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The Yeast SWI/SNF Complex Structure and Function

Dissertation Presented

by

Joan Frances Flanagan

Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences,
Worcester

In partial fulfillment of the requirements for the degree of:

DOCTOR OF PHILOSOPHY

January 18, 2001

Biochemistry

Yeast SWI/SNF Complex Structure and Function

A Dissertation Presented

By

Joan Frances Flanagan

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DEDICATION

*In loving memory of my mother
Frances Monaghan Flanagan*

ABSTRACT

DNA is packaged within the cells' nucleus as a highly compact chromatin structure ranging between 100-400 nm fibers. The organization and alteration of this structure is mandatory in order to arbitrate DNA-mediated processes of the cell, including transcription, DNA replication, recombination and repair. Many different kinds of enzymes modify chromatin components and, in turn, regulate the accessibility of DNA. These multi-subunit enzymes have emerged as key regulators for several processes of the cell. Central to understanding how DNA-mediated processes are regulated is to comprehend the consequences of these modifications of chromatin, which lead to altered states of either activation or inactivation.

One class of factors known to modify chromatin structure is the ATP-dependent chromatin remodeling enzymes. This class of enzymes encompasses evolutionarily conserved multi-subunit enzymes, which appear to function by using the energy of ATP hydrolysis to disrupt histone-DNA interactions. The prototype of ATP-dependent chromatin remodelers is the *Saccharomyces cerevisiae* SWI/SNF complex. The yeast SWI/SNF complex is required for the full functioning of several transcriptional activators and for the expression of a subset of yeast genes, a notable number being inducible and mitotic genes. The purified complex is comprised of the following eleven different polypeptides: Swi2p/Snf2p, Swi1p, Swi3p, Snf5p, Snf6p, Swp73p, Arp7p, Arp9p, Swp82p, Swp29p and Snf11p. It has been established that a core of homologous subunits

(Swi2p, Swi3p, Swp73p, Snf5p and the Arp proteins) is conserved among the SWI/SNF-related complexes from several organisms (yRSC, hSWI/SNF, hRSC, *Drosophila* Brahma). However, the functional contribution of these polypeptides in the complexes for altering chromatin structure is largely unknown. In this study, biochemistry is used to examine the structure of the complex and function of individual subunits of the yeast SWI/SNF complex to understand better how these proteins are acting in concert to remodel chromatin. In addition, we examine a role for SWI/SNF complex in the process of DNA replication.

The relative stoichiometry of the SWI/SNF complex subunits was determined by *in vitro* biochemical studies. Co-immunoprecipitation has demonstrated that there is only one copy of Swi2p/Snf2p per complex. Subsequent radioactive labeling of the purified complex revealed that the complex contains one copy of each subunit per complex with the exception of Swi3p and Snf5p, which are present in two copies per complex.

The subunit organization of SWI/SNF complex has been more clearly defined by determining direct subunit-subunit interactions in the complex. The Swi3p component has previously been shown to be critical for complex function *in vivo* and essential for the integrity of the complex *in vitro*, and this study demonstrates that Swi3p serves as a scaffolding protein that nucleates SWI/SNF complex assembly. *In vitro* binding studies with Swi3p have revealed that Swi3p displays self-association, as well as direct interactions with the Swi2p, Snf5p, Swp73p, Swi1p and Snf6p members of the complex.

The direct interactions of the yeast SWI/SNF subunits with transcriptional activators, thought to be important for yeast SWI/SNF targeting, were examined. *In vitro* binding assays demonstrate that individual SWI/SNF subunits, Snf5p, Snf6p and Swi1p, and sub-complexes Swi2p/Swi3p and Swp73p/Swi3p can directly interact with specific domains of transcriptional activators of either the Swi5p zinc-finger DBD or VP16 acidic activation domain. This work begins to characterize the functional contribution of individual subunits, and cooperative sub-complexes that are critical for the SWI/SNF complex functional activities.

The yeast SWI/SNF complex was investigated for the ability to play a role in DNA replication. Interestingly, plasmid stability assays reveal that minichromosomes that contain DNA replication origin ARS121 is weakened when the SWI/SNF complex is non-functional. ARS121's SWI/SNF dependency is overcome by the over-expression of DNA replication regulatory protein, Cdc6p. Thus, this suggests SWI/SNF may either indirectly effect DNA replication by effecting the expression of Cdc6p, or has a redundant function with Cdc6p. In addition, several crippled derivatives of ARS1 acquire SWI/SNF dependence, and it is found that the SWI/SNF complex requires a transcriptional activation domain to enhance ARS1 function. These results reinforce the view that SWI/SNF play a role in two chromatin-mediated processes', transcription and DNA replication.

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LIST OF ABBREVIATIONS

aa- amino acid	EM- electron microscopy
ABF1- ARS binding factor 1	hSWI/SNF- <u>h</u> uman <u>s</u> witch/ <u>s</u> ucrose <u>n</u> on-fermentable complex
ACF- <u>A</u> TP-utilizing and <u>c</u> hromatin accessibility <u>f</u> actor	ISWI- imitation SWI
ACS- ARS consensus sequence	FACS- fluorescence assisted cell sorting
ARP- actin related protein	FPLC- fast performance liquid chromatography
BAF- BRG1 associated factors	GST- glutathione S-transferase
BAP- BRM associated proteins	GTF- general transcription factors
CEN3- yeast centromeric plasmid	H1- linker histone H1
CHD- <u>c</u> hromatin <u>h</u> elicase- <u>D</u> NA binding proteins	H5- linker histone H5
ChIP- chromatin immunoprecipitation	H2A-core histone H2A
CHRAC- <u>c</u> hromatin <u>r</u> emodeling and <u>a</u> ccessibility <u>c</u> omplex	H2B- core histone H2B
CiS - <u>c</u> onserved in <u>S</u> wi3p family members	H3- core histone H3
cpm- counts per minute	H4- core histone H4
CT- chloramine T	HDAC- histone deacetylase
DBD- DNA binding domain	HO- mating type switching endonuclease promoter
DUE- DNA unwinding element	IPTG- isopropyl- β -D-thiogalactopyranoside
EDTA- ethylenediaminetetraacetic acid	LacZ- β -galactosidase
<i>et al.</i> - et alibi (and others)	mcm- maintenance of minichromosomes

MDa- megadalton	TAE- tris acetic EDTA
NRD/ NURD - <u>n</u> ucleosome <u>r</u> emodeling and <u>d</u> eacetylase complex	TBP- TATA box binding protein
NURF- <u>n</u> ucleosome <u>r</u> emodeling <u>f</u> actor	TEA- triethanolamine
ORC- origin recognition complex	TCA- trichloroacetic acid
PAGE- polyacrylamide gel electrophoresis	URA3- yeast uracil 3 selectable marker
PMSF- phenylmethylsulfonyl fluoride	VP16- herpes simplex virus protein 16
pre-RC- <u>p</u> re- <u>r</u> eplication <u>c</u> omplex	WCRF- <u>W</u> illiams syndrome transcription factor-related <u>c</u> hromatin remodeling <u>f</u> actor
Rb- Retnioblastoma	WT- wild type
RNAPII- RNA Polymerase II	YEPD- yeast <u>e</u> xtract, <u>p</u> eptone, and <u>d</u> extrose media
RSC- remodels the <u>s</u> tructure of <u>c</u> hromatin	
SAGA- <u>S</u> pt- <u>A</u> da- <u>G</u> cn5- <u>A</u> cetyltransferase	
SANT- <u>S</u> wi3, <u>A</u> da2, <u>N</u> -CoR, <u>T</u> FIIB”	
SBF- Swi4-Swi6 binding factor	
SD- synthetic dextrose media	
SDS- sodium dodacyl sulfat	
SIN- <u>S</u> WI- <u>i</u> ndependent	
SPT- suppressor of Ty	
SV40- simian virus 40 DNA replication origin	
SWI/SNF- <u>s</u> witch/ <u>s</u> ucrose <u>n</u> on-fermentable complex	

CONTRIBUTIONS

During my graduate career I required the collaboration of many people within the University of Massachusetts Medical School as well as outside contributors. The assistance and/or support that were given were relevant to, and greatly influenced my thesis proposal. I have included some of the work of my collaborators in my thesis in order to present my data in a way that reflects this interplay. At this time I would like to describe these contributions.

In Chapter II, I iodinate the purified SWI/SNF complex and would like to thank Dr. Reid Gilmore's lab, especially many thanks to Dan Kelleher for his expertise and time, in providing the means to iodinate the SWI/SNF complex.

In Chapter III, I analyze the organization of the yeast SWI/SNF complex. Many thanks to Dr. Laurie Boyer for initiating the structure/function analysis of the yeast Swi3p subunit, which provides the basis of this chapter, including the *in vivo* functional data, as well as the *in vitro* gel filtration and Far Western analysis. I wish to thank Dr. Stephen Doxsey for his insight on binding experiments and providing the HA *in vitro* translation vector, as well as the anti-GST antibody. In addition, thanks to Dr. Bradley Cairns for the generous gift of ARP9 cloned in an *in vitro* transcription-translation vector.

In Chapter IV, the analysis of SWI/SNF subunit binding interactions with transcriptional activators is described. I would like to thank Dr. Brenda Andrews for providing the GST-SWI5 expression plasmid.

In Chapter V, I analyze the role of yeast SWI/SNF in DNA replication. I would like to thank Y. Marahrens, B. Stillman, J. F. Theis, C. Newlon, S. Eisenberg, and B. Tye for the various ARS plasmids used in this analysis and S. Johnston for GAL4 antibodies. In addition, I would like to thank Dr. J.F.X. Diffley for his insight on plasmid stability experiments.

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CHAPTER I

INTRODUCTION

Introduction

The capability of regulatory factors to access a specific DNA sequence is of critical importance for many routine cellular processes, such as transcription, DNA replication, recombination and repair. However, the packaging of DNA into chromatin fibers can be obstructive to these processes. Thus, DNA-mediated processes require the alleviation of chromatin repression for efficient function (Wolffe and Kurumizaka, 1998). Alterations of chromatin structure have been correlated to the process of transcription, in which chromatin displays either a less or more compact configuration when transcription is activated and inactivated, respectively (reviewed in Wolffe, 1994; Workman and Buchman, 1993; Workman and Kingston, 1998). So how is this chromatin structure alteration regulated and executed? The cell has evolved various classes of macromolecular protein complexes capable of modifying chromatin structure to either allow or prohibit DNA access (reviewed in Kuo et al., 1998; Struhl et al., 1998; Zhang et al., 1999).

The research described in this thesis investigates one such macromolecular protein complex involved in the alteration of chromatin structure, the *Saccharomyces cerevisiae* SWI/SNF ATP-dependent chromatin remodeling complex. Interestingly, this chromatin remodeling enzyme is highly conserved in evolution from yeast to man. Examination and dissection of the yeast SWI/SNF complex, with the amenability of yeast genetics and biochemical analyses, will provide a better understanding of the complex's

role in the alteration of chromatin structure, which is important for the regulation of DNA-mediated processes. Moreover, this study should be applicable to SWI/SNF-like complexes in higher eukaryotic systems. This chapter discusses the relationship between chromatin structure, DNA-mediated processes, and chromatin modifying enzymes, with emphasis on the yeast SWI/SNF complex.

Chromatin Structure

Historically it was thought that chromatin was a static structure, whose primary role was to function as a scaffold for the eukaryotic cell's enormous genome (average 3×10^9 bp of DNA). We now know that chromatin is highly dynamic and plays a large regulatory role in DNA-mediated processes (Belmont and Bruce, 1994).

Chromatin essentially consists of core histone proteins, linker histone proteins and non-histone proteins associated with the DNA filament (Thomas and Furber, 1976). Chromatin of interphase cells has many levels of compaction, ranging from the 10nm fibers, known as "beads on a string", to greater than a 400nm fiber, or chromenema filament (Belmont and Bruce, 1994). The most fundamental unit of chromatin is the nucleosome core particle, consisting of 147 base pairs of DNA wrapped ~ 1.75 turns in a flat, left-handed superhelix around a histone octamer. The histone octamer is comprised of four highly conserved core histone proteins, H2A, H2B, H3, H4. During nucleosome assembly, an H3-H4 heterotetramer is assembled onto DNA, and this initial structure is subsequently flanked by two H2A-H2B heterodimers (for review Fletcher and Hansen, 1996). All four of these highly basic histone proteins have a central tertiary structural

motif known as the histone fold that mediates histone-histone interactions required for octamer and nucleosome assembly (Arents et al., 1991; Luger et al., 1997). Each core histone also contains an N-terminal domain (21-45 amino acids) and in some cases a C-terminal domain (e.g. H2A), called “tails”, which flank the central domain. The tail domains are less structured and extend from the histone octamer core (Bohm and Crane-Robinson, 1984; Luger et al., 1997; see Figure I.1). It has been determined that the tails are not required for assembly of the nucleosome, but rather they are essential for both intramolecular and intermolecular folding of nucleosomal arrays into higher order compacted structures *in vitro* (Fletcher and Hansen, 1996). The association of linker histones, such as H1 and H5, as well as other non-histone proteins with the nucleosomal array lead to further compaction and stabilization of chromatin. The end result is a stable 30nm fiber, which can further self-associate into 100-400nm structures as visualized by EM in interphase cells or detected *in vitro* by biophysical methods (Carruthers et al., 1998; Fletcher and Hansen, 1996).

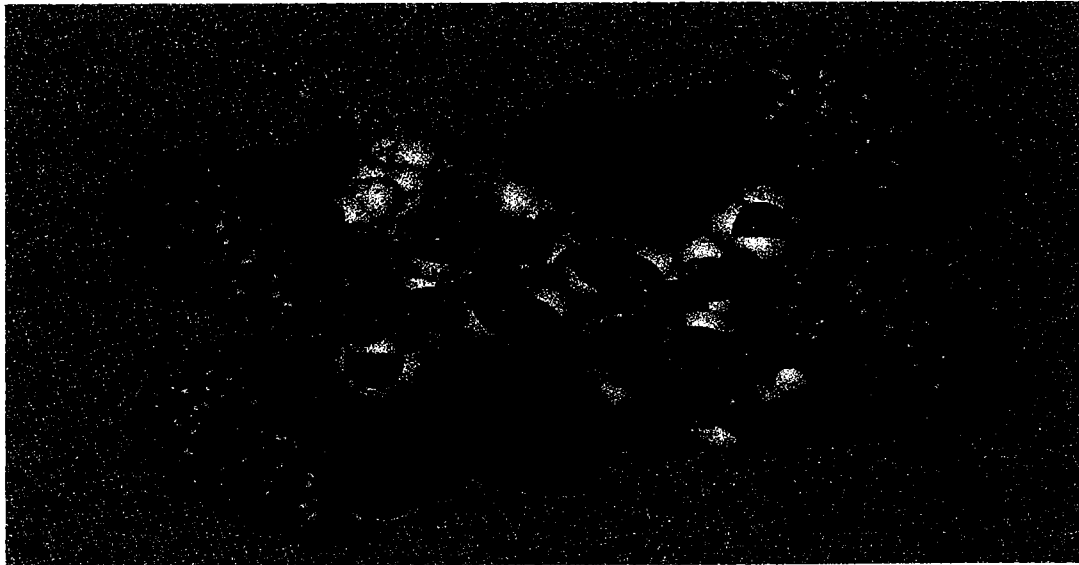
Notably, the N-terminal tail domains of the core histone are pertinent targets of regulation for many biological processes. The tails have been shown to be target sites for many post-translational modifications such as acetylation, phosphorylation, methylation, ADP-ribosylation and ubiquitination (reviewed in Fletcher and Hansen, 1996; Spencer and Davie, 1999; Strahl and Allis, 2000). These modifications have been linked to alterations in chromatin structure, apparently providing signals important for the regulation of DNA-mediated processes. In addition, these tail domains also serve as binding sites for transcriptional repressors and other non-histone proteins and can directly interact with the

Figure I-1. Representation of Nucleosome Structure. **A.** The nucleosome core particle is composed of 147 bp of DNA (gray) wrapped nearly two times around an octamer of histone proteins consisting of two copies of each core histones H2A (light blue), H2B (dark blue), H3 (green), and H4 (white). **B.** The histone octamer is composed of a tripartite structure; a central tetramer of histone H3 and H4 (white) flanked on each side by histone H2A/H2B heterodimers (blue). The phosphate backbone of DNA contacts charged residues on the surface of the histone octamer (shown in Red). Strategically placed arginine residues stabilize DNA on the surface of the histone octamer by interacting with the minor groove to create a left-handed superhelix (Garcia-Ramirez et al., 1992; Luger et al., 1997). Not shown here are the amino terminal domains, common sites of post-translational modifications, which exit the nucleosome through minor groove channels on all sides of the nucleosome. This figure was adapted from *Chromatin Structure and Gene Expression* 1995 Sarah C. R. Elgin, ed. Oxford University Press, NY.

A



B



tail domains (Johns, 1982; Roth, 1995; Dhalluin et al., 1999; Ornaghi et al., 1999).

Overall, it is believed that the N-terminal tail domains are key arbitrators in chromatin structure and function.

DNA-mediated Processes and Chromatin Alteration

In the interphase cell, chromatin is primarily packaged into 100-400nm condensed fibers, which is highly repressive to DNA-mediated processes. In fact, *in vivo* and *in vitro* studies have demonstrated that the most elementary level of chromatin, the nucleosome, is obstructive to the DNA-binding of regulatory factors to their specific sites (Axel et al., 1974; Lacy and Axel, 1975; Lorch et al., 1987; Simpson, 1978; Workman and Kingston, 1998; Workman and Roeder, 1987). Evidence has shown that alterations of nucleosome structure, which result in the enhancement of gene-specific activator binding, are essential in order for transcriptional activation to occur (reviewed in Fedor and Kornberg, 1989; Pazin et al., 1998; Workman and Kingston, 1998). In fact, it is generally recognized that disruption of chromatin is the rate-limiting step for most DNA-mediated processes. Therefore, the cell implements chromatin modifying enzymes in order to rapidly and reversibly unfold or de-compact chromatin at specific loci to facilitate DNA accessibility (or vice versa).

Transcription-associated Nucleosome Alterations. Chromatin structure plays an active role in the regulation of transcription activation. For many years it was known that transcriptionally active genes possess a more open or accessible chromatin state than inactive genes. Early evidence showed that although nucleosomes remain associated

with DNA at the actively transcribed genes, the chromatin structure existed in a more permissible state (Axel et al., 1974; Lacy and Axel, 1975). Eventually, nucleosome disruption was monitored by the use of nucleases, DNase I or micrococcal nuclease (MNase), which detected increased general sensitivity and hypersensitivity sites (Wu, 1980). The appearance of hypersensitive sites within regulatory regions mark active versus inactive genes for numerous inducible, developmentally expressed genes (reviewed in Krebs and Peterson, 2000), (Groudine et al., 1983; Groudine and Weintraub, 1982; Weintraub and Groudine, 1976; Wu, 1980). Today, the use of endonucleases in chromatin remodeling assays is also employed (Logie and Peterson, 1997; Hamiche et al., 1999; Langst et al., 1999), however little is known about the remodeled nucleosome conformations that occur (Groudine and Weintraub, 1982; Groudine et al., 1983; Wu, 1980; Schnitzler et al., 1998).

Chromatin Modifying Enzymes

Two broad classes of chromatin modifying enzymes are conserved throughout evolution (reviewed in Kuo and Allis, 1998; Struhl et al., 1998). One class is involved in post-translationally modifying the N-terminal tail domains of the core histones. For instance, histone acetyltransferases (HATs) modify chromatin by covalently adding an acetyl group to lysine residues at the N-terminal tails of the core histones (reviewed in Kuo and Allis, 1998). Histone acetylation can disrupt or promote interactions of non-histone proteins with the chromatin fiber, or it can destabilize the folding of nucleosomal arrays to promote a more "open", active state of chromatin (Hong et al., 1993; Krajewski

and Becker, 1998; Kuo et al., 1998; Lee et al., 1993; Tse et al., 1998). Conversely, histone deacetylase complexes (HDAC s) perform the opposite biochemical reaction, in which they remove acetyl groups from the N-termini of core histones establishing a more repressive state of chromatin (Kuo et al., 1998). Other enzymes in this class function to modify the N-terminal tail domains by phosphorylation, methylation or ADP-ribosylation (reviewed in Fletcher and Hansen, 1996; Spencer and Davie, 1999; Strahl and Allis, 2000). A second class of multi-subunit enzymes is the ATP-dependent chromatin remodelers, which use the energy derived from ATP hydrolysis to alter chromatin structure. These enzymes can disrupt the structure of the nucleosome, or influence the mobility and/or spacing of nucleosomes within arrays (Kingston and Narlilar, 1999; Tsukiyama et al., 1999; Kornberg, 1999). Moreover, genetic, biochemical, and genome expression analyses have demonstrated that these two classes of enzymes do not necessarily act independent of one another (Cosma et al., 1999; Holstege et al., 1998; Krebs et al., 1999; Pollard and Peterson, 1997; Sudarsanam et al., 2000). Collectively, chromatin-modifying enzymes greatly influence chromatin structure. Thereby investigating how these enzymes alter and regulate chromatin structure in such a dynamic fashion to allow DNA-mediated processes to occur properly and efficiently, is a fundamental question in understanding the mechanisms of DNA-mediated processes.

Importantly, human chromatin remodeling components have been correlated with the prevention of human disease. Specifically, histone acetyltransferases have been broadly implicated in cancer. For example, human CBP/p300 have been suggested to be transcriptional integrators that coordinate cues critical to cell cycle regulation, differentiation, DNA repair and

apoptosis. Disruption of these interactions could lead to alteration of one or more of these processes, and hence the progression of cancer or other diseases (reviewed in Jacobson and Pillus, 1999). The ATP-dependent SWI/SNF complex has also been implicated in human cancers. Mutations in the human Snf5p/Ini1p component of hSWI/SNF complex has been shown to be causative for some aggressive pediatric cancers, suggesting that Ini1p, and thus hSWI/SNF, is acting as a tumor suppressor (Versteeg et al., 1998). In addition, BRCA1, a tumor suppressor linked to breast cancer, is associated with a human SWI/SNF-related chromatin remodeling complex. Collectively, chromatin modifying enzymes regulate the access of specific DNA sequences during growth and development, which may be critical in the prevention of cancer (Wolffe and Hayes, 1999; Workman and Kingston, 1998).

ATP-Dependent Chromatin Remodelers. The ATP-dependent chromatin remodeling enzymes, which is the focus of this study, hydrolyze ATP in order to alter conformation of nucleosome structure, or alter the mobility of nucleosomes (for review see Kingston and Narlikar, 1999). This phenomenon is referred to as "chromatin remodeling" and is based on *in vivo* and *in vitro* studies that demonstrate that these enzymes facilitate perturbations of chromatin structure. These perturbations include changes in DNA-histone contacts as monitored by increased DNase I or MNase endonuclease digestion. Other functions for these chromatin remodeling enzymes include: the ability to promote activator-dependent transcription from assembled chromatin templates, the ability to influence nucleosome mobility resulting in either irregular or regular spaced nucleosomes, the ability to assemble chromatin by depositing nucleosomes onto DNA (Armstrong et al., 1998; Corona et al., 1999; Hamiche et al.,

1999; Ito et al., 1997; Jaskelioff et al., 2000; Kingston and Narlikar, 1999; Langst et al., 1999; LeRoy et al., 1998; Mizuguchi et al., 1997; Yudkovsky et al., 1999). Moreover, ATP-dependent chromatin remodeling has been coupled to other chromatin-modifying activities, such as histone acetylase/deacetylase and DNA repair activities (Brehm et al., 2000; Ebbert et al., 1999; Solari and Ahringer, 2000; Tong et al., 1998; Wade, 1998; Xue et al., 1998; Zhang et al., 1998).

The hallmark of ATP-dependent chromatin remodeling complexes is their SWI2/SNF2-like ATPase component. The SWI2/SNF2 family of proteins is based on sequence homology to the conserved nucleic acid-stimulated ATPase domain, which is a member of the DEAD/H super-family (see Figure I.2; Eisen et al., 1995; Pollard and Peterson, 1998). This ATPase domain contains seven (I-VII) regions, which are strongly conserved among family members, and in the case of Swi2p/Snf2p, each region is crucial for SWI/SNF function *in vivo* (Gorbalenya et al., 1989; Okabe et al., 1992; Richmond and Peterson, 1996). For example, a single residue change in the putative nucleotide binding loop (*swi2K798A*) behaves like a dominant negative mutant, which leaves the complex assembled yet eliminates SWI/SNF function (Cote et al., 1994; Laurent et al., 1993; Peterson and Herskowitz, 1992; Richmond and Peterson, 1996). The analogous mutation made in other SWI2 homologues (*BRG1K798R*; *sth1-K501R*; *brmK804R*; *iswi-K159R*, *isw1-K227A*, *isw2-K214A* or *ino80K737R*), also result in assembly-competent, dominant negative mutant phenotypes (Corona et al., 1999; Deuring et al., 2000; Ebbert et al., 1999; Gorbalenya et al., 1989; Laurent et al., 1993; Richmond and Peterson, 1996; Tsukiyama et al., 1999).

Figure 1.2 SWI2/SNF2-like ATPase Homologues. The hallmark of ATP-dependent chromatin remodeling complexes is a Swi2/Snf2p protein homologue. All homologues contain the SWI2/SNF2-like ATPase motif (red), a domain that is comprised of seven (I-VII) motifs, which are strongly conserved among all family members. Notably, motifs I and III contain the putative nucleotide binding and ATP hydrolysis sites, respectively. This class of chromatin remodelers can be further subdivided into three groups based on conservation to either yeast SWI2/SNF2, *Drosophila* ISWI or human Mi-2. Other motifs conserved in SWI2/SNF2-like homologues are the bromodomain (BrD, green), the SANT domain (yellow), the zinc-finger PHD motif (purple), and chromodomains (ChrD, blue).

ATP-dependent chromatin remodeling complexes can be further sub-divided into three groups based on whether their ATPase subunit is similar to either the yeast Swi2p, *Drosophila* ISWI (Imitation SWI), or human Mi-2/CHD proteins. The SWI/SNF-like sub-group is characterized by ATPase activity, which is stimulated by both nucleosomal and naked DNA substrates. This sub-group constitutes five members, including the yeast SWI/SNF and RSC complex, the human hbrm and hBRG1 complexes, and the *Drosophila Brahma* complex (Table I.2; Cairns et al., 1996; Dingwall et al., 1995; Wang et al., 1996).

Another sub-group is based on the *Drosophila* ISWI protein, which also contains a Swi2p ATPase domain, but its ATPase activity is only stimulated by nucleosomal DNA. This suggests that ISWI recognizes unique structural features of the nucleosome, which are contributed by both the DNA and core histones (see Figure I.2; Cairns et al., 1994; Corona et al., 1999; Cote et al., 1998; Cote et al., 1994; Georgel et al., 1997; Tsukiyama and Wu, 1995; Varga-Weisz et al., 1997). In support of this view, it has been shown that the N-termini of histones are critical for the ATPase and remodeling activity of ISWI-containing complexes (e.g. NURF). Presently the largest family, ISWI-like complex members include: *Drosophila* NURF, CHRAC and ACF (reviewed in Tsukiyama et al., 1999), human SNF2L containing complex, RSF, HuCHRAC and hACF/WRCF (Bochar et al., 2000; LeRoy et al., 2000; LeRoy et al., 1998; Poot et al., 2000; Wang et al., 1996) as well as, four distinct yeast complexes ISW1, ISW2, CHD1 and INO80, and variant ISWI-containing *Xenopus laevis* complexes (Tsukiyama et al., 2000; Guschin et al., 2000a; Table I.3).

The Mi-2/CHD-containing complexes define their own sub-group, since they contain both ISWI-like and SWI/SNF-like functions. Similar to ISWI, Mi-2 ATPase activity is stimulated best by nucleosomal DNA and less so by free DNA (Brehm et al., 2000; Guschin et al., 2000b; Tran et al., 2000). But similar to SWI/SNF, the ATPase domain and remodeling activity of Mi-2 remodeling function is histone tail-independent (Boyer et al., 2000; Brehm et al., 2000; Corona et al., 1999; Guschin et al., 2000) (Table I.4). Mi-2-like chromatin remodeling complexes include human NRD/NuRD, *Xenopus* Mi-2, *Drosophila* Mi-2 and *Caenorhabditis elegans* NuRD complexes (Kehle et al., 1998; Lu and Horvitz, 1998; Shi et al., 1999; Solari and Ahringer, 2000; Tong et al., 1998; Wade et al., 1998; Zhang et al., 1998; Table I.4). All Mi-2 complexes contain both ATP-dependent remodeling activity and histone deacetylase activity. The deacetylase activity of Mi-2 complexes is contributed by the histone deacetylase subunits (e.g. HDAC1, HDAC2, and Rpd3p). The ATP-dependent remodeling activity has been shown to enhance its deacetylation activity, and furthermore if histone deacetylase activity is inhibited, nucleosome remodeling is not effected (Hartzog and Winston, 1997; Hassig et al., 1998; Tong et al., 1998; Xue et al., 1998; Zhang et al., 1998). This indicates that these activities may be interdependent. Conserved subunits of Mi-2-containing complexes have also been shown to bind methylated DNA, suggests that SNA methylation may target these enzymes *in vivo*. Thus, Mi-2 complexes provide a functional link between different classes of chromatin modifying enzymes (ATP-dependent chromatin remodeling, deacetylase and DNA methylation). However the biochemical mechanism by which these activities contribute to generalized and specific

control of DNA-mediated processes is not known (Wade et al., 1998; Tong et al., 1998; Zhang et al., 1998; Xue et al., 1998).

SWI/SNF-like Chromatin Remodeling Complexes.

The yeast SWI/SNF complex, considered the prototype for ATP-dependent chromatin remodeling enzymes, is required for many transcriptional activators to enhance transcription in yeast (for reviews see Peterson and Tamkun, 1995; Winston and Carlson, 1992). Originally, five of eleven SWI/SNF subunits were identified in two independent genetic screens. One screen was designed to identify regulatory factors of the HO gene, which encodes an endonuclease required for mating type switching, and these mutants were termed SWI (mating type switching) (Stern et al., 1984). In an independent screen, factors involved in the regulation of the SUC2 gene, which encodes the enzyme invertase that is required for growth on non-fermentable carbon sources, were identified and called SNF (for sucrose non-fermenting) (Neigeborn et al., 1987). A subset of mutant genes identified by these two genetic screens, *swi1-*, *swi2-*, *swi3-*, and *snf2-*, *snf5-*, *snf6-*, respectively, share similar mutant phenotypes, such as slow growth, inefficient growth on non-fermentable carbon sources and gene-specific transcriptional defects (Abrams et al., 1986; Estruch and Carlson, 1990; Happel et al., 1991; Laurent et al., 1990; Peterson and Herskowitz, 1992). A genetic suppressor screen of *swi/snf* mutants revealed that mutations affecting core histones and non-histone chromatin associated proteins could bypass the need of SWI/SNF complex. This implied that the SWI/SNF complex counteracts chromatin structure in order to facilitate gene activation (for review see

Pollard and Peterson, 1998; Vignali et al., 2000; Winston and Carlson, 1992; Hirschhorn et al., 1992; Matallana et al., 1992).

The cloning of *swi2* and *snf2* revealed that they were the same gene, and biochemical purification of Swi2/Snf2p led to the discovery that Swi2p/Snf2p, Swi1p, Swi3p, Snf5p and Snf6p proteins are part of a large (~2 MDa) multi-subunit complex. The purified complex also contains six additional polypeptides- Arp7p, Arp9p, Swp82p, Swp73p, Swp29p and Snf11p (Cairns et al., 1994; Cairns et al., 1996; Cote et al., 1994; Muller, 1995; Peterson et al., 1994; Treich et al., 1995). Consistent with the genetics, numerous subsequent biochemical studies have shown that the SWI/SNF complex uses the energy derived from ATP hydrolysis to disrupt nucleosome structure *in vivo* and *in vitro* (reviewed Kornberg, 1999).

With the exception of the Swi2p ATPase, the biochemical functions of the individual SWI/SNF components remain unknown (for review see Vignali et al., 2000). Two subunits, Arp7p and Arp9p, show extensive sequence similarities to the actin fold domain, which in the case of actin is important for ATP binding and ATP hydrolysis regulation. Hypothetically, the ARPs may function by regulating Swi2/Snf2p's ability to hydrolyze the ATP needed for SWI/SNF remodeling activity. To date, site-directed mutagenesis of the conserved, putative ATP binding domain of Arp7p or Arp9p has shown no phenotypic defect (Cairns et al., 1998). Thus the role(s) of Arp7p and Arp9p are unknown. However, the remaining subunits fail to reveal sequence motifs that may be important for the catalytic mechanism of SWI/SNF chromatin remodeling activity.

Several of the yeast SWI/SNF components (Swi2p, Swi3p, Snf5p, Swp73p and the Arp subunits) have homologues that are constituents of other SWI/SNF-like chromatin remodeling complexes implying functional conservation among these complexes (Table I.2; see Figure III.1). Recently, it was shown that the human Swi2p, Swi3p, and Snf5p homologues constitute the minimal core of subunits that are required for efficient remodeling activity (Phelan et al., 1999). This suggests that the other conserved components (e.g. Swp73p and Arp proteins) are possibly needed to regulate the minimal core remodeling activities. The remaining subunits either show homology in a subset of complexes or are unique to their complex. For instance, yeast Swi1p shows homology to the OSA and Baf250p components of *Drosophila* Brahma and hSWI/SNF (complex A), respectively, whereas Snf6p, Swp82p, Swp29p and Snf11p appear to be unique to the yeast SWI/SNF complex (see Figure III.1).

Although little is known about the functional role of individual subunits of the SWI/SNF-like complexes, the size and complexity of these complexes suggest that they perform multiple functions. SWI/SNF complex displays various biochemical activities, which are either ATP-independent or ATP-dependent. In an ATP-independent manner, SWI/SNF-like complexes have the ability to bind naked and nucleosomal DNA with high affinity (Cote et al., 1998; Moreira and Holmberg, 1999; Quinn et al., 1996). SWI/SNF binding properties are similar to high mobility group (HMG)-box containing proteins, which recognize structured DNA without sequence specificity in a DNA length-dependent manner (>130 bp) (Bianchi et al., 1990; Bruhn et al., 1992; Cote et al., 1998; Grosschedl et al., 1994; Pil et al., 1993; Quinn et al., 1996).

