Isw2 complex slides nucleosomes to create repressive chromatin structure in vivo

Thomas G. Fazzio

A dissertation submitted in partial fulfillment of the
requirements for the degree of

Doctor of Philosophy

University of Washington

2004

Program Authorized to Offer Degree:
Molecular and Cellular Biology

INFORMATION TO USERS

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleed-through, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

ProQuest Information and Learning Company
300 North Zeeb Road
P.O. Box 1346
Ann Arbor, MI 48106-1346

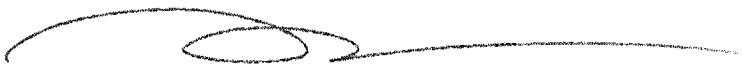
University of Washington
Graduate School

This is to certify that I have examined this copy of a doctoral dissertation by

Thomas G. Fazzio

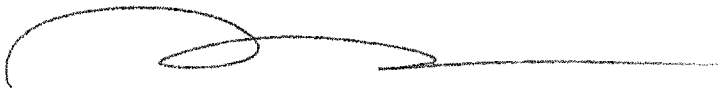
and have found that it is complete and satisfactory in all respects, and that any and all revisions required by the final examining committee have been made.

Chair of Supervisory Committee:

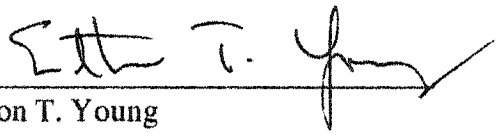


Toshio Tsukiyama

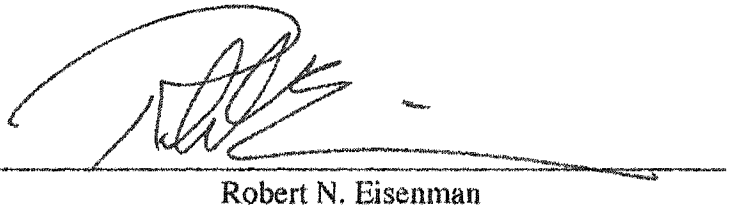
Reading Committee:



Toshio Tsukiyama



Elton T. Young



Robert N. Eisenman

Date: 6/1/2004

In presenting this dissertation in partial fulfillment of the requirements for the Doctoral degree at the University of Washington, I agree that the Library shall make copies freely available for inspection. I further agree that extensive copying of the dissertation is allowable only for scholarly purposes, consistent with "fair use" and prescribed in the U.S. copyright law. Requests for copying or reproduction of this dissertation may be referred to Proquest Information and Learning, 300 North Zeeb Road, Ann Arbor, MI 48106-1346, to whom the author has granted "the right to reproduce and sell (a) copies of the manuscript in microform and/or (b) printed copies of the manuscript made from microform."

Signature: Thao G Fogg

Date: 6/1/2004

University of Washington

Abstract

Isw2 complex slides nucleosomes to create repressive chromatin structure in vivo

Thomas G. Fazzio

Chairperson of the Supervisory Committee:
Toshio Tsukiyama
Associate Member
Division of Basic Sciences
Fred Hutchinson Cancer Research Center
Adjunct Professor, Department of Biochemistry

Each eukaryotic cell stores a tremendous amount of DNA (measuring about two meters in length) within its microscopic nucleus. To achieve this unlikely feat, eukaryotes compact their DNA into a dense, proteinaceous structure called chromatin. However, chromatin is generally inhibitory to essential processes that require access to DNA, such as transcription, DNA replication and recombination. Therefore, cells require active mechanisms to regulate chromatin structure. The imitation switch (ISWI) family of ATP-dependent chromatin remodeling factors are conserved throughout eukaryotes and utilize the energy of ATP to mobilize nucleosomes (the fundamental units of chromatin) on DNA. Members of the ISWI family have been shown to catalyze nucleosome sliding, spacing and loading activities in vitro. However, the in vivo functions of ISWI complexes, and the mechanisms by which they remodel chromatin in vivo, remain largely unknown. To gain some insight into these questions, I have undertaken an analysis of the Isw2 complex, using yeast as a model system. I found that

Isw2 complex functions to repress transcription of early meiotic genes in a parallel pathway to the Sin3-Rpd3 histone deacetylase (HDAC) complex in vivo. This was the first example of two mechanistically different chromatin remodeling complexes functioning in parallel to repress transcription. Using whole genome expression analysis, I found that the parallel pathways of repression by the Sin3-Rpd3 and Isw2 complexes extends to a substantial number of yeast genes in many functional categories. By mapping the positions of nucleosomes in the presence or absence of Isw2 complex, I found that Isw2 complex represses transcription by creating compact, nuclease-inaccessible chromatin structure near the promoters of target genes. To understand the mechanism by which Isw2 complex creates this compact chromatin structure, I created an inducible allele of the *ISW2* gene. Upon induction, I found that Isw2 complex slides nucleosomes unidirectionally toward the promoters of two target genes, reducing the spacing between nucleosomes as a result. This was the first demonstration of nucleosome sliding in vivo by an ATP-dependent chromatin remodeling factor, and has set the stage for experiments to probe the molecular details of nucleosome sliding by ISWI complexes at their physiological targets.

Table of Contents

List of Figures

List of Tables

Acknowledgements

I thank my advisor Toshio Tsukiyama for being an excellent mentor. Toshi encouraged me to pursue a variety of different projects that employed a broad range of genetic, molecular and biochemical techniques, which made for an excellent training experience. In addition, Toshi allowed me to pursue some of my own ideas and projects, while also providing significant input, advice and direction. I also thank my advisory committee: Ted Young, Dan Gottschling and Bob Eisenman for their excellent advice and criticism, all of which contributed to my growth as an independent scientist. In particular, special thanks go to Bob, Ted and Toshi for reading my dissertation and providing excellent comments. I thank all members of the Tsukiyama lab, past and present, for excellent scientific interactions, advice and critical comments throughout the time I have been in the lab. In particular, I thank Jesse Goldmark, with whom I collaborated during his short time in the lab to complete the experiments in Chapter I. Special thanks go to Jay Vary, Marnie Gelbart, Iestyn Whitehouse, Kim Wachter, Jack Vincent, Audrey McConnell and Cedar McKay for their insightful comments and ideas, as well as contributing to the collegial atmosphere of the lab. Our lab also benefited from joint lab meetings with a number of labs at the Fred Hutch, especially the Biggins and Gottschling labs. I especially would like to thank Sue Biggins for always taking time to answer questions and offer advice. Finally, I thank Mom, Dad, Troy, Brenda, and Jenny for their love and support, without which none of this work would have been possible.

Introduction

Chromatin Organization

Due to the contributions of genes, introns, transposons and other genomic elements, eukaryotic cells store an enormous amount of DNA in their nuclei. For example, the human genome consists of approximately 5 billion nucleotides which, if stretched end to end, would measure over one meter in length. In addition to the fact that it must be packed very tightly to fit within a nucleus measuring about 10 microns in diameter, DNA must be compacted in an orderly fashion to avoid extensive tangling within and among the different chromosomes. This requirement is most evident during the process of mitosis, when one maternal and one paternal copy of every chromosome (for a total of 46 chromatids in human cells) must be segregated to each of two daughter cells. In this case, tangling of the chromatin would likely result in chromosome breakage and, as a result, aneuploidy.

To accomplish this task of orderly compaction, DNA is packaged into chromatin. This process begins with the wrapping of DNA around an octamer of two each of the histone proteins H2A, H2B, H3 and H4 to form the nucleosome (Kornberg and Lorch 1999b). Since the histones are highly positively charged, the outer surface of the histone octamer provides a surface to which the negatively charged DNA double helix binds very tightly. The histone octamer makes over 100 ionic contacts with one face of the DNA double helix for each nucleosome, all of which are directed to the phosphate-rich backbone of the DNA (Luger et al. 1997). Therefore, the histone octamer is capable of binding the large variety of DNA sequences found in eukaryotic cells, with little sequence specificity. Each nucleosome contains DNA wrapped approximately 1.65 times around the histone octamer, and many nucleosomes are strung together along the long stretches of DNA that constitute each chromosome. These arrays of nucleosomes, resembling "beads on a string," are folded upon themselves extensively to form progressively thicker chromatin fibers, such as the 30 nm and 300 nm chromatin fibers (for review, see (Adkins et al. 2004)). The precise mechanisms by which these "higher

order" fibers are generated and maintained is an open question and active area of research. The net result of these many levels of compaction is a highly dense protein-DNA structure that is resistant to tangling and well suited to long-distance movement, which is critically important during cellular processes such as chromosome pairing, anaphase I and anaphase II during meiosis, as well as chromosome alignment and anaphase duting mitosis.

Chromatin Regulation: Histone Modifying Enzymes

While several processes involved in maintaining genomic integrity require chromatin to be in its most highly compact form, chromatin structure inhibits the vast majority of DNA-binding proteins from forming stable interactions with their DNA targets (Kornberg and Lorch 1999a). Since protein-DNA interactions are required for a number of essential processes, such as transcription, DNA replication and DNA repair, cells require active mechanisms to allow access to DNA. As a result, a sizeable number of proteins have evolved catalytic activities to modify chromatin structure. Since some factors modify chromatin to be more accessible to DNA-binding proteins, while others render chromatin less accessible, these proteins function as a group to dynamically regulate the structure of chromatin. While there is much to be learned about the mechanisms and components of the chromatin regulatory machinery, two main classes of enzymes have been discovered that regulate chromatin on the level of individual nucleosomes: histone modifying enzymes and ATP-dependent chromatin remodeling factors (Kingston and Narlikar 1999; Becker and Horz 2002; Neely and Workman 2002b; Lusser and Kadonaga 2003; Allard et al. 2004).

Histone modifying enzymes catalyze the covalent modification of histone proteins and belong to a number of protein families in several functional categories. The first nuclear histone modifying enzyme cloned and characterized was the *Tetrahymena* homolog of the yeast protein Gcn5 (Brownell et al. 1996). As Gcn5 had already been implicated in activation of transcription, the connection between histone modifying enzymes and gene regulation was immediately obvious. Since then the chromatin field

has experienced an explosion of new histone modifications and histone modifying enzymes. To date, methylation, acetylation, ubiquitylation, ADP-ribosylation, glycosylation and phosphorylation activities have been described (van Holde 1988; Berger 2002). These modifications are found to be directed toward an ever increasing number of serine, lysine and arginine residues on each of the four core histones (Zhang et al. 2003). The vast majority of known histone modifications lie on the amino terminal tails of the histone proteins that extend from the inner core of the histone octamer. However, the recent discovery of histone H3 methylation within a solvent-exposed surface of its core domain raises the possibility of an entirely new set of histone modifications that remain to be discovered (Ng et al. 2002; van Leeuwen et al. 2002; Zhang et al. 2003). Due to the convenient and powerful tools for studying gene regulation, the roles of histone modifications in gene silencing and activation have been studied most extensively. However, histone modifications have also recently been found to play roles in processes such as DNA repair and cell cycle control (Carrozza et al. 2003). As we learn more about the mechanisms by which nuclear processes are regulated, it is becoming increasingly clear that chromatin structure imparts some level of control on essentially every function in which DNA is involved.

So how do these covalent modifications function to regulate nuclear processes like transcription? The addition of acetyl and methyl groups to lysine residues and methyl groups to arginine residues results in neutralization of positive charges on these amino acids and may therefore reduce the affinity of histones for DNA (Allfrey et al. 1964). A second, non-mutually exclusive hypothesis was proposed by Allis and colleagues who observed that particular modifications were often associated with active transcription, while others were associated with silent chromatin. This led to the "histone code hypothesis," which postulates that specific modifications located on specific sites serve as specific binding sites for proteins that can function by further remodeling chromatin, recruiting the general transcription machinery or by other mechanisms (Strahl and Allis 2000). This hypothesis is supported by several recent discoveries. For example, protein domains called bromodomains, which are found in a

number of proteins that function in regulation of chromatin structure and transcription, bind specifically to acetylated lysine residues (Dhalluin et al. 1999). Therefore, histones marked with specific patterns of acetylation recruit bromodomain-containing proteins to specific loci on chromatin. In most cases, histone acetylation is associated with regions or specific genes that are actively transcribed. It is therefore not surprising that many well characterized bromodomain-containing proteins are known to be involved in activated transcription, such as the RSC and Swi/Snf complexes (ATP-dependent chromatin remodeling factors) and TAFII250 (a general transcription factor) (Zeng and Zhou 2002). Similarly, chromodomain-containing proteins have been shown to specifically bind methylated lysines, and are recruited to sites on chromatin with specific histone methylation marks. To date, the best studied chromodomain protein is HP1, which was identified as a protein that is enriched in heterochromatic regions of the genome (James et al. 1989). HP1 binds specifically to histone H3 methylated at lysine-9 (Bannister et al. 2001; Lachner et al. 2001); not surprisingly, this mark is also found to be enriched in heterochromatic regions and overlaps well with the pattern of HP1 localization. The mechanisms by which H3 lysine-9 methylation and HP1 binding contribute to heterochromatin formation continue to be actively pursued.

Chromatin Regulation: ATP-Dependent Chromatin Remodeling Factors

The second major class of factors that regulate chromatin structure are the ATP-dependent chromatin remodeling factors, which harness the energy of ATP hydrolysis to mobilize nucleosomes on DNA. ATP-dependent chromatin remodeling was first discovered as a biochemical activity in *Drosophila* embryo extracts that facilitates binding of the GAGA transcription factor to reconstituted chromatin in vitro (Tsukiyama et al. 1994). Subsequently, a number of chromatin remodeling factors have been purified and their biochemical activities characterized. ATP-dependent chromatin remodeling factors catalyze a number of activities involving nucleosome movement in vitro, including movement of nucleosomes along DNA molecules in cis (nucleosome sliding), movement of a histone octamer to a new DNA molecule (trans-displacement)

and disruption of the nucleosome to generate nuclease-accessible DNA (Becker and Horz 2002). In addition, two new related functions have been described: the exchange of H2A-H2B dimers on nucleosomes for dimers containing H2A-Z and H2B (Mizuguchi et al. 2004), and the exchange of H2A-H2B dimers between chromatin fragments (Bruno et al. 2003).

All known ATP-dependent chromatin remodeling factors are members of the Swi/Snf superfamily, due to the shared homology of the catalytic ATPase subunits present in each complex. The ATPase domain of Swi/Snf superfamily ATPases contains an array of motifs that are homologous to the PcrA family of helicase proteins (Eisen et al. 1995), raising the possibility that these factors may function in part by translocating along DNA (Saha et al. 2002; Whitehouse et al. 2003). Four main classes of this rapidly expanding ATPase superfamily have been identified, the Swi/Snf, ISWI, CHD1/Mi-2 and Ino80 families (Table I.1). While these four classes are homologous within their ATPase domains, they share no homology outside of this domain. Furthermore, most members of these families are complexed in vivo with additional subunits, with very few of these subunits shared between complexes (Kingston and Narlikar 1999). These two observations suggest that, while the biochemical activities of Swi/Snf superfamily members may be similar, these factors likely have distinct functions in vivo, as well as different mechanisms of remodeling chromatin. The biological and biochemical similarities and differences between the two most well studied classes of ATP-dependent chromatin remodeling factors, the Swi/Snf and ISWI classes, will be discussed in detail below.

The Swi/Snf Class of ATP-Dependent Chromatin Remodeling Factors

The founding member of the Swi/Snf superfamily of ATPases, yeast *SWI2/SNF2*, was discovered genetically in two independent screens for mutants defective in activation of transcription (Neigeborn and Carlson 1984; Breeden and Nasmyth 1987). Subsequently, Swi/Snf complexes have been purified and characterized biochemically from a number of organisms, including yeast (Swi/Snf, RSC), *Drosophila* (Brahma), and

Table I.1: Classes (subfamilies) of ATP-dependent chromatin remodeling factors within the Swi/Snf superfamily of ATPases and their constituents

Organism:	Swi/Snf	ISWI	Chd1/Mi-2	Ino80
S. cerevisiae	Swi/Snf RSC	Isw1a Isw1b Isw2	Chd1	Ino80 Swr1
Drosophila	BRM	NURF ACF CHRAC		
Xenopus		xACF xCHRAC xWSTF xISW1-D	xMi-2	
Human	Brg1 hBrm bBAF	hACF NoRC RSF hNURF hCHRAC WICH unnamed[a]	NURD	

[a] An unnamed Snf2h-comtaining complex has recently been described that binds to and helps load cohesin on to chromatin (see text).

human cells (Brm, Brg1). In vitro, Swi/Snf complexes exhibit a number of chromatin remodeling activities. Yeast Swi/Snf complex has been shown to reposition an end-positioned nucleosome to a more internally located site on the same DNA molecule without removing the histone octamer from DNA, a process known as nucleosome sliding (Whitehouse et al. 1999). However, the direction of nucleosome sliding is the opposite for human BRG1p (Fan et al. 2003), suggesting that the direction of nucleosome sliding varies with the specific factor and/or template DNA used. Later it was shown that this sliding was accomplished by the disruption of histone-DNA contacts throughout the nucleosome to form a "persistently altered nucleosome" which is granted greater mobility to migrate to a new position on the DNA molecule (Lorch et al. 1998; Narlikar et al. 2001). Interestingly, human Swi/Snf complexes and one yeast complex of the Swi/Snf family, RSC complex, have been shown to allow displacement of the histone octamer and transfer to a new DNA molecule, provided a high enough concentration of enzyme in the reaction (Lorch et al. 1999; Phelan et al. 2000). While these experiments shed light on the range of biochemical activities Swi/Snf complexes are capable of, it is unclear what roles Swi/Snf-dependent nucleosome sliding or trans-displacement play in vivo, or if these activities even occur in a physiological context.

One of the major in vivo functions of Swi/Snf complexes is in activation of transcription. Growth of yeast cells in low glucose induces transcription of the *SUC2* gene, encoding invertase, and this induction requires the Swi/Snf complex (Neigeborn and Carlson 1984). Further characterization of the *SUC2* locus revealed that chromatin structure is disrupted near the promoter upon induction, rendering DNA in the region nuclease-accessible (Hirschhorn et al. 1992). This disruption is dependent upon the Swi/Snf complex, but independent of transcription, suggesting that Swi/Snf complex is directly responsible for this chromatin disruption. Since this time, a large number of additional roles for Swi/Snf complex have been identified in yeast and other organisms. Mammalian Swi/Snf complexes have been shown to contribute to cell growth, transcriptional activation of the hsp70 gene and MyoD-mediated differentiation, among other things (Khavari et al. 1993; de La Serna et al. 2000; de la Serna et al. 2001). In

addition, Swi/Snf complexes appear to play some roles in repression of transcription. In yeast, deletion of the *SWI2/SNF2* gene results in increased transcription of a considerable number of genes by whole genome expression analysis (Holstege et al. 1998; Sudarsanam et al. 2000; Martens and Winston 2002). Mammalian Brm complex interacts physically with the transcriptional repressor Rb, and contributes to repression of E2F target genes (Trouche et al. 1997). The precise mechanism by which Swi/Snf complexes direct transcriptional repression, and how the decision is made between repression and activation at specific loci is currently unknown.

The ISWI Class of ATP-Dependent Chromatin Remodeling Factors

In contrast to the Swi/Snf class of factors, the field of research into the ISWI class of factors has, until recently, been driven mainly by biochemical experiments designed to characterize the enzymatic activities of purified complexes in vitro. The first ISWI complex identified and biochemically characterized was *Drosophila* NURF complex, which was purified by following the ATP-dependent chromatin disruption activity found in embryo extracts during chromatographic separations (Tsukiyama et al. 1995; Tsukiyama and Wu 1995). Shortly thereafter, a number of additional ISWI complexes were identified in *Drosophila* (ACF, CHRAC) (Ito et al. 1997; Varga-Weisz et al. 1997), yeast (Isw1a, Isw1b and Isw2) (Tsukiyama et al. 1999; Vary et al. 2003), *Xenopus* (xACF, xCHRAC, xWSTF, xISW1-D) (Guschin et al. 2000) and mammals (hACF, NoRC, RSF, hCHRAC and WICH) (Aihara et al. 1998; Bochar et al. 2000a; Poot et al. 2000; Bozhenok et al. 2002; Collins et al. 2002; Hakimi et al. 2002). Unlike Swi/Snf complexes, which tend to be large (10 or more subunits; >2 MDa in size), ISWI complexes tend to be much smaller (2-4 subunits).

Interestingly, the biochemical activities exhibited by ISWI complexes vary greatly from one ISWI complex to the next; these activities are summarized in Figure I.1. While the first ISWI complex discovered, NURF complex, was identified on the basis of its activity to disrupt chromatin structure in vitro, several other ISWI factors analyzed do not appear to exhibit this activity. All ISWI complexes tested exhibit the

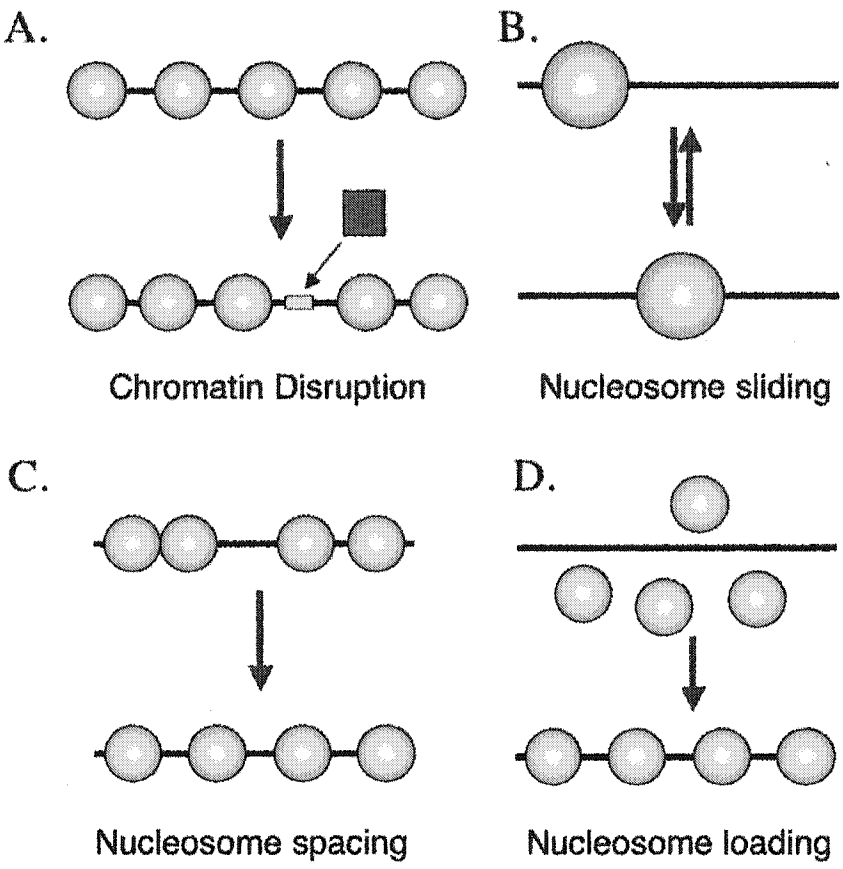


Figure I.1. Summary of chromatin remodeling activities identified for ISWI complexes in vitro. See text for details.

ability to slide nucleosomes along DNA in cis, although the direction of sliding (end-positioned nucleosome to the center, or vice versa) differs in some cases (Langst et al. 1999). Therefore, nucleosome sliding activity appears to be a general feature of ISWI complexes. In addition, most, but not all ISWI complexes tested have the ability to convert a randomly spaced array of nucleosomes to a regularly spaced array in which the lengths of linker DNA between nucleosomes are roughly equal. Finally, a subset of ISWI complexes, including ACF and RSF complexes, exhibit a nucleosome loading/chromatin assembly activity, in which the ISWI complex can catalyze nucleosome formation from free DNA and histone octamer, in an ATP-dependent reaction (Ito et al. 1997; Ito et al. 1999; Loyola et al. 2001). In contrast, several other ISWI complexes tested appear to lack this function.

By comparison with the nucleosome sliding activity of Swi/Snf complexes, ISWI complexes appear to slide nucleosomes by a substantially different mechanism. Whereas the ATPase activities of Swi/Snf complexes are stimulated by free DNA as well as chromatin, ISWI complexes are stimulated only by chromatin (though they bind chromatin and free DNA equally well) (Laurent et al. 1993; Corona et al. 1999). As mentioned above, DNA throughout the sliding nucleosome becomes accessible to nucleases early during sliding by the Swi2/Snf2 homolog BRG1. However, nucleosomal DNA remains nuclease-resistant during nucleosome sliding by Snf2h (a mammalian ISWI homolog) and yeast Isw2 complex (Kassabov et al. 2002; Fan et al. 2003). In addition, nucleosomes previously remodeled by BRG1 cannot be remodeled by Snf2h, indicating BRG1 leaves nucleosomes in an altered state upon sliding. It will be of considerable interest to identify the molecular basis for the mechanistic differences between these related classes of factors.

Much less is known about the direct in vivo functions of ISWI complexes. Homozygous *Drosophila iswi* mutants die early in development, at the late larval or early pupal stages, likely the stage at which embryos run out of maternal ISWI pools (Deuring et al. 2000). This phenotype is mimicked by flies bearing homozygous mutations in the *nurf301* gene, encoding the largest subunit of NURF complex

(Badenhorst et al. 2002). Therefore, one or more of the essential functions of ISWI during early development appear to be carried out by the NURF complex. However, homozygous null mutations of the fly ACF1 gene, encoding a subunit common to the other two fly ISWI complexes ACF and CHRAC, result in the death of most embryos at the same developmental stages, indicating essential developmental functions of one or both of these complexes (Fyodorov et al. 2004). Mutations in *nurf301* or *iswi* result in loss of *engrailed* and *ultrabithorax* expression, however, it is unclear if NURF complex functions directly in activation of these genes. *acf1* mutations show synthetic phenotypes with mutations in the histone chaperone *nap1*, and cells lacking ACF1 have chromatin with a shorter nucleosome repeat length and reduced nucleosomal periodicity in vivo, consistent with the in vitro chromatin assembly activity of ACF1 complex.

The most prominent phenotype of mutations in *Drosophila iswi*, which is common to homozygous *nurf301* mutants, is an abnormal X-chromosome morphology in males, in which the chromosome appears shorter and broader than normal (Deuring et al. 2000; Badenhorst et al. 2002). Dosage compensation (the process of equalizing the number of transcripts from genes located on the X-chromosome between males and females) occurs in flies by increasing transcription throughout the entire male X-chromosome by two fold. The MSL complex, containing the histone H4 lysine 16-directed acetylase MOF, is responsible for this chromosome-wide stimulation of transcription (Birchler et al. 2003). Interestingly, expression of the MSL complex in *iswi* mutant females results in the same X-chromosome abnormalities (Corona et al. 1999). Furthermore, overexpression of MOF enhances the phenotypes of a dominant-negative *iswi* mutant in vivo. These data suggest that ISWI complexes may have general repressive functions that are opposed by H4 lysine 16 acetylation in *Drosophila.* This model is supported by the finding that ISWI and RNA Polymerase II have mutually exclusive staining patterns on polytene chromosomes.

