ABSTRACT

Histone acetylation plays an important role in the assembly and modulation of chromatin structure. Two main types of Histone Acetyltransferase (HAT), HAT-A and HAT-B, are responsible for the acetylation of histones. The HAT-A enzymes catalyze the acetylation of nucleosomal core histones and are located in the nucleus. The HAT-B enzymes are primarily cytoplasmic and specific for the acetylation of free histones. The only HAT-B identified to date is comprised of two subunits, Hat1p and Hat2p. Hat1p is the catalytic subunit and Hat2p is required for the high affinity binding of Hat1p to histone H4 and for full catalytic activity. While the Hat1p/Hat2p complex was originally isolated from cytoplasmic extracts, evidence suggested that it is present in the nucleus as well. To characterize the nuclear form of this enzyme, we tagged Hat1p and Hat2p with a protein A-TEV-calmodulin binding protein (CBP) tag. By using the tandem affinity purification (TAP) method combined with other chromatographic techniques, we were able to identify several proteins that associate with Hat1p and Hat2p in the yeast nucleus. These proteins include the uncharacterized open reading frame YLL022C (named Hif1p), histone H3, and histone H4. The functional significance of the association of Hif1p with the Hat1p/Hat2p complex is confirmed by the observation that hif1Δ and hat1Δ strains display similar defects in telomeric silencing and DNA double strand break repair. Functional analysis revealed that Hif1p is a novel histone chaperone that selectively
interacts with histones H3 and H4. Hif1p is also a chromatin assembly factor, promoting
the deposition of histones in the presence of a yeast cytosolic extract. In vivo, the nuclear
Hat1p/Hat2p/Hif1p complex is bound to acetylated histone H4, as well as histone H3.

The association of Hif1p with acetylated H4 requires Hat1p and Hat2p to provide a direct
link between type B histone acetyltransferases and chromatin assembly. In addition, the
histone H4 associated with the nuclear Hat1p/Hat2p/Hif1p complex contains novel post-
translational modifications in the core domain. One site of core domain acetylation,
lysine 91, lies at the interface between the H3/H4 tetramer and H2A/H2B dimers, and
might be critical for the chromatin assembly.
Dedicated to my husband Jianxin, my son Dylan, and my parents
I would like to express my deepest appreciation to my advisor, Dr. Mark Parthun, for his friendly and unfailing support, and for being open to new ideas and discussions. Two years ago, I was frustrated and had doubt about my ability to do research after having had a difficult time with projects in my former laboratory. It is Mark’s endless encouragement and trust that helped me re-establish my confidence and led to the accomplishments of this PhD. His kindness, patience, and thoughtfulness have made all this time a wonderful learning experience. Mark’s keen insight and thoughtful reflection on science are abilities to which I aspire.

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CHAPTER 1

INTRODUCTION

The DNA of eukaryotes is known to be packaged into a nucleoprotein complex called chromatin. The basic unit of chromatin is nucleosome, in which 146 bp of DNA are wrapped around a protein core of histones H2A, H2B, H3, and H4. Nucleosomes are assembled into periodic arrays along eukaryotic DNA with a repeat length of 180-200 bp. Nucleosomes are further coiled to form a 30 nm chromatin fiber with the help of linker histone H1. Next, the 30 nm chromatin fibers form looped domains, which are attached to a scaffold of non-histone proteins. Finally, the chromatin folds into the maximally compacted chromosome seen at metaphase (Wolffe, 1998b).

Chromatin assembly refers to the process in which histone H3/H4 tetramers and H2A/H2B dimers are deposited sequentially onto newly synthesized DNA to form periodic arrays of nucleosomes (Haushalter and Kadonaga, 2003; Tyler, 2002). An extended definition includes chromatin condensation and the formation of higher-order structures, and will not be addressed here. Chromatin assembly is necessary for the replication of eukaryotic chromosomes (Haushalter and Kadonaga, 2003). Factors involved in the chromatin assembly, or more strictly, nucleosome assembly, include
histone chaperones (most of which are also known as chromatin assembly factors) and ATP-dependent chromatin assembly factors (Haushalter and Kadonaga, 2003; Tyler, 2002).

Nucleosome assembly occurs in either DNA replication-dependent or –independent manners (Haushalter and Kadonaga, 2003; Tyler, 2002). DNA replication-dependent nucleosome assembly happens immediately after DNA replication or DNA repair during the cell cycle. Parental histones are randomly distributed to two daughter DNA strands at the replication fork, and the newly synthesized histones are deposited onto the remaining replicated DNA strands (Haushalter and Kadonaga, 2003; Krude and Keller, 2001; Mello and Almouzni, 2001; Tsurimoto, 1999; Tyler, 2002). In contrast, DNA replication-independent nucleosome assembly occurs in differentiated cells that do not replicate (Ahmad and Henikoff, 2002; Ray-Gallet et al., 2002; Wolff and Hansen, 2001). Histone variants are thought to be involved in replication-independent nucleosome assembly pathway (Ahmad and Henikoff, 2002; Tagami et al., 2004). These histone variants, such as H2A.Z and H3.3, are synthesized outside of S phase and can be deposited onto DNA throughout the cell cycle (Haushalter and Kadonaga, 2003). DNA replication-independent nucleosome assembly has been proposed to provide a potential mechanism for histone turnover and therefore the switch of epigenetic states (i.e. the modification marks on histones) (Bannister et al., 2002; Haushalter and Kadonaga, 2003; Tagami et al., 2004)

In this chapter, histone acetylation related to nucleosome assembly (i.e. acetylation of nascent histones) will be discussed first; then the identification and
functions of histone chaperones and ATP-dependent factors will be reviewed; last, the
current working model of the nucleosome assembly *in vivo* will be introduced.

1.1 Nucleosome Assembly Related Histone Acetylation

The core histone proteins are highly conserved throughout eukaryotes. NH$_2$-teminal tails of histones are flexible and protrude outward from the nucleosome, while the central domain forms the core structure of the nucleosome (Annunziato and Hansen, 2000). Histones undergo a variety of post-translational modifications. Histone modifications include acetylation of lysine residues, methylation of lysine and arginine residues, phosphorylation of serine and threonine residues, and ubiquitination and sumolation of lysine residues (Annunziato and Hansen, 2000; Jenuwein and Allis, 2001; Strahl and Allis, 2000). Most of the modifications occur at the NH$_2$-termilal tail domains of histones and have been shown to play critical roles in chromosomal processes including gene regulation, chromosome condensation, recombination, and replication (Jenuwein and Allis, 2001; Strahl and Allis, 2000).

Histone acetylation is a reversible modification that takes place primarily on lysine residues located in the NH$_2$ terminal tail domains of each core histone, in which the acetyl group is added to the ε-NH$_2$ group of the side chain of the lysine residue, thus neutralizing the positive charge of the lysine (Holde, 1989; Wolffe, 1998a).

Newly synthesized histones H3 and H4 are found to be acetylated (Annunziato and Hansen, 2000). The acetylation pattern on the newly synthesized H4 is highly conserved from *Tetrahymena*, *Drosophila* to mammals, in which only K5 and K12 are acetylated (Sobel et al., 1995). However, newly synthesized H3 exhibits a more variable
acetylation pattern. There are five lysine residues on the NH₂-terminal tail of H3 that can be acetylated. In *Saccharomyces cerevisiae*, H3 K9, K14, K23, and K27 are acetylated with K9 and K23 predominating (Kuo et al., 1996). In *Drosophila*, K14 and K23 are primarily acetylated, while in *Tetrahymena*, K9 and K14 are the favorite sites (Sobel et al., 1995). In Hela cells, K14, K18, and/or K23 are preferentially acetylated (Sobel et al., 1995). The reasons behind this variability are still not clear. No special patterns of transient acetylation of newly synthesized H2A and H2B have been detected (Annunziato and Hansen, 2000).

Acetylation is catalyzed by histone acetyltransferases (HAT). There are two categories of HATs: type A and type B (Carrozza et al., 2003; Kurdistani and Grunstein, 2003; Roth et al., 2001). Both localization and histone substrate differ between these two types of HATs. Type A HATs are located in the nucleus and acetylate histones in the context of chromatin. Type A HATs are better characterized and are believed to be related to the regulation of gene transcription; On the other hand, type B HATs are primarily located in the cytoplasm, although evidence shows they also exist in the nucleus. Type B HATs are defined as specifically acetylating free histones which suggests that they modify nascent histones (Brownell and Allis, 1996).

Hat1p is the only type B HAT that has been identified to date. Hat1p was originally isolated from the budding yeast *Saccharomyces cerevisiae* (Parthun et al., 1996). The specific substrates for Hat1p are lysine residues 5 and 12 of free histone H4 (Kleff et al., 1995; Parthun et al., 1996). It has been noticed that native yeast Hat1p only acetylates H4 K12, which might reflect the *in vivo* situation (Parthun et al., 1996). Interestingly, recombinant Hat1p expressed in *E. coli* acetylates not only K5 and K12 of
H4, but also H2A with less activity (Parthun et al., 1996). Both SDS-PAGE and “in-gel”
acetyltransferase assay confirmed that yeast Hat1p is a 42 kDa protein (Parthun et al.,
1996). When purified from yeast cytoplasmic extracts, Hat1p is found in a complex with
Hat2p, a yeast homolog of the Rbap46/48 proteins which bind to the retinoblastoma
protein Rb (Parthun et al., 1996; Qian and Lee, 1995; Qian et al., 1993). The molecular
weight of yeast Hat2p is ~50 kDa. Hat2p is essential for high level Hat1p catalytic
activity. Incorporation of Hat2p increases Hat1p activity 10-fold. It has been
demonstrated that Hat2p binds directly to helix 1 of histone H4, and therefore mediates
the high-affinity binding of Hat1p to H4 (Ma et al., 1998; Verreault et al., 1998).
However, the stable H4 tail binding requires Hat1p/Hat2p complex in vitro (Parthun et
al., 1996). Homologs of the Hat1p/Hat2p complex have been identified in variety of
organisms from human, calf, and maize to *Xenopus*. Hence, both the acetylation pattern
on newly synthesized histone H4 and the Hat1p/Hat2p complex are highly conserved
throughout eukaryotic evolution (Eberharter et al., 1996; Imhof and Wolffe, 1999a;
Verreault et al., 1998).

Structural analysis of the Hat1p/acetyl-CoA complex helped interpret the
specificity of the acetylation pattern from the structural point of view. In this 2.3 Å
structure, Hat1p shows an elongated curved shape, and the acetyl CoA binds to a cleft on
the concave surface of the enzyme (Dutnall et al., 1998a; Dutnall et al., 1998b). An
enzyme-substrate interaction model has been proposed based on the crystal structure. In
the model, H4 K8-K16 binds to a channel capable of lodging 6-7 amino acids. The
energy favorable alignment was arranged in the way that K12 is adjacent to the carbonyl
group of acetyl CoA, thus K8 and K16 lie in two acidic regions opposite each other at the
end of the channel. The alignment with K5, which lies in proximity to acetyl CoA, is acceptable, but less energy favorable; whereas fitting K8 or K16 adjacent to acetyl CoA produces a steric conflict with the channel (Dutnall et al., 1998a; Dutnall et al., 1998b). Therefore, Hat1p preferentially acetylates K12, takes K5 as a less favorable substrate, but does not modify K8 and K16 at all.

Although the Hat1p/Hat2p complex was originally identified as a cytoplasmic protein complex, it is not restricted to the cytoplasm. Yeast Hat1p/Hat2p complex has been reported in both cytoplasmic and nuclear extracts (Parthun et al., 1996). Evidence from *Tetrahymena* and maize embryos also indicated that their HAT-B may shuttle between cytoplasm and nucleus (Lusser et al., 1999; Richman et al., 1988). However, in human cells, FLAG-tagged Hat1p appears to be primarily nuclear as detected by immunofluorescence (Verreault et al., 1998). In *Xenopus*, cellular fractionation experiments showed that the Hat1p/Hat2p complex can be either cytoplasmic or nuclear depending on the developmental state of the animal (Eberharter et al., 1996). For example, the majority of Hat1p is localized in nuclei in *Xenopus* oocytes, whereas the localization of it changes to the cytoplasm during embryogenesis. Thus, cytoplasmic localization may not be a defining property of type B histone acetyltransferases. The argument for the “amphi” localization of the Hat1p/Hat2p complex is that it may leak to the cytoplasm when cells are disrupted. On the other hand, immunofluorescence experiments visualized a transiently expressed, epitope tagged Hat1p and it is not clear how these factors might have affected the localization of the protein. Less ambiguous and more direct evidence is needed to clarify the issue. A related question then would be where the acetylation actually occurs, in the cytoplasm or nucleus?
While both the Hat1p/Hat2p complex and the acetylation pattern are highly conserved throughout eukaryotic evolution, neither is essential for viability in yeast. Deletion of the *HAT1* and/or *HAT2* genes does not result in any significant phenotypes (Kleff et al., 1995; Parthun et al., 1996). Deletion of the NH$_2$ terminus of any of the core histones causes slow growth, but cells are viable (Kayne et al., 1988; Mann and Grunstein, 1992; Morgan et al., 1991; Schuster et al., 1986; Wallis et al., 1983); however, mutants that combine the deletion of the NH$_2$ termini of both H2A and H2B or H3 and H4 are inviable (Ling et al., 1996; Morgan et al., 1991; Schuster et al., 1986). The substitution of lysine with arginine mimics the constitutively unacetylated state. It seems that at least one of four lysine residues of H4 can be changed to arginine without affecting yeast cell growth lysine residue (Durrin et al., 1991; Megee et al., 1990). There is only little effect on cell growth when both H4 K5 and K12 are changed to arginine (Ma et al., 1998; Megee et al., 1990). Nevertheless, yeast cannot tolerate the mutations on both the H3 and H4 NH$_2$-termini. For example, deletion of the histone H3 NH$_2$-terminus in combination with alteration of H4 K5, K8 and K12 to arginine causes a loss of nucleosome assembly and cell death (Ma et al., 1998). These observations may be explained, at least in part, by structural/functional redundancy between the NH$_2$-terminal tails of histones H3 and H4. Recent findings that Hat1p and certain sets of mutations on histone H3 NH$_2$-terminal tail function redundantly on telomeric silencing and DNA double-strand break repair further support this point (Kelly et al., 2000; Qin and Parthun, 2002). In yeast, *hat1Δ* combined with the H3 K14R substitution causes loss of gene silencing at telomere, whereas either one of the mutations alone has only minor or no effect (Kelly et al., 2000). The effect is more significant when *hat1Δ* is combined with
H3 K9R/K14R or K14R/K18R double mutations. Acetylation of H4 K12 has been shown to be responsible for mediating the function of Hat1p in telomeric silencing as expected (Kelly et al., 2000). Furthermore, when pairing with H3 K9, 18, 27R, hat1Δ mutant shows the defects in the recombinational repair of DNA double strand breaks (Qin and Parthun, 2002). These phenotypes also suggest a deficiency in chromatin assembly. However, the exact function of the Hat1p/Hat2p complex in chromatin assembly has yet not been elucidated.

Newly synthesized histones must be transported into the nucleus for nucleosome assembly. A pathway mediated by Kap123p, a karyopherins/importins, has been demonstrated to be the primary nuclear import pathway of histones H3 and H4; Kap121p and two other Kaps (Kap119p and Kap108p) could also mediate the nuclear import of H3 and H4 (Mosammaparast et al., 2002b). Based on the observation that the cytosolic Hat1p/Hat2p complex is stably associated with histone H4, it has been proposed that the Hat1p/Hat2p complex is co-transported into the nucleus with nascent H3 and H4, or even is involved in the transport mechanism (Mosammaparast et al., 2002b). Apparently similar to the issue of the localization of Hat1p/Hat2p, this is another dilemma requiring more convincing evidences.

1.2 Histone Chaperones

Since the positively charged histones and negatively charged DNA have a tendency to form insoluble aggregates when mixed directly in vitro at physiological conditions, acidic proteins are required to bind free histones to shield their positive charges, so that a regulated and ordered nucleosome assembly can occur (Akey and
These acidic proteins are called histone chaperones. They bind specifically to certain types of histones, and accompany the bound histones to the sites of chromatin assembly. A common feature of histone chaperones is an E/D rich long acidic tract (Akey and Luger, 2003). Outside of the acidic tract, there is normally no sequence similarity/homology among chaperones. Different chaperones show different binding preference toward the histones. For example, some specifically bind H3/H4, while others have higher affinity to H2A/H2B. Several histone chaperones have been identified in the last two decades (Haushalter and Kadonaga, 2003; Loyola and Almouzni, 2004). Five major histone chaperones are discussed below.

1.2.1 Chromatin Assembly Factor 1 (CAF-1)

Chromatin assembly factor 1 (CAF-1) complex is the only histone chaperone identified so far that exclusively mediates DNA replication-dependent nucleosome assembly (Kaufman, 1996; Smith and Stillman, 1989; Stillman, 1986). It consists of three subunits, p150, p60, and p48. The CAF-1 complex has been found in human, Drosophila, Xenopus, as well as yeast (Loyola and Almouzni, 2004; Ridgway and Almouzni, 2000). In budding yeast, these three subunits correspond to Cac1p, Cac2p, and Cac3p (Ridgway and Almouzni, 2000). CAF-1 is primarily nuclear, but p60 and p48 are also rich in the cytoplasm. CAF-1 associates with acetylated histone H3/H4 in the nucleus, and can be extracted with H3/H4 in a complex called CAC (chromatin assembly complex) (Annunziato and Hansen, 2000).

CAF-1 dependent nucleosome assembly is medicated by PCNA, proliferating cell nuclear antigen (Shibahara and Stillman, 1999). PCNA is a DNA polymerase sliding
clamp protein. It is loaded onto the DNA polymerase recognition site by RFC (replication factor C) before DNA replication. After the DNA replication is finished, DNA polymerase dissociates from DNA while PCNA stays on the newly synthesized DNA and is removed later by RFC (Jonsson and Hubscher, 1997; Kelman and Hurwitz, 1998; Lee et al., 1991; Tsurimoto, 1999; Tsurimoto and Stillman, 1989; Tsurimoto and Stillman, 1991). The largest subunit of CAF-1, p150, has been shown to bind directly to PCNA and they co-localize at DNA replication sites (Shibahara and Stillman, 1999). The binding of PCNA to the replicated DNA has been suggested to provide an “imprint” for CAF-1 mediated nucleosome assembly. The removal of PCNA by RFC would eliminate the “imprint” thus preventing the subsequent chromatin assembly (Shibahara and Stillman, 1999).

It is clear that CAF-1 specifically binds to and carries acetylated (H3-H4)$_2$ tetramers to nucleosome assembly sites (Kaufman, 1996; Loyola and Almouzni, 2004). Examination of the acetylation pattern of CAF-1 associated histones using human 293 cells revealed that 60% of H3 in CAC are unacetylated, and the rest are monoacetylated; while in the case of H4, ~66% are either monoacetylated or diacetylated, 33% are unacetylated, and trace amount are triacetylated with acetylations on K5, K12 and K8 (Verreault et al., 1996). It is proposed that the acetylated H4 K8 and the relatively large percentage of unacetylated H3 might be the result of post-deposition events in which newly deposited histones undergo deacetylations and acetylations (Annunziato and Hansen, 2000).

The p150 and p60 subunits of CAF-1 directly contact each other in the complex. Co-immunoprecipitation experiments using anti-acetylated H4 antibody showed that
p150 is necessary for p60 to interact with H3/H4 (Kaufman et al., 1995). However, later evidence from GST-histone pull down assays showed that p60 is a histone binding protein as well (Shibahara et al., 2000). The p48 subunit is identical to Rbap48, a protein that has WD-repeats and binds to the retinoblastoma gene product Rb (Annunziato and Hansen, 2000). Rbap46/48 is homologous to Hat2p, a subunit of the type B HAT complex (as discussed in 1.1). Its homologues have also been found to be components of histone deacetylase and chromatin remodeling complexes. One of the roles of Rbap46/48 that have been verified is its H4 binding capacity (Vermaak et al., 1999; Verreault et al., 1998). Consistent with this, p48 of the CAF-1 complex is capable of binding H4 in the absence of p150 and p60. Notably, the NH2 terminal tails of H3 and H4 are not required for CAF-1 binding (Shibahara et al., 2000). Therefore, histone acetylation may not be necessary for histone deposition onto replicated DNA mediated by CAF-1. It has been suggested that histone acetylation may be required for marking the newly replicated chromatin after deposition, or be necessary for a CAF-1 independent pathway (Shibahara et al., 2000).