ATP-dependent functions of the SWI/SNF-like complexes include alteration of nucleosome structure, enhancement of gene-specific activator binding, as well as movement of histone octamers on DNA in *cis* and in *trans* (Cote et al., 1994; Jaskelioff et al., 2000; Logie and Peterson, 1997; Logie et al., 1999; Lorch et al., 1998). Alteration of nucleosome structure by these complexes results in disruption of histone-DNA contacts and alterations in the path and length of DNA bent around the histone octamer (Bazett-Jones et al., 1999; Cote et al., 1998; Cote et al., 1994; Imbalzano et al., 1994; Imbalzano et al., 1996; Kwon et al., 1994; Logie and Peterson, 1997; Schnitzler et al., 1998; Wang et al., 1996). These data indicate that SWI/SNF-like complexes specifically perturb the rotational orientation of DNA on the surface of the nucleosome core in an ATP-dependent manner. *In vitro* biochemical studies have shown that human and yeast SWI/SNF can stimulate binding of an activator (including TBP) to nucleosomal binding sites in an ATP-dependent manner (Cote et al., 1994; Kwon et al., 1994; Imbalzano et al., 1994). SWI/SNF is able to disrupt DNase I digestion patterns of rotationally phased nucleosomal DNA, and it can stimulate nucleosome core binding of GAL4 derivatives, TBP, Sp1, NF-KB, and USF (Cote et al., 1994; Imbalzano et al., 1994; Kwon et al., 1994; Utley et al., 1997; Wang et al., 1996). Also in an ATP-dependent manner, SWI/SNF-like complexes have the ability to induce sliding of histones in *cis* and displacement of histone octamers in *trans* (Jaskelioff et al., 2000; Lorch et al., 1999; Owen-Hughes et al., 1996; Whitehouse et al., 1999). Overall, *in vitro* biochemical studies have revealed that these chromatin remodeling complexes have similar ATPase and nucleosome disruption activities.

Although it is known that chromatin remodeling complexes function in an ATP-dependent manner to remodel nucleosome structure, the mechanism by which the energy of ATP hydrolysis achieves these chromatin remodeling activities is not clear. Three models posit different mechanisms for how ATP hydrolysis induces disruption of nucleosome structure. One model suggests that ATP hydrolysis may be used to track along the DNA causing destabilization of histone-DNA interactions (discussed in Pazin et al., 1997). However, it is known that SWI/SNF lacks ATP-dependent tracking activity in DNA supercoiling assays (Quinn et al., 1996). A second model suggests that ATP-dependent remodeling generates a (H3-H4)₂ tetramer by displacing the H2A-H2B dimers (Lugar et al., 1997). This model predicts that accessibility of DNA within a (H3-H4)₂ tetramer should be equivalent to that of a nucleosomal DNA after chromatin remodeling, and chromatin remodeling will not be able to enhance the accessibility of (H3-H4)₂ tetramers. Yet, Boyer et al. (Boyer et al., 2000) demonstrate that in the remodeled state, DNA incorporated onto a (H3-H4)₂ tetramer is more accessible than the intact octamer, and furthermore SWI/SNF can remodel nucleosomal arrays that harbor disulfide-linked (H3-H4)₂ tetramers. Thus, SWI/SNF action does not convert a nucleosome into a tetramer, nor does it involve an obligatory disruption of the tetramer. A third model postulates that ATP hydrolysis changes the writhe or path of nucleosomal DNA, or peels DNA from the surface of the nucleosome (Bazett-Jones et al., 1999; Cote et al., 1998). This model posits that ATP hydrolysis changes the DNA topology by twisting or rotating nucleosomal DNA along its axis (Boyer et al., 2000; Gavin et al., 2001; Haves et al., 2000). Recently, it was shown that the ability of yeast SWI/SNF to enhance accessibility

of nucleosomal DNA is nearly eliminated when DNA topology is constrained in small circular nucleosomal arrays (Gavin et al., 2001). In addition, it has been demonstrated that SWI/SNF-like enzymes and recombinant Swi2p-like ATPases can generate superhelical torsion on (naked) DNA and chromatin (Haves et al., 2000). These data support the hypothesis that chromatin remodeling complexes use the energy of ATP hydrolysis to drive local changes in DNA topology, perhaps by propagating writhe or twist throughout the nucleosome which would lessen DNA-histone contacts.

Another topic of interest is how SWI/SNF-like complexes are targeted to specific chromatin loci in the cell. In general, three models propose how the SWI/SNF-like complexes are targeted to their gene-specific promoters - RNAPII holoenzyme-mediated targeting, transcriptional activator targeting, or no targeting. Yudkovsky et al. (Yudkovsky et al., 1999) used *in vitro* transcription extracts and immobilized DNA templates to demonstrate that yeast SWI/SNF can be recruited to a target gene by a transcriptional activator in an activation domain-dependent yet RNAPII- independent manner. In addition, recent *in vitro* and *in vivo* studies have demonstrated that SWI/SNF-like complexes are targeted to promoters via transcriptional activators (Agalioti et al., 2000; Cosma et al., 1999, Cosma, 2000, Krebs, 1999; Dimova et al., 1999; Fryer and Archer, 1998; Kowenz-Leutz and Leutz, 1999; Krebs et al., 2000; Lee et al., 1999; Natarajan et al., 1999; Neely et al., 1999; Recht and Osley, 1999; Wallberg et al., 2000; Yoshinaga et al., 1992; Yudkovsky et al., 1999). In addition to activation-domain dependent targeting, SWI/SNF complex also appears to be targeted by selected zinc-finger DNA binding domain. *In vivo* chromatin immunoprecipitation (ChIP) assays have

shown that SWI/SNF is recruited to the HO promoter by the gene-specific transcriptional activator, Swi5p (Krebs et al., 1999; Cosma et al., 1999). Swi5p contains a zinc-finger DBD, and this domain is sufficient to bind to SWI/SNF *in vitro*. Likewise the human SWI/SNF complex interacts with several transcriptional activators that contain a zinc-finger DBD (Kadam et al., 2000). Moreover, a human Swi2p-Swi3p sub-complex interacts with the zinc-finger DBD domain of transcriptional activator EKLF, and this interaction is sufficient to target chromatin remodeling in a gene-selective manner (Kadam et al., 2000).

Other DNA-mediated Processes and ATP-Dependent Chromatin Remodeling Enzymes. Not all SWI2/SNF2 family members are involved in transcription. For instance, *Drosophila lodstar* is involved in chromosome stability and RAD54, RAD5, RAD16 and ERCC6 are required for in DNA damage repair (Eisen et al., 1995). Moreover, at least 17 open reading frames in *Saccharomyces cerevisiae* have been identified as Swi2p homologues, and the SWI2/SNF2 family is growing due to the genome sequencing projects. (Pollard and Peterson, 1998). It appears that this family of proteins is involved in diverse DNA-mediated biological processes, and that these enzymes are structurally and evolutionarily conserved in a variety of organisms (Eisen et al., 1995).

Several studies have demonstrated that SWI/SNF-like chromatin remodeling complexes have distinct physiological roles in the cell (Cao et al., 1997; Cote et al., 1994; Laurent et al., 1993; Treich and Carlson, 1997). For instance, it appears that ySWI/SNF, *Drosophila* Brahma, and hSWI/SNF (complex A) are involved in transcriptional

activation. More specifically, yeast SWI/SNF appears to be required for the expression of a number of mitotic genes, and it has been postulated that SWI/SNF is needed to disrupt the condensed state of mitotic chromatin (Krebs et al., 2000). Alternatively, recent studies have shown that yeast RSC complex and hSWI/SNF (complex B)/hRSC/PBAF complex are involved in kinetochore function (Cao et al., 1997; Xue et al., 2000).

Nucleosomal structure also imposes an impediment to DNA-binding factors that recognize chromosomal replication origins. Nucleosomes positioned over essential *cis*-acting sequences of DNA replication origins abolish origin firing *in vivo* (Simpson, 1990), and the assembly of DNA into chromatin leads to a general repression of DNA replication efficiency *in vitro*. ATP-dependent chromatin remodeling enzymes have been shown to overcome the nucleosomal repression of DNA replication. For instance, nucleosome disruption by the *Drosophila* CHRAC complex is required for subsequent DNA replication initiation at the SV40 DNA replication origin as shown by MNase cleavage analysis (Alexiadis et al., 1997; Alexiadis et al., 1998; DePamphilis, 1993).

Other cellular processes such as, sporulation, meiosis, chromosomal segregation, and DNA recombination and repair, also require the involvement of ATP-dependent chromatin remodelers. For instance, yeast ISW2 is required for sporulation (Trachtulcova et al., 2000) and the *isw2* null mutant has a meiotic G1 cell, indicative that ISW2 may be required for early stages of meiosis (Trachtulcova et al., 2000). Additionally, STH1 the ATPase subunit of yeast RSC has been shown to be required for the activation of gene expression at the initiation of meiosis (Yukawa et al., 1999).

Mutations in *Drosophila* ISWI lead to multiple pleiotropic phenotypes, suggesting that ISWI may function in many cellular processes (Deuring et al., 2000). Since ISWI is a subunit of at least three distinct complexes (NURF, CHRAC, ACF), it is not clear which *Drosophila* ISWI-containing complex contributes to which ISWI phenotype.

Collectively, several lines of evidence indicate that ATP-dependent chromatin remodeling complexes are involved in regulating chromatin structure, not only for transcription, but many DNA-mediated processes in the cell.

Concluding Statement

The emerging theme in the chromatin field is that different ATP-dependent chromatin remodeling enzymes demonstrate similar *in vitro* biochemical activities. In general, these complexes alter chromatin structure in order for DNA-mediated processes to occur properly. Furthermore, subunits of different the chromatin remodeling complexes display a high degree of conservation. Dissecting the prototype of ATP-dependent chromatin remodelers, the *Saccharomyces cerevisiae* SWI/SNF complex, will help to illustrate how these complexes are assembled, and to ascribe basic functional roles for individual subunits in order to explain how they contribute to altering chromatin structure.

Table I.1 SWI/SNF-like ATP-Dependent Chromatin Remodeling Enzymes

SWI2/SNF2				Activities
Complex	ATPase Homologue	# of Subunits	MW (MDa)	
ySWI/SNF	SWI2/SNF2	11	~2	{ DNA-stimulated ATPase Activity, Nucleosome Disruption Activity, Nucleosome Mobility in <i>cis</i> and <i>trans</i>
yRSC	STH1	15	~1.5	
hSWI/SNF	{ hbrm hBRG1 }	>9	~2	
dSWI/SNF	<i>brm</i>	>8	~2	
<hr/>				
dNURF	ISWI	4	~0.5	{ Nucleosomal-stimulated ATPase Activity Nucleosome Disruption Activity Nucleosome Mobility in <i>cis</i> Nucleosome Spacing Activity Nucleosome Assembly Activity
dCHRAC	ISWI	4	~0.67	
dACF	ISWI	2	~0.22	
HuCHRAC	hSNF2h	4	~0.6-0.7	
hACF/WRCF	hSNF2h	2	~0.3-0.7	
hRSF	hSNF2h	2	~0.4-0.5	
hSNF2L	hSNF2L	?	?	
xACF/CHRAC	xSNF2h	?	?	
yISW1	ISW1	4	?	
yISW2	ISW2	2	?	
yINO80	Ino80	?	~2	
<hr/>				
NuRD/NRD	hMi-2	9	~1.5-2	{ Nucleosomal-stimulated ATPase Activity Nucleosome Disruption Activity Nucleosome Mobility in <i>cis</i>
xMi-2	xMi-2	6	~1.5-2	
dMi-2	dMi-2	?	~1.5-2	
CeNuRD	Chd3/Chd4	?	?	

Table I.2 Conserved Chromatin Remodeling Components (SWI/SNF-like)

ySWI/SNF (11 subunits)	yRSC (15 subunits)	hSWI/SNF Complex A (>10 subunits*)	hRSC/PBAF Complex B (12 subunits)	dBrahma (>9 subunits*)
Swi2/Snf2p	Sth1p	Brg1 hbrm	BRG1	dbrm
Swi3p	Rsc8p	Baf170p Baf155p	Baf170p Baf155p	moira
Snf5p	Sfh1p	Ini1p	Ini1p	snr1
Swp73p	Rsc6p	Baf60p (a,b,c)	Baf60p (a,b,c)	Bap60p
Arp7p Arp9p	Arp7p Arp9p	Baf53 Actin	Baf53 Actin	Baf74p Bap55p
Swi1p		Baf250p		osa/eyelid
Snf6p				
Swp82				
Swp29p				
Snf11p				
	Rsc1p Rsc2p Rsc4p		Baf180p	
		Baf110p Baf57	Baf110p Baf57	Bap111p
			Baf240	
			Baf200	

* Indicates only identified subunits are listed

Table I.3 Conserved Chromatin Remodeling Components (ISWI-like)

<u>dNURF</u> (4 subunits)	<u>dCHRAC</u> (4 subunits)	<u>dACF</u> (2 subunits)	<u>yISW2</u> (2 subunits)	<u>yINO80</u> (>12 subunits*)
ISWI	ISWI	ISWI	ISW2	Ino80p
	CHRAC-16p	dACF1		Arp4p
	CHRAC-14p			Arp5p
Nurf55p				Arp8p
				Actin
Nurf215p			p140	
Nurf18p	p175			Rvb1p
				Rvb2p
<u>yISW1</u> (4 subunits)	<u>hCHRAC</u> (4 subunits)	<u>hACF/WRCF</u> (2 subunits)	<u>hRSF</u> (2 subunits)	<u>hSNF2L</u> (?*)
ISW1	hSNF2h	hSNF2h	hSNF2h	hSNF2L
	hACF1	hACF		Ini1p
	hCHRAC-17p			
	hCHRAC-15p			
p110			p325	
p105				
p74				

*Indicates only identified subunits are listed

Table I.4 Conserved Chromatin Remodeling Components (Mi-2-like)

HumanMi2/ Nurd/NRD (9 subunits)	Xenopus Mi-2 (6 subunits)	Drosophila Mi-2 (>10 subunits*)	C. elegans NuRD (?*)
Mi-2α/CHD3 Mi-2β/CHD4	xMi-2	dMi-2	Chd3p Chd4p
HDAC1 HDAC2	xRpd3p	dRpd3	Hda-1
RbAp46 RbAp48	xRbAp48		Lin-53 Rba-1
Mta1p Mta2p	Mta1-like		Egl-27 Egr-1
Mbd3p	Mbd3p		
p66	p66		

* Indicates only identified subunits are listed

CHAPTER II

Subunit Stoichiometry of the *Saccharomyces cerevisiae* SWI/SNF complex

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Introduction

Many cellular processes require regulated access to the DNA template. Recently, several different classes of large multi-subunit enzymes have been shown to regulate transcription by modifying chromatin. One class of enzymes that alleviates chromatin repression is the ATP-dependent chromatin remodeling complexes (reviewed in Kingston and Narlikar, 1999). This class of remodelers uses the energy of ATP hydrolysis to alter histone-DNA contacts and thereby enhance the accessibility of nucleosomal DNA (Cote et al., 1994; Jaskelioff et al., 2000; Logie and Peterson, 1997).

The *Saccharomyces cerevisiae* SWI/SNF complex is considered the prototype of ATP-dependent remodeling complexes, which are evolutionarily conserved from yeast to human. Purified yeast SWI/SNF is a large multi-subunit complex (~2MDa by gel filtration) comprised of the following eleven different polypeptides: Swi2p/Snf2p, Swi1p, Swi3p, Snf5p, Snf6p, Swp73p, Arp7p, Arp9p, Swp82p, Swp29p and Snf11p (Cairns et al., 1994; Cote et al., 1994; Peterson et al., 1994). Disruption of either the SWI2, SWI1, SWI3, SNF5, SNF6, SWP73, ARP7 or APR9 genes yields a similar battery of *in vivo* phenotypes, including slow growth on different carbon sources and transcriptional defects for a subset of genes (Biggar and Crabtree, 1999; Holstege et al., 1998; Peterson

et al., 1994, Cairns et al., 1994; Peterson and Herskowitz, 1992; Sudarsanam et al., 1999). In contrast, inactivation of SNF11, SWP29 and SWP82 do not yield appreciable transcriptional defects, and thus their contribution to SWI/SNF function is not known. Moreover, Swi2p/Snf2p, Swi3p, Snf5p, Swp73p and the Arp proteins appear to represent an evolutionarily conserved core that is found in all SWI/SNF-like chromatin remodeling complexes. Dissecting how these large multi-subunit machines are assembled will provide important insights into the mechanism of chromatin remodeling.

Although it is clear that yeast SWI/SNF is composed of eleven different polypeptides, the relative stoichiometry of these components is not known. Gel filtration analyses estimate the native molecular weight of the purified complex to be approximately 2MDa (Peterson et al., 1994). However, if we assume that the complex is composed of one copy of each subunit, then the complex would have a molecular weight of only ~1 MDa. Furthermore, SDS PAGE analysis of purified yeast SWI/SNF suggests that the subunits are present in a roughly 1:1 stoichiometry, with the exception of the Swi3p subunit, which appears to stain more intensely with Coomassie or silver (Cairns et al., 1994; Cote et al., 1994). Thus, one question that arises is whether the large size detected by gel filtration is a result of multiple copies of each subunit per complex, or to an anomaly of the gel filtration method. One assumption that is made by gel filtration analysis is that the molecule is spherical. Thus, if SWI/SNF has an elongated structure, gel filtration will yield an anomalous molecular weight, and the true native molecular weight may be 1 MDa. Alternatively, SWI/SNF complex may be globular, but it may

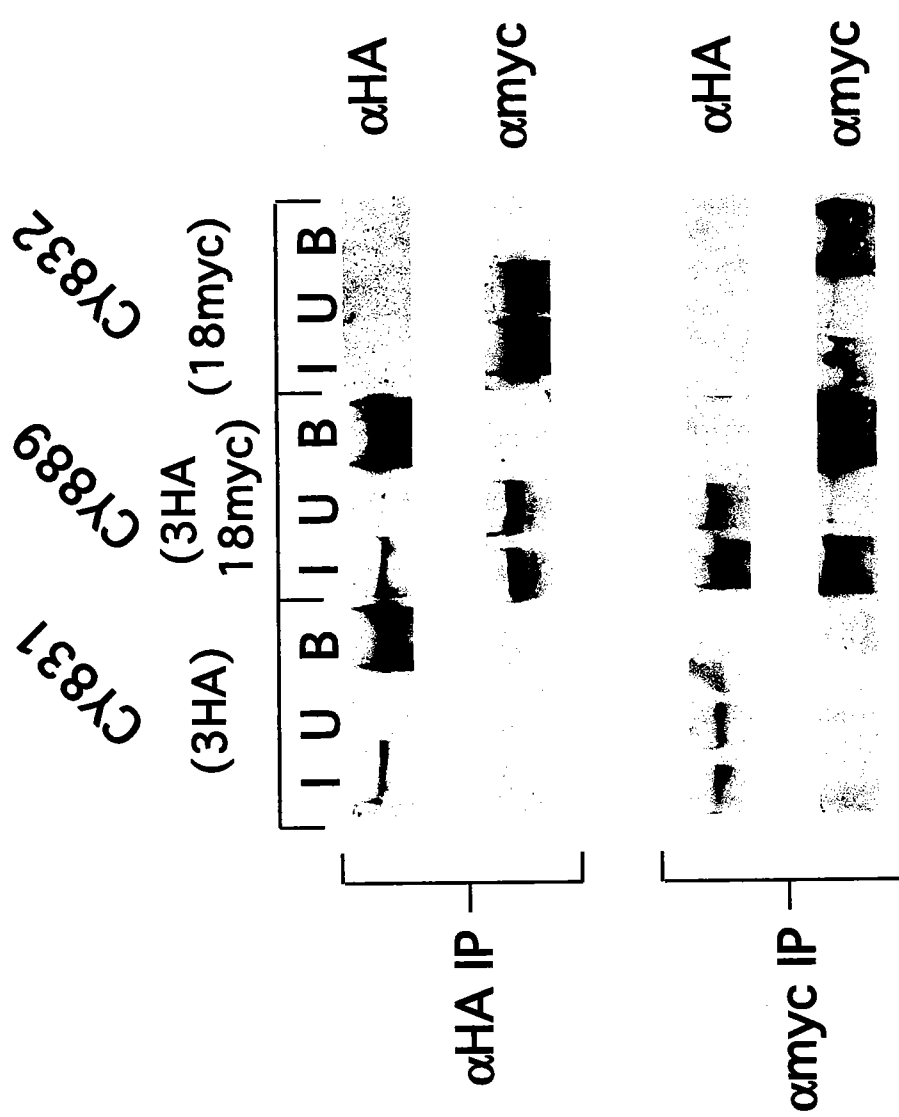
exist as a dimer of two 1 MDa complexes, each of which contains one copy of each subunit.

In this study, we determined the subunit stoichiometry of the conserved subunits of yeast SWI/SNF complex. First, we determined the number of Swi2p subunits per complex by constructing a haploid yeast strain harboring two different epitope-tagged versions of Swi2p, and by performing reciprocal co-immunoprecipitation assays. In addition, we purified SWI/SNF complex and determined the relative stoichiometry of the conserved subunits by ^{125}I -labeling of tyrosine residues.

Results

There is only one copy of Swi2p per SWI/SNF complex. As a first step towards understanding the stoichiometry of SWI/SNF subunits, we sought to determine the copy number of the Swi2p subunit(s) present in each yeast SWI/SNF complex. To address this, we used two different epitope-tagged alleles of SWI2 to create three different haploid yeast strains. The two control strains contained either one triple HA-tagged allele (SWI2-3HA) integrated at the URA3 locus (CY381), or one eighteen myc-tagged allele (SWI2-18myc) integrated at the chromosomal SWI2 locus (CY832). The test strain contained two epitope-tagged alleles of SWI2, Swi2-3HA is integrated at the URA3 locus and Swi2p-18myc integrated at the chromosomal SWI2 locus (CY889). We made whole cell extracts from each strain and SWI/SNF was immunoprecipitated using antibodies specific to either the HA or myc epitopes (Figure II.1). In the case of the control strains, which contained only one epitope-tagged version of Swi2p, each anti-sera

Figure II.1 One Copy of Swi2/Snf2p per SWI/SNF complex. Swi2p/Snf2p was immunoprecipitated from whole cell extracts made from yeast strains CY832 (SWI2-18myc), CY889 (SWI2-18myc; SWI2-3HA) and CY831 (SWI2-3HA). Lanes are labeled I, U or B for input (2.5%), unbound (2.5%) or bound (100%), respectively. immunoprecipitation experiments were performed with either monoclonal anti-HA (Babco) or anti-myc (Santa Cruz) immune sera. The presence of Swi2p was detected with either anti-HA (Babco) or anti-myc (Santa Cruz) by Western analysis.



immunoprecipitated only the expected epitope-tagged SWI2 allele, confirming the specificity of these sera (Figure II.1). In the whole cell extract that harbors two different epitope-tagged versions of Swi2p (Swi2-3HA and Swi2-18myc), immunoprecipitations with the α -HA antibody only immunoprecipitated the HA-tagged allele of SWI2. Likewise, immunoprecipitation with the α -myc sera did not immunoprecipitate the HA-tagged allele (Figure II.1). These results indicate that the yeast SWI/SNF complex contains only one copy of the Swi2p subunit.

The stoichiometry of SWI/SNF subunits. To further assess the number of each subunit(s) per complex, we determined the relative stoichiometry of the other polypeptides in the complex. First, I prepared highly purified SWI/SNF complex using a combination of Ni-2⁺ affinity chromatography, ion exchange, and Flag antibody affinity chromatography (see Experimental Procedures). Once isolated, we tested whether this purified SWI/SNF complex was active in an *in vitro* nucleosomal remodeling assay (Figure II.2B). In this assay, a nucleosomal array is reconstituted in a DNA fragment that contains 11 head-to-tail repeats of a 5S rRNA nucleosome positioning sequence, the central repeat bearing an unique Sall/HincII restriction enzyme site (Logie and Peterson, 1997). Nucleosome remodeling is detected by the enhanced Sall cleavage of the nucleosomal array in the presence of SWI/SNF and ATP (Figure II.2B; Logie and Peterson, 1997). The nucleosome remodeling activity of the highly purified SWI/SNF complex was found to be similar to the remodeling activity of SWI/SNF complex purified by our standard fractionation scheme (Peterson et al., 1994).

To address the question of stoichiometry of SWI/SNF subunits, the purified SWI/SNF complex was denatured in SDS, and tyrosine residues were quantitatively iodinated using a chloramine T oxidation procedure (see Experimental Procedures; Figure II.2A). ^{125}I -labeled SWI/SNF was then electrophoresed on SDS-PAGE. Radioiodination revealed the presence of the SWI/SNF subunits as well as additional polypeptides. To confirm the identity of each iodinated SWI/SNF species, *in vitro* translated ^{35}S -methionine labeled SWI/SNF subunits were electrophoresed in parallel with the iodinated SWI/SNF complex. Each SWI/SNF subunit was quantified for the amount of iodine incorporation by PhosphoImager analysis by using two methods. First, a vertical bar was drawn through the entire lane, and PhosphoImager analysis produced a pixel value for each band (Figure II.2D). Alternatively, each SWI/SNF subunit was boxed and a larger pixel area was quantified by PhosphoImager analysis (Table II.1). Each method produces pixel values for the individual SWI/SNF subunits that are then divided by the number of tyrosine residues in each subunit, and then normalized to one Swi2p per complex (Figure II.2D; Table II.1). From multiple experiments with different SWI/SNF preparations, the data consistently gave similar ratios of Swi1p, Swi3p, Snf5p, Swp73p, Arp7p, Arp9p, and Snf6p subunits. Quantified results indicated that the Swi1p, Swp73p, Arp7p, Arp9p and Snf6p subunits are present in one copy, and Swi3p and Snf5p are present in two copies per complex (Figure II.2 and Table II.1). The ratios for the remaining subunits (Swp82p, Swp29p and Snf11p) were either undetectable due to minimal tyrosines per polypeptide, or varied in their relative ratios to Swi2p from preparation to preparation.

Figure II.2 Iodination and Staining of SWI/SNF. A. SWI/SNF Iodination Scheme.

Purified SWI/SNF complex was denatured in SDS at room temperature. To the denatured complex Na^{125}I and chloramine-T were added. Na^{125}I is oxidized in the presence of chloramine-T, and a cationic $^{125}\text{I}^+$ is incorporate onto tyrosine side-chains.

The reaction is stopped in the presence of metabisulfite and the iodinated SWI/SNF complex precipitated in potassium chloride to remove free label. **B. SWI/SNF**

Remodeling Activity. SWI/SNF purified from yeast strain CY891 (SWI2-HA-6HIS; pSNF6-Flag) and tested for remodeling activity in comparison to complex purified from strain CY337 (SWI2-HA-6HIS; Logie and Peterson, 1997). The remodeling activity of the SWI/SNF purified from CY891 was at least 50% as active as complex purified from CY337, suggesting that the SWI/SNF complex is functional following purification. **C.**

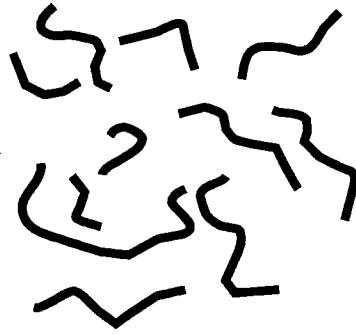
Iodinated SWI/SNF Complex. ^{125}I -labeled SWI/SNF (SWI2-HA; pSNF6-Flag) was electrophoresed and analyzed on a 10-20% gradient SDS-PAGE gel (BioRad). Iodinated SWI/SNF polypeptides were identified by alignment of individual, full-length ^{35}S -methionine *in vitro* translated SWI/SNF polypeptides electrophoresed in parallel. Shown is a 1 and 6 hour exposure of the same iodinated SWI/SNF preparation. Arrows identify SWI/SNF subunits and the number of tyrosine residues per polypeptide is indicated in brackets. * indicates additional ^{125}I -labeled bands in the preparation. The SWI/SNF subunits were quantified by PhosphoImager analysis (see Figure II.2D [vertical bar analysis] and Table II.1 [boxed analysis]). **D. Vertical Bar PhosphoImager Analysis of Iodinated SWI/SNF Complex.** One method for quantifying the iodination of SWI/SNF

was by drawing a vertical line through the sample to provide a pixel value for each subunit. Iodination pixel values were divided by the number of tyrosine residues per polypeptide and the counts for each SWI/SNF subunit were normalized to Swi2/Snf2p (see Table II.1).

Purified yeast SWI/SNF complex



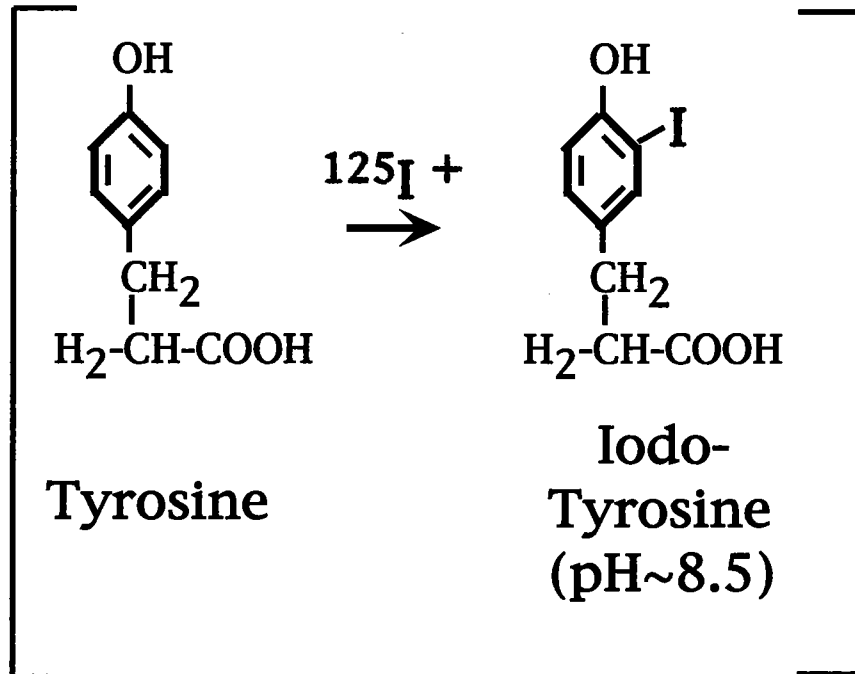
pH 7.5
4.4mM SDS
@ RT



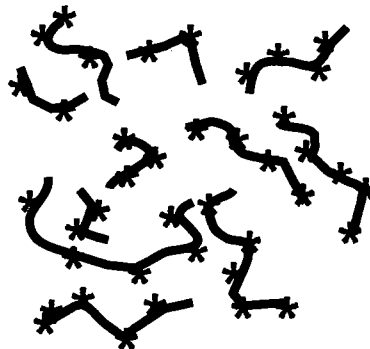
Denatured SWI/SNF

↓ Na¹²⁵I
(0.1μCi)

