silenced (FY3249: ade6-DN/N, leu1-32, ura4-D18, mat3-M(RV)::ade6t). Crosses were also carried out with a suitable strain to test telomeric silencing where the wild type ade6** gene is positioned adjacent to telomeric sequences and carried on a minichromosome (FY3180: ade6-DN/N, leu1-32, ura4-D18 [Ch16LEU2 ade6**-tel1]). Both of these strains harbour a truncated version of the endogenous ade6 gene (ade6-DN/N). In a wild type background, where the introduced ade6** marker genes are transcriptionally repressed, the ade6-DA//A/ allele confers a darker red colour compared to that of ade6-210 allele of the strains described in above.

The presence of the ade6** gene was checked by PCR (Fig. 3-3B). These various histone h3-h4 deleted strains were then serially diluted and plated on YE medium with limiting adenine to assess colony colour, which is indicative of the silencing state (Fig. 3-4A and B).

Mating type silencing (Fig.3-4A) was maintained in all of the h3/h4 deleted strains at 25°C. However at 32°C it was partially impaired in the strain h3.3/h4.3Δ (.3Δ) and in all three histone h3-h4 double deleted strains.
Fig. 3-5. Diagrams of the cent1 ade6\textsuperscript{−} insertions and the RT-PCR silencing assay.
A) Schematic diagram representing the structure of centromere 1 and the ade6\textsuperscript{−} gene insertion sites tested in the centromere silencing assays.
B) Schematic diagram showing the strategy used for the quantitative RT-PCR based silencing assay.
Telomeric silencing (Fig.3-4B) was functional in all of the histone h3-h4 deleted strains at 25°C. At 32°C the wild type strain showed variegated colony colour. Compared to the wild type strain the h3.1/h4.1Δh3.3/h4.3Δ strain showed increased alleviation of telomeric silencing, as more colonies were white, while strains h3.1/h4.1Δh3.2/h4.2Δ and h3.2/h4.2Δh3.3/h4.3Δ showed enhanced repression of the telomeric ade6· gene, as colonies were red.

Silencing is less stable at higher temperatures [Alishire et al., 1994] and a clear difference in silencing was observed in the histone h3-h4 deleted backgrounds grown at 25°C and at 32°C. At 25°C silencing was not impaired both at mat-3 and at tel1 in any of the histone h3-h4 deletions, whereas at 32°C, some combinations resulted in silencing defects. The alterations observed could be due to a direct effect of decreased ability to recruit silencing factors at heterochromatic sites, or to an indirect effect of transcriptional downregulation of such factors.

These results suggest that the three different h3-h4 gene pairs contribute with different degrees to silencing at the mat-3 and tel1 heterochromatic sites.

3.4.2. Quantification of centromeric silencing by RT-PCR.

In contrast to the tight silencing at the outer repeats of the centromere, the central core of the centromere displays less transcriptional repression. Silencing in the central core (TM1) is subject to variegation [Alishire et al., 1994]. Depending on the promoter, marker genes inserted at the central core are repressed, but not completely, leaving a basal level of expression resulting, in the case of the ade6· gene, in mainly white colonies even in a wild type background. The colony colour assay is thus not suitable to monitor central core silencing. A more quantitative assay was therefore used to monitor defects in central core chromatin structure. This more precise method was also employed to assay silencing at the outer repeats of the centromere. All strains with deleted histone gene pairs were crossed into a suitable background for a quantitative transcription assay. Like the strains described in paragraph 3.4.1, these strains harbour a non-functional truncated version of the ade6· gene (ade6-DN/N) at the endogenous ade6· locus, and a functional ade6· gene inserted either within the outer repeats of the centromere (otrIR), or at the central core of the centromere (TM1), (Fig. 3-5A).

All histone h3-h4 deleted mutants were crossed with these strains and the progeny were PCR checked as described previously for the strains used in paragraph 3.4.1.
Fig. 3-6. Quantitative RT-PCR silencing assay of histone h3-h4 deleted strains.
RNA extracted from the strains constructed as described in paragraph 4.2, was subject to RT-PCR and to radioactive competitive-PCR. Transcription of the ade6+ gene relative to the euchromatic minigene ade6-D/NN was quantified by phosphorimaging. The ratio ade6+/ade6-D/NN was normalized to 1 for the random integrant (R.Int). For each panel the top part shows the polyacrylamide gel of the radioactive PCR. A primer pair amplified both the ade6+ gene inserted at the silent locus (top band) and the minigene inserted at the endogenous ade6+ locus, ade-6D/NN (bottom band). The bottom diagram shows quantification of the amount of ade6+ versus ade6-D/NN transcripts. A) Results obtained for the outer repeat silencing. B) Results obtained for the central core silencing.
The transcript level of the fully transcribed \emph{ade6-DN/N} truncated gene was compared with transcripts of the full-length gene inserted at silent sites. As a control for these experiments \emph{ade6} transcription was measured in a strain retaining a complete set of \emph{h3-h4} genes harbouring the \emph{ade6} gene in a random position in the euchromatin where it is fully transcribed (R.Int). In this strain the ratio between the two \emph{ade6} transcripts (\emph{ade6}/ \emph{ade6-DN/N}) was normalised to one. In addition, a strain wild type for \emph{h3-h4} genes with the \emph{ade6} inserted at the silent locus in question was used as a control for the repressed state. All ratios obtained from the tested strains were normalised to the random integrant (R.Int). Total RNA was extracted from cells and subject to reverse-transcription (RT). The cDNA obtained was used to perform radioactive competitive PCR and quantified by phosphorimaging (see Chapter 2). The primers used to amplify the \emph{ade6} gene encompass the region that is deleted in the \emph{ade6-DN/N} truncated gene thus one primer pair amplifies two DNA products of different size providing a robust internal control for each reaction. See diagram in Fig. 3-5B.

The strong silencing at the \emph{otf/rim} junction is efficiently maintained in the histone \emph{h3-h4} deleted mutants. No alleviation of this centromeric \emph{ade6} gene silencing was observed compared to the wild type strain (Fig. 3-6A). As described previously, the colony colour assay (see Fig. 3-3A) showed some variegation of \emph{ade6} transcription in the two strains: \emph{h3.1/h4.1A} and \emph{h3.1/h4.1A}h3.3/h4.3A. The degree of alleviation in these strains must then be very subtle and not detectable by RT-PCR.

Silencing at the central core is less stable and the wild type strain showed a significant amount of \emph{ade6} transcription (Fig. 3-6B). No defect in central core silencing was detected compared to the wild type strain: the histone \emph{h3-h4} deleted mutants did not significantly affect \emph{ade6} transcription. In conclusion, these results suggest that by quantitative RT-PCR analysis, no defects in centromeric silencing can be detected.

To recapitulate, the strain \emph{h3.1/h4.1A}h3.3/h4.3A, retaining just the \emph{h3.2/h4.2} gene pair, was shown to be: a) the least growth defective, b) least sensitive to TBZ, c) and to only show slight variegation of outer repeat silencing by the colony-colour assay. The \emph{h3.1/h4.1A}h3.3/h4.3A strain is thus a good background in which to generate H3 and H4 specific mutations (see Chapter 4).
Fig. 3-7. Chromosome stability in the histone h3-h4 deleted strains.

Table A. Estimation of the frequency of lagging chromosomes in strains with reduced histone h3-h4 gene dosage.
Exponentially growing cells from histone deleted strains, wild type and swi6Δ were stained with anti-tubulin antibodies, to follow spindle elongation during mitosis, and DAPI to follow chromosome behavior. Cells with spindle longer than 2/3 of the cell length (late mitotic cells) were scored for the presence of lagging chromosomes. A high number of laggards (approx. 20%) were observed in the swi6Δ mutant as seen previously [Ekwall et al, 1995] and no increased number of lagging chromosomes was detected in the histone h3-h4 deleted strains, compared to the wild type strain.

B) Minichromosome loss rate in histone h3-h4 deleted strains.
A half-sectoring assay was carried out as described in paragraph 5.2. An increased minichromosome loss rate indicates increased chromosome instability. As predicted the mutant strain swi6Δ (FY 1317) showed an increased rate of minichromosome loss (2.2%) compared to a wild type strain (FY 538, less than 0.05%) [Allshire et. al, 1995]. Strain .2Δ (h3.2/h4.2Δ) showed a rate of about 1%, strain .1/.3Δ (h3.1/h4.1Δ h3.3/h4.3Δ) showed a rate of 0.6% compared to the wild type strain.
3.5. Chromosome stability.
Proper centromere function results in high fidelity chromosome transmission. During mitosis, condensed chromosomes segregate towards the two poles of the spindle and genetic material is equally distributed to the two daughter cells. Correct segregation and consequent equal distribution of chromosomes can be compromised in genetic backgrounds with a defective centromere such as swi6Δ. If histone h3-h4 gene dosage plays a role in building a fully functional centromere, then chromosome segregation might be disrupted in mutants with reduced histone h3-h4 genes. To address this possibility, the effect of reduction of histone h3-h4 gene number on chromosome segregation was assessed.

3.5.1. Lagging chromosomes.
Mutant strains that fail to silence the outer repeats of the centromere (eg. Swi6Δ) display defective chromosome segregation, with high rates of lagging chromosomes on late anaphase spindles [Allshire et al., 1995]. This phenotype can be exacerbated at the cold temperature of 18°C. All histone h3-h4 deleted strains were grown at 25°C and then shifted for about 2-3 division times to 18°C. Cells were then stained with anti α-tubulin, for microtubules, and DAPI for DNA. A wild type strain (FY1180) and the swi6Δ::his3" (FY 1200) strain were stained for comparison. Lagging chromosomes were scored in cells with spindles longer than two thirds of the cell length, when the chromosomes are clearly separated at the two opposite poles in a wild type background. Results are summarised in Table A in Fig. 3-7. In this experiment the swi6Δ::his3" mutant behaved as predicted [Ekwall et al., 1995] showing a high percentage of lagging chromosomes in about 20% of late mitotic cells, while no lagging chromosomes were observed in the wild type strain. Histone deleted strains showed relatively normal mitoses and no increase of lagging chromosomes was measured compared to the wild type strain. In conclusion, deletion of histone h3-h4 does not result in phenotypes seen in many mutants affecting the function of silent chromatin [Ekwall et al., 1996].

3.5.2 Chromosome loss rate.
A more sensitive assay to detect defective chromosome segregation was employed. The frequency of chromosome loss in the histone h3-h4 deleted strains was measured using an accurate half-sectoring assay. The 530Kb linear minichromosome Ch16 is marked with LEU2
and carries a non-functional ade6 allele (ade6-216) [Allshire et al., 1995]. The minichromosome was introduced in the histone h3-h4 deleted mutants by crossing and progeny were checked by PCR (not shown). The histone h3-h4 deleted strains carry a different non-functional ade6 allele (ade6-210). In the presence of the minichromosome, intragenic complementation takes place between the two non-functional ade6 products, resulting in growth of white ade6+ colonies on limiting adenine plates. In the absence of selection, Ch16 is very stable through mitosis in wild type cells (lost at 1/2000 division), but it is lost at high frequency in mutants that disrupt centromere function such as swi6Δ (4.9%). If the minichromosome is lost in the first division, the resulting colony has a red half-sector. Consistently with the literature, in this experiment 2% of the swi6Δ colonies were half- sectored and less that 0.1% of wild type colonies were half-sectored. A slightly increased rate of half-sectored colonies, compared to the wild type, was observed in all histone h3-h4 deleted mutants. In particular two strains showed an increased number of half sectors: h3.2/h4.2Δ (approx. 1%) and h3.1/h4.1Δh3.3/h4.3Δ (approx. 0.4%) (Fig. 3-7B). These results indicate that the h3-h4 gene dosage is important for proper chromosome transmission through cell division in fission yeast.

**DISCUSSION**

Fission yeast displays three histone h3-h4 gene loci. The effects of histone h3-h4 gene dosage with respect to silencing and centromere function have been investigated. The data presented show that any single h3-h4 gene pair is sufficient for viability. Cells retaining a reduced number of h3/h4 gene pairs in various combinations were affected differently for the parameters tested. Many complex interactions such as transcriptional dosage compensation and feedback regulation may take place in mutants with reduced h3-h4 gene dosage, making the phenotypes observed difficult to interpret. The phenotypic differences observed in the histone h3-h4 depleted strains can most easily be explained by differences in the levels of transcription of these loci. Not much is known about histone h3-h4 transcription in fission yeast. It has been reported that histone H3 mRNA peaks early in S phase [Takahashi et al, 2000], and it is very likely that all of the h3-h4 pairs will be expressed at the same time. Nevertheless it is possible that the three gene pairs are expressed at different levels. This possibility could be addressed for instance by performing northern blotting analysis of RNA from synchronised cells using
different histone h3-h4 gene pairs as probes, although this assay may not be sensitive enough to measure small differences of transcript level from the different genes.

Deletion of the h3.2/h4.2 pair in combination with any other pair caused either temperature sensitivity or sensitivity to TBZ, suggesting a major role for this gene pair in cellular functions.

With respect to centromeric silencing at the outer repeats two strains were affected in the colony colour assay: strain h3.1/h4.1Δ and strain h3.1/h4.1Δh3.3/h4.3. This suggests that the h3.1/h4.1 pair is important for silencing, however when the h3.2/h4.2 pair is also deleted in the h3.1/h4.1Δ background silencing was not affected. Possibly this is due to the upregulation of the h3.3/h4.3 pair in the double mutant background. The RT-PCR assay is a less sensitive but more quantitative measure of the silencing at the otr1 site, and suggested that the silencing of the outer repeats was hardly affected in any of the h3-h4 depleted strains.

The counting of late mitotic cells did not reveal a defect in chromosome segregation in the histone h3-h4 deleted backgrounds. However a more accurate assay, the minichromosome loss, showed that chromosome stability was affected to some extent suggesting a role for histone h3-h4 gene dosage in chromosome segregation. The strain h3.2/h4.2Δ showed a significant increase in minichromosome loss (1%), but surprisingly the strain h3.1/h4.1Δh3.2/h4.2Δ showed lower levels of minichromosome instability. This result perhaps suggests that histone gene deletion causes feedback regulation in S. pombe as it does in other organisms. Deletion of two h3-h4 gene pairs may promote enhanced transcription of the remaining gene pair, resulting in a milder defect if compared to loss of a single pair. Two different results were obtained by monitoring minichromosome loss and chromosome segregation in mitosis. This could be explained by the different number of mitotic events that were assessed in the two approaches. While in the minichromosome loss assay 3000 cell divisions per strain were measured, approximately 60 mitotic events per strain were scored by counting lagging chromosomes. Clearly, defective minichromosome propagation is a more general assay for chromosome segregation, not necessarily linked to proper centromere function, as increased rates of minichromosome loss could reflect other processes, for instance DNA replication or repair.

Mating type silencing appears to be slightly affected at 32°C in all combinations of double h3-h4 gene deletions and in the single h3-h4 deletion h3.3/h4.3Δ. This suggests that the presence of
two gene pairs, one of which must be the $h3.3/h4.3$, are required for proper mating type silencing.

At 32°C, in strains retaining only the $h3.1/h4.1$ or the $h3.3/h4.3$ pairs, ade6' silencing adjacent to a telomere insertion sites was stronger than at wild type telomeres. A strain lacking these two gene pairs ($h3.1/h4.1$$h3.3/h4.3$) showed alleviation of the ade6' gene, suggesting that the $h3.1/h4.1$ and the $h3.3/h4.3$ pairs play a role in telomeric silencing.

Overall these results suggest that at 32°C a reduced number of histone $h3$-$h4$ genes may affect proper silencing perhaps by reducing the number of nucleosomes available to recruit silencing factors or by altering their level in the cell.

The strains generated in this study provide useful tools for further investigation of the roles of histones H3 and H4 in centromere function. In particular a strain bearing one $h3$-$h4$ gene pair only has been generated. Such a strain can be utilised to generate $h3$ and $h4$ mutations that may specifically affect centromere function. As discussed previously, the strain retaining only the $h3.2/h4.2$ gene pair is clearly the best for this purpose. In Chapter 4 this gene pair was subject to site-directed mutagenesis of conserved residues in the histone N-termini. In addition, the histone $h3$-$h4$ deleted strains described here allowed strains to be constructed bearing a non stochiometric number of $h3$ and $h4$ genes: their description is presented in Chapter 7.
CHAPTER 4
SITE-DIRECTED MUTAGENESIS OF HISTONE H3 AND H4 N-TAILS

INTRODUCTION

Distinct modifications of histone amino termini, such as acetylation, phosphorylation and, more recently, methylation have been proposed to underlie a chromatin-based regulatory mechanism that modulates the accessibility of genetic information [reviewed by Jenuwein and Allis, 2001]. In addition to histone modifications that stimulate gene activity, it is of similar importance to restrict inappropriate gene expression if cellular processes are to proceed unperturbed. Fission yeast centromeres are transcriptionally silent and this silent state is thought to reflect the association of a complex of proteins involved in proper centromere function. Histone H3 and H4 are underacetylated in chromatin coating the outer repeats of the centromere and altering this underacetylated status by transient treatment of fission yeast cells with TSA caused heritable centromere defects such as lagging chromosomes, chromosome loss, and delocalisation of the heterochromatic protein Swi6 [Ekwall et al., 1997]. Chip analysis using antibodies raised against specific acetylated lysine residues of histone H3 and H4 emphasised that three acetylated lysines were particularly enriched at the centromere following TSA treatment: H4 K5, K8 and K16 [Ekwall et al., 1997]. In addition, mutations in the histone deacetylases CIR6 and CIR3 also affect centromeric silencing and are sensitive to TSA [Grewal et al., 1998]. Mutated backgrounds, such as cir4Δ, swi6Δ, rik1Δ that affect centromeric function also alleviate silencing at the outer repeats, thus transcriptional silencing is a convenient tool to rapidly assess whether a particular mutation is affecting centromere function.

The histone H4 N-terminal tail has been shown to be required for the recruitment of the Sir3 complex at the budding yeast telomeres and mating type locus [reviewed by Grunstein, 1998]. This mechanism requires that the histone H4 K16 residue must be in an underacetylated state to promote Sir3 recruitment. Altering K16 to G, which is a residue mimicking an hyperacetylated state, leads to loss of Sir3 binding and consequently, loss of mating type locus silencing [Johnson et al., 1990]. It is possible that histone H4 K16 is also important for silencing in the silent heterochromatic regions of fission yeast chromosomes. Mutations in the budding yeast
MSGRGKKGLGKGGAKRHRKILRDNIQG... Wild type
5 8 12 16
MSGRGKKGLGKGGAGRHRKILRDNIQG... h4.2 K16G
5 8 12 16
MSGRGKGGAGLGGGGGVRHRKILRDNIQG... h4.2 K8A
5 8 12 16
MSGRGKGGAGLGGGAGHRKILRDNIQG... h4.2 K8A/K16G
5 8 12 16
MSGR... h4.2ΔN(4-28)

ARTKQTARKSTGGKAPRKQLASKAARKKAA... Wild type
910
ARTKQTARASTGGKAPRKQLASKAARKKAA... h3.2 K9A
910
ARTKQTARRSTGGKAPRKQLASKAARKKAA... h3.2 K9R
910
ARTKQTARKATGGKAPRKQLASKAARKKAA... h3.2 S10A
910
ARTKQTARRATGGKAPRKQLASKAARKKAA... h3.2 K9R/S10A
910

Fig. 4-1. Summary of the histone H4 and H3 N-tail mutants generated.
histone H3 N-terminus tail only mildly affect telomeric silencing: deletions of the histone H3 residues 4-35 caused alleviation of telomeric silencing at a very low level compared to point mutations of the histone H4 K16 [Thompson et al., 1994].

Fission yeast cells lacking the Clr4 protein display alleviated silencing at the three silent heterochromatic regions [Allshire et al 1995] in addition to defective chromosome segregation and delocalisation of Swi6 [Ekwall et al., 1996].

Recently it was demonstrated that the SET domain of Clr4 and its mammalian counterpart Suvar3-9 is a histone H3 methyltransferases specific for K9 [Rea et al., 2000] and a histone H3 methyltransferase has been identified also in Neurospora crassa where histone H3 K9 methylation controls DNA methylation [Tamaru and Selker, 2001]. In addition, methylated histone H3 K9 provides a binding site for Swi6 and its mammalian homologue HP1 [Lachner et al, 2001; Bannister et al., 2001]. ChIP experiments using antibodies recognising methylated histone H3 showed that the outer repeats of the centromere and the mating type locus contain methylated H3 [Nakayama et al., 2001]. These findings point to histone H3 K9 as being a key residue responsible for mediating recruitment of "Swi6" silencing complexes at fission yeast centromeres.

Fission yeast is the ideal genetic system in which to explore whether a specific conserved lysine of histone H3 or H4 N-tails plays a role in mediating silencing at centromeres. Strains carrying a single H3/H4 gene are viable (see chapter 3), thus fission yeast strains can be generated in which the only source of histone H3 or H4 bears site-specific mutations. These strains should allow dissection of the in vivo role of particular modified histone tails in transcriptional silencing and chromosome segregation in fission yeast.

In Chapter 3, fission yeast cells harbouring only one histone H3-H4 gene set were described. These cells were viable at 25°C, displayed normal central core silencing and normal or mildly altered silencing of the outer repeats of the centromere. In particular of all the strains containing a single histone H3-H4 gene set the one retaining only the h3.2/h4.2 gene set behaved the most like a wild type strain therefore it was chosen for site-directed mutagenesis. The histone H3 and H4 N-tails mutations generated are summarised in Fig. 4-1.
Fig. 4-2. Fission yeast centromeres contain underacetylated histone H4 and H3 N-tails.
Chromatin immunoprecipitations were carried out on strains harbouring the ura4" gene inserted at three locations: 1) random euchromatic location (R.Int::ura4*); 2) outer repeats of centromere 1 (otrIR::ura4*); 3) central core of centromere 1 (TM1::ura4*). The strains also contain a truncated ura4 gene (ura4-D/SE) inserted at the ura4* locus which is used as an internal control for the PCR. Aliquots from each strain were immunoprecipitated using highly specific antibodies recognising acetylated lysines [Suka et al., 2001]. The antibodies used were against acetylated K5, K8, K12 and K16 of histone H4 and acetylated K9, K14, K18, K23 and K27 of histone H3. Whereas R.Int::ura4* was efficiently immunoprecipitated by all the anti-AcK antibodies, both otrIR::ura4* and TM1::ura4* were not, indicating lack of histone acetylation in these regions. These results were consistent with previous observation [Ekwall et al., 1997]. R.Int::ura4* and otrIR::ura4* but not TM1::ura4* were immunoprecipitated by an antibody that recognise the unmodified H3 N-tail (H3N-see Chapter 6) (from M. Grunstein). Anti-Cnp1 antibodies (see Chapter 6) immunoprecipitated TM1::ura4* but not R.Int::ura4*.
RESULTS

4.1. Centromeres are underacetylated.

ChIP experiments using antibodies recognising acetylated histone H3 and H4 lysines [White et al., 1999] showed that fission yeast centromeric outer repeat chromatin is largely composed of underacetylated histone H3 and H4 [Ekwall et al., 1997].