Very recently, a number of reports have indicated roles for mammalian ISWI complexes in a diverse array of nuclear processes. A human ISWI complex, containing a homolog of Acf1 and the ISWI homolog hSnf2h, is required for DNA replication

through pericentric heterochromatin (Bozhenok et al. 2002). As a result, cells depleted of Acf1 have delayed cell cycle progression through the late stages of S phase. Another, as yet unnamed Snf2h-containing complex has been purified and found to contain cohesin, as well as components of the NURD complex, an ATP-dependent chromatin remodeling complex of the CHD1 family (Hakimi et al. 2002). Interestingly, Snf2h and cohesin were found to co-localize on chromatin and the ATPase activity of Snf2h was required for cohesin loading, suggesting coordinate ATP-dependent chromatin remodeling and cohesin deposition by this complex. A third Snf2h-containing complex, NoRC, functions to recruit HDAC1 to human ribosomal gene promoters and promotes repression of RNA Polymerase I-mediated transcription (Strohner et al. 2001; Zhou et al. 2002; Strohner et al. 2004). Only one complex has been identified to date that carries the second mammalian ISWI homolog, Snf2l. Upon purification, this complex was also found to contain homologs of the *Drosophila* NURF complex, suggesting that NURF complex is evolutionarily conserved (Barak et al. 2003). Human NURF complex is enriched in the brain and, like *Drosophila* NURF, appears to regulate the *Engrailed* gene, indicating a remarkable conservation of function from flies to mammals.

The budding yeast, *Saccharomyces cerevisiae*, contains two ISWI homologs, *ISW1* and *ISW2*. Unlike ISWI genes in other organisms, neither *isw1* nor *isw2* single mutants have any detectable phenotype (Tsukiyama et al. 1999). However, an *isw1 isw2 chd1* triple mutant is temperature sensitive, indicating some degree of functional overlap among the three ATPases. Isw1 protein forms two complexes in vitro, Isw1a (Isw1p-Ioc3p) and Isw1b (Isw1p-Ioc2p-Ioc4p) (Vary et al. 2003). Biochemically, both complexes are active in nucleosome sliding and spacing assays, but the Isw1a complex has much higher specific activity in each case. While Isw1 complexes appear to have only minor roles in transcriptional regulation, the expression profiles of mutants lacking either Isw1a or b are distinct, indicating that the two complexes have some non-overlapping functions. Isw1 has been shown to be required for displacement of TATA-binding protein (TBP) from the promoter of the *PHO8* gene upon glucose depletion (Moreau et al. 2003), however the mechanism for this requirement is unknown.

Recently, *isw1* deletion was found to exhibit a synthetic temperature-sensitive phenotype in combination with mutations in non-essential components of the NuA4 histone acetylase complex (Vary and Tsukiyama unpublished data). Furthermore, mutations in the histone H4 tail were found to phenocopy the NuA4 mutations, indicating that histone H4 acetylation by NuA4 was the function required for viability in *isw1* mutant cells. The functional overlap between H4 acetylation and Isw1 complexes is currently under investigation.

In addition, Isw1 complexes appear to regulate transcriptional initiation, elongation and termination of some genes. Whereas Isw1a complex was found to repress initiation of transcription of the *MET16* gene, Isw1b complex appears to control elongation (Morillon et al. 2003a). In a separate assay, an *isw1* mutation enhanced the transcriptional termination defect observed in a *chd1* mutant (Morillon et al. 2003b). Interestingly, Isw1 function appears to be required for histone H3 lysine-4 trimethylation, a mark of active transcription, at some loci. However, the same group also found that lysine-4 trimethylation was required for Isw1 to associate with chromatin (Santos-Rosa et al. 2003), suggesting the possibility of a positive feedback loop involving the functions of one or both Isw1 complexes and the COMPASS complex, which contains Set1p (the H3 lysine-4 methylase).

Isw2 complex was originally purified from yeast as a two subunit complex and was found to exhibit nucleosome spacing activity in vitro (Tsukiyama et al. 1999). Later, Isw2 complex was shown to exhibit nucleosome sliding activity in vitro, sliding an end positioned mononucleosome to the center of a short DNA fragment (Kassabov et al. 2002). Interestingly, nucleosome sliding by Isw2 complex did not alter the rotation of the DNA with respect to the histone octamer (i.e., the same face of the double helix remained in contact with the histone octamer during the sliding reaction). Subsequently, two very small subunits, Dpb4p and Dls1p were also found to be part of the complex and found to be required for some, but not all Isw2 function in vivo (McConnell et al. 2004). Interestingly, the two small subunits are homologous to subunits of the *Drosophila* CHRAC complex. Since the fourth subunit of Isw2 complex, Itc1p, shares some

homology with the fourth subunit of CHRAC, Acf1 (which is also found in ACF complex), the two complexes appear to be orthologous.

By probing chromatin structure at a group of randomly selected genes from chromosome I, Kent et al. observed a number of changes in chromatin structure in *isw1* and *isw2* mutant cells relative to wild type. (Kent et al. 2001) Since none of the genes identified as possible targets of Isw2 complex had altered expression in an *isw2* mutant, the function of Isw2-dependent chromatin remodeling at these sites is unclear. The fact that *ISW1*, *ISW2* and *CHD1* were previously found to be synthetic temperature sensitive raised the possibility that Isw2 complex may also function in processes identified for Isw1 complex such as transcription termination, however *isw2* mutations appeared to have little effect on these functions (Alen et al. 2002).

Description of Dissertation

The goal of my thesis project has been to identify the processes in which yeast Isw2 complex functions in vivo and then to ask how, mechanistically, it functions in those processes. The broader goal of this work is to contribute to our understanding of how ATP-dependent chromatin remodeling factors function in general and, in doing so, contribute to our understanding of how chromatin structure is dynamically regulated in vivo, a process that we are just beginning to understand. Chapter I describes work done to examine the role of Isw2 complex in repression of early meiotic genes. This project was carried out in collaboration with Jesse Goldmark, a former technician in the Tsukiyama lab. We found that Isw2 complex was recruited to the promoters of early meiotic genes by the sequence-specific DNA binding protein Ume6p, which also recruits the Sin3/Rpd3 histone deacetylase complex to the same promoters. Isw2 complex functions in parallel with Sin3/Rpd3 complex to repress transcription of these genes. Furthermore, we found that Isw2 complex catalyzes formation of repressive chromatin structure at the promoter of one early meiotic gene, *REC104*. Chapter II describes a series of whole genome expression analyses I performed in order to determine the extent

to which Isw2 and Sin3/Rpd3 complexes function in parallel. This work identified a large number of new target genes that are repressed by these two complexes. Furthermore, these experiments revealed that Isw2 complex functions in general to create compact (nuclease-resistant) chromatin structure in vivo. In Chapter III, I asked the question: How does Isw2 complex remodel chromatin to create compact chromatin structure in vivo? To get at this question, I created an inducible allele of *ISW2* and isolated intermediate states of chromatin structure, as the Isw2-dependent remodeling reaction progressed in vivo. Analysis of these intermediates indicated that Isw2 complex utilizes a nucleosome sliding mechanism. Furthermore, I found that Isw2 complex slides nucleosomes unidirectionally toward the promoters of two independent target genes, as revealed by the directional migration of nuclease sensitivity at these sites. At the *REC104* promoter, to which we previously found Isw2 complex is recruited by Ume6p, an upstream nucleosome is slid by Isw2 complex into a position adjacent to, or possibly overlapping the Ume6p binding site. This led us to propose the model that Isw2 complex slides nucleosomes by pushing linker DNA into the proximal edge of the nucleosome, with the excess DNA bulge propagated through the nucleosome and out the other end (see Figure 3.6, Chapter III). Chapter IV illustrates a set of experiments I performed to elucidate the substrate requirements on chromatin for Isw2 complex. I identified the "basic patch" region of the histone H4 tail as a critical determinant for Isw2 complex to function and I found that this region is required for proper localization of Isw2 complex to its chromatin targets in vivo. Finally, I found that purified Isw2 complex binds directly to this epitope on reconstituted nucleosome core particles in vitro. I conclude in Chapter V by attempting to place this work into the broader context of the chromatin field and discussing future questions that this work has brought to light.

Chapter I: The Isw2 chromatin remodeling complex represses early meiotic genes upon recruitment by Ume6p

Summary

The ISWI class of chromatin remodeling factors exhibits potent chromatin remodeling activities *in vitro*. However, the *in vivo* functions of this class of factors are unknown at a molecular level. We have found that *S. cerevisiae* Isw2 complex represses transcription of early meiotic genes during mitotic growth in a parallel pathway to Rpd3-Sin3 histone deacetylase complex. This repressor function of Isw2 complex is largely dependent upon Ume6p, which recruits the complex to target genes. Nuclease digestion analyses revealed that Isw2 complex establishes nuclease-inaccessible chromatin structure near the Ume6p binding site *in vivo*. Based on these findings, we propose a model for the mechanism of transcriptional repression by two distinct chromatin remodeling complexes.

Introduction

The packaging of DNA into chromatin inhibits a wide variety of protein-DNA interactions. As a result, chromatin structure is generally inhibitory to cellular processes that require specific protein-DNA interactions, such as transcription. This implies that regulation of chromatin structure can modulate transcription both positively and negatively. For transcriptional activation, chromatin structure must be actively remodeled at the promoters of genes to counteract the repressive effect of chromatin. On the other hand, repressive chromatin structure can be established at the regulatory regions of genes to inhibit transcription. Recent studies have revealed that two major classes of factors, histone modifying enzymes and ATP-dependent chromatin remodeling complexes, play key roles in the regulation of chromatin structure (Tsukiyama and Wu 1997; Suka et al. 1998; Workman and Kingston 1998; Belotserkovskaya and Berger 1999; Kingston and Narlikar 1999; Kornberg and Lorch

1999b; Maldonado et al. 1999; Struhl 1999; Tyler and Kadonaga 1999; Wade and Wolffe 1999; Strahl and Allis 2000).

Among the histone modifications described so far, histone acetylation has been the most extensively studied because of its close correlation with transcription. Acetylation of histone tails is generally observed in transcriptionally active regions of chromatin. The level of histone tail acetylation is regulated by histone acetyltransferase (HAT) and histone deacetylase (HDAC) activities *in vivo*. The fact that many previously identified transcriptional co-activators and co-repressors possess HAT and HDAC activities, respectively, reinforces the idea that chromatin structure plays a critical role in the regulation of transcription (Struhl 1998; Suka et al. 1998; Strahl and Allis 2000). Budding yeast has five putative catalytic subunits of HDAC complexes; Rpd3p, Hda1p, Hos1p, Hos2p and Hos3p (Carmen et al. 1996; Rundlett et al. 1996). In addition, proteins related to a silencing factor, Sir2p, were recently found to comprise a novel class of HDAC (Tanny et al. 1999; Imai et al. 2000; Landry et al. 2000; Smith et al. 2000).

Rpd3 proteins from yeast to mammals associate with Sin3 protein. Rpd3-Sin3 complex is recruited to target genes by transcriptional repressors (Hassig et al. 1997; Heinzel et al. 1997; Kasten et al. 1997; Laherty et al. 1997; Nagy et al. 1997). In yeast, the Rpd3-Sin3 complex is recruited to target genes by Ume6p, a sequence-specific DNA binding protein (Kadosh and Struhl 1997; Kadosh and Struhl 1998b; Rundlett et al. 1998). This may account for the observation that Rpd3p-dependent deacetylation of histone H3 and H4 tails is highly local *in vivo*; only one or two nucleosomes at the promoters of target genes are affected (Kadosh and Struhl 1998b).

In addition to histone modifying enzymes, biochemical and genetic studies have identified several ATP-dependent chromatin remodeling complexes from a wide variety of eukaryotic organisms (Cairns 1998; Kadonaga 1998; Peterson 1998; Kingston and Narlikar 1999). They are grouped into three classes according to the ATPase subunit found in each complex, SWI/SNF, ISWI, and CHD1. Members of the ISWI class of ATPases identified so far are *Drosophila* ISWI, human SNF2L and SNF2H, and yeast

ISW1p and ISW2p. *Drosophila* ISWI protein has been found in three distinct chromatin remodeling complexes, NURF (Nucleosome remodeling factor) (Tsukiyama et al. 1995; Tsukiyama and Wu 1995), CHRAC (Chromatin accessibility complex) (Varga-Weisz et al. 1997), and ACF (ATP-utilizing chromatin assembly and remodeling factor) (Ito et al. 1997). The hSNF2H protein forms distinct complexes, RSF (remodeling and spacing factor) (LeRoy et al. 1998), WCRF (Williams syndrome transcription factor-related chromatin remodeling factor) / human ACF (Bochar et al. 2000b; LeRoy et al. 2000) and human CHRAC (Poot et al. 2000). Yeast Isw1p and Isw2p also form distinct complexes (Tsukiyama et al. 1999).

Extensive biochemical characterization revealed that each member of the ISWI class of remodeling factors has potent activity to alter chromatin structure *in vitro*. However, the functions of this class of factors *in vivo* are just beginning to be uncovered. *Drosophila ISWI* is essential for development as well as cell viability (Deuring et al. 2000). The expression of *engrailed* and *Ubx* genes in a *Drosophila ISWI* mutant is greatly reduced, suggesting that *ISWI* is required for transcription of some developmentally regulated genes. However, immunostaining of polytene chromosomes revealed mostly mutually exclusive localization of *Drosophila* ISWI protein and RNA polymerase II. This led to the proposal that ISWI-containing complexes in flies may have both positive and negative roles in transcription (Deuring et al. 2000). *Drosophila ISWI* is also essential for maintenance of the integrity of the male X chromosome (Deuring et al. 2000). A yeast *isw2* mutant exhibits defects in the early stages of meiosis (Trachtulcov et al. 2000), but the mechanism of this defect is unknown.

In a search for genes regulated by ISWI complexes in *S. cerevisiae*, we found that Isw2 complex represses transcription of early meiotic genes during mitotic growth in a parallel pathway to Rpd3-Sin3 histone deacetylase complex. Chromatin analyses revealed that Isw2 complex creates nuclease-inaccessible chromatin structure upstream of the Ume6p-binding site. Based on genetic experiments as well as protein-protein interactions *in vitro* and *in vivo*, we propose that Isw2 complex, like Rpd3-Sin3 complex, is recruited by Ume6p to repress early meiotic genes.

Results

Isw2 complex is required for repression of transcription *in vivo*

Because of their biochemical activities to alter chromatin structure *in vitro* (Tsukiyama et al. 1999), we hypothesized that Isw1 and Isw2 complexes may affect transcription *in vivo*. We attempted to identify genes that are regulated by these complexes using DNA microarray analysis. We enriched for candidate genes that might be regulated by these complexes, comparing wild type and an *isw1 isw2* double mutant. While we are uncertain of the relevance of the entire genome transcription profile in *isw1 isw2* mutant cells obtained (see Experimental Procedures), we found and confirmed by Northern blotting that several meiotic genes are derepressed in an *isw2* single mutant relative to wild type (Figure 1.1, lane 2). RNA samples were prepared from haploid (Mat **a**) cells grown in rich medium during early log phase, conditions under which meiotic genes are repressed in wild type cells (lane 1). These data suggest that Isw2 complex is involved in the repression of some meiotic genes during mitotic growth of yeast cells.

Derepression of meiotic genes in an *isw2* mutant is independent of known meiotic activators

We set up genetic tests to investigate the mechanism of meiotic gene derepression in *isw2* mutant cells. The signals required for initiation of meiosis, such as nitrogen and glucose starvation, lead to the activation of a key regulator, Ime1p (Mitchell 1994; Kupiec et al. 1997), which directly activates most early meiotic genes (Bowdish et al. 1995; Rubin-Bejerano et al. 1996). An *IME1* target gene, *IME2*, also activates early meiotic genes through an *IME1*-independent pathway (Smith et al. 1990). For middle and late meiotic genes, Ndt80p was recently identified as a transcriptional activator (Chu et al. 1998). Overexpression of these activators in mitotic cells induces expression of meiotic genes (Smith et al. 1990; Chu et al. 1998). We therefore tested whether derepression of meiotic genes in an *isw2* mutant is due to activation or induction of these meiotic activators by epistasis analysis (Fig. 1.1). We made null mutations of *IME1*, *IME2* and *NDT80* genes in both wild type and *isw2* mutant backgrounds, and tested

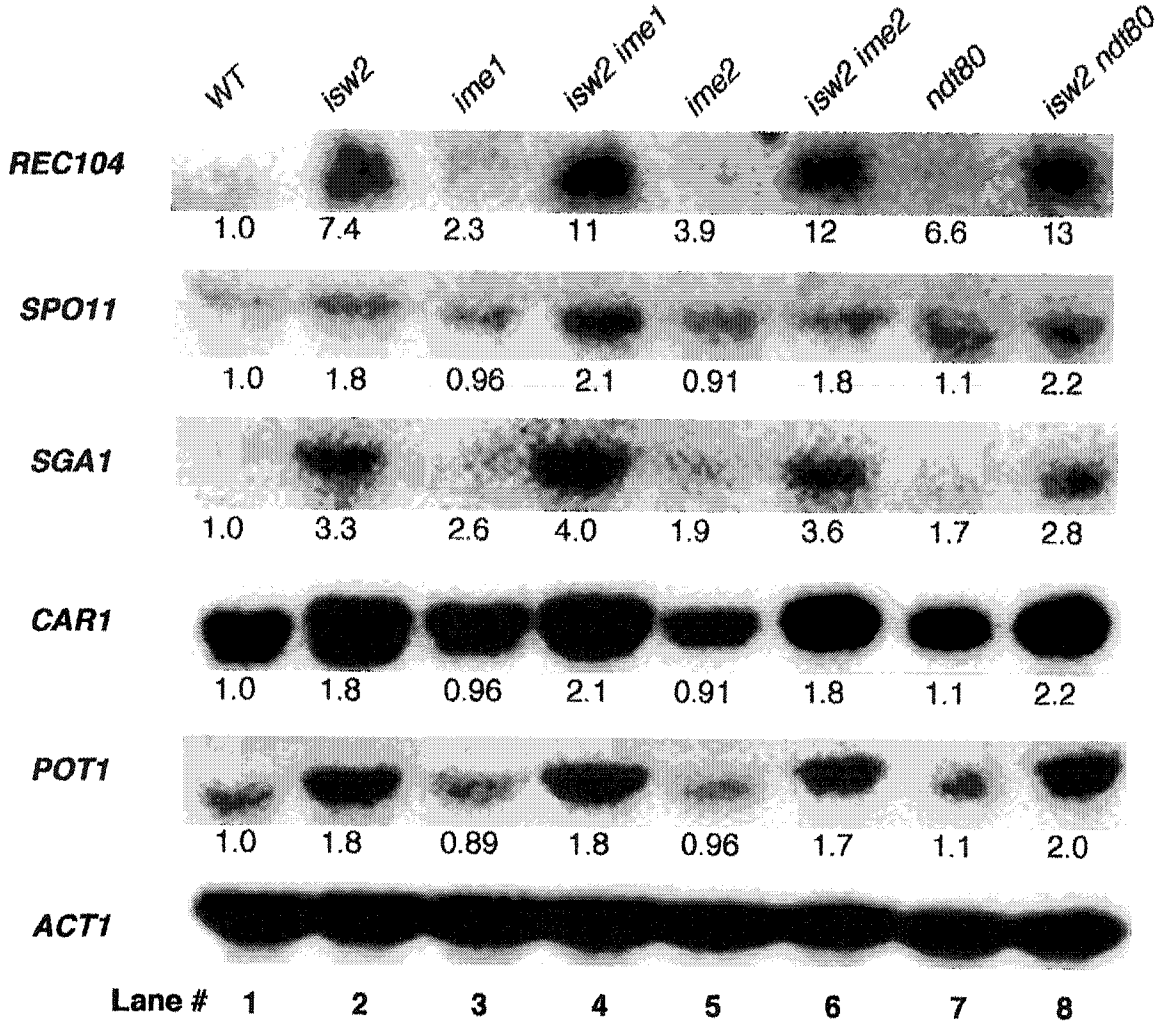


Figure 1.1. Activator-independent derepression of meiotic genes in an isw2 mutant. The genotype of the cells used is listed at the top of each lane. Listed vertically to the left of each blot is the gene being probed. The number under each band indicates the fold change in expression level relative to wild type cells as determined by phosphorimager. For all epistasis analyses, it should be noted that the levels of RNA in wild type cells are used as standards to calculate relative levels of RNA in mutants. Since the measured RNA levels for many meiotic genes in wild type cells are very close to background, calculated RNA levels in wild type cells (RNA signals minus background signals) are very sensitive to fluctuating background levels. This causes some fluctuation of the calculated fold change in RNA levels in mutants among different blots. However, epistatic interactions among mutants within each blot are not affected.

expression of meiotic genes during mitotic growth. As seen in Figure 1.1, derepression of all meiotic genes tested was not significantly affected in any of the double mutants (lanes 4, 6, 8). Thus, *IME1*, *IME2* and *NDT80* are not required for the derepression of meiotic genes in an *isw2* mutant during mitotic growth, although it is likely they are still required for activated transcription in *isw2* cells.

Isw2 complex represses URS1-containing genes in a parallel pathway to Rpd3-Sin3 complex

The vast majority of early meiotic genes have a common *cis* element, URS1, in their promoter sequences (Mitchell 1994; Kupiec et al. 1997); these genes are repressed by the Rpd3-Sin3 histone deacetylase complex during mitotic growth (Strich et al. 1989; Vidal and Gaber 1991; Bowdish and Mitchell 1993; Kasten et al. 1997; Kupiec et al. 1997). We tested whether Isw2 complex functions in the Rpd3-Sin3 pathway by epistasis analysis (Figure 1.2A). Some, but not all URS1-containing genes were derepressed in the *isw2* single mutant (lane 2). In an *rpd3* single mutant, all URS1-containing genes tested were moderately derepressed, as previously reported (lane 3). In contrast to both single mutants, all URS1-containing genes tested were strongly derepressed in an *isw2 rpd3* double mutant (lane 4). The level of derepression in the double mutant was significantly higher than in each single mutant (compare lanes 2 and 3 with lane 4), indicating that *ISW2* and *RPD3* function independently to repress URS1-containing genes. In addition, we noticed that expression of URS1-containing genes in the *isw2 rpd3* double mutant is generally much higher than the sum of the expression levels in each single mutant. This reveals that *ISW2* and *RPD3* function in parallel, partially compensatory pathways to repress these genes. Sin3p is proposed to tether Rpd3p to the promoters of URS1-containing genes (Kadosh and Struhl 1997; Kasten et al. 1997; Rundlett et al. 1998). Consistent with this model, *sin3* and *rpd3* mutations showed very similar genetic interactions with an *isw2* mutation (compare lane 2 with lanes 3-6), showing that Isw2 and Rpd3-Sin3 complexes function independently in parallel pathways.

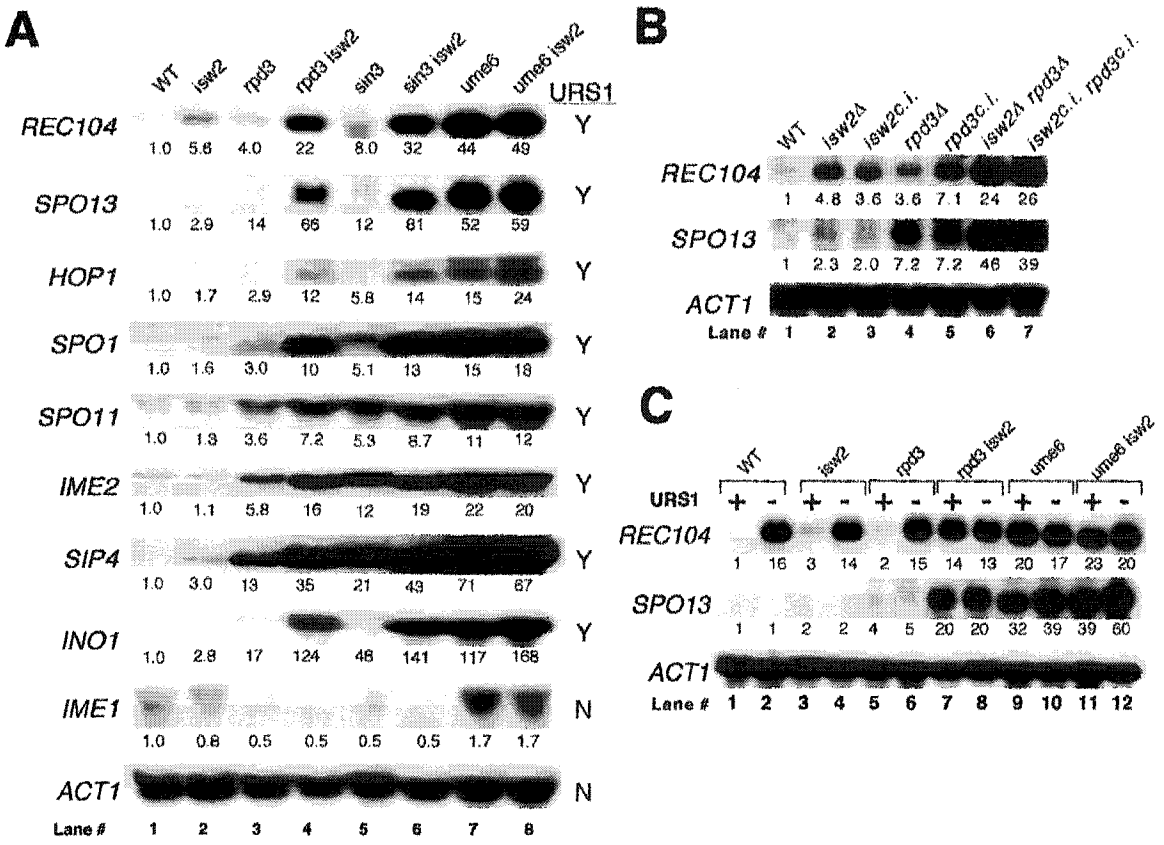


Figure 1.2. Epistasis analysis of *ISW2* with *RPD3*, *SIN3*, and *UME6*.
(A) Epistasis analysis using null mutants. The presence (Y) or absence (N) of a URS1 sequence in the promoter of each gene is indicated to the right.
(B) Catalytic activities of both Isw2 and Rpd3-Sin3 complexes are essential for repression. c.i. and _ denotes catalytic inactive- and null-mutants, respectively.
(C) Both *ISW2* and *RPD3* are epistatic to *UME6*. RNA samples for lanes 2, 4, 6, 8, 10, and 12 were prepared from cells with a mutated *REC104* URS1 sequence, while cells for lanes 1, 3, 5, 7, 9, and 11 have wild type *REC104* URS1 sequence. The *SPO13* URS1 sequence is wild type in all samples.

We next tested whether the catalytic activities of the two chromatin remodeling complexes are necessary for repression of early meiotic genes (Figure 1.2B). A K214R mutation (lysine to arginine substitution at amino acid 214) in the *ISW2* gene, introduced at the same residue as the previously published K214A mutation (Tsukiyama et al. 1999), inactivates all ATP-dependent activity of Isw2 complex without significantly affecting the stability of Isw2p (data not shown). An H151A mutation in Rpd3p has been shown to inactivate the histone deacetylase activity of Rpd3 complex (Kadosh and Struhl 1998a). As shown in Figure 1.2B, derepression of two early meiotic genes, *REC104* and *SPO13*, in the catalytically inactive mutants was very similar to that of the deletion mutants. *REC104* is more highly expressed in the catalytically inactive *rpd3* mutant than in the null mutant (lanes 4, 5). This may be due to partial compensation for *RPD3* function by other HDACs in the null mutant or dominant negative functions of the catalytically inactive *rpd3* mutant at the *REC104* promoter.