+
↓ 2mg/ml
chloramine-T

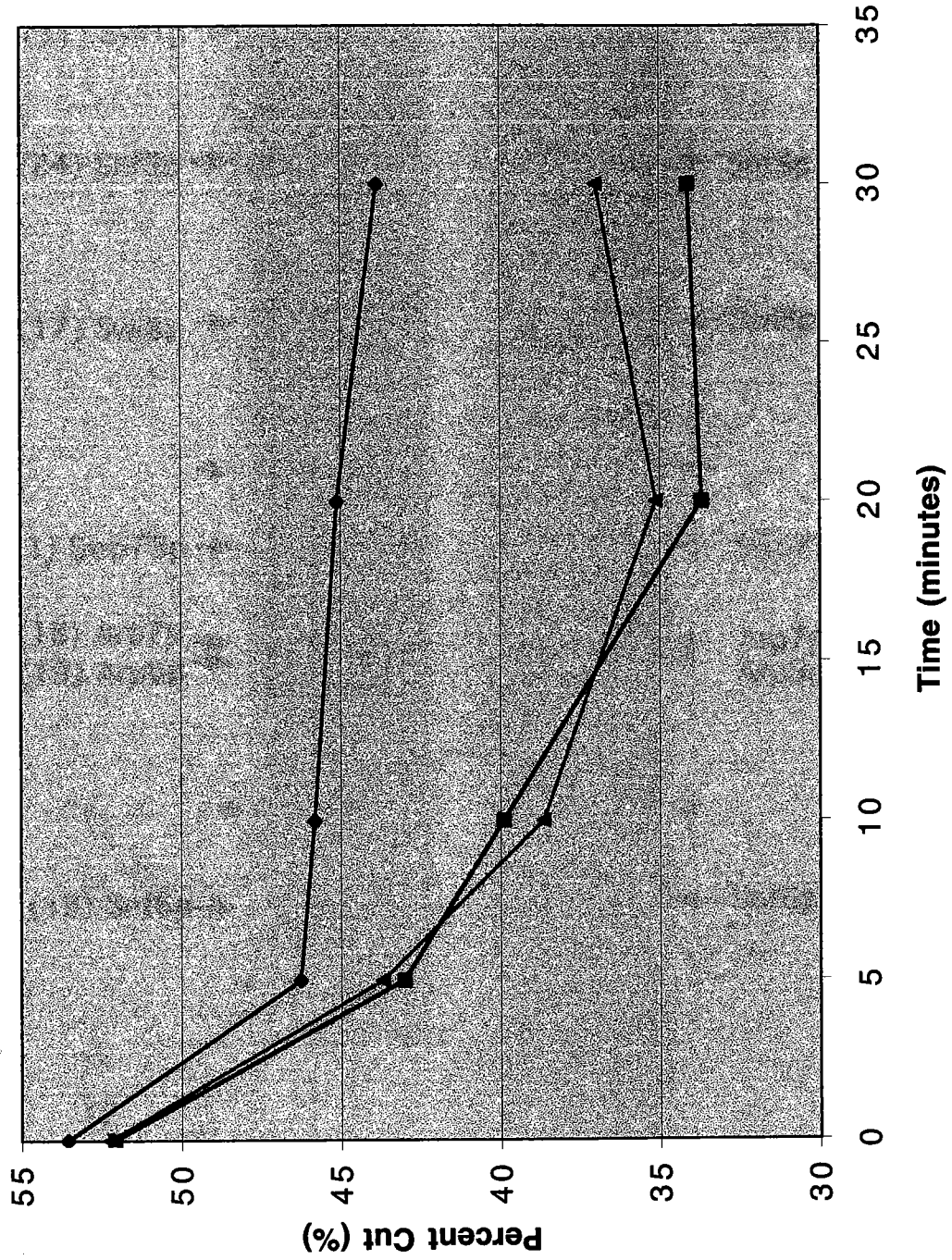


Na₂S₂O₅

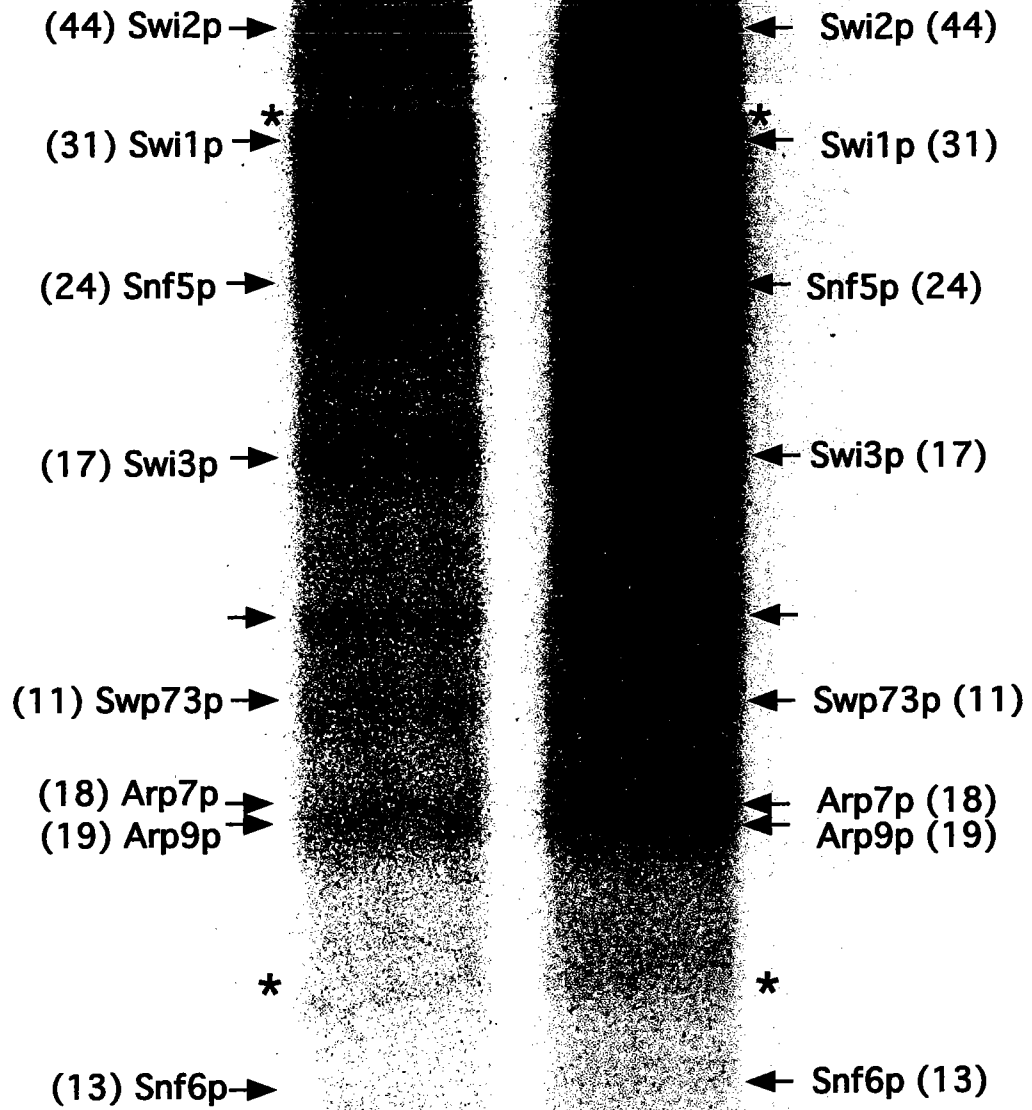


→ ppt in
145mM KAc

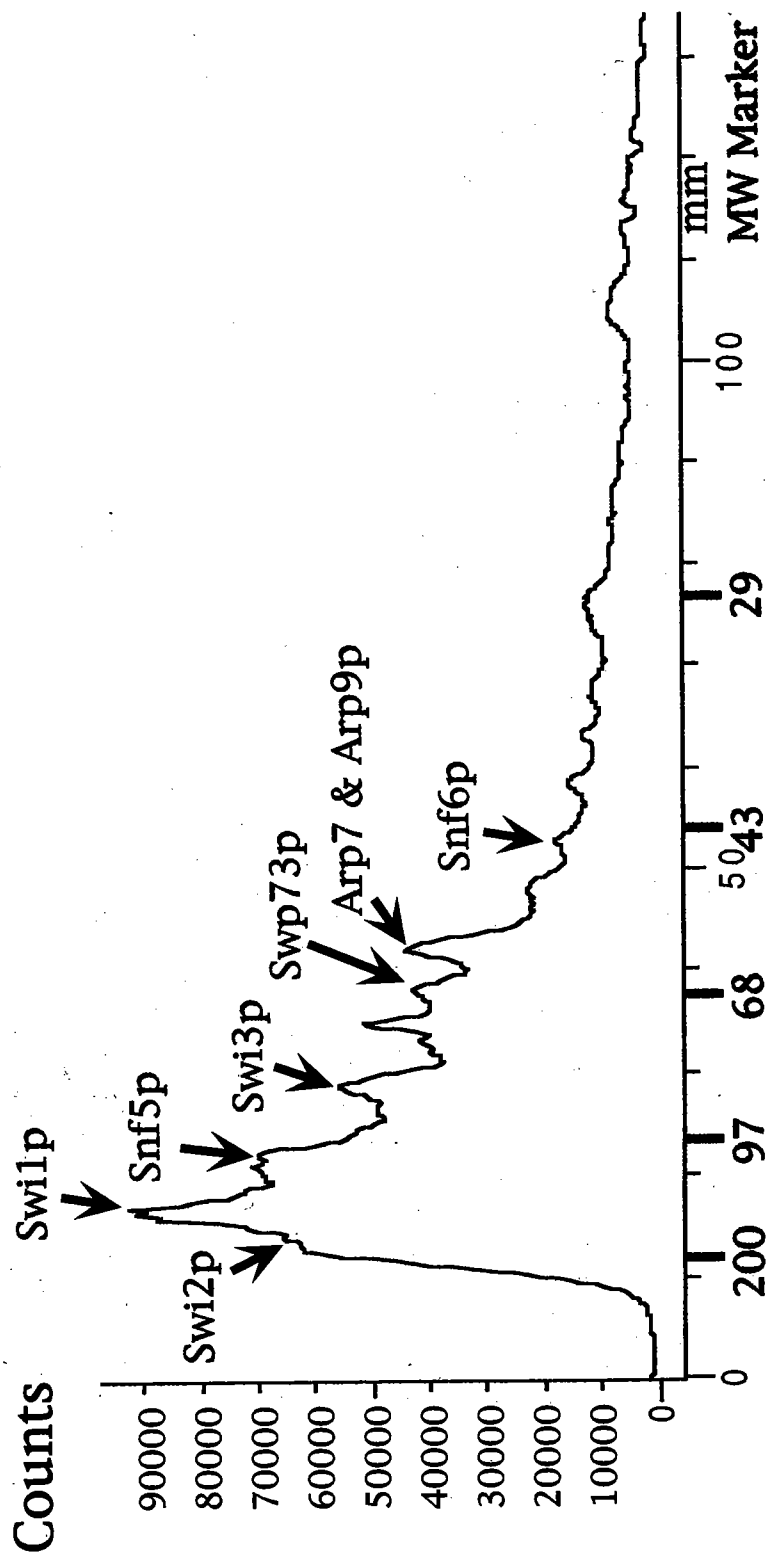
Nucleosome Remodeling Assay



125 I-Flag-SWI/SNF



C



D

TABLE II.1 RELATIVE SWI/SNF SUBUNIT STOICHIOMETRY

SWI/SNF Subunit	MW (Kd)	# Tyr per polypeptide	Exp 1		Exp 2		Exp 3		Ratio to Swi2/Snf2p
			Normalized ¹²⁵ I Incorporation Tyr/polypep.	Relative Ratio to Swi2p	Normalized ¹²⁵ I Incorporation Tyr/polypep.	Relative Ratio to Swi2p	Normalized ¹²⁵ I Incorporation Tyr/polypep.	Relative Ratio to Swi2p	
SWI2	194	44	122230	1	438435	1	22350	1	1
SWI1	148	31	181089	1.48	368286	0.84	14528	0.65	1
SNF5	102	24	196116	1.60	1008400	2.30	47134	2.11	2
SWI3	93	17	193757	1.59	1126019	2.57	48002	2.14	2
SWP82	nd	nd	nd	nd	nd	nd	nd	nd	nd
SWP73	64	11	89486	0.73	407218	0.93	37597	1.68	1
ARP7	54	19	373829	1.50	375695	0.86	19516	0.87	1
ARP9	50	18			378023	0.86	17541	0.78	1
SNF6	38	13	56775	0.46	358070	0.81	16805	0.75	1
SWP29	31	5	nd	nd	nd	nd	nd	nd	nd
SNF11	18	3	nd	nd	nd	nd	nd	nd	nd

Discussion

The *Saccharomyces cerevisiae* SWI/SNF ATP-dependent chromatin remodeling complex is a large multi-subunit complex that consists of eleven different tightly associated polypeptides (Peterson et al., 1994; Cairns et al., 1996). Here we have shown that yeast SWI/SNF contains only one Swi2p ATPase subunit per complex. Furthermore, we demonstrated that Swi1p, Swp73p, Arp7p, Arp9p and Snf6p subunits are also present in one copy and that the Swi3p and Snf5p subunits are present in two copies per SWI/SNF complex. We propose that other SWI/SNF-like chromatin remodeling complexes may maintain these relative stoichiometries for the conserved homologues.

One Swi2p per SWI/SNF Complex. The Swi2p protein, a DNA-stimulated ATPase, is the only SWI/SNF subunit with a defined biochemical activity. In this study, we determined that there is only one Swi2p per SWI/SNF complex (Figure II.1). This is intriguing mechanistically, since Swi2p is crucial for the ATP-dependent functions of the complex. For instance, a single residue change in the putative nucleotide binding loop (*swi2K798A*) of the ATPase domain behaves as a dominant negative mutant (see Figure I.2; Peterson and Herskowitz, 1992, Laurent et al., 1993; Cote et al., 1994; Gorbalenya et al., 1989; Richmond and Peterson, 1996). Moreover, the native complex can hydrolyze ATP at a rate of ~1000 ATPs per minute, leading to disruption of DNA-histone contacts (Cairns et al., 1996; Logie and Peterson, 1997). Additionally, a minimum core of human SWI/SNF subunits, BRG1 (Swi2 homologue), BAF155, BAF170 (Swi3p homologues),

and INI1 (Snf5p homologue), can carry out nucleosome remodeling activity in the absence of other SWI/SNF components (Phelan et al., 1999). Collectively, this suggests that a single Swi2p polypeptide in the complex is solely responsible for all ATP-dependent functions of the native complex.

Molecular Mass of the Yeast SWI/SNF Complex. Here, we determined the stoichiometry of SWI/SNF subunits by ^{125}I -labeling experiments (Figure II.2 and Table II.1). Swi2p, Swi1p, Swp73p, Snf6p, Arp7p, and Arp9p are stoichiometric and present in one copy per complex. In addition, our results indicate that there are two copies of Swi3p, which is compatible with studies that have shown that Swi3p, and Swi3p homologues appear to self-associate (Treich et al., 1995; Treich and Carlson, 1997; Crosby et al., 1999). To our surprise, our data indicate that there are two copies of Snf5p. Presently there are no additional data that either supports or refutes this result. If the other subunits (Swp82p, Swp29p, and Snf11p) are assumed to be present at one per complex, then our data suggests that the true molecular weight of SWI/SNF is ~ 1 MDa.

Experimental Procedures

Yeast strains and media. Standard genetic methods were followed to generate the appropriate strains. Yeast cultures were grown at 30°C in yeast extract-peptone (YEP) media containing 2% glucose (Difco Laboratories). For reciprocal immunoprecipitation experiments, an isogenic derivative of W303 (CY832) was obtained from K. Nasmyth

(Cosma et al., 1999) which contains a C-terminal 18-myc epitope tagged SWI2 at its original chromosomal locus. CY889 is isogenic to CY832 and contains an additional SWI2-3HA allele integrated at URA3 by one-step loop-in method. CY831 is a wild type strain that contains SWI2-3HA integrated at the URA3 locus by one-step loop-in method. CY388 is isogenic to CY382 and contains an additional SWI2-3HA integrated at the URA3 by one-step loop-in method. SWI/SNF was purified from either CY337 (SWI2-HA-6HIS; Logie and Peterson, 1997) or strain CY891 (SWI2-HA-6HIS; pSNF6-Flag), which is isogenic to CY337, but contains an episomal Flag-tagged copy of Snf6p.

Preparation of whole-cell extracts. Strains were grown in 100 ml of media at 30°C to an OD₆₀₀ of 0.8-1.0. Cells were harvested, washed and resuspended in 0.8 ml of E Buffer (20 mM Hepes, 350 mM NaCl, 10% glycerol, 0.1% Tween-20) with protease inhibitors. Cells were disrupted with glass beads using a mini bead beater (Biospec Products) for 5 pulses of 1 minute each, cooling on ice in between pulses. Extracts were clarified with a 10 minute spin at 14K in a microfuge, and the supernatants were centrifuged for 45 minutes, 48K (4°C) in a Beckman TL100 ultracentrifuge.

Reciprocal immunoprecipitation. Whole cell extracts were made from CY831 (SWI2-3HA), CY832 (SWI2-18myc), and CY889 (SWI2-18myc; SWI2-3HA) yeast strains. 100 ul of whole cell extract was added to 300ul of buffer (20 mM Tris, pH 8.0, 200 mM NaCl, 1 mM EDTA, 0.1% Triton-X 100, 1 mM DTT, 0.1 mg/ml BSA, 1 mM PMSF), each extract was immunoprecipitated with either anti-HA (HA.11; Babco) or anti-myc (9E10; Santa Cruz) anti-sera for 1hr on ice, subsequently adding Protein A beads for 1hr at 4°C and nutating. Immune complexes were isolated by centrifugation, washed 5 times

in binding buffer, and then bound proteins were eluted in 2xSDS sample buffer. Binding was analyzed by SDS-PAGE, and Western analysis with α -HA (HA.11; Babco) or α -myc (9E10; Santa Cruz) anti-sera. Westerns were visualized by ECL.

Flag-SWI/SNF Purification. The SWI/SNF complex was purified from strain CY891 (SWI2-HA-6HIS; pSNF6-Flag), which harbors an integrated SWI2-HA-6HIS and an episomal copy of a flag-epitope tagged SNF6 gene. A 20 L of culture was grown to $OD_{600}=1.0$ in SD-leucine (6.2g/L yeast nitrogen base without amino acids and 2% glucose). Cells were harvested at 4K, (4°C) for 10 minutes, washed in E Buffer (20 mM Tris, pH7.5, 350 mM NaCl, 10% glycerol, 0.1% Tween-20, 1 mM PMSF, 2 ug/ml of leupeptin and pepsatin A), and then lysed in a Biospec bead beater for 5x 30 seconds with 1' rest intervals in an ice bath. Lysate was centrifuged at 4°C for 45' at 40K in a Beckman Ultracentrifuge using a Ti-40 rotor. Cleared lysate was bound to 20ml Ni^{2+} -NTA agarose (Qiagen) for 3 hours, washed 1x in 250 ml E Buffer, 1x in 50 ml 20 mM Imidazole buffer (75 mM NaCl, 10% glycerol, 0.1% Tween-20, 1 mM PMSF, 2 ug/ml of leupeptin and pepsatin A), and eluted in 1x50 ml 500 mM Imidazole buffer. Elute was fractionated on a MonoQ (HR 5/5 Amersham Pharmacia Biotech) chromatography column. The complex was eluted from the column with a linear gradient from 10-50% Mono Q buffer B (50 mM Tris, pH 8.0, 10% glycerol, 0.5 mM DTT, 0.1% Tween-20, 0.1 mM EDTA). Fractions were collected and 10ul aliquots were analyzed by 6% SDS-PAGE, Western analysis with an α -HA antibody (16B12; Babco). Peak SWI2-HA fractions were pooled and then sedimented on a 30-13% glycerol gradient (20 mM HEPES, pH 7.5, 350 mM NaCl, 0.1% Tween-20, 0.5 mM DTT, 0.1% Tween-20, 0.1 mM

EDTA, 1 mM PMSF, 2 ug/ml of leupeptin and pepstatin A). Half ml fractions were collected and 10ul aliquots were analyzed by 6% SDS-PAGE analysis and Western analysis with α -HA antibody (16B12; Babco). Peak fractions were pooled and concentrated for ATPase, remodeling and iodination experiments.

¹²⁵I-labeled SWI/SNF experiment. 100 ul SWI/SNF complex purified from yeast strain CY891 was denatured in 6.25% SDS (BDH) at room temperature (RT) for 30 minutes, then labeled with 0.1 μ Cu ¹²⁵NaI in the presence of 2 mg/ml chloramine T (Aldrich) for 3 minutes at RT. Labeling reaction was stopped by the addition of 3 mg/ml Na₂S₂O₅ (sodium metabisulphite) in 20 mM TEA (triethanolamine). ¹²⁵I-labeled SWI/SNF was precipitated by the addition of 1 M potassium acetate and incubation for 30 minutes in an ice bath. Precipitates were isolated by centrifugation and then resuspended in 50 ul SDS mix (3% SDS, 3.5% glycerol, 0.2 mM DTT, 100 mM Tris, pH 6.8, 0.05 mM EDTA and bromophenol blue). 10% of the final ¹²⁵I-labeled SWI/SNF and 10 ul ³⁵S-methionine labeled SWI/SNF subunits were analyzed on a 10-20% gradient SDS-PAGE (BioRad) electrophoresed at 15 mV. Gels were fixed (35% methanol, 10% acetic acid) for 30 minutes, dried and then analyzed on a PhosphorImager.

Chromatin Remodeling Assay. 1 nM reconstituted 208-11s nucleosomal arrays (Logie and Peterson, 1997) were incubated at 37°C with *Sa*II restriction enzyme (5000U/ml) in a solution containing (100 ug/ml BSA, 1 mM ATP, 50 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 5% glycerol), and in the presence or absence of SWI/SNF. Samples were taken at the indicated time points, extracted with a 1:1 solution of phenol:chloroform and resolved by electrophoresis in a 1.2% agarose gel. The cut and uncut fractions, representing the

accessibility of the nucleosomal SalI site, were determined by PhosphoImager analysis using the ImageQuant software. Experiments were repeated independently at least three times.

CHAPTER III

Swi3p Functions as a Scaffold Subunit for SWI/SNF Assembly

Chapter III

Swi3p Functions as a Scaffold Subunit for SWI/SNF Assembly

Introduction

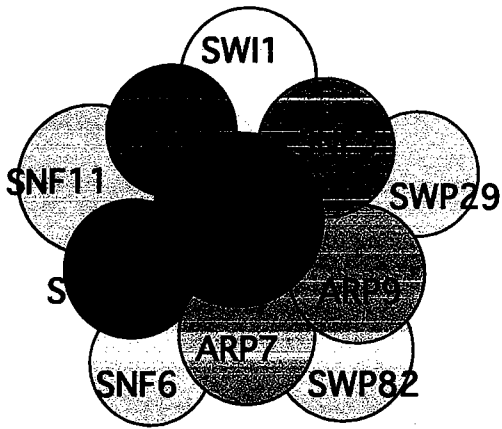
The ATP-dependent class of chromatin remodeling enzymes encompasses a group of highly conserved multi-subunit complexes, which appear to function by using the energy of ATP hydrolysis to disrupt histone-DNA interactions (reviewed in Kingston and Narlikar, 1999; Muchardt and Yaniv, 1999; Workman and Kingston, 1998).

Although the mechanism of remodeling by this class of enzymes is unknown, it has been established that a subset of components (Swi2p, Swi3p, Swp73p, Snf5p and the Arp proteins) are conserved among the SWI/SNF-like chromatin related complexes γ SWI/SNF, γ RSC, hSWI/SNF (Complexes A and B) and *Drosophila* Brahma (Cairns et al., 1996; Dingwall et al., 1995; Kwon et al., 1994; Papoulas et al., 1998; Phelan et al., 1999; Treich and Carlson, 1997; Wang et al., 1996; Wang et al., 1996) (see Figure III.1 and Table I.2). This implies functional conservation. To date, only the conserved DNA-stimulated ATPase subunit Swi2p has a known biochemical activity.

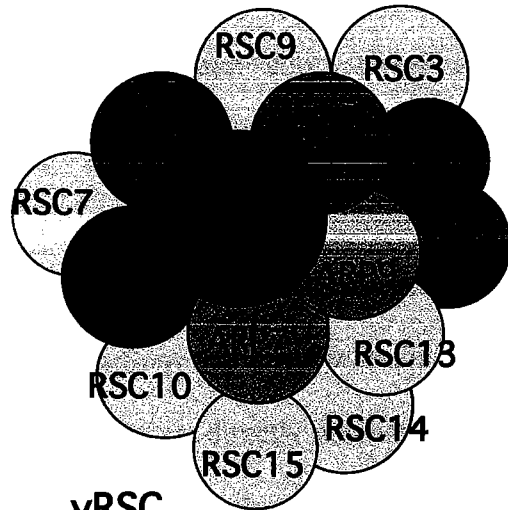
We do know that other subunits of the SWI/SNF-like complexes contribute to function *in vivo* and *in vitro* (Stern et al., 1984). In yeast, the integrity of SWI/SNF complex is compromised by the deletion of one or more of the SWI/SNF genes, with the exception of three subunits, Swp82p, Swp29p, and Snf11p, which neither demonstrate *swi/snf* mutant phenotypes, nor influence complex assembly (Peterson et al., 1994). Hence, their contribution to the complex's function, if any, is not known. Recently,

Figure III.1 Homologous Subunits in SWI/SNF-like ATP-dependent Chromatin

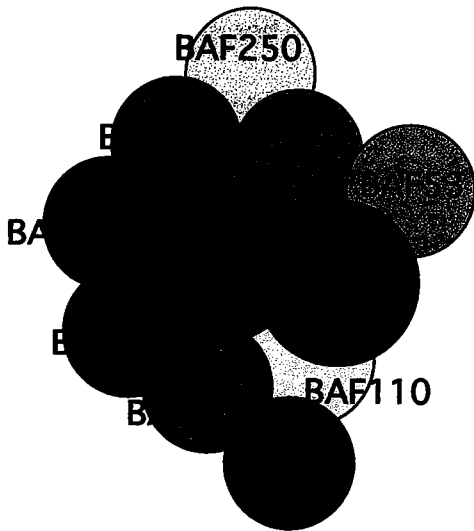
Remodeling Complexes. This is a schematic of SWI/SNF-like complexes identified in yeast (ySWI/SNF, yRSC), *Drosophila* (*Brahma*) and human (hSWI/SNF Complex A, hRSC/PBAF Complex B). The evolutionarily conserved “core” subunits consist of Swi2p (red), Swi3p (orange), Snf5p (green), and Swp73p (purple) homologues. In addition, the more divergent actin related proteins (ARPs, light blue) are also present in all complexes. Moreover, some subunits are only conserved in a subset of ATP-dependent complexes, such as ySwi1p (yellow), hBaf180 (pink), and the dBap111p (lavender). Unique subunits (gray) are present in all the complexes, perhaps defining specificity to each complex’s function. Note: this schematic only depicts conserved homologues among SWI/SNF-like complexes, and does not represent direct subunit-subunit interactions that occur within the native complexes.



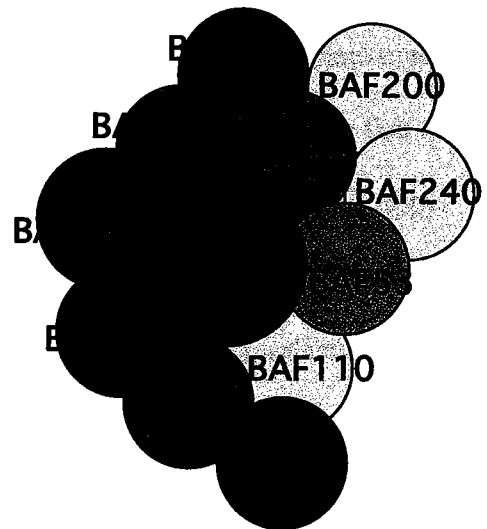
ySWI/SNF



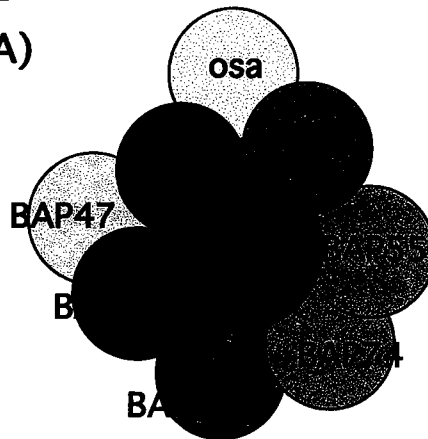
yRSC



hSWI/SNF
(Complex A)



hRSC/PBAF
(Complex B)



dBrahma

three of the conserved human subunits BRG1 (Swi2p), BAF170/BAF155 (Swi3p) and INI1 (Snf5p) have been shown to constitute the minimum "core" sub-complex that possesses the ability to disrupt DNA-histone interactions *in vitro*, albeit not as efficiently as native complex (Phelan et al., 1999).

In this study, we further characterize the organization of the components within SWI/SNF complex by analyzing subunit interactions that occur with the Swi3p subunit. We investigate whether individual SWI/SNF subunits directly interact with Swi3p by *in vitro* pull-down and co-immunoprecipitation assays. Both assays concur, indicating that Swi3p directly interacts with itself, as well as with the Swi2p, Snf5p, Swp73p, Swi1p, and Snf6p subunits. We further delineated the domains of Swi3p that mediate contact with each of these interacting SWI/SNF subunits. Moreover, we demonstrate that self-association of Swi3p requires the interdependent function of an intact leucine zipper motif and an N-terminal region of Swi3p. We propose a model for SWI/SNF subunit organization where Swi3p serves to nucleate the foundation for intramolecular subunit interactions for the SWI/SNF complex.

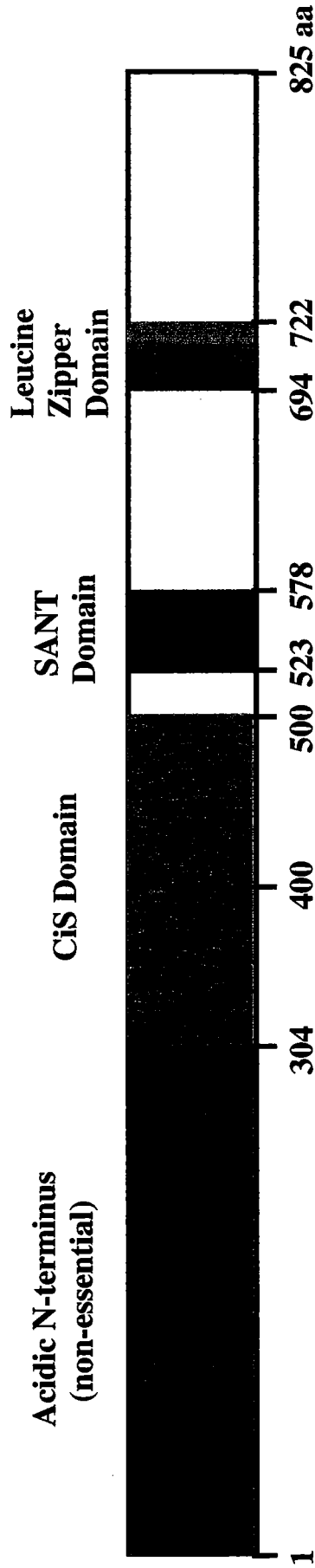
Results

Swi3p directly interacts with itself, Swi2p, Snf5p, Swp73p, Snf6p, and Swi1p.

Swi3p contains several different motifs. The amino terminus (1-304 aa) of Swi3p is highly acidic and non-essential, since deletion of this domain does not alter function *in vivo* and SWI/SNF complex harboring such a Swi3p deletion remains fully assembled *in vitro* (L. Boyer, thesis; Figure III.2). In addition to this non-conserved N-terminal **Figure**

III.2 Schematic Representation of Swi3p. The Swi3p polypeptide is 825 aa in length and contains multiple domains, including a highly acidic amino terminus (amino acids 1-304; orange), a region rich in prolines, hydrophobic, and aromatic amino acids (amino acids 300-500; green), a SANT (Swi3, Ada2, N-CoR, TF_{III}F"; amino acids 523-578; purple) domain and a putative hydrophobic leucine zipper (amino acids 694-722; light blue) (Aasland et al., 1996; Peterson and Herskowitz, 1992). Excluding the acidic N-terminal domain, the other three motifs are conserved in all Swi3p family members (for review see Vignali et al., 2000).

Swi3p



Motif	Amino Acids	Description	Function
Acidic N-terminus	1-304	Rich in aspartic and glutamic acids; net negative charge -60	Dispensable
Cis (Conserved in <u>Swi3p</u> Family members)	~300-500	Rich in hydrophobic and aromatic residues	Mediates interactions among components of SWI/SNF
SANT (<u>Swi3</u> , <u>Ada2</u> , <u>N-CoR</u> , <u>IFIIB</u>)	523-578	Homology to Myb-DNA binding domain	Possible histone binding domain*
Leucine zipper	694-722	Predicted hydrophobic coiled-coil	Mediates self-association of Swi3p

domain, three conserved motifs are found in all Swi3p family members; a region rich in prolines, hydrophobic, and aromatic amino acids (~300-500 aa), a SANT (Swi3, Ada2, N-CoR, TF_{III}F"; 523-578 aa) domain, and a putative hydrophobic leucine zipper (694-722 aa) (Figure III.2). All of these conserved domains are required for SWI/SNF function and assembly *in vivo* and *in vitro* (Aasland et al., 1996; Peterson and Herskowitz, 1992; Vignali et al., 2000).

Knowing that Swi3p is crucial for SWI/SNF function *in vivo*, and for integrity of the assembled SWI/SNF complex *in vitro*, we sought to investigate which SWI/SNF subunits directly interact with Swi3p. To address this question, we performed *in vitro* GST pull-down and HA co-immunoprecipitation assays. The first 306 amino acids of Swi3p can be removed without any deleterious effect on SWI/SNF function or assembly, thus our *in vitro* binding studies used a derivative of Swi3p that lacks this N-terminal domain. For the *in vitro* GST pull-down assays, recombinant GST-SWI3₂₄₀₋₈₂₅ and a control GST protein were over-expressed from the strong Gal1p promoter and purified from yeast (Figure III.3A). Fusion of GST to the N-terminus of Swi3 did not disrupt its function, since this fusion protein was able to fully complement the defect in transcription of a HO-lacZ reporter gene in a *swi3* deletion mutant (Figure III.3B). Furthermore, over-expression of GST-SWI3₂₄₀₋₈₂₅ displays no dominant negative phenotype (data not shown). Purification of this GST-SWI3 fusion protein on glutathione-agarose led to a highly pure preparation that did not contain other SWI/SNF subunits, as demonstrated by Coomassie staining and Western analysis (Figure III.3A; data not shown).

To test for direct Swi3p-subunit interactions, we incubated individual ^{35}S -methionine, *in vitro* translated SWI/SNF subunits with recombinant GST or GST-SWI3₂₄₀₋₈₂₅, and then we monitored for association with Swi3p by GST pull-down assays (Figure III.4A). For all binding assays in this study, two criteria were used to evaluate whether binding occurred. First, we required that the ^{35}S -radioactive signal for each SWI/SNF subunit in the bound fraction (e.g. bound GST-SWI3₂₄₀₋₈₂₅) must be greater than the control bound fraction (e.g. bound GST) and secondly, that this signal must be greater than the input signal (e.g. input GST-SWI3₂₄₀₋₈₂₅ which equals 2.5% of the total reaction). In this assay, GST-SWI3₂₄₀₋₈₂₅ interacted with itself, an N-terminal domain of Swi2p, SNF5, SWP73, SNF6, and SWI1. No significant binding was observed to the ATPase domain of SWI2, SWP29, ARP7, or ARP9 (Figure III.4A).

To confirm these direct interactions with an independent method, we complemented these data with *in vitro* co-immunoprecipitation assays. We incubated *in vitro* translated HA epitope-tagged alleles of Swi3 (HA-SWI3) with individual *in vitro* translated SWI/SNF subunits, followed by immunoprecipitation with α -HA immune sera (see Experimental Procedures; Figure III.4B). In agreement with the GST pull-down results, HA-SWI3 Δ 306 interacted with itself, an N-terminal domain of SWI2, SNF5, SWP73, SNF6, and SWI1. Likewise, no significant binding was observed to the ATPase domain of SWI2, SWP29, ARP7, or ARP9 (Figure III.4B).

Swi3p N-terminal Region Influences SWI/SNF Subunit Interactions.

Previous studies have shown that deletion of amino acids 300 to 500 of Swi3p eliminates

SWI/SNF complex assembly *in vitro* and its function *in vivo* (L. Boyer, thesis). For example, in extracts prepared from strains harboring the *SWI3Δ403* or *SWI3Δ506* alleles (which remove the first 403 and 506 amino acids of Swi3p, respectively), Swi3p and Swi2p do not co-fractionate by gel filtration analysis. This suggests that this conserved region of Swi3p is required for complex assembly or stability (L. Boyer, thesis; Figure III.2). To investigate whether amino acids 300-500 of Swi3p are required for Swi3p-SWI/SNF subunit interactions, Swi3p N-terminal deletion constructs, HA-SWI3Δ405 and HA-SWI3Δ506, were used in co-immunoprecipitation assays (Figure III.4B). For the HA-SWI3Δ405 construct, SWI2N, SNF5, SWP73 and SNF6 were still able to interact, whereas SWI3 and SWI1 could no longer interact (Figure III.4B). An additional deletion of 100 amino acids from the N-terminus of Swi3p, HA-SWI3Δ506, eliminates binding of SWP73 and SNF6, but has no effect on SWI2N or SNF5, which were still able to bind (Figure III.4B). These data suggest that amino acids 306 to 506 are critical for Swi3p-subunit interactions with Swp73p, Snf6p and Swi1p. These data further suggest that Swi2p and Snf5p require the C-terminal region (amino acids 506-825) to interact with Swi3p. Interestingly, these N-terminal deletions also eliminate the self-association of Swi3p (Figure III.B). Both N-terminal deletion derivatives were unable to associate with either full-length Swi3p or with analogous deletion derivatives. This was not expected, since previous data has implied that the putative, C-terminal leucine zipper motif contributed to Swi3p self-association (discussed below, L. Boyer, thesis, Treich et al., 1995; Treich and Carlson, 1997).