Deletion of \textit{CAC1}, \textit{CAC2}, or \textit{CAC3}, or all three simultaneously has no effect on yeast cell viability or nucleosome assembly, indicating the presence of other nucleosome assembly factors with redundant function (Kaufman et al., 1997). However, the yeast deletion strains show increased sensitivity to ultraviolet irradiation and defects on gene silencing at telomeres and the silent mating loci (Enomoto and Berman, 1998; Enomoto et al., 1997; Game and Kaufman, 1999; Kaufman et al., 1998; Kaufman et al., 1997; Monson et al., 1997). The role of CAF-1 in heterochromatin is conserved. In mouse, p150 of CAF-1 has been shown to bind directly to HP1 (heterochromatin protein 1), a
structural component of pericentric and telomeric heterochromatin (Murzina et al., 1999).
It appears that the nucleosome assembly function of CAF-1 is distinct from its functions
in heterochromatin maintenance as the HP1 binding sites of p150 are located at the NH₂-
terminal domain, which is dispensable for DNA replication-dependent nucleosome
assembly (Murzina et al., 1999). In addition, the specific acetylation pattern of newly
synthesized H4 (K5/K12) may not be as critical as was once thought in this context.

CAF-1 has been indicated to be involved in the nucleosome reassembly during
nucleotide excision repair (NER) after UV damage (Gaillard et al., 1996). There are four
steps involved in NER: recognition of lesion, excision of the damaged nucleotides and
flanking region, resynthesis of DNA to fill the gap, and ligation (Friedberg, 1995). CAF-
1 has been demonstrated to be required for the restoration of chromatin structure at the
final step (Green and Almouzni, 2003). As in its role during DNA replication, PCNA is
necessary to mediate the interaction between CAF-1 and DNA at repair sites through its
direct binding with p150 (Martini et al., 1998; Ridgway and Almouzni, 2000).
Furthermore, it has been shown that the recruitment of CAF-1 and PCNA definitely
requires NER. The existence of DNA damage itself is not enough for the recruitment
(Green and Almouzni, 2003). During DNA damage repair, chromatin structure needs to
be disrupted and rearranged both locally and globally (Wolffe, 1998a). The recruitment
of CAF-1 and PCNA to the damage sites has been determined to be directly linked to
local chromatin rearrangement by using a localized UV irradiation method (Green and
Almouzni, 2003). In addition, CAF-1 coupled nucleosome assembly has also been shown
to be triggered by DNA single-strand breaks or gaps (Ridgway and Almouzni, 2000).
However, CAF-1 is not responsible for the maintenance of chromatin structure during
1.2.2 Replication-Coupling-Assembly Factor (RCAF)

Replication-coupling-assembly factor (RCAF) comprises the anti-silencing function 1 (Asf1) and acetylated (H3-H4)$_2$ tetramers (Tyler et al., 1999). RCAF was first isolated from a crude Drosophila embryo extract (S190 extract) which was found to be able to promote DNA replication-dependent nucleosome assembly when supplemented to SV40 DNA-replication-chromatin-assembly reactions (Tyler et al., 1999). Interestingly, unlike acetylated H3/H4 in CAC, H3/H4 of RCAF show exactly the same acetylation pattern as nascent acetylated histones. In the case of Drosophila, H3 of RCAF is acetylated on K14, and H4 K5 and K12 are acetylated (Tyler et al., 1999).

Asf1, the major subunit of RCAF, was first identified as a factor that de-represses transcriptional silencing when over-expressed (Le et al., 1997; Singer et al., 1998). Phenotypes of the asf1Δ strain of budding yeast include: slow cell growth, silencing defects, and sensitivity to DNA-damage agents (Le et al., 1997; Singer et al., 1998; Tyler et al., 1999). However, the fission yeast homolog of Asf1 (CIA1) is essential (Umehara et al., 2002). It has been noticed that yeast asf1 and cac1 mutant show different sensitivities to DNA damage agents (Tyler et al., 1999). For example, asf1 mutants are sensitive to DNA damage induced by the mutagen methyl methane sulphonate (MMS) while cac1 mutants are not, meaning Asf1 (RCAF) but not CAF-1 is involved in the repair of MMS-induced DNA damage. On the other hand, both asf1 and cac1 mutants are sensitive to DNA damage induced by UV light.
UV light, but double mutants show greater sensitivity to UV than either of the single mutants (Tyler et al., 1999). Taken together, these suggest that Asf1 (RCAF) and CAF-1 function in both distinct and overlapping ways.

Asf1 has been demonstrated to be directly involved in the nucleosome assembly after DNA replication or during DNA repair in several biochemical studies (Krawitz et al., 2002; Mello et al., 2002; Tyler et al., 1999; Tyler et al., 2001). Notably, Asf1 alone is not able to promote nucleosome assembly in vitro. It functions synergistically with CAF-1 to assemble nucleosomes in a DNA replication-dependent manner (Tyler et al., 1999; Tyler et al., 2001). It has been shown that Asf1 binds directly to the p60/Cac2 subunit of CAF-1 and thus delivers histones H3/H4 to CAF-1 (Mello et al., 2002; Tyler et al., 2001). Asf1 has also been found to interact directly with the S phase damage checkpoint protein Rad53, thus to mediate nucleosome assembly after DNA damage (Emili et al., 2001; Hu et al., 2001). According to the proposed model, Rad53 binds Asf1 at normal conditions to prevent it from binding histones and assembling nucleosomes; after DNA damage, Rad53 is phosphorylated and releases Asf1, which then binds to histones and promotes nucleosome assembly (Emili et al., 2001).

Asf1 also interacts directly with Hir/HirA, another family of chromatin assembly factors that have been implicated in DNA replication independent nucleosome assembly pathway (Kaufman et al., 1998; Ray-Gallet et al., 2002; Sharp et al., 2001; Sutton et al., 2001; Tagami et al., 2004). The role of Asf1 here is believed to support the formation of silent chromatin and to regulate histone gene transcription and S phase progression. Asf1
has been found to co-purify with histone H3 deposition machineries that are involved in either DNA replication-dependent or –independent nucleosome assembly (Tagami et al., 2004).

In addition, Asf1 interacts with some other factors including a HAT complex SAS1 (Something About Silencing 1) (Meijsing and Ehrenhofer-Murray, 2001; Osada et al., 2001; Sutton et al., 2001), the chromatin remodeling factor Brama (Moshkin et al., 2002), and the basal transcription factor TFIID component TAF$_{II}$250 (Chimura et al., 2002; Sanders et al., 2002). Therefore, Asf1 also functions in other parts of DNA metabolism. It appears that these factors compete with histones for binding sites on Asf1 (Daganzo et al., 2003).

The crystal structure of Asf1 core region (Asf1N) has recently been solved at 1.5 Å resolution (Daganzo et al., 2003). The first 155 amino acids of yeast Asf1 are functional both \textit{in vitro} and \textit{in vivo} (Daganzo et al., 2003). A long acidic amino acids tract of Asf1 is located at the COOH terminal domain (156-279), and seems not to be critical for Asf1 function. The crystal structure shows that Asf1N folds into an extended β sandwich domain (ten β strands) with three α helices on the top. The domain adopts an immunoglobulin-fold structure (Daganzo et al., 2003). Three regions have been proposed to provide potential protein-protein interaction sites. The first region is a hydrophobic concave groove formed on the front of the molecule. Close to this hydrophobic groove is a highly conserved electronegative region, which might represent a histone interaction site. The third potential protein binding region lies opposite to the groove and forms a cleft. Aspartate 37 is located in the acidic region and has been shown to play an important role in mediating the binding between Asf1 and Hir1 protein (Daganzo et al., 2003).
Further investigation is necessary to determine whether other residues that are critical for the Hir1 binding belong to the groove domain or the acidic region.

Overall, Asf1 has been proposed to act as a histone donor that delivers histones to either the CAF-1 complex using a DNA replication-dependent pathway, or Hir/HirA proteins using a DNA replication-independent pathway. Asf1 might activate one assembly pathway over the other based on the variation in the histone pools in a cycling cell, thus is able to guarantee a constant supply of histones at nucleosome assembly sites, and also to provide a link with checkpoint control (Loyola and Almouzni, 2004).

1.2.3 Histone Regulation Proteins (Hir/HirA)

Originally identified as transcriptional repressors of histones H2A and H2B gene expression, $HIR$ genes are conserved throughout eukaryotes (Sherwood and Osley, 1991). Two major proteins are encoded by $HIR$ genes in yeast, Hir1p and Hir2p (Sherwood et al., 1993). Their homolog in higher eukaryotes is called HIRA (Lorain et al., 1996). Deletion of $HIR$ genes alone has no effect on gene silencing at telomeres or the silent mating type loci in yeast, but has a synergistic effect when combined with CAF-1 mutations (Qian et al., 1998; Sharp et al., 2002). Thus, Hir proteins are critical for a substitute silencing pathway when the CAF-1 mediated pathway does not function. $hir\Delta cac2\Delta$ double mutants also show accumulation of mis-segregated chromosomes during mitosis which reflects defects in kinetochore function (Sharp et al., 2002). Furthermore, mutants that have a combination of $hir\Delta cac2\Delta$ and $cse4\Delta$ (the centromeric histone variant gene) or $cbf3\Delta$ (the kinetochore gene) are lethal (Sharp et al., 2002).
Recombinant *Xenopus* HIRA has been shown to bind specifically to histones H3/H4 and deposit them onto DNA *in vitro* (Ray-Gallet et al., 2002). Further experiments using HIRA immuno-depleted high-speed *Xenopus* egg extract (HSE) revealed that HIRA is responsible for DNA replication-independent nucleosome assembly (Ray-Gallet et al., 2002). In these experiments, HIRA depleted HSE is not able to mediate nucleosome assembly that is independent of DNA replication, but keeps the nucleosome assembly ability in a DNA replication-dependent manner (Ray-Gallet et al., 2002). The role of HIRA in this aspect is further demonstrated by Almouzni and colleagues (Tagami et al., 2004). They showed that deposition machinery co-purifying with histone H3.3 specifically promotes DNA replication-independent nucleosome assembly. HIRA is the only component in the complex that different from the machinery co-purifying with H3.1 which mediates DNA replication-dependent nucleosome assembly (Tagami et al., 2004).

Hir proteins have also been indicated to facilitate nucleosome assembly in yeast (Sharp et al., 2001). FACT is a RNA polymerase II transcription elongation factor that mediates RNA polymerase II elongation on a chromatin template through binding and removing H2A/H2B dimers from nucleosomes (Orphanides et al., 1999). The yFACT complex is proposed to help passage of polymerase through nucleosomes by disrupting the nucleosome first and reassembling it afterwards. Mutations that affect the reassembly activity would accumulate abnormally disrupted chromatin (Formosa et al., 2002; Orphanides et al., 1999). Under this circumstance, Hir proteins are recruited to perform a nucleosome reassembly function as indicated by the inviability of *spt16ΔhirΔ* mutants (Formosa et al., 2002).
1.2.4 Nucleosome Assembly Protein 1 (NAP1)

Nucleosome assembly protein 1 (NAP1) was originally identified as an H2A/H2B histone chaperone that promotes nucleosome assembly in vitro in the presence of purified core histones (Ishimi et al., 1984). Nap1 is conserved from yeast to human (Loyola and Almouzni, 2004).

Deletion of NAP1 gene in yeast does not affect cell growth but has an effect on expression of about 10% of all genes genome-wide. Among these affected genes, 35% are clustered, indicating that Nap1 functions in large chromosomal domains instead of local sites (Kellogg and Murray, 1995; Ohkuni et al., 2003). In yeast and Xenopus, Nap1 has been found to interact with Clb2, a cyclin B family protein, to control mitotic events (Kellogg and Murray, 1995). In mouse, Nap1 has been indicated to be involved in the regulation of neural cell proliferation as nap1/2 knockout mice overproduced neural precursor cells and died at the mid-gestation stage (Ito et al., 1996; Rogner et al., 2000). Overall, these data suggest that Nap1 plays a role in cell cycle control.

One unique property of Nap1 is its shuttle function (Ito et al., 1996). Microscopic studies showed that Nap1 can shuttle histones from cytoplasm to nucleus as cells progress from G2 phase to S phase (Ito et al., 1996). This phenomenon has been observed in both Drosophila and human cells. Further evidence showed that Nap1 in fact participates in the nuclear import of H2A/H2B through direct binding with Kap114, a karyopherins/importin protein responsible for the nuclear import of H2A/H2B (Mosammaparast et al., 2002a).

Even though Nap1 preferentially binds to H2A/H2B in higher eukaryotes, it seems to specifically bind H3/H4 in yeast (McBryant et al., 2003). Therefore, the
H2A/H2B histone chaperone still needs to be identified in yeast. Furthermore, deletion of
*NAP1* in conjunction with the loss of *CAF-1* and *ASF1* in yeast does not add more growth
defects or transcriptional silencing defects than *cac1asf1* double mutants. This indicates
the existence of another unknown histone chaperone which very likely functions
redundantly with Nap1 (Tyler, 2002).

**1.2.5 Nucleoplasmin (Np) and N1/N2**

Nucleoplasmin (Np) and N1/N2 are the most abundant proteins in the nuclei of
*Xenopus* oocytes (Mills et al., 1980). They are both acidic proteins. Np specifically binds
to H2A/H2B, while N1/N2 is an H3/H4 chaperone (Akey and Luger, 2003). *Xenopus*
oocytes have large amounts of stored histones for packaging the chromatin in about 10
thousand somatic cells (Akey and Luger, 2003; Woodland and Adamson, 1977). Np and
N1/N2 therefore serve as a storehouse for these core histones in oocytes and allow the
progressive release of histones and nucleosome assembly right after fertilization. It is
possible that Np and N1/N2 transfer histones from the pool to other chaperones first (for
example, N1/N2 can transfer H3/H4 to CAF-1) instead of directly to DNA (Loyola and
Almouzni, 2004).

Homologues of *Xenopus* Np and N1/N2 have only been found in higher
eukaryotes in the animal lineage, but not in yeast, worms, or plants (Akey and Luger,
2003). They are mostly present in germinal cells and have more specialized functions
compared to other histone chaperones such as CAF-1 or Nap1 (Akey and Luger, 2003;
Loyola and Almouzni, 2004). Interestingly, somatic forms of NASP, the human N1/N2
homolog, have been shown to bind specifically to linker histone H1 instead of core
histones (Richardson et al., 2000). Furthermore, NASP is a component of the deposition machinery associated with both H3.1 and H3.3, indicating the human N1/N2 homolog may play different roles in nucleosome assembly than those of *Xenopus* (Tagami et al., 2004).

It has been observed that Np and N1/N2 are able to promote nucleosome assembly in the absence of each other, implicating different mechanisms from nucleosome assembly at replication forks, where H3/H4 tetramer and H2A/H2B dimers are considered to be deposited sequentially by different chaperones (Cotten and Chalkley, 1987; Laskey et al., 1978; Zucker and Worcel, 1990). The crystal structure of the NH2-terminal domain of Np (Np core) provides a model to interpret this (Dutta et al., 2001). Np core has been shown to be present as a pentamer in the crystal. Two Np core pentamers are found to be packed face to face to form a decamer that can accommodate five H2A/H2B dimers (Dutta et al., 2001). According to the proposed model, a Np decamer and five H2A/H2B dimers form a complex first, this complex then binds five H3/H4 tetramers to form five pre-assembled histone octamers that dock around the periphery of the decamer, and last, an octamer is directly transferred onto DNA by the Np decamer without the help of H3/H4 specific chaperones (Dutta et al., 2001). How it works when only N1/N2 is present still needs to be established.

### 1.3 ATP Dependent Chromatin Assembly Factors

Histone chaperones deliver histones onto DNA to assemble nucleosomes, but they are not able to produce periodic arrays of nucleosomes with 180-200 bp spacing. This process requires ATP dependent chromatin assembly factor (Haushalter and Kadonaga,
Two major ATP dependent chromatin assembly factors are to be discussed in this section: ATP-utilizing chromatin assembly and remodeling factor (ACF) and remodeling and spacing factor (RSF).

1.3.1 ATP-utilizing Chromatin Assembly and Remodeling Factor (ACF)

ATP-utilizing chromatin assembly and remodeling factor (ACF) was identified from crude *Drosophila* embryo extracts by biochemical fractionation (Ito et al., 1997). It plays roles not only in chromatin assembly but also in chromatin remodeling (i.e. any detectable change in histone-DNA interactions in a nucleosome) (Alexiadis and Kadonaga, 2002; Ito et al., 1997). ACF contains two subunits, ISWI (*Drosophila* imitation switch) ATPase which is essential in *Drosophila*, and Acf1 (Ito et al., 1999). ISWI is the catalytic subunit of ACF. It is a member of the SNF2-like family of ATPases (Ito et al., 1999). ISWI has been indicated to be a component of several complexes in *Drosophila* including CHRAC (chromatin accessibility factor) (Varga-Weisz et al., 1997), NURF (nucleosome remodeling factor) (Tsukiyama et al., 1995; Tsukiyama and Wu, 1995), and TRF2 (TATA-box-binding protein-related factor 2) (Hochheimer et al., 2002). ISWI serves as a motor to catalyze the movement of nucleosomes on DNA, whereas Acf1 can regulate/enhance ISWI activity (Haushalter and Kadonaga, 2003). It has been shown that the activity of ISWI increases about 30-fold when associated with Acf1 (Ito et al., 1997). Additionally, Acf1 also changes the function of ISWI in chromatin remodeling (Haushalter and Kadonaga, 2003). For example, ISWI catalyses the movement of nucleosomes from the centers to the ends of DNA fragments in a nucleosome sliding assay (Eberharter et al., 2001).
Another ISWI-containing complex CHRAC is closely related to ACF except that CHRAC contains two more small subunits, CHRAC14 and CHRAC16, which are not required for nucleosome assembly \textit{in vitro} (Corona et al., 2000; Eberharter et al., 2001; Poot et al., 2000; Varga-Weisz et al., 1997). In fact, these two complexes were identified at the same time through similar approaches by two different groups (Ito et al., 1997; Ito et al., 1999; Varga-Weisz et al., 1997).

The mechanism through which regularly spaced nucleosomes are generated has been proposed from \textit{in vitro} assays (Ito et al., 1997; Ito et al., 1999). After histones have been deposited onto DNA by histone chaperones, many of the interactions between histones and DNA are temporarily broken by energy from ATP hydrolysis of ACF/CHRAC, enabling histone octamers to move along the DNA and re-anneal to form regularly spaced nucleosomes. The process appears to need close cooperation among histones, DNA, ACF/CHRAC, and a histone chaperone (Haushalter and Kadonaga, 2003; Tyler, 2002).

1.3.2 Remodeling and Spacing Factor (RSF)

Remodeling and spacing factor (RSF) is another complex that mediates ATP dependent nucleosome assembly (Haushalter and Kadonaga, 2003; Loyola et al., 2001; Tyler, 2002). RSF was first identified from human cells, and has also been found in \textit{Xenopus} (LeRoy et al., 1998). RSF has two subunits, Rsf1 and hSNF2H (LeRoy et al., 1998). The latter is a \textit{Drosophila} ISWI homolog. The original function of RSF is to promote transcription initiation by RNA polymerase II on chromatin templates (LeRoy et al., 1998; Loyola et al., 2001). RSF has been shown to interact with histones H3/H4
directly (Loyola et al., 2001). The Rsf1 subunit could be responsible for this histone chaperone activity (Loyola et al., 2001). *In vitro* assays showed that purified RSF is able to deposit histones and generate regular arrays of nucleosomes during chromatin assembly without other histone chaperones (Loyola et al., 2001). Furthermore, each RSF only assembles one nucleosome whereas each ACF/CHRAC can assemble several nucleosomes (Ito et al., 1999; Loyola et al., 2001).