Both Isw2 and Rpd3-Sin3 complexes function in the Ume6p pathway of repression

During a systematic search for *in vivo* target genes of Isw2 complex by Northern blotting, we found that every URS1-containing gene tested was repressed by Isw2 and Rpd3-Sin3 complexes, including those do not have known meiotic functions, such as *INO1* and *SIP4* (Figure 1.2A). On the other hand, *IME1*, a gene that is induced early in meiosis but does not have a URS1 sequence, is not repressed by Isw2 complex. Since the URS1 sequence is a binding site for Ume6p (Strich et al. 1994; Anderson et al. 1995), we tested whether Isw2 complex and Ume6p function in the same pathway. Addition of an *isw2* mutation in the *ume6* background did not cause a large change in the level of derepression (Figure 1.2A, lanes 7 and 8). This demonstrates that Isw2 complex is not able to repress these genes in the absence of Ume6p, arguing that Isw2 complex and Ume6p act largely in the same pathway. We observed essentially the same genetic interactions among *ISW2*, *RPD3*, and *UME6* regarding repression of URS1-containing genes in another standard yeast strain background, S288C (data not shown).

A null mutation of *UME6* may cause pleiotropic effects on transcription since many genes are regulated by Ume6p (Strich et al. 1994; Steber and Esposito 1995; Jackson and Lopes 1996; Sweet et al. 1997). We therefore asked if Ume6p itself acts in the Isw2 pathway by an independent test. A triple base pair substitution, TGGCGGCT to TGTACGCT, in the URS1 element completely abolishes Ume6p binding *in vitro* (Jackson and Lopes 1996). This mutation was introduced within the promoter of the endogenous *REC104* gene in wild type and mutant cells. As shown in Figure 1.2C, the *REC104* gene containing the mutant URS1 sequence exhibited uniformly strong transcription in all genetic backgrounds tested. Thus the presence or absence of Isw2 and Rpd3-Sin3 complexes does not affect transcription of *REC104* in the absence of the UME6p binding site (compare lanes 4, 6, 8 with lane 2). Taken together, these results show that Isw2 and Rpd3-Sin3 complexes, working in parallel pathways, converge on the Ume6p pathway of repression.

Ume6p binds the URS1 independently of Isw2 and Rpd3-Sin3 complexes *in vivo*

At least two distinct models can explain the results of our epistasis analyses. In one model, Ume6p acts directly to repress transcription, while Isw2 and Rpd3-Sin3 complexes are needed for this repressive function of Ume6p (model 1). In this case, both complexes may facilitate binding of Ume6p to the URS1 sequence *in vivo* by parallel mechanisms. Alternatively, Isw2 and Rpd3-Sin3 complexes may repress transcription in parallel, and Ume6p is needed for both complexes to function (model 2). In this case, Ume6p may recruit both complexes to URS1-containing genes. We tested model 1 with a chromatin immunoprecipitation (CHIP) assay (Figure 1.3). Ume6p was tagged with FLAG epitope in wild type and *isw2 rpd3* double mutant cells. Sheared chromatin was prepared from these cells and Ume6p was immunoprecipitated using anti-FLAG antibody. PCR signals for the *ACT1* promoter, a gene that is not regulated by Ume6p, were uniformly low among immunoprecipitates from all cells, including control cells that do not have FLAG epitope on Ume6p. In contrast, signals specific to the promoter regions of Ume6 targets, *SPO13*, *INO1* and *SIP4*, were significantly higher in

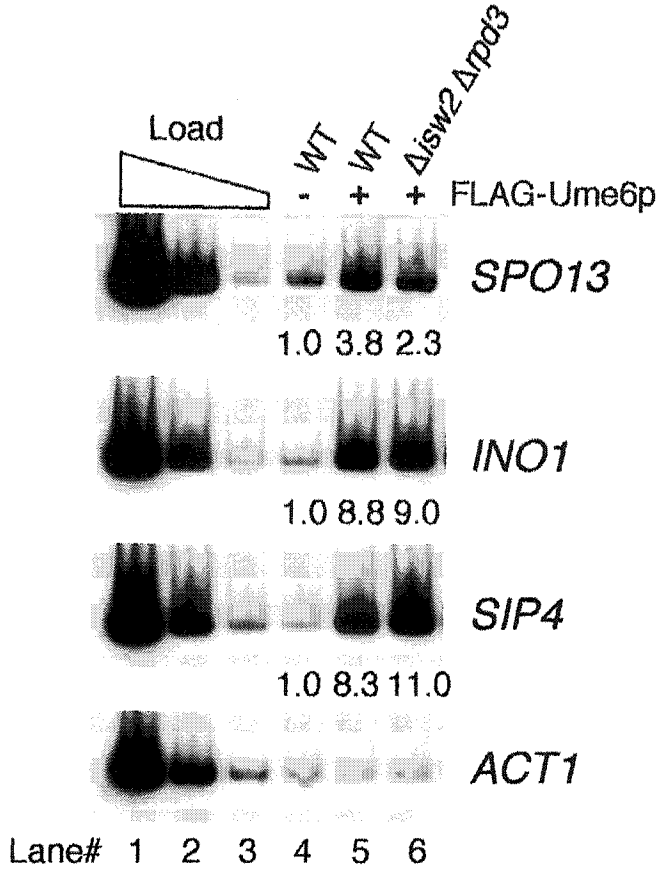


Figure 1.3. Binding of Ume6p to targets in wild type and *isw2 rpd3* cells in vivo. Chromatin associated with FLAG-tagged Ume6p was immunoprecipitated. Lane 4 shows signals from control cells containing untagged Ume6p. Numbers below lanes 4-6 denotes intensity of signals relative to background (lane 4). Lanes 1-3 are serial dilution (5-fold) of starting materials to demonstrate that the samples did not saturate PCR reactions.

cells with FLAG-tagged Ume6p (lane 5) than in control cells (lane 4), demonstrating binding of Ume6p at these promoters. The signals from Ume6p targets did not decrease in an *isw2 rpd3* double mutant for *INO1* and *SIP4*, the two genes that are the most strongly repressed by Isw2 and Rpd3-Sin3 complexes (lane 6). Signal for the promoter region of *SPO13* showed a small decrease in the *isw2 rpd3* mutant but remained significantly higher than background. These results show that Ume6p binds to the promoter of target genes independently of Isw2 and Rpd3-Sin3 complexes *in vivo*. This finding is inconsistent with model 1.

Ume6p and Isw2 complex interact *in vivo* and *in vitro*

To test model 2, recruitment of Isw2 complex by Ume6p, we first asked whether Ume6p and Isw2 complex stably interact *in vivo*, using a co-immunoprecipitation (co-IP) assay (Figure 1.4A). Whole cell extract was prepared from a strain containing FLAG-tagged Ume6p, HA-tagged Sin3p and Myc-tagged Itc1p, the second subunit of Isw2 complex. Itc1p, previously referred to as p140 (Tsukiyama et al. 1999), is encoded by the open reading frame YGL133W and exists exclusively in Isw2 complex *in vivo* (Gelbart et al. 2001). A control extract was also prepared from a strain containing HA-tagged Sin3p and Myc-tagged Itc1p but without a tag on Ume6p. Ume6p from the triple-tagged strain was quantitatively (80 to 90%) immunoprecipitated by anti-FLAG antibody. Sin3p was co-precipitated with Ume6p, consistent with a model that Rpd3-Sin3 complex is recruited by Ume6p. Similarly, precipitate from the triple tagged strain, but not from the control strain, contained Itc1p, demonstrating that Isw2 complex and Ume6p interact *in vivo*. It should be noted that the same amount of samples were loaded for Sin3p and Itc1p western analysis. This shows that the populations of Itc1p and Sin3p that interact with Ume6p are within an order of magnitude. Since Sin3p-Ume6p interaction has been well established (Kadosh and Struhl 1997; Kadosh and Struhl 1998b; Rundlett et al. 1998), roughly comparable co-IP of Itc1p with Ume6p strongly supports recruitment of Isw2 complex by Ume6p. We noticed that co-IP of Itc1p with Ume6p enriched a population of Itc1p that migrates significantly slower than the major population,

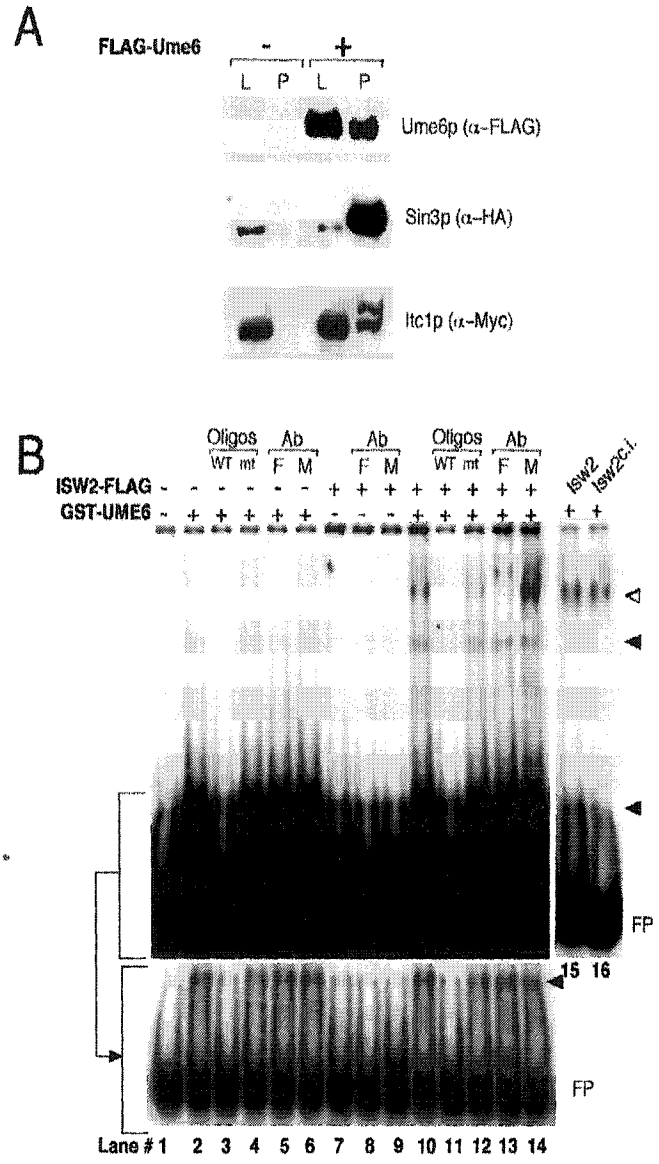


Figure 1.4. Isw2 complex is recruited by Ume6p.
(A) Co-immunoprecipitation of Isw2 complex with Ume6p. Whole cell extract was prepared from cells containing epitope tags as indicated, and immunoprecipitation was done using anti-FLAG antibody. The antibodies used for western blotting are indicated on the right.
(B) GST-Ume6p and Isw2 complex interact in an URS1-dependent manner on the REC104 promoter in vitro. FP indicates the location of free probe. The lower panel is a shorter exposure of the bracketed region of the gel, showing the faster migrating Ume6p complex. Arrowheads indicate positions of the complexes created by Ume6p, while the empty triangle denotes the position of the ternary complex. Ab indicates that antibodies were added in reactions; anti-FLAG-M2 (F) and anti MYC-9E10 (M) antibodies were used. Lanes 15 and 16; gel shift assay using wild type (lane 15) and catalytically inactive (lane 16) complexes.

indicating that a specific population of Itc1p preferentially interacts with Ume6p. We believe that the interaction between Ume6p and Itc1p can occur independently of DNA, since co-IP was also detected in the presence of 50 μg / ml ethidium bromide (data not shown). Since we were unable to quantitatively immunoprecipitate Itc1p from crude extract, reciprocal co-IP was not technically feasible. For the same reason, we could not determine whether Sin3p and Itc1p simultaneously interact with Ume6p.

We next asked whether Ume6p is able to directly interact with Isw2 complex on the URS1 sequence *in vitro* using purified proteins. Recombinant Ume6p forms two distinct complexes with an oligonucleotide probe corresponding to the *REC104* promoter (Figure 1.4B, lane 2, marked by arrowheads). Unlabeled wild type, but not mutant oligonucleotides containing a mutation in the URS1 sequence, compete Ume6p binding to the probe (lanes 3,4), demonstrating sequence specificity of the binding. Purified Isw2 complex has nonspecific DNA binding activity (Gelbart et al. 2001). However, because the reaction mixture contains excess nonspecific DNA, purified Isw2 complex alone does not show detectable binding to the probe under these conditions (lane 7). In the presence of both Ume6p and Isw2 complex, a slowly migrating complex appears (lane 10, marked by an empty triangle). The anti-FLAG antibody (lane 13), but not the control anti-Myc antibody (lane 14), super-shifts this new complex, demonstrating the presence of Isw2 complex in this slowly migrating complex. This new complex is dependent on the URS1, since it is competed by wild type (lane 11), but not mutant (lane 12) oligonucleotides. The formation of this complex is ATP-independent (lane 15). In addition, catalytically inactive Isw2 complex forms the same slowly migrating complex with Ume6p (lanes 15 and 16), showing that the formation of this complex does not require the ATPase activity of Isw2 complex.

Isw2 complex forms inaccessible chromatin structure proximal to the Ume6p binding site *in vivo*

If model 2 is correct for explaining the epistatic interactions between *ISW2* and *UME6*, Isw2 complex must be involved in repression of transcription *in vivo*, rather than

facilitating Ume6p binding. To test this possibility, we analyzed chromatin structure around the promoter region of an *ISW2* target, the *REC104* gene, in wild type and mutant cells by DNase I and micrococcal nuclease (MNase) digestion of chromatin followed by indirect end-labeling. DNase I preferentially digested chromatin at around nucleotides (nt) -400 and -120 of the *REC104* promoter in wild type cells (Figure 1.5A). In an *isw2* mutant, DNase I digestion at around nt -120 was strongly enhanced, showing significantly enhanced accessibility of chromatin in this area in the absence of Isw2 complex. In addition, DNase I digestion at around nt -350 and -510 was strongly induced in an *isw2* mutant, and the digestion at around nt -400 in wild type cells is strongly suppressed in the mutant. This indicates that the positions of nucleosomes change in the *isw2* mutant. In contrast to the *isw2* mutant, chromatin prepared from the *rpd3* mutant showed a DNase I digestion pattern very similar to that of wild type cells (compare lanes 10-12 with 2-4), suggesting that Rpd3-Sin3 complex does not significantly affect the positions of nucleosomes at target genes. This also argues that the changes in chromatin structure in the *isw2* mutant are not a consequence of increased transcription, since *REC104* is expressed at similar levels in *isw2* and *rpd3* single mutants. These data reveal that in wild type cells, Isw2 complex changes the positions of nucleosomes near the URS1 sequence (located at nt -93 for the *REC104* promoter) and establishes and / or maintains nuclease-inaccessible chromatin structure. Chromatin from the *isw2 rpd3* mutant shows a DNase I digestion pattern that is very similar to that of the *isw2* single mutant (lanes 14-16), further supporting the idea that Rpd3-Sin3 complex does not significantly affect local nucleosome positions. Chromatin from the *ume6* and *isw2 ume6* mutants shows DNase I digestion patterns similar to that of the *isw2* mutant (compare lanes 18-20, and 22-24 with 2-4). This supports our earlier conclusion that Isw2 complex and Ume6p function in the same pathway.

Results of chromatin structure analysis using MNase digestion were consistent with the observations above. The *rpd3* mutant shows a very similar digestion pattern to that of wild type cells, while the *isw2* mutant shows an altered digestion pattern. In particular, MNase accessibility was strongly induced at around nt -150 in the *isw2*

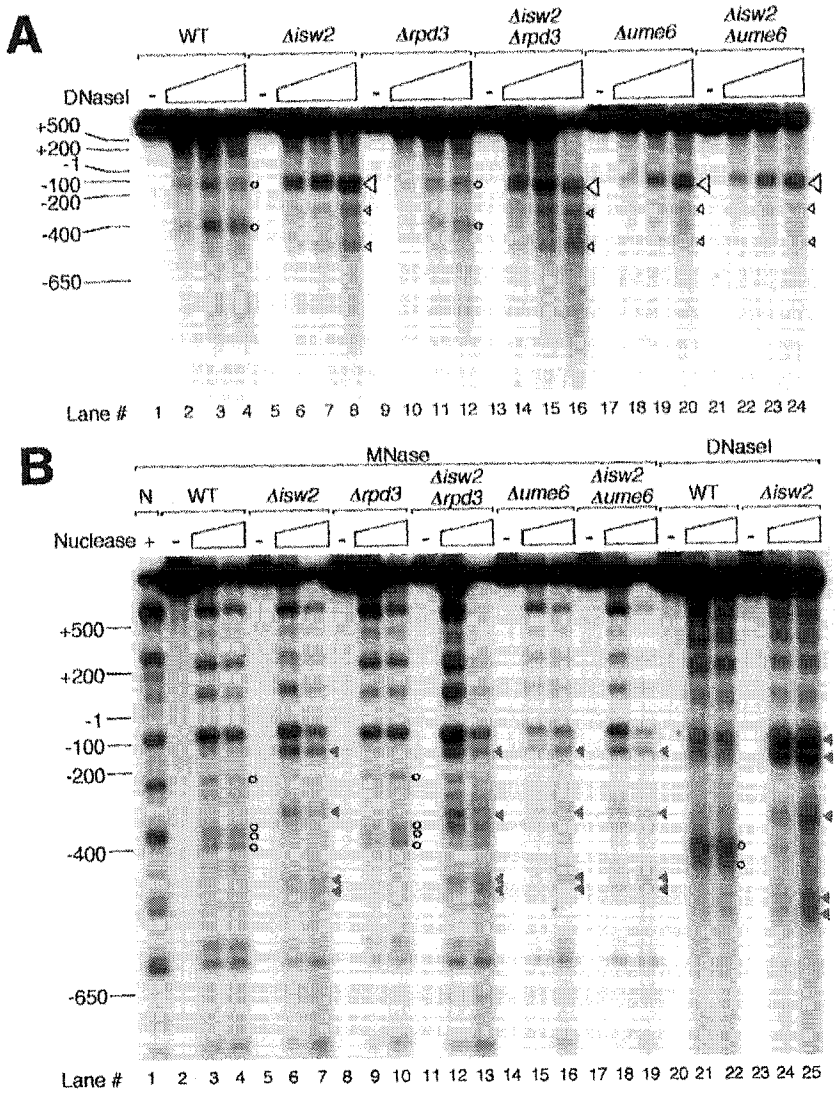


Figure 1.5. Isw2 complex regulates chromatin structure at the *REC104* promoter.
(A) Analysis of chromatin structure at *REC104* gene using DNase I followed by indirect endlabelling. Major hypersensitive sites in wild type (WT) or *rpd3* mutant cells are marked by circles; those found in cells with mutations in *ISW2* or *UME6* are marked by small triangles ; those enhanced in cells with mutations in *ISW2* or *UME6* are marked by large triangles.
(B) Nucleosome mapping by MNase digestion followed by indirect end-labeling. MNase cleavage sites enhanced in wild type (WT) or *rpd3* mutant cells are marked with open circles; those enhanced in cells with mutations in *ISW2* or *UME6* are marked with closed triangles. N denotes naked DNA control.

mutant, again showing that Isw2 complex is needed for establishment and / or maintenance of inaccessible chromatin structure near the Ume6p binding site. MNase digestion was also induced at around nt -350 and -500 in the *isw2* mutant (marked by filled triangles). These changes are in good agreement with the results of DNase I analysis (compare lanes 6 and 7 with 24 and 25). Consistent with the epistasis analyses, *ume6* and *isw2 ume6* mutants show MNase digestion patterns very similar to that of an *isw2* mutant.

Synthetic growth defects of the *isw2 rpd3* double mutant

Neither *isw2* nor *rpd3* mutations cause significant growth defects. However, during strain construction for our epistasis analysis, we found that the *isw2 rpd3* double mutant shows slow growth and temperature sensitive (ts) phenotypes (Figure 1.6). The double mutant grew very slowly at 35.5 °C, and could not form single colonies at 37 °C. The *ume6* single mutant had similar growth defects, and the *isw2 ume6* double mutant was more severely affected, suggesting *UME6*-independent functions of Isw2 complex *in vivo*. In contrast, the *isw2 sin3* double mutant did not exhibit detectable growth defects despite expression of early meiotic genes at levels comparable to that of the *isw2 rpd3* double mutant. This argues that the synthetic growth defects of the *isw2 rpd3* double mutant are not due to derepression of meiotic genes, and that *ISW2* and *RPD3* have unidentified, parallel functions *in vivo* that are *SIN3*-independent. This also argues that derepression of meiotic genes is not caused by slow growth of cells. We observed similar, but weaker, synthetic growth defects of an *isw2 rpd3* mutant in the S288C background (data not shown).

Discussion

Our report presents the first evidence that a member of the ISWI class of ATP-dependent chromatin remodeling factors negatively regulates transcription. These results are consistent with a recent report that an *isw2* mutant exhibits defects during the early stages of sporulation (Trachtulcov et al. 2000). Results of CHIP assay for Ume6p,

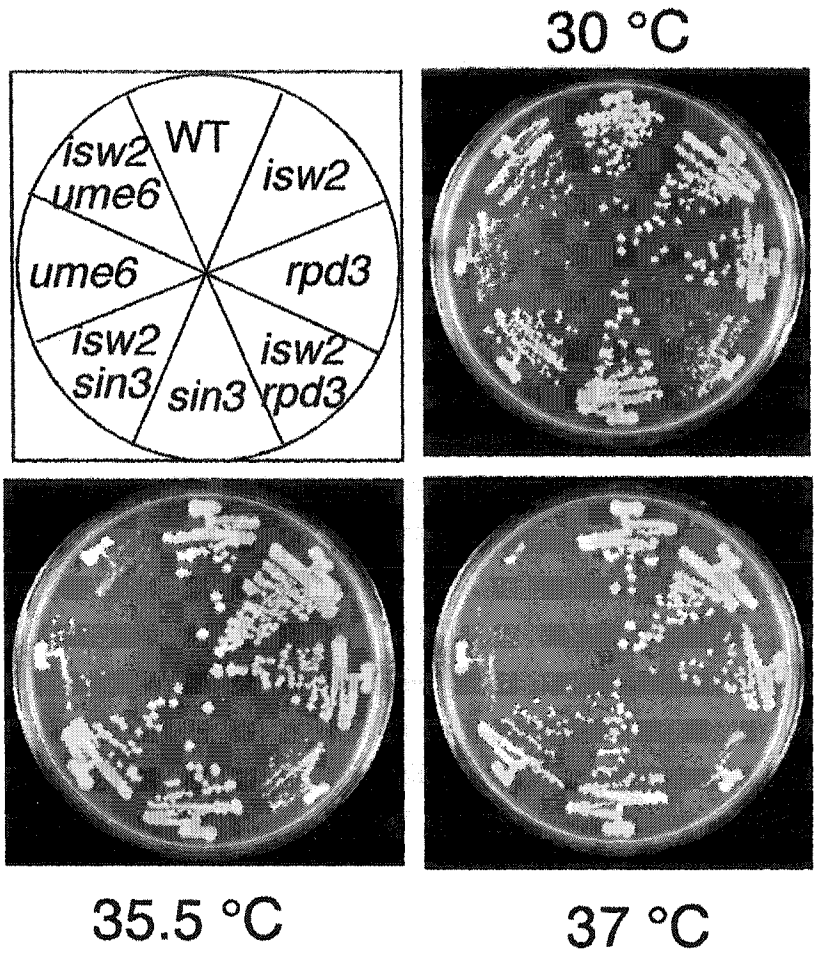


Figure 1.6. An *isw2 rpd3* mutation causes synthetic growth defects. Wild type and mutant yeast cells were streaked on rich (YEPD) plates and incubated at the temperatures indicated. The genotypes of yeast cells are shown in the upper left corner.

co-IP of Itc1p with Ume6p, and chromatin analyses are all consistent with a model in which Isw2 and Rpd3-Sin3 complexes function in parallel and partially compensatory pathways to repress transcription of genes containing URS1 sequences, and both complexes are dependent on recruitment by Ume6p. Based on these data, we propose a model for repression of URS1-containing genes by Isw2 and Rpd3-Sin3 complexes (Figure 1.7). In wild type cells, Isw2 complex promotes formation of nuclease-inaccessible chromatin structure upstream of the URS1 sequence at target genes by changing nucleosome positions. In addition, Rpd3-Sin3 complex removes acetyl groups from histone tails in nucleosomes as reported (Kadosh and Struhl 1998a; Rundlett et al. 1998), further enhancing the repressed state of chromatin at the promoter region. Nucleosomes are mispositioned in an *isw2* single mutant, but deacetylation of histone tails keeps URS1-containing genes moderately repressed. In an *rpd3* single mutant, properly positioned nucleosomes with acetylated histone tails can also repress transcription to some extent. However, in the *isw2 rpd3* double mutant, mispositioned nucleosomes with acetylated histone tails allows a high level of target gene transcription. Since both Isw2 and Rpd3-Sin3 complexes are recruited by Ume6p, chromatin structure and expression of many URS1-containing genes in the *ume6* mutant are similar to those of the *isw2 rpd3* double mutant. Finally, addition of an *isw2* mutation in the *ume6* background does not significantly change expression and chromatin structure of target genes since Isw2 function is largely dependent on Ume6p. The nuclease inaccessible chromatin structure may be directly responsible for Isw2-mediated repression. Alternatively, an unidentified mechanism may utilize this inaccessible chromatin structure for repression.

The repressor function of Isw2 complex *in vivo* was somewhat unexpected because of the biochemical activities of other ISWI-containing complexes. Two *Drosophila* ISWI-containing complexes, NURF (Tsukiyama and Wu 1995) and ACF (Ito et al. 1997), and a human complex, RSF (LeRoy et al. 1998), activate transcription from chromatin templates *in vitro* (Ito et al. 1996; Mizuguchi et al. 1997; LeRoy et al. 1998). However, our results are consistent with a recent report that *Drosophila* ISWI

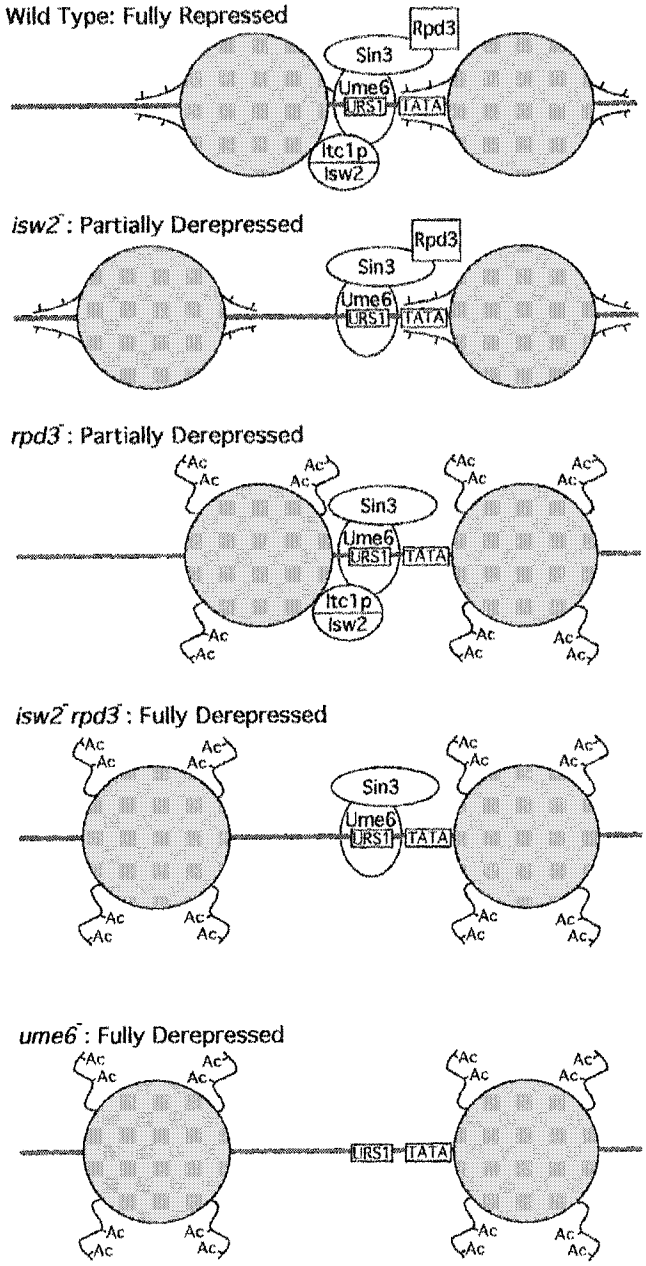


Figure 1.7. Model for repression of URS1-containing genes by Isw2 and Rpd3-Sin3 complexes. See text for details.

protein and RNA polymerase II localize on polytene chromosomes in a mutually exclusive manner (Deuring et al. 2000). There are at least two potential explanations for this apparent discrepancy. As reported (Tsukiyama et al. 1999), Isw2 complex does not exhibit *in vitro* activity to establish accessible chromatin structure at promoter regions in the presence of transcription factors (promoter-specific chromatin remodeling activity). Since promoter-specific chromatin remodeling activities are associated with NURF, ACF, and RSF, it is possible that Isw2 complex functions differently from these ISWI-containing complexes both *in vitro* and *in vivo*. If this is the case, there may be at least two functionally distinct classes of ISWI-containing complexes. In this regard, it should be noted that another *Drosophila* ISWI-containing complex CHRAC also does not exhibit promoter-specific chromatin remodeling (Varga-Weisz et al. 1997). It is also possible that Isw2 complex has unidentified positive roles in transcription *in vivo* as proposed for fly ISWI-containing complexes (Deuring et al. 2000). In either case, our present results and the mutually exclusive localization of *Drosophila* ISWI protein and RNA polymerase II on polytene chromosomes suggest that the repressor function of the ISWI class of chromatin remodeling factors has been conserved during evolution.