Figure III.3 Purification of GST and GST-SWI3 Proteins. A. Coomassie staining.

Expression of yeast GST-SWI3₂₄₀₋₈₂₅ (CY618) and GST (CY685) recombinant proteins.

Arrows indicate respective full length GST-SWI3_{240-825 a} (~94 kDa) and GST (~29 kDa)

proteins. * indicates an amino terminal deletion of GST-SWI3₂₄₀₋₈₂₅ determined by

Western analysis with Swi3 and GST immune sera. **B. Complementation Assays.** Yeast

strains that harbor a SWI/SNF-dependent HO-LacZ reporter construct in which

SWI/SNF components are wild type (CY150), swi3Δ (CY165; Peterson and Herskowitz,

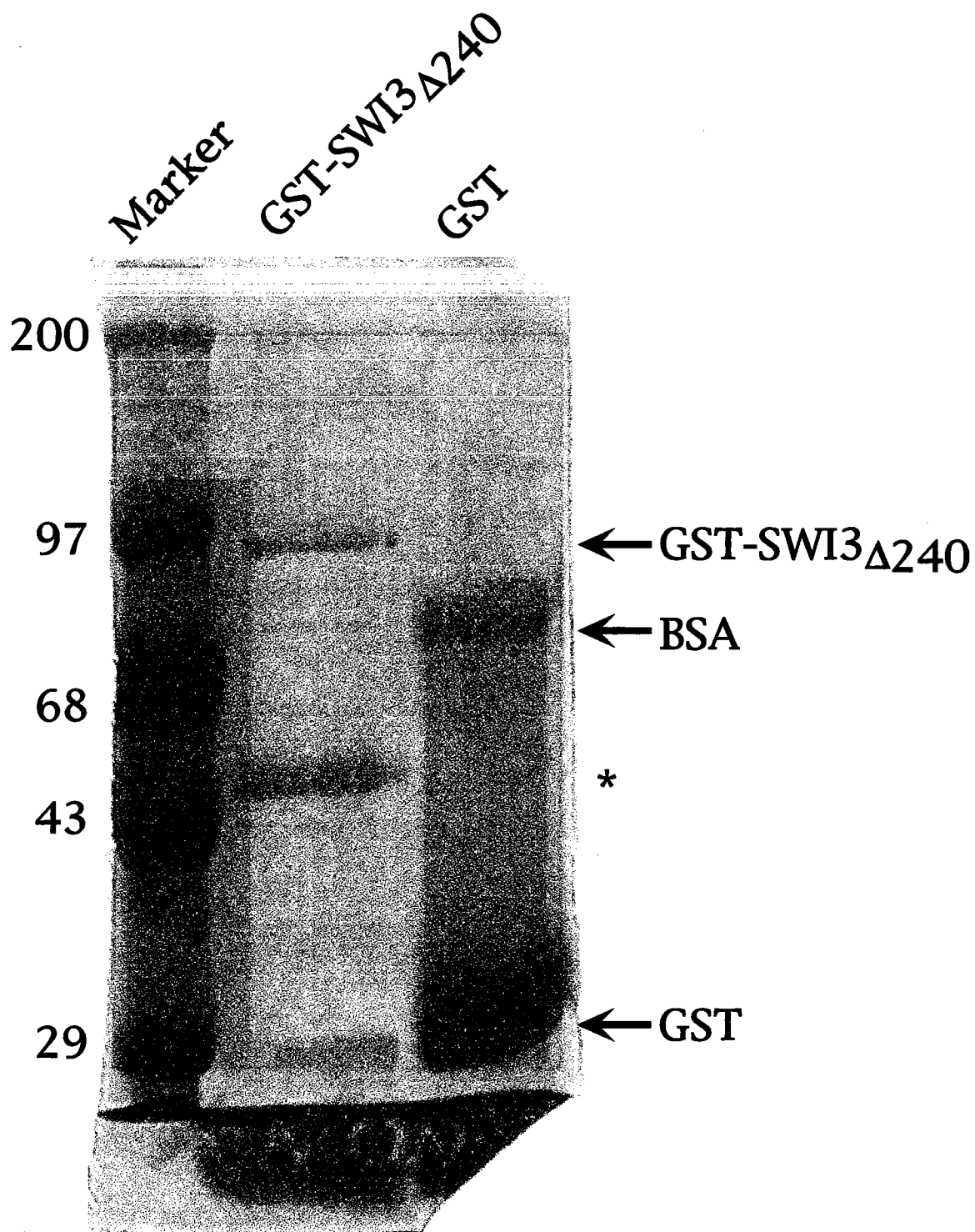
1992) or over-expressed GST-SWI3₂₄₀₋₈₂₅ (CY665), were constructed. Expression levels

for each strain were determined by measuring β-galactosidase activity (nmoles of *O*-

nitrophenyl-D-galactosidase per milligram of protein per minute) by spectrophotometric

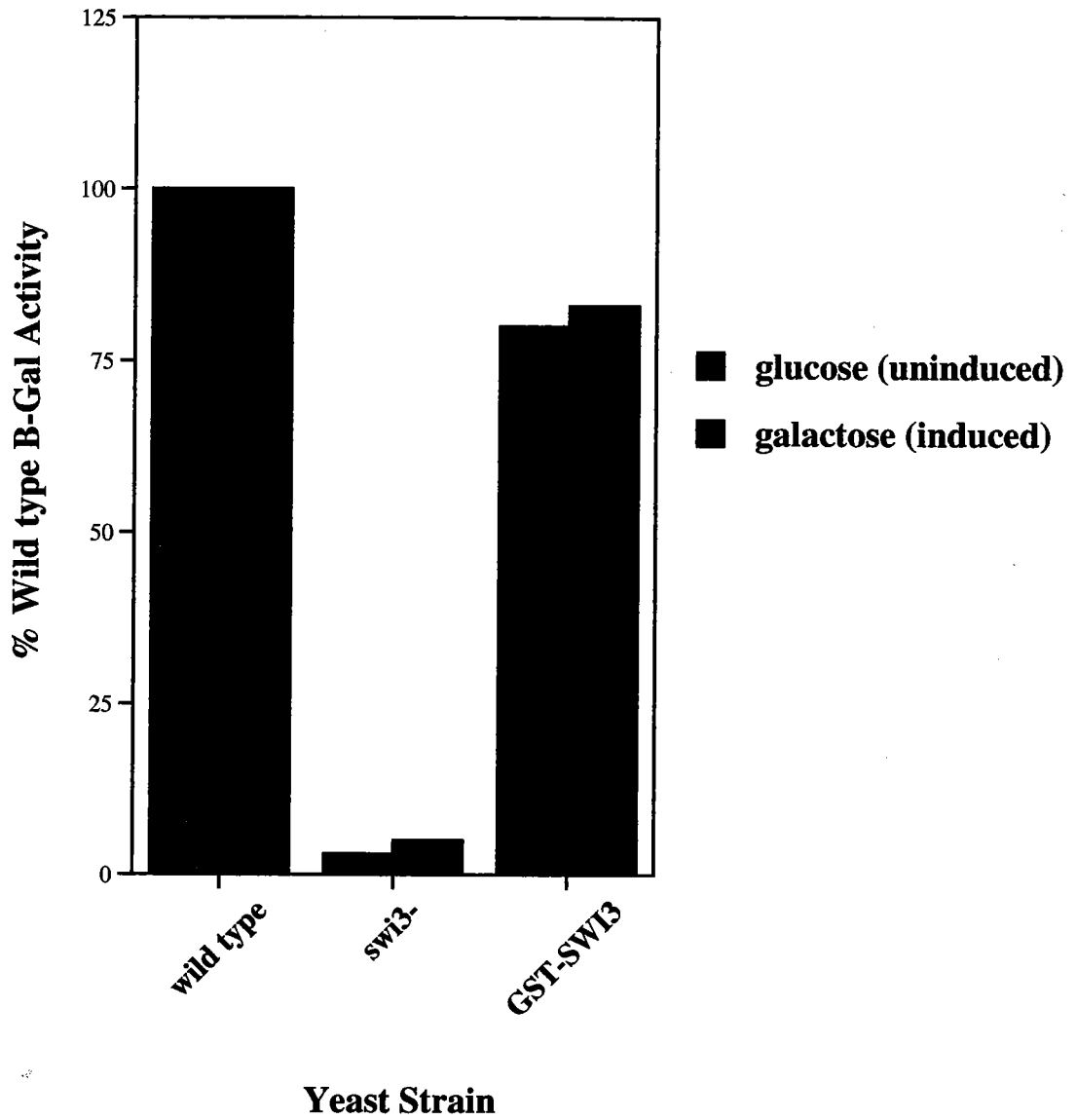
colormetric assay. The graphs show mean values of three individual experiments

normalized to the wild type activity (100%).



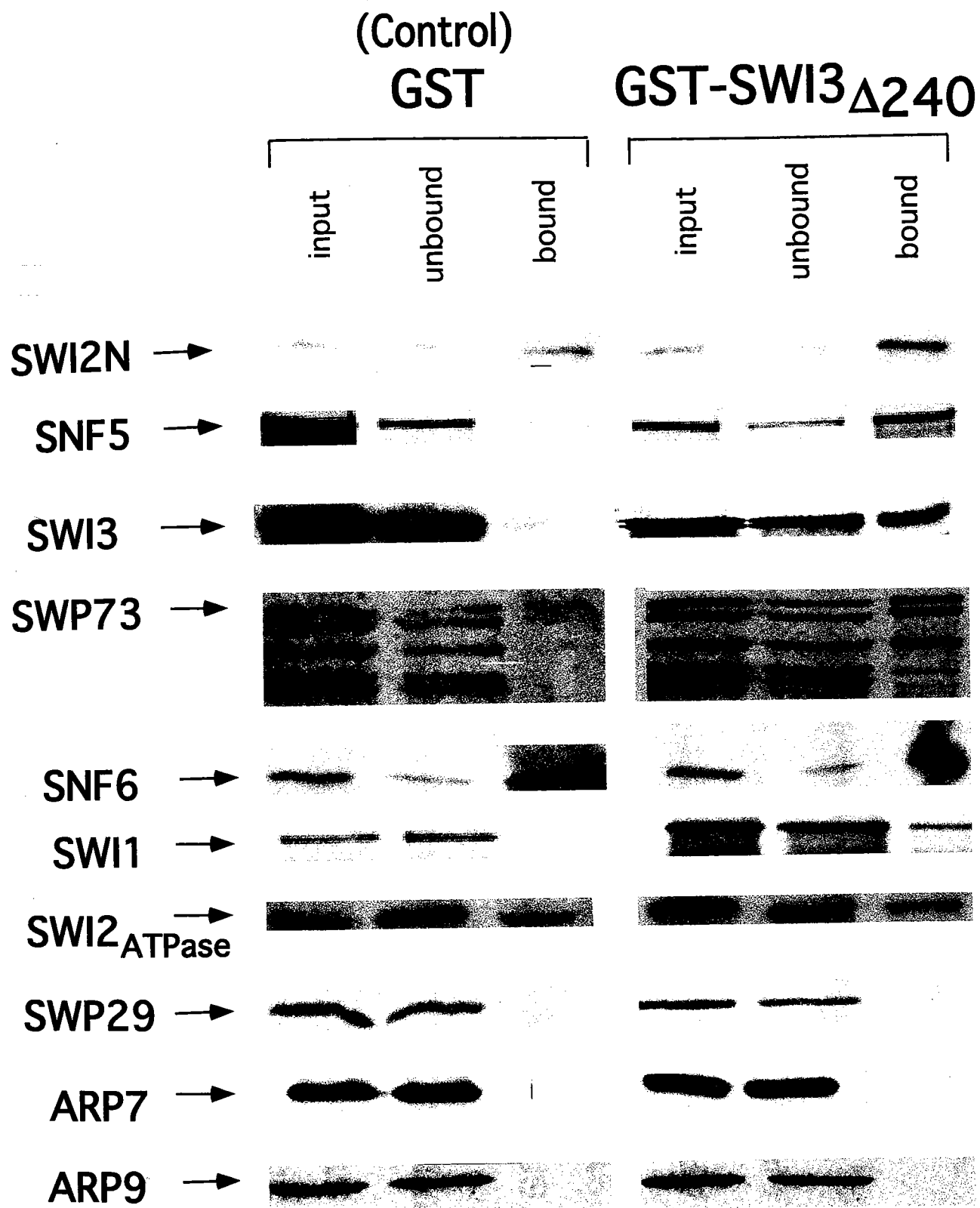
A

Complementation Assay



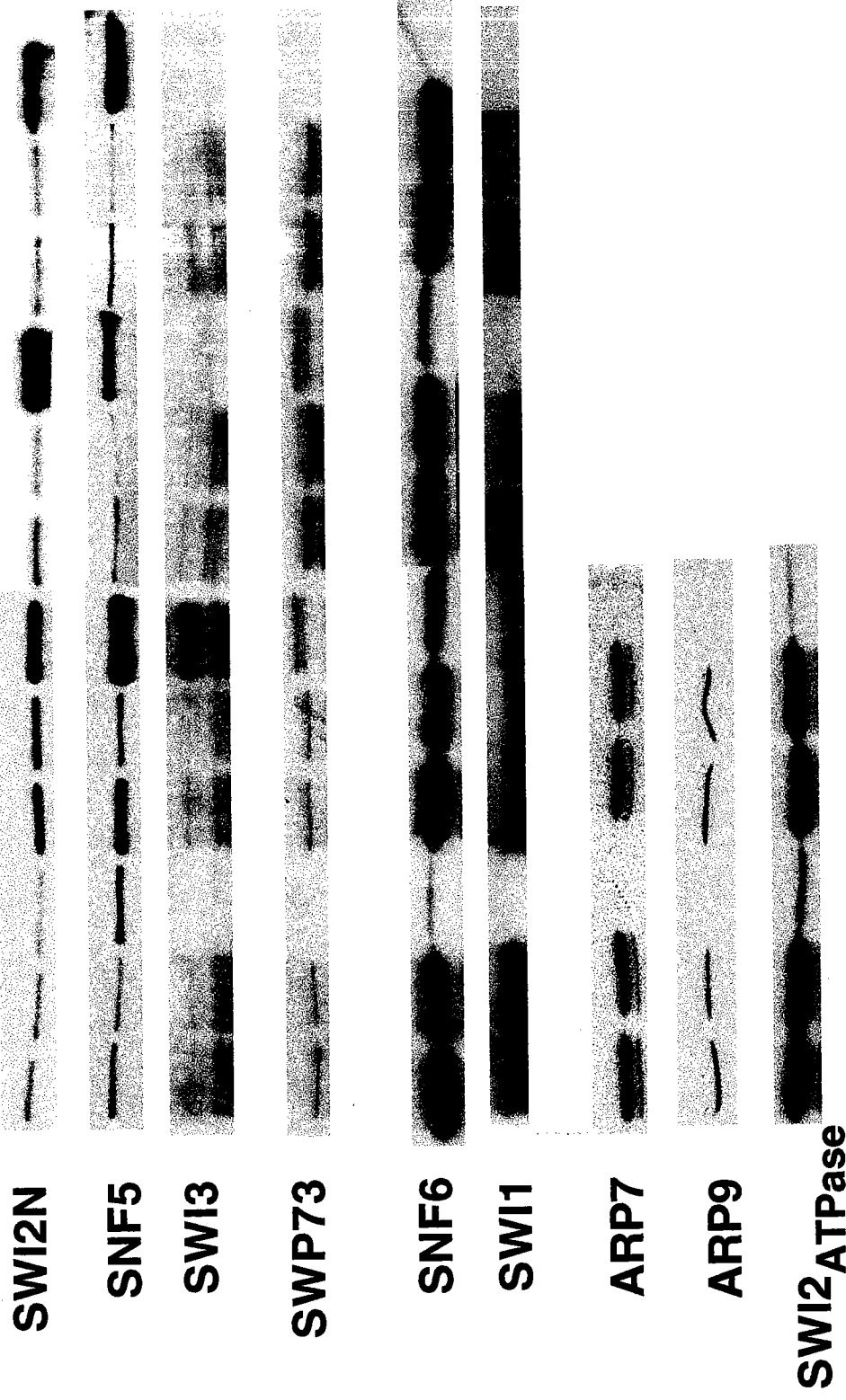
B

Figure III.4 Swi3p N-terminal Subunit Interactions. **A.** *In vitro* GST pull-down assays. Yeast recombinant GST (control) or GST-SWI3₂₄₀₋₈₂₅ was tested for direct binding to individual *in vitro* translated ³⁵S-methionine labeled SWI/SNF polypeptides. Reactions were immunoprecipitated with 7ul anti-GST-Sepharose-4B (Amersham), and the reaction inputs (2.5%), unbound fractions (2.5%) and bound fractions (100%) were run on SDS-PAGE, fixed, Amplified and autoradiographed. **B.** Co-immunoprecipitation assays. HA- epitope tagged *in vitro* translated SWI3 (control), HA-SWI3Δ306, HA-SWI3Δ405, HA-SWI3Δ506 were tested for direct binding to individual *in vitro* translated ³⁵S-methionine labeled SWI/SNF polypeptides. Reactions were assayed as described in Panel A. **C.** N-terminal region of Swi3p is important for Swi3p Self-Association. Amino acids 300-500 contribute to mediating self-association of Swi3p. HA co-immunoprecipitation assays were performed with *in vitro* transcribed SWI3 (control), HA-SWI3Δ306, HA-SWI3Δ405 or HA-SWI3Δ506, and binding was carried out with *in vitro* transcribed ³⁵S-methionine labeled Swi3p's N-terminal deletions SWI3Δ405 and SWI3Δ506. Reactions were immunoprecipitated and assayed as described in Panel A.

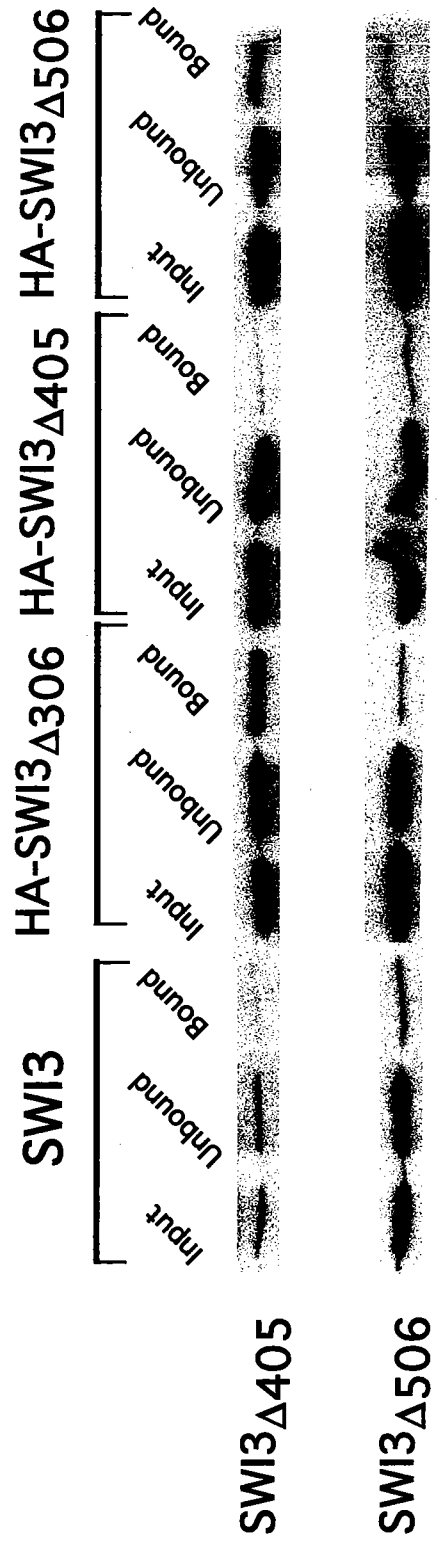


A

	HA-			HA-			HA-					
	Control			SWI3 Δ 306			SWI3 Δ 405			SWI3 Δ 506		
	I	U	B	I	U	B	I	U	B	I	U	B



B



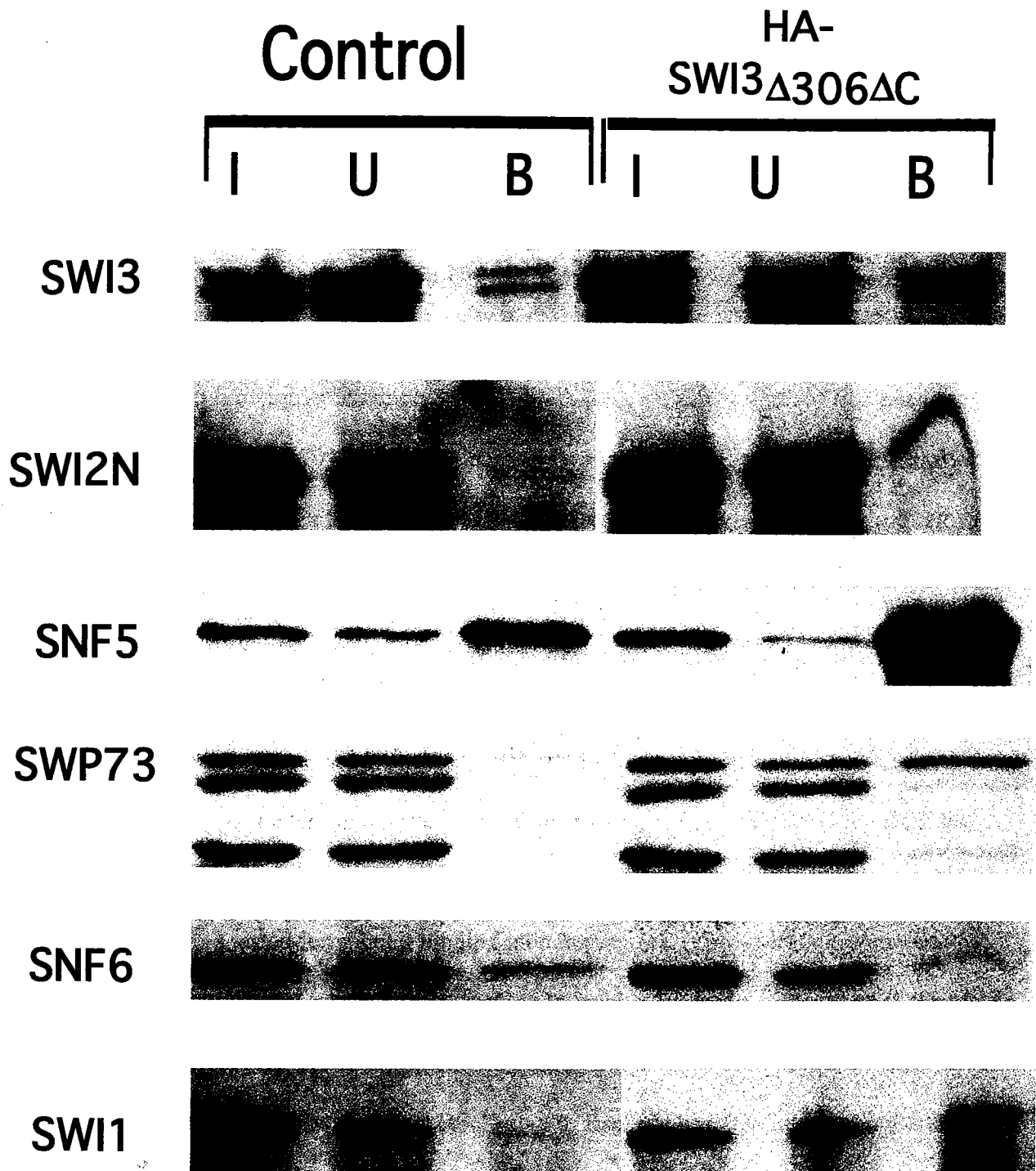
C

Swi3p C-terminal region influences SWI/SNF subunit interactions.

Knowing that the N-terminal domain of Swi3p is important for several intramolecular complex interactions (Figure III.4), we sought to determine whether the C-terminal domain of Swi3p also mediates SWI/SNF subunit interactions. A C-terminal deletion was constructed, HA-SWI3 Δ 306 Δ C (amino acids 306-540), which removes the leucine zipper domain and disrupts the SANT domain (see Figure III.2). This derivative was then tested for subunit interactions by the co-immunoprecipitation assay (Figure III.5). The results indicate that SNF5, SWP73 and SWI1 are able to interact with this C-terminal deletion, whereas SWI3, SWI2N and SNF6 subunits no longer bind (Figure III.5). This suggests that the C-terminal region (540-825 aa) of Swi3p is required for Snf6p, Snf5p and Swi2p binding.

Collectively, the C-terminal deletion and the N-terminal deletions (Figure IV.4B and Figure III.5) lead to a rough map of Swi3p-SWI/SNF subunit binding interactions. First, Swi1p and Swp73p only require the N-terminal region (a.a 306-506) to bind Swi3p, and Swi2p only requires the C-terminal region (a.a 540-825) to bind Swi3p. However, both the N-terminal region (a.a 405-506) and the C-terminal region (a.a. 540-825) of Swi3p are required for Snf6p association. Second, the minimal interaction domain needed for Snf5p was mapped to amino acids 506-540 of the Swi3p protein. Third, both the N-terminal and C-terminal regions contribute to Swi3p oligomerization. Furthermore, these data indicate the binding of SWI/SNF subunits to Swi3p does not require Swi3p oligomerization of Swi3p.

Figure III.5 Swi3p C-terminal Subunit Interactions. Swi3p's C-terminal region (amino acids 540-825) is required for Swi2p, Snf6p, and Snf5p association. HA co-immunoprecipitation assays were performed with either *in vitro* transcribed SWI3 (control) or HA-SWI3 Δ 306 Δ C (amino acids 306-540), and binding was carried out with individual *in vitro* transcribed 35 S-methionine labeled SWI/SNF subunits. Reactions were immunoprecipitated with 7 μ l anti-HA-Sepharose, and the reaction inputs (2.5%), unbound fractions (2.5%) and bound fractions (100%) were run on SDS-PAGE, fixed, Amplified and autoradiographed.



Leucine Zipper Domain Mediates Self-Association of Swi3p. Swi3p harbors a putative leucine zipper domain composed of four heptad repeats of leucine residues interspersed with one heptad repeat of methionine. Swi3p's leucine zipper motif lacks the basic residues characteristic of bZip-type domains, suggesting that it is involved in protein-protein rather than protein-DNA interactions (Peterson and Herskowitz, 1992). Previous studies have shown a single leucine to proline change in this motif (L714P) has no effect on function or assembly of SWI/SNF, whereas substitution of two leucines to prolines at positions 701 and 714 reduced *in vivo* transcription activity 10-fold and causes the SWI/SNF complex to disassemble *in vitro* (see Experimental Procedures; L. Boyer, thesis). This indicates an important role for the leucine zipper domain in Swi3p function.

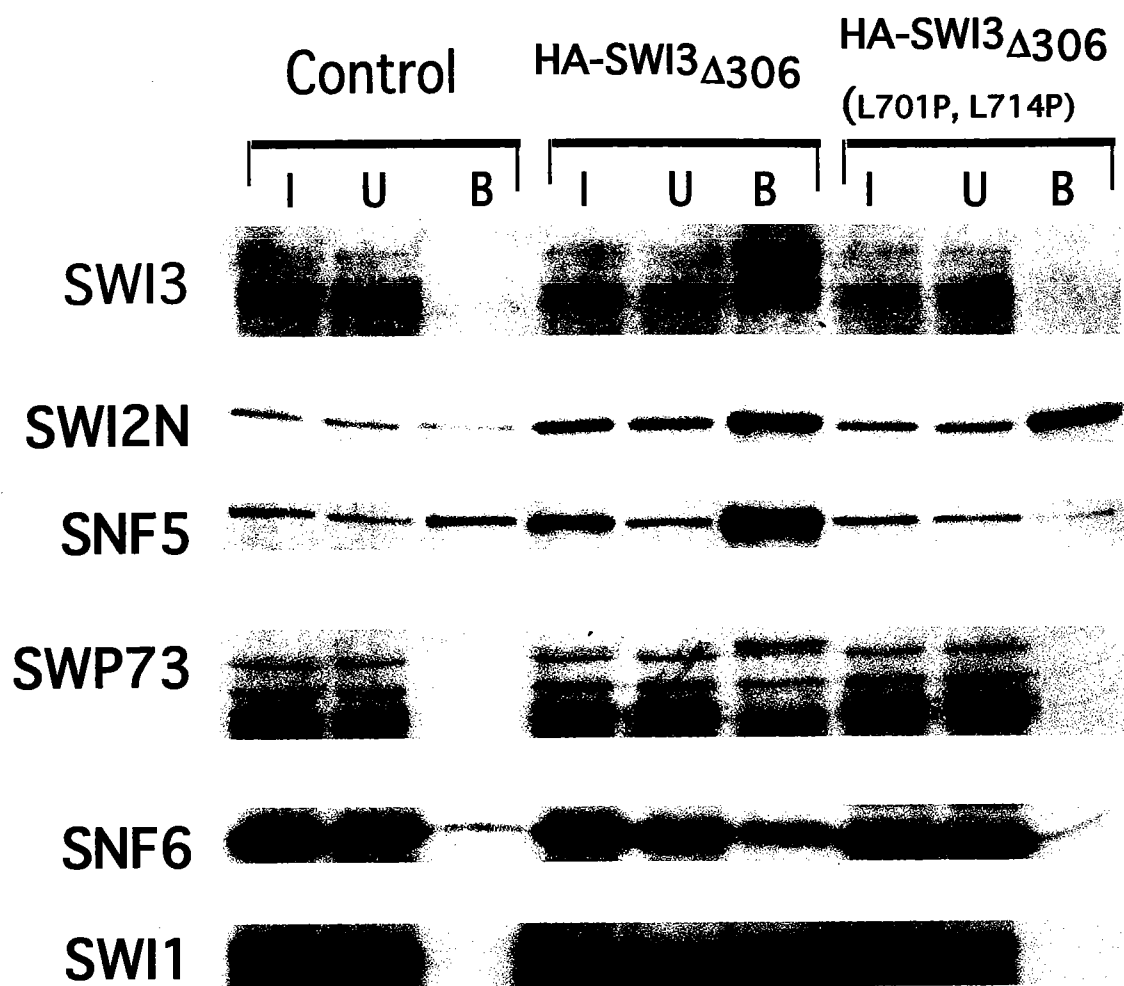
Knowing that Swi3p self-association is influenced by the deletion of the C-terminal region of Swi3p (Figure III.4 and Figure III.5), we sought to determine whether a more subtle C-terminal leucine zipper mutant had any influence on the self-association of Swi3p, or on the ability of other subunits to directly interact with Swi3p. Both the wild type (HA-SWI3 Δ 306) and double leucine zipper mutant (HA-SWI3 Δ 306 (L710P, L714P)) were tested for subunit binding interactions using the co-immunoprecipitation assay (Figure III.6A). As expected, the Swi3p leucine zipper mutant abolishes the ability of Swi3p to self-associate (Figure III.6A). This is consistent with previous two-hybrid data, which showed that the yeast RSC subunit, Rsc8/Swh3p (Swi3p homologue), requires the leucine zipper domain to self-associate (Treich and Carlson, 1997). Thus, an intact leucine zipper domain, as well as residues within the N-terminal domain, are required for Swi3p-self-association.

Surprisingly, the binding of SNF5, SWI1, and SWP73 subunits were all disrupted by the double leucine zipper mutant (Figure III.5 and Figure III.6A). This data contrasts our results showing that these subunits can interact with a Swi3p derivative, which lacks the entire C-terminal domain. Thus, these data suggest that the double leucine zipper mutant may alter the Swi3p protein, such that this domain interferes with the binding of subunits to the N-terminal region of Swi3p. However, Swi2p, which binds the C-terminal region of Swi3p, remains able to bind the leucine zipper mutant, suggesting that the structure of Swi3p is not completely abolished. Our data suggests that two distinct domains are required for Swi3p self-association - an N-terminal domain that contains residues 300-540 and the C-terminal leucine zipper domain. One possibility is that the N-terminal domain is required for the proper folding of the leucine zipper domain. To test this possibility we examined whether a small C-terminal derivative of Swi3p, which contains just the leucine zipper domain, might be able to interact with the N-terminal deletion alleles of Swi3p. *In vitro* translated ³⁵S-methionine labeled wild type or mutant leucine zipper C-terminal domains (amino acids 540-825) of Swi3p were monitored for direct binding to the HA-SWI3 N-terminal deletion constructs by co-immunoprecipitation assays (Figure III.6B). The results indicated that wild type the ³⁵S-Swi3p leucine zipper domain could bind to all the HA-SWI3 constructs with the exception of the leucine zipper mutant HA-SWI3Δ306 (L701PL714P) (Figure III.6B). Accordingly, the mutant SWI3 leucine zipper (L710P L714P) domain did not bind to any of the Swi3p N-terminal deletion constructs (Figure III.6B). These data confirm that the leucine zipper domain is critical for Swi3p self-association, and that folding of this domain does not require

Figure III.6 Double Leucine Zipper Mutant Abolishes Swi3p's Self-Association. A.

Only SWI2N can bind the double leucine mutant zipper derivative. Co-immunoprecipitation assays were performed with either *in vitro* translated SWI3 (control), HA-SWI3 Δ 306, or HA-SWI3 Δ 306(L710P, L714P) and individual *in vitro* translated ³⁵S-methionine labeled SWI/SNF polypeptides. Reactions were immunoprecipitated with 7ul anti-HA-Sepharose (Babco), and the reaction inputs (2.5%), unbound fractions (2.5%) and bound fractions (100%) were run on SDS-PAGE, fixed, Amplified and autoradiographed. **B.** Swi3p's leucine Zipper Domain is Important for

Leucine Zipper Self-Association. An intact leucine zipper, but not mutant leucine zipper domain, self-associates. HA co-immunoprecipitation assays were performed with either *in vitro* translated SWI3 (control), HA-SWI3 Δ 306, HA-SWI3 Δ 306 (L701P, L714P), HA-SWI3 Δ 405, or HA-SWI3 Δ 506, and binding was carried out with *in vitro* translated ³⁵S-methionine labeled Swi3p's wild type leucine zipper domain (amino acids 523-825) or a mutant leucine zipper domain (amino acids 523-825, L701P, L714P). Reactions were immunoprecipitated with 7ul anti-HA-Sepharose (Babco), and the reaction inputs (2.5%), unbound fractions (2.5%) and bound fractions (100%) were run on SDS-PAGE, fixed, Amplified and autoradiographed.