**1.4 Current Model of Nucleosome Assembly *In Vivo***

The current working model of nucleosome assembly *in vivo* is summarized in Fig 1.1. First, newly synthesized histones are acetylated by type B HATs. H4 is specifically acetylated at K5 and K12 by the Hat1p/Hat2p complex, whereas the HAT that catalyses the acetylation of nascent H3 still needs to be identified. In the next step, two molecules each of acetylated H3 and H4 form a tetramer. It is still controversial where the acetylation and tetramerization occur. Biochemical evidence suggests it to be a cytoplasmic event as both Hat1p/Hat2p complex and modified nascent histones H3 and H4 have been isolated in cytosolic fractions (Annunziato and Hansen, 2000). Following the acetylation and tetramerization is the nuclear transport of H3/H4. Meanwhile, H2A and H2B form a heterodimer and are transported into nucleus as well. In the nucleus, histone chaperones such as CAF-1 or ASF1 bind to and deposit acetylated H3/H4 onto newly synthesized DNA followed by the deposition of H2A/H2B possibly by Nap1. The acetylations on histones are removed by histone deacetylase soon after the deposition during the chromatin maturation.
Figure 1.1 Histone acetylation and chromatin assembly *in vivo*
CHAPTER 2

ISOLATION AND IDENTIFICATION OF NUCLEAR Hat1p/Hat2p complex

2.1 Abstracts

Histone acetylation plays an important role in the assembly and modulation of chromatin structure. Two main types of Histone AcetylTransferase (HAT), HAT-A and HAT-B, are responsible for the acetylation of histones. The HAT-A enzymes catalyze the acetylation of nucleosomal core histones and are located in the nucleus. The HAT-B enzymes are primarily cytoplasmic and specific for the acetylation of free histones. The only HAT-B identified to date is comprised of two subunits, Hat1p and Hat2p. Hat1p is the catalytic subunit and Hat2p is required for the high affinity binding of Hat1p to histone H4 and for full catalytic activity. While the Hat1p/Hat2p complex was originally isolated from cytoplasmic extracts, evidence suggested that it is present in the nucleus as well. To characterize the nuclear form of this enzyme, we tagged Hat1p and Hat2p with a protein A-TEV-calmodulin binding protein (CBP) tag. By using the tandem affinity purification (TAP) method combined with other chromatographic techniques, we were able to identify several proteins that associate with Hat1p and Hat2p in the yeast nucleus. These proteins include the uncharacterized open reading frame YLL022C (named Hif1p), histone H3, and histone H4. The functional significance of the association of Hif1p with
the Hat1p/Hat2p complex is confirmed by the observation that the hif1∆ and hat1∆ strains display similar defects in telomeric silencing and DNA double strand break repair. In addition, the histone H4 associated with the nuclear Hat1p/Hat2p/Hif1p complex contains novel post-translational modifications in the core domain. One site of core domain acetylation, lysine 91, lies at the interface between the H3/H4 tetramer and H2A/H2B dimers, and might be critical for the chromatin assembly.

2.2 Introduction

Nearly 30 years ago, histones H3 and H4 were shown to be rapidly acetylated post synthesis (Jackson et al., 1976; Louie et al., 1974; Ruiz-Carrillo et al., 1975). Following the deposition of these histones during de novo chromatin assembly, these acetyl groups are removed during the process of chromatin maturation (Annunziato et al., 1981; Annunziato and Seale, 1983; Worcel et al., 1978). While the timing of this cycle of acetylation and deacetylation suggested that it functions in the process of chromatin assembly, the precise role of these modifications remains unknown (Annunziato and Hansen, 2000).

Enzymes known as type B histone acetyltransferases (HATs) catalyze the acetylation of newly synthesized histones. As originally defined, type B HATs differ from the better characterized type A HATs in their substrate specificity and cellular localization. These enzymes specifically acetylate free histones and are located in the cytoplasm (Brownell and Allis, 1996). To date, Hat1p is the only type B HAT that has been identified. Originally isolated from S. cerevisiae, this enzyme is specific for free histone H4 and can acetylate lysine residues 5 and 12 (Kleff et al., 1995; Parthun et al.,
These sites of acetylation match the evolutionarily invariant pattern of acetylation seen on newly synthesized histone H4 molecules (Chicoine et al., 1986; Sobel et al., 1995). When purified from yeast cytoplasmic extracts, Hat1p is found in a complex with Hat2p, a yeast homolog of the Rbap46/48 proteins (Parthun et al., 1996; Qian and Lee, 1995; Qian et al., 1993). The Hat1p/Hat2p enzyme is highly conserved, as complexes containing homologs of these two proteins have also been isolated from human, maize and *X. laevis* extracts (Eberharter et al., 1996; Imhof and Wolffe, 1999a; Verreault et al., 1998).

Curiously, while both the acetylation pattern on newly synthesized histone H4 and the Hat1p/Hat2p complex are highly conserved throughout eukaryotic evolution, neither is essential for viability in yeast. Mutating histone H4 lysines 5 and 12 to arginine, which mimics the constitutively unacetylated state, has little effect on yeast cell growth (Ma et al., 1998; Megee et al., 1990). Likewise, deletion of the HAT1 and/or HAT2 genes does not result in any significant phenotypes (Kleff et al., 1995; Parthun et al., 1996). These observations may be explained, at least in part, by functional redundancy between the NH$_2$-terminal tails of histones H3 and H4. Deletion of the histone H3 NH$_2$-terminus in combination with either deletion of the H4 NH$_2$-terminus or alteration of H4 lysines 5, 8 and 12 to arginine causes a loss of nucleosome assembly and cell death (Ma et al., 1998). In addition, pairing a *hat1*Δ with mutations in specific sets of lysine residues in the H3 NH$_2$-terminus causes a loss of telomeric silencing and a deficiency in the recombinational repair of DNA double strand breaks, phenotypes consistent with defects in chromatin assembly (Kelly et al., 2000; Qin and Parthun, 2002).
In an effort to better understand the link between histone modification and chromatin assembly, we have purified the nuclear form of the yeast Hat1p/Hat2p complex. Nuclear Hat1p and Hat2p were found in a high molecular weight complex containing a novel histone chaperone (named Hif1p) and histones H3 and H4. Genetic analysis strongly suggests the \textit{in vivo} association of Hif1p with Hat1p and Hat2p. In addition to the expected acetylation in its NH$_2$-terminal tail domain, the histone H4 associated with the nuclear Hat1p/Hat2p complex also contained six modifications in its core domain, including acetylation of lysine 91. The crystal structure of nucleosome showed that this residue lies at the interface between the H3/H4 tetramer and the H2A/H2B dimer, so it might affect the process of chromatin assembly.

2.3 Materials and Methods

\textit{Yeast strains}

Yeast culture and genetic manipulation were done according to standard methods (Adams, 1998). \textit{HAT2} was TAP tagged at the COOH-terminus in strain UCC1111 (wild type) to generate strain NKY2 (Kelly et al., 2000; Puig et al., 2001). The presence of the epitope tags was confirmed by both PCR and Western blot using peroxidase-antiperoxidase complex (PAP, Sigma) or antibodies against Hat2p. Gene deletions were generated by PCR-mediated gene disruption with \textit{HIS3} unless otherwise indicated (Brachmann et al., 1998). \textit{ASF1} and \textit{CAC1} were disrupted in UCC1111 to generate SQY114 and TKY108, respectively (Kelly et al., 2000; Qin and Parthun, 2002). \textit{HIF1} was disrupted in UCC1111 and ASY50 (UCC111 with hat1::LYS2,(Parthun et al., 1996))
to generate XAY4 and XAY5, respectively. SSA2 and YBR139W were disrupted in UCC1111 to generate XAY6 and XAY17, respectively.

Protein purification

Yeast nuclear extracts were prepared essentially as described (Ponticelli and Struhl, 1990). NKY2, UCC1111, or XAY4 cells were harvested at mid-log phase. Spheroplasts were generated using Zymolyase 100T and lysed by resuspension in lysis buffer (10 mM HEPES [pH 6.0], and 18% [w/v] Ficoll 400) followed by dilution with 2 volumes of buffer A (10 mM HEPES [pH 6.0], 50 mM NaCl, 1 mM MgCl2, 0.5 mM PMSF) (Parthun et al., 1996). The nuclei and cell debris were pelleted by centrifugation at 1,500×g for 15 min. The supernatant was used as cytosolic extracts. The pellet was resuspended in buffer A and laid on a 60% sucrose cushion, which was centrifuged at 6,000×g for 8 min. The resulting band of nuclei at the interface was collected, resuspended in buffer A and lysed with 1/3 volume of 3 M of ammonium sulfate (final concentration of ammonium sulfate is 0.5 M). Nuclear lysate was centrifuged at 140,000×g for 90 min. The supernatant was kept as nuclear extracts and was precipitated with solid ammonium sulfate at the concentration of 516 mg solid per ml of supernatant. The precipitated nuclear extracts were pelleted at 20,000×g for 20 min. The pellet was resuspended with buffer C (20 mM HEPES [pH 7.6], 10 mM MgSO4, 10% glycerol, 0.5 mM PMSF, 10 mM β-mercaptoethanol) and dialyzed against Buffer C including 75 mM ammonium sulfate before chromatography. PMSF and β-mercaptoethanol were added into the buffers right before use.
Each 10 ml of NKY2 nuclear extract from 200 L of cells (~100 mg of protein) was bound to 400 µl calmodulin sepharose 4B (Amersham) and eluted with IPP150 Calmodulin Elution Buffer (10 mM Tris, [pH 8.0], 150 mM NaCl, 0.02 % NP-40, 1 mM MgAc₂, 1 mM Imidazole, 2 mM EGTA, 10 mM mercaptoethanol) (Puig et al., 2001). Up to 20 parallel purifications were performed at this step. UCC1111 nuclear extract was run through calmodulin sepharose 4B column as negative control. All fractions eluted from calmodulin resin were concentrated with Centriprep/Centricon YM-30 (Millipore) to about 1 ml. 250 µl was applied to a Superose 6 HR 10/30 column (Amersham). The column was equilibrated with buffer DN(50) (25 mM Tris [pH 7.0], 0.1 mM EDTA, 10% glycerol, 50 mM NaCl). Peak fractions from the Superose 6 column were pooled and loaded onto a Mono Q HR 5/5 column (Amersham). A 20 ml linear gradient from 50 mM to 1000 mM of NaCl was applied to elute the bound proteins (buffers used are DN(50) and DN(1000) (25 mM Tris [pH 7.0], 0.1 mM EDTA, 10% glycerol, 1000 mM NaCl), respectively). Peak fractions from the Mono Q column were concentrated with a Centricon YM-30. Concentrated protein samples were resolved by SDS-PAGE, visualized with Coomassie blue and excised for mass spectrometry analysis. The HAT activities of fractions from each column were monitored by liquid HAT assays (Parthun et al., 1996).

**Western Blots**

Western blots were performed and visualized using either an ECL chemiluminescent detection kit according to manufacturer’s instructions (Amersham) or premixed BCIP/NBT solution (Sigma) as a substrate for alkaline phosphatase.
Telomeric silencing and DNA damage assays were performed as described previously (Kelly et al., 2000; Qin and Parthun, 2002).

Immunofluorescence microscopy

Immunofluorescent localization was performed as described with modifications (Adams, 1998). Yeast cells were fixed with 3.7% formaldehyde at early exponential phase for 1 h at 30°C before harvesting. Cells were then incubated with 0.5 % sodium borohydride for 10 min at room temperature before spheroplasting to eliminate the fluorescence background. The spheroplasts were prepared by incubating cells with Zymolyase 100T in spheroplasting buffer for 30-45 min at 30°C. Spheroplasts were then fixed onto positively charged slides by immersing the slides into ice-cold methanol for 6-7 min and acetone for 30 s. The cells were blocked and probed with primary antibody (α-Hat2p or α-Myc, Sigma). Hat2p and Hif1p-Myc were visualized with Alexa and CY3 conjugated secondary antibodies, respectively. Nuclear DNA was visualized by DAPI staining. The fluorescence images were taken with a Zeiss LSM510 multiphoton confocal inverted microscope. A hat2Δ strain (TKY102) was used as negative control.

2.4 Results

2.4.1 Cellular Distribution of the Yeast Hat1p/Hat2p Complex

Type B HATs were originally defined as cytoplasmic enzymes. However, their actual cellular distribution remains unclear. Numerous cellular fractionation experiments
identified type B HAT activities in cytoplasmic extracts (Annunziato and Hansen, 2000). Conversely, immunofluorescent localization of Hat1p in human cells indicated that the enzyme was almost exclusively nuclear (Verreault et al., 1998). In *Xenopus*, Hat1p could be isolated from either cytoplasmic or nuclear extracts depending on the developmental stage of the animal (Imhof and Wolffe, 1999a; Imhof and Wolffe, 1999b). While yeast Hat1p and Hat2p have been reported to be components of HAT activities in both the cytoplasm and the nucleus, but the relative distribution of Hat1p and Hat2p between these cellular compartments has not been determined (Kleff et al., 1995; Parthun et al., 1996; Ruiz-Carrillo et al., 1975; Ruiz-Garcia et al., 1998).

To obtain an accurate picture of the cellular localization of the Hat1p/Hat2p complex, we performed indirect immunofluorescent imaging of native Hat2p in whole yeast cells. As seen in Figure 2.1, Hat2p was clearly detectable throughout the cytoplasm of yeast cells in a speckled pattern. In addition, there was a significant accumulation of Hat2p in nuclei as shown by co-localization with DAPI staining. We were not able to use immunofluorescence to examine the localization of Hat1p as antibodies against native Hat1p are not of sufficient specificity and titer. However, as seen below, there appeared to be little, if any, Hat2p in yeast cells that is not complexed with Hat1p allowing the localization of Hat2p to serve as an excellent indicator of the cellular distribution of Hat1p-containing complexes. Therefore, our results indicated that the yeast Hat1p/Hat2p complex was primarily nuclear but with a significant cytoplasmic component as well.
2.4.2 Purification of a Nuclear Hat1p/Hat2p Complex

While the cytoplasmic localization of the Hat1p/Hat2p complex is consistent with its proposed role in the acetylation of newly synthesized histone H4, the function of the nuclear form of the enzyme is not obvious. To shed light on the function of nuclear Hat1p/Hat2p, we purified the complex from nuclear extracts. The purity of our extracts was analyzed by probing Western blots containing cytosolic and nuclear extracts with Hat1p, Hat2p and known cytoplasmic and nuclear proteins (Figure 2.2). While both Hat1p and Hat2p are found in both extracts, Ded1p (a cytoplasmic RNA helicase) is found only in the cytosolic extract and the type A HAT Gcn5p is found exclusively in the nuclear extract (Chuang et al., 1997). This result further confirmed the localization of Hat1p and Hat2p as in both cytoplasm and nucleus. To facilitate isolation of the nuclear complex, we incorporated a tandem affinity purification (TAP) tag at the COOH-terminus of Hat2p. The TAP tag includes two IgG binding domains of *staphylococcus aureus* protein A and a calmodulin binding peptide connected by a TEV protease cleavage site (Puig et al., 2001; Ruiz-Garcia et al., 1998).

It is important to know whether the addition of the TAP tag to Hat2p results in defects in Hat1p/Hat2p *in vivo* function. We inspected this by examining whether NKY2 showed the same phenotype as seen in UCC1111 in telomeric silencing assay. Hat1p/Hat2p complex has been shown to be redundantly required for telomeric silencing with specific lysine residues in the histone H3 NH$_2$-terminal tail (i.e. lysines 14 and 23) (Kelly et al., 2000). UCC1111 and strains derived from it (for example, NKY2, TKY102) incorporate a telomeric *URA3* reporter gene and have been engineered to allow the expression of specific histone H3 alleles. Telomeric silencing was assayed by comparing
the growth of cells in the presence and absence of the drug 5-fluoorotic acid (5-FOA). Normal telomeric chromatin structure transcriptionally silences the \textit{URA3} reporter gene preventing the conversion of 5-FOA into a toxin by the \textit{URA3} gene product. Loss of telomeric silencing allows for the expression of \textit{URA3} and a resultant decrease in viability in the presence of 5-FOA (Gottschling et al., 1990). As seen in Figure 2.3, both wild type cells and Hat2p tagged cells grow normally in the presence of 5-FOA. \textit{hat1Δ} has no effect on telomeric silencing. Likewise, a histone H3 K14, 23R allele expressed in either UCC1111 or NKY2 does not significantly disrupt telomeric silencing. However, combining the \textit{hat1Δ} with the H3 K14, 23R allele causes a 100- to 1000-fold decrease in viability in the presence of 5-FOA. These results verified that the addition of a TAP tag to Hat2p does not affect the \textit{in vivo} function of the Hat1p/Hat2p complex.

The association of Hat1p and Hat2p in nuclei was assessed by passing nuclear extracts over calmodulin resin. As shown in Figure 2.4 (top), HAT activity bound to the calmodulin resin only in the presence of TAP-tagged Hat2p. The Hat1p/Hat2p complex was probably responsible for this HAT activity as it was specific for histone H4, and Western blot analysis indicated the presence of Hat1p in the bound fraction (Figure 2.4, bottom).

The strategy used for the purification of the nuclear Hat1p/Hat2p complex, outlined in Figure 2.5, employed both affinity and conventional chromatography. Following elution of the TAP-tagged Hat1p/Hat2p complex from the calmodulin resin, the sample was concentrated and applied to a Superose 6 column. Both Hat1p and Hat2p co-fractionated with a strong peak of HAT activity that was primarily specific for histone H4 but which also modified histone H2A at a much lower level (Figure 2.6). The ability
of the Hat1p/Hat2p complex to acetylate histone H2A was consistent with the activity of recombinant yeast Hat1p (Parthun et al., 1996; Verreault et al., 1998). The elution volume of this HAT activity indicated that the bulk of the nuclear Hat1p and Hat2p were found in a high molecular weight complex of ~500 kDa.

Peak fractions from the Superose 6 column containing the Hat1p/Hat2p complex were pooled and further fractionated on a Mono Q column. Figure 2.7 shows the peak of HAT activity that eluted from the Mono Q column along with a silver stained SDS-polyacrylamide gel of the corresponding fractions. Inspection of the protein pattern indicated that three polypeptides, with molecular weights of 42, 52 and 54 kDa, co-eluted with the peak of HAT activity (indicated by asterisks in Figure 2.7). The peak fractions were pooled, resolved by SDS-PAGE and visualized with Coomassie blue staining. A band corresponding to the 42 kDa protein and one encompassing the 52 and 54 kDa proteins were then excised. Following digestion with trypsin, the proteins were identified by mass spectrometry. As expected, the 42 kDa band was identified as Hat1p and the section spanning 52 to 54 kDa contained Hat2p. In addition, the second protein in the 52 to 54 kDa segment was identified as the product of the uncharacterized yeast ORF YLL022C. The gene name HIF1 (Hat1p Interacting Factor 1) had been previously reserved for this ORF.

Evaluation of the Coomassie blue stained gel used to identify the above three peptides indicated that these fractions also contained a number of other polypeptides including two low molecular weight ones (Figure 2.8). These bands were excised, digested with trypsin or chymotrypsin, and analyzed by mass spectrometry. As indicated in Figure 2.8 and Tables 2.1 and 2.2, three other high/medium molecular weight peptides
are identified as Sse1p, Ssa2p, and the product of the uncharacterized yeast ORF YBR139W. Sse1p and Ssa2p are members of heat shock protein family. Peptide fragments were isolated that definitively identified the most prominent low molecular weight species as histone H4 and a second, somewhat less abundant protein, as histone H3. It is unusual that the Hat1p/Hat2p/Hif1p complex appeared to be associated with more histone H4 than histone H3, as they are generally found in a tetramer of equal stoichiometry. However, it is likely that this discrepancy was related to the fact that histone H3 is much more susceptible to proteolysis and, therefore, may have been subject to degradation during purification of the complex.

2.4.3 Hif1p is linked to Hat1p Function In Vivo

In order to determine whether the co-purification of Hif1p with the nuclear Hat1p/Hat2p complex was functionally relevant, we examined whether deletion of HIF1 resulted in the same spectrum of phenotypes as seen in a hat1Δ. We first determined the role of HIF1 in telomeric silencing using the approach as discussed in 2.4.2. As seen in Figure 2.9, a hat1Δ or hif1Δ has little or no effect on telomeric silencing. Likewise, a histone H3 K14, 23R allele does not significantly disrupt telomeric silencing. However, combining either the hat1Δ or the hif1Δ with the H3 K14,23R allele causes a 100- to 1000-fold decrease in viability in the presence of 5-FOA. In addition, combining hat1Δ and hif1Δ does not result in a further decrease in viability.