More recently, highly specific antibodies have been developed that also recognise specific acetylated lysines [Suka et al., 2001]. To confirm previous observations, and to determine the acetylation status of central domain chromatin at centromeres, ChIP analysis was performed with these new antibodies on three ura4+ insertions: a random euchromatic expressed insertion (R.Int::ura4'), the otrIR::ura4' and the TM1::ura4' centromeric insertions (Fig. 4-2). Whereas the euchromatic ura4' insertion (R.Int::ura4') contained acetylated H3 and H4, as predicted very little acetylation of histone H4 was detected in the outer repeats (otrIR::ura4'). In particular, histone H4 K12 appears underacetylated in agreement with what reported by Suka et al., 2001. Notably, enrichment of acetylated H4 was very low at the central core of the centromere (TM1::ura4'). The central core ura4' insertion was not enriched in ChIP with anti histone H3 antibodies (raised against the N terminus of H3), and no enrichment was observed with antibodies recognising any of the acetylated forms of H3. This result is most likely due to the replacement of histone H3 in the central domain of the centromere, by the histone H3 like protein Cnp1. The composition of central core chromatin is explored in more detail in Chapter 6.

In contrast, since anti N-terminus H3 antibodies immunoprecipitated otrIR::ura4' this outer repeat centromeric chromatin does contain histone H3, however, there was no clear evidence for enrichment of this chromatin in ChIP with any of the acetylation specific antibodies.

4.2. Generating strains suitable for site-directed mutagenesis.

The histone H3 and H4 N-terminal tails from centromeric chromatin are underacetylated. If specific lysines have to be in this underacetylated state in order to allow association of factors involved in centromeric heterochromatin formation, then mutations mimicking an acetylated state could interfere with this association and cause alleviation of silencing.

In budding yeast, an allele of sir3 was isolated as an extragenic suppressor of the H4 K16 mutation [Johnson et al., 1990]. Site-directed mutagenesis of fission yeast histone H3 and H4
A. h4.2 and h3.2 tagged with the \textit{ura4}* gene, no other h3 and h4.

\begin{center}
\begin{tabular}{ll}
\text{h3.1/h4.1\Delta::his3} & \text{h3.1/h4.1\Delta::his3} \\
\text{h4.2 h3.2} & \text{h4.2 h3.2} \\
\text{h3.3/h4.3\Delta::arg3} & \text{h3.3/h4.3\Delta::arg3} \\
\text{ura} & \text{ura} \\
\end{tabular}
\end{center}

B. h4.2 and h3.2 replaced by the \textit{ura4}* gene, all other h3 and h4 present.

\begin{center}
\begin{tabular}{ll}
\text{h4.1 h3.1} & \text{h4.1 h3.1} \\
\text{h3.2} & \text{h3.2} \\
\text{h3.3 h4.3} & \text{h3.3 h4.3} \\
\text{ura4} & \text{ura4} \\
\end{tabular}
\end{center}

\textbf{Fig. 4-3.} Diagram of strains constructed to perform site-directed mutagenesis. 

\textbf{A) h3.2-\textit{ura4}* and h4.2-\textit{ura4}* tagged strains.}

The \textit{ura4}* gene was inserted immediately downstream of the h3.2 or the h4.2 genes. These strains do not contain any other histone genes as the h3.1/h4.1 gene pair is disrupted with \textit{his3}* and h3.3/h4.3 gene pair is disrupted with \textit{arg3}*.

These two strains were mainly used to cross out the h3.1/h4.1 and the h3.3/h4.3 pair after mutagenesis which was performed using the strains illustrated in B. Having the h3.2 or h4.2 gene tagged with \textit{ura4}* allowed selection against the wild type copy by growing the spores deriving from the cross on medium containing 5-FOA that selects for \textit{ura4}- cells. These two strains also contain the otIR::\textit{ade6}* insertion, the presence of which can be checked by PCR of the progeny, to allow rapid screening for silencing defects (not shown).

\textbf{B) h4.2\Delta::\textit{ura4}* and h3.2\Delta::\textit{ura4}*.

These two strains contain the genes to be mutagenised replaced by the \textit{ura4}* gene. Transformation with a fragment of DNA containing the mutated histone h3.2 or h4.2 causes replacement of the \textit{ura4}* and this event can be selected by growth on medium containing 5-FOA. Transformants were first checked by PCR and sequenced and were then crossed to the appropriate h3.2 or h4.2-\textit{ura4}* tagged strain to cross out the two remaining histone gene pairs. This allowed a high efficiency of incorporation of the mutated gene and assessment of whether a mutation is lethal.
N-tails could lead to the identification of conserved residues involved in mediating centromeric silencing. Such mutants could then be used in screens for extragenic suppressors to help identify new components of centromeric chromatin.

To generate site-directed histone H3 and H4 N-tail mutants a PCR based strategy was designed (see Chapter 2) to replace the wild type histone h3.2 or h4.2 with a mutated gene by transformation. To select for the replacement event, a convenient approach is to place the *ura4* gene just downstream of the wild type histone h3.2 or h4.2 genes. Site directed N-tail mutants are then generated by transforming these strains with a DNA fragment containing the point-mutated gene flanked by sequences that are identical to those adjacent to the histone gene. Transformants are then selected on medium containing 5-FOA, which selects for cells where the *ura4* gene has been deleted and likely to have been transplaced by the point-mutated histone gene.

To allow this strategy to be used, the *ura4* gene initially employed to perform the histone h3.x/h4.x deletions was first replaced with different marker genes. To achieve this, the *ura4* gene was substituted with the *his3* gene in h3.1/h4.1A::ura4 and with the *arg3* gene in h3.3/h4.3A::ura4. The two strains were then crossed together to generate h3.1/h4.1A::his3 h3.3/h4.3A::arg3. This double deletion strain was then transformed with the *ura4* gene flanked by sequences identical to either the h3.2 or the h4.2 downstream regions (*h3.2-ura4* and *h4.2-ura4*). The two resulting strains contained only one histone h3.2/h4.2 gene pair in which either the h3.2 or h4.2 had the *ura4* (*ura4* "tag") gene inserted at the 3' end (Fig. 4-3A) 60 and 20 base pairs from the stop codon respectively.

Two problems arose with the subsequent generation of the site specific histone mutations in these strains:

A) the *ura4* marker is located downstream of the histone gene and the desired point mutations affect the 5' coding DNA sequence. However, recombination can occur within the body of the histone gene itself, resulting in strains where the *ura4* marker has been replaced, but that have not incorporated the mutation in the coding sequence of the gene. Such events occurred frequently.

B) The tagged strains do not allow the recovery or identification of lethal mutations as the other two histone loci (h3.1/h4.1 and h3.3/h4.3) have been disrupted.
To overcome the disadvantages of the ura4\(^{+}\) tagged histone strains a different strategy was devised. The histone h3.2 and h4.2 genes were individually replaced by the ura4\(^{+}\) gene (Fig. 4-3B) and retained all other histone H3 and H4 genes. These strains were generated by gene replacement with a DNA fragment, obtained by PCR, containing the ura4\(^{+}\) gene flanked by sequences with homology to regions 5\' and 3\' to the h3.2 or h4.2 ORFs. The resulting strains were designated h3.2\(\Delta::\)ura4\(^{+}\) and h4.2\(\Delta::\)ura4\(^{+}\). These were then used for generating mutations using mutated h3.2 or h4.2 DNA. Growth on 5-FOA indicated that the ura4\(^{+}\) gene had been replaced with mutated h3.2 or h4.2. This was confirmed by sequencing. Subsequently, the clones were crossed to the h3.2 or h4.2-ura4\(^{+}\) "tagged" strains (described above), which are in the h3.1/h4.1\(\Delta::\)his3\(^{+}\) h3.3/h4.3\(\Delta::\)arg3\(^{+}\) background. Recombinants were selected on medium containing 5-FOA and lacking histidine and arginine allowing the growth of cells with h3.1/h4.1\(\Delta::\)his3\(^{+}\), h3.3/h4.3\(\Delta::\)arg3\(^{+}\) and not containing the wild type histone tagged with ura4\(^{+}\). This should result in strains bearing only a mutated H3 or H4 gene. This final cross also allows assessment of whether a particular mutation results in cell lethality. The h3.2-ura4\(^{+}\) and h4.2-ura4\(^{+}\) tagged strains conveniently harbour the ade6\(^{+}\) gene inserted within the outer repeats of centromere 1 (otrI\(R::\) ade6\(^{+}\)) to allow a rapid indication of defective outer repeats silencing.

4.3. Generating the histone H4K16G mutation.

To address a possible role for specific conserved lysines in silencing at the outer repeats a candidate approach was taken. The first residue that was altered by site-directed mutagenesis was histone H4 K16.

To mimic a constitutively hyperacetylated state, K16 was changed into a glycine (G). The neutral amino acid glycine is highly enriched in the H4 N-tail (30% in the first 30 amino acids) and can be regarded as a mimic of an acetylated lysine.

Primers were designed to introduce a NarI restriction site when the mutation K16 to G is correctly incorporated, allowing a rapid verification of the mutation event.

Two strains were transformed in parallel with the h4.2 K16G DNA fragment:

1) h4.2-ura4\(^{+}\) h3.1/h4.1\(\Delta::\)his3\(^{+}\) h3.3/h4.3\(\Delta::\)arg3\(^{+}\)
2) h4.2\(\Delta::\)ura4\(^{+}\) h3.1/h4.1\(\Delta::\)his3\(^{+}\).
**Fig. 4-4. Analysis of the histone H4 K16G mutant.**

A) PCR check of h4.2 K16G. The mutated h4.2 K16G gene was obtained by PCR (see Chapter 2) using the B1/B2 primer pair. To check the mutation event the h4.2 gene was amplified from genomic DNA from a wild type and a 5-FOA resistant transformant using the B2/W645 primer pair. The mutation K16G also introduces a *NarI* restriction site. The h4.2 gene was then digested with the *NarI* restriction enzyme and run on an ethidium bromide stained gel. A diagram of the h4.2 gene is also shown.

B) Three independent h4.2 K16G h3.1/h4.1Δ h3.3/h4.3Δ mutants were plated on medium containing limiting concentrations of adenine (10mg/ml) to assess silencing of the otriR::*ade6* gene. Colonies of h4.2 K16G were red indicating that outer repeat silencing was not affected. The control strain h4.2-*ura4* h3.1/h4.1Δ h3.3/h4.3Δ was also red, whereas the swi6 Δ mutant was white.
To reduce the background on 5-FOA plates, cells were co-transformed with a LEU2 based plasmid (pREP3X). A high proportion of LEU2 transformants will have also taken up the mutant histone DNA. The transformation mixture was grown overnight in medium lacking leucine and cells were plated on medium containing 5-FOA and lacking leucine. Eighteen LEU2/FOA\(^6\) transformants were analysed by PCR to check that the h4.2 locus had lost ura4' (the tag or the replacement). PCR products of the correct size were gel-purified and digested with the Nai restriction enzyme (Fig. 4-4A) to identify strains bearing the h4.2 K16G mutation. This test showed that all eighteen of the transformants coming from the h4.2 ura4' replacement strain \{h4.2 A::ura4' h3.1/h4.1A::his3\} contained the mutation, whereas only three out of seventeen transformants from the h4.2 ura4' tagged strain \{h4.2-ura4* h3.1/h4.1A::his3 h3.3/h4.3A::arg3\} contained the mutated residue. This confirmed that the use of strains harbouring the deletion of the histone gene to be mutagenised is far more efficient than using the ura4' tagged strains.

Three potential H4K16G mutants from the d4.2 tagged with ura4' \{h4.2-ura4' h3.1/h4.1A::his3 h3.3/h4.3A::arg3\} were sequenced and were confirmed to contain the K16G mutation. The fact that K16G mutants could be derived in this otherwise h4 deficient background shows that mutation of K16 to mimic the acetylated residue is not a lethal mutation.

4.4. The H4K16G mutation does not affect proper centromere function.

Three independent strains harbouring the H4 K16G mutation were plated on medium containing limiting concentrations of adenine to test for expression of the ade6\(^6\) gene inserted in the outer repeats of the cen1. All strains formed red colonies (Fig. 4-4B), indicating that the silencing over the outer repeat region of centromere 1 is not affected by the H4 K16G mutation. The K16G mutants were also tested for other possible phenotypes, such as growth at different temperatures and in the presence of the microtubule-poison TBZ (Fig. 4-5C). The mutant grew well at the elevated temperature of 36°C and was not sensitive to 10μg/ml TBZ at 25°C. However, cells from all three H4K16G mutants were slightly cold-sensitive at 18°C.

These data indicate that the conserved K16 of histone H4 is not required for the function of silent chromatin over the outer repeat region of fission yeast centromeres.
Fig. 4-5. Analysis of the histone H4 mutations.
A) Ethidium bromide stained gel showing the *NarI* digestion carried out on the DNA fragments used to introduce the H4 N-tail mutations into the genome. A cartoon of the expected restriction fragments is shown on the right and a map of the *h4.2* gene with the *NarI* sites is shown at the bottom.

B) Cells containing mutated H4 N-tail were plated on medium containing limiting adenine (10mg/ml) to assess silencing of the *otrR::ade6* gene. As already shown in Fig. 4-4B *h4.2* K16G formed red colonies and so did *h4.2* K8A, indicating that outer repeats silencing was not affected. The double mutant *h4.2* K8AK16G showed some rare white colonies amongst the red ones, indicating a slightly metastable silencing. The *h4.2-ura4* h3.1/h4.1A h3.3/h4.3A strain also showed slightly variegated colony colour. Consistently with previous observations the wild type control was red and the *swi6A* mutant was white in this experiment.

C) Strains containing mutated H4 N-tail were checked for growth phenotypes. Cells were plated on rich medium at different temperatures and on medium containing 10μg/ml TBZ at 25°C. All mutants grew well at 25°C and at 32°C; at 36°C colonies were consistently smaller in all three *h4.2* mutants. At 18°C K16G was slightly cold sensitive. The growth of K8A was significantly affected at 18°C and K16GK8A was also affected, but to a slightly lesser degree. Finally, K8A showed a mild sensitivity to 10μg/ml TBZ.
4.5. Generating the histone H4 K8A and K8AK16G mutations.
The next conserved lysine that was mutated to mimic an hyperacetylated state was histone H4 K8, as this residue is also hyperacetylated at the outer repeats following TSA treatment [Ekwall et al., 1997]. To mimic acetylation this residue was changed into an alanine (A), as it is also a neutral amino acid and it was convenient to design primers containing this mutation and the Narl restriction site. The double mutant H4 K8AK16G was also generated at the same time (see Chapter 2). The predicted fragment sizes produced by the Narl digestion are indicated in Fig. 4-5A.

The PCR products containing either K8A or K8AK16G histone H4 mutants were introduced by transformation into the strain h4.2A::ura4* h3.3/h4.3 A::arg3". Again, cells were co-transformed with a LEU2 plasmid, selected on medium containing 5-FOA lacking arginine and PCR checked for loss of the ura4" gene. The PCR products from colonies giving the correct size were digested with Narl. Potential mutants were sequenced to confirm the incorporation of the mutation and to ensure no other mutations were present. The K8A and K16GK8A mutants were crossed with the strain h4.2-ura4" h3.1/h4.1A::his3 h3.3/h4.3A::arg3" otrlR::ade6" and the progeny were selected on medium containing 5-FOA and lacking histidine and arginine. The presence of the H4 mutation was again checked by PCR of the h4.2 gene and digestion of the product with Narl. The mutants were also checked by PCR for the disruption of the h3.1/h4.1 and h3.3/h4.3 histone loci and for the presence of the ade6" gene inserted at the outer repeats of the centromeres. The only source of histone H4 in these strains is the mutated histone H4.

Both the single mutant H4 K8A and the double mutant H4 K8AK16G were plated on medium containing limiting concentrations of adenine to determine whether outer repeats silencing was defective in these backgrounds (Fig. 4-5B). This simple assay revealed that silencing was unaltered in the H4 K8A background, as all colonies were red. Silencing of cen1 otr was very mildly affected in the double mutant H4 K8AK16G as only a few white colonies grew amongst the red population. These rare white colonies remained white on further restreaking (not shown). It is important to point out that the control strain (h4.2-ura4" h3.1/h4.1A::his3 h3.3/h4.3A::arg3" otrlR::ade6") also gives rise to a few white colonies, but these became pink on restreaking. This suggests that the rare white colonies observed in the H4 K8AK16G mutant
stably alleviated *otr* silencing whereas those deriving from the control strain did not. The fact that no white colonies were observed in both the H4K16G and K8A single mutants suggests that silencing of the centromeric *ade6* gene was not affected in these backgrounds. Perhaps mutations of these two lysines block their involvement in transcriptional activation, rather than transcriptional silencing or they might individually affect the balance between activating and silencing proteins.

These histone H4 mutants were also plated on rich medium at different temperatures and in the presence of 10μg/ml TBZ at 25°C (Fig. 4-5C). The K8A mutant was slightly TBZ sensitive and K8A and K8AK16G were both cold-sensitive at 18°C. Altogether these data indicate that the two highly conserved lysines, K8 and K16 of histone H4 are not important for the formation of silent chromatin over the outer repeats of centromere 1. Nevertheless, single and double mutants caused cold sensitivity, K8A caused mild TBZ sensitivity and K8AK16G showed slight variegation in colony colour that appears different from that of the starting strain. As both cold sensitivity and TBZ sensitivity often affects centromeric mutants, these results suggest that these two residues may contribute to proper centromere function. Alternatively, these mutations could be affecting the expression of genes involved in cytoplasmic microtubules, spindle dynamics or centromere function.

4.6. Deletion of the histone H4 N-terminal tail.

Site directed mutation of two conserved N-terminal lysines, K8 and K16, of histone H4 to mimic hyperacetylated lysines did not significantly affect the silencing over the outer repeats regions of the centromere. There are three possible explanations for this result:

A) the two remaining conserved lysines (K5 and K12) play a role in mediating outer repeat silencing.

B) the four conserved lysines play redundant roles, thus is necessary to mutate all of them at the same time to impair silencing.

C) the histone H4 tail does not play a major role in silencing of the outer repeat region.

To address the first two possibilities it would be necessary to generate more single site-directed mutations and, perhaps, generate several combinations of them. A more rapid test is to delete the entire N-terminus of histone H4. If the histone H4 tail plays a role in recruiting a putative silencing factor deletion of the tail should disrupt this process resulting in a silencing defect.
Previous studies showed that in budding yeast the removal of the histone H4 N-terminal tail (residues 4-28) is viable and causes derepression of the silent mating type loci, \textit{HMRx} and \textit{HMRa} [Kayne et al, 1988].

This truncation is thought to leave intact the very amino-terminal residues that may be important for protein stability [Gonda et al., 1989] yet delete most of the hydrophilic amino terminus in which the conserved lysines are found (Fig. 4-6A). A strategy was therefore devised to remove the same residues from the N-terminal tail of H4 in fission yeast.

The H4ΔN(4-28) mutant was generated using a PCR based approach (see Chapter 2). The truncated \( h4.2 \) was then inserted within the genome into the \( h4.2 \) locus by transformation of the strain \( h4.2\Delta::ura4^* \) (FY4756) and selection on medium containing 5-FOA. Four transformants were analysed by PCR (Fig. 4-6B) for loss of the \( ura4^* \) gene from the \( h4.2 \) locus and for the presence of a shorter \( h4.2 \) compared to wild type \( h4.2 \) at the wild type locus. PCR analysis confirmed that all four transformants were correct integrants. These were crossed with the strain \( h4.2::ura4^* h3.1/h4.1\Delta::his3^* h3.3/h4.3\Delta::arg3^* \text{otr1R::ade}^* \) to generate a strain harbouring H4ΔN(4-28) as the only source of histone H4. Recombinant spores were selected on medium containing 5-FOA and lacking histidine and arginine and checked by PCR. Two independent clones of H4ΔN(4-28) \( h3.1/h4.1\Delta::his3^* h3.3/h4.3\Delta::arg3^* \) were analysed, one of which carried the \( \text{ade}^6 \) marker at otr1R.

To test for possible defects associated with the H4ΔN(4-28) mutation the two independent mutants were plated on rich medium at different temperatures, on medium containing 10μg/ml of the microtubule poison TBZ and on medium containing limiting adenine to assess silencing at the outer repeats of the centromere (Fig. 4-6C). The H4ΔN(4-28) mutant grew well at all temperatures tested and was not sensitive to TBZ. Importantly the H4ΔN(4-28) containing the \( \text{otr1R::ade}^6 \) gene did not alleviate silencing of \( \text{ade}^6 \) (Fig. 4-6C). Colonies showed a slightly variegated colony colour, to a degree which is comparable to that of the starting strain \( h4.2::ura4^* h3.1/h4.1\Delta::his3^* h3.3/h4.3\Delta::arg3^* \).