A

Control HA-SWI3 Δ 306 HA-SWI3 Δ 306 HA-SWI3 Δ 405 HA-SWI3 Δ 506

(L701P, L714P)

I U B I U B I U B I U B I U B

Wild Type
Leu Zipper →
Domain

Mutant
Leu Zipper →
Domain
(L701P,L714P)

B

N-terminal sequences. Furthermore, these data indicate that the role of the leucine zipper domain is to mediate Swi3p's oligomerization and not to mandate Swi3p-SWI/SNF binding interactions. Moreover, these data suggest that both the N-terminal region and the leucine zipper domain function interdependently for Swi3p self-association.

Discussion

Here we have shown that Swi3p, a conserved subunit in SWI/SNF-like ATP dependent remodeling complexes, functions as a scaffolding protein for the yeast SWI/SNF complex (for recent review Vignali et al., 2000). The SWI/SNF subunits that interact with Swi3p include Swi2p, Snf5p, Swp73p, Swi1p, and Snf6p. Swi3p also self-associates. In addition, we demonstrate that the leucine zipper domain and an N-terminal region of Swi3p are critical for Swi3p self-association, and that these domains function interdependently. Collectively, these data, and previous *in vivo* and *in vitro* studies, have indicated that Swi3p is critical for SWI/SNF complex function and assembly (L. Boyer, thesis). We propose that the Swi3p nucleates the assembly of the yeast SWI/SNF complex.

Direct Swi3p-Subunit Interactions within the SWI/SNF Complex. The 825 amino acid yeast Swi3p polypeptide consists of three highly conserved domains, as well as additional unique sequences (see Figure III.2). Deletion analysis of Swi3p roughly mapped the Swi3p binding domains important for direct interactions with SWI/SNF

components (Figure III.4B and Figure III.5). Swi1p binds to the N-terminal region of Swi3p at amino acids 306-405 (Figure III.4 and Figure III.5). Swp73p appears to bind an adjacent region of Swi3p between amino acids 405 to 540. Snf6p binding requires part of the N-terminal region (405-506 aa; Figure III.4), as well as the C-terminal region of Swi3p (amino acids 540-825; Figure III.5). Snf5p can bind all Swi3p N-terminal deletions, as well as the C-terminal deletion that removed amino acids 540-825. This suggests that amino acids 506 to 540 may provide a crucial domain for Snf5p interaction with Swi3p. Interestingly, the Snf5p binding domain overlaps a portion of the SANT domain. Perhaps the SANT domain may function as a sensory domain, providing Snf5p and Swi3p the ability to convey intramolecular signaling within the complex important for SWI/SNF activities. In support for this notion, it is known that the SANT domain is essential to SWI/SNF function and that it is involved in histone N-terminal domain interactions (L. Boyer, thesis). In addition, our studies show that an interaction between Swi2p and Swi3p occurs in the C-terminal region of Swi3p (amino acids 540 and 825; Figure III.5). Furthermore, disruption of the leucine zipper motif does not effect this binding interaction. Since the leucine zipper double mutant causes a secondary structure distortion of this domain, our data suggests that Swi2p binding may be occurring outside the leucine zipper motif. This data is consistent with studies showing an interaction between Swi2p and Swi3p by two hybrid analysis and with studies showing that an interaction between Sth1p (Swi2p homologue) and Rsc8/Swh3p (Swi3p homologue) is independent of Rsc8/Swh3p self-association (Treich et al., 1995; Treich and Carlson, 1997). In addition, it has previously been shown that the wild type or mutant

(L701PL714P) Swi3p leucine zipper domains (amino acids 540-825) bind to subunits of purified SWI/SNF complex by far-Western analysis. More specifically, the wild-type C-terminal domain bound to Swi3p, Swi2p and Snf5p, and binding to Swi2p and Snf5p was insensitive to disruption of the leucine zipper, whereas interaction with Swi3p was lost (L. Boyer, thesis).

Self-Association of Swi3p. Our study has shown that Swi3p can self-associate and that both the N-terminal region of Swi3p (amino acids 305-506) and the leucine zipper domain (amino acids 694-722) are important for this interaction (Figure, III.4B, Figure III.6, Figure III.7). Our data also suggests that these two domains are interdependent. If either domain is disrupted the remaining domain is insufficient for self-association of Swi3p. However, the leucine zipper domain present in such N-terminal deletion derivatives is still able to self-associate with a small, C-terminal domain of Swi3p, which contains only the leucine zipper domain. Thus, when N-terminal deletion derivatives are present in two copies, it appears that the partial N-terminal domain of Swi3p can block the function of the leucine zipper domain. Likewise, a double mutation in the leucine zipper domain can abolish the functions of the N-terminal domain, both in terms of self-association and binding of the other subunits. The simplest model to explain such interdependence is to propose that the N-terminal and C-terminal domains interact with each other.

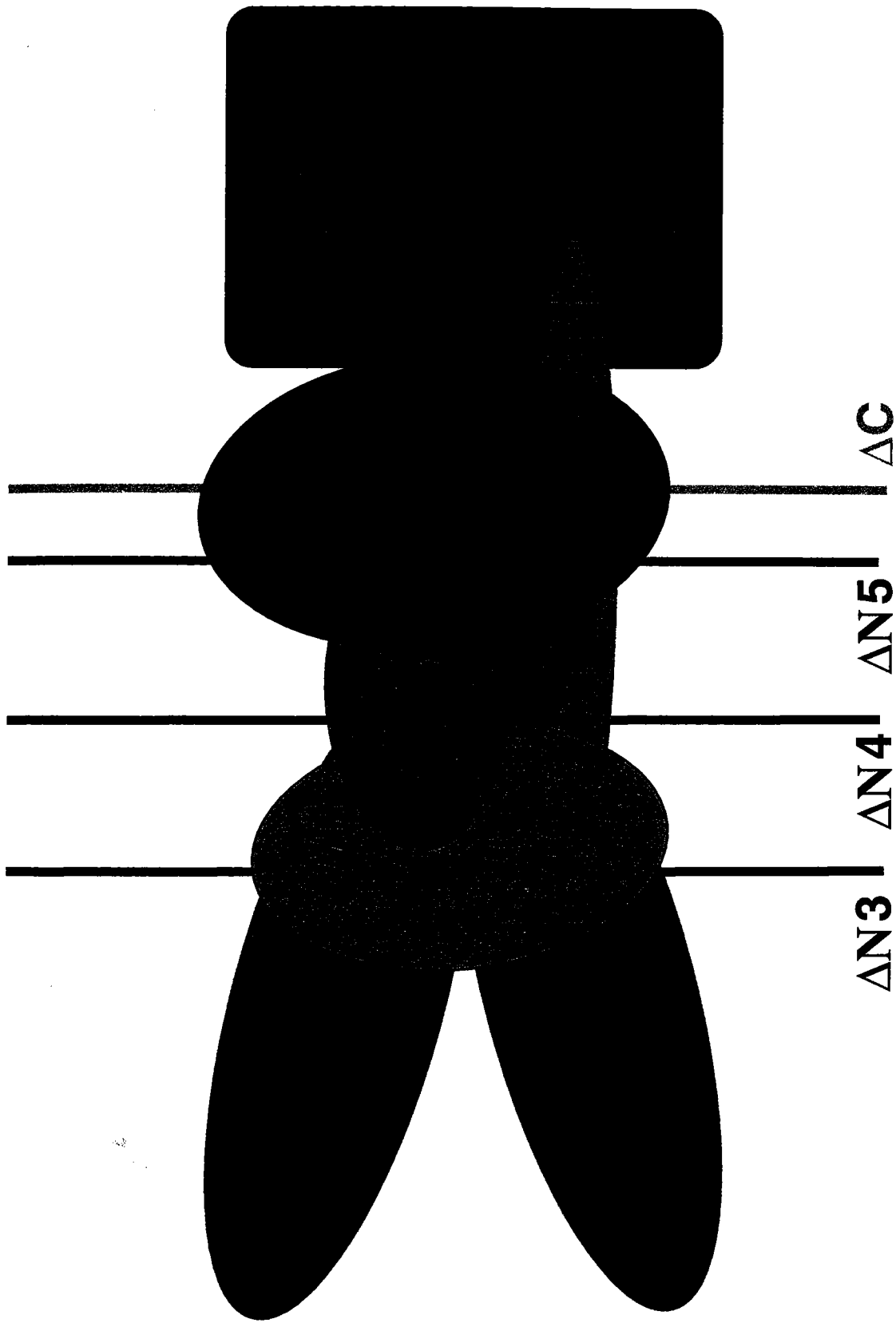
The Yeast SWI/SNF Complex Organization. Previously, little was known about the subunit organization of the SWI/SNF complex. Many of the ATP-dependent SWI/SNF-like chromatin remodelers have several conserved subunits, including Swi2p, Swi3p, Snf5p, Swp73p and Arp proteins. Some binding interactions among conserved components of SWI/SNF-like complexes have been described. For instance, the yeast RSC subunit, Rsc8/Swh3p (Swi3p), can self-associate as well as interact with Sth1p (Swi2p) and Rsc6p (Swp73p) (Treich and Carlson, 1997; Treich et al., 1998). Analogously, the *Drosophila* Swi3p homologue, MOR, has also been shown to self-associate and interact with BRM (Swi2p). Direct interaction has also been detected for human Baf170p (Swi3p) and Baf47/Ini1p (Snf5p) subunits. For the yeast SWI/SNF complex, a two-hybrid screen using the N-terminus of Swi2p (1-767 amino acids), which excludes the ATPase domain, interacted with both Swi3p and Snf11p subunits. It is also known that Swp29 interacts with Snf5p (Cairns et al., 1996; Treich and Carlson, 1997). Moreover, human BRG1 (Swi2p), BAF155, BAF177 (Swi3p) and INI1 (Snf5p) can be reconstituted and are the minimal subunits required for efficient chromatin remodeling (Phelan et al., 1999). Collectively, these data illustrate that the conserved homologues of the SWI/SNF-like chromatin remodeling complexes interact with each other.

This study describes a more detailed understanding of how the yeast SWI/SNF subunits interact intramolecularly within the complex and we propose a model that depicts the organization of the yeast SWI/SNF complex, based on data from Chapter II and Chapter III (see Figure III.8). Here we show that yeast Swi3p mediates self-association by the interdependent N-terminal region and conserved leucine zipper motif.

Swi3p's ability to self-associate indicates that SWI/SNF contains more than one copy of Swi3p per complex (Figure III.4B and Figure III.7A). Previous studies have shown that Swi3p homologues can self-associate. For instance, both the yeast and the *Drosophila* Swi3p homologue, Rsc8/Swh3p (Swi3p) and MOR can self-associate (Crosby et al., 1999; Treich and Carlson, 1997). We propose Swi3p is a dimer within the SWI/SNF complex. Furthermore, this study determines the stoichiometry of the SWI/SNF complex, which shows Swi3p is present in two copies, and the other subunits are present in one copy per SWI/SNF complex (Chapter II).

The Swi3p dimer provides a platform for the Swi1p, Swp73p, Snf6p, Snf5p, and Swi2p subunits to bind (Figure III.7). Both Swi1p and Swp73p bind the N-terminal domain of Swi3p exclusively. Binding of Snf6p and Snf5p appear to require both the N-terminal domain as well as a C-terminal region of the Swi3p polypeptide. In addition, Snf5p is present in two copies per complex (see Figure II.2). Finally, Swi2p, only present in one copy, binds exclusively to the C-terminal region of Swi3p. Previous data have reported binding interactions between Swi2p and Snf11p, as well as between Snf5p and Swp29p (Treich and Carlson, 1997, Cairns et al., 1996). Collectively, this produces the fundamental foundation for the yeast SWI/SNF complex, which we propose may be preserved for other SWI/SNF-like complexes.

Figure III.7 SWI/SNF Complex Organization Model. We propose Swi3p nucleates the assembly of the yeast SWI/SNF complex. N-terminal and C-terminal deletion of Swi3p charted the SWI/SNF subunit binding interaction domains. Swi3p directly interacts with SWI/SNF subunits Swi2Np₁₋₇₆₇, Snf5p, Swp73p, Snf6p and Swi1ps. In addition, two domains, the N-terminal domain (amino acids 300-540) and the leucine zipper motif (amino acids 694-722) function in an interdependent manner to mediate self-association of Swi3p.



Experimental Procedures

GST-fusion protein expression and purification. CY618 is wild type CY337 protease minus strain with Gal1-GST-SWI3₂₄₀₋₈₂₅ (CP291; Swi3p EcoRV fragment) was partially linearized with restriction enzyme XbaI and integrated at the SWI3 locus by one-step loop-in method and verified by Western. CY685 is wild type CY337 protease minus strain with Gal1-GST (CP311) was digested with restriction enzyme StuI and integrated at the URA3 locus by one-step loop-in method and verified by Western. Yeast cultures were grown at 30°C in yeast extract-peptone (YEP) media containing 2% galactose (Difco Laboratories). Recombinant GST-SWI3₂₄₀₋₈₂₅ (CY618) and GST (CY685) recombinant proteins were expressed and purified from 20 L and 5 L of culture, respectively. Cells are harvested at 4K, 4°C for 10 minutes and are washed in E Buffer (20 mM Tris, pH7.5, 350 mM NaCl, 10% glycerol, 0.1% Tween-20, 1 mM PMSF, 2 ug/ml of leupeptin and pepstatin A), lysed in Biospec bead beater for 5x 30 seconds with 1' intervals cooled in an ice bath. Lysate was spun at 4°C for 45' at 40K in Beckman Ultracentrifuge using a Ti-40 rotor. Cleared lysate was bound to 5-10 mls GST-agarose (Qiagen) for 3 hours, washed 1x in 250 ml E Buffer, eluted in 50 ml 50 mM glutathione buffer (75mM NaCl, 10% glycerol, 0.1% Tween-20, 1mM PMSF, 2ug/ml of leupeptin and pepstatin A). 100 ul of elute was TCA precipitated and electrophoresed on 10% SDS-PAGE analysis and Western hybridization with GST or Swi3 antibody.

β-Galactosidase assays. Yeast strains that harbor a SWI/SNF-dependent HO-LacZ reporter construct in which SWI/SNF components are wild type (CY150), swi3Δ (CY165; Peterson and Herskowitz, 1992) or over-expressed GST-SWI3₂₄₀₋₈₂₅ (CY665),

were constructed by standard genetic methods. *In vivo* complementation β -galactosidase activity was assayed quantitatively in permeabilized cells as previously described (Stern et al., 1984). Transformants were grown to mid-log phase in the appropriate media for plasmid selection. Activity was measured in Miller units followed by normalization to per cent wild-type activity.

***In vitro* translations** Individual SWI/SNF polypeptides cloned into transcription/translation vectors were made *in vitro* in the presence of [³⁵S] methionine (Amersham; 10mCi/ml) using the T7 TNT-coupled rabbit reticulocyte or T7 TNT-coupled wheat germ extract systems (Promega) according to manufacturer's specifications. Products were visualized by autoradiography or immunoblotting to confirmed the quality of all *in vitro* translation reactions.

***In vitro* GST pull-down assays.** For binding studies 5 ul/50 ul of individual [³⁵S] methionine-labeled SWI/SNF polypeptides *in vitro* translated reaction were pre-cleared for one hour with yeast purified GST-agarose beads. In a final volume of 400 ul of binding buffer (20 mM Tris, pH 8.0, 200 mM NaCl, 1 mM EDTA, 0.1% Triton-X 100, 1 mM DTT, 0.1 mg/ml BSA, 1 mM PMSF) pre-cleared proteins (removing 1/40 for input fraction) were mixed with 20ul (1:1) of individual GST-tagged fusion proteins for 1 hour, 4°C, nutating. Subsequently, reactions were spun down for 1 minute, saving 1/40 for unbound, and washed 5 times in protein binding buffer eluting bound proteins in 2xSDS sample buffer. Binding was analyzed by SDS-PAGE, fixed 20 minutes (10% acetic acid, 20% methanol), Amplify (Amersham Life Sciences) 30-60 minutes, dried and autoradiographed.

HA co-immunoprecipitation binding assays. For binding studies 5 ul/50 ul of individual [³⁵S] methionine-labeled SWI/SNF polypeptides *in vitro* translated reaction were pre-cleared for one hour with Sepharose CL4B beads. In a final volume of 400ul of binding buffer (20 mM Tris, pH 8.0, 150-250 mM NaCl, 1 mM EDTA, 0.1% Triton-X 100, 1 mM DTT, 0.1 mg/ml BSA, 1 mM PMSF) the pre-cleared reaction was mixed with 5 ul/ 50 ul reaction HA-SWI3 derivatives or SWI3 (negative control) *in vitro* translated in the presence of cold methionine (1 mM final per reaction) using the T7 TNT-coupled rabbit reticulocyte or T7 TNT-coupled wheat germ extract systems (Promega). 1/40 of the supernatant was removed (input fraction) and allowed to bind for 1 hour on ice. Subsequently 7.5 ul of anti-HA Sepharose beads (HA.11 AFC-101P; Babco) were added and bound for 1 hour, 4°C nutating. Reactions were spun down for 1 minute, saving 1/40 for unbound and washed 5 times in appropriate NaCl protein binding buffer eluting bound proteins in 2xSDS sample buffer. Binding was analyzed by SDS-PAGE, fixed 20 minutes (10% acetic acid, 20% methanol), Amplify (Amersham Life Sciences) 30-60 minutes, dried and autoradiographed.

***In vitro* transcription/translation plasmid cloning.** Plasmid construction by PCR based cloning into pET vectors (Novagen) for *in vitro* translation are presented as follows:

CP606 (SNF5) 5'GCCGGAATTCATGAATAATCAGCCGCAG-3';

5'GCCGGAATTCGGCGCAAAGGGACTCCGC-3' full length SNF5 2.8 kb PCR (EcoR1) product was cloned into pET5a (EcoR1); CP623 (SWI1) 5'GCCGCCAT

GGATGGATTTCTTTTTGAAT-3'; 5'GCCGCCGGAACGATCCCTGCTT-3'

N-terminal of SWI1 1.6 kb PCR (NcoI/SacII) and 5'GCCGCAGCAGC

AGGCCGCTGCA-3'; 5'GCCGCCATGGTGTTTAGTTGAGCCGCCC-3'

2.5 kb C-terminal of SWI1 PCR (SacII/NcoI) products were cloned into pET11d (NcoI);

CP712 (SWI2N) 5'CGGCGGATCCATGAACATACCACAGCGT-3';

5'GCCGCCTAGGCAATAAAGTCTGTAATC-3' SWI2 1-767 amino acids 2.3 kb PCR (BamHI) product was cloned into pET11a (BamHI); CP708 (SWI2ATPase)

5'GCCGGATCCGGACTATCAAATCAAGGG-3'; 5'CGGCGGATCCTTGTAGGCTC TCTCTAG-3' SWI2 ATPase domain amino acids 767-1220 1.36 kb PCR (BamHI)

product was cloned into pET11a (BamHI); CP687 (ARP7) 5'CGGCGGATCCCGACC

CTTAGTCATGGCT-3'; 5'CGGCGGATCCCTGTTTACAGTCGGCCTC-3' 1.4 KB

PCR (BamHI) was cloned into pET5a (BamHI); CP607 (SNF6) 5'CGGCGAATTCAT

GGGTGTCATCAAGAAG-3'; 5'CGGCGAATTCCAGTAGCTGCACAAATCC-3' 1.15

kb PCR (EcoRI) was cloned into pET5a (EcoRI); CP687 (ARP7) 5'CGGCGGATCCCG

ACCCTTAGTCATGGCT-3'; 5'CGGCGGATCCCTGTTACAGTCGGCCTC-3' 1.4 kb

PCR (BamHI) was cloned into pET5a (BamHI); CP621 (SWP73) 5'CGGCGAATTCAT

GTCCAAAGTAATGAAACCC-3'; 5'CGGCGAATTCTCCTCTAGAGCATATCAA-3'

1.75 kb PCR (EcoRI) was cloned into pET5a (EcoRI); CP615 (SWP29) 5'GCCGGAAT

TCATGGTAGCTCTATCTTCG-3'; 5'GCCGGGTACCTGTTTAGTTGAGCCGCCC-3'

0.97 kb PCR (EcoRI) was cloned into pET5a (EcoRI). CP105 (SWI3) full length SWI3

(Bstul) was cloned into pET3d (NheI/N- filled); CP544 (SWI3 wild type leucine zipper)

and CP630 (SWI3 mutant leucine zipper; L701P and L714P) 0.85 kb (NheI/BamHI)

fragments were cloned into pET11a (NheI/BamHI) separately. Full length ARP9 is

cloned in pET5a as described by Cairns et al. (Cairns et al., 1998).

HA epitope tagged *in vitro* translation vector was used to make SWI3 deletion constructs as follows: CP746 (SWI3) 5'GCCGGGATCCGGAGAATACACTGGGTGAG-3'; 5'CGGCGAATTCCTAGAGAGAGCCTACAAG-3' 1.745 KB PCR (BamH1/EcoR1) SWI3 amino acids 1-582 and SWI3 (BamH1/Cla1) 0.85 kb amino acids 583-825 were cloned together to make full length HA-SWI3; CP738 (HA-SWI3 Δ C) 5'GCCGGGATCCGGAGAATACACTGGGTGAG-3'; 5'CGGCGAATTCCTAGAGAGAGCCTACAAG-3' 1.745 kb PCR (BamH1/EcoR1) SWI3 amino acids 1-582; CP739 (HA- Δ 3SWI3 Δ C) 5'GGCCGGATCCTCCAAGTTATTCTAAATGG-3'; 5'GGCCATCGATGCCAAGTCAAGTGACGCACC-3' 0.83 kb PCR (BamHi/EcoR1)SWI3 amino acids 306-582; CP748 (HA- Δ 3SWI3) 5'GGCCGGATCCTCCAAGTTATTCTAAATGG-3'; 5'GGCCATCGATGCCAAGTCAAGTGACGCACC-3' 1.83 kb PCR (BamH1/EcoR1) SWI3 amino acids 306-825; CP750 (HA- Δ 4SWI3) 5'GGCCGGATCCCCACTAACGTCTCAATATTCC-3'; 5'GGCCATCGATGCCAAGTCAAGTGACGCACC-3' 1.4 kb PCR (BamH1/Cla1) SWI3 amino acids 405-825; CP751 (HA- Δ 5SWI3) 5'GGCCGGATCCAGGCGAACTTCACGTCCTCTG-3'; 5'GGCCATCGATGCCAAGTCAAGTGACGCACC-3' 1.1 kb PCR (BamH1/Cla1) Swi3 amino acids 506-825.

All SWI3 PCR products were cloned into HA *in vitro* transcription/ translation vector (CP707) (BamH1/Cla1) fragment. All constructs were verified that they were in the correct orientation and in proper coding frame by USB Sequenase Kit II using a primer to the T7 promoter 5'TAATACGACTCACTATAGGG-3'.

CHAPTER IV

SWI/SNF Subunit Functions in Targeting

CHAPTER IV

SWI/SNF Subunit Functions in Targeting

Introduction

The yeast SWI/SNF complex is involved in ATP-dependent chromatin disruption, and it is required for the expression of a number of inducible and mitotic yeast genes (Holstege et al., 1998; Krebs et al., 2000; Sudarsanam et al., 2000). ATP-dependent functions of SWI/SNF complex include alteration of nucleosome structure, enhancement of gene-specific activator binding, as well as movement of histones on DNA in *cis* and *trans* (Cote et al., 1994; Jaskelioff et al., 2000; Logie and Peterson, 1997; Logie et al., 1999; Lorch et al., 1999). Although chromatin structure is altered in an ATP-dependent manner, the mechanism of how ATP hydrolysis achieves the chromatin remodeling activities is not clear. However, recent data support the hypothesis that chromatin remodeling complexes use the energy derived from ATP hydrolysis to drive local changes in DNA topology by propagating writhe or twist throughout the nucleosome and lessening DNA-histone contacts (Gavin, 2001, Boyer, 1999; Haves, 2000). Collectively, these data suggest that SWI/SNF ATP-dependent activity leads to an altered accessibility of DNA important for the regulation of nuclear processes (Brown et al., 1996; Cote et al., 1998; Kwon et al., 1994; Logie and Peterson, 1997; Logie et al., 1999).

In the case of the ySWI/SNF, *Drosophila* Brahma, and hSWI/SNF complexes, chromatin remodeling is required for transcriptional regulation of a subset of genes *in vivo* (de La Serna et al., 2000; Deuring et al., 2000; Holstege et al., 1998; Kingston and

Narlikar, 1999; Sudarsanam et al., 2000). How chromatin-modifying enzymes are targeted to specific gene promoters has been an area of intense investigation. Recently, many studies have supported the hypothesis that ATP-dependent chromatin remodeling complexes are targeted by transcriptional activators (Agalioti et al., 2000; Cosma et al., 1999d; Dimova et al., 1999; Kowenz-Leutz and Leutz, 1999; Krebs et al., 2000; Krebs et al., 1999; Natarajan et al., 1999; Yudkovsky et al., 1999; Neely et al., 1999). For instance, in the case of SWI/SNF complexes, two different domains within transcriptional activators have been shown to target remodeling activity- acidic activation domains and zinc-finger DNA binding domains (e.g. AD versus DBD). Yeast SWI/SNF can bind to acidic activation domains *in vitro* and these interactions are sufficient to target remodeling activity at reconstituted nucleosomal arrays (Yudkovsky et al., 1999; Boyer et al., 2000). *In vivo*, yeast SWI/SNF is recruited to the HO target gene via the zinc-finger activator, Swi5p, and *in vitro* studies indicate that the Swi5p zinc-finger domain may be sufficient to interact with SWI/SNF complex (Krebs, et al., 1999; Cosma et al., 1999; M. Samuels, unpublished data). Likewise, yeast SWI/SNF interacts with both the zinc-finger DBD and the acidic transcriptional activation domain of rat glucocorticoid receptor (Wallberg et al., 2000; Yoshinaga et al., 1992). In the case of human SWI/SNF, it is not recruited by acidic activators *in vitro* (Boyer et al., 2000), however, human SWI/SNF complex zinc-finger DBDs can recruit hSWI/SNF remodeling activity *in vitro* (Kadam et al., 2000). More specifically, the human SWI/SNF subunits BRG1 (Swi2p homologue) and BAF155/BAF170 (Swi3p homologues) cooperatively target chromatin remodeling

by interacting with structurally distinct zinc-finger DBDs of EKLF, Sp-1, and GATA-1, (Kadam et al., 2000).

It is not known if promoter-bound activators recruit SWI/SNF, or whether SWI/SNF associates with activators in solution, which then target the complex to specific sites. In fact, studies indicate that the binding of activators can occur either prior to or subsequent to the recruitment of chromatin-modifying complexes (Neely et al, 1999; Krebs et al, 1999; Krebs et al, 2000; Cosma et al, 1999; Natarajan et al, 1999; Yudkovsky et al, 1999; Kowenz-Leutz and Leutz, 1999; Agalioti et al, 2000; Dilworth et al, 2000; Gregory et al, 1998; Ryan et al, 1998; Wallberg et al, 2000; Lee et al, 1999). For example, yeast SWI/SNF facilitates the binding of the Gal4 activator to an artificial reporter (Burns and Peterson, 1997). In the opposite manner, the Swi5p activator is bound to its DNA binding sites prior to the recruitment of yeast SWI/SNF to the HO promoter in yeast (Krebs et al., 2000).

It is not known which subunits of yeast SWI/SNF interact with acidic activation domains or with zinc-finger DBDs. In the case of human SWI/SNF, a BRG1/BAF155 sub-complex appears to be sufficient to interact with a subset of zinc-finger DBDs (Kadam et al., 2000). Here we demonstrate that the gene-specific transcriptional activator Swi5p can directly interact with the individual SWI/SNF components Snf5p, Swi1p and Snf6p, and that Swi5p can synergistically interact with sub-complexes Swi2p/Swi3p and Swp73p/Swi3p. We show that these direct interactions occur with the zinc finger DBD of Swi5p. In addition, we find that the Swi2p/Swi3p sub-complex can specifically interact with the VP16 acidic activation domain.

Results

Interaction of yeast SWI/SNF subunit with the yeast transcriptional activator

Swi5p. Yeast Swi5p is a gene-specific DNA-binding factor that contains a zinc-finger DBD domain, and it has been shown to be involved in targeting yeast SWI/SNF *in vivo* and *in vitro* (Cosma et al., 1999; Krebs et al., 2000; Krebs et al., 1999; Neely et al., 1999). To determine which yeast SWI/SNF subunit(s) directly interact with Swi5p, we incubated recombinant GST-SWI5 with individual, *in vitro* translated ³⁵S-methionine labeled SWI/SNF subunits (see Experimental Procedures; Figure IV.1A). GST pull-down assays demonstrated that the SWI/SNF components, SNF5, SNF6 and SWI1, bind to GST-SWI5, but whereas no significant binding was detected to the SWI3, SWP73 or SWI2N subunits (Figure IV.1A). This suggests that three different subunits of yeast SWI/SNF can interact with the transcriptional activator Swi5p.

Kadam *et al.* have demonstrated that subunits of the human SWI/SNF complex can interact with the zinc-finger DBDs of some transcriptional activators rather than their activation domains (Kadam et al., 2000). We sought to determine whether yeast SWI/SNF interacts with Swi5p through its zinc-finger DBD. To test this idea, we bound ³⁵S-methionine labeled SWI/SNF subunits to a GST-SWI5 fusion protein [GST-SWI5(DEF)], which contains only the zinc finger DBD domain (McBride et al., 1999). We then examined association by GST pull-down assays (Figure IV.1B). Results showed

Figure IV.1 SWI/SNF Subunits Interact Specifically with Transcriptional

Activators. A. Transcriptional Activator Swi5p specifically interacts with individual subunits and sub-complexes of SWI/SNF. GST-Swi5p directly interacts with Swi1p, Snf5p and Snf6p and Swi2p/Swi3p and Swp73p/Swi3p sub-complexes. GST pull down assays were performed with either bacterially expressed GST-SWI5, GST-VP16 or GST-VP16 Δ 456F442P binding to individual or combinations of *in vitro* transcribed ³⁵S-methionine labeled SWI/SNF polypeptides. Inputs 2.5%, unbound 2.5% and bound 100% fractions were electrophoresed on SDS-PAGE, fixed, Amplified and autoradiographed.

B. The DEF domain of transcriptional activator Swi5p specifically interacts with SWI/SNF components. GST pull down assays were performed with either bacterially expressed GST-SWI5, or GST-SWI3(DEF) binding to sub-complexes of *in vitro* transcribed ³⁵S-methionine labeled SWI/SNF polypeptides. Inputs 2.5%, unbound 2.5% and bound 100% fractions run on SDS-PAGE, fixed, Amplified and autoradiographed.

GST-SWI5

GST-VP16

GST-VP16
ΔF442P

I U B I U B I U B

SNF5

SNF6

SWI1

SWP73

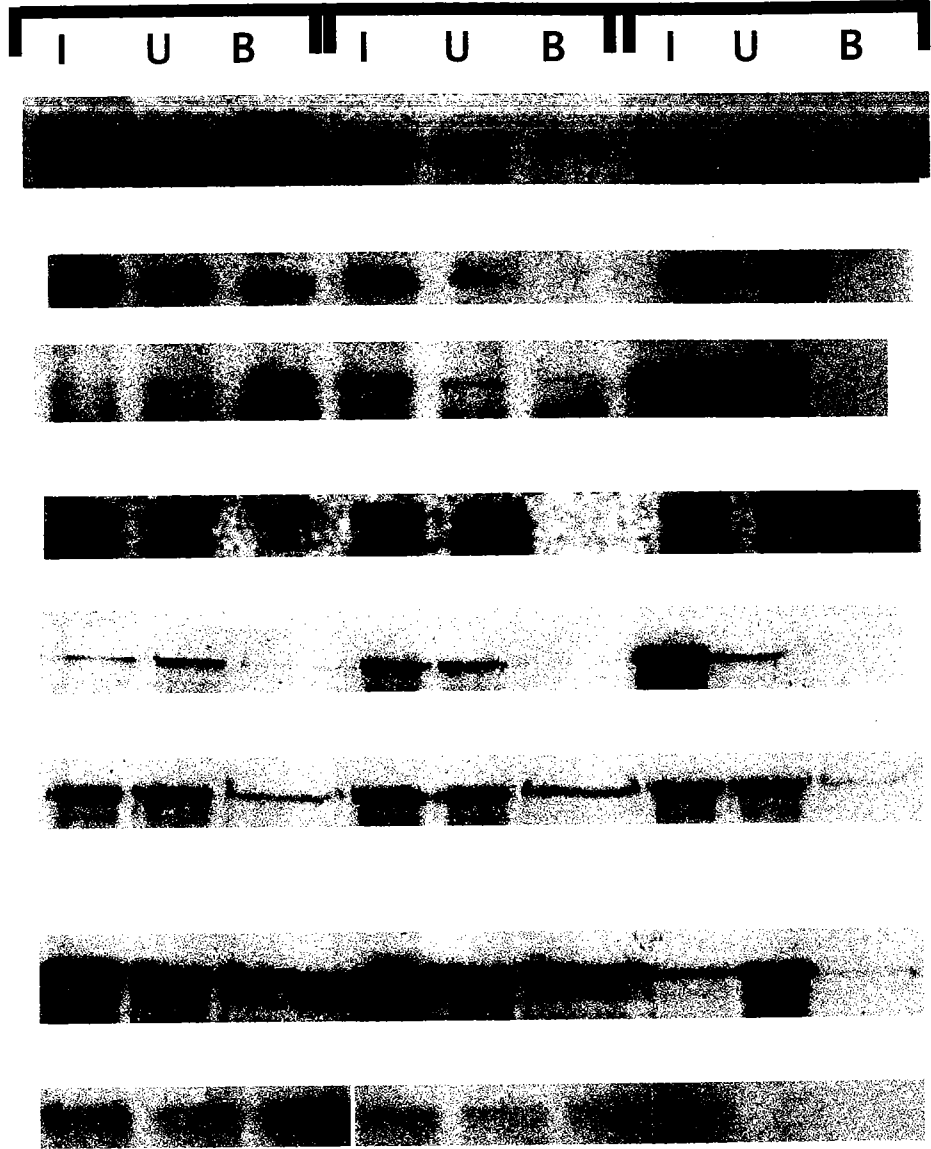
SWI2N

SWI3

SWI2N/SWI3

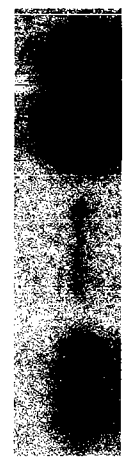
SWP73/SWI3

A



**GST-SWI15
(DEF)**

I U B



GST-SWI15

I U B



SNF5

SNF6

SWI1

SWI2N

SWI3

SWI3+SWI2N

B

that the Swi5p zinc-finger DBD, GST-SWI5(DEF), maintained binding to Swi1p, Snf5p and Snf6p. Thus, the SWI/SNF subunits, Snf5p, Snf6p, and Swi1p, can bind to Swi5p through its zinc-finger DBD domain (Figure IV.1B).