Mutations in HAT1 have also been linked to defects in the recombinational repair of DNA double strand breaks (Qin and Parthun, 2002). In this case, the phenotype of the hat1Δ is only manifested when combined with an allele of histone H3 in which lysines 9,
18 and 27 are converted to arginine (H3 K9, 18, 27R). The recombinational repair of DNA double strand breaks can be assayed by the introduction of a plasmid containing a galactose-inducible HO gene. The HO endonuclease introduces a single double strand break in the yeast genome at the MAT locus that is normally repaired by homologous recombination with sequences at the silent mating loci. Cells that are defective in the recombinational repair of the HO-induced double strand break display decreased viability on galactose-containing media (Herskowitz and Jensen, 1991). Cells containing a hat1Δ, hif1Δ or the H3 K9, 18, 23R allele grow normally on galactose (Figure 2.10). When the hat1Δ or hif1Δ was combined with the H3 K9, 18, 23R allele, a decrease in viability was evident when the cells were grown on galactose. Again, consistent with the telomeric silencing results, combination of hat1Δ and hif1Δ does not accentuate this phenotype.

The observation that hif1Δ mimics the highly specific H3-dependent phenotypes seen in conjunction with hat1Δ supports the biochemical evidence for a physical interaction between Hif1p and the nuclear Hat1p/Hat2p complex and demonstrates that this association is functionally relevant in the cell.

2.4.4 Nuclear Hat1p/Hat2p/Hif1p Complex Associates with Histones H3 and H4

In addition to identifying histones H3 and H4 as proteins that interact with the nuclear Hat1p/Hat2p/Hif1p complex in vivo, the mass spectrometry data in Tables 2.1 and 2.2 indicated the presence of specific post-translational modifications on these histones. For histone H3, we were unable to identify NH₂-terminal tail modifications as peptides encompassing this region were not recovered. However, methylation of a
residue in the H3 core domain was indicated by peptides encompassing residues 62-70 and 68-92 which have masses that precisely match the presence of a single methyl group (Table 2.2). It was not possible to precisely localize this modification, as there are multiple lysine and/or arginine residues in these peptides. However, there is only one methylatable residue, R69, which is found in both peptides.

The histone H4 associated with the nuclear Hat1p/Hat2p/Hif1p complex was found to possess multiple modifications. There were several peptides recovered that included residues 1-10 of the NH2-terminal tail domain. The masses of two of these peptides matched the molecular weight predicted for the addition of one and two acetyl groups, respectively. As the NH2-terminus of yeast H4 is blocked, one of these acetyl groups can be assigned to the NH2-terminal serine residue. The second acetyl group must then be found on either K5 or K8. A peptide with a mass of 1042.839 Da was also identified that was consistent with the triacetylation of peptide 1-10 (predicted mass of 1042.585 Da). However, this mass could not be used to confirm the acetylation of both K5 and K8 as this mass is also consistent with the molecular weight of a peptide containing H4 residues 89-97 (predicted mass of 1042.649 Da). Several modifications were also found in the core domain of the Hat1p/Hat2p/Hif1p associated histone H4. Dimethylation was observed for a peptide that encompassed residues 50-61 (Table 2.1). This peptide contains an arginine at position 55 and a lysine at position 59. Intriguingly, dimethylation of H4 K59 was recently detected in histones isolated from bovine thymus and mutations in yeast that alter H4 K59 disrupt transcriptional silencing at telomeres and the silent mating loci (Zhang et al., 2003). The acetylation of K91 is supported by three peptides, two spanning H4 residues 91-100 and a third spanning residues 91-97 (Table
In each case there is a mass shift indicative of addition of an acetyl group which can be unambiguously localized to K91. Two of these peptides indicated that the acetylation of K91 was associated with the presence of two methyl groups on arginine residues 92 and/or 95. Again, these sites of core domain modification on histone H4 appear to be highly conserved as the acetylation of K91 and the methylation of R92 are also observed in bovine thymus (Zhang et al., 2003). The localization of H4 K91 in the crystal structure of the nucleosome was examined. Figure 2.11 highlights in pink the location of this residue in the crystal structure of nucleosome (Luger et al., 1997). The left panel displays the full nucleosome where this site of modification appears buried in the interior of the structure. However, by looking at only the histone H3/H4 tetramer (right panel) it is apparent that this modification is in the region of histone H4 that is important for the docking of the H2A/H2B dimers with the H3/H4 tetramer (Akey and Luger, 2003). This striking location suggests the possibility that the acetylation of histone H4 K91 might be involved in the formation of histone octamers during chromatin assembly.

2.4.5 Heat Shock Proteins and YBR139W are not functionally associated with Hat1p

It has been reported that some proteins tend to co-purify with the targeted protein as background when the TAP purification method is applied (Shevchenko et al., 2002). Heat shock proteins are prominent among this group. As shown in Figure 2.8, Sse1p and Ssa2p, as well as the product of the uncharacterized open reading frame, YBR139W, were abundant in a pooled peak of HAT activity following Mono-Q fractionation. However, it is likely that the presence of these proteins in the purified preparation of the Hat1p/Hat2p complex is not meaningful, as inspection of Figure 2.7 indicated that these bands did not
precisely co-elute with the peak of HAT activity. To confirm that the heat shock proteins and \textit{YBR139W} are not linked to Hat1p function, \textit{ssa2}Δ and \textit{YBR139W}Δ strains were generated from UCC1111. Telomeric silencing assay was performed to test whether deletion of \textit{SSA2} displays the same phenotype as \textit{hat1}Δ strains. As shown in Figure 2.12, \textit{ssa2}Δ showed the same phenotype as the wild type strain instead of \textit{hat1}Δ strain, which indicates that Ssa2 is not involved in the telomeric silencing, therefore is not likely to associate with Hat1p/Hat2p complex functionally. This is consistent with what has been found before (Shevchenko et al., 2002). Thus, another heat shock protein, Sse1p, is also a background protein.

Similarly, \textit{YBR139W} was disrupted in UCC1111 and the resulted strain was used in telomeric silencing assay. The result is the same as seen with \textit{ssa2}Δ strain (Figure 2.13), therefore \textit{YBR139W} encoded protein is background as well.

2.4.6 Nuclear Hat1p/Hat2p complex acetylates chromatin histones

One property that distinguishes type B HATs from type A HATs is they specifically acetylate free histones. Strikingly, the purified nuclear Hat1p/Hat2p complex not only specifically acetylates free histones, but also catalyzes the acetylation of chromatin histones at relatively lower activity (Figure 2.14). In contrast, the Hat1p/Hat2p complex purified from cytosolic extracts (elutes from calmodulin column) and recombinant Hat1p only uses free histones as a substrate. This might indicate that the substrate specificity of Hat1p changes upon associating with different components.
2.5 Discussion

The results presented here provide a useful starting point to further investigate the function of type B HATs. We have demonstrated that the Hat1p/Hat2p type B HAT complex is found in both the cytoplasm and nucleus. The nuclear form of the enzyme associates with Hif1p, an uncharacterized protein, and modified histones H3 and H4. A number of these modifications occur within the core domains of H3 and H4.

2.5.1 Where Does the Acetylation of Newly Synthesized Histones Occur?

The original definition of type B HATs as cytoplasmic enzymes was logical given their function in the acetylation of newly synthesized histones. However, the speed with which newly synthesized histones are transported into the nucleus and assembled into chromatin complicates the definitive assignment of the point at which these molecules are modified. Evidence for cytoplasmic acetylation comes from biochemical fractionation experiments showing that newly synthesized histones H3 and H4 that have been modified can be separated away from nuclei into a cytosolic fraction (Chang et al., 1997). The caveat to the interpretation of these results is that preassembly H3/H4 tetramers may be easily extractable from nuclei following cell disruption. Similar difficulties attach to the interpretation of the numerous studies that have identified H4-specific type B histone acetyltransferase activities in cytosolic extracts (Chang et al., 1997; Eberharter et al., 1996; Lusser et al., 1997; Lusser et al., 1999; Mingarro et al., 1993; Richman et al., 1988; Wiegand and Brutlag, 1981). Indeed, immunofluorescent localization of Hat1p in human cells indicated that the enzyme was almost exclusively nuclear (Verreault et al., 1998). However, these experiments visualized a transiently expressed, epitope tagged Hat1p and
it is not clear how these factors might have affected the localization of the protein. In the present study, immunolocalization of native Hat2p expressed from its own promoter clearly indicated that this protein was both cytoplasmic and nuclear. Coupled with the observation that the cytosolic Hat1p/Hat2p complex is stably associated with histone H4, these data strongly support a model in which newly synthesized histone H4 is acetylated in the cytoplasm prior to nuclear import (Mosammaparast et al., 2002b).

2.5.2 The Nuclear Role of Type B Histone Acetyltransferases

The original definition of type B histone acetyltransferases as cytoplasmic enzymes was logical given their function in the acetylation of newly synthesized histones. The demonstration that these enzymes are also nuclear raises the question of their role in this cellular compartment. There are a number of possibilities, several of which are related to the observation that both the cytoplasmic and nuclear forms of the Hat1p/Hat2p complex are stably associated with histones H3 and H4. This association suggests that the enzyme is shuttled into the nucleus with its substrate. As such, the Hat1p/Hat2p complex may play a role in facilitating import of the histones into the nucleus. Following histone deposition, the Hat1p/Hat2p complex could then shuttle back to the cytoplasm where it would be free to repeat the cycle with another newly synthesized histone molecule. The Hat1p/Hat2p complex may also accompany newly synthesized histones into the nucleus to protect their pattern of acetylation by either shielding the H4 NH$_2$-terminal tail from the action of nuclear HDACs or by recreating their acetylation state following deacetylations (Imhof and Wolffe, 1999a; Verreault et al., 1998). An intriguing aspect of these models is that the Hat1p/Hat2p complex would
be acting in a stoichiometric rather than catalytic capacity consistent with the observation that there are very high levels of HAT1 protein in the nuclei of *X. laevis* oocytes where large quantities of histones are stored prior to embryogenesis (Imhof and Wolffe, 1999a). The Hat1/Hat2p complex may also act on substrates other than newly synthesized histones when in the nucleus. While the vast majority of nuclear histones are packaged into chromatin, free histones may be transiently liberated by processes, such as transcription and DNA repair, which disrupt chromatin structure. An interaction between these histones and the Hat1p/Hat2p complex may serve a role in re-establishing chromatin structure following these events. Alternatively, association of the Hat1p/Hat2p complex with other factors in the nucleus may alter the substrate specificity of the enzyme such that it can now acetylate chromatin substrates and function as a type A histone acetyltransferase (as seen in Figure 2.14).

### 2.5.3 Histone Modification and Chromatin Assembly

The histone H3 and H4 molecules that are associated with the nuclear Hat1p/Hat2p/Hif1p complex are likely to represent newly synthesized histones. The unexpected observation that these histones contain modifications in their core domains suggests that the evolutionarily conserved acetylations on the H3 and H4 NH₂-terminal tails are not the only modifications that play a role in the process of chromatin assembly.

While histone H4 molecules associated with the Hat1p/Hat2p complex can be acetylated on lysine 91 there are a number of reasons why it is unlikely that Hat1p is responsible for the acetylation of this residue. First, mutating H4 lysine 91 causes much more severe phenotypes than loss of Hat1p, indicating that if Hat1p does acetylate H4
lysine 91 it is certainly not the only enzyme responsible for the modification of this site (unpublished data, Parthun group). Second, Hat1p has a recognition sequence, GXGKXG, which directs its activity toward histone H4 lysines 5 and 12 and histone H2A lysine 5. The basis for this recognition sequence was apparent from the crystal structure of Hat1p where the contours of the substrate binding surface are complementary to a model peptide representing this recognition sequence. In particular, the pattern of glycine residues was important as steric hindrance would likely prevent the binding of substrates with large side chains at those positions. Hence, the sequence around H4 lysine 91, YALKRQ, is unlikely to promote the acetylation of this residue by Hat1p.

The results presented here raise the interesting question of why core domain modifications were not previously observed on newly synthesized histones. One explanation, that is particularly relevant for H4 lysine 91 acetylation, involves the kinetics of core domain versus NH2-terminal tail modifications. Acetylation of the NH2-terminal tail occurs rapidly following synthesis and is retained through deposition of the H3/H4 tetramers and completion of the octamer by H2A/H2B dimer addition. The NH2-terminal tail acetylation is then removed during the process of chromatin maturation (Annunziato and Hansen, 2000). Modifications such as lysine 91 acetylation (or arginine 92/95 methylation) are also generated on newly synthesized histones prior to assembly. However, the position of these modifications in the nucleosome suggests that they are likely to be removed prior to the addition of H2A/H2B dimers to the H3/H4 tetramer. Hence, these core domain modifications may be more transient, and more difficult to detect, than NH2-terminal tail acetylation.
The position of histone H4 lysine 91 in the nucleosome structure suggests that modification of this site could influence other processes in addition to chromatin assembly. For example, there is evidence that transcription by RNA polymerase II can result in disruption of nucleosome structure through the displacement of H2A/H2B dimers (Baer and Rhodes, 1983; Bazett-Jones et al., 1996; Belotserkovskaya et al., 2003; Formosa et al., 2002; Kireeva et al., 2002; Nacheva et al., 1989; Ruone et al., 2003; Sathyanarayana et al., 1999; Saunders et al., 2003). By preventing the reassembly of histone octamers, acetylation of lysine 91 could serve as a mechanism by which actively transcribed chromatin is kept in an open configuration. Deacetylation of this residue would allow for the reassembly of normal chromatin structure as part of the process of transcriptional repression. While a potential role for H4 lysine 91 acetylation in transcription remains speculative, it would provide an example of histone modifications regulating gene expression through specific structural alterations in chromatin rather than through modulating the binding of chromatin associated proteins.
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Table 2.1 Band 1 chymotrypsin digestion fragments
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<td>1184.675</td>
<td>1184.7632</td>
<td>H3 62IRKLPFQRL70</td>
<td>Monomethylation</td>
</tr>
<tr>
<td>2876.223</td>
<td>2876.5741</td>
<td>H3 68QRLVREIAQDFKTDLRFQSSAIGAL92</td>
<td>Monomethylation</td>
</tr>
</tbody>
</table>

Table 2.2 Band 2 chymotrypsin digestion fragments
Figure 2.1 Hat2p is both nuclear and cytoplasmic.

The subcellular localization of Hat2p was determined by indirect immunofluorescence and confocal microscopy. The experiment was performed with wild type (UCC1111, Top) and hat2Δ cells (TKY102, Bottom) (Kelly et al., 2000). Both strains were stained with DAPI and with an affinity-purified primary antibody raised against Hat2p. Hat2p was visualized with an ALEXA-conjugated secondary antibody. The images shown are A and E, differential interference contrast; B and F, DAPI; C and G, ALEXA and D and H, merge.
Figure 2.2 Purity of cytosolic and nuclear extracts is confirmed.

Yeast cytoplasmic and nuclear extracts, prepared from a wild-type strain (UCC1111), were probed with antibodies against Hat1p, Hat2p, Ded1p (cytoplasmic protein), or Gcn5p (nuclear protein) as indicated for a Western blot assay. Hat1p, Hat2p, Ded1p, and Gcn5 are indicated by arrows.
Figure 2.3 HAT2-TAP tag has no effect on the function of Hat1p in telomeric silencing.

Telomeric silencing was measured by spotting 10-fold serial dilutions of cells on synthetic complete plates (HC) and synthetic complete plates containing 5-FOA. Plates were photographed after 3-4 days of growth at 30°C. The relevant genotypes of strains are indicated on the left. WT, wild type.
Figure 2.4 Hat1p associates with nuclear Hat2p.

Nuclear extract generated from a wild type (UCC1111) and HAT2-TAP tag strain (NKY2) were passed over a calmodulin Sepharose column. Liquid HAT assays, using free histones as substrate, from the input (IN), flow-through (FT) and three successive Ca^{2+} elution fractions (E1-E3) are shown on the top. The HAT assays from the indicated NKY2 samples were resolved by SDS-PAGE and visualized by fluorography (\(^{3}\text{H}\), middle). The migration of histone H3 and H4 are indicated by lines on the right. The indicated NKY2 fractions were analyzed on a Western blot probed with anti-Hat1p antibodies. The arrow indicates the migration of Hat1p.
Figure 2.5 Schematic diagram of the purification scheme used for the nuclear Hat1p/Hat2p complex.
Figure 2.6 HAT assays of fractions from the Superose 6 column.

Elution positions of size standards (in KDa) are indicated by arrows. HAT assays were resolved and visualized as in Figure 2.4 ($^3$H). Duplicate western blots were probed with anti-Hat1p and anti-Hat2p antibodies as in Figures 2.4 and 2.2.
Figure 2.7 Nuclear Hat1/Hat2 Mono-Q fractionation

HAT assays of the indicated fractions from the Mono-Q column (Top). The proteins present in the corresponding fractions were resolved by SDS-PAGE and visualized by silver staining (Bottom). Migration positions of molecular weight standards are as indicated. Asterisks mark the polypeptides that co-elute with HAT activity.
Purified nuclear Hat1p/Hat2p complex was resolved by SDS-PAGE. The gel was stained with Coomassie blue and then analyzed by mass spectrometry. YLL022C (Hif1p), Histone H3, H4, YBR139W, Ssa2p, and Sse1p were identified to be co-purified with Hat1p and Hat2p.
Figure 2.9 Hif1p is involved in telomeric silencing.

UCC1111 (wild type), ASY50 (hat1Δ), XAY4 (hif1Δ), and XAY5 (hat1Δhif1Δ) cells were transformed with plasmids containing the indicated histone H3 alleles (Kelly et al., 2000). Telomeric silencing was assayed as in Figure 2.3. Plates were photographed after 3-4 days of growth at 30°C. The relevant genotypes of strains are indicated on the left.
Figure 2.10 Hif1p participates in the DNA double strand break repair

UCC1111 (wild type), ASY50 (hat1Δ), XAY4 (hif1Δ), and XAY5 (Hat1Δhif1Δ) cells containing the indicated histone H3 alleles, were transformed with pGALHO-pRS412. Sensitivity to the HO endonuclease was determined by spotting ten-fold serial dilutions of cells on synthetic complete plates lacking adenine and containing either glucose (GLU-ADE) or galactose (GAL-ADE) as the sole carbon source. Plates were photographed after 3-4 days of growth at 30°C. The relevant genotypes of strains are indicated on the left.
Figure 2.11 Crystal structure of the nucleosome.

Crystal structure of the nucleosome shows the full histone octamer (Left) or the H3/H4 tetramer (Right). Histone H4 lysine 91 is highlighted in pink. Structure was generated with MOLSCRIPT and RASTER 3D using atomic coordinates from PDB code 1ID3.
Figure 2.12 Ssa2p is not involved in telomeric silencing

UCC1111 (wild type) ASY50 (hat1Δ), and XAY6 (ssa2Δ) cells were transformed with plasmids containing the indicated histone H3 alleles (Kelly et al., 2000). Telomeric silencing was assayed as in Figure 2.3. Plates were photographed after 3-4 days of growth at 30°C. The relevant genotypes of strains are indicated on the left.
Figure 2.13 YBR139W is not involved in telomeric silencing

UCC1111 (wild type) ASY50 (hat1Δ), and XAY17 (YBR139WΔ) cells were transformed with plasmids containing the indicated histone H3 alleles (Kelly et al., 2000). Telomeric silencing was assayed as in Figure 2.3. Plates were photographed after 3-4 days of growth at 30°C. The relevant genotypes of strains are indicated on the left.
Figure 2.14 Nuclear Hat1p/Hat2p complex acetylates both free histones and chromatin histones

The HAT assays from the indicated samples were resolved and visualized as in Figure 2.4 ($^3$H). The samples are: recombinant Hat1p (lane 2, 3), Hat1p complexes purified from cytosolic extract (lane 4, 5) or from nuclear extract (lane 6, 7). The histone substrates are either free chicken histones (lane 2, 4, 6) or chromatin histones (lane 3, 5, 7). Negative control is shown on lane 1. The migration of histone H4 is indicated by lines on the left.
CHAPTER 3

HIF1p IS A NOVEL HISTONE CHAPERONE AND CHROMATIN ASSEMBLY FACTOR

3.1 Abstract

In eukaryotes, chromatin assembly is essential for the replication of chromosomes. Chromatin assembly factors are required to deposit histones onto DNA during chromatin assembly. A new protein, Hif1p has been demonstrated to be a component of the nuclear Hat1p/Hat2p complex. Functional analysis revealed that Hif1p is a novel histone chaperone that selectively interacts with histones H3 and H4. Hif1p is also a chromatin assembly factor, promoting the deposition of histones in the presence of a yeast cytosolic extract. In vivo, the nuclear Hat1p/Hat2p/Hif1p complex is bound to acetylated histone H4, as well as histone H3. The association of Hif1p with acetylated H4 requires Hat1p and Hat2p, providing a direct link between type B histone acetyltransferases and chromatin assembly.