From these data it can be concluded that histone H4 N-tail does not appear to play a significant role in centromeric silencing in fission yeast. Nevertheless it could play a role at other silent sites. Consequently, the focus of these studies was shifted to the histone H3 N-tail.
A

\[ \Delta(4-28) \]

MSGRGKGGKGLGKGGAKRHRKILRDNIQGITPAIRRLARRG... H4

B

\[
\begin{align*}
T952/P683 & \\
\text{1000} & \text{900} & \text{800} & \text{700} & \text{1} & \text{2} & \text{3} & \text{h4A Wt} & 0
\end{align*}
\]

\[
\begin{align*}
\text{h4A} & \\
\text{T592} & \text{ura}^4 & \text{P683}
\end{align*}
\]

\[
\begin{align*}
F655/W645 & \\
\text{1000} & \text{900} & \text{800} & \text{700} & \text{1} & \text{2} & \text{3} & \text{h4A Wt} & 0
\end{align*}
\]

\[
\begin{align*}
\text{R653} & \text{h4.2} & \text{W645}
\end{align*}
\]

C

\[
\begin{align*}
\text{25°} & \text{32°} & \text{36°} & \text{10μg/ml TBZ 25°C} & \text{25°C} & \text{1/10 Ade}
\end{align*}
\]

\[
\begin{align*}
\text{wt} & \\
\text{h4.2tag} & \\
\text{swi6A} & \\
\text{h4.2AN} & \\
\text{h4.2ΔN} & \\
\text{otfi::ade6} & \\
\end{align*}
\]
Fig. 4-6. Deletion of residues 4-28 of the histone H4 N-tail.
A) Amino acids sequence of the N-terminal tail of histone H4. The residues deleted in the H4ΔN(4-28) mutant are highlighted in red.

B) PCR check of four 5-FOA resistant colonies obtained after transformation of a h4.2Δ::ura4" strain with the deleted h4.2 allele.
Top panel: PCR to check the loss of the ura4" using one primer in ura4" (T952) and one downstream of the h4.2 locus (P683). Only the starting strain showed an amplification product.
Bottom panel: PCR to check integration of the deleted h4.2 in the correct location using one primer upstream of the h4.2 gene (R653) and one downstream (W645). The PCR amplified a product of 750bp from the four h4.2ΔN and a product of 850bp from a h4.2" wild type strain. As predicted, the h4.2Δ::ura4" strain did not show a PCR product.

C) Two independent H4 ΔN(4-28) h3.1/h4.1Δ h3.3/h4.3Δ strains were plated on rich medium at different temperatures and on medium supplemented with 10µg/ml TBZ. The two mutants grew well under all conditions. The H4 ΔN(4-28) h3.1/h4.1Δ h3.3/h4.3Δ mutant plated at the bottom of the plates contains the ohR::ade6" insertion. Silencing at the outer repeats was tested by plating cells on medium containing limiting adenine. The colonies from the mutant strain showed only some variegation of colour, similar to the h4.2-ura4" h3.1/h4.1Δ h3.3/h4.3Δ strain.
Fig. 4-7. Tetrads dissection analysis of H3 N-tail mutants.
Analysis of the cross between h3.2K9RS10A h3.1/h4.1Δhis3* and h3.2-ura4* tag h3.1/h4.1Δhis3* h3.3/h4.3Δarg3* (crosses using K9R only gave similar results). Ten ascii were dissected and, after spore germination, replica-plated onto different selective plates to assess the genotype. No recombinants could be isolated which were FOA+, thus containing the mutated histone h3.2, and arg*, thus deleted for the h3.3/h4.3Δarg3* indicating that the h3.2 mutations were lethal and could only be carried by strains containing wild type h3.3/h4.3. Plates were incubated at 25°C.
4.7. Histone H3 K9A and K9R mutants are lethal and cause a dominant silencing defect.

The recent discovery that Clr4/Suvar3-9 proteins are histone H3 K9 methyltransferases [Rea et al., 2000] provided new insight into how histone H3 rather than the histone H4 N-tail might mediate recruitment of silencing components over the outer repeats of the centromere. As discussed previously, this model is supported by the fact that ura4" inserted at the outer repeats of centromere 1 is packaged in methylated K9 histone H3 and Swi6 [Nakayama et al., 2001], Fig. 4-7A.

To directly test in vivo if histone H3 K9 is required to mediate silencing over centromeric outer repeats, this residue was subject to site-directed mutagenesis. Two mutations were made: K9A and K9R. Whereas alanine can be regarded as mimicking an acetylated lysine, arginine is known to not be a substrate for methylation by Suvar3-9/Clr4 methyltransferases and since is a positively charged residue it can be regarded as equivalent to a non acetylated lysine.

These mutations were generated by the same strategy adopted for the histone H4 mutations (see Chapter 2). The mutated h3.2 alleles were inserted at the h3.2 locus by transforming the h3.2::ura4" strain and selecting for 5-FOA transformants. PCR was used to check that the ura4" gene had been replaced by a mutated h3.2 and those that were positives were then sequenced. The resulting correct mutants were crossed to the h3.2-ura4" h3.1/h4.1::his3" h3.3/h4.3::arg3" strain to generate a strain containing only the desired mutated histone H3. Spores from the cross were plated onto medium containing 5-FOA to select for the mutated h3.2 gene, and replica-plated on medium lacking arginine to select for h3.3/h4.3::arg3" and medium lacking histidine to select for h3.1/h4.1::his3". Surprisingly, none of the 5-FOA resistant colonies (H3 K9A or K9R) were able to grow on medium lacking arginine, suggesting that mutated h3.2 alleles need the presence of the h3.3/h4.3 gene set for viability. In contrast, 5-FOA resistant recombinants containing the h3.1/h4.1::his3" deletion were isolated. The presence of the h3.1/h4.1::his3" deletion and of otr1R::ade6" was checked by PCR. Those derivatives were also sequenced again after the cross to ensure the presence of the H3 K9A or K9R mutation. Tetrad dissection analysis (see materials and methods) was carried out on ten ascii generated from the cross of h3.2-ura4" h3.1/h4.1::his3" h3.3/h4.3::arg3" x h3.2K9R h3.1/h4.1::his3" and it confirmed that mutations of K9 were lethal and that the h3.3/h4.3
**A**

anti-H3 MeK9 ChIP

R.int::ura4* chfR::ura4'

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**B**

h3.2-tag
h3.1/h4.1Δ
h3.3/h4.3Δ

h3.2K9A
h3.1/h4.1Δ

swi6Δ

h3.2K9R
h3.1/h4.1Δ

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**C**

*h3.2 K9A h3.1/h4.1Δ*

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5μ
Fig. 4-8. Histone H3 K9 to A and K9 to R mutations affect centromere function.
A) ChIP with anti-MeK9 antibodies (Upstate) shows that methylated histone H3 is enriched at the outer repeats (otlr::ura4' is enriched compared to the ura4-D/SE euchromatic truncation) but is absent from a random euchromatic location. This result is consistent with the findings of Nakayama et al., 2001.
B) The two K9 mutants generated, h3.2 K9A and h3.2 K9R, both containing the h3.3/h4.3' gene set and the otlr::ade6' gene, were plated on medium containing limiting concentration of adenine to assess silencing of the outer repeats of the centromere. Both mutations caused the formation of white colonies indicating that mutations in K9 cause a silencing defect.
C) Immunostaining of the h3.2 K9A h3.1/h4.1A with anti α-tubulin (red) to stain microtubules and DAPI (blue) to stain the chromosomes. 1) and 2) chromosomes failed to segregate to the two opposite poles. 3) Lagging chromosome. 4) Missegregation of chromosomes in two cells.
Fig. 4-9. Immunostaining of wild type cells using an anti-phospho H3 S10 (from D. Allis).

A) Double staining of the anti-phospho H3 (green) with the Mis6-HA (red) centromeric protein [Saitoh et al., 1995] shows an increased anti-phospho H3 staining colocalising with the centromere signal.

B) Double staining of the anti-phospho H3 (green) with the heterochromatin protein Chp1 (red) shows the co-localisation of the anti-phospho H3 signal with the heterochromatic foci.
gene set was required in combination with the K9R mutant h3.2 for viability (Fig. 4-7).

When plated on medium containing limiting concentrations of adenine (Fig. 4-8B) both mutants (H3 K9A, or H3 K9R h3.1/h4.1A) were white, demonstrating that both K9 mutations cause alleviation of silencing of ottrR::ade6' even though they retained a wild type h3.3/h4.3. This indicates that these mutations are dominant with respect to silencing. Immunostaining with anti-tubulin performed on the H3K9A h3.1/h4.1A mutant revealed the presence of defective mitoses in the large majority of mitotic cells (Fig. 4-8C).

4.8. Mutations of S10 reveal a link between S10 phosphorylation and silencing of the outer repeats.

Histone H3 S10 phosphorylation, which is regulated by Ipl1 in budding yeast and by Aurora B kinase in mammals could be connected with K9 post-translational modifications such as acetylation or methylation. In vitro experiments have demonstrated that peptides containing methylated K9 block S10 phosphorylation by Aurora kinase whereas unmodified or acetylated K9 are better substrate for S10 phosphorylation [Rea et al., 2001]. Moreover, Suvar3-9h1/h2 double knockout mouse cell lines display hyperphosphorylation of histone H3 [Rea et al., 2001]. S10 phosphorylation is a well-characterised modification that is important for proper chromosome segregation during mitosis and meiosis in a number of organisms [Hendzel et al., 1997; Wei et al., 1998]. Genetic studies in Tetrahymena showed that a point mutation of S10 (S10A) leads to abnormal chromosome segregation and extensive chromosome loss during mitosis and meiosis of micronuclei [Wei et al., 1999]. In budding yeast, however, the S10A mutation does not impair chromosome segregation [Hsu et al., 2000]. The role of histone H3 S10 phosphorylation has only very recently been characterised in fission yeast. Phosphorylation of S10 increases during mitosis and is mediated by the essential kinase Ark1 [Petersen et al., in press]. Preliminary results obtained from staining wild type cells with affinity-purified anti-phoshoS10 antibodies obtained from the Allis laboratory (Fig. 4-9) suggest that S10 phosphorylation takes place during mitosis in fission yeast as in other organisms. Co-localisation of the signal with the centromeric Mis6-HA protein (Fig. 4-9A) and with the Chp1 heterochromatin protein (Fig. 4-9B) suggests that the centromeres may be decorated with the anti-phosho-S10 antibodies. It was recently demonstrated that upon commitment to mitosis
Ark1 associated with chromatin and was particularly concentrated at several sites including kinetochore/centromeres [Petersen et al., 2002].

In vitro, several budding yeast HAT enzymes acetylate K14 more efficiently on H3 peptides which are phosphorylated on S10 [Lo at al., 2000]. Thus phospho-S10 H3 appears to be a better substrate for K14 acetylation.

It is possible that in fission yeast N-terminal tails containing the K9A and K9R mutations are better substrates for S10 phosphorylation by the Aurora kinase homologue, Ark1. This could stimulate acetylation of the H3 N-tail by HAT activity resulting in transcriptional activation.

To address whether the dominant alleviation of silencing observed in the K9R mutant was due to hyperphosphorylation of S10, two new mutants were generated to mimic a non-phosphorylatable state of S10: H3 S10A and K9RS10A. Again these mutations were lethal and the presence of the wild type h3.3/h4.3 gene pair allowed viability. Interestingly, the double mutant K9RS10A h3.3/h4.3 showed partially restored silencing of the otr1R::ade6" gene as colonies were pink on limiting adenine medium (Fig. 4-1OA). This suggests that in the K9R mutant the dominant alleviation of silencing involved the neighbouring S10 residue. Thus, S10 hyperphosphorylation could play a dominant-role in causing histone H3 hyperacetylation and, consequently, alleviation of silencing.

The S10A mutation itself when in the presence of h3.3/h4.3 showed partially alleviated silencing (Fig. 4-1OA) as colonies were pink on limiting adenine medium. This suggests that S10 might be important for outer repeats silencing. Alternatively, the mutation may disrupt interactions of silencing components with the centromere by interfering with methylation of K9 or with Swi6 binding to H3.

The histone H3 mutants in the h3.3/h4.3 background were also plated on rich medium at different temperatures and in the presence of the microtubule poison TBZ (Fig. 4-10B). In this experiment the two mutants K9A and K9R were sensitive to 10μg/ml TBZ at 25°C. Interestingly, sensitivity of the K9 mutation was partially suppressed in the K9RS10A double mutant as this only showed sensitivity to higher levels of TBZ (25μg/ml) at 25°C.
Fig. 4-10. Analysis of the histone H3 N-tail mutants.
A) Cells from H3 N-tail mutants were plated on medium containing limiting adenine to assess silencing of the \textit{otriR::ade6}\textsuperscript{+} gene. Whereas K9A and K9R formed white colonies, the double mutant K9RS10A showed partially restored silencing as it formed pink colonies. The S10A mutant on its own partially affects silencing as it also formed pink colonies. On the right a larger view of the cells plated on limiting adenine is shown (not an enlargement of the same plate).
B) The H3 N-tail mutants were plated on rich medium at different temperatures and on medium containing 10\(\mu\)g/ml and 25\(\mu\)g/ml TBZ at 25°C to test for growth defects. All four H3 N-tail mutants grew well at the temperatures tested. K9A and K9R showed sensitivity to 10\(\mu\)g/ml TBZ and K9RS10A showed sensitivity to 25\(\mu\)g/ml TBZ.
Fig. 4-11. Localisation of the Swi6-GFP fusion protein in the H3 N-tail mutant backgrounds in living cells.

A version of the pREP81XSwi6-GFP strain (derived from FY2214) was crossed into the histone H3 N-tail mutants and into the h3.1/h4.1Δ control. Cells were grown in thiamine free medium (PMG) to allow induction of the nmt promoter and were mounted live on glass slides (see Chapter 2). Swi6-GFP localised in heterochromatic foci in the wild type and in the h3.1/h4.1Δ control and it was delocalised in the clr4Δ (FY3300) control. Swi6-GFP was delocalised in the K9R mutant, although it
4.9. Localisation of Swi6 in the H3 N-tail mutants.
The localisation of Swi6 is dependent upon Clr4 [Ekwall et al., 1996] which methylates histone H3 on K9 providing a binding site for the chromodomain of Swi6 [Bannister et al., 2001]. Although strains bearing histone H3 N-tail mutations also contained a wild type histone H3, they display a silencing defect. The localisation of Swi6 was next assessed in these mutated backgrounds.

The localisation of Swi6 was examined in live cells expressing the GFP-Swi6 fusion protein expressed under the control of the attenuated pREP81 nmt promoter [Pidoux et al., 2000] in the histone H3 mutated backgrounds (Fig. 4-11). GFP-Swi6 appeared delocalised in the K9R background: the green signal filled the nucleus and only rarely was a single brighter spot detected. A similar loss of Swi6 localisation is seen in the clr4A mutant, which as previously described lacks H3 K9 methylation at centromeres and the mating type locus [Nakayama et al., 2001]. In contrast, Swi6-GFP appeared localised in the S10A and K9RS10A backgrounds although it often formed fewer spots. This indicates that H3 K9RS10A suppresses (at least partially) the delocalisation of GFP-Swi6.

Swi6 localisation was also tested by immunostaining using anti-Swi6 antibodies (Fig. 4-12). This experiment gave a different result, as in all three mutants (K9R; S10A; K9RS10A) Swi6 appeared only partially localised in a fewer number of spots, most of the time with one brighter spot, compared to the wild type control.

Considering that in these mutants only some of the available histone H3 is mutated, it is possible that Swi6 localisation is only partially affected. The live cells analysis has the advantage of being less prone to artefacts than immunostaining which requires cell fixation. However, the GFP-Swi6 fusion may not be 100% functional due to the GFP tag and the fact that it is overexpressed. Thus, it is possible that the delocalisation of GFP-Swi6 in the H3 K9R mutant is due to a combined effect between the mutation itself and the level of GFP-Swi6.

To establish whether Swi6 association is lost in any of the silent regions where it is normally present, such as at the centromeric outer repeats, mating type locus or telomeres, it will be worth performing anti-Swi6 ChIP on these histone H3 mutant strains.
Fig. 4-12. Localisation of Swi6 in H3 N-tail mutants.
Immunostaining was performed using affinity-purified anti-Swi6 antibodies on h3.2 K9R, S10A and K9RS10A mutants. A wild type strain, h3.1/h4.1Δ and clr4Δ were stained for comparison. In this experiment Swi6 formed 4-5 foci in the wild type strain, consistent with previous observations [Ekwall et al., 1995] whereas the clr4Δ mutation caused complete delocalisation of Swi6 [Ekwall et al., 1996]. The h3.1/h4.1Δ control showed staining identical to the wild type. In the K9R mutant Swi6 formed only one spot, occasionally the staining filled the nucleus as seen in the clr4Δ mutant. In S10A and K9RS10A Swi6 formed one brighter spot most of the times and occasionally few weaker spots were also detected.
DISCUSSION

Regions of the chromosomes where important developmental or cellular programmes reside often exclude gene activity. In particular cell lineages such repression is tightly controlled and must be stably maintained through cell division. One important mechanism used by cells to achieve this gene silencing is through post-translational modifications of histone H3 and H4 N-tails. The underacetylated state is required for centromere function in fission yeast [Ekwall et al., 1997] and in mammalian cells [Taddei et al., 2001]. In fission yeast, the fact that histone H4 lysines 8 and 16 appeared to be hyperacetylated at the outer repeats following TSA treatment [Ekwall et al., 1997] led to the hypothesis that H4 could be mediating recruitment of a silencing factor through one of these conserved lysines. One possibility was that fission yeast shared a silencing mechanism similar to budding yeast, where K16 of histone H4 is required for the recruitment of a Sir3/Sir4 like silencing component at heterochromatic regions [reviewed in Grunstein, 1998]. The data presented in this chapter indicate that the N-terminus of histone H4 is not required to mediate silencing at the outer repeats of fission yeast centromeres. Point mutations of K8 and K16 did not significantly alter outer repeats silencing and even the deletion of the entire hydrophilic H4N-tail had little or no effect on centromere silencing. The phenotypes associated with the H4 point mutations could be explained by changes in gene expression due to altered "acetylated" H4 N-tails. It would be of interest to determine which genes are affected in histone H4 N-tail mutants by microarray analyses.

The completion of the fission yeast sequencing project has now shown that there are no homologues of Sir3 or Sir4 in S. pombe. Interestingly, the budding yeast genome does not contain a Swi6 or aClr4 homologue. Thus, perhaps two completely divergent mechanisms have evolved to mediate heterochromatin formation: a H4/Sir3/Sir4 type, forming budding yeast heterochromatin and a H3/Swi6 type, composing fission yeast and higher eukaryotes heterochromatin.

In fission yeast, Clr4 transfers methyl groups onto K9 of histone H3 [Rea et al., 2000] providing a binding site for Swi6 at heterochromatic sites [Bannister et al., 2001; Nakayama et al., 2001]. Point mutations of H3 K9 mimicking either an acetylated state or a non-methylated state were found to be lethal. This result was unexpected as the clr4Δ mutant is viable as is the swi6Δ mutant [Allshire et al., 1995] or the combination of these mutants (Borgstrom and Allshire,
unpublished observations). However, the K9 point mutations caused alleviation of silencing even in the presence of a wild type H3. The fact that the silencing defect was dominant suggests that the mutation present on one H3 tail can affect the normal function of the wild type tail. It is possible that similar levels of WT and mutant H3 are present, and thus most nucleosomes contain one mutant and one wild type histone H3. The histone N-tails extend outward from the nucleosome structure and are flexible and disordered [Luger et al., 1997] thus the possibility that two H3 N-tails interact with each other cannot be excluded. The double mutant K9RS10A partially rescued the alleviation of silencing suggesting that phosphorylation of S10 might be involved in the dominant silencing defect of K9R. This is consistent with the in vitro studies that showed that acetylated or unmodified K9 peptides are better substrates for Ipl/Aurora kinase responsible for S10 phosphorylation [Rea et al., 2000]. The mutated K9 could promote S10 phosphorylation and, in a mechanism reminiscent of that observed in budding yeast [Lo et al., 2000], S10 phosphorylation could then be a signal to promote the recruitment of a HAT complex that acetylates the histone tail causing transcriptional activation. As already discussed, in budding yeast Gcn5 recognises phospho-S10 and acetylates H3 K14 activating transcription [Lo et al., 2000]. It would be interesting to test whether in fission yeast mutation of histone H3 K14 to G would also partially restore silencing in a K9R background or if K14 is acetylated in the K9R mutant by ChIP. The K9A/K9R-induced S10 phosphorylation could involve just the N-tail carrying the mutated K9 or both the mutated and the wild type N-terminal tails. In both cases this event causes delocalisation of Swi6-GFP.

Since H3 K9 acetylation blocks the in vitro methyltransferase activity of Suvar3-9 [Rea et al., 2000], it is possible that the centromeric defects observed upon TSA treatment [Ekwall et al., 1997] were due to hyperacetylation of H3 K9 which consequently blocked H3 K9 methylation causing loss of Swi6 binding.

But, since neither Clr4 nor Swi6 are essential, why are the K9 mutations lethal? There are several possibilities to explain this:

A) if unmethylated N-tails are better substrates for the Ark1/Aurora kinase the reduction of H3 K9 methylation in the H3 K9R h3.3/h4.3 mutant could cause increased S10 phosphorylation. This hyperphosphorylation of chromosomes may lead to cell death. However, the clr4Δ mutation also reduces H3 K9 methylation but it does not cause cell lethality.
B) Methylation of K9 could be not just involved in the formation of constitutive heterochromatin (at centromeres, telomeres and mating type loci), but it could also play a role in repression of genes involved in a number of cellular functions. However, this implies the existence in fission yeast of a K9 methyltransferase different from Clr4, as clr4 is not an essential gene and perhaps of an enzyme that de-methylates histone H3 to turn genes back on. In mammalian cells, the existence of another H3 K9 methyltransferase has been postulated [Peters et al 2001], and the mammalian G9a methyltransferase shows some specificity for K9 of histone H3 [Tachibana et al., 2001]. To date, there is no evidence supporting or refuting the existence of another K9 methyltransferase in fission yeast, even though the S. pombe genome contains probably 6 SET domain proteins. Also to date there is no evidence of the existence of demethylase activity.

C) The H3 N-tail K9 could be required for gene activity through binding of regulative factors. Similarly, the lethality of H3 S10A suggests that S10 phosphorylation is essential for cell viability and/or that S10 is required for binding of an essential factor. Also Ark1 is essential, but since its functions comprise those of both mammalian Aurora A and B it is possible that its essential function is due to defects in chromosome segregation and spindle assembly and dynamics.