While recent DNA microarray analyses revealed that yeast Swi/Snf complex may also have repressor activity (Holstege et al. 1998; Sudarsanam et al. 2000), the major function of this complex is to facilitate activation of transcription. Our report reveals that two ATP-dependent chromatin remodeling complexes in yeast, Isw2 and Swi/Snf complexes, have opposing functions in transcriptional regulation. This is consistent with the fact that Swi/Snf complex creates an open chromatin structure at the promoter regions of target genes *in vivo* (Hirschhorn et al. 1992; Gregory et al. 1999), whereas Isw2 complex establishes nuclease-inaccessible chromatin structure. The contrast between Isw2 and Swi/Snf complexes goes further: Isw2 and Rpd3-Sin3 histone deacetylase complexes function in parallel pathways in repression, whereas Swi/Snf and histone acetyltransferase complexes containing Gcn5p function cooperatively in activation (Pollard and Peterson 1997; Roberts and Winston 1997; Biggar and Crabtree 1999; Sudarsanam et al. 1999). This contrast is most evident in the case of the *INO1*

gene, repression of which is governed by Isw2 and Rpd3-Sin3 complexes whereas activation is facilitated by Swi/Snf and Gcn5 complexes (Peterson et al. 1991; Pollard and Peterson 1997). Furthermore, both the *isw2 rpd3* double mutation and the *swi2/snf2 gcn5* double mutation cause synthetic growth defects. These data reveal that the two major classes of chromatin remodeling factors, ATP-dependent factors and histone modifying enzymes, work together in both positive and negative regulation of transcription.

Experimental procedures

Strains

All yeast strains used in this study are derived from W1588-4C (ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100). This strain is congenic to W303-1A, except that a weak *rad5* mutation in the original W303 is repaired (Zhao et al. 1998). Null mutations of the *IME1*, *IME2*, *NDT80*, *RPD3*, *SIN3*, and *UME6* genes were created by replacing the coding region of each gene with the kanMX marker as described (Guldener et al. 1996). The *isw2* null mutation was made by replacing the coding sequence of the *ISW2* gene with the *hisG* cassette (Alani et al. 1987).

Catalytically inactive mutations of *ISW2* and *RPD3*, and mutations of the URS1 element upstream of the *REC104* gene were introduced into the genome by the 'pop in-pop out' method (Rothstein 1991).

Itc1p, the second subunit of Isw2 complex, was fused to 13 copies of the myc epitope immediately before the termination codon as described (Longtine et al. 1998). To tag proteins with the FLAG epitope, we constructed a plasmid which serves as a template for PCR-based tagging by homologous recombination (Gelbart et al. 2001).

RNA analyses

RNA samples for DNA microarray analysis were prepared from wild type cells, W1588-4C, and congenic YTT199 (*isw1 isw2*) (Tsukiyama et al. 1999) grown in YEPD (2% bacto peptone, 1% yeast extract, 2% glucose) at 30 °C during early log phase (O.D.

660=0.7) using acid phenol extraction. Microarray analysis using GeneChip Ye6100 (Affymetrix) was performed as described (Wodicka et al. 1997). We concentrated on the data most consistent with Northern analyses (e.g. *POT1, SPR3, SGA1*, and *REC104*). Since the array data were intended as a prescreen, only one dataset for each of the two RNA samples was collected. The data set is available at http://arep.med.harvard.edu/ExpressDB (Aach et al. 2000). For Northern blotting, RNA samples were prepared as above, and 25 μg of total RNA were loaded per lane. The signals were quantified by a phosphorimager (Molecular Dynamics).

Chromatin immunoprecipitation (CHIP)

CHIP assay was done essentially as described (Dudley et al. 1999) using strains containing three copies of FLAG tag on Ume6p, except that SDS was omitted from all buffers.

Protein purification

Isw2 complex was purified as described (Tsukiyama et al. 1999). Recombinant GST-Ume6 fusion protein was expressed in BL21 (DE3) pLysS cells. The expression plasmid pGST-Ume6 was constructed by ligating a BglII fragment from pMM225 (a generous gift of Michael Marolly and Randy Strich) containing the full length open reading frame of *UME6* into the BamH1 site of pGEX-4T1. GST-Ume6 protein was purified using Glutathione agarose beads.

Gel retardation assay

Oligonucleotides corresponding to nucleotides –60 to –114 bp of the *REC104* promoter were annealed and radiolabeled using [γ^{32}P] ATP and T4 polynucleotide kinase. Purified proteins (approximately 3.6 ng of GST-UME6, and 19.2 ng of Isw2 complex) were added to a 10 μl binding reaction containing 25mM Hepes-KOH pH 7.6, 10% glycerol, 5 μM $ZnCl_2$, 5 mM $MgCl_2$, 50 mM KCl, 0.1 mg/ml BSA, 1 mM ATP, 0.25 ng/μl poly(dA-dT)•poly(dA-dT) (Amersham Pharmacia Biotech), 0.5 ng/μl sonicated *E. coli* DNA,

and approximately 1.32 fmole/μl of radiolabeled probe. For competition, unlabeled double stranded oligonucleotides were added to a final concentration of 50 fmole/μl. The wild type oligonucleotide competitor is identical to that used for the probe. The mutant oligonucleotide competitor contained a triple base pair substitution within URS1, TTGGCGGCT to TTGtacGCT (mutated nucleotides are in lower case letters). For antibody-mediated supershift, 0.5 μg of purified anti-FLAG-M2 (Sigma) or anti-MYC (9E10, Covance) antibody was preincubated with Isw2 complex for 10 minutes at room temperature before adding to the binding reaction. Protein-DNA complexes were resolved on a 4% polyacrylamide gel in 1/4X Tris-Borate.

Chromatin structure analyses

Nuclei were prepared from 1 liter YPD cultures of early log phase yeast cells (OD_{660}=0.7) as described previously (Hirschhorn et al. 1992). For MNase digestions, aliquots of nuclei (1 mL ea) were digested with 0, 1.5, 5, 15, 50, 150, or 500U of MNase (Roche Molecular Biochemical) for 10 minutes at 37°C. DNA was purified from each and resuspended in 100 μL TE. Approximately 9 μL of the 0, 50, and 150U samples were digested with EcoRI (-1050) and PstI (+1020), and subjected to indirect end-labeling of the *REC104* locus. Naked DNA was prepared from identical nuclear preparations and digested with 5 U of MNase in 500 μl volume for 10 minutes at 37 °C. DNase I analysis was done essentially identically, except that SPC buffer was supplemented with 3 mM $MgCl_2$ and nuclei were digested with 0, 1, 2, or 4 units of DNase I at 37 °C for 20 minutes. The probe used for indirect end-labeling of the *REC104* locus was a PCR product extending from -809 to -998 with respect to the initiation codon.

Chapter II: Widespread collaboration of Isw2 and Sin3-Rpd3 chromatin remodeling complexes in transcriptional repression

Summary

The yeast Isw2 chromatin remodeling complex functions in parallel with Sin3-Rpd3 histone deacetylase complex to repress early meiotic genes upon recruitment by Ume6p. For many of these genes, the effect of *isw2* mutation is partially masked by functional Sin3-Rpd3 complex. To identify the full range of genes repressed or activated by these factors and uncover hidden targets of Isw2-dependent regulation, we performed full genome expression analyses using cDNA microarrays. We find that Isw2 complex functions mainly in repression of transcription in a parallel pathway with Sin3-Rpd3 complex. In addition to Ume6-target genes, we find that many Ume6-independent genes are derepressed in mutants lacking functional Isw2 and Sin3-Rpd3 complexes. Conversely, we find that *ume6* mutants, but not *isw2 sin3* or *isw2 rpd3* double mutants, have reduced fidelity of mitotic chromosome segregation, suggesting one or more functions of Ume6p that are independent of Sin3-Rpd3 and Isw2 complexes. Chromatin structure analyses of two non-meiotic genes reveals increased DNaseI sensitivity within their regulatory regions in an *isw2* mutant, as seen previously for one meiotic locus. These data suggest that Isw2 complex functions at Ume6-dependent and –independent loci to create DNaseI-inaccessible chromatin structure by regulating the positioning or placement of nucleosomes.

Introduction

Regulation of RNA synthesis is a complex process affected by many factors that influence initiation, elongation and termination of transcription. These factors include transcriptional activators and repressors, as well as general transcription factors. In eukaryotes, the compaction of DNA into chromatin provides an additional level of complexity, due to the fact that chromatin structure is inhibitory to many protein-DNA

interactions required for transcription (Struhl 1999). Consequently, chromatin structure must be dynamically regulated for many genes whose transcription is repressed or induced at different times of the cell cycle, phases of development, or changes in growth conditions.

A large number of proteins from several organisms have been found to modify chromatin structure. These factors can be grouped into two classes based on their biochemical activities: histone modifying enzymes and ATP-dependent chromatin remodeling complexes (for reviews see (Suka et al. 1998; Workman and Kingston 1998; Kingston and Narlikar 1999; Knoepfler and Eisenman 1999; Maldonado et al. 1999; Wade and Wolffe 1999; Strahl and Allis 2000)). Histone modifying enzymes employ several different enzymatic mechanisms to covalently modify histone proteins. These modifications include acetylation, phosphorylation, ubiquitination and methylation. The second class of factors utilizes the energy of ATP hydrolysis to alter histone-DNA contacts, which often leads to repositioning of histone octamers on DNA (Wu et al. 2000).

The role of histone acetylation in regulation of transcription has been studied extensively (Grunstein 1997; Strahl and Allis 2000; Wu and Grunstein 2000). With some exceptions, high levels of histone acetylation correlate with transcriptionally active regions of chromatin, while lower levels of acetylation are found in transcriptionally inactive regions. Histone acetylation is regulated by the activities of histone acetyltransferase (HAT) and histone deacetylase (HDAC) complexes, many of which function as transcriptional activators or repressors, respectively (Struhl 1998; Suka et al. 1998; Strahl and Allis 2000).

Three major families of HDACs have been conserved in eukaryotes, represented by Rpd3, Hda1, and Sir2 proteins (Kuo and Allis 1998; Guarente 2000). The *RPD3* gene was first identified genetically in yeast as a transcriptional repressor of several genes (Vidal and Gaber 1991; Vidal et al. 1991). It was later found that Rpd3 proteins in several species associate with Sin3p and other Sin3-associated proteins (SAPs) to form a multiprotein complex (Alland et al. 1997; Kadosh and Struhl 1997; Zhang et al. 1997).

Recruitment of this complex by transcriptional repressors leads to local deacetylation and repression of transcription (Hassig et al. 1997; Heinzel et al. 1997; Kadosh and Struhl 1997; Kasten et al. 1997; Laherty et al. 1997; Nagy et al. 1997; Zhang et al. 1997).

A variety of ATP-dependent chromatin remodeling factors have also been identified in several organisms and can be grouped into three classes based on the ATPase subunit present in each complex; SWI/SNF, ISWI and CHD1 (Cairns 1998; Kadonaga 1998; Peterson 1998; Kingston and Narlikar 1999). Members of the ISWI class of chromatin remodeling factors were first identified biochemically in *Drosophila*, following their ATP-dependent activity to disrupt nucleosomes or impart regular spacing on nucleosome arrays in vitro. These studies led to the identification of three ISWI-containing complexes, NURF (Tsukiyama et al. 1995; Tsukiyama and Wu 1995), CHRAC (Varga-Weisz et al. 1997) and ACF (Ito et al. 1997). Subsequently, several other ISWI-containing complexes were identified in humans (LeRoy et al. 1998; Bochar et al. 2000a; LeRoy et al. 2000; Poot et al. 2000), yeast (Tsukiyama et al. 1999) and Xenopus (Guschin et al. 2000; Kikyo et al. 2000), based on sequence homology to *Drosophila* ISWI.

Although the biochemical activities of several ISWI complexes have been studied in detail, the in vivo functions of this class of factors are only beginning to be identified. The *Drosophila ISWI* gene is essential for development and cell viability (Deuring et al. 2000). *Drosophila ISWI* mutants also have reduced expression of the *Ubx* and *engrailed* genes, suggesting the requirement of one or more ISWI-containing complex for expression of these genes. In addition, *ISWI* mutants are compromised for male X-chromosome integrity (Deuring et al. 2000).

We recently found that one of the two yeast ISWI complexes, Isw2 complex, functions during vegetative growth to repress genes induced early in meiosis (Goldmark et al. 2000). Many early meiotic genes are repressed under these conditions by the Sin3-Rpd3 histone deacetylase complex, upon recruitment by the sequence-specific DNA-binding protein, Ume6p (Kadosh and Struhl 1997; Kadosh and Struhl 1998b; Rundlett et

al. 1998). Like Sin3-Rpd3 complex, Isw2 complex appears to be recruited to the promoters of early meiotic genes by Ume6p; however, repression by Isw2 complex occurs independently of Sin3-Rpd3 complex. Analysis of chromatin structure of one Ume6-target gene in wild type and various mutant cells reveals that Isw2 complex functions to create DNaseI-inaccessible chromatin structure by altering the positions of nucleosomes upstream of the Ume6p-binding site. These data reveal that Isw2 complex functions in parallel with Sin3-Rpd3 complex to repress transcription of common target genes.

While it is clear that Isw2 and Sin3-Rpd3 complexes collaborate to repress Ume6-target genes, it is not clear whether this collaboration extends to any Ume6-independent genes. To investigate this possibility, we compared the full genome transcriptional profiles of mutants defective in one or more of these factors. We also competed single mutants of *sin3* and *rpd3* with *isw2 sin3* and *isw2 rpd3* double mutants directly on microarray slides to identify genetic interactions between Isw2 and Sin3-Rpd3 complexes that are not always detectable in traditional mutant versus wild type competitions. We find that, while Ume6-targets represent a major group of genes repressed by these complexes, a large number of Ume6-independent genes are derepressed in mutants defective in Sin3-Rpd3 and Isw2 complex functions. Comparison of deletion and catalytically inactive mutants of *isw2* and *rpd3* reveal differences in both phenotype and transcriptional profiles which may result from functions of these proteins independent of their known catalytic activities, inhibition of related factors by catalytically inactive protein, or a combination of the two. We also find that *ume6* mutants have reduced fidelity of chromosome segregation which results in higher rates of chromosome gains and losses relative to wild type cells, suggesting a role for Ume6p in chromosome segregation that is independent of Isw2 and Sin3-Rpd3 complexes. Chromatin structure analyses of two Ume6-independent genes that require Sin3-Rpd3 and Isw2 complexes for proper regulation reveal that *ISW2* function is required in both cases for formation of DNaseI-inaccessible chromatin structure, as previously observed for the Ume6-target gene, *REC104* (Goldmark et al. 2000). These

data suggest that Sin3-Rpd3 and Isw2 complexes collaborate to repress transcription of Ume6-dependent and some Ume6-independent genes and that Isw2 complex functions to create DNaseI-inaccessible chromatin structure at the promoters of many of these genes.

Results

Overlapping functions of Isw2 and Sin3-Rpd3 complexes

Recently we found that Isw2 complex functions during mitotic growth in Ume6p-dependent repression of early meiotic genes in a parallel pathway to Sin3-Rpd3 histone deacetylase complex (Goldmark et al. 2000). While it is clear that Sin3-Rpd3 and Isw2 complexes function in repression of Ume6-target genes, it is unknown whether this collaboration extends to genes not regulated by Ume6p. To determine the full extent to which Sin3-Rpd3 and Isw2 complexes function in parallel pathways of transcriptional repression, we used spotted cDNA microarrays representing >96% of all yeast genes to analyze the genome-wide expression profiles of mutants defective in one or both of these complexes relative to wild type cells. For each mutant, four independent mutant versus wild type microarray hybridizations were carried out (two dye-swap pairs) and the average expression ratio and standard error for each spot was determined (see Materials and Methods for details; the full data set can be obtained at ftp://milano.fhcrc.org/ArrayLab/Fazzio/). Initially we focused on *isw2*, *rpd3*, and *sin3* single null mutants, as well as *isw2 rpd3* and *isw2 sin3* double null mutants. In addition, we analyzed expression profiles of *ume6* and *isw2 ume6* null mutants; these data will be discussed in a later section. We observed relatively few changes in transcript levels in the *isw2* single mutant, as previously described for an *isw2/isw2* homozygous mutant diploid strain (Hughes et al. 2000a). Because we previously found that Isw2 and Sin3-Rpd3 complexes can each partially compensate for the lack of the other in repression of common target genes, we focused on genes that require defects in both complexes for moderate levels of derepression. As shown in Table 2.1, a larger number of genes are moderately derepressed (>3 fold) in *isw2 rpd3* and *isw2 sin3* double mutants than in single mutants. Similarly, more genes are derepressed to higher levels (> 5 fold, >10

Table 2.1. Number of genes with increased expression in indicated deletion mutants[1]

	≥3 fold[2]	≥5 fold	≥10 fold
Δisw2	3 (0)	0	0
Δrpd3	35 (8)	9 (3)	1 (1)
Δisw2 Δrpd3	114 (21)	24 (13)	6 (2)
Δsin3	42 (10)	8 (3)	2 (1)
Δisw2 Δsin3	93 (22)	31 (12)	9 (5)
Δume6	113 (40)	45 (20)	17 (10)
Δisw2 Δume6	320 (52)	80 (24)	22 (13)

[1]Indicated in parenthesis is the number of genes in each group that contain the URS1 core sequence (GGCGGC) within 500 base pairs upstream of their initiation codon.

[2]720 genes were more than 3-fold derepressed in one or more of the seven mutants listed above. Of these, the expression ratios of 630 were statistically significantly higher than 2 at the 0.05 level; the ratios of 178 of the 197 genes that were more than 5-fold derepressed were statistically significantly higher than 3 at the 0.05 level; the expression ratios of 53 of the 57 genes that were more than 10-fold derepressed were statistically significantly higher than 5 at the 0.05 level.

fold) in the double mutants than in single mutants, revealing that Sin3-Rpd3 and Isw2 complexes function in parallel pathways to repress transcription of a considerable group of genes. As expected, many genes found to be repressed by Sin3-Rpd3 and Isw2 complexes include known targets of Ume6p and genes predicted to be Ume6 targets by virtue of an upstream Ume6p binding site (URS1) (Table 2.1). We also searched for a second Ume6-binding sequence previously identified in the promoter of the *PHR1* gene (Sweet et al. 1997); however, this sequence was not overrepresented in the promoters of genes derepressed in any of our mutants. In addition to genes repressed by Ume6p, we observed many genes that require Sin3-Rpd3 and Isw2 complexes for repression that lack URS1 sequences. The majority (82%) of these genes are not substantially derepressed in the *ume6* mutant, suggesting that derepression in *isw2 sin3* and *isw2 rpd3* mutants was not a secondary consequence of upregulation of early meiotic genes (see full data set for details). Together, these data reveal a substantial group of genes that require Sin3-Rpd3 and Isw2 complexes for repression, independently of Ume6p.

In addition to the role of Isw2 complex in repression, we also found that transcription of a small number of genes was reduced in the *isw2* mutant (see full data set for details). For these genes, the relationship between Isw2 complex and Sin3-Rpd3 complex in activation or maintenance of basal transcription is less clear. Few genes have significantly reduced expression in the *isw2* deletion mutant (0 genes reduced > 3 fold, 1 gene reduced > 2 fold). In addition, many genes with reduced expression in the *sin3* or *rpd3* single mutants are unaffected or oppositely affected by the addition of an *isw2* mutation in these backgrounds. These data suggest a relatively minor role of Isw2 complex in transcriptional activation or maintenance of basal transcription under the conditions used (logarithmic growth on rich glucose medium).

Hidden functions of Isw2 complex revealed in *sin3* and *rpd3* mutant backgrounds

The data in Table 2.1 suggest that many targets of Isw2-mediated repression may be masked in *isw2* mutant cells by the presence of functional Sin3-Rpd3 complex. To identify *ISW2*-target genes affected in this way, we performed two experiments. First,

we divided *isw2 rpd3*:wild type expression ratios by *rpd3*:wild type ratios for all genes (Table 2.2, second column); similarly, *isw2 sin3*:wild type expression ratios were divided by *sin3*:wild type ratios (Table 2.2, fifth column). In theory, the ratios obtained through these calculations should reveal defects in transcriptional regulation resulting from loss of Isw2 function in a *rpd3* or *sin3* mutant background. However, when we compared these data to those obtained by Northern blotting (Table 2.2, first and fourth columns),we noticed several inconsistencies. For several meiotic genes previously found to be targets of Isw2-dependent repression, the ratios derived from one or both calculations described above do not reflect known derepression due to *isw2* mutation (c.f. *HOP1, SPO11, REC104, SPO1*). Under the growth conditions for this experiment (logarithmic growth of haploid cells in rich glucose medium) many genes are tightly repressed in wild type cells. In microarray experiments, expression ratios for genes with low signal in the wild type channel are often underestimated relative to ratios measured by Northern blotting (Kooperberg et al. 2002). As a result, *isw2 rpd3*:wild type expression ratios for these genes measured using microarrays can be similar to *rpd3*:wild type ratios; likewise, *isw2 sin3*:wild type ratios can be similar to *sin3*:wild type ratios. Consequently, when double mutants are divided by single mutants to compare their expression profiles, some differences observed by Northern blotting fail to be uncovered.

To circumvent this problem, we measured *isw2 rpd3*:*rpd3* and *isw2 sin3*:*sin3* ratios directly by labeling single and double mutant RNA samples with opposing dyes and hybridizing them to the same microarray slide. In contrast to the typical method of measuring mutant:wild type ratios, this approach uses *rpd3* or *sin3* single mutants as "references" to which double mutant expression levels are compared. As a result, the problem of underestimated expression ratios resulting from minimal wild type expression is significantly reduced for genes derepressed in *rpd3* or *sin3* mutants. For both the *isw2 rpd3: rpd3* and the *isw2 sin3*:*sin3* comparisons, the expression ratio obtained from the direct (mutant versus mutant) hybridization is larger for most genes examined than the one obtained by dividing two mutant:wild type expression ratios (Table 2.2). In addition, when expression ratios obtained by direct hybridization are

Table 2.2. Direct competition of double mutants with single mutants reveals hidden functions of Isw2 complex[a]

Gene	*isw2 rpd3/ rpd3* (Northern[b])	*isw2 rpd3*:wt/ *rpd3*:wt (array/array[c])	*isw2 rpd3*:*rpd3* (direct hybridization[d])	*isw2 sin3/ sin3* (Northern[b])	*isw2 sin3*:wt/ *sin3*:wt (array/array[c])	*isw2 sin3*:*sin3* (direct hybridization[d])
SPO11	2.0	1.1 (0.3)	1.6 (0.2)	1.6	1.4 (0.3)	1.6 (0.3)
SPO1	3.3	1.3 (0.2)	1.7 (0.2)	2.5	1.6 (0.1)	1.8 (0.2)
HOP1	4.1	1.5 (0.1)	1.9 (0.1)	2.4	1.8 (0.1)	2.2 (0.3)
REC104	5.5	2.5 (0.2)	2.9 (0.4)	4.0	1.4 (0.4)	3.3 (0.3)
IME2	2.8	2.2 (0.5)	1.8 (0.1)	1.6	1.8 (0.2)	1.9 (0.2)
SGA1	1.7	1.5 (0.2)	1.8 (0.1)	1.4	1.9 (0.2)	1.8 (0.2)
SIP4	2.7	1.9 (0.3)	2.9 (0.4)	2.0	1.6 (0.3)	1.9 (0.4)
POT1	3.7	3.1 (0.5)	6.1 (3.2)[e]	3.2	3.3 (0.7)	4.7 (0.8)
SPO13	4.7	4.2 (1.1)	3.4 (0.3)	6.8	2.9 (0.4)	3.0 (0.4)
INO1	7.3	6.4 (0.8)	8.3 (0.4)	2.9	3.6 (0.7)	5.0 (0.6)

[a] Means and standard errors (between parenthesis) of ratios were computed as means and standard errors of log-ratios and backtransformed to the ratio scale assuming that the log-ratios have a normal distribution, so that the ratio has a log-normal distribution (Johnson and Kotz 1970). Shaded boxes indicate genes for which derepression due to isw2 mutation is more evident in direct hybridization experiments (third and sixth columns) than in array versus array comparisons (columns two and five).

[b] Ratios of Northern quantitations of genes indicated to the left. mRNA levels measured in *isw2 rpd3* and *isw2 sin3* double mutants were divided by mRNA levels measured in *sin3* and *rpd3* single mutants, respectively.

[c] Ratios obtained by dividing one mutant:wild type microarray experiment by another. *isw2 sin3*:wild type ratios were divided by *sin3*:wild type ratios for each gene. Similarly, *isw2 rpd3*:wild type ratios were divided by *rpd3*:wild type ratios.

[d] *isw2 sin3*:*sin3* expression ratios were measured directly on microarray slides. Similarly, *isw2 rpd3*:*rpd3* ratios were measured directly on microarray slides.

[e] For one of the replicates of the isw rpd3:rpd3 hybridization for POT1 the background level was very high, and as a result the posterior variance of the estimate of the expression log-ratio was very high. If this one realization is excluded the estimate of the expression ratio changes to 3.4 with a standard error of 0.8.

compared to ratios calculated from mutant versus wild type experiments, we find that direct hybridization more accurately detects derepression observed by Northern blotting. While the values of expression ratios measured for mutant versus mutant experiments were not always identical to those calculated from Northern data, this method qualitatively identified derepression due to the *isw2* mutation for all ten genes analyzed, in both *rpd3* and *sin3* mutant backgrounds.