3.2 Introduction

DNA is packaged into chromatin in eukaryotes. The basic unit of chromatin is the nucleosome, which consists of 146 bp of DNA wrapping around core histones H2A, H2B, H3 and H4. Nucleosome assembly is required for the replication of eukaryotic
chromosomes (Wolffe, 1998a). Two steps are involved in nucleosome assembly. In the first step, histone H3/H4 tetramers are loaded onto DNA through the action of various chromatin assembly factors such as CAF-1 (chromatin assembly factor 1) and Asf1p (anti-silencing factor). The octamer is then completed by the addition of two histone H2A/H2B dimers (Haushalter and Kadonaga, 2003; Tyler, 2002). Most of these chromatin assembly factors are also known as histone chaperones. They specifically bind to certain types of histones, and accompany the bound histones to sites of chromatin assembly (Haushalter and Kadonaga, 2003; Loyola and Almouzni, 2004; Tyler, 2002).

Chromatin assembly occurs in either a DNA replication dependent or independent manner (Haushalter and Kadonaga, 2003; Tyler, 2002). Distinct chromatin assembly factors appear to participate in the different modes of chromatin assembly. CAF-1 exclusively mediates DNA replication-dependent nucleosome assembly (Kaufman, 1996; Smith and Stillman, 1989; Stillman, 1986). It is a heterotrimer containing p150, p60 and p48 subunits (Kaufman, 1996; Smith and Stillman, 1989; Stillman, 1986). All three subunits are histone binding proteins (Shibahara et al., 2000). The function of CAF-1 is associated with PCNA (proliferating cell nuclear antigen), a DNA polymerase sliding clamp protein. PCNA stays on the newly synthesized DNA following the replication (Shibahara and Stillman, 1999). Upon direct binding of p150 with PCNA, CAF-1 is recruited to the replication sites and deposits histones to replicated DNA (Shibahara and Stillman, 1999). Deletion of the three genes of the CAF-1 complex either separately or simultaneously does not affect yeast viability, but causes the increased sensitivity of cells
to UV irradiation and defects on gene silencing (Kaufman et al., 1997). In mammalian cells, CAF-1 has been shown to be essential for normal S-phase progression (Hoek and Stillman, 2003).

Hir/HirA proteins (histone regulation proteins), on the other hand, are responsible for DNA replication independent chromatin assembly (Ray-Gallet et al., 2002). Deletion of HIR genes alone does not affect cell growth, but has a synergistic effect on gene silencing when combined with CAF-1 disruption (Qian et al., 1998; Sharp et al., 2002). Therefore, Hir proteins are critical for a substitute silencing pathway when the CAF-1 mediated pathway does not function. Using HirA immuno-depleted Xenopus high-speed egg extracts (HSE), Almouzni and colleagues observed the decrease of the nucleosome assembly activity which could be restored upon addition of HirA and (H3-H4)$_2$ tetramers (Ray-Gallet et al., 2002). However, HirA depletion has no effect on the DNA replication-dependent assembly activity of HSE. Taken together, they demonstrated that HirA is required for the DNA replication-independent chromatin assembly (Ray-Gallet et al., 2002). In addition, the expression of HirA has also been shown to block the S-phase progression in mammalian cells (Hall et al., 2001).

Asf1 is involved in both DNA replication-dependent and -independent chromatin assembly (Kaufman et al., 1998; Krawitz et al., 2002; Mello et al., 2002; Ray-Gallet et al., 2002; Sharp et al., 2001; Sutton et al., 2001; Tagami et al., 2004; Tyler et al., 1999; Tyler et al., 2001). It associates with H3 and H4 to form a complex known as RCAF (replication-coupling assembly factor) (Tyler et al., 1999). Asf1 functions synergistically with CAF-1 to assemble nucleosomes (Tyler et al., 1999). It also has been shown that Asf1 interacts directly with Hir/HirA proteins (Kaufman et al., 1998; Ray-Gallet et al., 2002).
65

2002; Sharp et al., 2001; Sutton et al., 2001; Tagami et al., 2004). Hence Asf1 may not
directly deposit histones onto DNA, instead, it may act as a histone donor at the
intersection of different assembly pathways (Loyola and Almouzni, 2004). Deletion of
\textit{ASF1} in yeast causes slow cell growth, silencing defects, and sensitivity to DNA damage
agents (Le et al., 1997; Singer et al., 1998; Tyler et al., 1999).

Nap1 (nucleosome assembly protein 1) is an H2A/H2B chaperone (Ishimi et al.,
1984). Nap1 has a shuttle function. It shuttles histones from the cytoplasm to the nucleus
as cells progress from G2 phase to S phase (Ito et al., 1996). Deletion of the \textit{NAP1} gene
has no effect on cell growth but affects the expression of about 10% of the genome
(Kellogg and Murray, 1995; Ohkuni et al., 2003).

Some histone chaperones function in histone storage. Nucleoplasmin (Np) and
N1/N2 are this type of histone chaperone (Akey and Luger, 2003; Woodland and
Adamson, 1977). They are the most abundant proteins in the nuclei of \textit{Xenopus} oocytes
(Mills et al., 1980). Np specifically binds H2A/H2B, whereas N1/N2 is an H3/H4
chaperone (Akey and Luger, 2003). \textit{Xenopus} oocytes have large amount of stored
histones for the packaging of chromatin in about 10 thousand somatic cells (Akey and
Luger, 2003; Woodland and Adamson, 1977). Np and N1/N2 therefore store these core
histones in oocytes and allow the progressive release of histones and nucleosome
assembly right after fertilization. Homologues of \textit{Xenopus} Np and N1/N2 have only been
found in higher eukaryotes in the animal lineage, but not in yeast, worms, or plants (Akey
and Luger, 2003). They are mostly present in germinal cells and have more specialized
functions compared to other histone chaperones such as CAF-1 or Nap1 (Akey and
Luger, 2003; Loyola and Almouzni, 2004). Interestingly, somatic forms of NASP, the
human N1/N2 homolog, have been shown to bind specifically to linker histone H1 instead of core histones (Richardson et al., 2000). Furthermore, NASP is a component of the deposition machinery associated with both H3.1 and H3.3, indicating that human N1/N2 homologs may play different roles in nucleosome assembly than those of *Xenopus* (Tagami et al., 2004).

*In vivo* nucleosome assembly starts with the acetylation of newly synthesized histones, catalyzed by type B HATs (Haushalter and Kadonaga, 2003; Tyler, 2002). The Hat1p/Hat2p complex specifically acetylates free histone H4 suggesting a connection between Hat1p/Hat2p and nucleosome assembly (Annunziato and Hansen, 2000). However, no direct evidence to support this connection has been provided. We have purified and identified the nuclear form of the yeast Hat1p/Hat2p complex (Chapter 2). Nuclear Hat1p and Hat2p were found in a high molecular weight complex containing Hif1p and acetylated histones H3 and H4. Characterization of the nuclear Hat1p/Hat2p complex revealed that Hif1p is a novel histone chaperone. Hif1p shows sequence similarity to the *X. laevis* histone chaperone N1. Recombinant Hif1p binds specifically to histones H3 and H4. In addition, *in vitro* assays identify Hif1p as a chromatin assembly factor. *In vivo*, Hif1p binds to acetylated histone H4 (as well as histone H3) in a Hat1p/Hat2p dependent manner, thus providing a direct physical link between type B HATs and the process of chromatin assembly.
3.3 Materials and Methods

Yeast strains and plasmids

Yeast culture and genetic manipulation were done according to standard methods (Adams, 1998). HIF1 was Myc tagged at the COOH-terminus in UCC1111, ASY50 (hat1Δ), TKY102 (hat2Δ) and TKY105 (hat1Δhat2Δ) to generate XAY10, XAY12, XAY14 and XAY15, respectively (Kelly et al., 2000; Longtine et al., 1998). The presence of the epitope tags was confirmed by both PCR and western blot using antibodies against c-Myc. Gene deletions were generated by PCR-mediated gene disruption with HIS3 unless otherwise indicated. ASF1 and CAC1 were disrupted in UCC1111 to generate SQY114 and TKY108, respectively (Kelly et al., 2000; Qin and Parthun, 2002). HIF1 was disrupted in NKY2 to generate XAY7 (See “Materials and Methods” in Chapter 2).

pTrcHis2A (Invitrogen) was used as the expression vector for recombinant Hif1p. The HIF1 gene was obtained by PCR. An Ncol site and HindIII site were introduced at the 5’ and 3’ end of the HIF1 gene respectively for cloning purpose. The HIF1 gene was cloned into pTrcHis2A between the Ncol and HindIII sites to generate pXA4. The mutated sequences on HIF1 were changed back to wild type on pXA4 using a site-directed mutagenesis kit (Stratagene) to generate pXA5. The HIF1 gene sequence on pXA5 was confirmed by DNA sequencing.

Protein expression and purification

Purification of the nuclear Hat1p/Hat2p complex is performed as in Chapter 2 (Materials and methods).
Recombinant Hif1p was expressed using pXA5. [His]$_6$ and Myc tags were introduced at the COOH-terminus of Hif1p with this expression strategy. 1 mM IPTG was added to the *E. coli* culture at the O.D$_{600}$ of 0.4, and sat at 18°C for 1 h to allow the culture temperature drop from 37°C to 18°C. The inducted cells were grown at 18°C for 24 h for protein expression.

Purification of [His]$_6$ and Myc-tagged recombinant Hif1p proceeded from 1 L of *E.coli* cells. The cell pellets were resuspended in Start buffer (20 mM sodium phosphate [pH 7.4], 500 mM NaCl) and incubated with lysozyme (1 mg/ml cells) on ice for 30 min. Cells were lysed by an alternating sonication and freeze-thaw procedure, and pelleted at 3,000×g at 4°C for 15 min. Protease inhibitors (PMSF, Pepstatin, Leupeptin, and protease inhibitor cocktail, Sigma) were added to prevent the degradation of rHif1p. The supernatant of the lysate was applied to a Ni$^{2+}$ charged HiTrap Chelating HP column. The column was washed with 3 CV (column volume) of Wash buffer 1 (Start buffer + 20 mM Imidazole) and Wash buffer 2 (Start buffer + 50 mM Imidazole) before elution. Recombinant Hif1p was eluted with 500 mM of Imidazole and confirmed by western blot using anti-c-Myc antibodies. Purified rHif1p was dialyzed against DN(50) and concentrated to 0.5 mg/ml with Centricon YM-30 before use.

*Western Blots*

Western blots were performed and visualized using either an ECL-PLUS chemiluminescent detection kit (Amersham) according to manufacturer’s instructions or a premixed BCIP/NBT solution (Sigma) as a substrate for alkaline phosphatase.
**Histone Binding Assay**

The rHif1p/histone binding assay was performed as follows: Anti-c-Myc agarose conjugate (Sigma) was equilibrated with PBS, and incubated with binding buffer (25 mM HEPES, 200 mM KCl, 13 mM MgCl$_2$, 10 % glycerol, 0.1% NP-40, and 0.3 % β-Mercaptoethanol (Munakata et al., 2000), Myc-tagged rHif1p (1 µg) and/or purified chicken core histones (12.5 µg) at 4°C for 2.5 h with rotation. Unbound proteins were removed by extensive washing with binding buffer. The bound proteins were eluted with 20 µl 2×SDS loading buffer and analyzed by 18% SDS-PAGE and Coomassie blue staining.

**Immunofluorescence microscopy**

Immunofluorescent localization was performed as described in Chapter 2. The fixed cells were blocked and probed with primary antibodies (α-Hat2p or α-Myc, Sigma). Hat2p and Hif1p-Myc were visualized with Alexa and CY3 conjugated secondary antibodies, respectively. Nuclear DNA was visualized by DAPI staining. The fluorescence images were taken with a Zeiss LSM510 multiphoton confocal inverted microscope. A *hat2Δ* strain (TKY102) was used as negative control.

**Co-immunoprecipitation Assay**

Yeast whole cell extracts were used in Hif1p co-immunoprecipitation assay. Yeast whole cell extracts from UCC1111, XAY4, XAY10, XAY12, XAY14, and XAY15 cells were prepared as described (Schultz et al., 1991). Briefly, cells were harvested at mid-log phase and washed with cold H$_2$O and extraction buffer (100 mM
HEPES, pH7.9, 245 mM KCl, 5 mM EGTA, 1mM EDTA, 0.5 mM PMSF and 0.3% β-mercaptoethanol were added before use). The cell pellets were squirted through a 3 ml syringe into a 50 ml tube containing liquid N₂. The frozen pellets were ground to fine powder in the presence of liquid N₂ and incubated with a certain amount of extraction buffer on ice for 20 min (150 µl buffer/g cells) before the centrifugation was applied at 100,000×g for 1 h at 4°C. Supernatant was collected as whole cell extracts and dialyzed against dialysis buffer (20 mM HEPES, pH 7.9, 100 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, 20% Glycerol) before use.

Hif1p co-immunoprecipitation assay was performed with the same protocol as histone binding assay unless indicated. 0.25 ml of whole cell extracts from UCC1111, XAY4, XAY10, XAY12, XAY14, and XAY15 were incubated with binding buffer equilibrated anti-c-Myc agarose conjugate (Sigma) and 0.25 ml binding buffer at 4°C for 2.5 h with rotation. Bound proteins were eluted with 20 µl 2×SDS loading buffer and loaded on 18% SDS-PAGE. The protein gel was transferred to supported nitrocellulose membrane blot (Bio-Rad) and probed with anti-acetylated-H4 antibody. The blot was visualized using premixed BCIP/NBT solution (Sigma) as a substrate for alkaline phosphatase.

**In vitro chromatin assembly**

*In vitro* chromatin assembly assays were performed with 0.2 µg pUC18 DNA that had been relaxed with DNA topoisomerase I as a substrate. The assembly pre-reactions (20 µl total volume) contained 10 mM Tris-HCl, pH 8.0/1.0 mM EDTA/100 mM NaCl/100 µg/ml BSA and 0.6 µg purified chicken core histones, 3.0 µg purified rHif1p, 1
µl yeast nuclear extract or 1 µl yeast cytosolic extract as indicated. The pre-reactions were incubated on ice for 1 h, followed by addition of 0.2 µg relaxed DNA (4 µl) and 2 mM ATP (1 µl). The 25 µl of assembly reactions were incubated at 37°C for 1 h after which they were stopped with Stop Buffer (0.2% of SDS, 200 µg/ml protease K) at 37°C for 15 min. DNA was run on a 1.2 % agarose gel after phenol extraction and visualized with ethidium bromide staining.

3.4 Results

3.4.1 Cellular Distribution of Yeast Hif1p

Indirect immunofluorescence was used to better define the sub-cellular co-localization of Hif1p and the Hat1p/Hat2p complex. Visualization of Hif1p was facilitated by the genomic incorporation of a COOH-terminal Myc epitope. Importantly, the association of Myc tag onto Hif1p does not result in defects in telomeric silencing (Figure 3.1). Figure 3.2 shows the co-localization of Hat2p and Hif1p (visualized with anti-Hat2p or anti-Myc antibodies, respectively). As also seen in Chapter 2 (Figure 2.1), Hat2p is easily detectable in both the nucleus and the cytoplasm. Hif1p co-localized with Hat2p in the nucleus but there appeared to be little, if any, co-localization of Hat2p and Hif1p in the cytoplasm. Combined with the observation that Hif1p is not found in purified preparations of the cytoplasmic Hat1p/Hat2p complex, these results suggest that Hat1p and Hat2p are found in distinct complexes in the cytoplasm and nucleus and that the association of the Hat1p/Hat2p complex with Hif1p occurs following entry into the nucleus.
3.4.2 Expression and purification of recombinant Hif1p

We have shown that hif1Δ mimics the highly specific H3-dependent phenotypes seen in conjunction with hat1Δ (Chapter 2). This demonstrates that the association of Hif1p with the nuclear Hat1p/Hat2p complex is functionally relevant in the cell. To further investigate the biological function of Hif1p, we expressed and purified recombinant Hif1p in E. coli.

pXA5 plasmid derived from the pTrcHis2A expression vector (Invitrogen) was used to express recombinant Hif1p. The HIF1 gene was obtained by PCR and the sequence was confirmed by DNA sequencing. To optimize the expression of rHif1, different concentrations of IPTG (0.4 mM and 1 mM) were used for induction, different induction times (5 h and 24 h), and different expression temperatures (25°C and 37°C) were tested. The expressed proteins from induced cells were recovered by SDS buffer extraction and applied to 12% SDS PAGE for Western blot assay (Figure 3.3, top). Western blot bands stained for Hif1p were analyzed by volume quantitation using IMAGEQUANT software (Figure 3.3, bottom). The results showed that a longer induction time (24 h), lower temperature (25°C), and relatively higher IPTG concentration (1 mM) increased the expression of rHif1p. The degradation of expressed Hif1p had been an issue. As seen in Figure 3.3, there is even certain degree of degradation when Hif1p is expressed at basal level in non-induced cells. Lower inducing temperature appeared to decrease the level of degradation but could not prevent it completely. In an effect to minimize the proteolysis of recombinant Hif1p, 18°C was employed as inducing temperature in the experiment. Protease inhibitors, including protease inhibitor cocktail (Sigma), 1 µg/ml Pepstatin,
1 µg/ml Leupeptin, and 0.5 mM PMSF, were added to buffers to prevent further degradation during cell disruption and purification.

Cell lysate from pXA5 were loaded onto a Ni$^{2+}$ charged HiTrap Chelating HP column. Protein peak fractions were collected and loaded onto 12% SDS-PAGE and either visualized by Coomassie blue staining (Figure 3.4a, top) or analyzed by Western blot (Figure 3.4a, bottom). Pure recombinant Hif1p bands were obtained with elution. However, degraded Hif1p bands seen from the beginning of expression co-eluted with full length Hif1p. We tried to separate full length Hif1p from degraded fragments by further fractionating the elutes from a Ni$^{2+}$ affinity column on a Mono Q column, but were not able to separate them completely (Figure 3.4b). However, as seen below, the presence of degraded fragments does not affect the function of full length rHif1p. Therefore, this Ni$^{2+}$ affinity column purified rHif1p was used for the functional assays performed below.

### 3.4.3 Hif1p is a Histone H3 and H4 Specific Histone Chaperone that Functions in Chromatin Assembly

Initial comparisons with the NCBI protein sequence database did not identify any proteins with obvious similarity to Hif1p. However, a search of the ProDom protein domain database indicated that HIF1 displays similarity to the *X. laevis* histone chaperone N1 (Servant et al., 2002). The level of sequence similarity is relatively modest (20% identical, 42% similar) but extends across the length of Hif1p (Figure 3.5). In addition, Hif1p contains an acidic patch that is characteristic of many histone chaperones.
N1 is a highly abundant protein in *X. laevis* oocytes that binds specifically to histones H3 and H4 (Dilworth et al., 1987; Kleinschmidt et al., 1986; Kleinschmidt et al., 1985; Kleinschmidt and Franke, 1982). N1 appears to function in both the storage and assembly of histone H3/H4 tetramers during the rapid rounds of DNA replication that occur early in *X. laevis* embryogenesis (Philpott et al., 2000).

Hif1p, containing COOH-terminal [HIS]$_6$ and Myc tags, was expressed in and purified from *E. coli* (Figure 3.4 and 3.6, lane 2). Following incubation with core histones, the purified rHif1p was immunoprecipitated with anti-Myc antibodies. As seen in Figure 3.6, only histones H3 and H4 co-precipitated with rHif1p. Quantitation of the co-precipitated Hif1p and histone H3 and H4 indicated that a single H3/H4 tetramer was bound to each Hif1p molecule (Figure 3.6, bottom). It is unlikely that the binding of Hif1p to histones H3 and H4 was the result of non-specific electrostatic interactions as the positively charged histones H2A and H2B did not associate with Hif1p. Hence, consistent with the observed sequence similarity between Hif1p and *X. laevis* N1, Hif1p is a yeast histone chaperone specific for H3 and H4.