The analysis of cells containing the mutated H3 K9 and S10 as the only source of histone H3 would be of great interest to understand their function. This could be achieved by germination of spores originating from the cross of the h3.2-ura4+ h3.1/h4.1Δ h3.3/h4.3Δ strain with the H3 K9 h3.1/h4.1Δ or by turn off experiments where the wild type h3.2 is under the repressible nmt promoter.
**Fig. S-1. High conservation in the H2AF/Z family of proteins.**

Protein alignment of members of the H2AF/Z family from different organisms. High conservation is present throughout the protein. *S. pombe* *pht1* has an extended N-terminus tail of about 30 residues, which is absent in the other homologues. From the top down H2A variants from: *S. cerevisiae*, *S. pombe*, sea urchin (*Strongylocentrotus purpuratus*), *Drosophila melanogaster*, chicken (*Gallus gallus*), mouse (*Mus musculus*) and H2A from *S. pombe*. The histone fold domain is highlighted by the black box. Identical residues are boxed in red, similar residues are highlighted in gray. Highlighted in blue are the residues where the H2AvD unique features are thought to reside [Clarkson et al, 1999].
CHAPTER 5
INVESTIGATING THE ROLE OF PHT1 (AN H2A VARIANT) IN CENTROMERE FUNCTION

INTRODUCTION

Eukaryotic cells have evolved a number of ways to modulate nucleosome structure for regulation of gene expression. One way is to incorporate specific histone variants in a subset of nucleosomes. Members of the H2AF/Z family of histone H2A variants have been implicated in such processes in a number of different organisms. This family of proteins is highly conserved in a wide range of eukaryotes (see protein alignment in Fig. 5-1). The most highly conserved stretches are found throughout the histone fold domain, whereas more divergence is present at the N-terminus and at the C-terminus. In particular S. pombe H2AF/Z (Ph1) displays an extended N-terminal tail that is absent in its homologues.

The H2AF/Z protein is essential in Drosophila [van Daal et al., 1992] and in Tetrahymena [Liu et al., 1996] and is required for the early development of the mouse embryo [Faast et al., 2001]. In Drosophila a study where the null mutant for H2AvD was rescued by transgenes, obtained by mutating H2AvD residues to the equivalent H2A residues, identified a small region (residues 92-103, highlighted in Fig. 5-1) that when mutated could not rescue the null phenotype [Clarkson et al., 1999]. The authors suggest that in this carboxy terminal region resides the unique feature of the H2A variant. In budding yeast, the HTZ1 (H2AF/Z) null mutant is viable, but it is unable to grow at the elevated temperature of 37°C [Santisteban et al., 2000] and displays a slow growth phenotype, which is more pronounced at the low temperature of 20°C [Jackson et al., 2000]. Overexpression of H2A could not rescue the mutant phenotype [Santisteban et al., 2000] and plasmid shuffle experiments showed that the major H2A genes cannot provide the HTZ1 function and that HTZ1 cannot replace the essential function of the major H2As [Jackson et al., 2000]. Interestingly, the Tetraymena H2Av is able to rescue the phenotypes associated with disruption of the budding yeast HTZ1 gene, suggesting a functional conservation amongst H2Av proteins [Jackson et al., 2000]. It thus appears that the major H2A and H2Az genes play distinct roles in the cell and are not interchangeable.
Another possibly important difference is that in contrast with core histones, that are synthesised at S phase, H2Az synthesis does not appear to be cell cycle regulated in chicken [Dalton et al., 1989]. The H2Av transcript persists throughout the cell cycle, at a level of about 10% that of H2A; this is also the case in Chinese-hamster ovary cells [Wu et al., 1981].

The localisation of the H2Az proteins has been studied in three different systems. In Drosophila, H2AvD is widely but non-randomly distributed in the genome, being incorporated in nucleosomes of both active and inactive genes [Leach et al., 2000]. In budding yeast HTZ1 is present throughout the nucleus with increased localisation in subnuclear structures likely to be the nucleolus [Dhillon et al., 2000]. Finally, in Tetrahymena, hv1 is present at transcriptionally active macronuclei, but excluded from transcriptionally inactive micronuclei [Allis et al., 1986], implicating a role for H2Av in transcriptional activation. Further evidence supporting a role in transcriptional activation comes from the finding that deletion of HTZ1 in budding yeast makes cell viability highly dependent on the SNF/SWI remodelling complex and on the SAGA histone acetyltransferases complex [Santisteban et al., 2000]. Both SNF/SWI and SAGA complexes are involved in transcriptional activation of specific genes. Double mutants deleted for both htz1 and either snf2, a subunit of the SNF/SWI complex, or gcn5 histone acetyltransferase, a subunit of the SAGA complex, were impaired for growth. However, H2Av does not only play a role in transcriptional activation, in contrast it has also been implicated in silencing at the mating type locus (HMR) and at telomeres in budding yeast [Dhillon et al., 2000]. Overexpression of HTZ1 restored silencing at HMR in a sirt mutant strain and the htz1Δ deletion resulted in a partial loss of silencing at HMR and a profound loss of telomeric silencing. Thus, the H2Av family of proteins have been implicated both in transcriptional activation and repression.

In contrast to the lack of cell cycle regulation of H2Av expression in chicken hamster ovary cells, transcription of the H2Av of S. pombe, Pht1, is cell cycle regulated, with its mRNA peaking near the G1/S boundary, close to the time of the peak in histone transcript levels [Durkacz et al., 1986]. Cells deleted for the pht1 gene are viable, grow slowly and exhibit an altered colony morphology [Carr et al., 1994]. More interestingly, the stability of a minichromosome was found to be dramatically decreased in pht1 null cells implicating Pht1 in mechanisms required for chromosome stability and transmission. The fact that S. pombe strains lacking Pht1/H2AF/Z have an increased rate of minichromosome loss may be indicative of a defect in centromere
Fig. 5-2. Serial dilution assays of the pht1Δ mutant.

A) Silencing at the outer repeats was tested by colony colour assay. Red colonies indicated repression of the ade6° gene inserted at otr (see wild type and pht1Δ) while white colonies indicated derepression of the ade6° gene (see swi6Δ control).

B) Growth on medium lacking arginine was used to assay silencing at the central core in the strain pht1Δ TM1::arg3°. Two colonies one that was large (L) and one that was small (S) on medium lacking arginine were plated at the temperatures of 25°C and 32°C and growth compared with an arg3°, a wild type TM1::arg3° and an arg3° strain on medium lacking arginine. The wild type grew poorly and so did the (S) pht1Δ, whereas the (L) pht1Δ grew well.

C) Growth at 36°C and in the presence of 10μg/ml TBZ at 25°C. pht1Δ (L) and (S) were not temperature sensitive but they were sensitive to TBZ to a lesser degree than swi6Δ control. pht1Δ (L) was slightly more sensitive to TBZ than pht1Δ (S).
function, but might also be due to defects during DNA replication or chromosome integrity. However, if Pht1 is incorporated in centromeric chromatin, its absence could affect proper silencing either at the outer repeats or at the central core of the centromere.

As an initial test for a potential role of Pht1 at centromeres, cells lacking Pht1 were assessed for defects in silencing.

RESULTS

5.1. Monitoring \textit{pht1Δ} defects with respect to centromere function.

As previously discussed, fission yeast centromeric silencing is disrupted in mutants affecting centromere function. For instance, cells lacking Swi6 displayed alleviated silencing at the outer repeats of the centromere [Allshire et al., 1995], whereas mutated alleles of mis6 alleviated silencing at the central core of the centromere [Partridge et al., 2000]. To test whether lack of Pht1 has an effect on silencing at the outer repeats of the centromere, the \textit{ade6} gene inserted at the outer repeats of centromere 1 was introduced into the \textit{pht1Δ} mutant background \textit{pht1Δ ::ura4*} (FY 1067) from A. Carr. As described previously, cells expressing \textit{ade6} produce white colonies whereas nonexpressing cells form red colonies. Colonies formed by cells lacking Swi6 were white. In contrast, \textit{pht1Δ} cells, like wild type cells, formed red colonies therefore silencing of \textit{ade6} within the outer repeats was not affected, Fig. 5-2A. To test whether central core silencing is impaired in \textit{pht1Δ} cells, the \textit{pht1Δ ::ura4*} mutant was crossed to generate a strain where a crippled \textit{arg3} gene is inserted at \textit{TM1} in an \textit{arg3} background (\textit{arg3-D3}). In a wild type background the \textit{arg3} gene inserted within the central core (\textit{TM1}) is repressed and cells grow very poorly on medium lacking arginine. Centromere mutants affecting the central core of the centromere, such as the \textit{mis6} mutant, grow well on medium lacking arginine (A. Pidoux, unpublished observation). In contrast to wild type cells, when the \textit{pht1Δ TM1::arg3*} cells were plated on medium lacking arginine colony size was variable. This mixture of colony sizes suggests different levels of \textit{arg3} expression and thus variegated states with respect to central core silencing. Although in general silencing at the central core is not very strong, variegation of the crippled \textit{arg3} gene is not observed in a wild type background. The \textit{pht1Δ}
mutant alleviated and repressed states of the TM1::arg3\textsuperscript{+} gene are clearly stably inherited through cell division since cells from a single large (L) colony continued to grow well on plates lacking arginine whereas cells from the smaller colony (S) produced poorly growing small colonies, similar to the wild type strain TM1::arg3\textsuperscript{+}, Fig.5-2B. This result suggests that, once established, defective or functional silencing of central core chromatin is maintained in the pht1\textDelta mutant. It is possible that Pht1 plays a role in establishing silencing rather than maintaining it and thus in its absence the frequency of fully silent cells is reduced.

To test whether other defects were associated with the pht1\textDelta mutation the sensitivity of the two isolated pht1\textDelta (L) and pht1\textDelta(S) strains to microtubule depolymerising drug TBZ and to temperature was compared with a wild type strain and the swi6\textDelta mutant (Fig. 5-2C). pht1\textDelta(L) and pht1\textDelta (S) grew well at the elevated temperature of 36°C. As expected the swi6\textDelta mutant was sensitive to 10\textmu g/ml TBZ. pht1\textDelta(L) and pht1\textDelta (S) were also sensitive to TBZ, although to a lesser degree than the swi6\textDelta control. In particular it was noticed that the pht1\textDelta(L) colonies were slightly more sensitive to TBZ than the pht1\textDelta(S) colonies. In conclusion, fission yeast cells lacking the H2AF/Z gene product, Pht1, display alleviation of silencing at the central core of the centromere (TM1) and sensitivity to the microtubule poison TBZ. These results support a possible role of Pht1 in centromere function.

5.2. Chromosome segregation defects in pht1\textDelta.

To further investigate the possible role of Pht1 in chromosome segregation, the mitotic behaviour of chromosomes was observed in the pht1\textDelta mutant (Fig. 5-3). Cells with impaired centromere function often display defective mitotic configurations for example swi6\textDelta [Ekwall et al., 1995] and mis6 [Takahashi et al., 1994] which are rarely observed in a wild type strain. Wild type and Pht1\textDelta cells were stained with anti tubulin to follow spindle elongation during mitosis and with DAPI to follow chromosome behaviour. Chromosome segregation defects such as unequal segregation, failure to segregate chromosomes toward the poles and lagging chromosomes were observed very frequently (approximately 50\%) in the mutant but not in the wild type strain (wild type not shown, see Fig. 5-3). Given the nature of the defects it is possible that Pht1 directly affects centromere function. However, these defects might also arise if
Fig. 5-3. Chromosome segregation defects in the *pht1Δ* mutant.
Immunostaining of the *pht1Δ* mutant with anti-tubulin (green) to stain the mitotic spindle and DAPI (red) to stain the DNA. Chromosome segregation defects were observed at high frequency. A) and B) unequal segregation. C) Lagging chromosome (left) and failure to segregate chromosomes (right). D) (Top) failure to segregate chromosomes. E) Unequal segregation (left) and hypercondensed and not separated chromosomes. F) Lagging chromosome. G) Unequal segregation.
chromosome integrity (decondensation/condensation, replication, repair, telomere structure) is impaired.

5.3. Generation of an antibody against the N-terminus of Ph1.

To determine if Ph1 plays a direct role at centromeres requires reagents to detect the Ph1 protein. Such reagents would allow the study of Ph1 association with specific domains within chromosomes. For example, is it incorporated in centromeric chromatin, or widely associated along the chromosomes? Two techniques can be employed to address this: immunostaining and chromatin immunoprecipitation. Since Ph1 displays an extended non-conserved N-terminal tail the first 20 residues of the protein were used to raise polyclonal antibodies. A KLH conjugated peptide (MILRHAPRVHESAFLTHKT) was synthesised with a terminal cysteine which would allow coupling to a sulfolink matrix for affinity purification. The peptide was injected into one rabbit and bleeds were tested by both western blotting and immunostaining in wild type versus Ph1Δ cells. Total protein extract and histone preparations were carried out on the two strains (see Chapter 2) and were used to check whether successive bleeds could recognise a band of the predicted size of approximately 19KDa in a western blot, which should be absent in the ph1Δ extract. No specific band was observed with 1st, 2nd, 3rd and 4th (last) bleeds with any of the dilutions tested. It is possible that Ph1 is low in abundance and therefore difficult to detect, overexpression of Ph1 would address this possibility. Immunostaining of both wild type and ph1Δ cells was also performed using a dilution of 1:50 of the 4th bleed. The staining resulted in speckles in the nucleus, which were observed also in the ph1Δ strain, but not in the preimmune sera control. An attempt to clean up the 4th bleed was made. A 1:50 dilution of the 4th bleed was repeatedly incubated against ph1Δ mutant cells and then employed for immunostaining of wild type and ph1Δ cells. Unfortunately, this type of procedure did not improve specificity of the immunostaining. Affinity purification of the crude sera may improve the specificity of these antibodies. It is possible that no antibodies were produced that recognise the peptide utilised. Alternatively, it is possible that the predicted ORF does not correspond to the real one. The amino acids utilised to generate antibodies could not be part of the Ph1 protein either because not transcribed at all or because cleaved off after translation. The real start of transcription remains to be investigated.
DISCUSSION
In this chapter, the possible role of Pht1 in centromere function has been addressed. The interest arose from previous studies where Pht1 was implicated in proper minichromosome transmission [Carr et al., 1994]. Members of the H2AF/Z family of proteins have been implicated in silencing and in chromosome function in previous studies. Clearly, cells lacking pht1 display variegated silencing at the central core of *S. Pombe* centromere 1 in addition to sensitivity to the microtubule poison TBZ and a high frequency of chromosome segregation defects. The results presented support the possibility that Pht1 may play a role in centromere function. Since silencing is only destabilised in the central core and not in the outer repeats it is possible that Pht1 contributes to the function of chromatin underlying the kinetochore. It is known that the central core of the centromere contains a specialised H3 variant, Cnp1, the counterpart of human CENP-A [Takahashi et al., 2000]. One possibility is that Pht1 could be a specialised H2A that is incorporated specifically at the central core of fission yeast centromeres. Testing this will require the localisation of Pht1 and its association with centromeric chromatin to be investigated. Given the failure to raise antibodies which recognise the N-terminus an obvious next step is to tag the open reading frame (ORF) with HA or other epitope.
CHAPTER 6
CNP1 ASSOCIATES WITH THE CENTRAL DOMAIN CHROMATIN AND CAN SPREAD OVER NON-CENTROMERIC DNA

INTRODUCTION
Centromeres differ from the rest of the nuclear chromatin in that they possess a unique histone H3-like protein, first identified in humans as CENP-A [Earnshaw et al., 1985; Palmer et al., 1991] and in budding yeast as Cse4p [Stoler et al., 1995]. CENP-A homologues have now been identified in a number of eukaryotes, demonstrating that this highly conserved histone H3-like protein is characteristic of highly divergent centromeres throughout the eukaryotic kingdom. All CENP-A homologues identified to date share the same basic organisation as histone H3, consisting of an amino-terminal domain and a carboxy-terminal histone-fold domain. CENP-A only associates with active centromeres and is an essential component of centromeric chromatin of all eukaryotes [Earnshaw et al., 1985; Howman et al., 2000; Stoler et al., 1995; Buchwitz et al., 1999; Blower and Karpen, 2001; Takahashi et al., 2000]. Thus CENP-A is a key player in centromere structure and function in many organisms.

Fission yeast centromeres are organised into two clearly distinct domains, the outer repeats (otr) surrounding the central domain composed of the innermost repeats (imi) and the central core (cnt).

The association of Swi6 and Mis6 with centromeres has been previously described [Partridge et al., 2000]. This extensive study based on ChiP showed that the outer repeats of centromere 1 (otr1L and otr1R) contain the Swi6 protein [Ekwall et al, 1995], while the central domain contains the Mis6 protein [Saitoh et al., 1997, Partridge et al, 2000] and no Swi6. The tRNA genes are found between these two domains and they may act as boundary barriers between the two domains [Partridge et al., 2000].

Fission yeast Cnp1 has been shown to associate with the central core and the innermost repeats, but is not associated with the outer repeats of the centromere [Takahashi et al., 2000].
In that study, the authors utilised C-terminal HA and GFP tagged versions of Cnp1 under its own promoter, which may not be fully functional. The localisation was performed both by examining cells expressing Cnp1-GFP and by ChIP analysis using cells expressing Cnp1-HA. The immunoprecipitated DNA was analysed using primers designed to amplify a small region of the central core and of the innermost repeats of the centromeres. The extent of Cnp1 association with the central core and the innermost repeats was not investigated further, for example the boundaries between the Cnp1 containing chromatin with the Swi6 containing chromatin were not defined and it was not addressed whether the domain of association of Mis6 overlaps with that of Cnp1.

The central domain of the centromere, containing the central core (cen) and the two surrounding innermost repeats (iml), contains a specialised chromatin structure characterised by a smeared micrococcal nuclease (MNase) digestion pattern [Polizzi and Clarke, 1991; Takahashi et al., 1992]. This central core specific chromatin is disrupted in mutants such as mis6 [Saitoh et al., 1997] and mis12 [Goshima et al., 2000]. Cnpl is also required for the formation of this central domain-specific chromatin structure. Takahashi et al. (2000) have proposed that Mis6 is required for the deposition of Cnpl at centromeres, implicating Mis6 as a potential Cnpl specific loading factor. However, no evidence for physical or genetic interactions between the two proteins has been reported. In addition, it is not known whether Cnpl replaces histone H3 in central domain nucleosomes or whether they are both constituents of this chromatin.

In S. cerevisiae, genetic studies have shown that Cse4 interacts with histone H4 through the histone fold [Smith et al., 1996; Glowczewski et al., 2000] and biochemical studies indicate that both Cse4 [Chen et al., 2000] and human CENP-A [Shelby et al., 1997] form homodimers. This suggests that CENP-A like nucleosomes are homotypic, containing CENP-A homodimers and no histone H3.

Therefore a more favoured view is that CENP-A completely replaces histone H3 in the nucleosomes that underlie the kinetochore. Alternatively, kinetochore chromatin could simply be enriched in CENP-A containing nucleosomes. Histone H3 containing nucleosomes could be interspersed in this specialised chromatin or they could be organised in alternating stretches with CENP-A containing nucleosomes. Such scenarios are hard to address in the mammalian
system, where the sequences associated with CENP-A are composed of large tandem arrays of satellite DNA and where it is difficult to define specific centromeric sequences.

In the well-defined fission yeast centromere it is possible to ask directly whether a protein is associated with a specific centromeric region. The chromatin immunoprecipitation (ChIP) technique was employed to address whether histone H3 is incorporated in the central core Cnp1 containing chromatin. ChIP has been extensively used to study the association of proteins with centromeres and telomeres in budding yeast and fission yeast [Hecht et al., 1996; Ekwall et al., 1997; Meluh and Koshland, 1997; Saitoh et al., 1997; Goshima et al., 1999; Partridge et al., 2000, Takahashi et al., 2000]. In this Chapter ChIP has been employed to explore the relative association of histone H3 and Cnpl with the central core region and the results suggest that histone H3 is largely underrepresented, if not excluded, from the central core chromatin.

How CENP-A-like proteins are deposited specifically at active centromeres is a major goal of centromere research. As discussed in Chapter 1, this could involve cis-acting DNA elements, replication timing or differential CENP-A/histone H3 expression. It is also possible that the heterochromatic environment or the repetitive nature of centromeric associated DNA rather than specific sequences constitute a signal for CENP-A/Cnpl deposition.

In this chapter, fission yeast cen1 was manipulated to test if Cnpl could associate with non-centromeric DNA inserted in the central core. The results indicate that Cnpl can be loaded onto this non-centromeric DNA. The implications of these data are discussed.

RESULTS

6.1. Cnpl is the S. pombe counterpart of human CENP-A.
Because of the complexity of its centromeres and its genetic tractability, fission yeast is a good model in which to study centromeres. The identified key players at fission yeast centromeres, such as for instance Swi6 or Cir4, have counterparts in multicellular organisms including human.

It was thus of great interest to identify the CENP-A counterpart of fission yeast. One approach to identify this gene was to exploit the high conservation shared between CENP-A like proteins
Fig. 6-1. Multiple alignment of the protein sequences of *S. pombe* Cnpl, human CENP-A and *S. pombe* histone H3.1.

Protein sequences were retrieved using Entrez Protein (www.ncbi.nlm.nih.gov/Entrez/protein.html) and were aligned using ClustalX. Identical residues are highlighted in red, similar residues in grey. The α-helices and β-loops in the histone fold domain are shown above the residues involved in those structures. The three proteins are highly conserved particularly within their histone fold. Cnpl displays a shorter N-tail. At position 58 Cnpl harbours a small insertion of 4 residues that is absent in CENP-A. Cnpl and CENP-A share the insertion of a Valine at positions 66 and 81, respectively, which is absent in histone H3.1.
to design primers to be used in degenerate PCR. Efforts to identify the fission yeast CENP-A with this strategy were unsuccessful. In parallel, since the *S. pombe* sequencing project was close to completion, the database was frequently searched for open reading frames with homology to human CENP-A and *S. pombe* H3. Such an open reading frame was identified in the *S. pombe* database in August 1999 by the lab by TBLASTN (protein versus translated DNA) using both *S. pombe* histone H3 and human CENP-A as queries. A protein alignment between human CENP-A, *S. pombe* histone H3 and *S. pombe* CENP-A homologues is shown in Fig. 6-1.

In parallel, the same open reading frame was identified as a clone, which complemented the *sim2* mutant. *sim2* was subsequently found to be a temperature-sensitive allele of fission yeast CENP-A, identified in a mutagenic screen for central core factors affecting central core silencing (A. Pidoux, manuscript in preparation). However, during the course of this work Takahashi et al. (2000) presented their observations on *S. pombe* CENP-A, which they named Cnp1 (Centromeric Protein 1).