In an attempt to categorize targets of Isw2-dependent repression, we analyzed more closely the set of genes identified above that are derepressed in *isw2 rpd3* or *isw2 sin3* double mutants relative to *sin3* or *rpd3* single mutants. This class of genes requires Isw2 complex for repression in the absence of functional Sin3-Rpd3 complex. We focused on the set of genes derepressed at least 1.7 fold in one or both of the direct hybridization experiments, as this cutoff level allowed for inclusion of ~90% of genes known to be repressed by Isw2 complex (as measured by Northern blotting) (Table 2.2), but did not include any genes found by Northern blotting to be unaffected in *isw2* mutant cells (data not shown). By these criteria, 315 genes (~5% of all yeast genes) belonging to many different functional categories were found to be derepressed when an *isw2* mutation was present in *rpd3* and/or *sin3* mutant backgrounds; representatives of this group of genes are listed in Table 2.3. For comparison, only 112 genes met this 1.7 fold threshold in the *isw2* single mutant. As expected, a significant portion (~20%) of the 315 genes in this group contained the core Ume6p-binding site, 5'-GGCGGC-3', in the 500 base pairs upstream of their initiation codon; the majority (~72%) of these genes were also found to be derepressed in *ume6* and *isw2 ume6* mutant backgrounds (see full data set on the web). No other sequences were significantly overrepresented in the promoters of these genes. We also observed 80 genes whose expression is decreased more than 1.7 fold in one or both double mutants relative to *rpd3* or *sin3* single mutants, consistent with a lesser role for Isw2 complex in activation of transcription. The majority of these genes are uncharacterized open reading frames and no functional category of genes is highly represented in this group.

Table 2.3. Subset of genes that require *ISW2* function for repression in *sin3* or *rpd3* mutant backgrounds[1]

ATP synthesis	Cu2+ ion homeostasis	Glucose Metabolism	Phosphate Metabolism	Transport	Unknown (cont.)
STF1	SLF1	HSP12	PHO11	FUR4	EBP2
ATP10	CUP1-1	GIP2	PHO89	AGP2	FKS3
ATP20	CUP1-2	PIG2	PHO12	BPH1	FRE7
		GPH1		SIT1	GIT1
Secretion	Cell cycle	GPM2	Meiosis/ Sporulation	FTR1	KIN2
BFR2	PCL5		SPO13	JEN1	PGU1
		Amino Acid Synthesis			
SED5	PCL1		MEK1	TPO1	PIR3
NCE103	SYF2	GCV1	SPO1	SUL2	SIP18
	CDC26	LYS1	HOP2	THI7	SMT4
Transcriptional regulation		MET3	NDJ1	FET3	SOL4
SMP1	DNA repair		MSH4	ALP1	SPI1
CAT8	MSH5		REC104	YOR071C	YCL035C
SIP4	MAG1	Protein degradation/	HOP1	CTP1	YDR374C
MTH1	RNR3	modification	MEI5	AQY2	YER037W
KAR4	DIN7	SMT3	SHC1	AQY1	YET1
SPO1	ALK1	RPN4	SPS100		YFR026C
RTG1	MGT1	SRT1	SGA1	Unknown	YGL117W
PHD1	RAD51		SPS19	BOP2	YGR079W
YAP6			NOS1	BTN2	YIL037C
CIN5		Cytoskeleton		COS1	YIR043C
CYC8	Ethanol utilization	SRO9	TCA cycle	COS4	YJL045W
SUB1	ALD3	TPM2	IDP1	COS7	YKL071W
NRD1	ALD2	BNR1	IDP2	COS8	YLR179C
FUR4	ALD4	HSP42	CIT3	CTL1	YMR269W

[1]For full list of genes that require *ISW2* function for repression in *sin3* and *rpd3* mutant backgrounds, see ftp://milano.fhcrc.org/ArrayLab/Fazzio/

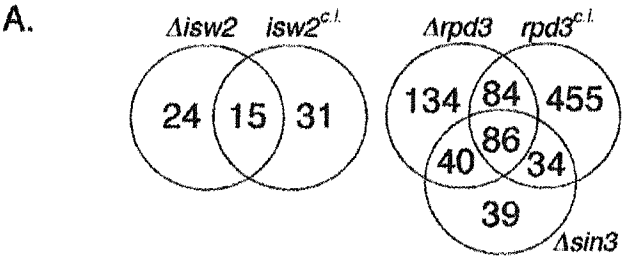
Catalytically inactive *isw2* and *rpd3* mutants differ from deletion mutants in phenotype and transcriptional profiles

Isw2p has nucleosome stimulated ATPase activity that is required for both chromatin remodeling in vitro (Tsukiyama et al. 1999), as well as repression of early meiotic genes in vivo (Goldmark et al. 2000). Similarly, histone deacetylase activity of Rpd3p is required for normal levels of repression of target genes (Kadosh and Struhl 1998a; Chen et al. 1999; Goldmark et al. 2000). However, several deacetylase-defective mutants of *rpd3* were previously found to be only partially defective in repression of a LexA reporter construct (Kadosh and Struhl 1998a), suggesting one or more functions of Rpd3p that are independent of its deacetylase activity. In addition, overexpression of catalytically inactive Rpd3 protein in wild type cells results in a partial dominant negative phenotype (Kadosh and Struhl 1998a). It is therefore possible that catalytically inactive mutants of *isw2* and *rpd3* may exhibit defects in transcriptional regulation not seen in deletion mutants. In deletion mutants, related chromatin remodeling factors may partially compensate for the deleted proteins, whereas catalytically inactive proteins may prevent these factors from accessing chromatin. In addition, accessory proteins shared by multiple chromatin remodeling factors may be titrated by catalytically inactive protein. Alternatively, deletion mutants may have defects in transcriptional regulation not observed in catalytically inactive mutants, due to functions of these proteins that are independent of their known catalytic activities.

To determine the differences between catalytically inactive and deletion mutants of *isw2* and *rpd3*, we analyzed the transcriptional profiles of single *isw2* and *rpd3* catalytically inactive mutants, as well as the *isw2 rpd3* double catalytically inactive mutant. For this purpose, we analyzed an *rpd3* mutation in which a conserved histidine residue at position 151 is substituted to alanine; this mutation was previously found to eliminate deacetylase activity of Rpd3p (Kadosh and Struhl 1998a). Similarly, we analyzed a previously characterized *isw2* substitution mutation (lysine 214 to arginine), known to eliminate the ATPase activity of Isw2p.

While many of the same genes were misregulated in deletion and catalytically inactive mutants of *isw2* and *rpd3*, in each case there were considerable differences in transcriptional profiles between the two types of mutants (Figure 2.1a). While relatively few genes are derepressed at least two-fold in either isw2 mutant, a substantial amount of non-overlap is apparent for the two transcriptional profiles. A moderate number of genes are derepressed at least two-fold in the *rpd3* deletion mutant but not the catalytically inactive mutant. More strikingly, a large number of genes are derepressed at least two-fold in the catalytically inactive *rpd3* mutant that do not meet this threshold in the deletion mutant (Figure 2.1a, right panel). Most of these genes are not significantly derepressed in *sin3* mutant cells, suggesting that Rpd3p is required for repression of this group of genes independently of Sin3p. In contrast, most genes derepressed in the *sin3* mutant are also derepressed in one or both *rpd3* mutants, with the largest single group of *sin3*-responsive genes being those derepressed in all three mutants. These data suggest that Sin3p acts primarily in conjunction with Rpd3p to repress transcription of common target genes, whereas Rpd3p appears to be required for repression of some genes independently of Sin3p.

To further investigate the differences in transcriptional profiles between deletion and catalytically inactive mutants of *rpd3*, we searched the 489 genes derepressed in the catalytically inactive mutant, but not in the deletion mutant, for common sequences within their presumed regulatory regions (within 500 base pairs upstream of their initiation codon). While we did not find any known regulatory elements, several elements with overlapping sequences were overrepresented in this group to a high level of significance (Figure 2.1b). The consensus of these sequences, 5'-GNGATGAGNT-3', is present in the upstream 500 base pairs of 174 yeast genes. Of these, 56 (32%) are at least 2-fold derepressed and 131 (75%) are derepressed at least 1.5-fold in the catalytically inactive *rpd3* mutant. This sequence was previously identified by two independent computational approaches and found to be overrepresented in the regulatory regions of a group of genes with a common expression pattern during the cell cycle (Tavazoie et al. 1999; Bussemaker et al. 2001). These data cannot distinguish whether



B.

Sequence	Frequency	Confidence
GNGATGAG	(73/489)	log p < -12
GNGATNAG	(109/489)	log p < -5
GCNATGAG	(53/489)	log p < -8
GCGNTGAG	(51/489)	log p < -7
GCGANGAG	(51/489)	log p < -7
GCGATNAG	(53/489)	log p < -8
GCGATGNG	(54/489)	log p < -9
GCGATGAG	(49/489)	log p < -13
CGATGAG	(65/489)	log p < -9
CGATGA	(107/489)	log p < -3
GATGAG	(123/489)	log p < -6
GANGAGNT	(119/489)	log p < -3
GATGNGNT	(108/489)	log p < -5
GATGAGNT	(81/489)	log p < -11
Consensus: G[C/N]GATGAGNT	(51/489)	log p < -13

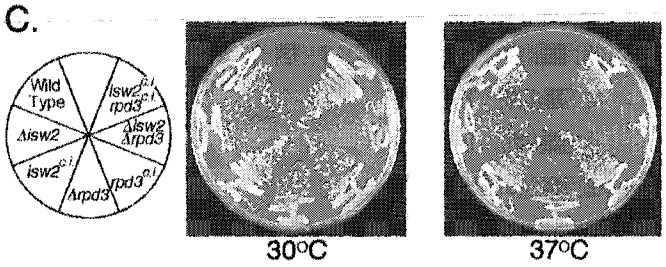


Figure 2.1. Catalytically inactive and deletion mutations of *isw2* and *rpd3* have different effects on transcription and growth.

(A) The number of genes derepressed at least two fold in catalytically inactive (c.i.) and deletion (Δ) mutants of *isw2* and *rpd3* are compared in Venn diagrams. The number of genes derepressed at least two fold in the *Δsin3* mutant are included for comparison. It should be noted that the Venn diagrams tend to overestimate the differences between two mutants since genes derepressed in both mutants can be derepressed slightly more than two fold in one mutant and slightly less than two fold in the other mutant. Nevertheless, real differences in transcriptional profiles between deletion and catalytically inactive mutants are evident for both *isw2* and *rpd3*.

(B) Summary of a regulatory element search of the 489 genes derepressed at least 2-fold in the catalytically inactive *rpd3* mutant, but not in the *rpd3* deletion mutant, as indicated in (A). The search parameters allowed for oligonucleotides of eight bases or fewer, permitting degeneracies. Highly significant elements belonging to the same consensus were aligned with their frequencies and confidence estimates (calculated by the GENESPRING software package) indicated.

(C) Wild type, deletion (Δ), and catalytically inactive (c.i.) mutant yeast cells were streaked on rich (YEPD) plates and incubated at 30^0C or 37^0C, as indicated. The genotypes of yeast cells are shown to the left.

this sequence functions directly in Rpd3-mediated repression, or the catalytically inactive *rpd3* mutation indirectly leads to derepression of these genes. However, the fact that this sequence is overrepresented in the regulatory regions of genes derepressed in the same mutant as well as coexpressed during the cell cycle suggests that it may function in transcriptional regulation. Among the genes that contain this sequence in their upstream regulatory regions are those encoding components of the RNA processing and degradation machinery, as well as subunits of RNA polymerases I and III ((Tavazoie et al. 1999) and data not shown). This element may therefore coordinate regulation of genes involved in several different aspects of RNA metabolism.

Consistent with the differences in transcriptional profiles, we also observed differences in growth phenotypes between deletion and catalytically inactive mutants of *isw2* and *rpd3*. Previously we found that the *isw2 rpd3* double deletion mutant exhibits a synthetic slow growth phenotype at 30°C and fails to form colonies at 37°C (Goldmark et al. 2000). In contrast, the double catalytically inactive mutant grows at a rate similar to wild type at 30°C and grows slightly slower than wild type at 37°C (Figure 2.1c). We also find that the *rpd3* single deletion mutant grows slightly slower than wild type at 37°C, while growth of the catalytically inactive *rpd3* single mutant is unaffected. These results suggest that the *isw2 rpd3* double deletion mutant is impaired in one or more functions required for cell growth or division, and this defect is enhanced at higher temperatures. The phenotype of the double catalytically inactive mutant suggests that it is less severely impaired in these functions. The partial growth defect of the double catalytically inactive mutant at 37°C is very similar to that observed previously for an *isw2 sin3* double mutant (Goldmark et al. 2000). These data, combined with the transcriptional data discussed above, suggest one or more functions of Rpd3p that require neither deacetylase activity nor Sin3p. However, the fact that many more genes are derepressed in the catalytically inactive *rpd3* mutant than in the deletion mutant suggests that catalytically inactive protein might inhibit other transcription or chromatin remodeling factors. The differences in growth phenotypes and transcriptional profiles

between deletion and catalytically inactive mutants may therefore result from a combination of these two effects.

Genomic instability of *ume6* mutants

Isw2 complex is recruited by Ume6p and functions in repression of genes that contain the Ume6p-binding site, URS1 (Goldmark et al. 2000). Since repression of early meiotic genes by Isw2 and Sin3-Rpd3 complexes is fully dependent upon Ume6p, we wished to determine whether *UME6* function is required for repression of non-meiotic genes by these complexes. To determine the extent to which Isw2 and Sin3-Rpd3 complexes depend on Ume6p, as well as the extent to which Ume6p functions through these complexes, we analyzed the expression profiles of *ume6* and *isw2 ume6* mutants.

Surprisingly, more genes were derepressed to high levels in the *ume6* and *isw2 ume6* mutants than in *isw2 rpd3* or *isw2 sin3* mutants (Table 2.1), suggesting the presence of a number of Ume6-targets that are not regulated by Isw2 and Sin3-Rpd3 complexes. In addition, we find many genes that do not have a nearby URS1 sequence derepressed in *ume6* and *isw2 ume6* mutants (Table 2.1). Upon closer inspection, a much higher than average density of derepressed genes were located on chromosome 16 (for the *ume6* mutant) or 9 and 16 (for the *isw2 ume6* double mutant) (data not shown). Recently, Hughes et al observed similar chromosomal expression biases in ~8% of a large number of expression studies (Hughes et al. 2000b). This group examined the nature of the expression biases for each of these mutants and found, for nearly every case examined, the biases resulted from duplications or deletions of chromosomal segments or whole chromosomes.

To determine whether *ume6* and *isw2 ume6* mutants contained chromosomal duplications, we isolated DNA from mutants and wild type cells, labeled each and performed competitive hybridizations on microarray slides as described previously (Hughes et al. 2000b). We analyzed the DNA content of two independent *ume6* mutants and three independent *isw2 ume6* mutants constructed in our laboratory in two different strain backgrounds, as well as one *ume6* mutant obtained from another laboratory (Table

2.4). In all cases, we found evidence that at least one, and sometimes two or more chromosomes were duplicated in these mutants. During the course of this work, independent expression data for a *ume6* mutant was published (Bernstein et al. 2000); analysis of these data reveals chromosomal expression biases that strongly suggest the presence of chromosomal duplication (data not shown), supporting our findings. Independent DNA isolates from the same *ume6* mutant revealed variability in which chromosomes were duplicated, suggesting that duplicated chromosomes were not always maintained during growth (Table 2.4). While the most common duplications were of chromosomes 9 and 16, we also observed, less frequently, duplications of chromosomes 1, 2, 3 and 8. Hughes et al found that, for several mutants, the duplicated chromosomes or chromosomal segments contained genes homologous to the gene mutated, suggesting that selection for extra copies of homologous genes might partially compensate for lack of the mutated gene. However, in the case of *UME6*, we have found no close homolog within the yeast genome. In addition, the high degree of variability in chromosomes duplicated in *ume6* mutants suggests against the possibility that these mutants are selected for extra copies of particular genes.

Alternatively, chromosomal duplications in *ume6* mutants may result from a general defect in chromosome segregation. In this case, chromosome loss would be predicted to occur in addition to chromosomal duplications. However, in our expression studies we would only have observed chromosomal duplications, since chromosome loss is lethal to haploid cells. To test this possibility, we constructed strains containing a minichromosome harboring the *SUP11* gene, which suppresses the *ade2-101* mutation in our yeast strains, rendering colonies white in color. Because this chromosome is dispensable for cell viability, chromosomal loss rates can easily be measured by monitoring differences in colony color after nonselective growth (Hieter et al. 1985; Spencer et al. 1990). By this assay, we find that *ume6* and *isw2 ume6* mutants lose the minichromosome roughly 26-62 fold more often than wild type cells (Table 2.5, compare wild type to *ume6* and *isw2 ume6* mutants). These results suggest that

Table 2.4. Chromosomal duplications in *ume6* mutants

Strain[1]	Background	Genotype	Chromosomal Duplications	
			partial[2]	full[3]
YTT570	W303	*ume6*	1, 6	9, 16
YTT570	W303	*ume6*	1, 3, 6	9, 16
YTT570	W303	*ume6*	1, 3, 6, 9	16
YTT570	W303	*ume6*	1, 3, 6	16
YTT570	W303	*ume6*	1, 3, 5, 6, 9	16
RSY431	W303 (R.S.)[4]	*ume6*	1, 3, 6	2, 9
YTT622	S288C	*ume6*	1, 6	9
YTT572	W303	*isw2 ume6*	3, 6	1
YTT572	W303	*isw2 ume6*	1, 3, 6	9, 16
YTT572	W303	*isw2 ume6*	1, 3, 6	9, 16
YTT572	W303	*isw2 ume6*	1, 3, 6	9, 16
YTT572	W303	*isw2 ume6*	1, 3, 6	9, 16
YTT624	S288C	*isw2 ume6*	1, 6	9
YTT625	S288C	*isw2 ume6*	1, 6	3, 8, 9

1. Five independent DNA isolates (listed individually) were analyzed for YTT570 (*ume6*) and YTT572 (*isw2 ume6*).
2. Chromosomes with DNA contents that were moderately higher than normal were considered to be partial duplications. In every case, the DNA content of genes along the entire length of the partially duplicated chromosome was moderately increased, suggesting that the entire chromosome was duplicated in a portion of cells from which DNA was prepared, rather than that a portion of the chromosome was duplicated in all cells.
3. Chromosomes with DNA contents that were substantially higher than average were considered to be full duplications (duplicated in all or nearly all cells).
4. RSY431 is a *ume6* mutnt in the W303 yeast background from the laboratory of Randy Strich.

Table 2.5. Increased rate of minichromosome loss in *ume6* mutants

Genotype	Red Colonies	Half-Sectored Colonies	Total Colonies	Loss Rate Generation[1]
Wild Type	19	4	6944	5.8×10^{-4}
isw2	47	5	5248	9.6×10^{-4}
ume6	183	36	1225	3.6×10^{-2}
isw2 ume6	154	26	1924	1.5×10^{-2}

[1]Loss rate per generation = half-sectored colonies / (total colonies – red colonies) (Yoon and Carbon, 1999)

chromosomal duplications observed in *ume6* mutants result from chromosome missegregation events.

Isw2 complex is required for formation of DNaseI-inaccessible chromatin structure at two Ume6-independent loci

Chromatin structure analysis of one gene (*REC104*) targeted for repression by Isw2 and Sin3-Rpd3 complexes via Ume6p revealed that Isw2 complex forms DNaseI-inaccessible chromatin structure upstream of the Ume6p-binding site (URS1) (Goldmark et al. 2000). In *isw2* mutants, increased accessibility of chromatin near the URS1 sequence appeared to be due to a shift in the positions of two or three nucleosomes directly upstream of this site. Formation of inaccessible chromatin structure at this site by Isw2 complex requires a functional *UME6* gene, but is unaffected by mutation of the *RPD3* gene.

Because formation of DNaseI-inaccessible chromatin structure by Isw2 complex occurs directly adjacent to the Ume6p binding site, it is possible that Ume6p modifies or regulates the activity of Isw2 complex upon recruitment, in a manner unique to Ume6-target genes. If this is correct, Isw2 complex may modify chromatin structure differently at loci where it functions independently of Ume6p. Alternatively, Isw2 complex may function similarly to create inaccessible chromatin structure at all genes for which it regulates chromatin structure, regardless of the mechanism of recruitment. To distinguish between these possibilities, we analyzed chromatin structure of the *POT1* gene, using micrococcal nuclease and DNaseI digestions of chromatin, followed by indirect end-labeling (Figure 2.2a). The *POT1* gene was selected because it is repressed in parallel pathways by Isw2 and Sin3-Rpd3 complexes, yet data from both microarray and Northern blotting experiments confirm little or no change in *POT1* expression is observed in a *ume6* mutant (Figure 2.2a; see microarray data set on the web). In addition, no Ume6p-binding site (URS1) is present in the *POT1* upstream regulatory region. As previously observed for the *REC104* gene, the positions of three nucleosomes at the *POT1* locus appear to change in the *isw2* mutant, while no change is observed in

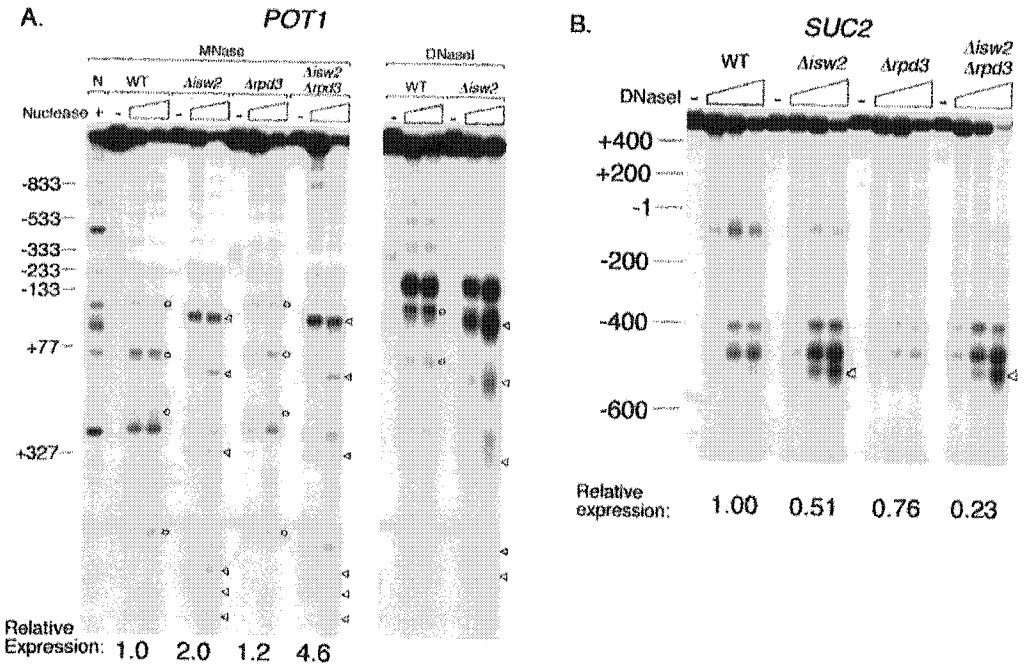


Figure 2.2. Isw2 complex is required for formation of DNaseI-inaccessible chromatin structure at two Ume6-independent loci.
(A) Increased DNaseI-accessibility and improperly positioned nucleosomes at the *POT1* locus in *isw2* mutant cells. Chromatin structure analysis was performed using MNase or DNaseI digestion followed by indirect-end labeling. MNase and DNaseI cleavage sites enhanced in wild type (WT) or *rpd3* mutant cells are marked with circles; those enhanced in *isw2* mutant cells are marked with triangles. N denotes naked DNA control. Relative expression levels indicated below each mutant were measured by Northern blotting. For reference, *POT1* expression measured by Northern blotting in a *ume6* mutant is 1.2 fold of wild type.
(B) Decreased expression of the *SUC2* gene in *isw2* mutants despite increased DNaseI accessibility of the upstream regulatory region. Chromatin structure analysis was performed using DNaseI digestion followed by indirect-end labeling. The DNaseI cleavage site enhanced in *isw2* mutant cells is marked with a triangle. Relative expression levels indicated below each mutant were measured by Northern blotting.

the *rpd3* mutant. As we observed previously for the *REC104* gene, these changes are accompanied by an increase in DNaseI hypersensitivity near the promoter. In contrast to the *REC104* locus, chromatin structure changes at the *POT1* locus extend well into the coding region in *isw2* mutant cells. Nevertheless, DNaseI-inaccessible chromatin structure is established at the *POT1* locus in the presence of Isw2 complex that is very similar to chromatin structure at the *REC104* locus. These data suggest that Isw2 complex may function similarly to remodel chromatin at these two loci, despite the fact that repression of *REC104* by Isw2 complex requires *UME6* function, while repression of *POT1* transcription is *UME6*-independent.

Although Isw2 complex appears to have only a small role in transcriptional activation or maintenance of basal transcription , we found that transcription of one gene (*SUC2*) was significantly reduced in *isw2*, *sin3*, *rpd3*, *isw2 sin3* and *isw2 rpd3* mutants (see full data set). Expression of *SUC2* was lower in *isw2 sin3* and *isw2 rpd3* double mutants than in any single mutant, indicating that Isw2 and Sin3-Rpd3 complexes affect *SUC2* transcription independently. These data were confirmed by Northern blotting (Figure 2.2b, "Relative Expression"). Under the conditions of RNA isolation for these studies (2% glucose media) the *SUC2* gene is actively repressed. Therefore, our results suggest that Isw2 and Sin3-Rpd3 complexes are required for low levels of basal *SUC2* transcription. Chromatin structure of the *SUC2* promoter has been studied extensively, revealing a role for Swi/Snf complex in creation of nuclease-accessible chromatin during transcriptional activation (Hirschhorn et al. 1992). Since increased transcription is often associated with more accessible chromatin structure, it is possible that Isw2 complex also functions to make chromatin more accessible at the *SUC2* locus, in contrast to its functions to create DNaseI-inaccessible chromatin structure at the *REC104* and *POT1* loci.

To test this possibility, we probed chromatin structure of the *SUC2* locus using DNaseI digestions of chromatin, followed by indirect end-labeling (Figure 2.2b). The DNaseI digestion patterns of wild type and *isw2* mutant chromatin are very similar for the *SUC2* open reading frame and much of the upstream regulatory region. However, a

notable increase in DNaseI cleavage is observed approximately 500 base pairs upstream of the initiation codon in the *isw2* mutant. As we previously found for the *REC104* and *POT1* loci, the DNaseI digestion pattern of *rpd3* mutant chromatin was very similar to that of wild type. Thus, at the *SUC2* locus, as with the *REC104* and *POT1* loci, Isw2 complex, but not Sin3-Rpd3 complex, is required for creation of DNaseI-inaccessible chromatin structure. However, unlike the *REC104* and *POT1* genes, the increase in DNaseI accessibility is associated with a decrease in *SUC2* transcription in the *isw2* mutant. These data suggest that Isw2 complex functions by a common mechanism at Ume6-dependent and –independent loci to create DNaseI-inaccessible chromatin structure, and that *ISW2*-dependent inaccessible chromatin structure can affect transcription both positively and negatively.