We also tested whether rHif1p could directly participate in chromatin assembly. rHif1p was incubated with purified core histones and a relaxed circular plasmid. In this assay, chromatin assembly activity is indicated by the introduction of supercoils into the relaxed plasmid as histones are loaded onto the DNA. As seen in Figure 3.7 (lane 7), rHif1p alone could not deposit core histones onto a relaxed plasmid. To determine whether additional factors were required for Hif1p-dependent chromatin assembly, yeast cytosolic or nuclear extracts were added to the reaction (Figure 3.7, lanes 8 and 9). While the addition of nuclear extract had no effect, combining rHif1p with a yeast cytosolic
extract led to robust chromatin assembly activity. In the absence of rHif1p, the cytosolic extract induced only a small increase in plasmid supercoiling (Figure 3.7, lane 11). Hence, Hif1p is a chromatin assembly factor capable of directly participating in the deposition of histones onto DNA.

Next, it was important to determine whether endogenous Hif1p could also function as a chromatin assembly factor. Purified nuclear Hat1p/Hat2p/Hif1p complex was tested in the same chromatin assembly assay (Figure 3.8). Just as seen with rHif1p, the Hat1p/Hat2p/Hif1p complex promoted the conversion of the relaxed circular plasmid into a highly supercoiled form in the presence of histones and yeast cytosolic extract (Figure 3.8, lane 7). On the contrary, nuclear Hat1p/Hat2p complex purified from hif1Δ strain (XAY7, HAT2 was TAP-tagged) was not able to promote supercoiling even in the presence of yeast cytosolic extract (Figure 3.8, lane 11). This result makes two important points. First, native Hif1p, in the context of the Hat1p/Hat2p/Hif1p complex, can directly function in chromatin assembly. Second, the observation that the chromatin assembly activity of the Hat1p/Hat2p/Hif1p complex still requires the addition of cytosolic extract indicates that this extract is providing a factor(s) other than Hat1p or Hat2p. This conclusion was further supported by the observation that cytosolic extracts from strains lacking Hat1p and/or Hat2p are still capable of promoting rHif1p-mediated chromatin assembly (Figure 3.9, lanes 6-11).

The synergistic increase in chromatin assembly seen with the combination of rHif1p and the factor(s) present in the yeast cytosolic extract is reminiscent of the interplay between Asf1p and the CAF-1 complex (Mello et al., 2002; Sharp et al., 2001; Tyler et al., 1999). To determine whether Hif1p was acting in conjunction with either of
these factors, chromatin assembly assays were performed with rHif1p, purified histones and yeast cytosolic extracts prepared from cells lacking the largest subunit of the CAF-1 complex ($\text{cac1}^\Delta$), Asf1p, or Hir2. As is apparent from Figure 3.10 (lanes 6-10), neither Cac1p nor Asf1p is required for the chromatin assembly activity of Hif1p suggesting that Hif1p may be functioning as part of an uncharacterized chromatin assembly pathway. Hir2 is not necessary for the chromatin assembly activity of Hif1p either (Figure 3.10, lane 11; Figure 3.11, lane 6). However, when rHif1 was omitted from the reaction, supercoiling was actually promoted in the presence of histones and cytosolic extract from $\text{hir2}^\Delta$ strain (Figure 3.10, lane 12; Figure 3.11, lane 7). The explanation could be that there is a distinct chromatin assembly pathway which is suppressed by Hir2. When $\text{HIR2}$ is deleted, the pathway would be activated and promote chromatin assembly. The activation of this pathway may or may not be related to Hif1p. Therefore, when cytosolic extract from $\text{hir2}^\Delta$ strain was used, the supercoiling observed on lane 10 (Figure 3.9) and lane 5 (Figure 3.10) could result from both Hif1p mediated nucleosome assembly and the unknown pathway. However, in the absence of Hif1p, the unknown pathway is exclusively responsible for the nucleosome assembly (Figure 3.9, lane 11; and Figure 3.10, lane 6). Moreover, when the cytosolic extracts from wild type cells were added back to the assembly reaction of lane 6 (Figure 3.10), the relaxed DNA was not able to be converted to supercoiling form (Figure 3.11, lane 7), indicating the block of the unknown pathway by Hir2 upon the addition of wild type cytosolic extract. To identify the components of this pathway and reveal the connection between this and Hif1 pathway would be of great importance and interest.
3.4.4 Hif1p Associates with Histones In Vivo in a Hat1p/Hat2p-Dependent Manner

The assertion that Hif1p functions as a chromatin assembly factor leads to the strong prediction that it should be associated with histones \textit{in vivo}. We have shown the \textit{in vivo} association of the nuclear Hat1p/Hat2p/Hif1p complex with histones H3 and H4 from the analysis of the purified complex (Chapter 2). To obtain additional evidence for the \textit{in vivo} association of Hif1p with acetylated histone H4, extracts from strains containing Myc-tagged Hif1p were immunoprecipitated with anti-Myc antibodies. Western blots containing immunoprecipitated proteins were then probed with antibodies that specifically recognize acetylated forms of histone H4. As seen in Figure 3.12, acetylated histone H4 was co-immunoprecipitated with anti-Myc antibodies in the presence of Hif1p-myc but not from an untagged control strain confirming the cellular interaction of acetylated histone H4 with Hif1p (Figure 3.12, lanes 1 and 2).

We next tested whether Hat1p and Hat2p were important for the interaction between Hif1p and acetylated histone H4. Myc-tagged Hif1p was introduced into \textit{hat1\Delta}, \textit{hat2\Delta} and \textit{hat1\Delta hat2\Delta} strains and co-immunoprecipitations were performed with anti-Myc antibody. Strikingly, while equal amounts of Hif1p-myc were precipitated in each reaction, both Hat1p and Hat2p were required for the association of Hif1p with acetylated histone H4 (Figure 3.12, lanes 3-5). Hence, at least one role of the Hat1p/Hat2p complex may be to provide the histones substrates used by Hif1p for deposition.

3.4 Discussion

The results presented here provide new insight into the function of type B HATs. While these enzymes have long been presumed to function in histone deposition, there
has been no direct evidence linking the acetylation of newly synthesized histones and the process of chromatin assembly. We have showed that the nuclear form of Hat1p/Hat2p type B HAT complex associates with an additional factor, Hif1p (Chapter 2). Here we have demonstrated that Hif1p is a novel histone H3/H4-specific histone chaperone that functions in chromatin assembly. Hif1p complex associates with acetylated histone H4 (as well as histone H3) \textit{in vivo} and this interaction is mediated by Hat1p and Hat2p. Thus, these results demonstrate a physical connection between type B HATs and factors directly involved in the process of chromatin assembly.

\textit{Where Does Hat1p/Hat2p Complex Function?}

We have demonstrated that the Hat1p/Hat2p type B HAT complex is found in both the cytoplasm and nucleus (Chapter 2). Our data support the conventional model in which newly synthesized histone H4 is acetylated in the cytoplasm prior to nuclear import (Chapter 2). As depicted in Figure 3.13, evidence now suggests an update to models describing events subsequent to the initial acetylation of newly synthesized histones (Chapter 1, Figure 1.1). The interaction between the Hat1p/Hat2p complex and newly synthesized histone H4 does not appear to be transient, but rather, this association may persist through the addition of histone H3 and import into the nucleus. Once in the nucleus, Hat1p and Hat2p interact with the chromatin assembly factor Hif1p which then functions with as yet unidentified factors to promote histone deposition. Whether the Hat1p/Hat2p complex functions in a similar capacity in delivering histones to other chromatin assembly factors, such as CAF-1 and Asf1p, remains to be determined.
This model raises the question of why a type B HAT complex would remain stably associated with its substrate in the nucleus. There are a number of possibilities. As has been discussed in Chapter 2, the Hat1p/Hat2p complex may facilitate import of the histones into the nucleus. Following histone deposition, the Hat1p/Hat2p complex could then shuttle back to the cytoplasm where it would be free to repeat the cycle with other newly synthesized histone molecules. The Hat1p/Hat2p complex may also accompany newly synthesized histones into the nucleus to protect their pattern of acetylation by either shielding the H4 NH$_2$-terminal tail from the action of nuclear HDACs or by recreating their acetylation state following deacetylation (Imhof and Wolffe, 1999a; Verreault et al., 1998). Combined with the results presented in this chapter, another possibility also exist, that is, the Hat1p/Hat2p complex may be more directly involved in histone deposition by actively facilitating the interaction of histones H3 and H4 with downstream chromatin assembly factors such as Hif1p. An intriguing aspect of these models is that the Hat1p/Hat2p complex would not be acting in a strictly catalytic capacity consistent with the observation that there are very high levels of HAT1 protein in the nuclei of *X. laevis* oocytes where large quantities of histones are stored prior to embryogenesis (Imhof and Wolffe, 1999a).

The association of the Hat1p/Hat2p complex with histones H3 and H4 in the nucleus may also reflect its action on substrates other than newly synthesized histones. While the vast majority of nuclear histones are packaged into chromatin, free histones may be transiently liberated by processes, such as transcription and DNA repair, which disrupt chromatin structure. An interaction between these histones and the Hat1p/Hat2p complex may serve a role in re-establishing chromatin structure following these events.
The N1 Family of Histone Chaperones

Members of the N1 family of histone chaperone proteins, originally identified in *X. laevis*, have been found in a number of animal species including humans and mice but have not been recognized in lower eukaryotes and plants (Kleinschmidt et al., 1986; O'Rand et al., 1992; Richardson et al., 2000; Welch and O'Rand, 1990; Welch et al., 1990). The identification of Hif1p as a histone H3/H4 specific histone chaperone suggests that this family may be more widely represented in eukaryotic evolution. In fact, database searches indicate that there are proteins in many organisms, such as *C. elegans*, *D. melanogaster* and *S. pombe*, that share a higher level of similarity with *X. laevis* N1 than does Hif1p. It will be interesting to determine whether these proteins also function as histone chaperones.

While *X. laevis* N1 and yeast Hif1p have shared physical characteristics, it is unclear whether they are true functional orthologs. The role of N1 in the storage of histones in *X. laevis* oocytes is a specialized function that does not have an obvious parallel in yeast. In addition, while both Hat1p and N1 are present at high levels in *X. laevis* oocyte nuclei, there is no evidence of any interaction between them (Imhof and Wolffe, 1999a; Kleinschmidt and Franke, 1982). However, the relevance of the nuclear Hat1p/Hat2p/Hif1p complex to higher eukaryotic chromatin assembly is suggested by the recent observation that each of their human counterparts, HAT1, p48 and NASP, was found to co-purify with the soluble forms of both histone H3.1 and H3.3 (Tagami et al., 2004).
Figure 3.1 HIF1-Myc tag has no effect on the function of Hif1p in telomeric silencing.

Telomeric silencing was measured by spotting 10-fold serial dilutions of cells on synthetic complete plates (HC) and synthetic complete plates containing 5-FOA. Plates were photographed after 3-4 days of growth at 30°C. The relevant genotypes of strains are indicated on the left. WT, wild type.
Figure 3.2 Co-localization of Hif1p with nuclear Hat2p

The co-localization of Hif1p and Hat2p was determined by indirect immunofluorescence and confocal microscopy. Cells of strain XAY10, in which Hif1p contains a COOH-terminal Myc tag were probed with anti-Hat2p and anti-Myc primary antibodies. Hat2p and Hif1p were visualized with ALEXA and CY3 conjugated secondary antibodies, respectively. *hat2Δ* cells were used as negative control. The images shown are A and F, differential interference contrast; B and G, DAPI; C and H, CY3; D and I, ALEXA and E and J, merge.
Figure 3.3 Optimization of recombinant Hif1p expression in *E.coli*

Plasmid that carries *HIF1* gene was expressed at the combination of different conditions as indicated. The expressed proteins from induced cells were recovered by SDS buffer extraction and applied to 12% SDS PAGE for Western blot assay (top). The Western blot was probed with anti-Myc antibody, visualized using ECL-PLUS chemiluminescent detection kit and analyzed with IMAGEQUANT. Migration positions of full length rHif1p and degraded Hif1p were indicated with arrows on the right side. The quantitation volume of each band is listed (bottom). The combination of 1 mM IPTG/24 h/25°C produced the optimal expression in terms of rHif1 yields and the ratio of full length rHif1 to proteolytic fragment Hif1p. Protein expressed in non-induced cells is shown on lane 1.
Figure 3.4 Purification of recombinant Hif1p from *E.coli*

Recombinant Hif1p was purified using Ni²⁺-affinity column. (A) The protein peak fractions at different wash conditions were collected and resolved by SDS-PAGE. The different Imidazole concentration was indicated on the top. The fraction number was indicated on the bottom. The protein was visualized with Coomassie blue staining (top) and analyzed with Western blot using anti-Myc antibody (bottom). Migration positions of full length rHif1p and degraded Hif1p were indicated with arrows on the right side. (B) rHif1 peak fractions were pooled and applied to NaCl linear gradient as indicated using Mono Q column. Migration positions of full length rHif1p and proteolytic fragment of Hif1p were indicated with arrows on the right side.
Figure 3.5 Schematic diagram showing the sequence similarity between *X. laevis* N1 and yeast Hif1p.
Figure 3.6 rHif1p binds specifically to H3 and H4

Immunoprecipitation assays were performed with purified proteins (Myc-tagged rHif1p and/or chicken core histones). After extensive washing, proteins that remained bound to the anti-c-Myc agarose resin were resolved by SDS-PAGE and visualized by Coomassie blue staining. Bands corresponding to full length and proteolytic fragments of rHif1p are indicated by asterisks. Lane 1 shows the pellet from a beads-only reaction, lanes 2 and 3 represent 50% of the respective input fractions and lanes 4-6 are the pellet fraction from reactions containing the indicated rHif1p and histones as indicated. Listed on the bottom is the quantitation volume of rHif1, H3 and H4 bands on lane 6.
Chromatin assembly activity of rHif1p was assayed by incubating the indicated factors with a relaxed circular plasmid. Following incubation, the plasmids were deproteinated, resolved by agarose gel electrophoresis and visualized by ethidium bromide staining. The migration of the supercoiled (S) and relaxed (R) forms of the plasmid are indicated by arrows. Core histones used for lane 14 was recombinant core histones. Lanes 1 and 2 show the template DNA before and after relaxation with topoisomerase, respectively.
Figure 3.8 Nuclear Hat1p/Hat2p/Hif1p complex promotes chromatin assembly \textit{in vitro}

Nuclear Hat1p/Hat2p complex purified from NKY2 or XAY7 (hif1Δ) was mixed with purified chicken core histones and yeast cytosolic extract as indicated. rHif1p was mixed with core histones and cytosolic extract as control (lane 3). Chromatin assembly activity was assayed as described in Figure 3.6.

<table>
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<tr>
<th></th>
<th>NUC. Hat1p COMPLEX</th>
<th>rHif1p</th>
<th>CORE HISTONES</th>
<th>CYT. EXTRACT</th>
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<td>WT</td>
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<td>hif1Δ</td>
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**Figure 3.8** Nuclear Hat1p/Hat2p/Hif1p complex promotes chromatin assembly \textit{in vitro}
Figure 3.9 *In vitro* chromatin assembly activity of Hif1p is independent of Hat1p and Hat2p

Core histones and rHif1p were incubated with cytosolic extracts derived from wild type, *hat1Δ* (ASY50), *hat2Δ* (TKY102), or *hat1Δhat2Δ* (TKY105) strains. Chromatin assembly activity was assayed as described in Figure 3.6.
Figure 3.10 In vitro chromatin assembly activity of Hif1p is independent of CAF-1, Asf1 or Hir2

Core histones and rHif1p were incubated with cytosolic extracts derived from wild type, \textit{cac1}\(\Delta\) (TKY108), \textit{asf1}\(\Delta\) (SQY114), or \textit{hir2}\(\Delta\) (ASY60) strains. Chromatin assembly activity was assayed as described in Figure 3.6.
Figure 3.11 Cytosolic extracts without Hir2p is capable of promoting chromatin assembly in the absence of Hif1p

Core histones were incubated with cytosolic extracts derived from wild type or hir2Δ (ASY60) strains with or without rHif1p. Chromatin assembly activity was assayed as described in Figure 3.6.
Equal amounts of whole cell extracts were incubated with anti-c-Myc agarose and proteins bound to the resin recovered by centrifugation. Bound proteins were resolved by SDS-PAGE and analyzed on Western blots probed with antibodies specific for acetylated histone H4 or Myc as indicated. Whole cell extracts were prepared from wild type strain (UCC1111) or strains containing Hif1p-myc (XAY10), or Hif1p-myc combined with hat1Δ (XAY12), hat2Δ (XAY14), or hat1Δhat2Δ (XAY15) as indicated above each lane.

Figure 3.12 *In vivo* association of Hif1p with acetylated histone H4 is dependent on Hat1p and Hat2p
Figure 3.13 A model describing the potential mechanism of action of the Hat1p/Hat2p type B HAT
In the current study, we purified and characterized the nuclear form of the yeast Hat1p/Hat2p complex. Nuclear Hat1p and Hat2p were found in a high molecular weight complex containing a novel histone chaperone, Hif1p, and acetylated histones H3 and H4. Hif1p binds specifically to histones H3 and H4. \textit{In vitro} assays identify Hif1p as a chromatin assembly factor. \textit{In vivo}, Hif1p binds to acetylated histone H4 (as well as histone H3) in a Hat1p/Hat2p dependent manner. In addition, new modification sites in the histone H4 core domain were identified. The results presented here demonstrate a physical connection between type B HATs and factors directly involved in the process of chromatin assembly, thus providing new insight into the function of type B HATs. More functional studies on Hif1p are necessary for us to further understand the exact mechanism of this histone chaperone. Furthermore, one of the novel modifications (H4 K91 acetylation) has been found to be involved in chromatin assembly and gene silencing. The mechanisms behind these are to be investigated.
4.1 Further investigation the Hif1p functions

4.1.1 Whether Hif1p is involved in DNA replication-dependent or –independent chromatin assembly

Hif1p has been shown to facilitate chromatin assembly \textit{in vitro} in the presence of yeast cytosolic extract. \textit{In vivo} chromatin assembly occurs in either a DNA replication-dependent or –independent manner. CAF-1 exclusively participates in DNA replication-dependent chromatin assembly, while Hir proteins are responsible for DNA replication-independent chromatin assembly. Asf1, on the other hand, is involved in both pathways. It would be interesting to reveal the pathway in which Hif1p participates. To investigate this, SV40 DNA replication reactions will be performed with rHif1, cytosolic extract, and core histones (Stillman, 1986; Tyler et al., 1999). SV40-origin containing plasmid, SV40 T-antigen and Hela extract containing all required replication factors are included in the replication reaction (Stillman, 1986). $[\alpha{}^{32}\text{P}]$dCTP is added to the reaction so that the newly replicated DNA is radiolabelled. The resulting gel would be visualized by ethidium bromide staining to show the supercoiling of bulk DNA. The supercoiling of newly replicated DNA will be revealed by autoradiography. If Hif1p mediated chromatin assembly is coupled with DNA replication, the preferential supercoiling of the newly synthesized DNA over the bulk DNA will be observed (Tyler et al., 1999). The fact that Hif1p is involved in DNA double-strand break repair (Chapter 2) suggests that Hif1p is very likely to promote chromatin assembly in a DNA replication-dependent manner.

CAF-1 functions in chromatin assembly through interactions with PCNA at the DNA replication site (Shibahara and Stillman, 1999). A co-immunoprecipitation experiment could be performed to examine whether PCNA co-immunoprecipitates with
anti-Myc antibodies using whole cell extract from a Hif1-myc tagged strain (XAY10, Chapter 3). A positive result will further confirm that Hif1p is involved in DNA replication-dependent chromatin assembly.

Recent work has shown that a human N1 homolog, sNASP, co-purifies with machineries responsible for either DNA replication-dependent or –independent chromatin assembly (Tagami et al., 2004). Hif1p may be an N1 homolog in yeast (Chapter 3, Discussion), thus, it is possible that Hif1p is involved in both chromatin assembly pathways.

4.1.2 Identify other proteins associated with Hif1p in yeast

We have shown that Hif1p associates both physically and functionally with nuclear Hat1p/Hat2p (Chapters 2 and 3). Hif1p also associates with H3/H4 in vivo. However, this would not exclude the possibility that Hif1p associates with other proteins as well in yeast. To investigate this, we can purify Hif1p complex from whole cell extract derived from a Hif1-myc tagged strain (XAY10) using immunoprecipitation with anti-Myc antibody. The resulting complex could be resolved on SDS-PAGE and the bands could be identified using mass spectrometry. One potential protein that may be identified with this strategy is the unidentified factor in the cytosolic extract that functions with Hif1p to promote histone deposition. Upon identification of other proteins that associate with Hif1p, more Hif1p functions could be revealed.
4.1.3 Determine the functional domains of Hif1p

*Saccharomyces cerevisiae* Hif1p comprises 385 amino acids. A predicted signal peptide cleavage site is at R219. The whole peptide would be divided into two domains at this site. A D/E rich domain is located in the COOH-terminal region. The expression pattern of recombinant Hif1 indicated the existence of at least two stable polypeptides upon proteolysis (See Chapter 3, Figure 3.4). Furthermore, proteolysis of Hif1p with trypsin might be able to reveal more stable polypeptides. To study the functions of different domains of Hif1p, truncated proteins such as 1-219, 220-385 could be produced and the functional studies including chromatin assembly assay and histone binding assay could be performed using these peptides.