6.2. Deleting the *cnp1* gene.

CENP-A like proteins have been shown to be essential for viability in all systems tested. To assess whether fission yeast CENP-A/Cnp1 was essential, the gene was deleted by homologous recombination. The *ura4* marker gene was used to replace the entire *cnp1* open reading frame. A PCR fragment was generated containing the *ura4* gene surrounded by approximately 60 base pair of homology to the *cnp1* locus on each side. The fragment was introduced into a diploid strain by transformation and selection on medium lacking uracil. Transformants were analysed by PCR for replacement of one copy of the *cnp1* gene with the *ura4* marker gene and for the presence of a *cnp1* wild type gene. Four transformants (A-D) contained the *ura4* correctly targeted as shown by PCR, Fig. 6-2A.

To assess the lethality of a *cnp1* gene knockout, tetrad dissection analysis of spores generated after meiotic division can be carried out. Unfortunately, all of the *cnp1/cnp1Δura4* transformants were unable to go through meiosis (sterile h+/h- diploids) thus this type of analysis was not possible. To overcome this problem, transformants can be crossed back to wild type haploid strains of the two opposite mating types (h+ and h) to generate a *cnp1/cnp1Δura4* diploid which is h+/h- and able to produce spores.
**Fig. 6-2. Deletion of the cnp1 gene.**

**A)** PCR check of the *cnp1Δ::ura4* knockout in a diploid strain.

Ethidium bromide stained agarose gel of four independent *ura4* transformants (A-D) PCR checked using primers to detect the *cnp1Δ::ura4* disruption (left) and a wild type *cnp1* copy (right).

**B)** *cnp1* is essential.

The diploid strain *cnp1/cnp1Δ::ura4* was broken down to the haploid state on medium containing 10μg/ml TBZ. Derivatives that formed red or pink colonies (i.e. haploids) on medium containing limiting adenine plate (left) were replica-plated onto medium lacking uracil (right) to assess the presence of the *cnp1Δ::ura4* deletion. None of the haploids grew on medium lacking uracil, suggesting that *cnp1* is an essential gene. This finding is consistent with the observations of Takahashi et al., 2000.
An alternative way to assess if the \( cnp1 \) gene is essential is to break down the diploid to a haploid state and test if any of the resulting haploid cells are \( \text{ura}^4 \) (\( cnp1\Delta \text{ura}^4 \)), Fig. 6-2B.

The diploid strain employed for the \( cnp1 \) knockout harbours two different mutated alleles of the \( \text{ade}^6 \) gene the \( \text{ade}^6-210 \) allele and the \( \text{ade}^6-216 \) allele at their loci. The \( \text{ade}^6-210 \) allele confers a red colour to haploid colonies on limiting adenine plates whereas the \( \text{ade}^6-216 \) allele confers a pink colour. In the diploid \( \text{ade}^6-210/\text{ade}^6-216 \) intragenic complementation takes place between these two alleles rendering these cells \( \text{ade}^6 \) and white on limiting adenine medium. To maintain the diploid state, which is rather unstable in fission yeast, it is necessary to maintain selection by growing cells on medium lacking adenine. In the absence of selection, the diploid cells frequently "break down" to the haploid state. This "break down" can be induced by plating diploid cells on medium containing the microtubule poison TBZ. TBZ exacerbates the instability of the diploid state by affecting microtubule polymerisation during mitosis. The generation of haploids can be monitored by the formation of pink (\( \text{ade}^6-216 \)) and red (\( \text{ade}^6-210 \)) colonies on limiting adenine plates.

Red and pink haploid cells obtained by break-down of the \( cnp1^+/cnp1\Delta::\text{ura}^4 \) diploid were all \( \text{ura}^4 \) demonstrating that cells harbouring the \( cnp1 \) deletion are not viable.

6.3. Epitope-tagging of Cnp1.

In order to detect the Cnp1 protein, the Cnp1 ORF was fused to a epitope tag expressed from an inducible promoter. Mammalian CENP-A is a histone H3-like protein which is targeted to centromeres via its C-terminal domain [Sullivan et al., 1994] and its N-terminal tail is likely to extend outward from the nucleosome. It is a reasonable assumption that N-terminal epitope tagging will be less likely to interfere with protein function than a C-terminal tag. A well characterised regulatable promoter available for \( S. \ pombe \) gene expression is the \( nmt \) (no message in thiamine) promoter [Maundrell, 1990]. In the absence of thiamine \( nmt \) drives the expression of the protein downstream, whereas the presence of thiamine inhibits transcription. Three different degrees of \( nmt \) strength are available: high strength, medium and low strength. To avoid possible problems that might arise from massive overexpression of a kinetochore-specific histone H3-like protein, Cnp1, the medium strength promoter (pREP42) was chosen.

Cnp1 was cloned under the \( nmt \) promoter and in frame with the epitope tag generating an N-terminal protein fusion. To allow expression of the epitope-tagged Cnp1 fusions, it is necessary...
Fig. 6-3. Localisation of the epitope-tagged Cnp1.
A) EGFP-Cnp1 co-localises with the Mis6-HA centromeric protein. Immunostaining was carried out on cells expressing the Mis6-HA protein and EGFP-Cnp1. The green (Cnp1) and the red (Mis6) signal co-localised at the centromeres.
B) ChIP reveals that HA-Cnp1 is associated with the central core of the centromere. ChIP experiments were carried out on cells expressing HA-Cnp1 from a plasmid using anti HA antibodies (12CA5). The IP DNA was analysed by multiplex PCR using three primer sets designed to amplify the outer repeats (top band-otr), the central core (middle band-cnt) and the fbp1 gene as an euchromatic control (bottom band). The two independent HA-Cnp1 expressing clones showed enrichment of the cnt band in the IP DNA, whereas the no tag control (left) showed no enrichment.
to grow cells in medium lacking thiamine. Two vectors were employed, one harbouring the EGFP epitope and one harbouring the HA epitope [Craven et al., 1998]. The vectors also bear an ARS (autonomously replicating sequence) and a selectable marker, ura4+. The EGFP-Cnp1 vector (pREP42X-EGFP-Cnp1) was linearised by digestion at a unique restriction site in the ars1 sequence.

The DNA fragment obtained was introduced by homologous recombination with the endogenous ars1 locus by yeast transformation and selection on medium lacking uracil. The pREP42X-EGFP-Cnp1 was inserted at the ars1 locus in a strain that harbours the Mis6-HA protein [Saitoh et al., 1997] to allow the co-localisation of the two centromeric proteins. Potential transformants were PCR checked using a primer within the nmt promoter and a primer within the ars1 sequence downstream from the site of integration. Positives were then analysed by southern blot to check for single integration events (not shown). In Fig.6-3A the immunolocalisation of Mis6-HA with EGFP-Cnp1 is shown. As predicted, the two proteins co-localised in a single spot representing the three centromeres of S. pombe clustered together in interphase. The diploid strain cnp1/cnp1Δ::ura4+ was transformed with the plasmid pREP41-HA-Cnp1. Diploid cells containing the plasmid and the cnp1::ura4+ deletion were selected on media lacking adenine, leucine and uracil. Positive transformants were then plated on limiting adenine medium containing 10μg/ml TBZ to induce diploid break down. The haploid pink and red colonies generated were tested for growth on medium lacking uracil and leucine. Some of the resulting haploid colonies contained both cnp1Δ::ura4+ and pREP41(LEU2)-HA-Cnp1. This indicates that the HA-Cnp1 tag is functional, because it can complement the lack of wild type cnp1.

To test the association of HA-Cnp1 with the centromere, chromatin immunoprecipitation was carried out. Wild type haploid cells were transformed with the episomal plasmid pREP41X-HA-Cnp1. Transformants were used to perform chromatin immunoprecipitation using anti-HA antibodies (12CA5) and analysing the immunoprecipitated DNA by multiplex PCR, Fig.6-3B. The position of the primers used in the PCR is described later in this chapter (section 4). Preliminary results showed that HA-Cnp1 was enriched in the cnt (central core) region and not in otr (outer repeats). However, the background in this experiment was surprisingly high as the
Fig. 6-4. Anti-Cnp1 antibodies detect the centromeres.

**A) Immunolocalisation of Cnp1 using the polyclonal anti-Cnp1 antibodies.**
Wild type cells were stained with a 1:500 dilution of crude anti-Cnp1 serum. The staining shows a clear, bright spot corresponding to the three centromeres clustered together in interphase cells. A) Green: Cnp1. B) Merge, blue: DNA.

**B) Co-localisation of Cnp1 with Mis6-HA.**
Double immunostaining using anti-Cnp1 (green) and anti-HA (red) antibodies on cells expressing the Mis6-HA fusion protein shows that the two signals co-localise.
CHAPTER 6 CNP1 ASSOCIATES WITH THE CENTRAL DOMAIN

euchromatic control (*tbp1*) was also amplified in the immunoprecipitated DNA, although not to the same extent as the *cnt* DNA. This experiment suggests that Cnp1 is primarily associated with the central core of the centromere and not with the outer repeats, consistent with published data [Takahashi et al., 2000]

6.4. Generation of antibodies against Cnp1 N-terminus.

The epitope-tagged Cnp1 proteins were expressed from an inducible promoter and are not regulated in a normal cell cycle fashion. To allow detection of the wild type endogenous Cnp1 protein, the generation of polyclonal antibodies was undertaken in parallel with the construction of epitope-tagged Cnp1. For this purpose the 19 non-conserved residues that form the N-terminus tail of Cnp1 were used to raise polyclonal antibodies. The KLH conjugated peptide MAKKSLMAEGPDPIPRPK was synthesised and injected into one sheep. Immunostaining with the second bleed (see Materials and Methods) detected a single spot in the nucleus corresponding to the three centromeres clustered together, Fig.6-4A. To confirm that the spot detected in the immunostaining experiment corresponded to the centromeres, co-localisation with the Mis6-HA [Saitoh et al., 1997] protein was also carried out, Fig. 6-4B.

Unfortunately, Cnp1 could not be detected by western blot analysis with this serum despite attempting several methods of protein extraction and filter blotting. To confirm that the serum does indeed contain antibodies which recognise the Cnp1 protein, immunoprecipitation experiments were carried out (Fig. 6-5A). The strain containing EGFP-Cnp1 and an untagged strain were lysed under mild conditions and extracts used for immunoprecipitation with anti-Cnp1 antibodies (see Chapter 2). The immunoprecipitated material was analysed by Western blotting with anti-GFP antibodies. A band corresponding to EGFP-Cnp1 (predicted size 43KDa) was efficiently immunoprecipitated in the tagged strain and was absent in the untagged strain.

The same immunoprecipitation experiment was also applied to two strains where the only source of Cnp1 is fused with the HA epitope (the endogenous *cnp1* gene is deleted, the strain contains the pREP41 (*LEU2*)-HA-Cnp1 plasmid) that was described earlier in this chapter (paragraph 3). Extracts from two strains containing the HA-Cnp1 fusion (expected size approximately 17.2KDa) and a wild type strain containing untagged *cnp1* were immunoprecipitated using anti-Cnp1 antibodies and analysed by Western blot using anti-HA antibodies (Fig. 6-5B). The western blot detected a band of the predicted size of HA-Cnp1 in
**Fig. 6-5. Anti-Cnp1 antibodies can immunoprecipitate Cnp1.**

A) IP was performed on the strain containing the EGFP-Cnp1 protein fusion under the pREP42 promoter and on a wild type strain (no tag). The IPs were analysed by Western blot using anti-GFP antibodies (Molecular probes) which detected a band of approximately 43KDa only in the WCE and IP from the EGFP-Cnp1 strain. *lgG heavy chain band.

B) IP was carried out on two strains containing the HA-Cnp1 protein fusion under the pREP41 promoter as the only source of Cnp1 and on a wild type strain (no tag). The IPs were analysed by Western blot using anti-HA antibodies which detected a band of approximately 17.2KDa only in the WCE and IP from the HA-Cnp1 strains, which was absent in the untagged strain. *lgG light chain band.
the IP from the HA-Cnp1 tagged strains that was undetectable in the control strain. These results indicate that the serum does contain anti Cnp1 antibodies.

A search of the *S. pombe* database with the 19 residues used for the immunisation did not identify any matching sequence apart from Cnp1. Together this suggests that the antibodies generated specifically recognise Cnp1.

### 6.5. Analysis of Cnp1 localisation at centromeres by ChIP.

In order to define the regions of the centromere associated with Cnp1, chromatin immunoprecipitation experiments (ChIP) were carried out as described in Chapter 2 using the polyclonal anti-Cnp1 antibodies.

In the experiment shown in Fig. 6-6B ChIP was carried out on a wild type strain using the anti-Cnp1 antibodies. The immunoprecipitated DNA was analysed using primers designed to amplify the outer repeats and the central core of *cen1* and an euchromatic control. Multiplex PCR analysis was carried out on chromatin before and after immunoprecipitation. The presence of DNA from three chromosomal regions was assessed using primer pairs designed to amplify the outer repeats of *cen1* (*otr*-top band), the central core of *cen1* (*cnt-middle band*) and the euchromatic control (*fbp1*-bottom band), see diagram in Fig. 6-6A. It is clear that the ratio between the three PCR products changed in the IP relative to the total input DNA after immunoprecipitation, showing an enrichment of the central core DNA band (*cnt-middle band*).

This result demonstrates that Cnp1 is associated with the central core and not with the outer repeats of the centromere, nor with the *fbp1* control, consistent with the observations contained in Takahashi et al., 2000.

Because of the sequence conservation between the three centromeres of fission yeast, and because of their repetitive nature, primers that specifically detect centromere 1 sequences are difficult to design. The primer pairs designed to amplify the outer repeats and the central core of the centromere [Partridge et al., 2000] were tested for specificity on DNA from three YACs (Sanger Centre), 3G9Y, 10E4Y and 11C3Y containing centromeres 1, 2 and 3, respectively.

The *otr pair* amplified DNA from all three centromeres, whereas the *cnt pair* amplified DNA from
A

Swi6p  Cnp1p/Mis6p  Swi6p

otr1L  otr  TM1  cnt  otr1R

R.Int

ura4^  —

ura4-D/SE

GEL

Quantitative PCR

Calculate ratio

B

WT

otr  cnt  fbp

GEL

695 bp  415 bp

Calculate ratio
Fig. 6-6. Defining the regions of the centromere associated with Cnp1 by ChIP.
Ethidium bromide stained agarose gels showing the chromatin immunoprecipitation (ChIP) experiments performed using anti-Cnp1 antibodies.
A) The *cen1-ura4* insertion strains [Allshire et al., 1995] were used to map Cnp1 association across the centromere. ChIP was carried out in parallel in strains harbouring the *ura4* marker inserted at 5 locations across the centromere and one random location in the euchromatin as control. Arrows on the centromere 1 diagram indicate the sites of insertion of the *ura4* gene. These strains also contain an internal deletion of the *ura4* gene (*ura4-D/SE*) at the *ura4* locus. DNA from the total crude preparation (C) and from the IP was analysed by PCR using a primer set that amplifies a region spanning the *ura4-D/SE* deletion, generating two PCR products. In this way the enrichment of the *ura4* band can be estimated relative to an euchromatic internal control. See diagram. The Eagleye quantification software was used for the quantification. The *ura4* at locations 3 to 5 was enriched indicating that Cnp1 is associated with the whole central domain. In particular, Cnp1 was more abundant in TM1 (5) and (4). No Cnp1 was detected at the random integrant location, a small enrichment was detected at the *imr/otr* junction (1). Note that this insertion is actually at the *imr/otr* junction on the right side of centromere 1.
B) DNA from the total crude extract (C) and from the immunoprecipitated material (IP) of a wild type strain was analysed by multiplex PCR. Three primer sets were used to amplify a region of the outer repeats (top band), one region of the central core (middle band) and the *fbp1* gene, as a non-centromeric control. The IP DNA showed enrichment of the middle band corresponding to the central core. These results are consistent with the work of others [Takahashi et al., 2000].
centromere 1 and 3 (not shown). Thus these primer pairs are not ideal to draw quantitative conclusions from ChiP experiments.

To examine in more detail and in a more quantitative fashion which specific regions of cen1 incorporate Cnp1, strains with the ura4* marker located at different positions within cen1 [Allshire et al., 1995] were utilised in ChiP experiments similar to previous analyses [Partridge et al., 2000]. The diagram in Fig. 6-6A shows the sites of insertion of ura4* within cen1 that were analysed for association with Cnp1. The ability of Cnp1 to associate with ura4* sequences located at different centromeric positions was compared with the ability to associate with the ura4-DS/E, a truncated version of the ura4* gene at the ura4* locus. A single primer pair was employed to amplify both ura4* and ura4-DS/E. This strategy provides more quantitative information as each PCR reaction contains an internal control. The enrichment of the ura4* PCR product is compared with that of the ura4-DS/E product, the resulting level of enrichment can be estimated in comparison with the ratio ura4*/ura4-DS/E in the crude DNA. The results are illustrated in Fig. 6-6B. Five centromeric ura4* insertion strains were used for this experiment: one within the otr/imr junction (1-FY648) three within the imr repeats (2-FY496, 3-FY525, 4-FY534) and one within the central core in TM1 (5-FY336). A strain harbouring the ura4* marker in a random euchromatic location (R.Int-FY4835) was used as control. While no enrichment of the ura4* was observed in the random integrant immunoprecipitated DNA, a clear enrichment of ura4* was observed in the three insertions in imr (2-4) and in TM1 (5). A small enrichment of the ura4 band was observed in the otr/imr junction insertion, this may be due to experimental variation since this region is expected to lack Cnp1 [Takahashi et al., 2000] Fig. 6-3A and 6-6B. Association of Cnp1 with this particular site needs to be examined in more detail.

The imr site (4) and the TM1 site (5) have been shown to contain a significant amount of Mis6 while lacking Swi6 [Partridge et al., 2000]. In contrast the site situated between the two tRNA genes in imr (3), does not contain Mis6 but shows significant enrichment in anti-Swi6 immunoprecipitations [Partridge et al., 2000]. Interestingly, Cnp1 was found in a region where Mis6 has been shown to be not highly represented. Verification of this will require performing ChiP on the same cell extract with anti-Swi6, anti Mis6-HA and anti-Cnp1 antibodies.
6.6. Cnp1 can associate with non-centromeric sequences inserted in the centromere.

ChIP experiments clearly show that Cnp1 efficiently immunoprecipitated the *ura4* gene inserted at all sites within the central domain of the centromere (*imr/cnt*). This result suggests that Cnp1, like Swi6 [Partridge et al., 2000], is able to spread from endogenous centromeric sequences over neighbouring non-centromeric sequences artificially inserted within the centromere. It was of interest to test how far this spreading of Cnp1 could be extended and with what efficiency. This approach was previously employed to examine the ability of Swi6 to associate with non-centromeric DNA [Partridge et al., 2000].

If Cnp1 were not able to efficiently coat a longer DNA fragment within the centromere context it might suggest that the centromeric sequences are required for Cnp1 loading. In contrast, if the spreading can take place, it would be a first indication that Cnp1 can be loaded at sites not containing normal centromeric sequences.

An extended fragment of DNA containing the *ura4* gene (1.7 Kb) flanked by 1.3 and 1.7 Kb of DNA from the *ade6* gene on the left and right side respectively [Partridge et al., 2000], was introduced at *TM1* by transformation, Fig. 6-7A. Homologous recombination allowed a central core insertion of *ade6* (*TM1::ade6*) to be replaced with the *ade6::ura4*::*ade6* fragment. The *ade6* gene inserted at *TM1* confers white colony colour on limiting adenine plates, loss of the *ade6* gene results in red colonies. Transformants were selected on limiting adenine medium lacking uracil to select for loss of the *ade6* gene and integration of the *ade6::ura4*::*ade6* fragment at *TM1* by homologous recombination (this strain was constructed by J. Partridge). The *ura4*/*red* transformants obtained were checked by PCR and crossed into a *ura4-D/SE* background to allow quantification in ChIP experiments (strains henceforth abbreviated *TM1::bigura4*).

In Fig. 6-7B, ChIP experiments were carried out using the polyclonal anti-Cnp1 antibodies on strains harbouring the *ura4* gene inserted at control loci and different positions in *cen1*. Recovered immunoprecipitated DNA (IP) was compared with the total input DNA (T) by competitive radioactive PCR of *ura4* (Chapter 2). The enrichment of the *ura4* band reflects the
A

B

C

- URA

5-FOA

complete

**A**

![Genetic Circuit Diagram]

**B**

<table>
<thead>
<tr>
<th></th>
<th>R.Int::ura4+</th>
<th>TM1::ura4+</th>
<th>Otr::ura4-</th>
<th>(A) TM1::bigura4+</th>
<th>(B) TM1::bigura4+</th>
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<td>T</td>
<td>T</td>
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<td>4.3</td>
</tr>
</tbody>
</table>

**C**

- URA

5-FOA

complete

- R.Int::ura4+
- ura4
- TM1::ura4+
- (1) TM1::bigura4+
- (2) TM1::bigura4-
- otr1R::ura4+
Fig. 6-7. Cnp1 can be incorporated in a large non-centromeric fragment inserted at TM1.
A) Diagram of the TM1::ade6::ura4^::ade6 strain (TM1::bigura4^). A fragment of approximately 5Kb, containing the ura4^ gene surrounded by 1.3 and 1.7Kb of the ade6 gene on either side was introduced in TM1::ade6^ strain by homologous recombination.
B) ChIP experiments were carried out using the anti-Cnp1 antibodies on the strain TM1::bigura4^ and on strains harbouring the ura4 inserted at TM1 (TM1::ura4^) at the otr/imr junction (otr::ura4^) and at a random euchromatic location (R.int::ura4^). DNA from the total input DNA (T) and from the immunoprecipitated samples (IP) was subjected to quantitative radioactive PCR. The products obtained were run on polyacrylamide gels and exposed to a phosphorimager screen. PCR bands were quantified using the ImageQuant software. The values obtained for the random integrant location were normalised to one and values for all of the other samples were calculated relative to it. As predicted, no enrichment of the ura4 band was measured in the otr::ura4^ strain. Strain TM1::ura4^ showed an enrichment of the ura4 band of 21 fold, indicating association of Cnp1 with this region. An enrichment was also measured in the TM1::bigura4^ strain indicating that Cnp1 can associate with this extended non-centromeric fragment. The enrichment was reduced to 4-6 fold compared with TM1::ura4^ suggesting that less Cnp1 was incorporated onto the ura4^ gene.
C) Serial dilution assay to compare silencing of the ura4^ gene in TM1::ura4^ and in TM1::bigura4^. Cells were plated on medium lacking uracil and on medium containing the drug 5-FOA, toxic for ura4^ expressing cells, and were incubated at 32°C. The R.int::ura4^ and a ura4 strain were plated as controls. While TM1::ura4^ grew well on both media, TM1::bigura4^ was more sensitive to 5-FOA, indicating a slight alleviation of silencing.
amount of Cnp1 associated with that DNA. Levels of enrichment of ura4* at each insertion site were quantified relative to ura4-D/SE and normalised to values obtained for a strain bearing a random integration of ura4* (R.Int::ura4*). In this experiment again a small enrichment of the ura4* band was measured in the ofrR:: ura4* insertion.