Discussion

Because we previously found that functional Sin3-Rpd3 complex could often compensate for loss of Isw2 function in repression of common target genes, we compared the transcriptional profiles of mutants defective in one or both complexes to determine the full extent of their overlap. In addition, we wished to determine the extent to which the repressive functions of Isw2 and Sin3-Rpd3 complexes depend on Ume6p. Our data point to four main conclusions. First, Isw2 complex functions in a parallel pathway to Sin3-Rpd3 complex to repress transcription of a substantial number of genes. We find that synthetic phenotypes (those observed only when an *isw2* mutation is combined with a *sin3* or *rpd3* mutation) are more consistently revealed using double mutant versus single mutant hybridizations, especially for genes that are tightly repressed in wild type cells. While the largest single group of genes repressed by these complexes consists of Ume6-target genes, the majority of genes that require Isw2 and Sin3-Rpd3 complexes for repression are Ume6-independent.

Second, chromatin structure analyses suggest that Isw2 complex is required to create DNaseI-inaccessible chromatin structure in the upstream regulatory regions of two Ume6-independent genes. At this time, we cannot exclude the possibility that the

observed changes in chromatin structure and transcription are due to indirect effects of *isw2* mutation, since we have been unable to localize Isw2 complex to specific chromosomal loci. The *rpd3* mutant is defective in regulation of the *POT1* and *SUC2* genes to a similar extent as the *isw2* mutant. However, only cells with the *isw2* mutation show the mutant nuclease digestion patterns at these loci, suggesting against the possibility that chromatin structure defects in *isw2* mutant cells result from misregulation of transcription. Because *SUC2* transcription is *decreased* in *isw2* mutant cells, it appears that Isw2-dependent DNaseI-inaccessible chromatin structure can have different effects on transcription at different loci. While it is not clear why inaccessible chromatin structure results in increased transcription of the *SUC2* gene, one possibility is that it partially inhibits binding of a transcriptional repressor of *SUC2*. Consistent with this possibility, a binding site for the Mig1 repressor is located very close (at –499 with respect to the initiation codon) (Bu and Schmidt 1998) to the DNaseI hypersensitive site present only in *isw2* mutant cells.

Recently, Kent et al showed that Isw2 complex is required for formation of wild type chromatin structure upstream of three randomly selected Ume6-independent genes: *FIG1, MET17* and *PHO3*, as revealed by differences in their MNase cleavage patterns in *isw2* mutant cells relative to wild type (Kent et al. 2001). However, transcription of the *FIG1* and *PHO3* genes is unaffected by *isw2* mutation and *MET17* transcription is only slightly increased in *isw2* mutant cells (see full data set). It is therefore possible that regulation of chromatin structure at these loci by Isw2 complex serves a function other than transcriptional regulation. Alternatively, additional chromatin remodeling factors may function in parallel to regulate transcription of these genes. In this case, loss of Isw2 function might be masked by these parallel factors, analogous to the relationship between Isw2 and Sin3-Rpd3 complexes in repression of some early meiotic genes. Taken together, the data suggest that Isw2 complex functions by a common mechanism at Ume6-dependent and –independent loci to create nuclease-inaccessible chromatin structure, and that *ISW2*-dependent inaccessible chromatin structure can affect transcription positively, negatively, or not at all, depending on the specific context.

Third, there are considerable differences in transcriptional profiles between deletion and catalytically inactive mutants of *isw2* and *rpd3*. These differences are most evident for *rpd3* mutants, which suggest the presence of deacetylase-independent and Sin3-independent functions of Rpd3p, as well as the possibility that catalytically inactive Rpd3p may inhibit other repressors of transcription. Consistent with these differences, *rpd3* deletion mutations show a synthetic growth defect in an *isw2* mutant background, whereas catalytically inactive mutations of *rpd3* and deletion mutations of *sin3* show less severe synthetic growth defects. In contrast, a recently published comparison of the transcriptional profiles of *rpd3* and *sin3* deletion mutants suggests that virtually all Rpd3p function requires Sin3p (Bernstein et al. 2000). The major difference between this report and our data is our finding that a large number of genes require *RPD3* but not *SIN3* for repression. These conflicting conclusions might be explained by differences in strain background or conditions utilized for growth of yeast cells. However, we previously observed phenotypic differences between *rpd3* and *sin3* deletion mutants in two different yeast strain backgrounds with regard to their synthetic growth defect in an *isw2* mutant background (Goldmark et al. 2000). These data support the conclusion that Rpd3 has some Sin3-independent functions in vivo.

Fourth, Ume6p has one or more roles in chromosome segregation during haploid mitotic growth that are independent of Isw2 and Sin3-Rpd3 complexes. As a result, haploid *ume6* mutants tend to accumulate chromosomal duplications. For this reason, expression data for the *ume6* and *isw2 ume6* mutants should be interpreted with caution, since chromosomal duplications result in artificial inflation of genes on the duplicated chromosomes and may indirectly affect expression of additional genes. Despite this fact, we find that a large group of genes appear to be regulated by Isw2 and Sin3-Rpd3 complexes independently of Ume6p. It is noteworthy that the expression profiles of *sin3, rpd3, isw2 sin3* and *isw2 rpd3* mutants show no signs of chromosomal duplications in these strains. In contrast, Hughes et al previously found chromosomal duplications associated with *rpd3/rpd3* and *sin3/sin3* homozygous mutant diploids (Hughes et al. 2000b). These differences may result from differences in strain background or growth

media, or possibly different effects of *sin3* and *rpd3* mutations in haploid and diploid cells. Nevertheless, our data suggest that Ume6p has one or more functions in chromosome segregation during haploid mitotic growth that are independent of Sin3-Rpd3 and Isw2 complexes under the conditions tested. It is possible that Ume6p regulates transcription of specific genes independently of Sin3-Rpd3 and Isw2 complexes. Alternatively, Ume6p may function more directly in some aspect of chromosome segregation. The bias observed for duplications of chromosomes 9 and 16 may reflect a growth advantage associated with extra copies of these chromosomes in *ume6* mutant cells. However, it is also possible that these chromosomes are more susceptible than others to missegregation in *ume6* mutants.

Experimental Procedures

Strains

Unless indicated, all yeast strains were derived from W1588-4C. This strain is congenic to W303-1A, except that a weak *rad5* mutation in the original W303 is repaired (Zhao et al. 1998). Deletion and catalytically inactive mutations of the *ISW2, SIN3, RPD3,* and *UME6* genes were described previously (Goldmark et al. 2000). The CFIII minichromosome (Spencer et al. 1990) was introduced into isogenic wild type and mutant strains by crossing an *isw2 ume6* double mutant (YTT1065) with SBY475 (courtesy of Sue Biggins, FHCRC). The colony sectoring assay for measuring rates of minichromosome loss was done as previously described (Hieter et al. 1985; Yoon and Carbon 1999).

RNA isolation

RNA samples were prepared from mutants and wild type cells grown at 30°C in YEPD medium (2% bacto peptone, 1% yeast extract, 2% glucose) to early log phase (OD_{660}=0.7) using acid phenol extraction. To ensure identical growth conditions, each strain was grown on media aliquoted from a common preparation. mRNA was prepared using Oligotex beads (Qiagen) as described by the manufacturer.

Production of Spotted Microarrays

Microarray construction and hybridization protocols were modified from those described elsewhere (DeRisi et al. 1997). Yeast microarrays were constructed using a set of ~6200 ORF-specific PCR primer pairs (Research Genetics, Huntsville, AL), which were used to amplify each open reading frame of the yeast genome. Individual PCR products were verified as unique via gel electrophoresis and purified using ArrayIt 96-well PCR purification kits (TeleChem International, Sunnyvale, CA). Purified PCR products were mechanically "spotted" in 3X SSC (450 mM sodium chloride and 45 mM sodium citrate, pH 7.0) onto poly-lysine coated microscope slides using an OmniGrid high-precision robotic gridder (GeneMachines, San Carlo, CA).

Microarray Hybridizations and Data Analysis

The protocol used for cDNA labeling was a modification of a protocol described elsewhere (http://cmgm.stanford.edu/pbrown/protocols/aadUTPCouplingProcedure.htm). Briefly, labeled cDNA targets were prepared by reverse transcription of 2 μg mRNA using oligo dT(18) primer in the presence of 0.2 mM 5-(3-aminoallyl)-2'-deoxyuridine-5'-triphosphate (aa-dUTP; Sigma-Aldrich Company, St. Louis, MO), 0.3 mM dTTP, and 0.5 mM each of dATP, dCTP,and dGTP. Following cDNA synthesis, either Cy3 or Cy5 mono-reactive fluors (Amersham Life Sciences, Arlington Heights, IL) were covalently coupled to the cDNA-incorporated aminoallyl linker in the presence of 50 mM Sodium Bicarbonate (pH 9.0). Two color expression profiles were generated using microarrays in which reference and experimental cDNA targets were labeled with different fluors. Following co-hybridization to the chip, a fluorescent image of the microarray was collected at both emission wavelengths using a GenePix 4000 fluorescent scanner (Axon Instruments, Inc., Foster City, CA) and image anaylsis was performed using GenePix Pro Microarray Acquisition and Analysis Software.

Four microarray hybridizations were carried out for each mutant versus wild type or mutant versus mutant comparison (two sets of two reverse fluor combinations). We

carried out a Bayesian background correction, described in detail in Kooperberg et al (Kooperberg et al. 2002). Briefly, we assume that both the foreground and background intensities of both channels for each spot come from a normal distribution with an unknown mean and variance. After estimating the variance parameters from the data, we can estimate for each channel the posterior distribution of the difference between the foreground and the background mean as well as their ratio, which is the quantity of interest. To do this, we assume an uninformative uniform prior on the intensities. The effect of this procedure is that for genes where the intensities are high, the estimated ratio is very similar to the traditional estimate, but that for spots where the foreground intensity is close to the background intensity the estimates for the ratios are shrunk slightly towards one, yielding a substantial reduction in the variance for these spots.

All calculations involving expression ratios were carried out on the (natural) log scale by computing averages and standard errors. Averages and standard errors were then converted to ratios by assuming that the average of the log-ratios follows the normal distribution (which seems reasonable given both the error distribution of log-ratios, and the central limit theorem). The average ratio then has a log-normal distribution. This average (ave) and its standard error (SE) relate to the average and the standard error of the log-ratios by:

$$ave_{ratio} = \exp(ave_{log\ ratio} + SE^2_{log\ ratio}/\ 2) \qquad SE_{ratio} = ave_{ratio} * [\exp(SE^2_{log\ ratio})-1]^{1/2}$$

as described (Johnson and Kotz 1970).

For analysis of the genomic location of misregulated genes and cis-element searches of promoter sequences, we used the GENESPRING software package (Silicon Genetics).

Chromatin Structure Analysis

Digestion of chromatin was performed on crude preparations of nuclei (Hirschhorn et al. 1992) as described previously (Goldmark et al. 2000). After digestion with micrococcal nuclease or DNaseI, DNA was purified, digested with EcoRI and PstI (for *POT1*) or EcoRI and XbaI (for *SUC2*) and subjected to indirect end-labeling. The probe used for

indirect end labeling of the *POT1* locus was a PCR product extending from +406 to +623 with respect to the initiation codon. The probe used for indirect end-labeling of the *SUC2* locus was a PCR product extending from –885 to –661 with respect to the initiation codon.

Chapter III: Chromatin remodeling *in vivo*: evidence for a nucleosome sliding mechanism

Summary

Members of the ISWI family of chromatin remodeling factors exhibit ATP-dependent nucleosome sliding, loading, and spacing activities *in vitro*. However, it is unclear which of these activities are utilized by ISWI complexes to remodel chromatin *in vivo*. We therefore sought to identify the mechanisms of chromatin remodeling by *Saccharomyces cerevisiae* Isw2 complex at its known sites of action *in vivo*. To address this question, we developed a method of identifying intermediates of the Isw2-dependent chromatin remodeling reaction as it proceeded. We show that Isw2 complex catalyzes nucleosome sliding at two different classes of target genes *in vivo*, in each case sliding nucleosomes closer to the promoter regions. In contrast to its biochemical activities *in vitro*, nucleosome sliding by Isw2 complex *in vivo* is unidirectional and localized to a few nucleosomes at each site, suggesting that Isw2 activity is constrained by cellular factors.

Introduction

Regulation of chromatin structure is crucial to many cellular processes that require access to DNA, such as transcription, DNA replication, and recombination. Chromatin structure is regulated on several levels, from chemical modifications of DNA and histones to proteins that affect higher order chromatin structure and nuclear localization. One class of chromatin regulators, ATP-dependent chromatin remodeling factors, functions by altering contacts between histone octamers and DNA, resulting in repositioning of nucleosomes, displacement of histones from DNA, loading of new nucleosomes, or alteration of the nucleosome structure (Havas et al. 2001; Varga-Weisz 2001; Becker and Horz 2002; Narlikar et al. 2002; Neely and Workman 2002a; Peterson

2002; Tyler 2002). ATP-dependent chromatin remodeling factors are classified into different families based on the ATPase subunit found within each complex, including SWI/SNF, ISWI, CHD1, INO80 and RAD54, each with distinct biochemical activities and in vivo functions.

Members of the ISWI class of factors have been purified from diverse eukaryotes ranging from yeast to humans (Langst and Becker 2001b). All ISWI complexes tested to date exhibit nucleosome sliding activities *in vitro* (Hamiche et al. 1999; Langst et al. 1999; Kassabov et al. 2002; Vary et al. 2003), the precise mechanism of which is unknown. In addition, subsets of ISWI complexes exhibit nucleosome spacing (Ito et al. 1997; Varga-Weisz et al. 1997; Tsukiyama et al. 1999; LeRoy et al. 2000; Loyola et al. 2001) and loading (Ito et al. 1997; LeRoy et al. 2000; Loyola et al. 2001) (chromatin assembly) activities which may function to create regular arrays of nucleosomes on newly synthesized DNA or to restore chromatin structure at regions where it has been disrupted by alternative chromatin remodeling complexes, transcription, or other processes. In contrast, the *in vivo* functions of ISWI complexes are just beginning to be discovered, with roles in regulation of transcription (Goldmark et al. 2000; Fazzio et al. 2001; Kent et al. 2001; Alen et al. 2002; Badenhorst et al. 2002), chromosome structure (Deuring et al. 2000), development (Deuring et al. 2000; Badenhorst et al. 2002; Yasui et al. 2002), DNA replication (Bozhenok et al. 2002; Collins et al. 2002) and chromosome segregation (Hakimi et al. 2002) recently identified. However, for few of these examples are the direct chromosomal loci targeted by ISWI complexes known.

We and others have previously identified several loci that require Isw2 complex for the formation of wild type chromatin structure in yeast (Goldmark et al. 2000; Fazzio et al. 2001; Kent et al. 2001; Alen et al. 2002); in an *isw2* mutant a few nucleosomes are shifted in position relative to wild type at each locus. While this type of analysis provides information about where Isw2 complex is acting and the consequences of *isw2* mutation, it provides little insight into the mechanism utilized by Isw2 complex to generate wild type chromatin structure. For example, at several Isw2 target genes, a strong nuclease-hypersensitive site is located near the promoter in an *isw2* mutant, but

not in wild type cells (Goldmark et al. 2000; Fazzio et al. 2001). Thus, Isw2 complex might function by loading a new nucleosome at this nucleosome-free site and repositioning surrounding nucleosomes to accommodate the addition. Alternatively, Isw2 complex could slide a few nucleosomes from immediately upstream or downstream regions to occupy the positions observed in wild type cells. Yet another possibility is that Isw2 complex shifts the positions of an extended array of nucleosomes each by a fixed distance, forcing a nucleosome at one end of the array into the previously nucleosome-free region. To test these possibilities, we examined the mechanism of chromatin remodeling by Isw2 complex at two distinct classes of target genes *in vivo*. We find that, in each case, Isw2 complex slides nucleosomes a short distance toward the promoter. In addition, we find that Isw2-dependent chromatin remodeling *in vivo* is directed to specific regions, in contrast to the broadly functioning activities described for ISWI complexes *in vitro*.

Results and Discussion

Nucleosomes migrate upstream at the *POT1* gene upon *ISW2* induction

To examine the mechanism of Isw2-dependent chromatin remodeling in vivo, we sought to identify intermediates of the chromatin remodeling reaction as chromatin structure is converted from the *isw2* mutant (*ISW2* off) state to the wild type (*ISW2* on) state as diagrammed in Figure 3.1a. To achieve rapid induction of the *ISW2* gene, we created a galactose-inducible allele of *ISW2* at its native chromosomal locus (Figure 3.1a). Cells grown in raffinose medium express the *ISW2* gene at a very low level. After galactose addition, Isw2p associates with chromatin at low levels after 30 minutes and at increasing levels thereafter (Figure 3.1b and c). After two hours of galactose induction, Isw2p is associated with chromatin at levels slightly lower than those found in wild type cells, indicating that Isw2 complex is not saturating chromatin during the time course.

Initially we focused on chromatin structure at the *POT1* locus, a previously identified target of Isw2 complex in which three nucleosomes appear to be shifted in position in *isw2* mutant cells, resulting in partial derepression of the gene

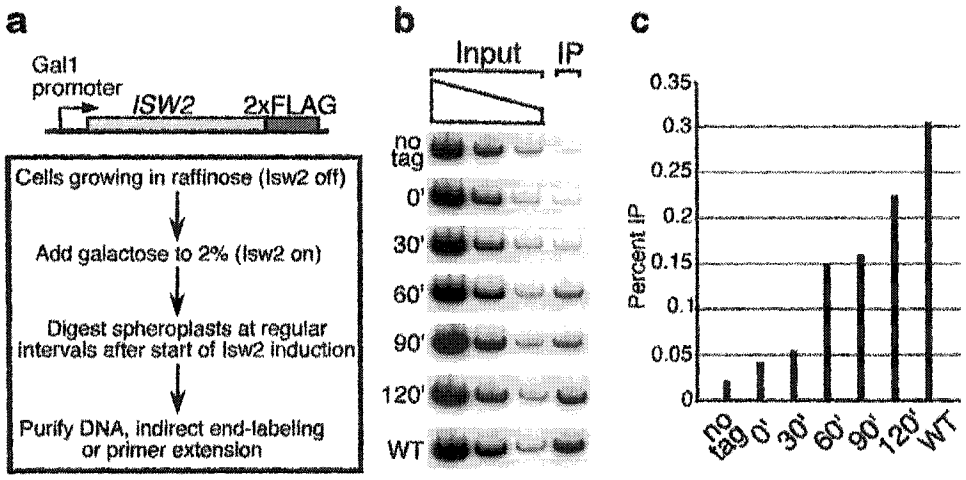


Figure 3.1. Association of Isw2 complex with chromatin upon induction with galactose. a, Diagram of the system. b, Kinetics of Isw2p association with the *POT1* locus as measured by chromatin immunoprecipitation. 50-, 250-, and 1250-fold dilutions of DNA isolated from input chromatin are shown for comparison. Shown is one of two independent experiments that gave similar results. c, Quantitation of the chromatin immunoprecipitation data shown in (b), expressed as a percentage of the input.

(Fazzio et al. 2001). We used low-resolution indirect end-labeling analysis to obtain a broad view of the changes in chromatin structure during *ISW2* induction. Three probes of chromatin structure were used for this analysis: DNaseI, micrococcal nuclease (MNase), and methidiumpropyl-EDTA-iron(II) (MPE-Fe^{2+}), in order to avoid cleavage-site biases that may be present in any one reagent. Each reagent cleaves DNA preferentially in non-nucleosomal regions; therefore, cleavage sites common to all probes represent linker DNA between nucleosomes while the protected sites are likely due to the presence of nucleosomes.

Before galactose addition, and shortly after, chromatin structure at the *POT1* locus appears identical to that of *isw2* null mutant cells (Figure 3.2a-c; compare Δ*isw2*, 0' and 20'), whereas chromatin structure is similar (but not identical) to wild type cells at later time points (compare 120', 140' with wild type). Close inspection of intermediate time points reveals a gradual upstream migration of three nucleosomes (as inferred by appropriately sized regions of nuclease protection) beginning at 40 minutes and proceeding throughout the time course until reaching the positions observed in wild type cells. Since chromatin remodeling is unlikely to occur synchronously in all cells, the intermediates observed likely reflect a mixture of cells at different stages of the chromatin remodeling reaction at each time point. This phenotype is particularly evident for the nuclease-sensitive region located at ~ +50 in the *isw2* mutant (near the indicated NcoI site), as revealed by a "smearing" upstream of nuclease sensitivity beginning 40 minutes after galactose addition. We therefore conclude that Isw2 complex functions to direct three downstream nucleosomes into slightly more upstream positions, closer to the *POT1* promoter. Chromatin remodeling during the time course did not appear to be due to indirect effects of galactose addition, as Δ*isw2* and wild type cells were unaffected by carbon source (data not shown).

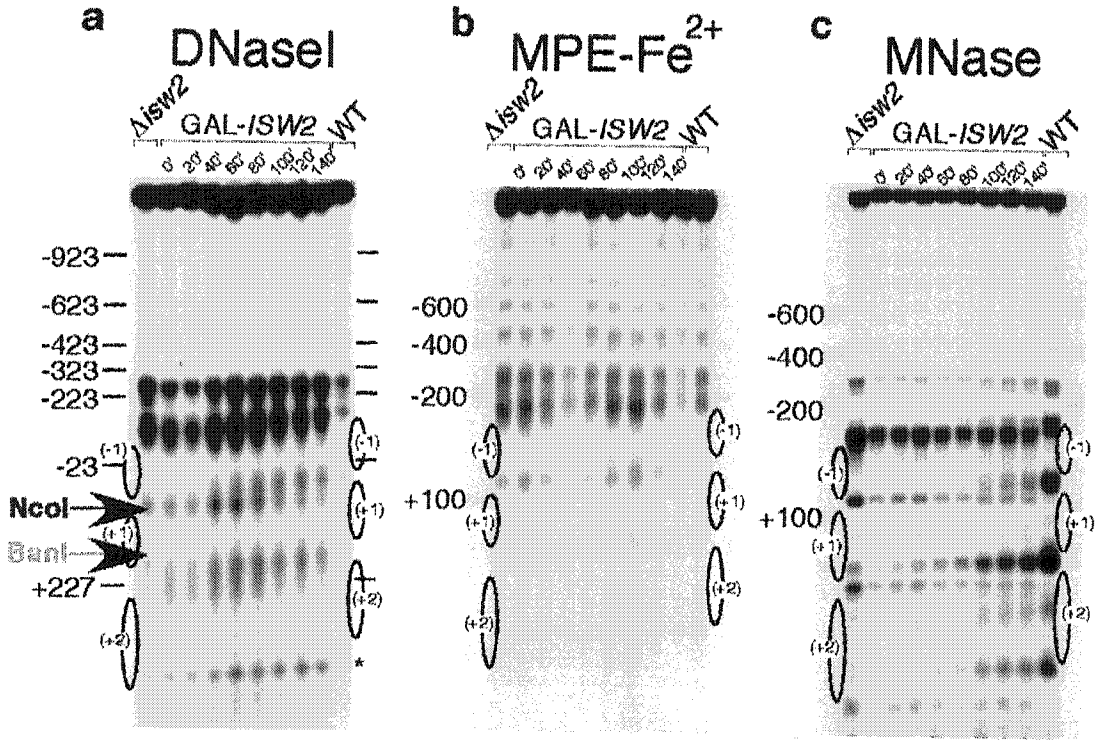


Figure 3.2. Nucleosomes migrate upstream toward the *POT1* promoter upon *ISW2* induction. Indirect end labeling of chromatin samples digested with a, DNaseI, b, methidiumpropyl-EDTA-iron(II) (MPE-Fe^{2+}) and c, micrococcal nuclease (MNase) during a time course of *ISW2* induction. Times after galactose addition are indicated. D*isw2* and wild type (WT) samples are shown for comparison. Inferred nucleosome positions for *isw2* and wild type cells are shown to the left and right, respectively, of each panel. The band marked by an asterisk is a cleavage or hybridization artifact also observed when cells untreated with nuclease are subjected to the same DNA isolation, restriction digestion and blotting procedure (Fazzio et al., 2001).

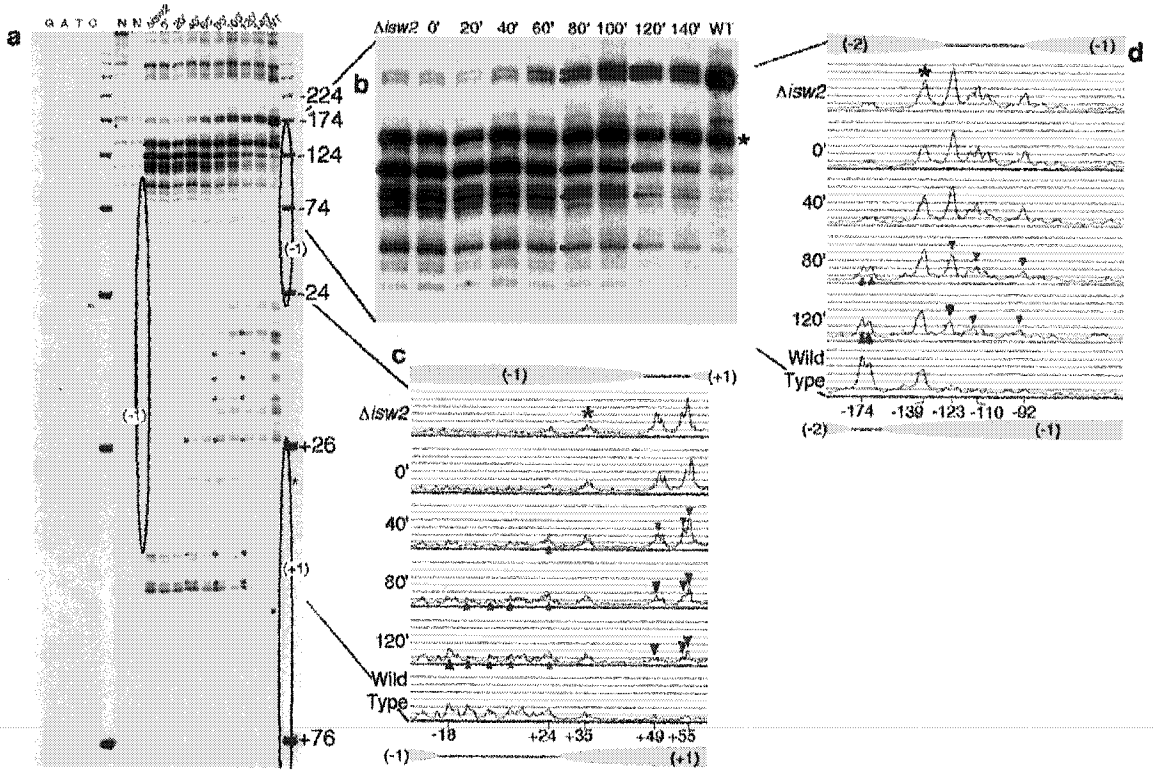


Figure 3.3. High resolution mapping of chromatin remodeling intermediates reveals Isw2-dependent nucleosome sliding at the *POT1* locus. a, DNA samples from micrococcal nuclease-digested chromatin were subjected to primer extension analysis using a primer corresponding to base pairs +126 to +96 with respect to the *POT1* initiation codon. Shown for reference is a 50 bp ladder, digestions of naked DNA (N), and dideoxy sequencing reactions (G, A, T, C), as well as Δ*isw2* and wild type (WT) samples. Small blue dots mark small to moderate increases or decreases in nuclease sensitivity, while large red dots indicate larger changes. Asterisks indicate MNase cleavage sites that persist throughout the time course. These sites are located within stretches of AT-rich sequences, which are hypersensitive to MNase cleavage. b, magnified view of the upstream region in (a). c and d. Densitometry analysis of MNase cleavage sites during the chromatin remodeling time course. Peaks represent the intensity of bands, normalized for loading. Blue and red arrows mark the peaks noted in (a) and (b) by dots of the same color. Upward and downward pointing arrows indicate increasing and decreasing MNase sensitivity, respectively. Inferred nucleosome positions in Δ*isw2* and wild type cells are indicated above and below each panel.