It has been shown that the NH$_2$ terminal domain of Asf1 is functional both *in vitro* and *in vivo* (Daganzo et al., 2003). On the other hand, its acidic COOH-terminal tail seems not to be essential for function (Daganzo et al., 2003). Similarly, studies on Nap1 indicated that deletion of COOH-terminal acidic region has little effect on chromatin assembly. However, this acidic region has been shown to mediate the binding of Nap1 with histones (McBryant et al., 2003). The acidic tails of Np and Np-type proteins have been indicated to be dispensable as well (Akey and Luger, 2003). We would expect that studies on Hif1p would reveal a similar result. NH$_2$-terminal domain might be functional, but COOH-terminal region would be required for the electrostatic interactions between Hif1p, histones, and the target DNA.

Based on the functional studies of different fragments of Hif1p, functional domains or the full length protein could be crystallized. A crystal structure of functional domains would help identify protein-protein or protein-DNA interaction sites on Hif1p.
In addition to sites for histone binding, these protein-protein interaction sites could also include Hat1p and/or Hat2p binding sites and sites for other potential bound proteins. These sites could be distinct, or different interacting factors may compete for a single binding site.

4.1.4 How does Hif1p affect telomeric silencing and DNA double-strand break repair

We have shown that Hif1p participates in telomeric silencing (Chapter 2). The repression of genes at telomeres starts with the interaction of SIR (silencing information regulators) proteins with RAP1 (repressor activator protein 1) and the NH$_2$ termini of histones H3 and H4 followed by spreading of Sir2p, Sir3p, and Sir4p proteins along chromatin from the initiation site (Hecht et al., 1995; Hecht et al., 1996; Kayne et al., 1988; Strahl-Bolsinger et al., 1997; Thompson et al., 1994). Acetylation states on the NH$_2$-terminus of histone H4 have been shown to affect the binding of Sir3 and thus regulate the formation of heterochromatin (Carmen et al., 2002). In addition, acetylation of H4 K12 has been shown to be responsible for mediating the function of Hat1p in telomeric silencing (Kelly et al., 2000). As Hif1p appears to be involved in the deposition of histones associated with Hat1p, it would be interesting to determine whether the loss of HIF1 alters the acetylation state of histones near the telomere or affects the association of silencing factors such as Sir3p.

Hif1p has been also shown to be involved in DNA double-strand break repair (Chapter 2). It has been suggested that Hat1p might influence DNA double-strand break repair through Asf1p-dependent chromatin assembly (Qin and Parthun, 2002). Hif1p and Asf1p may mediate chromatin assembly at DNA damage sites through the same pathway.
Asf1p has been shown to dynamically interact with Rad53, the DNA damage checkpoint protein, to mediate chromatin assembly after DNA damage (Emili et al., 2001; Hu et al., 2001). Since Hif1p could be involved in the same chromatin assembly pathway as Asf1p, Hif1p might interact with Rad53 as well.

4.2 Significance of the acetylation of H4 K91

Several core domain modification sites on histone H4 that associate with nuclear Hat1p/Hat2p/Hif1p complex have been identified in the study (Chapter 2). These sites of core domain modification on histone H4 appear to be highly conserved as the acetylation of K91 and the methylation of R92 are also observed in bovine thymus (Zhang et al., 2003). The localization of H4 K91 in the crystal structure of the nucleosome suggests that this modification might be important for the docking of the H2A/H2B dimers with the H3/H4 tetramer (Akey and Luger, 2003; Luger et al., 1997). Thus, the acetylation of histone H4 K91 might be involved in regulating the formation of histone octamers during chromatin assembly.

Preliminary data indicated that the K91A mutants have defects on the formation of silent chromatin at the telomeres and at the silent mating locus HMR (unpublished data, Parthun group). The mutant strains showed the sensitivity to agents causing DNA single or double strands breaks as well (unpublished data, Parthun group). These phenotypes are consistent with the participation of this residue in the process of chromatin assembly. The mechanisms that the acetylation of K91 affects gene silencing could be involved in several aspects. Microarray analysis has shown the increase of gene expression at the telomere in K91A (unpublished data, Parthun group). In addition, CHIP
assay data indicated that the association of Sir2p with silent chromatin is affected in K91A (unpublished data, Parthun group). Furthermore, the acetylation of K91 could be required for the interaction with Hif1p. Hif1p may not be able to bind histones with K91A mutations, thus affecting the assembly. A co-immunoprecipitation experiment would be able to verify or exclude this possibility.

One extremely valuable work would be to identify the specific HAT that acetylates H4 K91. One strategy is to use the antibody that specifically recognizes acetylated H4 K91 to screen each single gene deletion yeast strain. However, if there are multiple HATs which function redundantly on the acetylation of H4 K91, it would be difficult to identify these HATs using this approach. Another strategy could be to start with in vitro HAT activity assay with yeast cell fractions using a synthetic peptide that contains the core domain of yeast histone H4 as substrate. The fractions that show the K91 acetylation activity would be subjected to further purification.
CHAPTER 5

MEMBRANE PROTEIN CRYSTALLIZATION IN LIPIDIC MESOPHASES:
DETERGENT EFFECTS

5.1 Abstract

The “cubic phase method” for growing crystals of membrane proteins uses a complex mixture of water, lipid, protein, and other components. The current view is that the cubic phase is integral to the process. Thus additives from whatever source introduce the possibility of destabilizing the phase, thereby compromising the crystallization process. Detergents are used to solubilize membrane proteins and are likely to be ported into the cubic medium with the target protein. Depending on the identity and concentration of the detergent, the cubic phase, which itself is membranous, may be solubilized or destabilized in such a way as to render it unsuitable as a crystal growing system. The nonionic detergent n-dodecyl-β-D-maltopyranoside is commonly used in membrane protein work. In this study, we evaluate its effect on the cubic mesophase of hydrated monoolein. X-ray diffraction was used for phase identification and mesophase microstructure characterization. The results show that while low levels of the detergent are tolerated, increasing concentrations trigger a cubic-to-lamellar phase transition in a temperature-dependent manner. This finding is rationalized in the context of
complementary molecular shapes of the lipid and the detergent and has implications for the mechanism of crystallization in lipidic mesophases as discussed.

5.2 Introduction

The medium-chain-length alkyl glycosides are high-solubility, nonionic detergents (Warr et al., 1986). Because of their mild nature, they have found extensive use in the solubilization of membrane proteins for subsequent structure characterization, reconstitution, and crystallization studies. The alkyl glycoside dodecyl maltoside (DM) has been used in such applications and is currently being evaluated for its impact on membrane protein crystallization using lipidic mesophases, hereafter referred to as the \textit{in} meso method (Caffrey, 2000). The latter makes use of a multicomponent system in which hydrated monoolein, as a cubic mesophase, figures prominently (Rummel et al., 1998). While the exact role of the cubic phase in the crystallization process is still a mystery, it exists as the major phase before and subsequent to protein crystal formation. Thus it would appear that it is an essential feature of the method. Accordingly, adventitious materials introduced into the system along with the protein or from whatever source have the potential of destabilizing the host cubic phase. Given the assumption that the cubic phase serves an essential role in the process, such a destabilizing effect might be considered detrimental to the overall objective. By the same token, because we do not understand the very mechanism of \textit{in} meso crystallization, it is possible that such additives might well facilitate the process (Caffrey, 2000).

The purpose of the current study is to evaluate the effect that DM has on the phase behavior and microstructure of hydrated monoolein under conditions similar to those
used in a typical *in meso* crystallization trial. This takes the form of monoolein dispersed with 40% (w/w) water, which exists in the cubic phase at 20°C (Hyde et al., 1984; Briggs et al., 1996; Qiu and Caffrey, 2000). The detergent would normally accompany the protein in the *in meso* crystallization mix because it is a common membrane-solubilizing agent. The actual concentration of DM that ends up in the mixture depends on the protein and the particulars of the purification process. Accordingly, in this study we examine the effect of DM over a range of concentrations.

The original *in meso* method was developed with bacteriorhodopsin as the test membrane protein. Crystallization was performed at 20°C in a 3:2 (w/w) mixture of monoolein and aqueous medium. With reference to the temperature-composition phase diagram of the monoolein/water system, the phase expected to form under such conditions of hydration and temperature is of the cubic type (Fig. 1; Qiu and Caffrey, 2000). In the case of other membrane proteins, a temperature other than 20°C may be preferred. Accordingly, we have examined phase behavior above and below 20°C in the range from 0°C to 40°C in this study.

The equilibrium phase diagram of the monoolein/water system shows a solid (as opposed to a liquid) crystal phase, prevailing at temperatures below 17°C (Figure 5.1). The former referred to as the lamellar crystal or Lc phase. It consists of lipid bilayer sheets stacked one atop the other with rigid chains oriented normal or tilted with respect to the bilayer plane. The Lc phase is most unlikely to support reconstitution and crystallization of membrane proteins, and at this point it is considered undesirable as far as *in meso* crystallization is concerned. The question arises, then, of how to access the pure cubic phase below 17°C if a coexisting solid Lc phase represents the equilibrium
state for the system. The answer lies in the ability of liquid crystal phases to undercool in the same way that water remains liquid when cooled appropriately to below 0°C. Indeed, it has been shown that special care must be taken to ensure the expression of equilibrium behavior in lipidic systems and that without it, metastability or undercooling prevails (Qiu and Caffrey, 2000). In the current study, the response of the monoolein/water system to increasing concentrations of DM was examined under both equilibrium and metastable conditions.

In what follows, we show how DM modifies the phase behavior of the monoolein/water system over a range of temperatures under equilibrium and metastable conditions. The phases formed are identified and characterized structurally by means of low- and wide-angle x-ray diffraction. At sufficiently high concentrations, DM completely destabilizes the cubic phase and triggers formation of the lamellar liquid crystal (L\(\alpha\)) phase. This effect is rationalized in the context of the detergent and the lipid as amphiphiles having complementary molecular shapes.

5.3 Materials and Methods
Monoolein (356.54 g/mol) was purchased from Nu Chek Prep (Elysian, MN) with better than 99% purity, as determined by thin-layer chromatography (Qiu and Caffrey, 1998) and was used without further purification. n-Dodecyl-\(\beta\)-D-maltopyranoside (510.6 g/mol) was from Anatrace (Maumee, OH). It had a reported purity in excess of 99%, as determined by high-performance liquid chromatography, and was used without further purification. Dextrose (glucose) and D-\((+)-\)maltose were from Fisher Scientific
Degradation products (Pittsburgh, PA). Ultrapure water was obtained from a Milli-Q Water System consisting of a carbon filter cartridge, two ion exchange filter cartridges, and an organic removal cartridge.

Sample preparation

Dry solid monoolein (~20 mg) was mechanically mixed with appropriate amounts of water or DM solution (~13 mg) in a syringe-based mixing device as described (Cheng et al., 1998) to achieve the desired sample composition. The preparations were made at room temperature (~20°C). For most of the samples, the aqueous component represented 40% (w/w) of the sample. The remainder of the sample consisted of monoolein. The DM solutions used covered the range from 0 to 0.5 M, corresponding to a final DM concentration in the overall mixture ranging from 0 to 0.2 M. The homogeneously mixed samples were transferred to 1-mm diameter quartz capillaries (Charles Supper, Natick, MA), flame-sealed and glued with 5-min epoxy (Hardman, Belleville, NJ), and were stored before data collection at either 4°C (equilibrium measurements) or at room temperature (metastable measurements) for anywhere from one to several days before use in x-ray diffraction measurements. The actual water content of the samples was determined gravimetrically with a microbalance (M3P-000V001; Sartorius Corp., Edgewood, NY) (Cheng et al., 1998).

In a separate study, samples were prepared as above with a weight ratio of DM (0.25 M aqueous solution) to monoolein from 2:3 to 3:1. In another study, samples were prepared in which the DM/monoolein weight ratio was fixed at 1:12 (corresponding to a
sample with 60% (w/w) monoolein and 40% (w/w) 0.25 M DM solution), while the water content of the sample was increased from 35% to 58% (w/w).

**X-ray diffraction**

Copper Kα x-rays (1.5418 Å, nickel (0.025 mm thick) filtered) for use in diffraction measurements were produced using a two-beam port rotating anode x-ray generator (18 kW, RU-300; Rigaku U.S.A., Danvers, MA) as described (Qiu and Caffrey, 2000). The sample-to-detector distance (~250 mm) was calculated using silver behenate ($d_{001}=58.376$ Å; Blanton et al., 1995).

Equilibrium phase x-ray diffraction patterns were collected in the temperature range from -15°C to 40°C in increments of 5°C in the heating direction. Up to eight samples were accommodated at one time in the temperature-controlled beryllium sample holder. Eight diffraction patterns (covering a real space range from ~2 Å to 160 Å at a sample-to-detector distance of 200 to 250 mm) were collected side by side behind a 25.4-mm-wide lead slit on a 200 mm 3,250 mm image plate (Fuji HR-IIIN; Fuji Medical Systems, USA, Stamford, CT). Before diffraction measurements were taken, samples were incubated at -15°C for at least 2 h to fully develop the equilibrium Lc phase. This is what we refer to as the standard “subzero degree” (Celsius) incubation. Subsequently, the temperature was increased to and samples were incubated at a particular measurement temperature for 5–10 h before the 30-min x-ray exposure was made.

The metastable phase diffraction measurements were performed in the temperature range from 0°C to 40°C. In this case, samples were divided into two sets. The first set was incubated initially at 20°C for 5 h, followed by a 30-min exposure. The
temperature was then dropped to 0°C in steps of 5°C with a 5–10-h incubation at each intermediate temperature, followed by the 30-min exposure. The second set of samples was also incubated at 20°C for 5 h, followed by a 30-min data collection period. Subsequently, the temperature was increased to 40°C in increments of 5°C, with the same thermal equilibration and data collection protocols as above.

All other measurements were performed at 20°C with a preincubation period of 5 h and a 30-min exposure as above.

*Image analysis*

A phosphorimage scanner (Storm 840; Molecular Dynamics, Sunnyvale, CA) operating at a resolution of 100 mm/pixel and a dynamic range of $10^5$ was used to read images recorded on the image plates. The radial integration of diffracted intensity was performed on all images with the FIT2D program (Hammersley, 1997), the output of which was intensity versus scattering angle plots for each frame. Diffraction peaks were fitted by Gaussians, using the FIT2D and PEAKFIT programs (Jandel Scientific).

**5.4 Results**

**5.4.1 Temperature-composition phase diagrams**

Two types of phase diagrams were constructed in the course of this study. The first represents equilibrium behavior, while the second incorporates metastability and the natural tendency of liquid crystal phases to undercool.
5.4.1.1 Equilibrium behavior

The equilibrium phase diagram for the three-component system consisting of monoolein, DM, and water constructed in the range from -15°C to 40°C is shown in Figure 5.2A. In reality, this is a partial phase diagram wherein the concentration of monoolein is held constant at 60% (w/w), while the two other components are varied one against the other in the remaining 40% (w/w) of the sample. For purposes of mapping out the phase diagram, an aqueous solution was used in which the final DM concentration was increased from 0 to 0.2 M. To ensure equilibrium conditions, i.e., to set the system in the equilibrium Lc phase, all samples were preincubated at -15°C for at least 2 h before data collection. The Lc phase was identified by its characteristic diffraction pattern (Qiu and Caffrey, 2000). It consists of a series of equally spaced powder rings in the low-angle region and a group of sharp wide-angle reflections.

The equilibrium phase diagram is dominated by the Lc phase at temperatures below 20°C, regardless of DM concentration (Figure 5.2A). Furthermore, the lamellar repeat of the Lc phase is relatively insensitive to added DM in that it holds steady at ~49.5 Å in the range studied (Figure 5.3B, Table 5.1). At and above 20°C, the solid Lc phase no longer exists. It is in this region that the assorted liquid crystal phases emerge, the identity and characteristics of which depend on DM concentration and temperature. Thus, in the absence of detergent, the cubic-Pn3m phase exists in what amounts to a sample consisting of 60% (w/w) monoolein and 40% (w/w) water in the 20–40°C range. As the DM concentration increases, the cubic-Pn3m phase gives way to the cubic-Ia3d and finally to the Lα phase. Regions of pure cubic-Pn3m, cubic-Ia3d, and Lα phase are observed in the 20–40°C range, and phase coexistence is found between the pure phases.
The cubic-Pn3m/cubic-Ia3d and the cubic-Ia3d/Lα boundaries shift to higher temperatures with increasing DM concentration.

5.4.1.2 Metastable behavior

To allow for the full expression of metastable phase behavior, all samples examined in this part of the study were prepared at 20°C. Under this condition, the cubic-Pn3m, cubic-Ia3d, and Lα liquid crystal phases are observed over the range of DM concentrations used (Figure 5.2B). This is the same behavior that was reported on in the equilibrium phase diagram above (Fig. 5.2A). However, in contrast to the equilibrium conditions, these samples were cooled slowly and in a stepwise manner to 0°C. As expected, this treatment allowed the liquid crystal phases formed at 20°C to undercool and to do so down to 0°C. Thus, along the 0°C isotherm the same series of phases extending from the cubic-Pn3m to the cubic-Ia3d and Lα phases is observed with increasing DM concentration as was seen at 20°C. Furthermore, the cubic-Pn3m/cubic-Ia3d and cubic-Ia3d/Lα boundaries that existed above 20°C extend smoothly down to 0°C. Comparing the phase diagrams in Figure 5.2, A and B, we see that metastability prevails in the latter in the temperature range from 0°C to 20°C. Where the Lc phase represents equilibrium phase behavior below 20°C, we now find it replaced by one or another of three different liquid crystal phases under metastable conditions. Thus what characterizes the metastable phase diagram is a complete absence of the solid Lc phase and the persistence of liquid crystal phases down to the lowest temperature examined.
5.4.2 Lattice parameter temperature and composition dependence

The temperature and composition dependence of the lattice parameters of the solid and the different mesophases in the equilibrium and metastable phase diagrams are shown in Figure 5.3. Both systems exhibit typical liquid crystal phase thermal expansivities within the limits of measurement accuracy (Briggs et al., 1996; Qiu and Caffrey, 2000). Thus, for example, we see that with increasing temperature the lattice parameters of the liquid crystal phases tend to fall (Figure 5.3, A and C). The effect is more pronounced in the cubic phases and less so for the Lα phase. The Lc phase is essentially temperature insensitive in the range studied, as expected for the solid state.

The lattice parameters of both the Lc and the liquid crystal phases are relatively insensitive to DM concentration up to 0.2 M (Figure 5.3, B and D). This is true under both equilibrium and metastable conditions. Nonetheless, the detergent can induce dramatic changes in phase behavior, depending on temperature and on whether equilibrium or metastability prevails.

In the studies just described, the concentration of monoolein in the system remained constant, while the relative amounts of the two other components, DM and water, varied in opposite directions. We have also examined the effect of holding the relative amounts of monoolein and DM constant while changing the overall water content of the sample at 20°C. The starting point for this study was the system corresponding to 60% (w/w) monoolein and 40% (w/w) 0.25 M DM solution (final DM concentration in the overall mixture, 0.1 M). According to the phase diagrams in Figure 5.2, this should place the system in the cubic-Ia3d or the cubic-Ia3d plus Lα coexistence region. The experiment was performed twice. On one occasion the cubic-Ia3d phase alone was
observed. On the other, cubic-Ia3d plus Lα phase coexistence was found (Figure 5.4 A). The effect of increasing the water concentration to 58% (w/w) under these conditions was to induce the formation of the cubic-Pn3m phase.

In a separate study, we examined the effect of holding constant the relative amounts of DM and water while adjusting the concentration of monoolein at 20°C. Practically, this was carried out by dispersing monoolein with increasing amounts of a DM solution of fixed concentration, in this case, 0.25 M. As in the previous study, the starting point for the study corresponded to a sample with 60% (w/w) monoolein and 40% (w/w) 0.25 M DM solution. The effect of increasing the relative amount of DM solution was to destabilize the cubic-Ia3d plus La phase coexistence and to trigger Lα phase formation (Figure 5.4 B). The latter existed pure at a final concentration of 8–10% (w/w) DM.