Clearly, Cnp1 is able to coat the TM1::bigura4*, because the ura4* band is enriched in the IP DNA relative to the total, Fig. 6-7B. The conditions used for these ChIP experiments were identical to those used previously which normally result in sheared DNA of approximately 500-1000bp in size [Ekwall and Partridge, 1999]. The primer pair utilised for the competitive PCR is situated at approximately 1.9Kb from the left side and 2.1Kb from the right side of the centromeric sequences, respectively (Fig.6-7A). The PCR fragment generated is 695bp long, thus, assuming DNA fragments are on average 500bp, Cnpl must have spread between 2-2.5Kb.

However, while the ura4* band is enriched approximately 20 fold in the TM1::ura4* strain, in the TM1::bigura4* strain the enrichment is reduced to approximately 4-6 fold. Thus the amount of Cnp1 protein associated with ura4* in the TM1::bigura4* strain is reduced compared to the amount associated with ura4* in strains where only ura4* is inserted at TM1. Nevertheless, this result supports the idea that Cnp1 can spread over a long fragment of non-centromeric DNA and that specific centromeric DNA sequences may not be absolutely required in cis for Cnp1 loading.

It is possible that the spreading from the endogenous centromeric DNA flanking the bigura4* fragment takes place in a gradient fashion. This would imply less Cnp1 binding in the middle of ura4*, the part detected by the primer pair used in these experiments, but more Cnp1 binding toward the ura4* flanking sequences. This is an interesting issue which is worth investigating in the future.

6.7. TM1::bigura4* is less silenced compared to TM1::ura4*.

Other analyses have shown that Cnp1 plays a role in centromeric silencing since three alleles of Cnp1 (sim2-76, sim2-87 and sim2-169) were isolated in a random mutagenesis screen for factors affecting central core silencing (A. Pidoux, manuscript in preparation).

Although Cnp1 is able to coat almost 5kb of TM1::bigura4* the amount of protein associated appeared lower than the amount coating the TM1::ura4* DNA. If less Cnp1 is associated with
A

H3 N-terminus peptide (anti-H3N)

H3S.cerev MARTKQTARKSTGGKAPRKQLASKAARKSAPSTGGVKKPHRYKPGT
H3S.pombe MARTKQTARKSTGGKAPRKQLASKAARKAAPATGGVKKPHRYKPGT
Cnp1 MARSWKSLMAMPGDPARRPRKRYRPGTTALREIRKYQRSTDLLIQRL

B

H3 C-terminus peptide (anti-H3C)

human H3 CAIHAKRVTIMPKDQLARRIRGERS
H3S.pombe CAIHAKRVTIMPKDMQLARRIRGERS
Cnp1 CAIHAKRVTIMQRDMQLARRIRGERS

Fig. 6-8. Amino acid alignment of histone H3 N and C-termini.
A) Peptide alignment between the N-terminal peptide of S. cerevisiae histone H3 used to raise the anti-H3N antibodies [Suka et al., 2001] with the S. pombe histone H3 and Cnp1 N-termini. The N-termini of the two histone H3 proteins are very highly conserved, whereas Cnp1 N-terminus is divergent.
B) Alignment between the human histone H3 C-terminus peptide employed to raise the anti-H3C antibodies (A. Verreault, unpublished data) and S. pombe histone H3 and Cnp1 C-termini. High conservation is shared between all three sequences. Compared to the human histone H3 peptide, 3 amino acid changes are present in S. pombe histone H3 and 3 amino acid changes and a truncation of two residues are found in Cnp1.
ura4* flanked by larger inserts then perhaps this ura4* gene could be correspondingly less silent. To test this, the ability of TM1::ura4* and TM1::bigura4* strains to grow on medium lacking uracil (positive selection for ura4* expressing cells) and medium containing 5-FOA (positively selects cells with a silent ura4*) was compared. Cells with the small insertion in TM1 (TM1::ura4*) grew well on medium containing 5-FOA, in contrast cells from the larger insert TM1::bigura4* were slightly more sensitive to 5-FOA, Fig. 6-7C. Although subtle this observation was reproducible and indicates that the longer insert at TM1 expresses the ura4* gene at an increased level. This supports the involvement of Cnpl in centromeric silencing because when less Cnpl is associated, silencing is less efficient.

6.8. Lack of histone H3 in central core chromatin.

Cnpl containing nucleosomes appear to be exclusively present in the chromatin of the central domain of fission yeast centromeres. It is not known whether Cnpl is present in all central core nucleosomes or if nucleosomes containing histone H3 are also incorporated at the central domain of the centromere. The ura4* centromeric insertions are the ideal system and ChIP a useful technique to address this issue. Antibodies that recognise histone H3 were used to determine if H3 is incorporated in the ura4* chromatin in different contexts. Again, the random integrant ura4*, which will be packaged in normal histone H3 chromatin is used as control. Two different types of anti H3 antibodies were utilised:

a) an affinity purified polyclonal antibody raised against the first 46 residues of S. cerevisiae histone H3 (anti-H3N) [Suka et al., 2001]. This region of histone H3 is highly conserved between S. cerevisiae and S. pombe (Fig. 6-8A).

b) polyclonal antibodies (crude serum) raised against the last 26 residues of human histone H3 C-terminus (anti-H3C), Fig. 6-8B (A. Verreault, unpublished data).

Since there is no evidence that histone H3 is modified at the C-terminus the anti-H3C antibodies should be able to recognise all possible isoforms of histone H3. The alignment of the human C-terminus of H3 with that of S. pombe histone H3 shows good homology. ChIP experiments were carried out comparing enrichment of ura4* inserted at TM1 (TM1::ura4*) and of ura4* inserted in a random euchromatic location (R.Int::ura4*) using anti-Cnpl and anti-H3N antibodies. The result is shown in Fig. 6-9A. The anti-Cnpl antibodies can efficiently immunoprecipitate the ura4* in the TM1::ura4* strain but not in the R.Int::ura4* strain.
Conversely, the anti-H3N antibodies immunoprecipitated some *ura4^*, together with *ura4-D/SE* in a ratio of approximately 1:1, in the R.Int::*ura4^* strain, but only a very weak *ura4* band could be detected in the *TM1::ura4^* strain. This result is consistent with a situation where histone H3 is present throughout the euchromatin, but is absent from the central core chromatin. However, there are two caveats to this experiment. Firstly, the *ura4^* band is underrepresented in the total crude DNA from the *TM1::ura4^* strain, making the faint *ura4* band in the *TM1::ura4^* DNA immunoprecipitated with the anti-H3N difficult to interpret. It has been observed previously [Partridge et al., 2000] that for some unknown reason the extraction of the *ura4^* DNA when inserted in the central core of the centromere is relatively inefficient, as compared to that of *ura4^* inserted in euchromatin or even in the centromeric outer repeat chromatin. This experimental limitation is difficult to overcome and it is likely due to the incorporation of this centromeric region in a large complex of kinetochore proteins which causes a less efficient release in extracts from crosslinked cells. Nevertheless, the Cnp1 antibodies are able to efficiently immunoprecipitate the underrepresented *ura4^* DNA whereas the anti-H3N are not. The second caveat is that the anti-H3N antibodies were raised against a GST-fusion protein expressed in *E. coli*, thus they may not recognise, for instance, post-translational modifications present in the histone H3 N-tail. It is possible that the N-tail of the histone H3 present at the central core is modified, for example acetylated or methylated, and it is not recognised by the anti-H3N antibodies. To avoid this possible limitation of the anti-H3N antibodies, the second anti serum of antibodies recognising the H3 C-terminus were also employed in ChlP experiments. The experiment was carried out as described for the anti-H3N antibodies and results are shown in Fig. 6-9B. The fact that the anti-H3C chromatin immunoprecipitated from the random integrant contained equivalent amounts of *ura4^* and *ura4-D/SE* chromatin, but *TM1 ura4^* was undetectable is consistent with the lack of histone H3 at the central core of the centromere. It could be argued that histone H3 is present in central core chromatin, but that the C terminus is not accessible to the antibody due to steric hindrance by the kinetochore complex. However, since the same result was obtained using the anti-H3N antibody and the N-tail of histone H3 is expected to be more accessible, this possibility can be ruled out. A good control for accessibility would be to perform ChlP with an anti histone H4 antibody raised against the C-terminus.
Fig. 6-9. Histone H3 is excluded from TM1::ura4+ chromatin.

Chip experiments were carried out using two different anti histone H3 antibodies (H3N and H3C) to compare the amount of histone H3 associated with the ura4+ inserted at a random euchromatic location (R.Int::ura4+) and with the ura4+ inserted at the central core of centromere1 (TM1::ura4+). Chip using anti-Cnp1 antibodies was carried out in parallel for comparison.

A) Acrylamide gel of the radioactive competitive PCR from anti-H3N ChIP. These antibodies efficiently immunoprecipitated ura4 from the random integrant location whereas they failed to immunoprecipitate the ura4 inserted in the central core. This indicates a lack of histone H3 in this centromeric region. The Cnp1 antibodies worked as predicted.

B) Ethidium bromide stained agarose gel showing the PCR carried out on ChIP performed using the anti-H3C antibodies. Consistent with the anti-H3N ChIP, histone H3 was not detected on ura4 inserted in the central core of centromere 1, while it was detected on the ura4 at the euchromatic location.

C) Insertion of the extended ura4+ (TM1::bigura4+) fragment within the central core caused incorporation of histone H3. This experiment was carried out in parallel with 6-9B. The anti-H3C antibodies were able to detect some histone H3 coating ura4+ in the TM1::bigura4+ ChIP.
In conclusion, these data suggest a paucity of histone H3 in the central core chromatin, which is replaced by Cnp1.

**6.9. Increased distance from the centromeric sequences correlates with incorporation of histone H3 in central core chromatin.**

The data presented in this chapter show that the chromatin associated with central domain of fission yeast centromeres contains a specialised histone H3 like protein, Cnp1. This protein is not exclusively incorporated onto centromeric sequences, but it is able to coat a long fragment of non-centromeric DNA inserted within the central domain of centromere. The amount of Cnp1 associated with non-centromeric sequences appears to be reduced with the increasing size of this DNA (TM1::bigura4*) and correlates with a slight decrease in the silencing of that DNA. Finally, histone H3 is largely underrepresented in the central domain ura4* insertion (TM1::ura4*).

It is possible that the decreased silencing and association of Cnp1 with longer central domain insertions might be mirrored by increased incorporation of histone H3. This issue was addressed in ChIP experiments using anti-H3C antibodies on chromatin extracted from the TM1::bigura4* strain in parallel with the experiment shown in Fig.6-9B. In contrast with the result obtained for the TM1::ura4* strain (Fig. 6-9B), where no ura4* was detected in the immunoprecipitated DNA, some ura4* was detected in DNA immunoprecipitated from the TM1::bigura4* strain with anti-H3C antibodies, Fig. 6-9C. This data suggest that the larger non-centromeric fragment is packaged in nucleosomes containing mostly Cnp1, but that some histone H3 is also incorporated.

**DISCUSSION**

Cnp1, the fission yeast counterpart of human CENP-A, was identified by homology searches of the *S. pombe* database and as a mutant alleviating silencing at the central core of the centromere (A. Pidoux, unpublished observations). The EGFP-Cnp1 protein co-localised with the centromeric factor Mis6-HA and the HA-Cnp1 protein was shown to associate with the central core sequences by chromatin immunoprecipitation (ChIP). To allow the detection of the endogenous wild type Cnp1, polyclonal antibodies were raised against the non-conserved N-terminal tail of Cnp1. These antibodies were employed in immunostaining experiments and detected a single spot in interphase cells, which corresponds to the three *S. pombe*
centromeres clustered at the nuclear periphery [Funabiki et al., 1993]. The anti-Cnp1 antibodies were also employed in ChIP experiments to define the regions of the centromere associated with Cnp1. Consistent with the results of others [Takahashi et al., 2000] Cnp1 was enriched at the central core and absent from the outer repeats of the centromere. The ura4 \(^{+}\) cen1 insertion strains [Allshire et al., 1995] were employed as previously [Partridge et al., 2000] to perform a more detailed mapping of Cnp1 localisation by ChIP. The results indicated that Cnp1 is largely underrepresented at the atr/imr junction, but it is associated with all sites tested within the imr repeats and with the central TM1.

The two centromeric factors Swi6 and Mis6 have been shown to be associated with centromeric sequences in a reciprocal fashion. While Swi6 is preferentially associated with the outer repeats of the centromere, Mis6 is specifically associated with the central domain. The region spanning the two tRNA genes present in the imr repeats has been proposed to be a transition point between Swi6 and Mis6 associated sequences [Partridge et al, 2000]. The fact that Cnp1 appears to be abundant across this transition region, where Mis6 is poorly represented, suggests that perhaps Cnp1 and Mis6 association with centromeric sequences may not overlap completely. Mis6 has been proposed to be a loading factor for Cnp1 [Takahashi et al., 2000]. However, it is not known how Mis6 could act as a loading factor and if this function would necessarily imply that the two components must occupy the same centromeric sequences. Further analysis is required to precisely map the position of these two proteins relative to each other.

To address whether Cnp1 is associated with the endogenous regions on either side of the tRNA genes, anti-Cnp1 immunoprecipitated DNA from a wild type strain was analysed by PCR using two primer sets designed to amplify sequences internal or external to the tRNA genes [Partridge et al., 2000]. Preliminary results indicated that Cnp1 is associated with both these regions, thus the association of Cnp1 over the ura4 insertions 3 and 4 shown in Fig. 6-4A is not simply due to spreading of the protein from the more internal imr sequences (not shown).

The rare occurrence of neocentromeres (which contain CENP-A), indicates that CENP-A loading onto DNA does not appear to require specific cis-acting DNA sequences. To test if in S. pombe Cnp1 can only associate with centromeric sequences, an extended ura4 fragment of approximately 5kb was inserted within the central core region, and ChIP experiments were
performed to assay the presence of Cnp1. The results obtained showed that Cnp1 is capable of spreading over non-centromeric sequences from the flanking endogenous centromeric DNA, suggesting that centromeric DNA itself is not the only DNA that can be packaged in Cnp1 containing chromatin. The surrounding centromeric repeats and central domain could play a role in driving Cnp1 loading onto intervening DNA regardless of their sequence.

To test whether the central domain sequences themselves are required, it will be necessary to replace the entire central domain with a large DNA fragment, leaving only the flanking outer repeats of the native centromere.

Quantification of the immunoprecipitated DNA showed that the amount of Cnp1 on the extended *ura4* fragment (*TM1::bigura4*) is reduced by approximately 4-6 fold, compared to that on *TM1::ura4*. There are several possible explanations for this:

a) Cnp1 could be limiting in the cell, therefore the spreading over bigura4 is not very efficient. A direct way to address this possibility would be to assess whether the spreading is more efficient when Cnp1 is overexpressed.

b) The inefficient spreading of Cnp1 could be partially incompatible with the *ura4* gene activity. If this is the case, it is not clear why Cnp1 can be deposited efficiently on *TM1::ura4*, which is also transcribed albeit at a lower level. One way to address the negative role of *ura4* transcription on Cnp1 spreading could be to replace the *ura4* in the *TM1::ura4* and *TM1::bigura4* with a non-transcribed *ura4* lacking the whole promoter region (J. Partridge). This approach is in progress.

c) Loss of Cnp1 from *TM1::bigura4* could be due to a general low affinity for non-centromeric sequences for Cnp1 loading. The central core region is relatively A+T rich and perhaps Cnp1 preferentially associates with such sequence. This could be tested by placing other A+T rich sequences within the central domain.

d) Finally, the distance of *ura4* from the endogenous centromeric sequences, which might play a role in targeting Cnp1 loading at centromeres, may limit the ability of Cnp1 to be assembled. Again, overexpression of Cnp1 may rescue this.

In humans neocentromeres only arise occasionally on DNA sequences that do not contain any centromeric DNA. The data presented showed that fission yeast Cnp1 can be incorporated onto DNA from regions encoding the *ura4* and the *ade6* gene, thus not belonging to
centromeric sequences. Together these results indicate that Cnp1 is associated with the whole central domain and that this domain is not a rigid structure, rather, it can be extended by adding exogenous non-centromeric sequences. The limit to which the Cnp1 spreading phenomenon can be extended has not been defined, although it seems likely that larger fragments will probably contain less Cnp1 and perhaps confer instability to the whole centromere.

The ChIP experiments presented in Fig. 6-6A showed that Cnp1 is associated with TM1 sequences. The next question addressed was whether Cnp1 is the only histone H3 like component of the central core chromatin or, rather, if histone H3 is also a component. Using two different types of antibodies, one recognising the N-tail and one the C-terminus of histone H3, ChIP experiments indicated that histone H3 is largely excluded from the central core. Thus, by the resolution provided by the ChIP technique, Cnp1 appears to be the major if not the only H3 like histone of central core nucleosomes, which presumably also contain histone H4, H2A and H2B. Future analyses should assess whether histone H3 is excluded from the entire central domain or if some histone H3 can be found toward the edge of the region, for instance in the region spanning the tRNA genes.

The extended central domain, in the TM1::bigura4* strain, not only incorporates Cnp1 nucleosomes, but it also incorporates some histone H3. The reason for this is not clear, the inefficient spreading of Cnp1 over TM1::bigura4* may somehow allow the incorporation of histone H3.

As discussed in Chapter 1, one model for CENP-A deposition is based upon assembly of CENP-A nucleosomes in regions of the genome in which they were previously contained. The application of these models to S. pombe remains speculative, as there is no evidence to exclude that Cnp1 assembly at centromeres is simply coupled to DNA replication. However, it is possible that the presence of a non-centromeric transcribed DNA fragment inserted within the central core interferes, for instance, with new Cnp1 deposition or removal of histone H3 after replication.
CHAPTER 7
ALTERED HISTONE H3/H4 GENE RATIO IMPAIRS CENTROMERE FUNCTION

INTRODUCTION
The fission yeast haploid genome contains three copies of genes encoding histone H3 and H4. In contrast there are two genes encoding histone H2A and only one gene for histone H2B. The consequences of reducing the number of histone H3/H4 gene pairs has been described in Chapter 3. Briefly, it was established that S. pombe cells were viable when harbouring only one H3/H4 gene pair out of the three present in the wild type haploid genome. Cells with only one H3/H4 gene pair showed different phenotypes with respect to growth and silencing at heterochromatic sites depending on which pair was retained. This suggested that the three different sets may slightly differ in their relative contribution to chromatin. However, the effect on centromere function in cells harbouring a reduced number of histone H3/H4 genes was very mild. These strains, which maintained the stoichiometry between the number of histone H3 and H4 genes, showed normal chromosome segregation and moderate increase in minichromosome loss rates. Previously, plasmids containing histone H3 were isolated in screens for factors that when overexpressed alleviate silencing at the central core of the centromere (A. Pidoux, unpublished observations) suggesting that an excess of histone H3 may disrupt central core chromatin function. In addition, histone H3.3 was identified in a screen for factors that cause chromosome loss when overexpressed in fission yeast [Halverson et al., 2000]. In budding yeast it has been shown that overexpression of histone H3 has a strong dominant-effect on three temperature-sensitive mutants of cse4 [Glowczewski et al., 2000]. The Cnp1 histone H3 like protein appears to replace histone H3 in the central domain chromatin. It is likely that this region is packaged in nucleosomes thus it is possible that altering the ratio of histone H3 to H4 proteins rather than their quantity will interfere to a greater extent with the assembly of this centromere specific chromatin. For example an excess of histone H3 might compete with Cnp1 for centromeric nucleosome assembly. The effect of altering the H3/H4 ratio is studied in this chapter.
A

\[ H4.1 / H3.1 \Delta::\text{his3}^+ \]

\[ H4.2 \ H3.2\Delta \]

\[ \text{ura}4^+ \quad \rightarrow \quad \text{H4.3 \ H3.3} \]

\[ h3.2\Delta::\text{ura}4^+, \ h3.1/h4.1\Delta::\text{his3}^+ \]

(H4>H3)

B

\[ h4.1/h3.1\Delta::\text{his3}^+ \]

\[ h4.2\Delta \quad h3.2 \quad h4.3 \ h3.3 \]

\[ \text{ura}4^+ \]

\[ h4.2\Delta::\text{ura}4^+, \ h3.1/h4.1\Delta::\text{his3}^+ \]

(H3>H4)

C

\[ h4.1 \quad h3.1 \]

\[ h4.2 \ h3.2\Delta \quad h4.3/h3.3 \Delta::\text{arg3}^+ \]

\[ \text{ura}4^+ \]

\[ h3.2\Delta::\text{ura}4^+, \ h3.3/h4.3\Delta::\text{arg3}^+ \]

(H4>H3)

D

\[ h4.1 \ h3.1 \]

\[ h4.2\Delta \quad h3.2 \quad h4.3/h3.3 \Delta::\text{arg3}^+ \]

\[ \text{ura}4^+ \]

\[ h4.2\Delta::\text{ura}4^+, \ h3.3/h4.3\Delta::\text{arg3}^+ \]

(H3>H4)
Fig. 7-1. Schematic diagram representing the histone $H3/H4$ genes in strains containing altered histone gene ratios.