Isw2 complex slides nucleosomes into their proper positions in *vivo*

To gain greater insight into the mechanism of Isw2-dependent chromatin remodeling at the *POT1* locus, we analyzed remodeling intermediates at higher resolution using primer extension analysis (Figure 3.3a, b). We focused on the (-1) and (+1) nucleosomes, separated by linker DNA spanning base pairs +49 to +55 in the *isw2* mutant and -18 to +24 in the wild type. In cells with the inducible *ISW2* allele, MNase sensitivity shifts gradually from downstream to upstream upon induction. The wild type linker region begins to become accessible to MNase at 40', at its downstream end (Figure 3.3a, marked by blue dot). Subsequently, the remainder of the wild type linker region becomes accessible (Figure 3.3a, 60'-80'). By the end of the time course (120'-140'), the upstream MNase cleavage site located at –18 is strongly enhanced (Figure 3.3a, red dot), as is seen in wild type cells. Complimentary changes are observed at the upstream edge of the nucleosome: MNase cleavage sites at -92, -110, and -123 are suppressed as the time course progresses, while two strong cleavage sites emerge at -174 and -173, indicative of the newly forming linker region upstream of the remodeled nucleosome.

To obtain an unbiased view of these data, we quantified and graphed the intensity of MNase cleavage at each site as the time course of *ISW2* induction proceeded by phosphorimager. These quantitative data confirm the progression of MNase sensitivity from downstream to upstream within the (-1) to (+1) linker region (Figure 3.3c), as well as the corresponding progression from downstream to upstream of MNase protection upstream of the (-1) nucleosome (Figure 3.3d). For example, the MNase cleavage site at -24 appears and reaches its maximum level of MNase-sensitivity 40 minutes after *ISW2* induction (Figure 3.3c). In contrast, the cleavage site at -18 begins to appear at 80 minutes and only reaches its maximum level of MNase-sensitivity after 120 minutes. Taken together, these data strongly suggest that Isw2 complex functions by a nucleosome sliding mechanism at the *POT1* locus, specifically by sliding nucleosomes from downstream to upstream. Alternative models, such as sliding nucleosomes from upstream regions into the wild type locations, or loading a new nucleosome in the

vicinity of the upstream hypersensitive site, are inconsistent with the observed gradual migration of nuclease accessibility.

Isw2 complex slides nucleosomes toward its site of recruitment

While nucleosome sliding could represent a general feature of Isw2-dependent chromatin remodeling *in vivo*, this mechanism may be unique to the *POT1* locus. To address this possibility, we analyzed changes in chromatin structure at the *REC104* locus during a time course of *ISW2* induction using primer extension analysis. In contrast to *POT1*, chromatin remodeling at *REC104* is dependent upon Ume6p, which binds to the URS1 sequence element (Park et al. 1992; Mitchell 1994; Anderson et al. 1995) and recruits both Isw2 complex and the Rpd3/Sin3 histone deacetylase complex to the promoters of early meiotic genes (Kadosh and Struhl 1997; Rundlett et al. 1998; Goldmark et al. 2000). As with *POT1*, chromatin remodeling at *REC104* results in nuclease-inaccessible chromatin near the promoter. However, at the *REC104* locus this is accomplished by moving upstream nucleosomes further downstream. The (-1) nucleosome migrates approximately 60 base pairs downstream into proximity of the URS1 sequence (Figure 3.4a). Quantitation of these data reveals that MNase sensitivity migrates incrementally from upstream to downstream during the time course (Figure 3.4b). The two most prevalent MNase cleavage sites upstream of the (-1) nucleosome in wild type cells are located at -210 and -259. Upon *ISW2* induction, the upstream site (-259) begins to emerge at 40 minutes and reaches its maximum level of MNase sensitivity by 80 minutes, whereas the downstream (-210) cleavage site begins to emerge at 80 minutes and reaches maximal sensitivity only after 120 minutes. We therefore conclude that Isw2 complex slides upstream nucleosomes downstream toward its location at the URS1 site. In light of the known biochemical activities of ISWI complexes in vitro, Isw2 complex might perform this function by forcing linker DNA upstream of the URS1 site into the nucleosome (Figure 3.6) by either a twisting or a bulging mechanism (Havas et al. 2000; Langst and Becker 2001a; Kassabov et al. 2002).

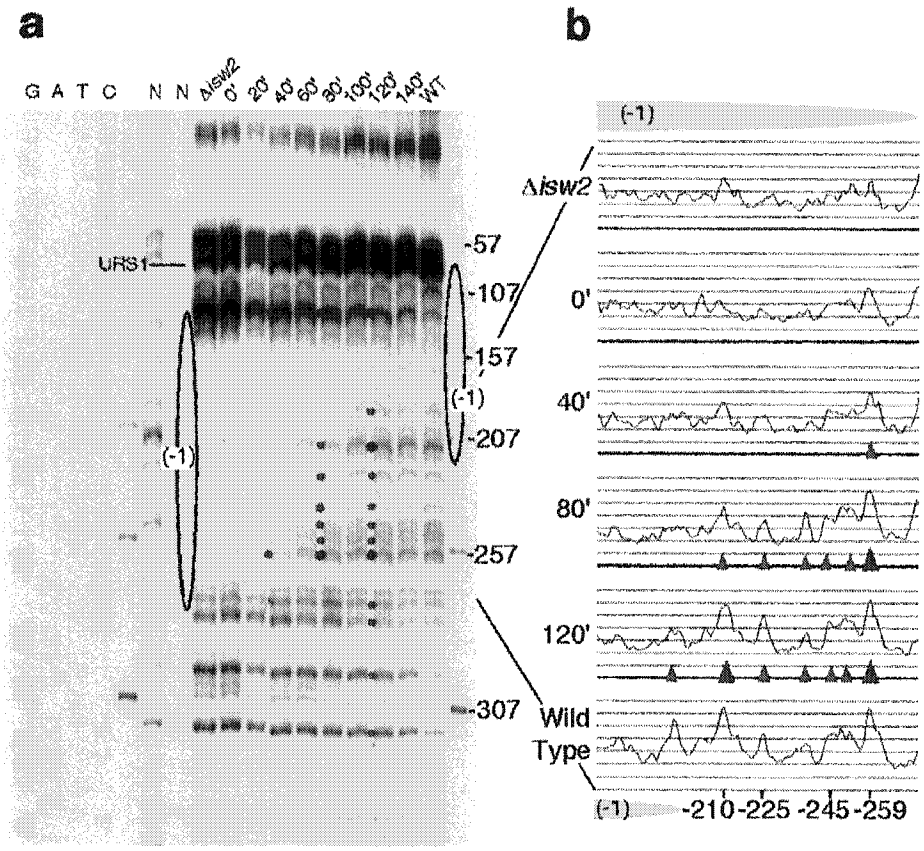


Figure 3.4. Analysis of chromatin structure at the *REC104* locus reveals Isw2-dependent nucleosome sliding at a second class of genes. a, Primer extension mapping of MNase cleavage sites at the *REC104* locus during a time course of *ISW2* induction. Labels are as in Figure 3a. The location of the Ume6p binding site (URS1) is indicated. b, Densitometry analysis of MNase cleavage sites during the chromatin remodeling time course. Labels are as in Figure 3c and d. The positions of labels in parentheses could not be precisely determined due to the resolution of the sequencing reactions, and were therefore estimated based on their positions.

Kinetics of Isw2-dependent nucleosome sliding

To obtain an independent and quantitative measure of the kinetics of chromatin remodeling during *ISW2* induction, we tested the accessibility of chromatin to the BanI and NcoI restriction enzymes, which have recognition sites near a remodeled nucleosome at the *POT1* locus (Figure 3.5a-c). In *Δisw2* mutant cells, the NcoI site is found in the linker region upstream of the (+1) nucleosome, while the BanI site is located within the (+1) nucleosome. Conversely, in wild type cells, the NcoI site is located within the (+1) nucleosome while the BanI site is exposed (Figure 3.2a). Due to the short digestion times used, BanI cleavage in wild type and NcoI cleavage in *Δisw2* mutant cells is partial (Figure 3.5c, bar graphs). During *ISW2* induction, NcoI accessibility remains high for the first 40 minutes, after which it decreases at a relatively steady rate throughout the remainder of the time course. BanI accessibility increases at a relatively steady rate following a similar 40-60 minute delay, upon addition of galactose. These kinetics appear to mirror the kinetics of Isw2p association with chromatin at the *POT1* locus (Figure 3.1c). This suggests that the rate of nucleosome sliding is limited by the levels of Isw2 complex in this system, which, in turn, are limited by the rate of induction of the *GAL1* promoter. These data are consistent with the rapid rate of chromatin remodeling observed in vitro for *Drosophila* NURF complex (Hamiche et al. 1999). The tight correlation between the rate of Isw2 association with chromatin and the rate of chromatin remodeling suggests that Isw2-dependent chromatin remodeling may occur independently of cell cycle stage. However, because these experiments have been performed on an asynchronous population of cells, we cannot rule out a possible requirement for passage through a particular cell cycle stage for remodeling to occur. Taken together, these data strongly suggest that the changes in nucleosome positions are the direct result of Isw2 complex activity.

Isw2 complex slides nucleosomes without disrupting nucleosomal integrity

The fact that the decrease in nuclease accessibility of DNA entering a sliding nucleosome (at the NcoI site) roughly mirrors the increase in accessibility of DNA

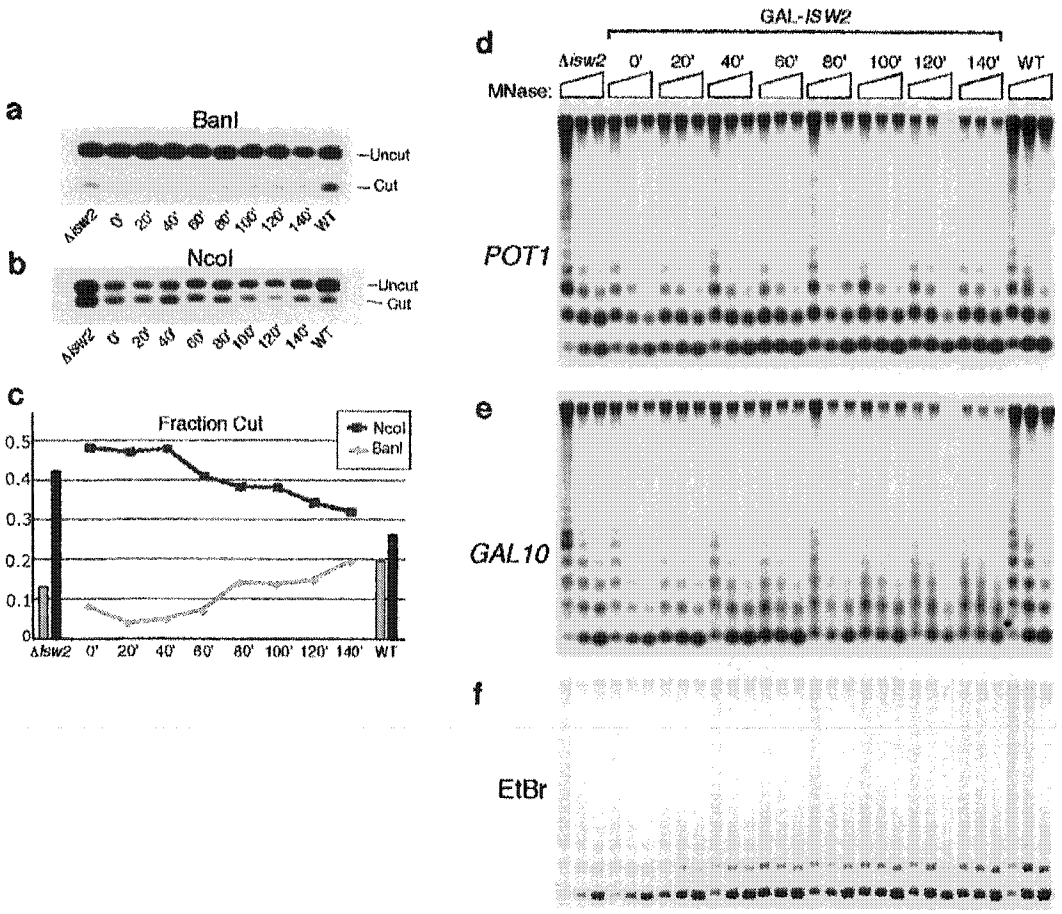


Figure 3.5. Isw2 complex slides nucleosomes immediately upon association with chromatin, without disrupting nucleosomal integrity. a and b, Accessibility of chromatin to the BanI and NcoI restriction enzymes over a time course of *ISW2* induction. Times after galactose addition are indicated. Locations of the BanI and NcoI recognition sites are indicated in Figure 2a. For each enzyme, one of two experiments with very similar results is shown. c, Quantitation of the data in (a) and (b) reveal the kinetics of chromatin remodeling at the *POT1* locus. d and e, Sensitivity of chromatin at the *POT1* (d) and *GAL10* (e) loci to extensive MNase digestion during galactose-mediated induction of *ISW2*. Three doubling concentrations of MNase are shown for each time point. Wild type (WT) and *Δisw2* control cells were grown in raffinose medium and processed identically to the inducible strain. f, Ethidium bromide stain of the digested DNA blotted in (d) and (e), indicating the bulk chromatin digestion pattern.

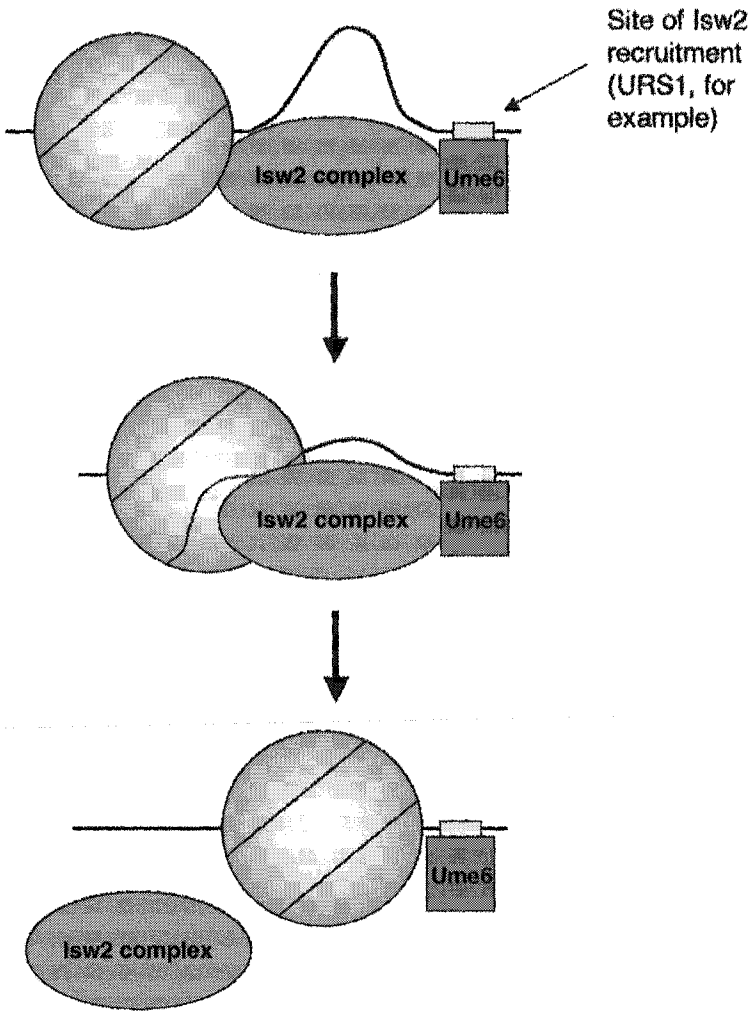


Figure 3.6 Nucleosome sliding model for Isw2 complex. Isw2 first binds to the nucleosome immediately adjacent to its site of recruitment (URS1 in this example). Isw2 complex then forces some of the intervening linker DNA into the nucleosome, forming a "bubble" of extra DNA that traverses the entire 1.65 turns of DNA wrapped around the nucleosome. After the bubble exits the nucleosome on the other side, the net result is sliding of the nucleosome toward the site of Isw2 recruitment.

exiting the nucleosome (at the BanI site), suggests that DNA remains tightly associated with the histone octamer throughout the sliding reaction. To test this possibility further, we digested chromatin with high levels of micrococcal nuclease during a time course of *ISW2* induction, and tested the degree to which DNA is protected at specific loci by the presence of intact nucleosomes. Since this experiment is performed on extensively digested chromatin in the absence of indirect end-labeling, it provides information only on the integrity of the nucleosomal array, rather than where nucleosomes are positioned (Almer and Horz 1986; Almer et al. 1986); to the extent that nucleosomes are disrupted or absent from a particular region, one expects to observe smearing of the DNA within the nucleosomal ladder when the region is probed (Gregory and Horz 1999). Upon probing the region of the *POT1* gene at which Isw2 complex acts, we observed no perturbation of the nucleosomal array at any point throughout the time course (Figure 3.5d), as the *POT1* digestion pattern closely resembles bulk chromatin (Figure 3.5f). In contrast, chromatin structure was disrupted within the *GAL10* coding sequence (probed as a control) at later time points upon activation by galactose (Figure 3.5e, 100'-140'), consistent with previous observations (Cavalli and Thoma 1993). We conclude that Isw2 complex slides nucleosomes *in vivo* without disrupting nucleosome integrity to a noticeable extent. These results are consistent with the *in vitro* observation that, unlike the Swi/Snf family of chromatin remodeling factors (Bazett-Jones et al. 1999; Aalfs et al. 2001; Lorch et al. 2001; Narlikar et al. 2001; Aoyagi et al. 2002), ISWI complexes slide nucleosomes along DNA without increasing the accessibility of DNA throughout the nucleosome or altering the physical properties of nucleosomes (Aalfs et al. 2001; Kassabov et al. 2002).

Conclusions

Many studies of ATP-dependent chromatin remodeling factors in vivo have analyzed chromatin structure of specific genes, comparing wild type cells to those lacking a particular chromatin remodeling complex (Hirschhorn et al. 1992; Goldmark et al. 2000; Fazzio et al. 2001; Kent et al. 2001), or comparing chromatin structure pre- and post-

induction of a target gene (Lohr 1984; Almer et al. 1986; Schmid et al. 1992; Wu and Winston 1997; Lomvardas and Thanos 2001). While these experiments have uncovered the end products of the chromatin remodeling reaction at these loci, they provide little information about the mechanism used to create this chromatin structure. To begin to address the mechanisms utilized by the ISWI class of ATP-dependent chromatin remodeling factors, we created a system that allowed us to successfully capture intermediates of the Isw2-dependent chromatin remodeling process. We find that Isw2 complex remodels chromatin structure at two different classes of target genes by a similar nucleosome sliding mechanism, differing only in the direction of nucleosome sliding with respect to the promoter. To our knowledge, this is the first example in which intermediates of a chromatin remodeling reaction have been identified *in vivo*, as well as the first direct demonstration of nucleosome sliding by a chromatin remodeling factor *in vivo*. In contrast to its *in vitro* nucleosome spacing activity, Isw2-dependent nucleosome sliding *in vivo* is directed toward a few nucleosomes and does not result in formation of a regularly spaced nucleosomal array, suggesting that the sliding activity of Isw2 complex is constrained by cellular factors *in vivo*.

Experimental Procedures

Yeast strains , Plasmids and Growth Conditions

All yeast strains were derived from W1588-4c, which is congenic to W303 (Zhao et al. 1998). Strains expressing Isw2p with two tandem copies of the FLAG tag at its C-terminus were constructed by subcloning ISW2-2xFLAG from pRS416-ISW2-2xFLAG (Tsukiyama et al. 1999) into pRS406 and introducing it into the genome by the 'pop-in-pop-out' method (Rothstein 1991). The *GAL1* promoter was placed upstream of the *ISW2*-2xFLAG gene by cloning the first 500 base pairs of the *ISW2* gene into pSB157 (courtesy of Sue Biggins, FHCRC) and integrating the plasmid at the *ISW2*-2xFLAG chromosomal locus. For *ISW2* induction time course experiments, cells were grown in YEP (2% Bacto peptone 1% Bacto yeast extract) +2% raffinose to $OD_{660} = 0.5$, at which

time galactose was added to 2%. The moment of galactose addition was considered time = 0'. Control strains were grown in YEP+2% Raffinose to OD_{660} = 0.6-0.7.

Chromatin Immunoprecipitations

Chromatin immunoprecipitation was performed as described previously for Ume6-3xFLAGp (Goldmark et al. 2000), with a few exceptions. Protein G Dynabeads (Dynal) were used to perform the immunoprecipitation reactions. Isw2-2xFLAG was eluted from beads by incubation with 3xFLAG peptide twice for 30 minutes each at room temperature. A detailed protocol is available upon request.

Nuclease Digestions and Southern Hybridizations

Micrococcal nuclease (MNase) and DNaseI digestions of chromatin were performed on permeabilized spheroplasts as described previously (Kent et al. 2001), with the following modifications. Zymolyase was used at 50 mg/ml and the spheroplasting step was extended to 2 minutes. For indirect end-labeling experiments, 75 units of MNase or 15 units of DNaseI were used per 200 µl aliquot of spheroplasts. Digestions were stopped with 800 µl of buffer G2 (Qiagen) after 4 minutes at 37°C. 4-5 identical 200 µl digestions performed in parallel were combined, treated with 20 units of RNaseA for 1 hour and proteinase K for 3 hours to overnight. DNA was purified on Qiagen genomic-tip 20/G columns and resuspended in 40 µl, of which 10 µl was used for indirect end-labeling as described (Fazzio et al. 2001). Methidiumpropyl-EDTA-iron(II) (MPE-Fe^{2+}) digestions were processed similarly, except hydrogen peroxide was added to 600 µl of spheroplasts to 0.015%, to which was added 24 µl of a freshly prepared mixture of 0.625 mM ferrous ammonium sulfate, 10 mM dithiothreitol, and 0.625 mM MPE. After digestion for 4 minutes at 37°C, reactions were terminated with 36 µl of 100 mM bathrophenanthrolinedisulfonic acid. 2.4 ml of buffer G2 was added and samples were processed as described above. For the nucleosome disruption experiment (Figure 3.5d-f), 200 µl aliquots of spheroplasts were digested with 150, 300 or 600 units of MNase for

4 minutes at 37°C. Reactions were terminated by adding SDS to 0.6% and EDTA to 15 mM. After overnight proteinase K digestion, lysates were extracted with phenol/chloroform, treated with RNaseA, ethanol precipitated and resuspended in 100 µl TE. Samples were then purified on Qiagen PCR purification columns, as described by the manufacturer, and eluted in 50 µl. 5 µl of each digestion were run on a 1% agarose gel, transferred to a nylon membrane and hybridized with a probe corresponding to base pairs –200 to +292 of the *POT1* gene. This blot was later stripped and rehybridized with a probe corresponding to base pairs +1492 to +1997 of the *GAL10* gene. Restriction enzyme digests were performed similarly, except 100 µl aliquots were digested with 200 or 100 units of BanI or NcoI, respectively for 20 minutes at 37°C and the reactions were stopped by adding SDS and EDTA as described above. After RNaseA and proteinase K treatment, DNA was purified by phenol/chloroform extraction and ethanol precipitation. Approximately 1/6 of each sample was digested with RsaI and AluI and subjected to Southern hybridization as described above. The fraction of total chromatin cut at each time point was quantitated by a phosphorimager.

Primer Extensions

Primer extensions were performed as described previously (Shimizu et al. 1991) on the same MNase-digested samples used for indirect end labeling, with a few modifications. Labeled primers were extended with Taq polymerase for 30 cycles, using 30 second denaturation, annealing and extension times. Dideoxy sequencing reactions were performed with a Sequenase v2.0 sequencing kit (USB). Densitometry of the bands in each lane was performed using a phosphorimager.

Chapter IV: The histone H4 basic patch directly binds Isw2 complex and is required for recruitment to target loci in vivo

Summary

Members of the ISWI family of ATP-dependent chromatin remodeling factors catalyze nucleosome sliding in vitro and in vivo. To perform this function, ISWI complexes are predicted to bind the histone octamer and DNA separately, in order to generate the force necessary to disrupt histone-DNA contacts during the process of sliding. To identify the binding site on histones for yeast Isw2 complex, we tested the effects of mutations in the histone tails, using chromatin structure at in vivo targets of Isw2 complex as a readout for Isw2 function. We found that the histone H4 "basic patch" was the only portion of any of the four histone amino terminal tails that was required for the function of Isw2 complex in vivo. Furthermore, we found that the basic patch was required for specific association of Isw2 complex with its target sites in vivo. Finally, we found that Isw2 complex requires the H4 basic patch for binding to nucleosome core particles in vitro. These data, along with previous data from others showing its requirement for stimulation of ATPase activity of *Drosophila* ISWI, support a role for the H4 basic patch as the nucleosome recognition module for ISWI complexes.

Introduction

Members of the ISWI class of ATP-dependent chromatin remodeling factors display a number of chromatin remodeling activities in vitro, including chromatin disruption, nucleosome sliding, nucleosome spacing and chromatin assembly activities (Langst and Becker 2001b). However, different ISWI complexes catalyze different subsets of this list of activities. For example, *Drosophila* NURF, the founding member of the ISWI class of chromatin remodeling factors, acts in concert with GAGA factor to disrupt chromatin structure in vitro, allowing access of GAGA to its binding site on DNA (Tsukiyama and Wu 1995). In addition, NURF complex has been shown to slide

nucleosomes in cis along DNA (Hamiche et al. 1999), but does not catalyze nucleosome spacing or chromatin assembly in vitro. In contrast, ACF and CHRAC (from *Drosophila* and humans) and RSF (from humans) catalyze chromatin assembly, nucleosome spacing and nucleosome sliding in vitro, but do not appear to catalyze chromatin disruption (Ito et al. 1997; Varga-Weisz et al. 1997; Langst et al. 1999; LeRoy et al. 2000; Loyola et al. 2001). In fact, these complexes are the only ATP-dependent chromatin remodeling factors reported to catalyze chromatin assembly, and therefore may represent a specialized subset of ISWI complexes dedicated to this purpose. All ISWI complexes tested to date catalyze nucleosome sliding in vitro, suggesting this process may be central to the functions of all ISWI complexes in vivo.

Recently, we showed that yeast Isw2 complex functions in vivo at its chromosomal targets by sliding nucleosomes into a repressive configuration (Fazzio and Tsukiyama 2003). At two independent classes of target genes, Isw2 complex slid three nucleosomes toward the promoter. However, the direction of nucleosome movement was different in each case. At the *REC104* promoter, Isw2 complex slid upstream nucleosomes into more downstream positions, whereas at the *POT1* promoter, nucleosomes extending from the promoter into the coding region were slid into more upstream positions. Previously, we found that Isw2 complex was recruited to the promoters of early meiotic genes by direct interaction with the sequence-specific DNA binding protein Ume6p (Goldmark et al. 2000). Upon close inspection, Isw2 complex appears to slide one upstream nucleosome to a position immediately adjacent to—or possibly overlapping—the Ume6p-binding site URS1 (Figure 3.4). This led us to propose a model in which Isw2 complex slides nucleosomes by forcing linker DNA adjacent to its site of recruitment into the nearest nucleosome (Figure 3.6). After the "bulge" of displaced DNA traverses 1.65 turns and exits out the other side of the nucleosome, the net result is what appears to be "sliding" of the nucleosome by a length of DNA equal to the bulge.