Human Swi2p (BRG1) and Swi3p (BAF155) function as a sub-complex that interacts with zinc-finger DBD domains (Kadam et al., 2000). To test whether Swi2p and Swi3p can also form a functional sub-complex, we analyzed combinations of SWI/SNF subunits for binding to GST-SWI5 and GST-SWI5(DEF) by the GST pull down assay. We determined that SWI3/SWI2N and SWI3/SWP73 sub-complexes could bind both the full length and zinc-finger domain of Swi5p (Figure IV.1). Importantly, binding was only observed if twice as much SWI3 compared to SWI2N or SWP73 was included in these reactions. These results are consistent with our results in Chapter II and Chapter III, which indicate that Swi3p is present in two copies in each yeast SWI/SNF complex. These data suggest that yeast Swi3p can cooperatively and functionally interact with Swi2p and Swp73p to mediate direct interaction with the Swi5p zinc-finger DBD transcriptional activator.

The Swi2p/Swi3p Sub-Complex Specifically Interacts with VP16. Yeast SWI/SNF complex can also be targeted by acidic activation domains of transcriptional activators (Krebs et al., 2000; Natarajan et al., 1999 ; Neely et al., 1999; Ryan et al., 2000; Yudkovsky et al., 1999). VP16, a potent acidic activation domain, and Gal4-VP16 have been shown to direct SWI/SNF mediated targeting *in vitro* (Neely et al., 1999; Yudkovsky et al., 1999, Boyer, 2000). Thus, we sought to determine if any of the

SWI/SNF subunits or sub-complexes could directly interact with the VP16 activation domain in a GST pull down assay (Figure III.1A). None of the individual SWI/SNF subunits interacted with either wild type GST-VP16 or mutant GST-VP16F442P. Interestingly, only the SWI3/SNF2N sub-complex could directly and selectively interact with the functional GST-VP16 acidic activation domain (Figure IV.1A).

Discussion

Here we have shown that several SWI/SNF subunits can directly interact with transcriptional activators. We show that the yeast SWI/SNF components (Swi1p, Snf5p, Snf6p), as well as sub-complexes (Swp73p/Swi3p and Swi2p/Swi3p) specifically and directly interact with the Swi5p transcriptional activator through the C-terminal region which contains the zinc-finger DBD domain. Additionally, we show that the Swi2p/Swi3p sub-complex can selectively interact with the functional transcriptional activation domain of VP16. These data are consistent with the idea that many subunits of SWI/SNF are involved in regulation rather than remodeling activity per se.

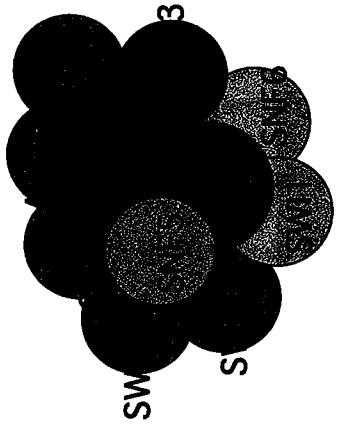
Selective Binding of SWI/SNF Subunits to Transcriptional Activators. *In vitro* and *in vivo* studies have demonstrated that transcriptional activators target chromatin remodeling complexes to their designated gene promoters (Ikeda et al., 1999; Krebs et al., 2000; Krebs et al., 1999; Massari et al., 1999; Natarajan et al., 1999; Neely et al., 1999; Utley et al., 1998; Yudkovsky et al., 1999, Cosmo, 1999; see Figure IV.3). Here we ascribe functional roles for individual subunits of the SWI/SNF complex that

directly interact with Swi5p. Two subunits that are unique to the yeast SWI/SNF complex, Swi1p and Snf6p, and one subunit that is conserved in all SWI/SNF complexes, Snf5p, specifically interact with Swi5p via the zinc-finger DBD domain (Figure IV.1). these results are consistent with the observation that Swi5p can interact with native SWI/SNF complex, via the zinc-finger DBD domain (M. Samuels and C.L.P., unpublished data). In addition, these data are analogous to results showing that human SWI/SNF interacts selectively with the zinc-finger DBD of several transcriptional activators. Additionally, the conserved subunits, Swi3p, Swi2p and Swp73p are only able to mediate interaction with transcriptional activators as sub-complexes (Swi2p/Swi3p and Swp73p/Swi3p). This suggests that these subunits are functioning cooperatively in a sub-complex, which may be a mechanistic requirement for the native complex. This notion is supported by the observation that human BRG1-BAF170/BAF155 is a minimal sub-complex that can recruit hSWI/SNF and mediate chromatin remodeling at the gene-specific EKLF-promoter (Kadam et al., 2000).

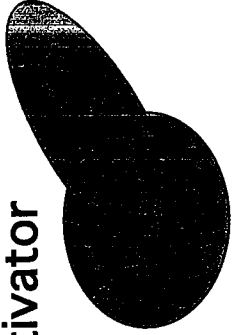
We also demonstrate that only the SWI/SNF sub-complex, Swi2p/Swi3p, displays specific and direct interaction with the intact VP16 acidic activation domain (Figure IV.1). This interaction was abrogated by a mutant derivative of VP16, suggesting that the interaction is selective. Collectively, these data suggest that targeting of chromatin remodeling complexes occurs through specific SWI/SNF components, and furthermore, selective and direct interactions occur with distinct moieties of transcriptional activators, such as acidic transcriptional activation domains or zinc-finger DBDs.

IV.3 SWI/SNF-Activator Targeting Model. We propose a model in which gene-specific transcriptional activators recruit/target SWI/SNF complex to their desired gene promoter. This figure depicts a nucleosomal array containing an activator binding site upstream of a transcriptional promoter site. Previous studies have shown that yeast SWI/SNF is able to bind activators in solution or after the transcriptional activator is bound to the promoter. Regardless, the activator is able to directly interact with SWI/SNF and recruit/target SWI/SNF to its gene-specific promoter. The yeast SWI/SNF components, including Snf5p, Snf6p, Swi1p, can directly bind the gene-specific transcriptional activator Swi5p. The Swi2p/Swi3p and Swp73p/Swi3p sub-complexes can cooperatively bind the gene-specific transcriptional activator Swi5p. In addition, the Swi2p/Swi3p sub-complex can interact with the VP16 acidic transcriptional activator.

SWI/SNF

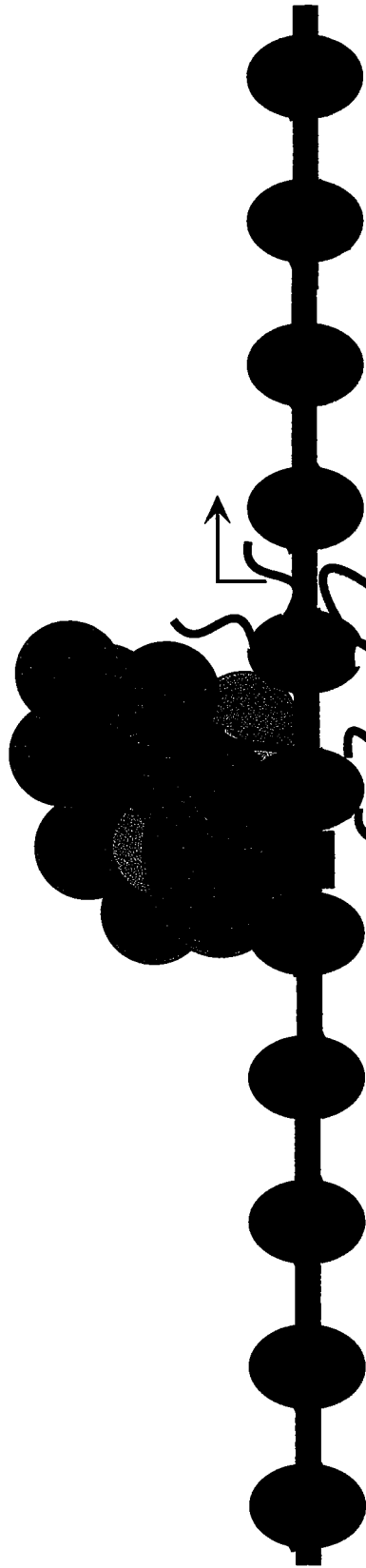


Activator



Activator binding site

Promoter start site



Activator binding site

Promoter start site

Experimental Procedures

***In vitro* translations.** ^{35}S -methionine-labeled SWI/SNF proteins or fragments (Amersham; 10mCi/ml) were obtained in each case using the TNT Coupled Wheat Germ or Rabbit Reticulolysate Extract System (Promega) according to manufacturer's specifications. Products were visualized by autoradiography or immunoblotting to confirm the quality of all *in vitro* translation reactions.

GST-fusion protein expression and purification. GST-fusion proteins were expressed in DH5a from the pGex-2T vector. CP867 (GST-SWI5), CP885 (GST-VP16), CP887 (GST-VP16 Δ 456_{FP442}) were provided by Brenda Andrews. Bacterial strains expressing GST fusions proteins were induced for 3hrs with 0.5mM IPTG at an OD₆₀₀~0.6, harvested at 4K for 10 min. Pellets were freeze-thawed, resuspended in 1/50 culture volume in MTPBS (150 mM NaCl, 16 mM Na₂HPO₄, 4 mM NaH₂PO₄, 1% TritonX-100 (pH 7.3), 1 mM PMSF, 2 ug/ml of leupeptin and pepstatin A), and lysed by sonication (550 Sonic Dismembrator, Fisher) for 10 sec pulses for 1 minute on ice until >80% lysis. Lysate was cleared by centrifugation at 10,000xg for 5' at 4°C and bound to glutathione-agarose beads (Amersham Pharmacia Biotech AB) for 30' at 4°C, nutating. Beads were washed in 50 mls of MTPBS, 100 mls MTPBS with 1 M NaCl, and additional 200 mls of MTPBS, and resuspended in 1:1 slurry of GST-fusion beads: MTPBS.

GST co-immunoprecipitation binding assays. For binding studies, 5 ul/50 ul reaction of individual labeled *in vitro* translated SWI/SNF polypeptides were pre-cleared for one hour with bacterial purified GST-agarose beads. In a final volume of 400 ul of binding buffer (20 mM Tris, pH 8.0, 200 mM NaCl, 1 mM EDTA, 0.1% Triton-X 100, 1 mM

DTT, 0.1 mg/ml BSA, 1 mM PMSF) pre-cleared proteins (removing 1/40 for input fraction) were mixed with 20 ul (1:1) individual GST-tagged fusion proteins for 1 hour, 4°C, nutating. Subsequently, reactions were spun down for 1 minute, saving 1/40 for unbound and washed 5 times in protein binding buffer eluting bound proteins in 2xSDS sample buffer. Binding was analyzed by SDS-PAGE, fixed 20 minutes (10% acetic acid, 20% methanol), incubation in Amplify (Amersham Life Sciences) for 30-60 minutes, dried and autoradiographed.

CHAPTER V

A Role for the Yeast SWI/SNF Complex in DNA Replication

CHAPTER V

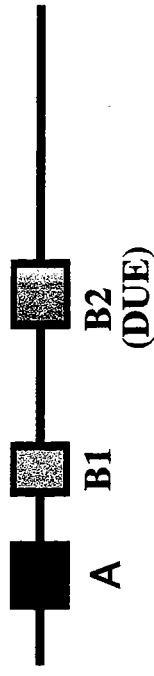
A Role for the Yeast SWI/SNF Complex in DNA Replication

Introduction

The organization of an eukaryotic genome into chromatin can lead to repression of many cellular processes that require accessibility of specific DNA sequences to enzymatic machinery. Nucleosomes, the basic building blocks of chromatin, are general repressors of transcription *in vivo* and *in vitro* (reviewed in Adams and Workman, 1993; Axel et al., 1974; Lacy and Axel, 1975; Lorch et al., 1987; Simpson, 1990; Workman and Roeder, 1987). Nucleosome assembly can block the ability of transcription factors to access their binding sites. Likewise, positioning of nucleosomes over crucial *cis*-acting sequences of DNA replication origins, can block origin firing *in vivo* (Simpson, 1990). Furthermore, assembly of DNA into chromatin leads to a general repression of DNA replication efficiency *in vitro*.

The replication of yeast chromosomes requires the activation of multiple *cis*-acting replication origins. Native yeast chromosomal origins (ORIs) were initially identified as sequence elements that allowed extrachromosomal maintenance of plasmids, and thus they were called autonomously replicating sequences (ARSs) (Brewer and Fangman, 1987; Deshpande and Newlon, 1992; Huberman et al., 1987). ARS elements are defined as A-T-rich sequences of 150-300 base pairs. Although ARSs are modular, usually containing at least three distinct sequence elements, no obvious homology exists, except the A element or ARS consensus sequence (ACS) (see Figure V.1). All ARS elements contain a match to an

Figure V.1. Autonomously Replicating Sequences (ARS) Schematic. A. Yeast origins ARS1, ARS121, ARS307 and ARS309 are schematically drawn to depict their modular *cis*-acting sequence elements. A denotes the ARS consensus sequence (ACS); B denotes the different *cis*-acting elements important for origin function. All B elements do not have known functions; some have been shown to be DNA unwinding elements (DUE) or enhancer elements (binding sites for ABF1 or other activators).



essential 11 bp ARS consensus sequence (ACS), WTTTAYRTTTW (where W is A or T, Y is T or C, and R is A or G), which is part of a larger 17 bp extended consensus element (Theis and Newlon, 1994; Broach et al., 1983). The ACS serves as a binding site for the conserved origin recognition complex (ORC) which plays a crucial role in origin function *in vivo* (Diffley and Cocker, 1992). In addition, each ARS contains sequences 3' to the T-rich strand of the ACS, called the B element, which is composed of at least two, non-redundant sequence elements (B1, B2, ...) (Celniker et al., 1984; Deshpande and Newlon, 1992; Marahrens and Stillman, 1992; Rao et al., 1994; Theis and Newlon, 1994; Walker et al., 1991). The B1 element is adjacent to the ACS, and it is required for ORC binding as well as an additional, uncharacterized function. In contrast, the B2 element does not appear to represent a protein binding site, but instead appears to function as a DNA unwinding element (DUE).

In addition to the ACS, B1, and B2 elements, many ARSs contain a third sequence element that functions as a replication enhancer. This sequence element can be located 3' of the B2 element, as is the case for ARS1 and ARS307, or the enhancer can be found 5' of the T-rich strand of the ACS, as is the case for ARS121. In the case of ARS1, the B3 element contains a binding site for the ABF1 transcription factor, and the role of ABF1 in ARS1 function can be provided by a host of other transcriptional activators, including RAP1, GAL4, and a LexA-VP16 chimeric activator (Diffley and Stillman, 1988; Sweder et al., 1988; Walker et al., 1989). Several other ARS elements, such as ARS121, also contain functionally important ABF1 binding sites, whereas other ARSs contain distinct sequence elements that presumably perform similar functions (i.e. B4 in ARS307 and REN1501 in ARS1501). Although the role of these elements in origin function is not clear, it has been proposed that one role for transcriptional activators in

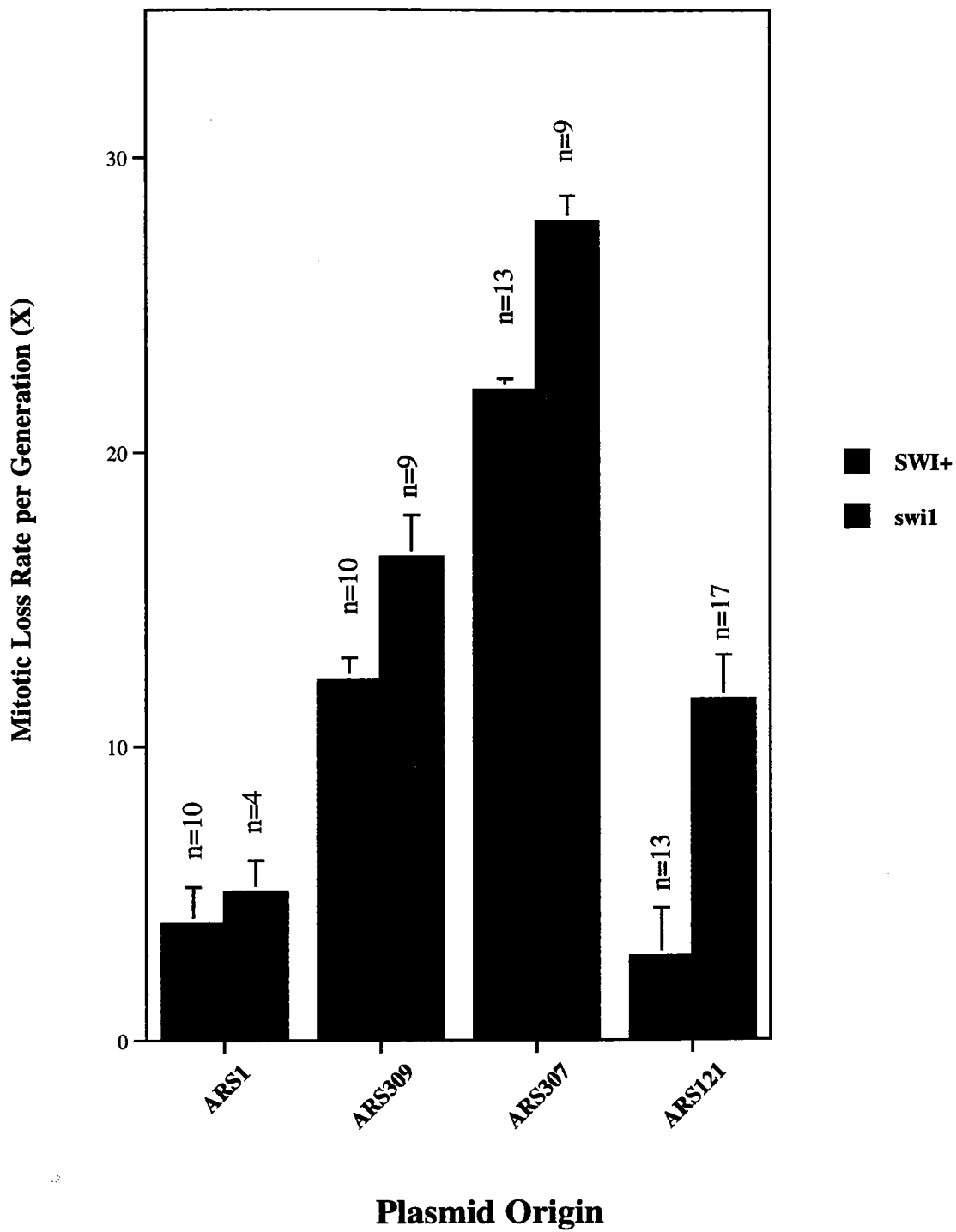
DNA replication may be to counteract nucleosomal repression. Consistent with this view, deletion of the ABF1 binding site within ARS1 (the B3 element) leads to an invasion of nucleosomes into the essential ACS element and a decrease in ARS1 function. Furthermore, *in vitro* studies have shown that many acidic transcriptional activators can stimulate *in vitro* DNA replication by antagonizing the repressive effects of nucleosomes (Cheng and Kelly, 1989; Cheng et al., 1992; Li and Botchan, 1994).

Since bulk chromatin in an interphase cell is primarily composed of large, 100-200 nm condensed fibers, mechanisms must exist to rapidly and reversibly unfold or decompact specific loci to facilitate DNA accessibility. The SWI/SNF complex is required for many transcriptional activators to enhance transcription in yeast (for reviews see Peterson and Tamkun, 1995; Winston and Carlson, 1992). Mutations that alter chromatin components partially alleviate the defects in transcription due to inactivation of the SWI/SNF complex (Hirschhorn et al., 1992; Kruger et al., 1995; Prelich and Winston, 1993), and SWI/SNF complex can use the energy of ATP hydrolysis to disrupt nucleosome structure *in vitro* (Cote et al., 1994; Jaskelioff et al., 2000; Logie and Peterson, 1997). Thus it has been proposed that the primary role of this complex may be to facilitate the function of gene regulatory proteins in a chromatin environment by remodeling chromatin structure. Since nucleosomal structure in eukaryotes imposes an impediment to the initiation of replication as well as transcription, in this study we have investigated whether SWI/SNF complex might also play a role in DNA replication *in vivo* in yeast.

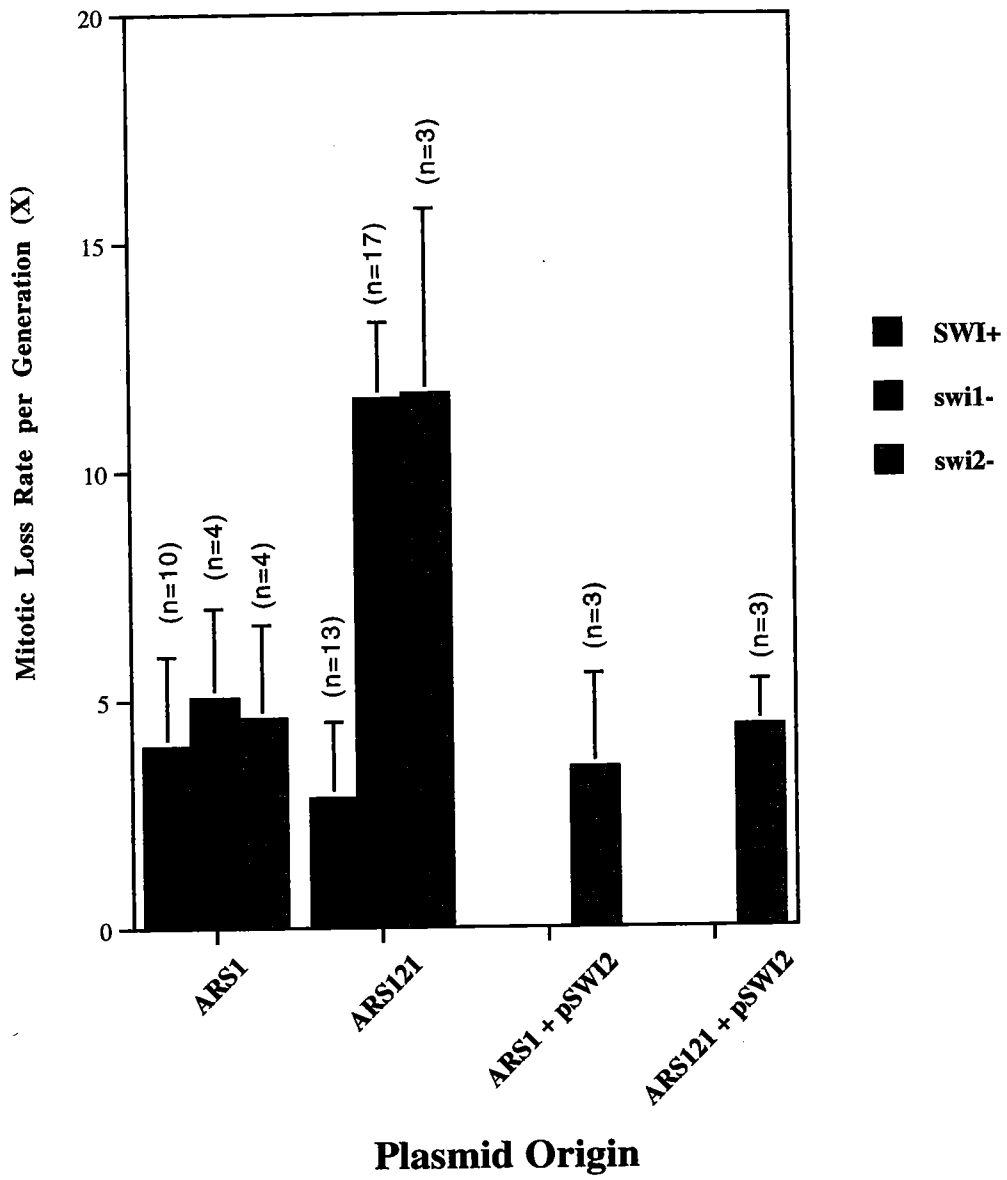
Results

SWI/SNF complex is required for efficient function of the yeast replication origin, ARS121. Yeast autonomous replication sequences (ARSs) are examples of cellular chromosomal sequences that can function as origins of DNA replication on plasmids and in a chromosomal context (Brewer and Fangman, 1987; Diffley et al., 1995; Huberman, 1987). To address the role of the yeast SWI/SNF complex in DNA replication, we measured the mitotic stability of plasmids that contain a yeast selectable marker (*URA3*), a cloned centromere (CEN3), and a single replication origin (ARS). Isogenic *SWI*⁺ or *swi1*⁻ cells harboring such minichromosomes were grown to saturation in media that selected for plasmid maintenance, diluted into nonselective media, and then allowed to grow for an additional 12 or 5 generations for *SWI*⁺ or *swi1*⁻ cells, respectively. The rate of plasmid loss per generation of growth in nonselective media is indicative of the functioning of the replication origin (Bouton and Smith, 1986; Celniker et al., 1984; Dani and Zakian, 1983; Fitzgerald-Hayes et al., 1982; Holmes and Smith, 1989; Marahrens and Stillman, 1992; Srienc et al., 1985). Figure V.2A presents mitotic stability assays for four minichromosomes that contain different ARS elements. The stability of minichromosomes that contain ARS1, ARS307, or ARS309 is not altered significantly by inactivation of the SWI/SNF complex (less than a 1.3-fold increase in rate of plasmid loss in the *swi1* vs. *SWI*⁺ strains). In contrast, the stability of a minichromosome that contains ARS121 is dramatically reduced in the *swi1* mutant (2.9% +/- 1.7 in *SWI*⁺ vs. 11.6% +/- 1.5 in *swi1*). This decrease in plasmid stability is similar in magnitude to defects due to a partial loss of function mutation in *CDC17*, which encodes a DNA polymerase (Hogan and Koshland, 1992). Thus,

Figure V.2 SWI/SNF complex is required for efficient maintenance of a minichromosome containing ARS121. A. Mitotic stability assays were performed in isogenic *SWI*⁺ (CY296) and *swi1*Δ (CY298) strains carrying minichromosomes pARS/WTA (ARS1), p309-326 (ARS309), pC2G1A (ARS307), or yCp5AB121 (ARS121). B. Mitotic stability assays in *SWI*⁺ (CY296), *swi1*Δ (CY298), and *swi2*Δ (CY120) strains carrying pARS/WTA (ARS1) or yCp5AB121 (ARS121). Assays were also performed in the *swi2*Δ strain (CY120) harboring a plasmid that contains *SWI2* (pSWI2). The number of experiments (n) is shown above each column, and the standard error is indicated by brackets.



A.



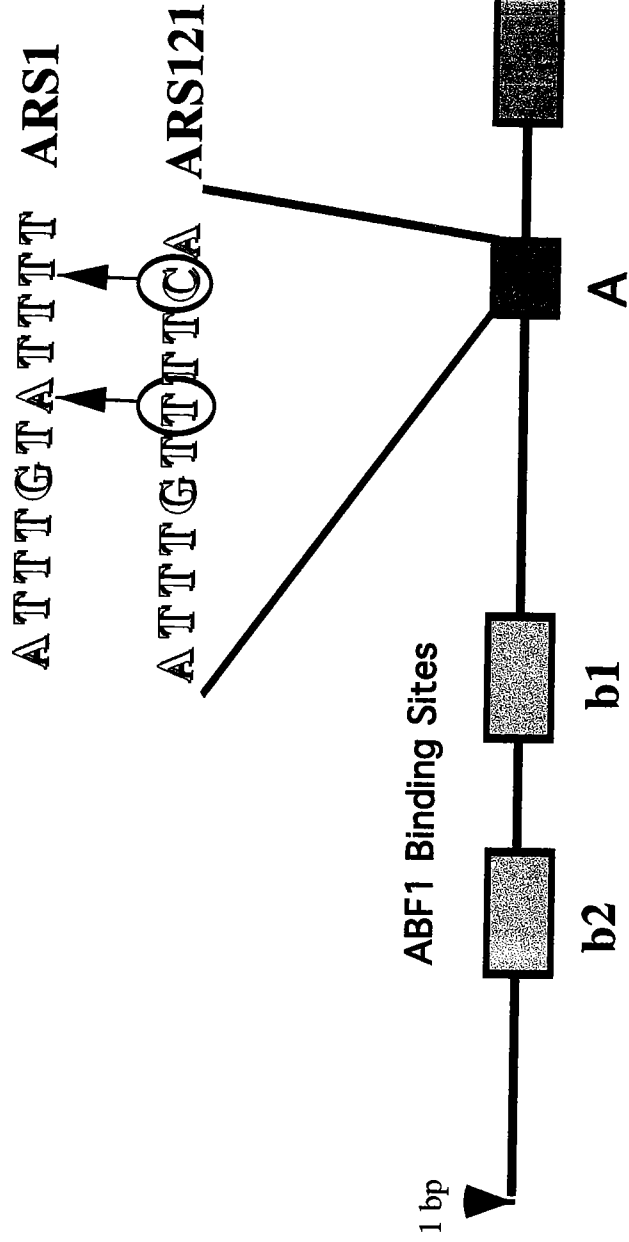
B.

these plasmid stability assays indicate that SWI/SNF is required for efficient functioning of at least one yeast replication origin.

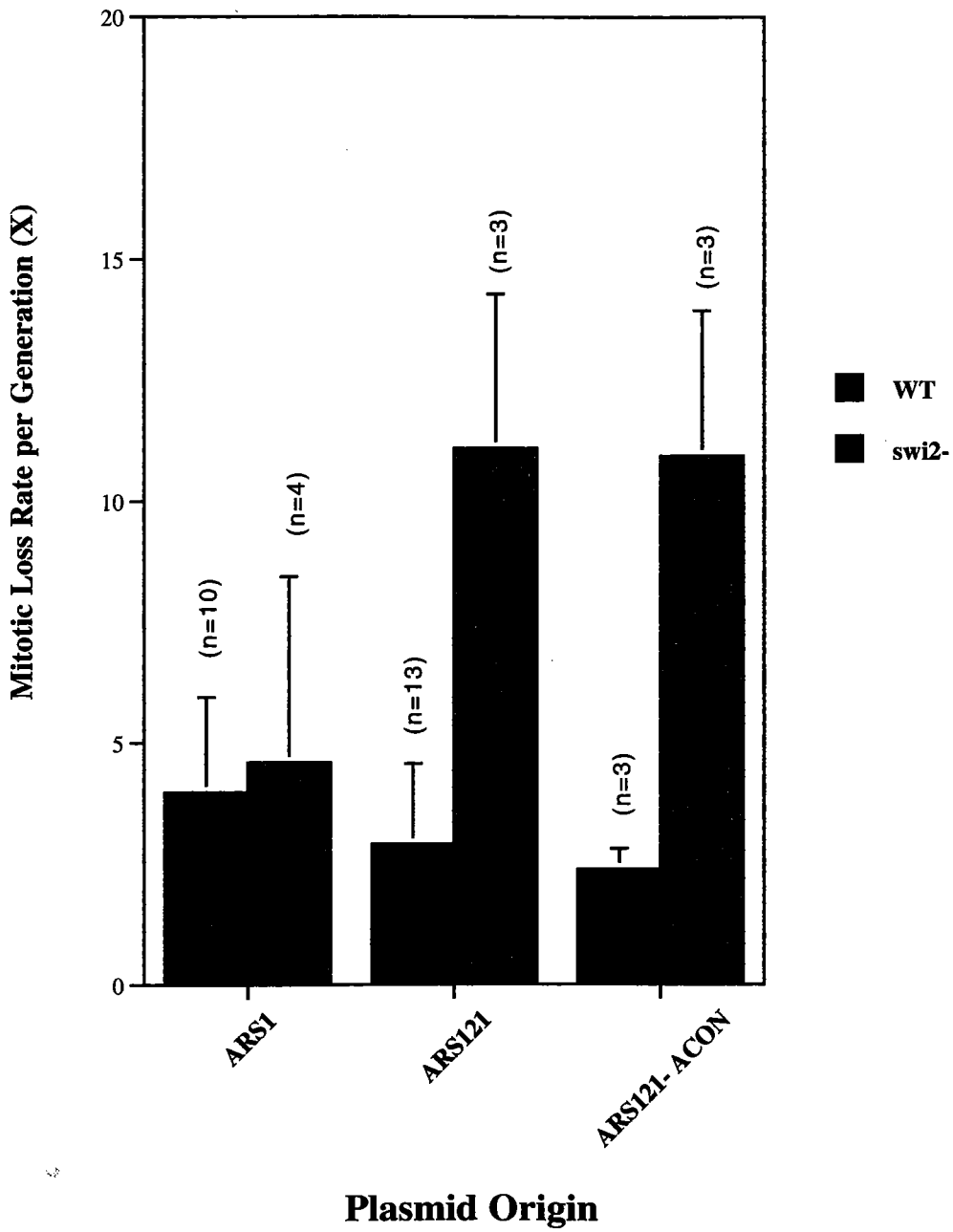
SWI/SNF function in transcription requires the ATPase activity of the SWI2/SNF2 subunit (Gorbalenya et al., 1989; Okabe et al., 1992; Richmond and Peterson, 1996). Mutations in this domain eliminate the ability of SWI/SNF to support transcriptional activation *in vivo* (Khavari et al., 1993; Laurent et al., 1992; Richmond and Peterson, 1996) and nucleosome disruption activity *in vitro* (Cote et al., 1994). To investigate whether full activity of the ARS121 replication origin also requires the ATPase subunit of the SWI/SNF complex, the mitotic stability of an ARS1 or ARS121 minichromosome was determined in isogenic *swi2Δ* (CY120) and *SWI2*⁺ (CY120 with a episomal copy of *SWI2*) cells (Figure V.2B). The stability of a minichromosome that contains ARS1 is not significantly altered in the *swi2* deletion mutant (3.5 +/- 2 in *SWI2*⁺ vs. 4.6 +/- 4 in *swi2Δ*, n=4); however, the stability of a minichromosome that contains ARS121 is decreased 3.4-fold (4.4 +/- 1 in *SWI2*⁺ vs. 11.7 +/- 4 in *swi2Δ*, n=3). Thus, these results suggest that an intact SWI2/SNF2 ATPase is required for the functioning of the SWI/SNF complex in DNA replication.