5.5 Discussion

The in meso method for growing crystals of membrane proteins makes use of hydrated monoolein in the cubic mesophase (Rummel et al., 1998; Luecke et al., 1999). Typically, the protein is stripped from the native biomembrane by solubilizing with a detergent. DM is commonly used for this purpose. The solubilized protein is dispersed and presumably reconstituted into the membranes of the cubic mesophase before crystal formation (Caffrey, 2000). On its journey into the monoolein-containing mixture, the protein is accompanied by the detergent and possibly by native membrane lipid. It is conceivable that the detergent plays a role in crystal growth. In the case of bacteriorhodopsin, the detergent used was octyl glucoside, an alkyl and a saccharide
homolog of DM. We are in the process of attempting membrane protein crystallization using proteins solubilized in DM. Accordingly, the purpose of this study was to determine first and foremost if the cubic phase formed by hydrated monoolein could tolerate DM at the levels expected to be present during a typical crystallization study. For this purpose, the partial phase diagrams in Figure 5.2 were constructed. A second objective was to evaluate the effect that temperature had on phase stability and the nature of the interaction between DM and the cubic phase. A final objective was to determine if metastability, which has been documented in the simple, two-component monoolein/water system (Qiu and Caffrey, 2000), would persist in the presence of DM. If so, then the range of temperatures in which the monoolein/DM/water system might be used for crystallization was to be determined. In all of these studies, x-ray diffraction was used. Thus, in addition to providing for an unequivocal identification of phase type, the method allowed for quantitation of phase microstructure.

5.5.1 Phase behavior

We begin our discussion of the effect that DM has on the phase behavior of monoolein/water by examining the equilibrium phase diagram of the latter two-component system (Figure 5.1). The \textit{in meso} method makes use of a system consisting of monoolein at 40\% (w/w) water. With reference to Figure 5.1 A, we see that this produces the cubic-Ia3d phase at 20°C. However, it is important to note that the phase boundary lines drawn in Figure 5.1 A are approximate and are a best visual fit to a set of phase identity coordinates in temperature-composition space (Qiu and Caffrey, 2000). Thus, in our experience a monoolein sample prepared with 40\% (w/w) water nominally will result
in the cubic-Pn3m phase and/or the cubic-Ia3d phase. This uncertainty has many origins. They include 1) errors in sample preparation involving relatively small (mg) quantities of lipid and water, 2) slight changes in temperature encountered during sample preparation, 3) the fact that phase behavior is very sensitive to temperature and composition in the vicinity of 20°C and 40% (w/w) water (see Figure 5.1 A), 4) the notorious capacity of the cubic phase to undercool, and 5) the minuscule differences in energy between the two cubic phases. Thus the sense is that reproducible behavior is expected only when extraordinary care is taken to ensure a high degree of accuracy in sample composition and a fixed thermal history during sample preparation. Many of these issues have been addressed (Hyde et al., 1984; Cheng et al., 1998; Qiu and Caffrey, 2000). Suffice it to say that samples prepared to a target composition of 40% (w/w) water can give rise to one and/or the other of the two cubic phases when prepared under standard conditions at 20°C.

The phase diagram in Figure 5.2 A shows how the detergent, DM, alters hydrated monoolein phase behavior as a function of temperature. At zero DM concentration, the phase change with temperature corresponds to the 40% (w/w) water isopleth (line of constant composition) in Figure 5.1 A. This is essentially what is observed. Specifically, the Lc phase persists up to 15°C at least and transforms to the cubic phase, in this case the cubic-Pn3m phase, with increasing temperature at and above 20°C. The cubic-Pn3m phase is stable up to 40°C. As DM is added to the aqueous phase, it has little impact on Lc phase behavior below 20°C. At and above 20°C, DM triggers a series of phase transformations. The first happens in the low millimolar DM concentration range and is of the cubic-Pn3m-to-cubic-Ia3d phase type. The second involves a cubic-Ia3d-to-La
phase transition, where the phase boundary rises from 0.1 M to 0.2 M DM with increasing temperature. To some degree, this behavior mimics what is happening in the simple two-component monoolein/water phase diagram as the water content of the sample is reduced isothermally (Figure 5.1A). We will revisit this point later.

The bulk of the in meso crystallization studies performed to date have been carried out at 20°C. Thus, we proceed to the question of the impact that DM has on cubic phase stability under such conditions and to its possible effects on membrane protein crystallization. The data in Figure 5.2B show that the cubic phase persists up to a concentration of ~0.1 M DM in the aqueous medium at 20°C and to ~0.2 M at 40°C. This is in excess, by a factor of 10, of the concentration of DM expected to be introduced along with protein to the in meso system. Our first conclusion therefore is that DM is likely to be compatible with membrane protein crystallization in meso in that it does not destabilize the cubic phase at low concentrations. Furthermore, the tolerance of the system for DM increases with increasing temperature, up to 40°C at least.

While the cubic phase is stable in the presence of low concentrations of DM, the system converts to the solid Lc phase below 20°C under equilibrium conditions. Thus, if equilibrium conditions were to prevail, the monoolein/water system could not be used for in meso crystallization below room temperature, simply because the cubic phase does not form there. This is where the phenomenon of metastability or undercooling becomes evident. We know from previous studies that the liquid crystal phases observed in the monoolein/water system undercool when treated appropriately (Qiu and Caffrey, 2000). This involves sample preparation at 20°C and subsequent slow cooling. The undercooled phases so formed have been known to persist for years. However, the equilibrium Lc
phase can be accessed by a low-temperature incubation at -15°C for as little as 2 h. Thus, by undercooling the cubic phase, we have an opportunity to carry out in meso crystallization all the way down to 0°C. The question that concerned us in the current study had to do with how DM might affect such undercooling behavior. The data in Figure 5.2B show clearly that undercooling persists over the entire DM concentration range examined and all the way down to 0°C. Thus, we proceed to our second conclusion that the monoolein/water system should prove useful in membrane protein crystallization over a relatively wide range of DM concentrations and down to 0°C.

5.5.2 Rationalizing the effects of DM

At low concentrations, DM has little effect on the phase stability of hydrated monoolein other than to induce a cubic-Pn3m-to-cubic-Ia3d phase transformation. At higher concentrations, however, DM destabilizes the cubic phase and triggers formation of the planar Lα phase. The concentrations of DM at which these effects occur are temperature dependent. However, temperature effects will be neglected for the moment. Qualitatively, we can understand the tendency of monoolein to form the highly curved cubic mesophase in light of its molecular shape (Figure 5A). The molecule has a relatively small polar headgroup consisting of glycerol and a single ester linkage. In contrast, the chain to which it is attached is long (18 carbons to be exact), with a kink in the middle of the chain arising from a cis double bond between the 9th and 10th carbon atoms. In the liquid crystal phase, the chain is considered “fluid,” with an abundance of trans/gauche isomers along the length of the chain. Thus the dynamically averaged shape of the molecule brings to mind a coneshaped object with the polar headgroup at the
pointed end of the cone and the methyl terminus of the acyl chain at the wider end. However, close packing of cones leads to a spherical object. The cubic phases have curved hydrocarbon/water interfaces that approximately parallel a minimal surface where the termini from adjacent monolayers in a bilayer meet (Hyde et al., 1984). Thus we should envision the dynamically averaged shape of an individual monoolein molecule more as a differentially curved wedge than as a cone. And now we proceed to the effect that DM has on the cubic mesophase of monoolein. If we examine the molecular structure of DM for a moment (Figure 5.5B), we immediately note a shape that is complementary to that of monoolein. DM, in contrast to monoolein, has a large polar headgroup consisting of two glucose moieties in a 1–4 glycosidic linkage, which in turn is glycosidically linked to a relatively short (12 carbon atoms long) alkyl chain. The corresponding dynamically averaged molecular shape that this conjures up is an inverted cone or wedge. Thus, if DM partitions into the lipid compartment of the monoolein mesophase, as is likely, given its amphipathic nature, one might expect that the tendency of the mixed system to create a curved hydrocarbon/water interface will be lessened as DM is titrated in. In the limit where curvature is completely removed, the bilayers flatten and a lamellar phase emerges. This is precisely the effect seen as DM is added to the cubic mesophase of hydrated monoolein (Figure 5.2).

There is an additional effect to consider as we attempt to rationalize DM’s effect on cubic phase stability as expressed in the phase diagrams of Figure 5.2. This has to do with the fact that the measurements were made under conditions where the concentration of monoolein remained constant while the relative amounts of DM and water varied in opposite directions. Thus, as the DM concentration was increased, the concentration of
water in the system decreased. Inspection of Figure 5.1 shows that lowering the water concentration destabilizes the cubic phase in favor of the lamellar phase. Thus it is possible that the effect of increasing DM concentration, as revealed in Figure 5.2, corresponds simply to a lowering of water concentration. However, while this effect may play some role, it cannot be the only one, for the following reason. At the highest concentration of DM used in the study, viz. 0.2 M DM in the aqueous medium (which constitutes 40% (w/w) of the overall sample, i.e., monoolein, water, and DM), the detergent accounts for 10% (w/w) of the overall sample, with water representing the remaining 30% (w/w). If we neglect the contribution of DM to the system, this corresponds to a monoolein/water mix with close to 33% (w/w) water. Referring back to the monoolein/water phase diagram in Figure 5.1, we see that a shift from 40% to 33% (w/w) water has little effect on phase behavior.

The foregoing assumes that there is no interaction between the detergent and water. In reality, we expect a large interaction because of the carbohydrate and, thus, waterloving nature of the DM headgroup. Accordingly, the addition of DM to the system will exert an osmotic or waterwithdrawing effect as water is entrained by noncovalent interactions in the immediate vicinity of the carbohydrate moiety and sequestered away from the water-loving glycerol headgroup of monoolein. Indeed, the effect may be to exaggerate the inverted wedge shape of the DM molecule and to enhance its ability to “unfurl” the bilayers that make up the cubic mesophase.

Indirectly, we have examined the effect that the carbohydrate portion of DM might have on the phase behavior of the monoolein system at 40% (w/w) water in the cubic phase at 20°C as follows. The lipid was dispersed with a solution containing
increasing amounts of either maltose, a disaccharide, or glucose, a monosaccharide, to mimic the sugar end of DM. The results in Figure 5.6 show that neither sugar destabilizes the cubic-Pn\textsubscript{3}m phase, which persists in the presence of 0.5 M maltose and 1.2 M glucose. In both cases, the sugar lowers the lattice parameter of the cubic phase, suggesting a water-withdrawing effect. It is interesting to note that the potency of the disaccharide, maltose, in this regard is approximately twice that of the monosaccharide, glucose. These data suggest therefore that the effect in which DM triggers the cubic-to-lamellar transition in hydrated monoolein is attributable, at least in part, to its amphipathic nature and to the complementarity of its molecular shape to that of the host lipid, monoolein.

In addition to mapping out the phase diagrams in Figure 5.2, the nature of the interaction between DM, monoolein, and water was examined in a system where a 0.25 M DM solution was mixed in increasing proportions with a fixed amount of monoolein. In this study, which was carried out at 20°C, the DM/water ratio was fixed (1:7 w/w), while the relative amounts of the aqueous solution and the lipid changed. The data are shown in Figure 5.4B, along with the relative amounts of all three components in the system. Moving from left to right in the figure, we find that the cubic-to-lamellar phase transition is induced. This happens despite the fact that the water content of the system has surpassed 60% (w/w). If the concentration of water were the only factor dictating phase behavior, such a large degree of hydration would have converted the system into the fully hydrated cubic-Pn\textsubscript{3}m (see Figure 5.1). Obviously, this is not the case, because the water carries detergent with it, which, at the end of the addition, has reached a
concentration of 10% (w/w) in the overall sample. Thus the system responds by adjusting phase more to the minor component, in this case the detergent, than it does to added water.

In a second and related study, the detergent/lipid ratio was fixed (at 1:12 w/w, 1:17 mol/mol) while the water content of the system was raised. The measurements were made at 20°C, and the results (Fig. 4 A) show that in this case increasing the water content caused a conversion to the cubic-Pn3m phase. By the end of the addition, the water content of the system is 58% (w/w) and the DM concentration is 3.3% (w/w) overall. Given the statement in the preceding paragraph that the detergent essentially dictates phase behavior, one might expect that a concentration of 3.3% (w/w) DM would stabilize the cubic-Ia3d phase or cubic-Ia3d plus Lα phase coexistence as opposed to the cubic-Pn3m phase (see Figure 5.2). We explain this apparent disparity by noting that the highest water content sample in Figure 5.4A has 58% (w/w) water. The detergent has reasonable water solubility (cmc = 0.2 mM, 0.01% (w/w)) and will partition into it. As a result, less is available for distributing into the lipid mesophase. In essence, having excess water in the system lowers the effectiveness of the detergent in altering lipid phase behavior.

The literature includes reports on the effects other additives have on the phase behavior of hydrated monoolein. In one from this group, NaCl was shown to profoundly stabilize the inverted hexagonal phase at the expense of the cubic-Pn3m phase (Caffrey, 1987). The effect continued up to 5 M NaCl. In a separate study, oleic acid was found to induce a cubic-Pn3m-to-cubic-Ia3d phase transition that could be reversed at high salt concentration (Aota-Nakano et al., 1999). Protonic equilibrium was examined in the
mixed monoolein/oleic acid system with the cubic-Ia3d, cubic-Pn3m, and inverted hexagonal phases stabilized at pH 6–7, 5.5–6, and <5.5, respectively.

5.5.3 Implications for membrane protein crystallization

As noted, our objective was to evaluate the effects that the detergent, DM, has on the phase properties of the system used for in meso crystallization of membrane proteins. What we find is that at low concentrations, and in the range expected for typical crystallization trials, DM does not have an impact on cubic phase stability. With increasing concentration, DM stabilizes the cubic-Ia3d over the cubic-Pn3m phase. It is not clear at this juncture whether this will affect crystallizability, because a careful study of the cubic phase type preference for crystallization has not yet been made. At sufficiently high concentrations, however, the detergent triggers a cubic-to-lamellar transition. It has been postulated that a lamellar phase represents the conduit from the bulk cubic phase to the crystal surface (Caffrey, 2000). The view is that the bulk cubic phase in which the protein is reconstituted gives way to a lamellar phase of the La type in the immediate environment of the crystal. Under conditions where crystal formation is favored, the protein migrates through the tortuosity of the cubic phase into a locally formed lamellar phase that serves as an epitaxial launch pad into the crystal. If indeed the lamellar phase figures in the crystallization process, then the presence in the in meso mix of a detergent such as DM, which ultimately favors Lα phase formation, may facilitate the process. But we have just shown that a relatively high concentration of DM is required in the monoolein/water system to induce Lα phase formation. It may be that such concentrations are achieved locally and transiently in a system with a low overall
detergent content and/or that the conditions prevailing at the time of crystallization lower the concentration of detergent needed to induce lamellar phase formation.

A partial temperature-composition phase diagram of the monoolein/water/DM system has been constructed in the temperature range from -15°C to 40°C under conditions of equilibrium (Figures 5.1 and 5.2). The cubic mesophase at 40% aqueous phase remains stable up to ~0.1 M DM at 20°C. At higher concentrations, the cubic phase is destroyed and a lamellar liquid crystal phase emerges. These effects have been explained on the basis of monoolein and DM having complementary molecular shapes. The postulated involvement of a local lamellar phase in the crystal growth process would suggest a beneficial role for the detergent in the in meso method of membrane protein crystallization. While the measurements reported herein refer to DM, it is likely that the results and conclusions apply to related detergents and have some generality. Similar measurements have been conducted under conditions that allow mesophase undercooling to occur. In this case, the cubic and lamellar phases persist all the way down to 0°C. This result suggests that phase metastability can be exploited to effect crystallization in meso with proteins that require low temperatures for stability.

5.6 References


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**Table 5.1 Equilibrium phase lattice parameter as a function of temperature and composition.**

This represents a tabulation of the data in Figure 5.3. Average values are reported where duplicate measurements were made.
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Table 5.2 Metastable phase lattice parameter as a function of temperature and composition.

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### Table 5.3 Phase lattice parameter as a function of temperature and composition representing DM solution effect

This represents a tabulation of the data in Figure 5.4. Average values are reported where duplicate measurements were made.

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Table 5.4 Phase lattice parameter as a function of temperature and composition representing excess water effect

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Table 5.5 Phase lattice parameter as a function of temperature and composition representing glucose effect

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<td>Pn3m: 100.3 Ia3d: 158.9</td>
</tr>
<tr>
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<td>Ia3d: 101.7</td>
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<td>Pn3m: 95.8</td>
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<td>Ia3d: 88.1</td>
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**Table 5.6 Phase lattice parameter as a function of temperature and composition representing maltose effect**

This represents a tabulation of the data in Figure 5.6. Average values are reported where duplicate measurements were made.
Figure 5.1 Phase behavior of monoolein, dodecyl maltoside, and water in various combinations.

A, temperature-composition phase diagrams of the monoolein/water (redrawn from Qiu and Caffrey, 2000); B, temperature-composition phase diagrams of the dodecyl maltoside/water systems (redrawn from Warr et al., 1986); C, the isothermal phase diagram for the monoolein/dodecyl maltoside/water system at 20°C. It was constructed based on the data in Figure 5.1, A and B, 5.2, and 5.4.
Figure 5.2 Identity and location in temperature-composition space of each phase and coexisting phases in the monoolein/dodecyl maltoside/water system as determined by x-ray diffraction.

Samples were prepared with 60% (w/w) monoolein and a 40% (w/w) aqueous solution of dodecyl maltoside. The molar concentration of DM on the lower abscissa as well as the DM and water % (w/w) concentration on the upper abscissa represent the final concentration in the overall mixture (lipid + water + DM). The diffraction measurements were made in the heating direction from -15°C to 40°C for the equilibrium phase diagram (A) and in the heating direction from 20°C to 40°C and in the cooling direction from 20°C to 0°C for the metastable phase diagram (B). The exposure time was 30 min, and sample incubation at each temperature was for a minimum of 5 h. The solid lines represent phase boundaries and are drawn to guide the eye. Phases: ◊, Lc; ○, Lα; △, cubic-Pn3m; ×, cubic-Ia3d. The solid symbols indicate the presence of ice. The high-temperature part of the phase diagram was repeated once, and the data points for the two sets of measurements are offset from one another slightly on the abscissa for clarity.
Figure 5.3 Temperature (A and C) and composition (B and D) dependence of the lattice parameters of the phases found in the monoolein/dodecyl maltoside/water system at the indicated temperatures and sample composition in units of molar dodecyl maltoside.

Measurements were made under equilibrium (A and B) and metastable (C and D) conditions. The phases and other conditions are as described in the legend to Figure 5.2.
Figure 5.4 Identity and lattice parameter of each phase and coexisting phases in the monoolein/dodecyl maltoside/water system as determined by x-ray diffraction in control experiments.

(A) The molar ratio of monoolein/dodecyl maltoside was fixed at 17:1 (12::1 w/w) corresponding to 40% (w/w) 0.25 M dodecyl maltoside and 60% (w/w) monoolein, while the water content of the sample was increased. The relative amounts of all three components in the system are shown along the upper abscissa. (B) The dodecyl maltoside concentration in the aqueous solution was fixed at 0.25 M, while the percentage aqueous medium in the overall mix was raised from 40% to 75%. The relative amounts of all three components in the system are shown along the upper abscissa. In this experiment, the dodecyl maltoside/water ratio was fixed. All measurements were performed at 20°C after samples were incubated for at least 5 h.
Figure 5.5 Space-filling models of monoolein (A) and dodecyl maltoside (B) in isolation and together (C).

The continuous line surrounding the models was drawn to give a sense of the dynamically averaged molecular volume occupied in the different arrangements. Light and dark spheres represent carbon and oxygen atoms, respectively.
Figure 5.6 Dependence of the cubic phase lattice parameter on glucose and maltose concentration in hydrated monoolein at 20°C.

Concentration refers to the final sugar content of the overall mixture (lipid + water + sugar). The maltose study was repeated once, and data from both data sets are shown. In one of the data sets the cubic-\textit{Ia3d} phase was observed under low sugar concentration. This fickle feature of cubic phase behavior is commented on in the text.


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