A single histone $h3.2$ or $h4.2$ was replaced by the $ura4^*$ marker gene. The strains obtained were combined with the deletions of either the $h3.1/h4.1$ ($h3.1/h4.1\Delta::his3^*$) or the $h3.3/h4.3$ gene set ($h3.3/h4.3\Delta::arg3^*$). The resulting mutants can be subdivided in two classes: $H4>H3$ containing two histone H4 and one histone H3 genes (A and C), and $H3>H4$ containing two histone H3 and one histone H4 (B and D).
RESULTS

One strategy to investigate the effect of increased levels of histone H3 versus H4 and vice versa on centromere function is to overproduce histone H3 or H4 from an inducible promoter. However, regulatable promoters such as nmt1 do not provide correct temporal control of histone gene expression. Therefore, a different approach was taken to construct strains bearing non-stoichiometric numbers of histone H3 and H4 genes. Instead of overexpressing the histones, the number of endogenous histone H3 and H4 genes was manipulated to obtain altered gene ratios. This strategy has the advantage that the histones are expressed from their endogenous cell cycle regulated promoters.

In Chapter 3 it was established that the h3.2/h4.2 pair was the most important for cell viability. Strains harbouring an unbalanced H3 versus H4 endogenous gene ratio were therefore generated by simultaneously disrupting a single h3.2 or h4.2 gene and a whole h3.x/h4.x gene set. In place of the wild type 3:3 ratio, these strains harbour a 2:1 ratio of histone H3 versus H4 and vice versa.

To build these unbalanced histone H3/H4 mutants, two strains (described in Chapter 4) were used which replaced h3.2 or h4.2 contained with the ura4* marker gene. These strains, h3.2A::ura4* and h4.2A::ura4*, were crossed with the strains h3.1/h4.1A::his3* and h3.3/h4.3A::arg3* (also described in Chapter 4). The four unbalanced histone H3/H4 strains generated are summarised in Fig. 7-1. These strains can be subdivided in two types:
A) H3:H4=2:1 ratio (h4.2Δ h3.1/h4.1 and h4.2Δ h3.3/h4.3)
B) H4:H3=2:1 ratio (h3.2Δ h3.1/h4.1Δ and h3.2Δ h3.3/h4.3Δ).
For simplicity A and B type strains will henceforth be referred to as H3>H4 and H4>H3 respectively.

7.2. Effect of H3:H4 ratio on viability and outer repeat silencing.
To test possible growth defects associated with unbalanced H3:H4 ratios, serial dilutions of cells were plated on rich medium at different temperatures (Fig. 7-2A).
Fig. 7-2. Phenotypic analysis of strains expressing unbalanced histone H3:H4 ratios.

A) Viability of the histone H3/H4 unbalanced strains at different temperatures.
Serially diluted cells were plated on rich medium and incubated at different temperatures. h3.2Δ h3.3/h4.3Δ, h4.2Δ h3.3/h4.3Δ and h4.2Δ h3.1/h4.1Δ were cold sensitive at 18°C. h4.2Δ h3.1/h4.1Δ grew poorly at 32°C. h4.2Δ h3.3/h4.3Δ and h4.2Δ h3.1/h4.1Δ were temperature sensitive at 36°C.

B) Viability of the histone H3/H4 unbalanced strains in the presence of the microtubule poison TBZ and outer repeats silencing.
Cells were plated on rich medium containing 10μg/ml TBZ and 15μg/ml TBZ and were incubated at 25°C and 32°C, respectively. The swi6Δ mutant was sensitive to both concentrations whereas the wild type control grew well. h4.2Δ h3.3/h4.3Δ was sensitive to 15μg/ml TBZ at 32°C and h4.2Δ h3.1/h4.1Δ was sensitive to both concentrations of TBZ.

The unbalanced histone strains contain the ade6Δ gene inserted within the outer repeats of centromere 1 (cot1R::ade6Δ). Cells were plated on rich medium containing limiting concentrations of adenine (10mg/L) at 25°C to test silencing at the outer repeats of centromere 1. The swi6Δ mutant was white as predicted, indicating alleviation of outer repeats silencing. The strains h4.2Δ h3.3/h4.3Δ and h4.2Δ h3.1/h4.1Δ were red as was the wild type, indicating that silencing at the outer repeats was maintained in these backgrounds. In contrast the strain h3.2Δ h3.3/h4.3Δ was pink and the strain h3.2Δ h3.1/h4.1Δ showed variegated colony colour.

C) Table summarising the phenotypes of the unbalanced histone mutants.
The four strains behaved differently. Strikingly, the H3＞H4 strain h4.2Δ h3.1/h4.1Δ formed only a few colonies at all temperatures tested. The other H3＞H4 strain, h4.2Δ h3.3/h4.3Δ, was cold sensitive at 18°C and temperature sensitive at 36°C.

The two H4＞H3 strains grew well at 25°C and 32°C, but were very mildly temperature sensitive at 36°C; in addition strain h3.2Δ h3.3/h4.3Δ was cold sensitive at 18°C.

Other phenotypes were also assessed in the unbalanced histone H3/H4 strains: cells were plated on medium containing the microtubule poison TBZ at the two concentrations of 10μg/ml and of 15μg/ml (Fig. 7-2B). As predicted, the swi6Δ control was sensitive to both concentrations of TBZ whereas the wild type strain grew well. Both histone H3＞H4 strains were sensitive to TBZ: h4.2Δ h3.1/h4.1Δ, was sensitive to 10 μg/ml TBZ at 25°C and to 15 μg/ml TBZ at 32°C, whereas h4.2Δ h3.1/h4.1Δ, was only sensitive to 15 μg/ml TBZ at 32°C. TBZ is most effective at lower temperatures and no colonies were formed by these strains on medium containing 15μg/ml TBZ at 25°C (not shown).

Finally, since all four unbalanced histone H3/H4 strains also contained the ade6° gene inserted at the outer repeats of centromere 1 (otr1R::ade6°), cells were plated on medium containing limiting concentrations of adenine to assess silencing in this region (Fig. 7-2B). Colonies from the wild type strain (FY1800) were red, indicating silencing of the otr1R::ade6° gene, whereas colonies from the swi6Δ strain were white, due to alleviation of silencing of the otr1R::ade6° gene. The two H3＞H4 strains were red on limiting adenine medium, indicating that silencing at the outer repeats of the centromere was maintained. In contrast, both histone H4＞H3 strains showed some degree of alleviation: strain h3.2Δ h3.3/h4.3Δ was pink and strain h3.2Δ h3.3/h4.3Δ showed variegated colony colour. A table summarising all of the different phenotypes associated with the unbalanced histone H3/H4 strains is shown in Fig. 7-2C.

In conclusion, strains harbouring an altered ratio of histone H3 versus histone H4 genes display a number of growth defects in different conditions. In particular, strains with H3＞H4 histones were most severely affected.

The defective growth observed in strains with altered H3:H4 gene ratio is presumably due to altered levels of H3:H4 proteins. To assess this, the level of histone proteins was compared. Histones were extracted at high concentration, electrophoresed through 16% polyacrylamide
Fig. 7-3. Protein levels and central core silencing in strains with unbalanced H3:H4 genes.

A) Altered histone gene level is reflected in altered histone protein level.
Core histones were extracted at high concentration using a histone preparation protocol (see materials and methods) from a control strain, lacking the h3.1/h4.1A gene set, and from the two unbalanced histone strains H3>H4 (h4.2A h3.1/h4.1A) and H4>H3 (h3.2A h3.1/h4.1A). The protein extract was electrophoresed through a 16% polyacrylamide gel and stained with Coomassie dye. In the control strain the histone bands did not stain equivalently. However, it can be observed that the histone H4>H3 contains more histone H4 compared to histone H3 whereas the histone H3>H4 contains more histone H3 compared to histone H4.

B) Central core silencing is alleviated in cells containing H3>H4.
Cells from a wild type strain containing the arg3+ marker gene inserted within the central core (constructed by A. Pidoux), strain h3.2A h3.1/h4.1A TM1::arg3+ and h4.2A h3.1/h4.1A TM1::arg3+ were plated on medium lacking arginine to assess silencing at the central core. While the wild type TM1::arg3+ and the histone H4>H3 strains grew poorly, strain with H3>H4 histones grew well on medium lacking arginine indicating defective central core silencing.
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gel and visualised by Coomassie staining. A histone preparation [Ekwall et al, 1997, see Chapter 2] was carried out on the H3>H4 strain h4.2Δ h3.1/h4.1Δ and on the H4>H3 strain h3.2Δ h3.1/h4.1Δ. These two strains were chosen because the histone H3>H4 containing the h3.1/h4.1 deletion was the most defective for growth under different conditions. The resulting stained gel is shown in Fig. 7-3A.

Histone bands can be easily identified within the resolution of the gel. The predicted size for the four core histones is indicated beside the gel in Fig. 7-3A. The identity of the histone H3 and H4 bands was confirmed by Western blot performed on histone preparations from wild type cells (not shown). The control strain is also disrupted for one H3/H4 gene set (h3.1/h4.1Δ) but retained an equal number of H3:H4 genes (2:2). Not all of the histone proteins stain to the same extent with Coomassie even in the control. However, in the H4>H3 extract, the band corresponding to histone H4 appears stronger, relative to the histone H3 band. In contrast, in the H3>H4 histone preparation, the histone H3 band appears stronger than the histone H4 band. This experiment indicates qualitatively that non-stoichiometric H3/H4 gene levels are mirrored by altered histone protein ratios. A more rigorous test would be to compare the levels of histone H3 and H4 in the unbalanced strains by Western blotting of cell extracts using antibodies against the C-terminus of histone H3 and H4.

7.4. Increased H3 relative to H4 alleviates silencing in the central core.

Cells with H4>H3 clearly showed partially defective silencing in the outer repeat region of cen1, whereas cells with H3>H4 showed no alteration of silencing in this region, Fig. 7-2B. Since the H3 like protein Cnp1/CenpA is known to associate specifically with the central domain it is possible that cells with H3>H4 would display defects in central core silencing. Plating assays were performed to assess central core silencing of strains with unbalanced histone H3:H4 ratios containing the crippled arg3* marker gene inserted in the central core region of cen1 (TM1::arg3*). As previously described in Chapter 5, wild type strains bearing this arg3* marker grow poorly on medium lacking arginine. The crippled arg3* gene lacks the TATA box making it more sensitive to the weaker form of silencing which occurs in the central core region. Strains expressing H3>H4 (h4.2Δ h3.1/h4.1Δ TM1::arg3*) and H4>H3 (h3.2Δ h3.1/h4.1Δ TM1::arg3*) bearing the TM1::arg3* marker were plated on medium lacking arginine, Fig. 7-3B. Since the strain h4.2Δ h3.1/h4.1Δ formed very few colonies even at 25°C (see Fig. 7-2A), approximately
Fig. 7-4. Cells expressing H3>H4 display chromosome segregation defects. Cells from a wild type strain and from the unbalanced H3>H4 and H4>H3 (not shown) were stained with α-tubulin (green) to follow mitotic progression and DAPI (red) to stain the DNA. The results for the unbalanced H3>H4 strain (h4.2Δ h3.1/h4.1Δ) are shown. The large majority of cells containing H3>H4 histones displayed chromosome segregation defects such as missegregation and lagging chromosomes (indicated by arrows). The histone H4>H3 behaved similarly to a wild type strain and showed normal chromosome segregation. 

H3>H4
h4.2Δ h3.1Δ/h4.1Δ

Green: tubulin  Red: DAPI  Merge

A

B

C

D

E
Fig. 7-5. Cnp1 staining is weakened in strains expressing more histone H3 than histone H4. Immunostaining was carried out on the unbalanced histone strains with anti-Cnp1 antibodies. Whereas the histone H4>H3 (h3.2Δ h3.1/h4.1Δ) showed normal Cnp1 localisation (top panel), the histone H3>H4 strain (h4.2Δ h3.1/h4.1Δ) showed very weak almost undetectable Cnp1 staining (bottom panel). Similar results were obtained with the H4>H3 (h3.2Δ h3.3/h4.3Δ) and the H3>H4 (h4.2Δ h3.3/h4.3Δ) strains (not shown).
three times more cells from this strain were used for the plating assay. The wild type TM1::arg3+ (FY3043), an arg3+ strain (FY1180) and an arg3 strain (FY1645) were plated for comparison. As expected, the wild type TM1::arg3+ grew poorly on medium lacking arginine, as did the strain expressing H4>H3. In contrast, the strain expressing H3>H4 grew well on medium lacking arginine. This indicates that the central core silencing is impaired in cells expressing more H3 than H4, but not in cells expressing more H4 than H3.

7.5. Increased histone H3 expression causes severe mitotic defects and weakened Cnp1 localisation.

The alleviation of central core silencing observed in the histone H3>H4 strain may interfere with centromere function and chromosome segregation. To test this, cells expressing H3>H4 (h4.2Δ h3.1/h4.1Δ) and H4>H3 (h3.2Δ h3.1/h4.1Δ) were fixed and stained with anti-α-tubulin, to reveal spindle length and with DAPI to stain chromosomes (Fig. 7-4). Strikingly, the cells with H3>H4 showed a high incidence of chromosome missegregation and chromosomes lagging on the spindle in late anaphase cells, whereas the H4>H3 cells (not shown) and the wild type control did not. This result was reproducible and defects were observed in the large majority of the H3>H4 (h4.2Δ h3.1/h4.1Δ) mitotic cells. Preliminary results also showed that the unbalanced histone H4>H3 strain harbouring the h3.3/h4.3Δ deletion (h3.2Δ h3.3/h4.3Δ) showed some lagging chromosomes and that the histone H3>H4 strain harbouring the h3.3/h4.3Δ deletion (h4.2Δ h3.3/h4.3Δ) was not as defective as the H3>H4 harbouring the h3.1/h4.1Δ deletion (h3.2Δ h3.1/h4.1Δ) strain (not shown).

The anti-tubulin immunostaining showed that mitotic chromosome segregation was impaired in the unbalanced H3>H4 strain (h4.2Δ h3.1/h4.1Δ). To check whether Cnp1 localisation was affected, both strains with H4>H3 histones (h3.2Δ h3.1/h4.1Δ and h3.2Δ h3.3/h4.3Δ) and with H3>H4 histones (h4.2Δ h3.1/h4.1Δ and h4.2Δ h3.3/h4.3Δ) were subject to immunostaining using anti-Cnp1 antibodies, Fig. 7-5 and 7-6. Surprisingly, whereas both histone H4>H3 strains showed wild type anti-Cnp1 staining, the H3>H4 strains showed very weak, almost undetectable staining indicating that the association of Cnp1 with centromeres is defective.
Fig. 7-6. Comparative Cnp1 staining in wild type and unbalanced H3:H4 cells. Cells from the unbalanced histone strains H3→H4 \((h4.2Δ h3.3/h4.3Δ::arg3^FY4292)\) and H4→H3 \((h3.2Δ h3.3/h4.3Δ::arg3^FY4300)\) were mixed in equal number with a wild type strain containing the Mis6-HA tagged protein \((FY2928)\). The samples were then fixed and subject to the immunostaining procedure. Cells were stained simultaneously with anti-Cnp1 and anti-HA \((12CA5)\) antibodies. While all cells from the mix containing H4→H3/Mis6-HA showed an anti-Cnp1 spot, only wild type cells, which contain the Mis6-HA spot, stained with anti-Cnp1 in the H3→H4/Mis6-HA mix. This confirms the result observed in Fig. 7-4. A) Green: anti-Cnp1. B) Red: Mis6-HA. C) Blue: DAPI. D) Merge.
Fig. 7-7. Increased dosage of *cnpl* complements the temperature sensitivity and the silencing defect of the unbalanced histone H3>H4 strain.

A multicopy plasmid (*LEU2* based) containing *cnpl* under its own promoter (pDB-cnp1) and the vector without any insert (pDB) were transformed into three strains containing the *arg3* gene inserted at TM1 (*TM1::arg3*): a wild type (FY3043), the histone H4>H3 (FY4318) and H3>H4 (FY4317).

A) Cells were plated on medium lacking leucine and incubated at 36°C. The presence of multicopy *cnpl* complemented the temperature-sensitivity of histone H3>H4 at 36°C, whereas the presence of the empty pDB vector did not. Wild type and H4>H3 not shown.

B) Cells were plated on medium lacking leucine and arginine, to assess silencing at the central core. Histone H3>H4 containing the empty pDB vector grew well on medium lacking arginine, but grew poorly when containing increased copies of *cnpl*. 
To be certain that Cnp1 staining itself was defective in these H3>H4 cells a direct comparison was performed by mixing wild type and H3>H4 cells (Fig. 7-6). Since Cnp1 staining co-localised with Mis6-HA in wild type cells (Fig. 6-4B), cultures from H3>H4 (h4.2Δ h3.3/h4.3Δ) and H4>H3 cells (h3.2Δ h3.3/h4.3Δ) were mixed in equal proportion with wild type cells containing the Mis6-HA tagged protein (FY2928). The mixed samples were then fixed and subject to the immunostaining procedure. Cells were stained simultaneously with anti-Cnp1 and anti-HA (12CA5) antibodies. The secondary antibodies employed were FITC anti sheep to label Cnp1 in green and Texas red anti-mouse to label Mis6-HA in red. This allowed wild type cells, displaying red Mis6-HA signal to be distinguished from the unbalanced histone mutants cells which lack the Mis6-HA protein. In Fig. 7-6 the top panel shows that all cells from the H4>H3/Mis6-HA sample exhibited Cnp1 staining. In contrast only cells containing a spot for Mis6-HA (i.e. wild type) showed a Cnp1 spot in the H3>H4/Mis6-HA sample. This confirms that cells expressing more histone H3 than H4 display impaired Cnp1 association with the centromere.

7.6. Multicopy cnpl complements the temperature sensitivity and the central core silencing defects in cells expressing H3>H4.

Cnp1 localisation is clearly disrupted in cells expressing H3>H4. It is possible that the excess of histone H3 competes with Cnp1 for deposition within the central core resulting in less Cnp1 association. If this is the case then increasing expression of Cnp1 in H3>H4 cells might correct this defect restoring normal viability and reimposing central core silencing on the H3>H4 strain. To test this, strains harbouring TM1::arg3' and unbalanced histone H3/H4 ratio were transformed, along with the wild type TM1::arg3', with a LEU2 based multicopy plasmid containing cnpl' under its own promoter (pDB-cnpl') and with the control vector (pDB). The pDB-cnpl' plasmid was isolated as a complementing plasmid of three sim2 alleles (temperature sensitive alleles of the cnpl gene) identified in a central core silencing screening (A. Pidoux, manuscript in preparation).

The unbalanced histone H3>H4 strain (h4.2Δ h3.1/h4.1Δ TM1::arg3') was temperature sensitive at 36°C and this phenotype was suppressed by the presence of multicopy cnpl', Fig. 7-7A. This H3>H4 strain also showed alleviation of silencing at the central core, as it grew on medium lacking arginine. Multicopy cnpl' clearly allowed the reimposition of central core silencing, since in its presence cells grew poorly on medium lacking arginine (Fig. 7-7B).
Fig. 7-8. Cnp1 is associated with the central core of the histone H3>H4 strains by ChIP. ChIP was carried out on the histone H3/H4 unbalanced strains and on a wild type strain with anti-Cnp1 antibodies. The experiment was carried out in duplicate. The total input DNA (T) and the immunoprecipitated material (IP) were analysed by multiplex PCR using three primer pairs designed to amplify the outer repeats of the centromere (otr-top band) the central core (cnt-middle band) and the fbp1 gene as a euchromatic control (bottom band). The cnt PCR product was enriched in all of the immunoprecipitated samples (IP) compared to the total input DNA samples (T), indicating that Cnp1 is associated with these sequences in all strains tested.
Multicopy \( cnp1^* \) did not affect central core silencing or growth of the histone H4>H3 and the wild type strains.

These data suggest that the defects observed in the strain expressing more histone H3 than H4 are caused by a defect in Cnp1 function, which is suppressed by increased expression of \( cnp1 \).

7.7. Cnp1 is still associated with the central core chromatin in cells expressing excess of histone H3.

Immunostaining experiments indicated that strains with H3>H4 showed significantly reduced Cnp1 at centromeres (section 5). However, since Cnp1 is an essential component of fission yeast centromeres [Takahashi et al., 2000], some Cnp1 must remain at centromeres otherwise H3>H4 cells would be unviable. To assess this, ChIP analysis was performed using anti-Cnp1 antibodies on extracts from cells with altered histone H3:H4 ratios: H3>H4 (\( h4.2\Delta \ h3.1/h4.1\Delta \)) and H4>H3 (\( h3.2\Delta \ h3.1/h4.1\Delta \)), Fig. 7-8. Immunoprecipitated DNA was analysed by multiplex PCR, using three primer pairs designed to amplify regions from the outer repeats of the centromere (top band) the central core (middle band) and the \( fbp1^* \) gene as an euchromatic control (bottom band). The PCR band corresponding to the central core was enriched in all of the immunoprecipitated samples. These analyses demonstrate that, although undetectable by immunostaining in cells expressing H3>H4 (Fig. 7-5), Cnp1 is still associated to some extent with the central core of centromeres in these cells. These ChIP data are mainly qualitative, it therefore remains possible that a reduced amount of Cnp1 is associated with the H3>H4 central core. This was next explored using quantitative ChIP.

7.8. Histone H3:H4 ratio modulates the amount of Cnp1 binding at the central domain.

In order to quantify the amount of Cnp1 associated with the central core, cells expressing unbalanced histone H3:H4 ratios carrying the \( cen1 \ ura4^* \) insertions [Allshire et al., 1995] were utilised. The unbalanced H3:H4 strains described so far carry a \( ura4^* \) marker gene used to replace the \( h3.2 \) and \( h4.2 \) genes. To utilise the \( cen1-ura4^* \) insertions in the unbalanced histone backgrounds it was necessary to swap the \( ura4^* \) gene used for the single histone gene replacements with a different marker, the \( LEU2 \) gene. This was achieved by transforming strains \( h3.2\Delta::ura4^* \) and \( h4.2\Delta::ura4^* \) with a DNA fragment containing the \( LEU2 \) gene flanked by
Fig. 7-9. Quantitative ChIP analysis in strains expressing unbalanced histone H3:H4 ratios. ChIP using anti-Cnp1 were carried out on four *ura4*^+^ *cen1* insertion strains in the unbalanced histone background. The enrichment of the *ura4* band in the immunoprecipitated DNA (IP) versus the total (T) from the *cen1* insertions was normalised versus the enrichment of the *ura4* inserted at a random euchromatic location from the same histone background. The quantification was performed using the EagleEye (Promega) quantification software. The values obtained revealed that in the histone H4>H3 backgrounds more Cnp1 appears associated with TM1::*ura4*^+^, TM1::*bigura4*^+^ and Imr::*ura4*^+^ whereas in the histone H3>H4 background the amount of Cnp1 at these sites appears decreased.
sequences of the *ura4* gene obtained by PCR. Cells were selected on medium containing 5-FOA and lacking leucine, to select for *ura4* LEU2 transformants. Transformants were then checked by PCR and crossed to strains harbouring the disrupted histone pairs: h3.1/h4.1Δ::his3* and h3.3/h4.3Δ::arg3*. Progeny were selected on medium lacking both leucine and either histidine (to obtain h4.2Δ::LEU2 h3.1/h4.1Δ::his3* and h3.2Δ::LEU2 h3.1/h4.1Δ::his3*) or arginine (to obtain h4.2Δ::LEU2 h3.3/h4.3Δ::arg3* and h3.2Δ::LEU2 h3.3/h4.3Δ::arg3*). Strains harbouring the disruption of h3.2 or h4.2 together with the disruption h3.1/h4.1Δ::his3* were then crossed with the cen1 *ura4* insertion strains.