ARS121 is distinct from other ARS elements as it only contains four 9/11 matches to the ACS. Only one of these partial matches is essential for ARS121 function (Walker et al., 1991), whereas the three non-essential 9/11 matches are contained within a B2/DUE element (denoted NTR) which is located 3' to the T-rich strand of the essential ACS (see Figure V.1). One possibility is that the SWI/SNF-dependence of ARS121 is due to its essential, partial match to the ACS. To test this possibility, PCR mutagenesis was used to change 2 bp within the essential ACS of ARS121 so that it matches the ARS consensus sequence contained within ARS1 (Figure

Figure V.3. An ARS121 derivative that contains a consensus ACS element remains SWI/SNF dependent. **A.** Sequence comparison of the ACS elements of ARS1 and ARS121. Site directed mutations introduced to change the ARS121 ACS element to a perfect consensus are indicated by arrows. **B.** Mitotic stability assays in *SWI*⁺ (CY296) and *swi2* Δ (CY120) strains carrying either yCp5AB121 (ARS121) or pCP561 (ARS121-ACON). The number of experiments (n) is shown above each column, and the standard error is indicated by brackets.



A.

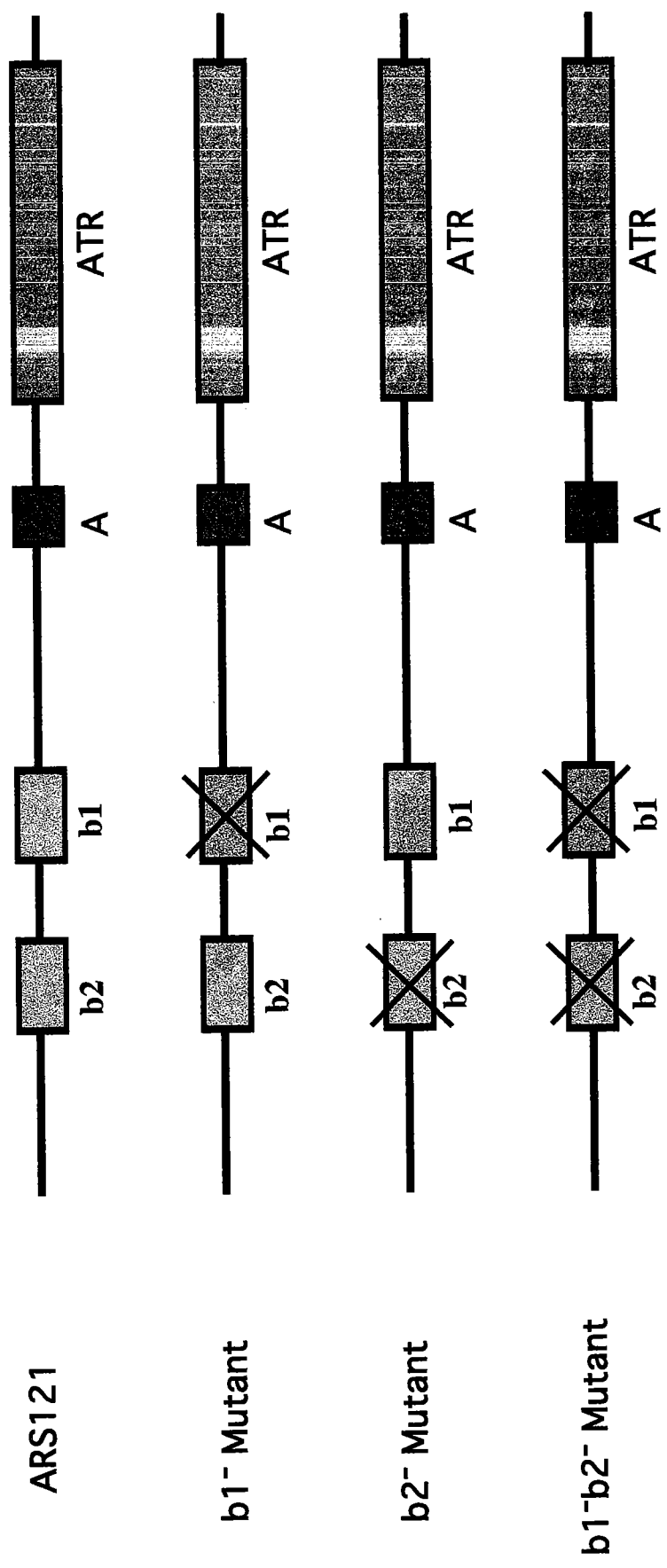


B.

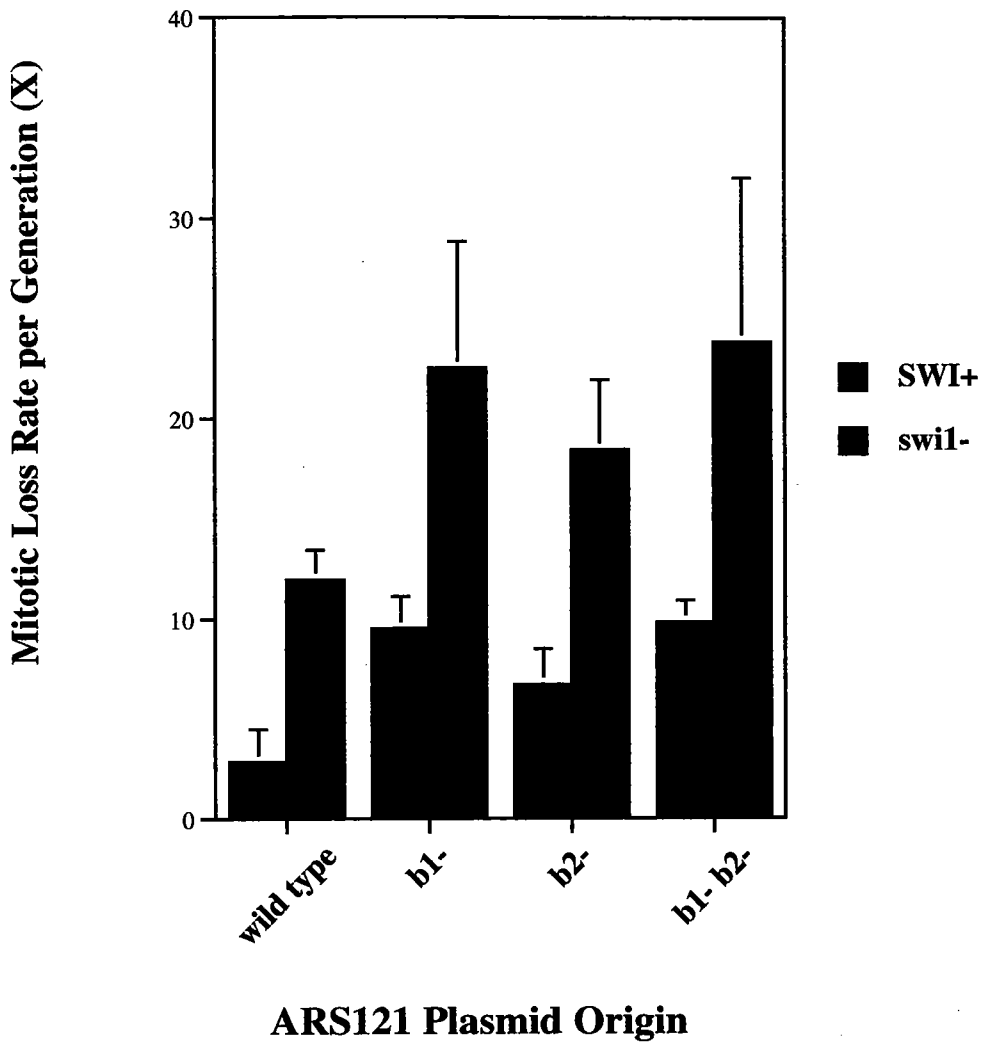
V.3A). We then tested if this ARS121 derivative (ARS121-ACON) was still dependent on SWI/SNF function. The results shown in Figure V.3B indicate that changing the essential ACS of ARS121 to a perfect 11/11 match has no effect on plasmid stability in wild-type cells nor does it change the SWI/SNF dependence of this origin (Figure V.3B). The stability of a minichromosome that contains this ARS121-ACON is still decreased 4.6 -fold in a *swi2* mutant (2.4 ± 0.4 in *SWI*⁺ vs. 10.9 ± 3.1 in *swi2*⁻, n=3). Thus these results suggest that the SWI/SNF dependence of ARS121 is not due to its essential, imperfect match to the ACS.

ARS121 contains a replication enhancer consisting of two binding sites (b1 and b2) for the ABF1 transcription factor located 5' of the T-rich strand of the essential ACS (see Figure V.1, Figure V.4A). Mutation of one or both binding sites leads to a 2- to 3-fold decrease in the stability of plasmids containing ARS121 (Walker et al., 1989). Since SWI/SNF plays an important role in the functioning of many transcriptional activators, we tested whether the SWI/SNF dependence of ARS121 involved ABF1. Plasmid stability assays were performed in *SWI*⁺ and *swi1*⁻ cells with ARS121 derivatives that harbor deletions in either one (b1- or b2-), or both (b1-b2-) in the ABF1 binding sites (Figure V.4B). Similar to previous studies (Walker et al., 1989), deletion of the ABF1 binding sites caused a 2- to 3-fold decrease in plasmid stability in *SWI*⁺ cells. Surprisingly, full functioning of these ARS121 derivatives remained dependent on SWI/SNF function. For example, the stability of the ARS121 derivative that contains a mutation of both ABF1 binding sites (b1-b2-) is still decreased 3-fold in a *swi1* mutant (10% loss per generation in *SWI*⁺ versus 29% in *swi1*⁻, n=3). Thus, SWI/SNF does not appear to facilitate the functioning of ABF1 at ARS121, since, if this were the case, removal of the ABF1 binding sites would be equivalent to inactivation of SWI/SNF.

Figure V.4. Derivatives of ARS121 have increased dependence on SWI/SNF function. A. Comparison of the wild type ARS121 sequence to the b1-, b2- and b1- b2- double mutants. **B.** Mitotic stability assays were performed in isogenic SWI+ (CY296) and *swi1*- (CY298) strains carrying minichromosomes yCp5AB121 (ARS121), AB121B1 (ARS121 b1- mutant), AB121B2 (ARS121 b2- mutant) and AB121B1B2 (ARS121 b1-b2- double mutant). The number of experiments (n) is shown above each column, and the standard error is indicated by brackets.



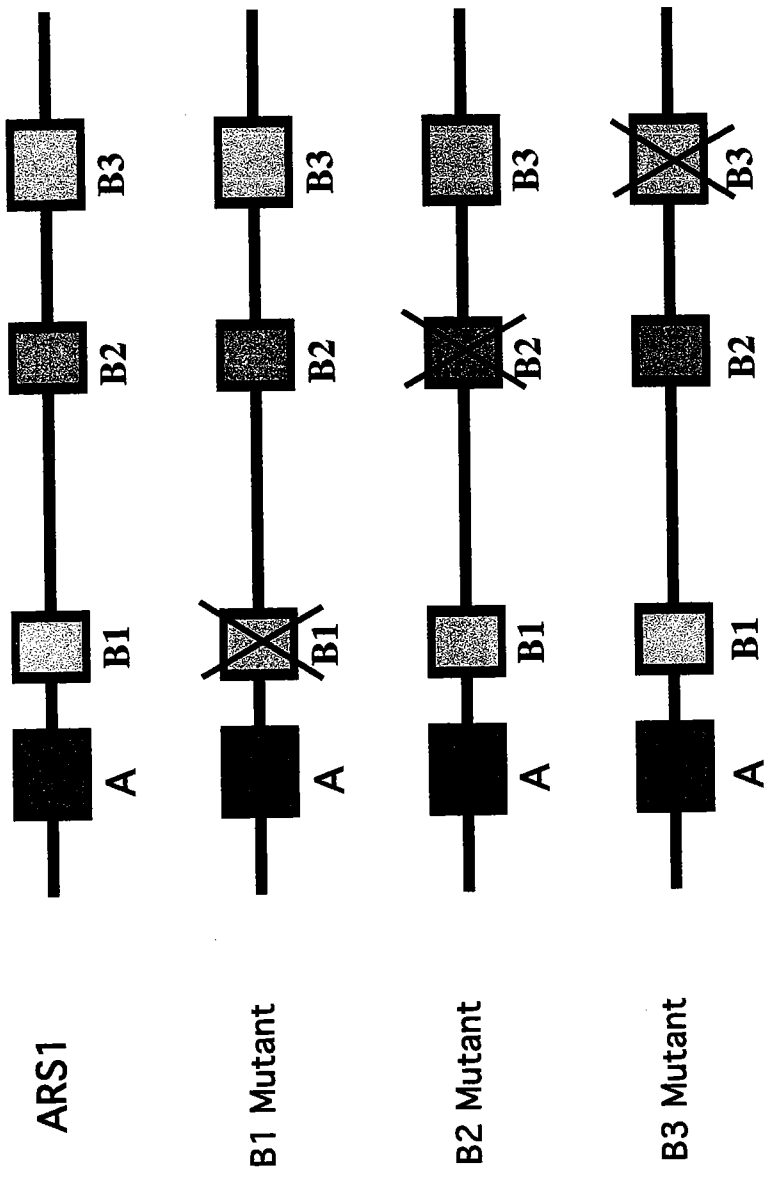
A.



B.

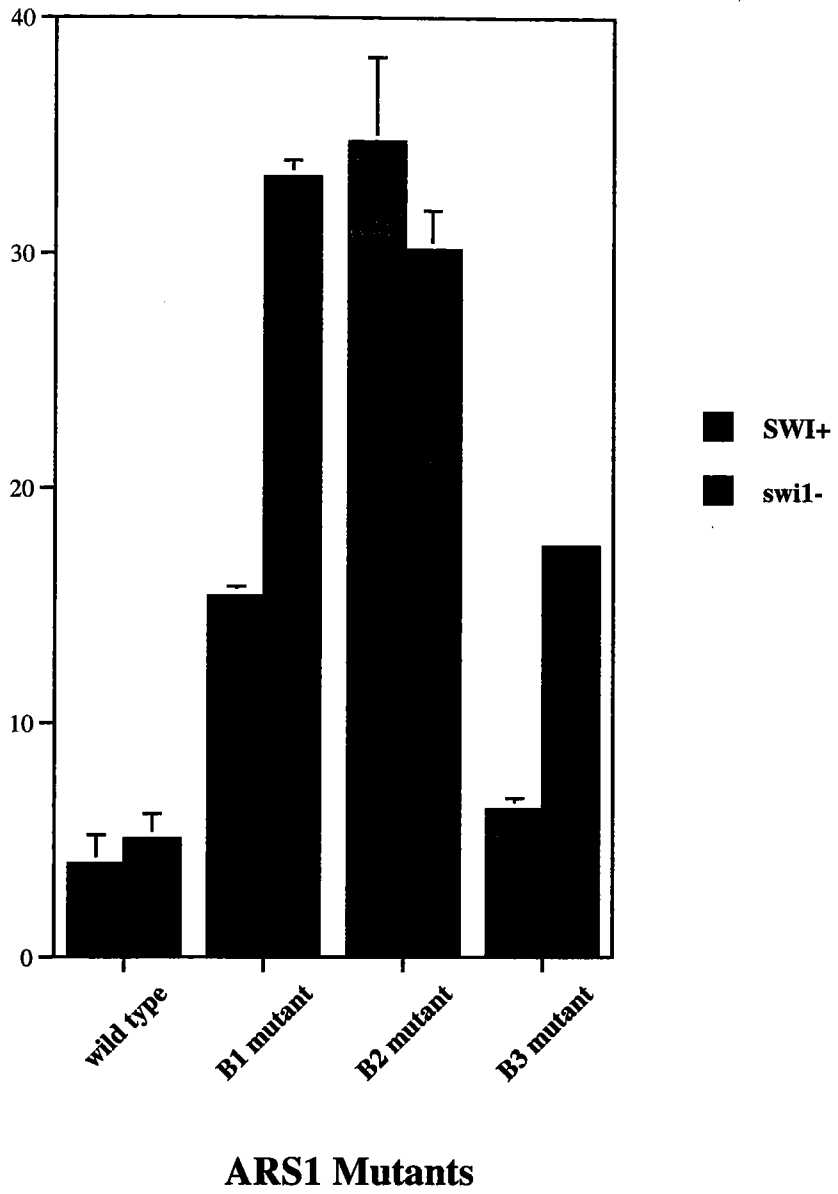
Figure V.5. Derivatives of ARS1 have increased dependence on SWI/SNF function. A.

Comparison of the wild type ARS1 sequence to linker scanning mutants B1, B2 and double point mutation in the B3 elements. **B.** Mitotic stability assays were performed in isogenic *SWT*⁺ (CY296) and *swi1*Δ (CY298) strains carrying minichromosomes pARS/WTA (ARS1), pARS/835-842 (ARS1 B1 linker scanning mutant), pARS/757-764 (ARS1 B3 linker scanning mutant), pARS/756,758 (ARS1 B3 double point mutant), or pARS/798-805 (ARS1 B2 linker scanning mutant). The number of experiments (n) is shown above each column, and the standard error is indicated by brackets.



A.

Mitotic Loss per generation (X)



B.

Derivatives of ARS1 have increased dependence on SWI/SNF function. Our deletion studies suggest that the role of SWI/SNF at ARS121 is to facilitate the functioning of the central core (ACS, B1 and B2/DUE/NTR elements), rather than the replication enhancer (ABF1 binding sites). However, the ACS, B1, and B2-like elements appear to be functionally interchangeable among different ARS elements (Rao et al., 1994; Raychaudhuri et al., 1997; Figure V.3), and thus it is not clear why only some ARS elements require SWI/SNF function. One possibility is that some ARS elements contain replication enhancers that are able to perform a function that is redundant with SWI/SNF. Some replication enhancer elements do appear to be ARS specific; for instance, the B4 enhancer element from ARS307 cannot be replaced with an ABF1 binding site (Lin and Kowalski, 1997), and the ABF1 binding sites in ARS121 cannot be replaced with binding sites for a variety of other transcriptional activators (Wiltshire et al., 1997). This hypothesis predicts that a SWI/SNF independent ARS might become dependent on SWI/SNF function in the absence of its replication enhancer.

We analyzed the mitotic stability of minichromosomes that carry ARS1 derivatives with linker scanning mutations in either the single ABF1 binding site (B3 element) or in the B1 or B2/DUE core elements (Figure V.5). The stability of a minichromosome that contains an intact ARS1 is not affected by inactivation of SWI/SNF (Figure V.2A and V.5B). However, an ARS1 derivative that contains a double point mutation in the single ABF1 binding site is ~3-fold less stable in the *swi1* mutant as compared to the *SWI*⁺ strain (6.3% +/- 0.5 in *SWI*⁺ vs. 17.5% +/- 0.3 in *swi1* cells; Figure V.5B). Thus, an ARS1 derivative that lacks a replication enhancer requires SWI/SNF function for full activity. Surprisingly, a minichromosome carrying a linker scanning mutation in the B1 element is also ~2-fold less stable in a *swi1* mutant (15.4% +/- 0.3 in

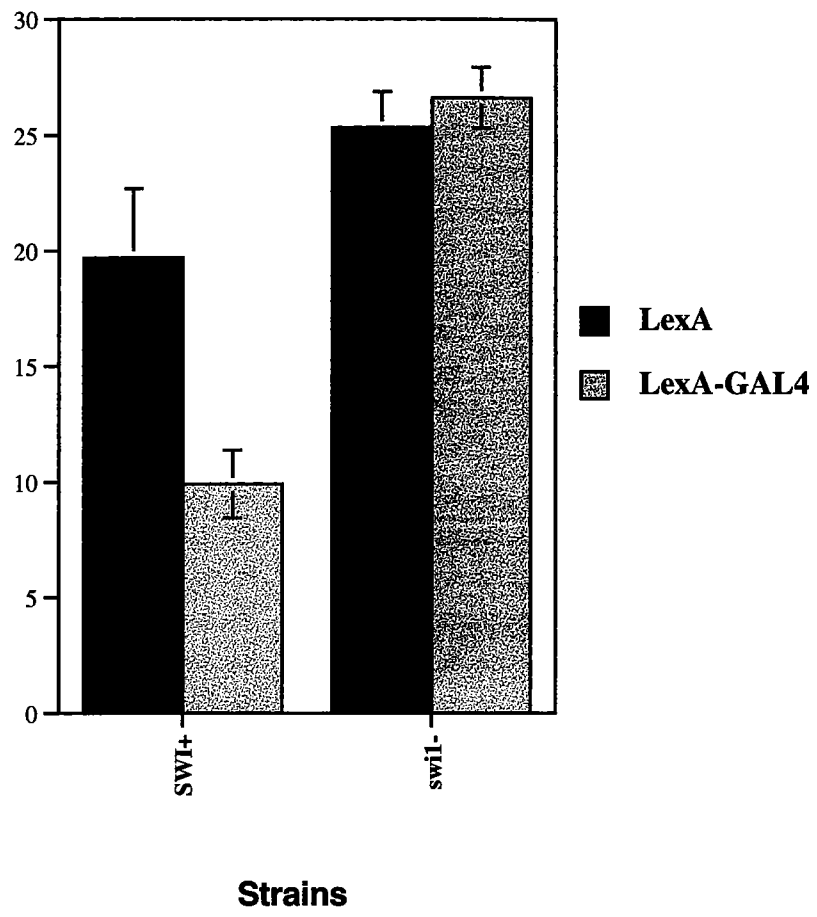
SWI⁺ vs. 33.2% +/- 0.7 in *swi1* cells), even though this derivative still contains the ABF1 binding site. In contrast, the stability of a minichromosome that contains a mutation in the B2/DUE element was not affected by inactivation of SWI/SNF (34.7 +/- 3.6 in *SWI*⁺ vs. 30.1 +/- 1.7 in *swi1* cells). Thus, these results suggest that, in the case of ARS1, a single ABF1 binding site, or the B1 element, can perform a function that is redundant with SWI/SNF action.

Marahrens and Stillman (Marahrens and Stillman, 1992) have demonstrated that a chimeric activator protein, LexA-GAL4, can functionally substitute for ABF1 when a consensus LexA binding site replaces the ABF1 site within ARS1. Since a single binding site for ABF1 was sufficient to make ARS1 independent of SWI/SNF function (Figure V.5), we tested whether the binding of LexA-GAL4 could also confer SWI/SNF independence. Mitotic stability assays were performed with cells that harbored a minichromosome with an ARS1 derivative that has a LexA binding site replacing the ABF1 site. When the LexA DNA binding domain was expressed, minichromosomes that contain this ARS1 derivative were lost from *SWI*⁺ cells at high frequency (Figure V.6A; 19.7% +/- 3.0%). If cells express a LexA fusion protein that contains the GAL4 transcriptional activation domain (LexA-GAL4), the mitotic stability of this minichromosome was enhanced (Figure V.6A; 9.9% +/- 1.5%; see also Marahrens and Stillman, 1992). In the absence of an intact SWI/SNF complex, however, this minichromosome remains very unstable even if LexA-GAL4 is expressed (Figure V.6A; 25.3% +/- 1.7% for LexA and 26.6% +/- 1.3% for LexA-GAL4). Importantly, the LexA-GAL4 fusion protein is expressed at equivalent levels in the *SWI*⁺ and *swi1* cells (Figure V.6B). Thus, LexA-GAL4 can only substitute for ABF1 in *SWI*⁺ cells; in the absence of SWI/SNF this chimeric activator is unable to enhance origin function.

Figure V.6. The SWI/SNF complex is required for the GAL4 transcriptional activation

domain to enhance origin function. **A.** Mitotic stability assays were performed in *SWT*⁺ (CY296) or *swi1Δ* (CY298) strains harboring the minichromosome pARS/LexA 798-805. Strains also contained a plasmid that expressed either the LexA DNA binding domain (pLEX[1-82]) or a LexA-GAL4 fusion protein (pMA411). Assays were performed as described in Materials and Methods except that selective medium lacked both uracil and histidine, and nonselective medium lacked only histidine. **B.** Western analysis of LexA expression. Whole cell extracts (Peterson and Herskowitz, 1992) were prepared from equal numbers of *SWT*⁺ (CY296) and *swi1Δ* (CY298) cells harboring plasmid pMA411, and expression of the LexA-GAL4 fusion protein (denoted by arrow) was identified by western blot using an antibody directed against the C-terminus of GAL4 (Peterson and Herskowitz, 1992).

Mitotic loss rate per generation (X)



A.

B.

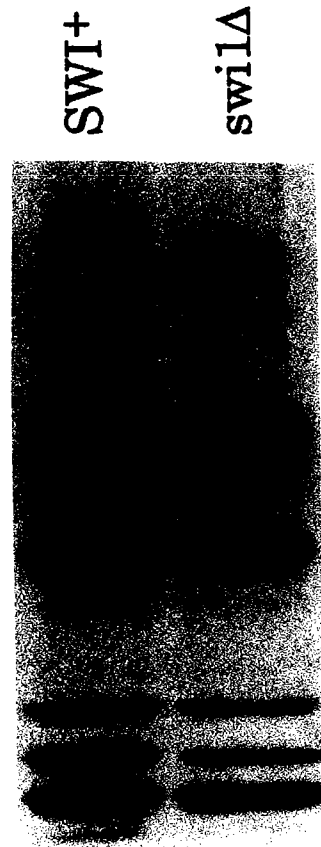
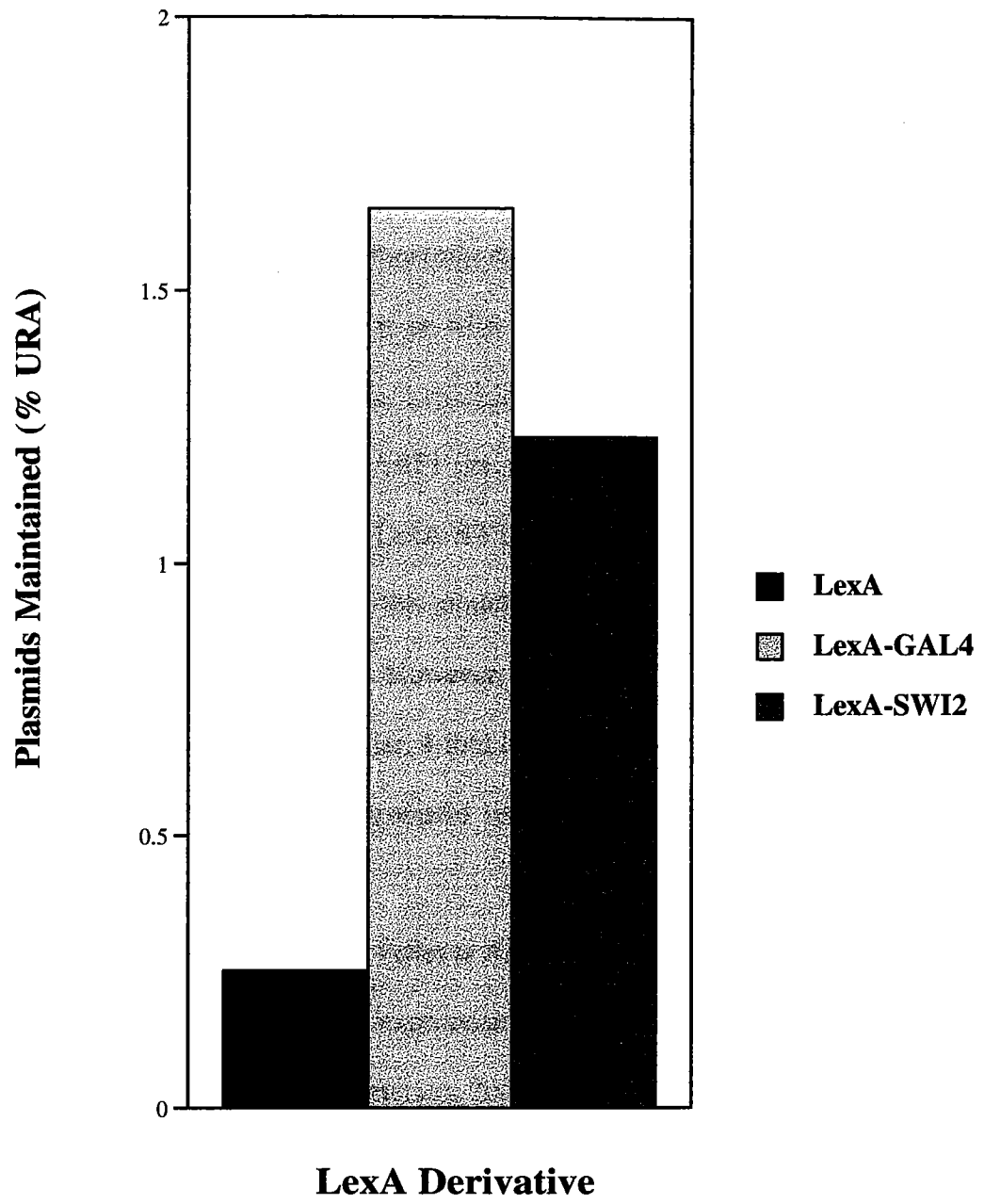


Figure V.7. Tethering the ATPase subunit of SWI/SNF to ARS1 enhances ARS function.

Mitotic stability assays were performed in *SWI*⁺ (CY296) or *swi1*Δ (CY298) strains harboring the minichromosome pARS/LexA 798-805. Strains also contained a plasmid that expressed either the LexA DNA binding domain (pLEX[1-82]) or a LexA-GAL4 fusion protein (pMA411) or LexA-SWI2 (CP337). Data shown are the averages of four independent experiments; the standard error was <10%. Assays were performed as in Figure V.6.

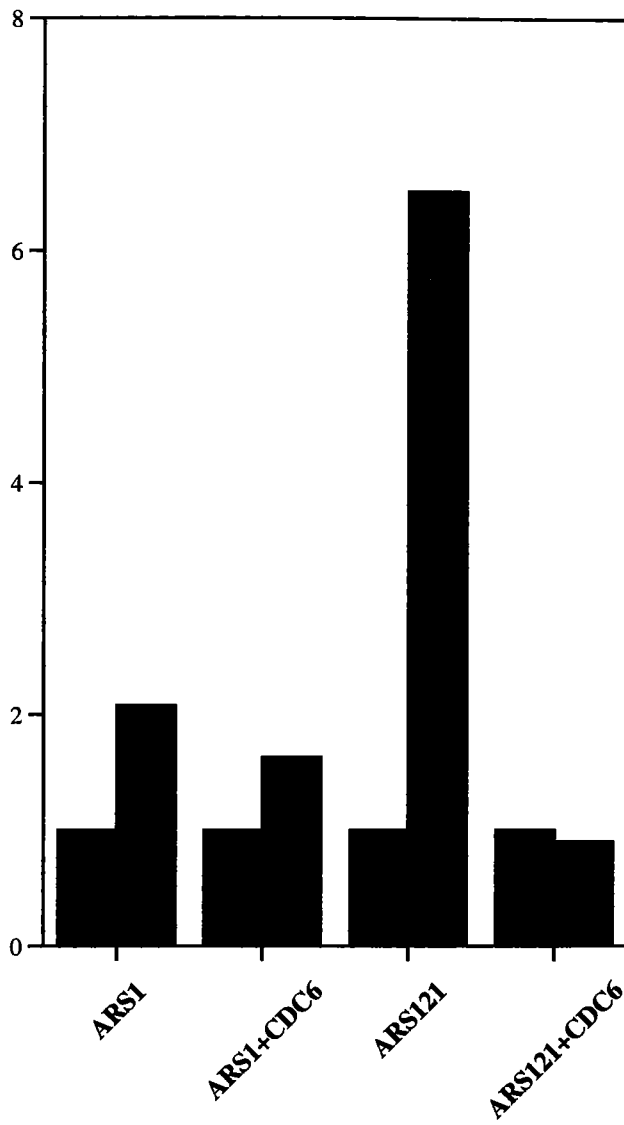


SWI/SNF is required for the full functioning of ARS121 and for ARS1 derivatives that lack a replication enhancer or contain a replication enhancer composed of a LexA-GAL4 binding site. The ability of SWI/SNF to enhance the functioning of these origins may represent a direct role for SWI/SNF, or alternatively, SWI/SNF may function via an indirect mechanism to stimulate replication. If SWI/SNF functions directly, then origin function might be enhanced by stably tethering SWI/SNF complex to an ARS element via a LexA DNA binding domain. Figure V.7 shows mitotic stability assays for a minichromosome with an ARS1 derivative that contains a LexA binding site in place of the ABF1 site, and with cells that express either the LexA DNA binding domain, LexA-GAL4, or a LexA-SWI2 fusion protein (Figure V.7). In the presence of only a LexA DNA binding domain, this minichromosome is maintained in only 0.5% of the cells after growth in nonselective media. However, expression of either the LexA-GAL4 or LexA-SWI2 fusion protein leads to a similar, 4-fold increase in mitotic stability. Thus, tethering the ATPase subunit of the SWI/SNF chromatin remodeling complex can enhance replication origin function *in vivo*.

Over-expression of Cdc6p relieves ARS121 SWI/SNF-dependency. The SWI/SNF complex is required for the optimal expression of a subset of yeast genes, including Cdc6p (Holstege et al., 1998; Krebs and Peterson, 2000). Cdc6p is expressed at both the end of mitosis and during G1 of the cell cycle, and is required for the establishment and maintenance of the pre-replication complex (pre-RC) (reviewed in Dutta and Bell, 1997; Tye, 1999; Coleman et al., 1996; Detweiler and Li, 1997; Piatti et al., 1996). For example, inactivation of Cdc6p using a *cdc6* temperature-sensitive mutant causes pre-RCs to become thermolabile and new pre-RCs are not formed (Detweiler and Li, 1997). Since Cdc6p expression is lowered in a SWI/SNF mutant,

Figure V.8 Over-expression of Cdc6p relieves ARS121 SWI/SNF-dependency. Mitotic stability assays were performed in isogenic *SWI*⁺ (CY318) (isogenic to CY26, *trp1Δ63*) and *snf6* deletion strain *snf6Δ* (CY332) strains carrying minichromosomes pARS/WTA (ARS1), or yCp5AB121 (ARS121) in the presence or absence of a high copy plasmid that harbors a Galp-HA-CDC6 fusion cassette as described in (Detweiler and Li, 1997). Three independent experiments were done for each strain.

Mitotic Loss Rate per Generation (X)



■ WT
■ $\Delta snf6$

Plasmid Origin

we tested whether SWI/SNF-dependence of ARS121 is due to lower levels of Cdc6p. The stability of minichromosomes that contain either wild type ARS1 (SWI/SNF-independent) or ARS121 (SWI/SNF-dependent) was analyzed in wild type or *snf6*- strains that also contained a plasmid that over-expresses Cdc6p from the strong Gal1 promoter (Figure V.8). In the case of the ARS1 minichromosome, CDC6 over-expression did not effect stability either in the presence or absence of SWI/SNF (Figure V.8). In contrast, over-expression of CDC6 completely eliminated the SWI/SNF requirement for stability of the ARS121 minichromosome. In the absence of the CDC6 plasmid, the ARS121 minichromosome was lost at a 6-fold higher rate than in the *snf6* mutant, whereas in the presence of Gal1-CDC6 a *snf6* deletion had no effect on ARS121 function (Figure V.8). Thus, over-expression of CDC6 can bypass the need for SWI/SNF in ARS121 function.

Discussion

Here we have shown that SWI/SNF function is not restricted to transcription, but it can also play a role in DNA replication. As is the case for transcription, most yeast replication origins (ARS elements) do not require SWI/SNF function. These SWI/SNF-independent origins include ARS1, ARP307, ARP309 and ARS1 derivative lacking the B2 *cis*-acting element. Origins that require SWI/SNF for optimal activity include ARS121, derivatives of ARS121 that lack ABF1 binding sites, ARS1 derivatives lacking either the ABF1 binding site or the B1 element, and an ARS1 derivative that requires a LexA-GAL4 chimeric activator. Genetic and biochemical studies have indicated that SWI/SNF complex may facilitate the function of transcriptional activators by contending with chromatin-mediated repression of transcription.

Interestingly, ARS121 origin is no longer SWI/SNF-dependent in which Cdc6p is over-expressed. By analogy, we propose that SWI/SNF complex may perform a similar role at replication origins in cases where chromatin structure impinges on their function.

Role of SWI/SNF in mitotic stability of minichromosomes. Mitotic stability of minichromosomes requires the efficient functioning of both a replication origin (ARS) and centromere (CEN). If a minichromosome does not replicate efficiently, then too few copies will be available to segregate to the daughter cell. Likewise, if segregation is impaired, the daughter cell will not receive a copy of the minichromosome. Several results demonstrate that SWI/SNF influences the function of the replication origin and not the centromere. First, minichromosomes that contain wild-type ARS elements all contain a cloned CEN element, but only the plasmid that contains ARS121 requires SWI/SNF function. Moreover, the activation of an ARS on a minichromosome is required for the complete replication of that plasmid. Second, each of the ARS1 derivatives is contained in the same plasmid backbone and has an identical CEN element. However, only ARS1 derivatives containing mutations in either the B1 or B3 element, or an ARS1 derivative that is dependent upon LexA-GAL4, require SWI/SNF function. In contrast, wild type ARS1 and an ARS1 derivative lacking the B2 element remain SWI/SNF-independent. Thus, the SWI/SNF dependence of minichromosome stability is a property of the replication origin, not the CEN element. Such specificity for SWI/SNF function also indicates that SWI/SNF does not simply govern expression of a general replication factor, but that it plays a more direct role in origin function. Furthermore, SWI/SNF does not appear to govern replication by controlling expression of ABF1, since the full functioning of ARS121 and ARS1 derivative

that lack ABF1 binding sites still requires SWI/SNF. Collectively, these results suggest that the SWI/SNF dependence on DNA replication is analogous to that in transcription, in which SWI/SNF alters chromatin structure for efficient origin firing.

Role of replication enhancers in determining SWI/SNF dependence of ARS

function. Why does the full functioning of some ARSs require SWI/SNF action, whereas others do not? In the case of ARS1, our data suggests that the single ABF1 binding site allows ARS1 to function in the absence of SWI/SNF. A double point mutant in this ABF1 site reduces ARS1 function and increases the requirement for SWI/SNF. Replacing the ABF1 site with a binding site for the LexA-GAL4 chimeric activator allows more efficient function in *SWI*⁺ cells, but LexA-GAL4 is unable to restore SWI/SNF independence to this ARS1 derivative. Although it is not known exactly how ABF1 enhances ARS1 function, it is known that the ABF1 binding site is required to keep nucleosomes from encompassing the essential ASC element (Venditti et al., 1998). Since one transcriptional role for SWI/SNF is to facilitate the binding of factors to nucleosomal sites (Burns and Peterson, 1997), it seems likely that a repositioning of nucleosomes over the ARS1 ACS is a likely cause for the increased dependence of this ARS1 derivative on the SWI/SNF chromatin remodeling complex. Mutations in the B1 binding site may also influence the chromatin context, which then influences the SWI/SNF dependence of this derivative; likewise ARS121 may have inherent chromatin context which makes this origin SWI/SNF dependent. This chromatin context may depend on elements within the plasmid or be an inherent feature of the chromosomal copy of ARS121.

The full functioning of the ARS121 origin also relies on ABF1 binding sites, however, in this case the two ABF1 sites are not sufficient to provide a function that is redundant with SWI/SNF action. Previous studies have demonstrated that the two ABF1 sites at ARS121 are not functionally equivalent to the single site at ARS1 (Fedor et al., 1988). Although the function of a single ABF1 site at ARS1 can be replaced by sites for RAP1, GAL4, p53 or LexA fusion activators (Detweiler and Li, 1997; Marahrens and Stillman, 1992), heterologous activators cannot substitute for the two ABF1 binding sites at ARS121 (Fedor et al., 1988). These two different ABF1-dependent replication enhancers differ with respect to their orientation and distance from the essential ACS element. The two ABF1 binding sites at ARS121 are located 160 and 220 bp upstream of the essential ACS element and 260-320 bp upstream of the B2/DUE/NTR element. In contrast, the single ABF1 binding site at ARS1 is adjacent to the B2/DUE element and it is only located 80 bp 3' to the T-rich strand of the ACS. Venditti and colleagues (Venditti et al., 1998) have proposed that the binding of ABF1 can act as a boundary that establishes the nucleosome-free state of ARS1; this proposed boundary function is likely to contribute to the SWI/SNF independence of ARS1. Boundary elements, however, can only propagate their effects over short distances (~80 bp; Fedor et al., 1988) and thus ABF1 may not be able to influence nucleosome positioning at ARS121, leading perhaps to the increased dependence of this ARS on the SWI/SNF chromatin remodeling complex.

The Role of SWI/SNF and Cdc6p at ARS-Specific DNA replication Origins.

SWI/SNF complex is only needed for the expression for a subset of genes (~6%/~6000 yeast genes), and similar to transcription regulation, SWI/SNF appears to be required for optimal firing

of at least one DNA replication origin, ARS121 (1/~400 yeast origins) (Figure V.8). It has been shown that SWI/SNF complex is needed for the expression of Cdc6p, an essential pre-RC assembly regulator (Holstege et al., 1998; Krebs et al., 2000; Piatti et al., 1996; Sudarsanam et al., 2000). And thus, one possibility is that SWI/SNF may be acting indirectly in DNA replication by governing the expression of pre-replication assembly factor, Cdc6p. Cdc6p mutations cause increased rates of plasmid loss due to a lower efficiency of origin firing and not due to defects in DNA synthesis or plasmid segregation (Hartwell, 1976; Hogan and Koshland, 1992). Thus, in a *swi/snf* mutant, low expression levels of Cdc6p may specifically influence the functioning of the ARS121 replication origin. In fact, Cdc6p over-expression in cells lacking a functional SWI/SNF complex stabilizes ARS121, rendering the origin no longer SWI/SNF-dependent. However, this model does not explain why the SWI/SNF-independent origin, ARS1, is unaffected by the absence of SWI/SNF and hence low Cdc6p expression. An alternative view suggests that over-expression of Cdc6p may non-specifically bypass the need of SWI/SNF at ARS121. Consistent with this view, Liang et al. (Liang et al., 1999) have shown that over-expression of Cdc6p can bypass the need for the key Mcm2 and Mcm5 (minichromosome maintenance) replication proteins at the H4 origin.

Experimental Procedures

Yeast Strains and Plasmids All strains are congenic to S288C and are isogenic derivatives of strain yPH274 (Sikorski and Hieter, 1989). The *swi1* and *swi2* deletion alleles are described in (Peterson and Herskowitz, 1992). For Cdc6p over-expression plasmid stability assays, wild type

yeast strain CY318 (isogenic to CY26, *trp1*Δ63) and *snf6* deletion strain CY332 (*snf6*Δ2, *ura3*-52, *lue2*-, *his3*Δ200, *trp1*Δ63) were used.

ARS plasmids pARS/WTA (ARS1), pARS/835-842 (ARS1 B1 linker scanning mutant), pARS/756,758 (ARS1 B3 double point mutant), pARS/798-805 (ARS1 B2 linker scanning mutant), and pARS/LexA 798-805, as well as high copy/*HIS3* plasmid that expressed either the LexA DNA binding domain (pLEX[1-82]) or a LexA-GAL4 fusion protein (pMA411) are described in (Marahrens and Stillman, 1992). Plasmid p309-326 (ARS309) and pC2G1A (ARS307) are described in (Theis and Newlon, 1994). Plasmid yCp5AB121 (ARS121) is described in (Walker et al., 1989) and plasmid AB121B1 (ARS121 b1 mutant), AB121B2 (ARS121 b2 mutant) and AB121B1B2 (ARS121 b1b2 double mutant) is described in (Wiltshire et al., 1997). Plasmid CP337 is SWI2 in RS315 (Sikorski and Hieter, 1989) as described in (Richmond and Peterson, 1996). Plasmid JL775 (CP877) Galp-HA-CDC6 fusion cassette in pRS314 as described in (Kroll et al., 1996).

Mitotic Plasmid Stability Assays For the plasmid stability assay, 5 ml cultures were grown in SD medium (6.7 grams/liter yeast nitrogen base without amino acids (Difco), 2% glucose) and supplemented with all amino acids except uracil (Stern et al., 1984) (selective medium) and grown to saturation at 30°C. Aliquots of these cultures were then diluted and plated in triplicate on SD -uracil and YEPD (1% yeast extract (Difco), 2% bacto-peptone (Difco), 2% glucose) media. Colonies were counted from both sets of plates to determine the value, A, which is the percentage of cells that maintain the minichromosome under selective conditions. An additional aliquot of the original cultures was diluted into 3 ml of YEPD medium (nonselective medium) to

a final OD₆₀₀ value of 0.0003 or 0.05 for *SWI*⁺ or *swi*⁻ cultures, respectively. These cultures were grown to saturation at 30°C (12 generations for *SWI*⁺ and 5 generations for *swi*⁻ cells), diluted, and plated in triplicate on SD - uracil and SD + uracil media. Colonies were counted from both sets of plates to determine the value, B, which is the percentage of cells that maintain the plasmid after nonselective growth (% URA⁺). The rate of plasmid loss per generation was calculated using the equation: $X = 1 - e^r$, where X is the rate of plasmid loss per generation, $r = \ln(A/B) / N$, and N is the number of generations in nonselective medium (Dani and Zakian, 1983). Colonies were counted from both sets of plates to determine the value, B, which is the percentage of cells that maintain the plasmid after nonselective growth (% URA⁺). The rate of plasmid loss per generation was calculated using the equation: $X = 1 - e^r$, where X is the rate of plasmid loss per generation, $r = \ln(A/B) / N$, and N is the number of generations in nonselective medium (Dani and Zakian, 1983). The only changes made for the Cdc6p over-expression plasmid stability assay was that the selective medium was SD medium supplemented with all amino acids except uracil and tyryptophan (Stern et al., 1984); nonselective medium was SD medium-tyryptophan and both *SWI*⁺ or *snf6* cultures were grown to saturation at 30°C for 6 generations.

Site Directed Mutagenesis of ARS121 Mutagenesis of ARS121 was performed by a two-step PCR-mediated mutagenesis procedure. Primer oligonucleotides (DNA International) were as follows:

ARS121-ACON: 5' GTTAAACATAAAAATCTCACTTC 3'

PBR-TAG: 5' GAGGATCCCCGGGTACGTATCACGAGGCCCTTTCG 3'

ARS121.HINDIII.3': 5' GCCGAAGCTTAGAATTTGGCTCTG 3'

HHF2.5SL 5' GAGGATCCCCGGGTAC 3'

Primers ARS121-ACON and PBR-TAG were used in a PCR reaction to generate the point mutations in ARS121 using yCp5AB121 (Walker et al., 1989) as a template. The PCR product, yCp5AB121, and primers ARS121.HINDIII.3' and HHF2.5SL were then used in a subsequent PCR reaction. The resulting 0.4kb PCR product and plasmid yCp5AB121 were digested with EcoRI/HindIII (New England BioLabs) and ligation yielded plasmid CP561. ARS121 mutations were confirmed by DNA sequence analysis (Sequenase, USB).

CHAPTER VI

Summary

Conclusions

Chromatin plays a large role in all DNA-mediated processes in the cell, including transcription, DNA replication, recombination and repair. The cell implements a number of diverse classes of chromatin modifying enzymes to alter chromatin structure. In the last decade, this class of multi-subunit complexes has begun to grow in multitudes, expounding on functional roles involved in a number of different DNA-mediated cellular processes. One class of chromatin modifying enzymes is the ATP-dependent protein machines that use the energy of ATP hydrolysis to perturb chromatin structure (see Table I.1; for review see Kingston and Narlikar, 1999; Workman and Kingston, 1998). This class of ATP-dependent chromatin remodeling complexes is conserved throughout evolution, and purification of these complexes has provided the means to further explore the mechanism of nucleosome remodeling. The research presented here focused on understanding the structure and function of the prototype of ATP-dependent chromatin remodelers, the *Saccharomyces cerevisiae* SWI/SNF complex.

Stoichiometry of the Yeast SWI/SNF Complex. ATP-dependent chromatin remodeling enzymes are typically large, macromolecular assemblies (reviewed in Muchardt and Yaniv, 1999; Vignali et al., 2000). The purification and characterization of several complexes has led to the remarkable finding that the SWI/SNF family of ATP-dependent chromatin remodelers consist of a core of conserved subunits with homology

to yeast SWI2/SNF2, SWI3, SNF5, and SWP73 (see Figure III.1 and Chapter III). Although several laboratories have purified SWI/SNF-like complexes from many organisms, different biochemical fractionation schemes have been employed to isolate these complexes, and the subunit stoichiometries have not been ascertained for any of the SWI/SNF-like ATP dependent remodelers. Purification and staining of the yeast SWI/SNF complex suggests that the subunits are stoichiometric, with the exception that Swi3p stains more intensely (Cairns et al., 1994; Peterson and Herskowitz, 1992; Treich et al., 1995). We know that the sum of the molecular weights of the polypeptides for the yeast SWI/SNF complex equals ~1 MDa, yet by gel filtration analysis the native SWI/SNF complex appears to be ~2MDa.

One obvious possibility is that the complex could consist of roughly two copies of each subunit. However, this study has shown that only one copy of Swi2p is present per complex (Chapter II). Subsequent iodination of the purified yeast SWI/SNF complex allowed us to determine the relative stoichiometry of the conserved components in the complex relative to the Swi2p polypeptide (Chapter II). Results consistently demonstrated stoichiometric levels of the Swi1p, Swp73p, Arp7p, Arp9p, and Snf6p subunits relative to the Swi2p polypeptide, whereas Swi3p and Snf5p appear to be in two copies per Swi2p polypeptide. This is consistent with previous *in vivo* and *in vitro* studies done which also suggested that the Swi3p and Rsc8/Swh3p (Swi3p homologue in RSC) were present in more than one copy per complex (Chapter III; L. Boyer, thesis; Treich and Carlson, 1997). In contrast, there are no additional data supporting or refuting our result, indicating that Snf5p is also present in two copies. Summation of the

SWI/SNF subunits by the relative ratios determined, and the assumption that there is only one copy of the remaining subunits per complex, gives a molecular mass equaling 1.1 MDa, suggesting this is the actual molecular weight of the yeast SWI/SNF complex. Future experiments, such as scanning transmission electron microscopy (STEM), may provide a more precise molecular mass of the purified yeast SWI/SNF complex.

Organization of the Yeast SWI/SNF Complex. The SWI/SNF family of ATP-dependent chromatin remodeling complexes has a subset of conserved subunits that are homologous to Swi2p, Swi3p, Snf5p, Swp73p and Arp proteins in yeast (see Figure III.1). Moreover, Swi2p, Swi3p, and Snf5p homologues appear to define the minimal functional core of these enzymes *in vitro* (Phelan et al., 1999). However, the organization of the components within these complexes is not known, and only a few subunit interactions have been described. It is known that the N-terminal region of Swi2p (amino acids 1-767) interacts with Swi3p and Snf11p by two hybrid analysis (Treich and Carlson, 1997) and that Swi3p appears to self-associate (L. Boyer, thesis, Treich and Carlson, 1997). Several studies also indicate that Swi3p family members can interact with both Swi2p and Swp73p family member homologues (Crosby et al., 1999; Phelan et al., 1999; Treich et al., 1995; Treich and Carlson, 1997; Treich et al., 1998). However, the overall organizations of the SWI/SNF-like chromatin remodeling complexes are not well depicted.

Here we further illustrated the yeast SWI/SNF complex organization depicting how the conserved core of subunits directly interact, and hence how they may

communicate on an intramolecular level for the regulation of SWI/SNF activity. We did this by analyzing direct binding to the Swi3p polypeptide for a number of reasons. First, Swi3p is part of a protein family, which contains a number of conserved motifs. All Swi3p family members include a region rich in prolines, hydrophobic, and aromatic amino acids (amino acids 300-500), a SANT (Swi3, Ada2, N-CoR, TF_{III}F'; amino acids 523-578) domain and a putative hydrophobic leucine zipper (amino acids 694-722) (see for recent review; Aasland et al., 1996; Peterson and Herskowitz, 1992; Vignali et al., 2000). Secondly, structure function analysis of Swi3p revealed regions of Swi3p that are required for function *in vivo* and for the assembly of SWI/SNF complex *in vitro* (L. Boyer, thesis). Furthermore, studies have suggested that Swi3p may be present in more than one copy, by Coomassie staining and far-Western analysis (L. Boyer, thesis, Treich and Carlson, 1997). Moreover this study has determined that there are two copies of Swi3p per SWI/SNF complex (Chapter II). Thus, we hypothesized that Swi3p serves as a scaffold for the subunits in the SWI/SNF complex.

Our binding studies confirm that Swi3p nucleates the assembly of a functional SWI/SNF complex (Chapter III). Namely, Swi3p can directly interact with Swi1p, Snf2p, Snf5p, Swp73p, and Snf6p, as well as self-associate. Collectively, Swi3p-subunit binding interactions are mediated throughout the entire length of the Swi3p protein, excluding the non-essential N-terminal amino acids, 1-304. Furthermore, deletion analysis of Swi3p mapped the Swi3p binding domains required for these subunit interactions as well as self-association (Chapter III). Identification of these domains sets a foundation for future studies to isolate either *swi/snf* mutants or *swi3* mutants in yeast

that can remain assembly competent, but dysfunctional for some aspect of SWI/SNF chromatin remodeling activities. These types of experiments would provide functional data in the context of the native SWI/SNF complex, further defining the functional roles for these subunits and Swi3p moieties, and may portray the necessity of these components in intramolecular communication for different intermolecular activities (discussed in Peterson, 1998).

We defined two domains, the conserved N-terminal domain (305-540 aa), and the leucine zipper motif (694-722), that are interdependent for the self-association of Swi3p (Chapter III). This is supported by data that shows that the Swi3p homologue Swh3p/Rsc8 requires the leucine zipper motif for self-association, but this domain was insufficient for complete self-association (Treich et al., 1997). Analogously, our data shows that if one domain is non-functional, the other intact domain is insufficient to function in self-association of Swi3p (Chapter III). For instance, the leucine zipper domain can self-associate with a small, C-terminal domain of Swi3p, which contains only the leucine zipper domain. However, when a leucine zipper domain is present in two functional copies, but one copy contains a truncated version of the N-terminal domain, self-association does not occur. Thus, it appears that the presence of the partial N-terminal domain of Swi3p can block the function of the intact leucine zipper domain. Likewise, a double point mutation in the leucine zipper domain can abolish the function of the N-terminal domain, both in terms of self-association and binding of the other subunits. These data indicate that the N-terminal domain and leucine zipper domain function interdependently.

Our analysis, as well as previous data, indicates that Swi3p makes direct contacts with various SWI/SNF subunits, and that the self-association of Swi3p provides an extensive platform for the assembly of these large multi-subunit complexes (Chapter III; Crosby et al., 1999; Phelan et al., 1999; Treich et al., 1995; Treich and Carlson, 1997; Treich et al., 1998). Collectively, these data suggest that Swi3p (and family members) may function to nucleate the assembly of a functional SWI/SNF complex. It is interesting to speculate that Swi3p may sensor intramolecular signals and thereby coordinate the complex activity.

Targeting of Yeast SWI/SNF Complex to Gene-Specific Promoters. Although it appears that a subset of conserved subunits are required for full activity of these chromatin remodeling enzymes *in vitro*, the remaining components (in addition to the conserved core subunits) are required for yeast SWI/SNF function *in vivo* (Cairns et al., 1996; Laurent et al., 1993; Peterson and Herskowitz, 1992; Phelan et al., 1999). This suggests that each subunit performs some critical function. Recently, much data has suggested that SWI/SNF complexes are targeted to gene promoters via gene-specific transcriptional activators (Yoshinaga et al., 1992; Muchardt and Yaniv, 1993; Ichinose et al., 1997; Ostund-Farrants et al., 1997; Fryer and Archer, 1998; Armstrong, 1998; Dunaief et al., 1999; Shanahan et al., 1999; de la Serna et al., 2000; Neely et al., 1999; Krebs et al., 1999; Krebs et al., 2000; Cosma et al., 1999; Natarajan et al., 2000; Yudkovsky et al., 1999; Kowenz-Leutz and Leutz, 1999; Agalioti et al., 2000). For example, the gene-specific transcriptional activator Swi5p appears to recruit yeast SWI/SNF the HO promoter *in vivo* (Cosma et al., 1999; Krebs et al., 2000; Krebs et al., 1999). Along these

lines Kadam *et al.* have recently shown that zinc-finger transcriptional activators (EKLF, GATA-1, Sp1) can specifically target human SWI/SNF in a gene-selective manner. More specifically, the human BRG1-BAF170/BAF155 sub-complex functions synergistically in transcription factor-targeted chromatin remodeling (Kadam *et al.*, 2000).

Our study demonstrated that the individual subunits, Swi1p, Snf5p, and Snf6p, as well as sub-complexes Swi2p/Swi3p and Swp73p/Swi3p, directly interact with the gene-specific activator Swi5p through its zinc-finger DBD (Chapter IV). These data attribute functional roles to the individual SWI/SNF subunits, Snf5p, Snf6p and Swi1p. The direct interaction of these individual subunits with transcriptional activators suggests that they are important for targeting/recruitment of the native SWI/SNF complex. In addition, we showed a cooperative effect of Swi3p/Swi2p and Swi3p/Swp73p sub-complexes for binding to the zinc-finger DBD of the Swi5p activator (Chapter IV). In addition, the Swi2p/Swi3p sub-complex cooperative binds with the VP16 activation domain (Chapter IV). Previous studies have shown that yeast SWI/SNF complex interacts with different moieties of transcriptional activators, such as the activation domain or a zinc-finger DBD domain (Kadam *et al.*, 2000; Krebs *et al.*, 2000; Natarajan *et al.*, 1999; Neely *et al.*, 1999; Yudkovsky *et al.*, 1999; M. Samuels and C. L. P., unpublished data). Perhaps these sub-complexes undergo a conformational change that promotes interactions with different DNA-binding factors that cannot be achieved independently (Chapter IV). These conformational changes may be important for targeting and recruiting activities of the SWI/SNF complex. It would be interesting to determine in the context of the native SWI/SNF complex, how such intramolecular interactions might affect SWI/SNF

functional activities. For instance, engineering mutants that form stable complexes, but are non-functional for interaction with different transcriptional activators may provide better insight into the mechanism of SWI/SNF targeting and recruitment.

Overall, it appears that the conserved subunits, Swi2p, Snf5p, Swi3p and Swp73p, are involved in the recognition of DNA-binding factors, and thus they may be a conserved targeting/recruiting function for homologues of the ATP-dependent chromatin remodelers. Analogous to human SWI/SNF, we would expect that the yeast SWI/SNF complex selectively regulates chromatin remodeling through direct interaction with specific DNA-binding proteins (Kadam et al., 2000). Future studies dissecting the functional relationship of these components in the native complex for facilitating targeted promoter recognition, and possibly complex-promoter stabilization, will further our understanding of the mechanism of targeting by SWI/SNF.

A Role for SWI/SNF in a DNA-mediated Process other than Transcription.

The SWI/SNF complex functions to alter chromatin structure in order to facilitate the activation of gene expression (reviewed in Winston and Carlson, 1992; Pollard and Peterson, 1998; Vignali et al., 2000). Similarly, it could be postulated that SWI/SNF complex is involved in other DNA-mediated processes, which require alteration of chromatin structure. In fact, many Swi2p ATPase family members are involved in diverse biological DNA-mediated processes (Eisen et al., 1995; Gorbalenya et al., 1989; Pollard and Peterson, 1998).

We have showed that SWI/SNF appears to play a role in origin specific DNA-replication activation (Chapter V). Specifically, a functional SWI/SNF complex is required for optimal efficiency of the ARS121 origin. Furthermore, the ATPase activity of the Swi2/Snf2p subunit is required to support efficient ARS121 DNA-replication (Laurent et al, 1992; Khavari et al, 1993; Richmond and Peterson, 1996; Cote et al, 1994). Moreover, our deletion studies suggest that the role of SWI/SNF at ARS121 is to facilitate the functioning of the central core (ACS, B1 and B2/DUE/NTR elements), rather than the replication enhancer (ABF1 binding sites). We also found that a SWI/SNF-independent DNA replication origin, ARS1, can be constructed into a SWI/SNF-dependent origin by altering replication enhancer elements (Chapter V). However, SWI/SNF may be playing more of an indirect role in replication origin efficiency, since we showed that over expression of *CDC6* in a *swi/snf* mutant, relieved SWI/SNF-dependence of ARS121. This may be a reflection of the fact that *CDC6* expression is lower in *swi/snf* mutant cells (Whitehouse et al., 1999; Krebs et al, 2000), and furthermore that Cdc6p is important for the assembly of the pre-replication complexes at DNA-replication origins for the subsequent S phase (Piatti et al., 1996). Alternatively, SWI/SNF may be acting redundantly with Cdc6p. In support of this notion, over-expression of *CDC6* also rescues the replication defects of MCM2 and MCM5 mutants (reviewed in Lee and Bell, 2000; Tye, 1999). Moreover, ATP-dependent chromatin remodeling by *Drosophila* CHRAC has been shown to be needed prior to SV40 DNA replication (Alexiadis et al., 1997; Alexiadis et al., 1998). It would be interesting to perform nucleosome mapping, two-dimensional gel analysis and ChIP

analysis to determine whether SWI/SNF is directly required for origins to efficiently function *in vivo* (Brewer and Fangman, 1987). These types of analyses would provide better insight of SWI/SNF role in DNA replication.

Synopsis

SWI/SNF was the first member of the ATP-dependent chromatin remodeling enzymes to be identified. It has been shown to be fundamentally important for the regulation of chromatin structure critical for a DNA-mediated process, namely transcription. Purified SWI/SNF can disrupt nucleosome structure leading to an enhanced accessibility of nucleosomal DNA to DNase I, restriction enzymes, and sequence specific DNA-binding proteins (Cote et al., 1998; Cote et al., 1994; Logie and Peterson, 1997; Logie et al., 1999; Owen-Hughes and Workman, 1996; Utley, 1999). The subunits in SWI/SNF-like chromatin remodeling enzymes may have evolved subtle differences in nucleosome cofactor requirements, or protein domains specific to each complex might contribute to interactions with the nucleosome structure or other transcriptional components. This may be the basis for these enzymes being categorized as functionally distinct, these distinctions are likely to reflect subtle differences in protein recognition, promoter targeting or in the regulation of the remodeling cycle (discussed in Peterson, 1998), rather than key differences in the basic remodeling mechanism. However, a subset of subunits in these complexes is conserved evolutionarily, suggesting the elementary mechanism for all these complexes is similar. One possibility of how ATP-

dependent enzymes may be regulated differently is through their differential recruitment to chromatin. This would be consistent with our data in which yeast SWI/SNF components selectively bind specific domains of different transcriptional activators. This is consistent with recent studies that recruitment of ySWI/SNF and hSWI/SNF occur via specific types of activators (Kadam et al., 2000; Kim, 1999; Lee et al., 1999, Natarajan et al., 1999; Neely et al., 1999; Yudkovsky et al., 1999; see below for further discussion).

The studies presented in this thesis address the fundamental question of how the subunits of the yeast SWI/SNF complex are physically organized in order to better understand the intramolecular structure of the complex that may be important for intermolecular activities. Our data support a view that the conserved core of homologous subunits provides an underlying similar organization among SWI/SNF-like ATP-dependent chromatin remodeling enzymes. It is important to remember that these enzymes ultimately act on chromatin structure. Naturally, this is an overly simplistic view, but it must be recognized that ATP-dependent chromatin remodeling enzymes are employed in many different processes of the cell to alter the accessibility of DNA in the context of chromatin. Hence, the unique subunits within these complexes may provide their functional specificity in the cell.

Yeast contains two distinct SWI/SNF-like ATP-dependent chromatin remodeling complexes, SWI/SNF and RSC. A number of studies suggest that the actions of yeast SWI/SNF complex are gene specific, notably required for mitotic gene expression (Holstege et al., 1998; Krebs et al., 2000; Sudarsanam et al., 2000). However, RSC is believed to be involved in kinetochore function, and not gene expression. Thus, each of

these enzymes may perform a similar function for some specific DNA-mediated process, or they may be regulated in such a way so that they are available only for specific time periods during the cell cycle. Alternatively, the activity of these enzymes may be targeted to chromosomal locations by virtue of their interaction with various DNA-binding factors. This interaction would provide the specificity and temporal regulation for these complexes. It is interesting to note that the same activators and repressors are able to recruit different remodeling complexes. Moreover, these same activators and repressors can target members of the same class of remodeling enzymes. Specificity would be governed by external cues such as growth factor or stress signals. Thus, it is interesting to speculate that the activity of chromatin remodeling enzymes may be regulated by cellular cues. This would allow for combinatorial control of gene regulation and would allow for the integration of multiple cellular signals to achieve the proper transcriptional state and serve as a dynamic mechanism for regulation of higher order chromatin structure. On the other hand, it is also possible that the function of chromatin remodeling enzymes is context dependent. Consistent with this idea, it has recently been shown that SWI/SNF is required for expression of a number of mitotic-specific genes (Krebs et al., 2000). Thus, the local chromatin structure may play a role in determining which enzymes are needed.

Little is known about how chromatin remodeling enzymes work *in vivo*. Although a great deal of genetic analyses and *in vitro* biochemistry suggests that these enzymes alter chromatin structure as a means to regulate DNA-mediated processes, it may not be that simple. Limited evidence links the *in vitro* studies to the *in vivo*

situation. For instance, SWI/SNF is required for the disruption of MNase I digestion patterns and presumably chromatin structure in the SUC2 promoter (Hirschhorn et al., 1992; Wu and Winston, 1997). Chromatin is clearly a target for ATP-dependent chromatin remodelers *in vivo*, however, it is not clear the limit of what these enzymes can do. Although the core of our knowledge about ATP-dependent chromatin is in the process of transcriptional regulation, it will be interesting to determine how chromatin remodeling enzymes contribute to other DNA-mediated processes such as DNA replication, recombination, and repair. Here we show that the yeast SWI/SNF complex plays a role in DNA replication. Future studies wait for the generation of mutants and biological tools that will allow for dissection of the activities of chromatin remodeling enzymes *in vivo*.

Understanding how chromatin remodeling enzymes function as transcriptional regulators is important to understanding the manifestation of disease. Many cases of cancer, often a result of aberrant or untimely gene expression are due to mutations in the chromatin remodeling enzymes that have been directly connected to transcriptional regulation (for review see Archer and Hodin, 1999; Jacobson and Pillus, 1999). There is extensive evidence that chromatin remodeling enzymes are causative agents for disease. For instance, somatic mutations that truncate the hSnf5/Ini1p gene have been identified in several aggressive pediatric malignant rhabdoid tumors (Versteeg et al., 1998). Thus, SWI/SNF in higher eukaryotes may contribute to cellular proliferation. SWI/SNF can enhance the transcriptional activation of the estrogen, retinoic acid, and glucocorticoid receptors as well (Fryer and Archer, 1998; Ichinose et al., 1997; Trouche et al., 1997).

Moreover, SWI/SNF has recently been found in a complex with Rb (retinoblastoma protein) and HDACs and that this complex promotes E2F-mediated G1 arrest (Zhang et al., 2000). Furthermore, recent work demonstrating an association between BRCA1 and a human SWI/SNF-related complex, provides a link between chromatin remodeling and breast cancer (Bochar et al., 2000). Hence, mutations in components of SWI/SNF appear to contribute to cancer progression. Importantly, histone acetyltransferases have also been broadly implicated in cancer. One example, supported by a number of laboratories, shows a role for CBP and p300 as transcriptional integrators for physiological cues to coordinate cell-cycle regulation, differentiation, DNA repair and apoptosis (discussed in Jacobson and Pillus, 1999). Thus, it is obvious that disruption of important interactions mediated by or functional abrogation of CBP/p300 could lead to alteration of one or more of these processes and thereby lead to cancer or a variety of other diseases. Clearly, continued investigations to further define the physiological role of chromatin remodeling enzymes will provide a molecular basis for understanding disease. Possibly, chromatin remodeling enzymes may become targets for gene therapy or targets for novel drug therapies in the future.

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