The sites tested for association of Cnp1 with the *ura4* insertions in the unbalanced histone H3/H4 background were TM1::ura4*, TM1::bigura4*, the imr1 insertion between the two tRNA genes (insertion 3 Fig. 6-4A) and the random insertion of *ura4* at an expressed non-centromeric site as a control. All strains were checked by PCR for the presence of the *ura4*-D/SE minigene control inserted at the endogenous *ura4* locus.

ChIP analyses were carried out using anti-Cnp1 antibodies on strains with the four different *ura4* insertions in wild type, H4>H3, and H3>H4 backgrounds. Results are shown in Fig. 7-9. The relative amount of *ura4*:*ura4*-D/SE PCR products was estimated using the quantification software of Eagleeye (Stratagene). Ratios were normalised with the random *ura4* insertion from the same genetic background.

In immunoprecipitates from wild type cells the TM1::ura4* insertion was strongly enriched (50X) relative to the *ura4*-D/SE minigene control. Interestingly, this enrichment was increased in cells expressing H4>H3 (63X versus 50X), but it was decreased in cells expressing H3>H4 (40X versus 50X). This suggests that more Cnp1 is associated with the central core in the histone H4>H3 background and less in the histone H3>H4.

A similar profile was observed in cells bearing the TM1::bigura4* insertion; in these cells there appears to be less enrichment of *ura4* and thus less Cnp1 associated in the wild type background compared to the smaller TM1::ura4* insertion (16X versus 50X). This is probably due to partially inefficient spreading of Cnp1 over this longer non-centromeric fragment as discussed in Chapter 6. In cells containing H4>H3 it appeared that the amount of Cnp1 associated with the *ura4* insertion was increased (24X versus 16X) whereas in the H3>H4 background it decreased (6 fold versus 16 fold).
Fig. 7-10. Silencing of *ura4* ^+^ cen1 insertion in the unbalanced histone background.

Cells from strains bearing *ura4* ^+^ inserted at a random euchromatic location, the central core (*TM1::ura4* ^+^), *TM1::bigura4* ^+^, the insertion between the two tRNA genes in the inner repeats (*imr::ura4* ^+^) and the outer repeats (*otr::ura4* ^+^) were plated onto medium containing 5-FOA, that selects for *ura4* ^+^ cells, and on medium lacking uracil.

Histone H3>H4 appeared to have a repressive effect on the random *ura4* ^+^ integration as these cells grew well on medium containing 5-FOA. Histone H3>H4 alleviated *ura4* ^+^ silencing at *TM1* (as it did for *TM1::arg3* ^+^, Fig. 7-3B) and it also slightly alleviated silencing at *imr*, while it had no effect on silencing at the outer repeats.

Only histone H4>H3 had a mild effect on silencing at the outer repeats, this is consistent with the fact that this strain background also caused alleviation of *ade6* ^+^ at *otr* (Fig. 7-2B).
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Cells with \textit{ura4}\textsuperscript{*} inserted at \textit{imr iL} gave a similar pattern of results. The amount of Cnp1 associated with \textit{ura4}\textsuperscript{*} in this region was lower than the amount present at \textit{TM1} (11X versus 50X). However, again Cnp1 association was slightly increased in cells expressing H4>H3 (13X versus 11X) and slightly decreased in cells expressing H3>H4 (6X versus 11X).

These quantitative analyses need to be confirmed utilising radioactive PCR in duplicate experiments before firm conclusions can be made, nevertheless they suggest that the ratio between histone H3 and H4 is able to modulate the amount of Cnp1 associated with the central domain.


Silencing of centromeric \textit{ura4}\textsuperscript{*} markers in cells with unbalanced histone H3:H4 dosage was analysed (Fig. 7-10).

For this purpose cells were plated on medium containing 5-FOA to select against cells expressing \textit{ura4}\textsuperscript{*} and on medium lacking uracil to select for expressing cells.

Surprisingly, the random integration of \textit{ura4}\textsuperscript{*} appeared more repressed in cells expressing H3>H4, as it grew better than the corresponding wild type on medium containing 5 -FOA. This suggests that increased dosage of histone H3 relative to H4 could have a general repressive effect on gene expression. However, consistent with results described earlier (Fig. 7-3) repression of centromeric \textit{TM1::ura4}\textsuperscript{*} was alleviated in cells containing H3>H4, since growth was very sensitive to 5-FOA.

Analyses described in Chapter 6 (Fig. 6-7C) showed that silencing of \textit{TM1::bigura4}\textsuperscript{*} is slightly defective relative to \textit{TM1::ura4}\textsuperscript{*}. However, contrary to expectation, expressing H3>H4 did not cause further alleviation of \textit{TM1::bigura4}\textsuperscript{*} silencing.

Expression of more histone H3 than H4 slightly affected silencing of \textit{imrlL::ura4}\textsuperscript{*} (insertion between the two tRNA genes) as cells showed a slightly increased sensitivity to 5-FOA than the wild type.

Finally \textit{otr::ura4}\textsuperscript{*} silencing was only mildly affected in the H4>H3 background. This is consistent with the observation that \textit{otr::ade6}\textsuperscript{*} was slightly alleviated in this background (Fig. 7-2B).
Fig. 7-11. Chromatin structure of the central core region of cells with unbalanced H3:H4.
MNase digestions (see Chapter 2) were carried out using serially more concentrated enzyme (0, 25, 50, 100, 150, 200, 300 and 500 units/ml indicated as 1-8) on cells from strains h3.2A h3.1/h4.1A (H4>H3 FY 5186); h4.2A h3.1/h4.1A (H3>H4 FY5185) and wild type (FY4837). All these strains also contain the TM1::ura4$^+$ insertion.
A) Ethidium bromide stained agarose gel showing the characteristic ladder pattern of the digested genomic DNA.
B) Southern blot using a TM1 probe reveals the unusual chromatin structure of the central core region [Polizzi and Clarke; 1991] which did not appear altered by unbalanced H3:H4 ratios.

The central domain of fission yeast centromeres is characterised by a unique chromatin structure [Takahashi et al., 1992] that can be monitored by micrococcal nuclease (MNase) digestion. Whereas the outer repeats display a regular digestion pattern, resulting in a normal nucleosomal ladder, the central domain digestion pattern results in a smeared signal. It is not known what the nature of this structure is, but there are several possibilities:

a) central domain nucleosomes could be irregularly spaced;

b) nucleosomes in this region could have increased mobility;

c) the central domain linker DNA could be less accessible to MNase digestion because of the number of proteins associated with this region;

d) the nucleosomes in this region differ from all the rest of the chromatin due to replacement of histone H3 with Cnp1 resulting in differential digestion pattern.

This peculiar chromatin organisation is lost in cells lacking Mis6 [Saitoh et al. 1997], Mis12 [Goshima et al., 1999] and Cnp1 [Takahashi et al., 2000]. The central domain chromatin structure in the unbalanced histone H3/H4 strains was analysed. MNase digestion (see Chapter 2) was performed on a wild type strain and on cells expressing H3>H4 and H4>H3 containing the \( TM1::ura4^+ \) insertion (Fig. 7-11). The digested chromatin was analysed by Southern blot, probing with a fragment of TM1. The digestion pattern of cells with altered H3:H4 ratios was very similar to that of the wild type control. Thus even though increased H3 dose relative to H4 affects centromere silencing and leads to less Cnp1 association with this region this “unusual chromatin” does not appear to be affected. All MNase experiments have been performed at 25°C. At this temperature Cnp1 is undetectable by immunostaining and shows reduced centromeric association by ChIP. However, perhaps growth at higher temperature would lead to a greater defect detectable by Mnase digestion, since H3>H4 expressing cells are temperature sensitive (Fig. 7-2). It is also possible that the difference in Mnase digestion at 25°C is very subtle, perhaps probing with \( ura4^+ \) could give a more pronounced result.

DISCUSSION

Fission yeast centromeric DNA is packaged in nucleosomes. The central domain region of the centromere is packaged in specialised nucleosomes containing Cnp1 histone H3-like protein...
Instead of normal histone H3. The fission yeast haploid genome contains an equal number of histone H3 and H4 genes (3:3). It is likely that the wild type ratio between histone H3 and histone H4 genes is important for a number of cellular processes. For example, altered H3:H4 ratios could affect chromatin assembly or transcription of a number of genes leading to pleiotropic phenotypes. It is also possible that unpaired histone gene ratios may interfere with the proper assembly of centromeric chromatin.

To investigate the role of impaired histone H3/H4 ratios in fission yeast centromere function, strains harbouring both histones H4>H3 and H3>H4 genes were constructed. Whereas a 2:2 ratio did not affect viability or centromeric function, see Chapter 3, the 2:1 ratio showed severe defects, especially in the case of cells expressing H3>H4. A change of just one histone H3 or H4 gene copy was sufficient to cause a change in histone proteins ratio detectable by Coomassie staining (Fig. 7-3A).

Both types of unbalanced histone strains (H3>H4 and H4>H3) showed impaired growth to different degrees. One of the two histone H3>H4 strains, h4.2Δ h3.1/h4.1Δ, was more defective than the other. For some undetermined reason cells lacking h4.2 are more sensitive to deletion of the h3.1/h4.1 than the h3.3/h4.3 gene set. This sensitivity may reflect different levels of transcription between these two gene sets. In Chapter 4 it was demonstrated that mutations in the histone H3.2 N-terminal tail require the h3.3/h4.3 gene set for viability. It is possible that the h3.3/h4.3 pair is more transcribed than the h3.1/h4.1 because viability was not restored by the presence of the h3.1/h4.1 gene set. Perhaps in the H3:H4 unbalanced strains the presence of the h3.3/h4.3 gene set in a h4.2Δ background causes more severe defects because h3.3 is transcribed to a higher degree.

Both strains containing excess of histone H3 (H3>H4) showed defects associated with centromere function. In this background, silencing at the central core is alleviated suggesting that excess of histone H3 is interfering with the correct assembly of this chromatin. Interestingly cells expressing more histone H4 than H3 showed slightly impaired silencing over the outer repeat regions of centromeres. It is possible that this is related to the assembly of outer repeats chromatin containing methylated histone H3 which is required for Swi6 mediated silencing [Bannister et al, 2000; Nakayama et al, 2001]. Increased H4 may interfere with the Swi6 silent chromatin which is dependent upon methylated histone H3.
The fact that cells expressing H3>H4 show severe chromosome segregation defects supports a role for H3:H4 ratio in normal centromere function. The finding that Cnp1 localisation is impaired in histone H3>H4 strains indicates that the alleviation of silencing and chromosome segregation defects were due to impaired Cnp1 function. This was confirmed by the fact that increased copies of the \textit{cnp1} gene can complement both the silencing defect and the temperature-sensitivity at 36°C of the histone H3>H4 strain.

ChIP analysis demonstrated that, although undetectable by immunostaining, Cnp1 was still associated to some extent with endogenous central core sequences (TMR) in a strain expressing H3>H4. However, a more quantitative ChIP analysis exploiting the \textit{cen1-ura4}\textsuperscript{*} insertions [Allshire at al., 1995] in the unbalanced histone background showed that the amount of Cnp1 associated with the \textit{ura4}\textsuperscript{*} insertions within the central domain of histone H3>H4 strain was consistently reduced (Fig. 7-9). Since Cnp1 was undetectable by immunostaining in cells containing H3>H4 a reasonable prediction would be that Cnp1 association measured by ChIP was significantly lower. However, it is also possible that excess of histone H3 relative to H4 could alter the kinetochore structure affecting the accessibility of Cnp1 to the anti-Cnp1 antibodies in immunostaining but not in ChIP analyses.

In the case of cells expressing H4>H3 the opposite observation was made, as more Cnp1 appeared associated with the central domain. These data suggest that the relative amount of histone H4 in the cell could play a role in modulating the amount of Cnp1 recruited at the centromere. The results could be explained as follows. The fission yeast genome is approximately 13.8Mb and the three central domains constitute an estimated 40Kb. Fission yeast nucleosomes occupy 147bp therefore the genome is packaged in approximately 94,000 nucleosomes. However, the three central domains are estimated to contain a maximum of 300 Cnp1 nucleosomes. Consequently, the majority of histone H4 protein is utilised by the cell to form normal H3-H4 nucleosomes that constitute 99.7% of total nucleosomes. Only 0.3% of all nucleosomes contain Cnp1-H4 and these associate with the central domain chromatin. If histone H4 levels within the cell are limiting for Cnp1 it is possible that when the amount of histone H4 is increased it facilitates the formation of Cnp1-H4 nucleosomes. Conversely, if the amount of histone H3 is increased the already limiting histone H4 available for Cnp1 will be
reduced. A lack of histone H4 for forming Cnp1 chromatin would decrease the amount of Cnp1 incorporated at centromeres and lead to impaired centromere function.

An alternative hypothesis is that excess of histone H3 could affect \textit{cnp1} transcription reducing the amount of Cnp1 protein in the cell and its association with the central domain. The fact that excess of histone H3 caused repression of transcription of the \textit{ura4} gene at a random euchromatic location (Fig. 7-10) makes this a distinct possibility, which is difficult to test since the anti-Cnp1 serum does not work in Western blot analysis. An epitope tagged version of Cnp1 under its own promoter (6XMyo epitopes fused with the N-terminus) has been constructed and attempts to integrate it into the genome to replace the endogenous Cnp1 are in progress.

Clearly, the amount of Cnp1 associated with the central domain is important for centromere function, as reduction of Cnp1 at the central domain by H3>H4 caused impaired silencing and high rates of chromosome missegregation. Cnp1 could be responsible for recruiting other centromere and kinetochore components. For example it is known that mouse Cenpa is required for Cenpc localisation [Howman et al., 2000]. It would be interesting to test whether the fission yeast Cenpc homologue, Cnp3 (A. Pidoux, unpublished data) is delocalised in the H3>H4 background.

Reduced association of Cnp1 with the central domain could partially compromise its normal function thus impairing the recruitment of other kinetochore components such as those responsible for attachment to the mitotic spindle. The precise effect caused by having reduced or increased amount of Cnp1 associated to the regions tested is not clear. One possibility could be that less or more Cnp1 nucleosomes are deposited leaving more or less naked DNA throughout the region. It is not known whether the number of Cnp1 nucleosomes increases or decreases by an equivalent factor across the entire central domain or if there is a gradient of Cnp1 nucleosomes across the central domain. To test these possibilities anti-Cnp1 ChiP of all of the \textit{ura4} insertions across the centromere could be carried out in strains expressing unbalanced histone H3:H4 ratios.

If less Cnp1 is recruited to the centromere, is histone H3 incorporated in its place? Such a possibility would imply that a hypothetical centromere-specific chromatin assembly factor could recognise and assemble H3-H4 nucleosomes at centromeres in place of Cnp1-H4. It can be
speculated that such a central process, such as Cnp1 assembly at the centromere, would not rely only on proper histone H3:H4 ratio and that the specificity of a centromere-specific assembly factor must be exclusive for Cnp1. Preliminary ChIP experiments using the anti-H3C antibodies suggest that histone H3 is not incorporated in the central domain chromatin in cells expressing H3>H4. The data presented suggest that a finely regulated histone H3 versus H4 ratio is central to allow proper Cnp1 association with the centromere and this could be relevant for higher organisms.
CONCLUSIONS AND FUTURE PERSPECTIVES

The role of histone H3 and H4 dosage, N-termini and the role of two histone variants, Pht1 and Cnp1, have been studied with respect to centromere function.

Role of histone gene dosage in centromere function.
Histone genes are redundant in the genome of a number of organisms [Hentschel and Birnstiel, 1981; Old and Woodland, 1984]. The reason for this reiteration of histone genes is not well understood. The fission yeast haploid genome contains three gene loci each encoding a histone H3 and a histone H4 gene [Matsumoto et al., 1985]. Is this normal dosage important for viability? This question was addressed in Chapter 3, where fission yeast strains have been generated which contained reduced numbers of histone gene sets. The results presented showed that, although cells could survive with only one set of H3-H4 genes, each set appeared to affect viability to a different degree. In addition, some combination of gene set deletions caused differential effects on gene silencing. These data altogether indicated that, although reiterated and almost identical in amino acid sequence, histone H3 and H4 transcribed from different gene sets contributed differently to cell viability and to the formation of chromatin at silent sites. One explanation for this phenomenon is that the three gene sets could be expressed at different levels in the cell and that different deletions caused complex feed back regulation.

The generation of one strain containing a single gene set which behaved almost like a wild type provided a genetic background in which to perform histone H3 and H4 site-directed mutations.

Histone modifications and their role in mediating centromeric silencing.
Histone H3 and H4 N-termini undergo a number of post-translational modifications [Jenuwein and Allis, 2001]. It has been known for a long time that heterochromatin domains, including centromeres, are characterised by an underacetylation of histone N-termini. Alterations of this state by treatment with histone deacetylase inhibitors disrupt heterochromatin function [Ekwail et al., 1997; Taddei et al., 2001]. More recently, it has become clear that the underacetylated state contributes to the introduction of another important mark characteristic of heterochromatin histone H3 methylation of K9 by the Suvar3-9 HMTase [Rea et al., 2000; Nakayama et al., 2001].
CONCLUSIONS AND FUTURE PERSPECTIVES

2001]. This modification creates a binding site for HP1 [Bannister et al., 2001; Lachner et al., 2001]. Fission yeast heterochromatin displays features and protein components very similar to those of higher eukaryotes and yet is a genetically tractable organism. In such a system it was possible to address the role of conserved lysines by altering them into residues mimicking an acetylated state. The results collected indicate that whereas histone H4 N-terminus plays a minor role in centromeric outer repeats silencing, histone H3 plays an essential role. Mutations of histone H3 K9 to A or R were found to be lethal and caused alleviation of outer repeats silencing even in the presence of a wild type histone H3. This dominant effect was suppressed following the alteration of the neighbouring S10 to A, indicating that this residue also plays a role in silencing. One hypothesis is that S10 is hyperphosphorylated in the K9A or K9R mutants and this activates transcription of the marker gene placed in the outer repeats. As this transcriptional activation could be triggered via acetylation, it would be interesting to perform more site-directed mutations of the remaining conserved lysines of histone H3 N-terminus (K4 and K14) to understand which of them is acetylated during this process. The lethality of the H3 mutations was unexpected, as cells lacking the HMTase activity (clr4Δ) are viable [P. Lord, PhD thesis, 1998]. In addition, S. cerevisiae mutants have been constructed which lacked the entire histone H3 or H4 tail. Lethality occurred only when both deletions were combined, suggesting a redundant role for these N-tails [reviewed by Verreault, 2000]. It is possible that in fission yeast the histone H3 tail plays an essential role, not necessarily related to centromere function, but perhaps related to transcriptional regulation. There are ways to uncover the terminal phenotype of the H3 N-tail mutants generated, which will be worth exploring in the future.

Possible involvement of Pht1 in centromere function.

Pht1, the fission yeast homologue of the H2AZ/F family of histone H2A variants, could play a role in centromere function. This possibility is supported by the fact that cells lacking Pht1, although displaying normal outer repeats silencing, showed variegated central core silencing and chromosome segregation defects in mitosis. Unfortunately, the generation of a polyclonal antibody directed against the N-terminus was not successful, thus in the course of this project it was not possible to address the localisation of Pht1. Protein tagging would be worth performing to further study the role of this protein.
Fission yeast CENP-A/Cnp1 function at kinetochores.

The CENP-A homologue of fission yeast, Cnp1, is an essential component of centromeres [Takahashi et al., 2000]. It is associated with the entire central domain of the centromere, being more concentrated in the central core. In contrast, histone H3 appears to be largely underrepresented in this chromatin.

As CENP-A can associate with neocentromeres, the ability of Cnp1 to be incorporated into a large fragment of non-centromeric DNA was tested. The results presented indicate that, as seen in mammals, fission yeast CENP-A/Cnp1 can be assembled in non-centromeric DNA. The amount of Cnp1 associated with this non-centromeric fragment was reduced on increasing the size of the inserted DNA, and possible explanations for this have been proposed. Perhaps, the cell needs to "adapt" to an increased centromere size by coating it with Cnp1, but this is an inefficient process. An interesting experiment would be to test if overexpression of Cnp1 can improve its spreading. This fragment of non-centromeric DNA was inserted within a normal centromere. It is still not known whether, for example, Cnp1 could be incorporated ectopically in a non-centromeric region of the genome and whether this would be sufficient to trigger kinetochore assembly.

Altering the ratio between histone H3 and H4 can perturb the proper packaging of the central domain of the centromere with Cnp1 nucleosomes. In particular, excess of histone H3 compared to histone H4 caused severe mitotic defects, alleviation of centromeric silencing and decreased Cnp1 loaded at centromeres. It is possible that the pool of histone H4 available to form Cnp1 nucleosomes is limiting in the cell and that excess of histone H3 titrates part of this pool from Cnp1. An interesting question that remains to be explored is what is the structure of this altered central domain chromatin containing less Cnp1? Does histone H3 get incorporated or are there simply less Cnp1 nucleosomes? Initial analysis with MNase digestion, suggested that this specialised chromatin is not different from that of a wild type. However, more detailed analyses, such further MNase experiments or ChIP, can be carried out to explore this chromatin further.
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