One prediction of this model is that Isw2 complex should require separate binding sites for the histone octamer and DNA, in order to generate the force necessary

to push DNA into the nucleosome. Since DNA makes extensive contacts with the histone octamer within the nucleosome, nucleosome sliding by this (or any) method would require breaking every ionic bond and reforming each in a new position. For example, one nucleosome at the *POT1* promoter is slid by Isw2 complex approximately 70 base pairs, from its location in the absence of Isw2 complex to a new upstream location closer to the *POT1* promoter (Fazzio and Tsukiyama 2003). Since core nucleosomes incorporate 146 bp of DNA, sliding of this nucleosome at the POT1 locus results in approximately 50% turnover of nucleosomal DNA: 70 bp is forced in one end and 70bp exits the other end. This model makes no predictions about whether all of the DNA is inserted in one step (one big bulge) or in multiple small steps (small individual bulges).

Several possible candidates for the Isw2 complex binding site on the histone octamer have emerged in recent years. Becker and colleagues previously identified a basic portion of the histone H4 N-terminal tail that is required for stimulation of the ATPase activity of recombinant *Drosophila* ISWI in vitro (Clapier et al. 2001; Clapier et al. 2002). A second group, studying the requirements for chromatin assembly by the human RSF complex, found that not only was the histone H4 tail required for chromatin assembly, but deletion of the H2A or H2B tails also decreased the efficiency of the assembly process (Loyola et al. 2001). Another group, focusing on yeast Isw1 complexes, found that association of Isw1p with chromatin in vivo requires methylation of lysine-4 on histone H3 by the histone methyltransferase Set1p (Santos-Rosa et al. 2003). Similarly, a recently described human Snf2h-containing complex was found to colocalize on chromatin with the H3 lysine-4 methylation mark in vivo, although it was unclear in this case whether lysine-4 methylation was required for Snf2h association with chromatin (Hakimi et al. 2002).

To identify the possible binding site on the histone octamer for Isw2 complex, we tested deletion mutants eliminating part or all of the N-terminal tails of each of the four core histones for their ability to promote Isw2-dependent chromatin remodeling. Using chromatin structure of Isw2 target genes as a readout for Isw2 complex function, we

determined that the histone H4 basic patch was the sole portion of any of the N-terminal tails required for chromatin remodeling by Isw2 complex. We mutated each of the five residues in the basic patch and found that lysines-16 and -20 were dispensable for Isw2-dependent chromatin remodeling. Using chromatin immunoprecipitation assays, we found that the basic patch was required for Isw2 complex to specifically associate with its target sites on chromatin, which was unexpected in light of the fact that Isw2 complex binds to nucleosomal arrays and free DNA equally well in vitro. In addition, we found that binding of Isw2 complex to core nucleosomes in vitro requires the basic patch, strongly suggesting that Isw2 complex binds directly to this epitope. These experiments support a model in which Isw2 complex binds DNA and the H4 basic patch independently to force DNA into the nucleosome during the process of nucleosome sliding.

Results

Chromatin remodeling by Isw2 complex in vivo requires amino acids 15-19 of histone H4 but no other N-terminal histone tails

We reasoned that histone mutations that prevented Isw2 complex from remodeling chromatin, due to an inability of Isw2 complex to bind histones or a failure of the mutant histones to stimulate the ATPase activitiy of Isw2p, would result in chromatin structure at Isw2 target genes similar or identical to that of an *isw2* mutant. Therefore, we decided to use chromatin structure at Isw2 target genes in vivo as a readout for function of Isw2 complex. We constructed yeast strains in which the sole copy of the histone to be mutated was carried on a plasmid with a *URA3* marker, transformed them with a *TRP1*-marked plasmid bearing a specific histone mutation and then selected against the wild type plasmid by plating on 5-FOA (plasmid shuffling). Chromatin structure in each mutant was assayed as described in Experimental Procedures.

Initially we focused on chromatin structure at the *POT1* gene, which is repressed by Isw2 and Sin3/Rpd3 complexes acting in parallel pathways, and is a target of Isw2-

dependent chromatin remodeling (Fazzio et al. 2001; Fazzio and Tsukiyama 2003). Using this assay, we found that the entire N-terminal tails of histones H3 (Figure 4.1a), H2A and H2B (Figure 4.1b) were dispensable for Isw2-dependent chromatin remodeling. In contrast, two of four deletions of the histone H4 N-terminal tail resulted in chromatin structure that appeared identical to that of *isw2* mutant cells (Figure 4.1a). The smallest of these, deleting amino acids 15-19, removes most of a run of basic residues previously referred to as the "basic patch" of the histone H4 tail. Importantly, a large deletion removing amino acids 4-14, representing nearly all of the H4 tail *except* the basic patch, resulted in wild type chromatin structure. Therefore, the histone H4 basic patch is the only portion of any histone tail that is required for the function of Isw2 complex. Similar results were obtained for a second Isw2 target gene, *REC104* (data not shown).

The requirement of Isw2 complex for the basic patch is limited to three residues: $R_{17}H_{18}R_{19}$

The H4 basic patch was previously found to be necessary for activation of the ATPase activity and facilitation of nucleosome spacing by *Drosophila* ISWI in vitro (Clapier et al. 2002). Since ISWI was able to bind nucleosomes lacking the H4 tail, the H4 tail was thought to be required for ATPase activity and chromatin remodeling at a step subsequent to nucleosome binding (Clapier et al. 2001). The authors found that nucleosomes lacking the first 15 or 16 amino acids were partially defective in ATPase stimulation, while nucleosomes lacking the first 19 amino acids were completely incapable of stimulating ATPase activity beyond the level of DNA alone (Clapier et al. 2002). In a later report, free histone tails acetylated at either lysine-16 or lysine-12 were incapable of inhibiting nucleosome-stimulated ATPase activity of ISWI in a competition assay, while unacetylated tails or tails acetylated at lysine-8 strongly inhibited this activity (Corona et al. 2002). These data suggest that the ability of the H4 basic patch to stimulate the ATPase activity of ISWI is regulated by the acetylation state of nearby lysines.

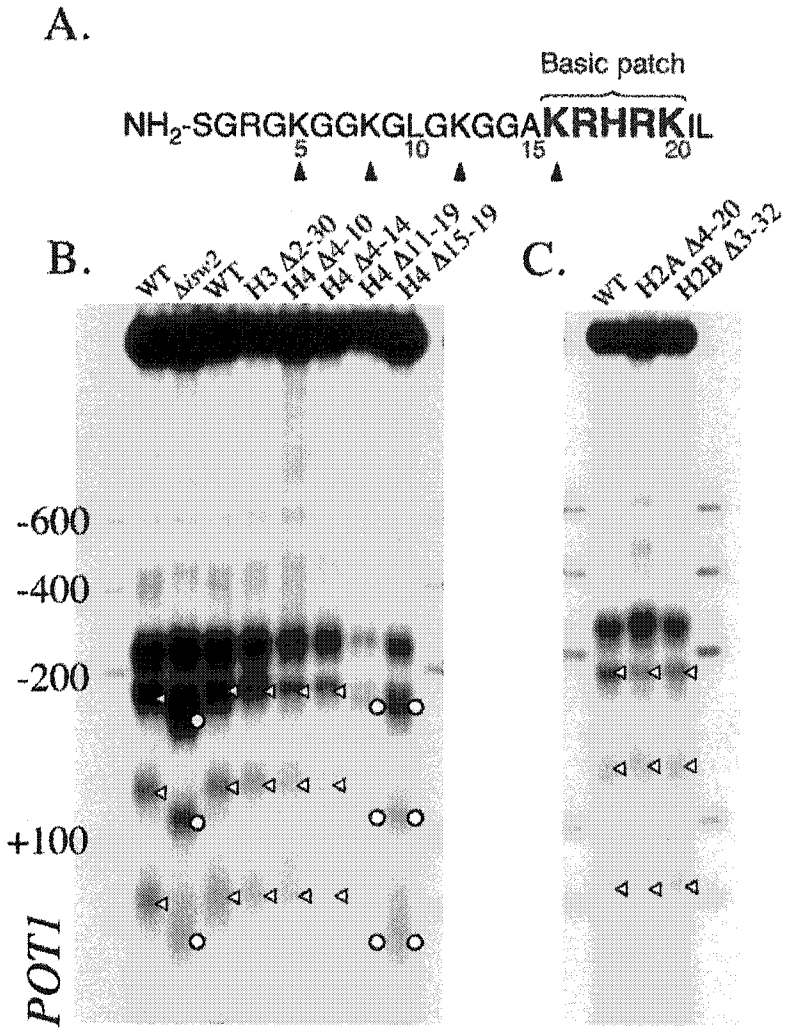


Figure 4.1. Amino acids 15-19 of histone H4 are the only necessary portion of any histone tail for Isw2-dependent chromatin remodeling. A. The histone H4 tail, with the basic patch region highlighted. Acetylatable lysines are indicated with arrowheads. B and C. Chromatin structure at the *POT1* gene was analyzed for the wild type and the indicated yeast mutants, using DNaseI as a probe of chromatin structure. Triangles mark DNaseI cut sites that follow the wild type pattern. Circles mark the DNaseI cut sites that resemble the *isw2* mutant pattern. Size standards, numbered with respect to the *POT1* initiation codon, are indicated to the left.

To further narrow the basic patch requirement of Isw2 complex in vivo, we tested a series of point mutations in the H4 basic patch (Figure 4.2). Alanine substitutions in one or both of the lysines at positions 16 and 20 within the basic patch had no effect on Isw2-dependent chromatin remodeling, indicating these residues were not required for Isw2 function. In addition, mutation of lysine-16 to glutamine (to mimic acetylation) or arginine (to conserve charge) also had no effect on chromatin structure (data not shown); however, this does not rule out a role for lysine acetylation in regulation of the basic patch in yeast cells (see Discussion).

A double mutation, in which both arginine-17 and -19 residues were previously mutated to alanines and found to destroy ATPase stimulation of *Drosophila* ISWI by H4 tail peptides (Clapier et al. 2002), resulted in chromatin structure identical to that of an *isw2* mutant. Interestingly, single alanine substitutions of arginine-17 or histidine-18 resulted in a partial defect in chromatin structure (Figure 4.2a; note brackets). This phenotype may result from a mixed population of cells in which some cells have wild type chromatin structure at the *POT1* gene and some have the *isw2* mutant chromatin structure. Alternatively, nucleosomes may be adopting a third, intermediate pattern of nucleosome positioning that we have not observed previously. The single arginine-19 to alanine substitution had no noticeable phenotype; however, since this mutation clearly enhances the phenotype of a single arginine-17 to alanine substitution, we conclude that arginine-19 plays a role in basic patch function.

Next we asked whether the basic patch needs only to be basic to promote Isw2-dependent chromatin remodeling, or whether the specific sequence arginine-histidine-arginine is is important for function of the basic patch. To distinguish between these two possibilities, we tested the effect of a double mutation changing both arginines to lysine (hereafter called R17K R19K) on Isw2-dependent chromatin remodeling. Surprisingly, this mutation resulted in an intermediate phenotype similar to that of the R17A and H18A single mutants (Figure 4.2b). This indicates that the specific arginine-histidine-arginine sequence of the wild type basic patch is required for its full function in Isw2-dependent chromatin remodeling, but that substitutions maintaining the positive charge

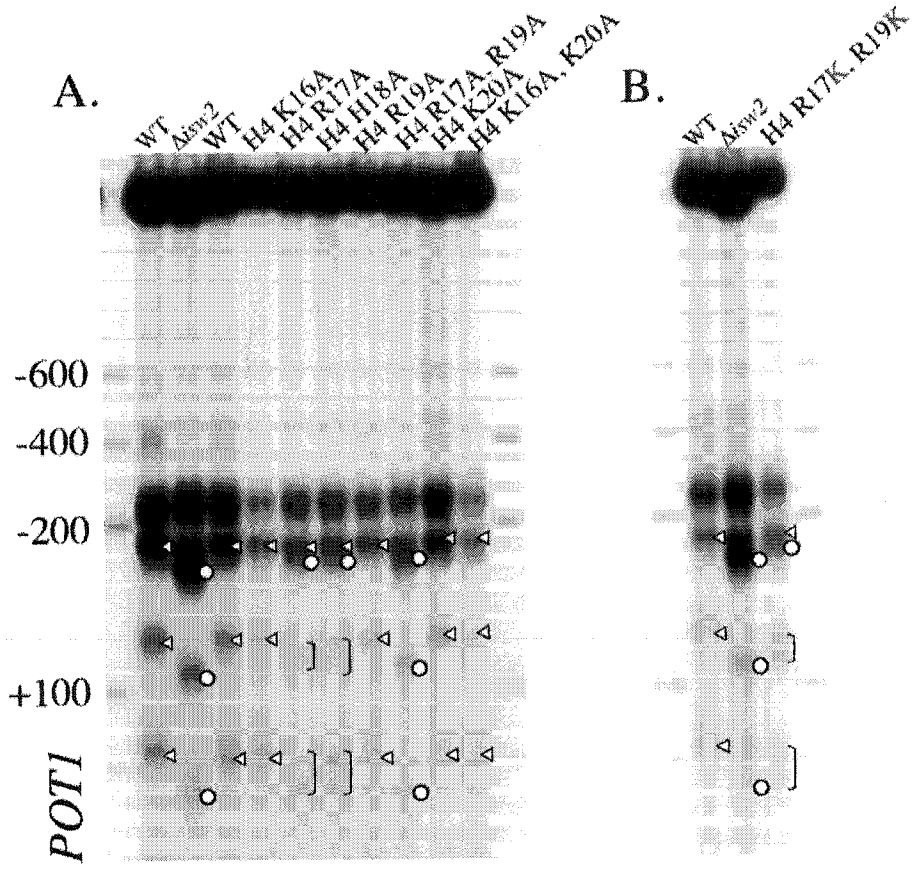


Figure 4.2. The basic patch requirement for function of Isw2 complex is limited to arginine-17, histidine-18 and arginine-19. A and B. Chromatin structure was analyzed as in Figure 4.1. Triangles and circles indicate DNaseI cut sites with the wild type and *isw2* mutant patterns, respectively. Brackets indicate DNaseI cut sites that resemble intermediate states between the wild type and *isw2* mutant pattern.

are partially functional. Together, these results point to a central role for the three interior basic patch residues, arginine-17, histidine-18 and arginine-19, in chromatin remodeling by Isw2 complex in vivo.

An H4 basic patch mutation prevents specific association of Isw2 complex with target loci in vivo

Previously, Becker and colleagues found that recombinant *Drosophila* ISWI bound to nucleosomes lacking the entire H4 N-terminal tail (Clapier et al. 2001), which led them to conclude that the H4 tail was required at a step subsequent to nucleosome binding. However, these experiments employed mononucleosomes assembled with extensive linker DNA on each end. Since Isw2 complex was previously found to bind chromatin and free DNA equally well (Gelbart et al. 2001), we decided to investigate the requirement of the H4 basic patch for Isw2 complex to bind to target genes on chromatin in vivo. Recently our laboratory has found that specific association of Isw2 complex with its known target sites is observed only with catalyticially inactive mutants of Isw2p (Gelbart and Tsukiyama manuscript in preparation). We therefore tested the ability of both wild type and catalytically inactive Isw2 (Isw2ci) to associate with its known sites of action in the presence or absence of an H4 basic patch mutation (Figure 4.3). As described previously (Gelbart and Tsukiyama manuscript in preparation), we observed for wild type Isw2 complex a level of nonspecific chromatin binding that was similar at the Isw2 target promoter *POT1* and a negative control promoter, *ACT1* (lanes 3-4). This level was essentially unchanged in an R17A R19A H4 basic patch mutant (compare lanes 3-6). Also consistent with previous results, we found a substantial enrichment of Isw2 localization at the *POT1* promoter over the *ACT1* promoter in the Isw2ci mutant (lanes 7-8). However, in the presence of the H4 basic patch mutant, localization of Isw2ci at *POT1* was reduced to similar levels as *ACT1* (lanes 9-10). Furthermore, these levels were similar to the levels of association with both genes by wild type Isw2 complex. Therefore, in the absence of a functional H4 basic patch, Isw2ci is capable of only general, nonspecific binding to chromatin. We found similar,

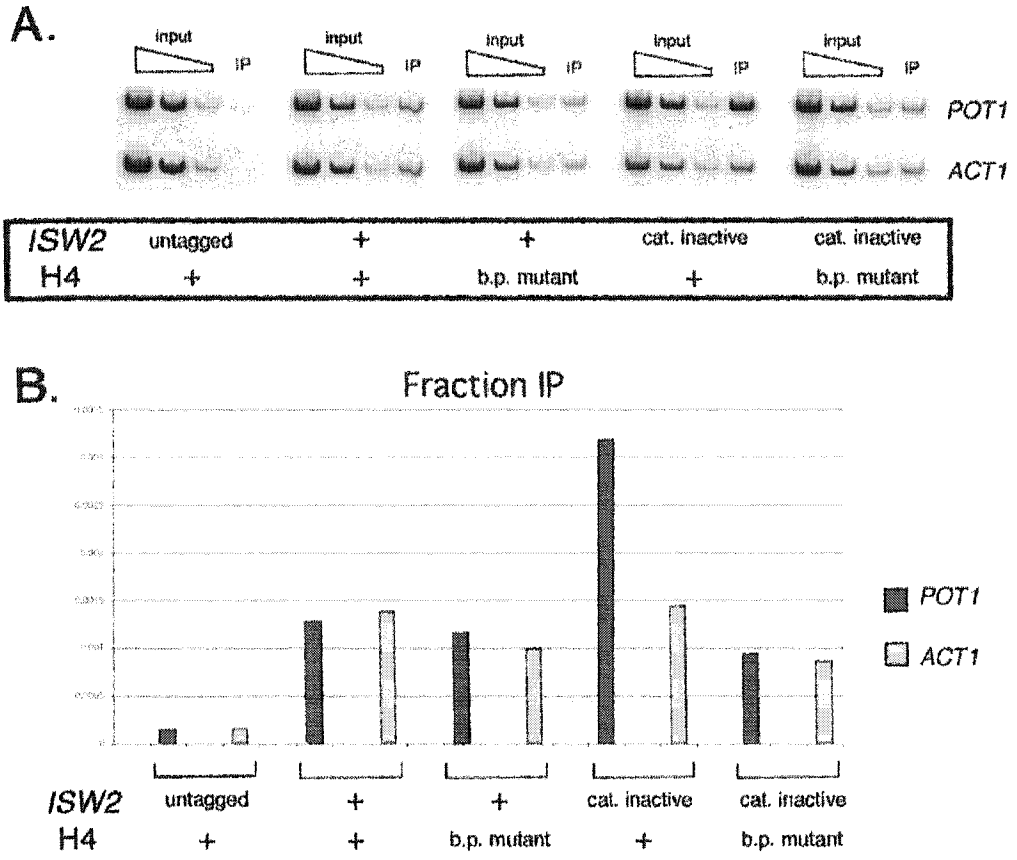


Figure 4.3. The basic patch is required for specific association of Isw2 complex with its chromatin targets in vivo. A. Chromatin Immunoprecipitation was performed as described in Experimental Procedures. Radioactive duplex PCR was carried out for the *POT1* and *ACT1* promoters, using a serial dilution of input chromatin (to ensure linearity of the PCR reaction) and precipitated chromatin. The genotypes of each sample are indicated in the box below the panel of PCR reactions. B. The fraction of the input immumuprecipitated was quantitated for each sample using a phosphorimager. Similar results were found for another Isw2 target, *INO1*.

yet stronger association of Isw2 complex with the *INO1* promoter that also requires the Isw2ci mutation and wild type H4 basic patch (data not shown). We conclude that the H4 basic patch is essential for specific association of Isw2 complex with its target sites in vivo, but is unnecessary for the general, ubiquitous binding of chromatin previously identified for Isw2 complex (Gelbart and Tsukiyama manuscript in preparation).

The H4 basic patch is required for Isw2 complex to bind core nucleosomes in vitro

The finding that the H4 basic patch is required for specific association of Isw2 complex with target genes in vivo suggests that Isw2 complex may directly bind to the basic patch. Since Isw2 complex was previously shown to bind DNA and chromatin equally well in vitro (Gelbart et al. 2001), we decided to test binding of Isw2 complex to nucleosome core particles (hereafter referred to as "core nucleosomes"), in which the histone octamer is wrapped with 146 base pairs of DNA. Core nucleosomes have only enough DNA to form 1.65 turns on the histone octamer, with no free DNA extending outside of the octamer to which Isw2 complex can bind (Luger et al. 1997). We reconstituted core nucleosomes as described in Experimental Procedures containing either wild type or the R17A R19A basic patch mutant histone H4, and assayed binding of Isw2 complex using native polyacrylamide gel electrophoresis (Figure 4.4).

Upon mixture of Isw2 complex with core nucleosomes we observed the appearance of a slower migrating species during native gel electrophoresis (Figure 4.4a). This slower migrating band was substantially more prominent for nucleosomes containing wild type histone H4 than those with a mutated H4 basic patch. Furthermore, the intensity of the slower migrating band increased with an increased dose of Isw2 complex. Upon quantitation of the core nucleosomes shifted in each lane, we found that the fraction of wild type nucleosomes shifted was 4.0-5.5 fold higher than that of the basic patch mutant. To confirm that the shifted species contained Isw2 complex, we added to binding reactions containing both Isw2 complex and wild type core nucleosomes either a control antibody (9E10) or an antibody directed against the FLAG epitopes fused to the C-terminus of Isw2 complex (Figure 4.4b). The control antibody

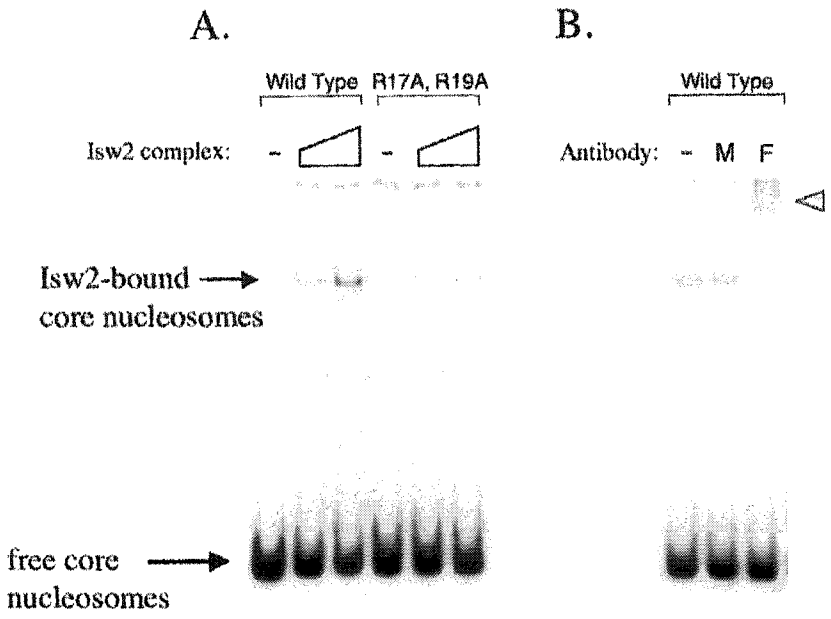


Figure 4.4. The H4 basic patch is required for binding of Isw2 complex to core nucleosomes. A. Electromobility shift assay (EMSA), measuring binding of Isw2 complex to wild type or mutant core nucleosomes. Core nucleosomes bound by Isw2 complex and unbound nucleosomes are indicated to the left. B. The slower migrating species contains Isw2 complex. Wild type core nucleosomes were incubated with Isw2 complex and the indicated antibody. The supershifted species is marked with an arrowhead. M: anti-Myc; F: anti-FLAG.

had no effect on either the unshifted core nucleosomes or the shifted species. In contrast, the anti-FLAG antibody super-shifted the slower migrating band to a very slowly migrating species, but had no effect on the unshifted majority of core nucleosomes. These data confirm that the shifted species contains Isw2 complex. We conclude that Isw2 complex binds directly to core nucleosomes and that this interaction is dependent upon the histone H4 basic patch.

Discussion

It is becoming increasingly clear that nucleosome sliding by ISWI complexes occurs without disruption of the nucleosome structure, implying that at any given time during the sliding process, the majority of DNA within the nucleosome remains tightly bound by histones (Kassabov et al. 2002; Fan et al. 2003). In the absence of massive disruption of histone-DNA contacts throughout the nucleosome, any model for nucleosome sliding must involve the ISWI complex actively forcing DNA outside of the nucleosome into a position that can be bound by the histone octamer, with concomitant disruption of histone-DNA contacts at the nucleosome edge. An implication of this model is that the ISWI complex should have to bind DNA and the histone octamer independently to generate the force necessary to disrupt histone-DNA contacts.

To identify the binding site on the histone octamer for yeast Isw2 complex, we used an assay that allowed us to screen through a number of histone mutants in vivo by asking whether or not they support Isw2-dependent chromatin remodeling. We found that the histone H4 basic patch, which was previously identified as an activatior of the ATPase activity of *Drosophila* ISWI, was required for Isw2 complex to function in vivo. In addition, we found that the basic patch was required for specific association of Isw2 complex with its target sites in vivo. Finally, we found that Isw2 complex binds core nucleosomes in vitro and that this binding requires a functional H4 basic patch. These data strongly suggest that Isw2 complex interacts directly with the basic patch. It should be noted that while these experiments demonstrate that the H4 basic patch is necessary for Isw2 interaction in vivo and in vitro, they do not demonstrate that this site is

sufficient for Isw2 binding. Due to technical problems, we were unable to test whether Isw2 complex binds to the basic patch of free histone H4 tails in the absence of the nucleosome. It is therefore possible that the basic patch is only part of a more complex binding site that includes contributions from the core domain of histone H4 and/or the other histone core domains.

Since substitution mutations of lysines-16 and -20 had no effect on Isw2-dependent chromatin remodeling we concluded that these residues are not required for function of the basic patch. However, these results do not necessarily contradict previous results for *Drosophila* ISWI both in vitro and in vivo showing that acetylation of lysine-16 of histone H4 disrupts ISWI function. For example, if a protein recognizes and specifically binds H4 tails that are acetylated at lysine-16, this protein may inhibit interaction of ISWI complexes with the basic patch and inhibit chromatin remodeling as a result. By substituting lysine-16 with alanine, glutamine or arginine, we likely would disrupt the binding site for this hypothetical ISWI inhibitor, rendering chromatin permissive for Isw2-dependent chromatin remodeling. We previously found that Isw2-dependent chromatin remodeling at common targets of the Sin3-Rpd3 histone deacetylase complex was independent of Rpd3 function, suggesting the acetylation state of chromatin had no effect on Isw2 function. However, Rpd3p appears to strongly deacetylate all lysines on histone H4 *except* lysine-16 (Suka et al. 2001). Deletion of the *SAS2* gene, encoding a histone H4 lysine-16 specific acetylase also had no effect on Isw2-dependent chromatin remodeling, but this result does not rule out the possibility of an alternative lysine-16 acetylase that acts at sites of Isw2 function. Therefore, lysine-16 acetylation remains a possible regulatory mechanism for determining whether the basic patch is accessible to Isw2 complex.

If the H4 basic patch is a binding site for Isw2 complex, how does Isw2 identify its specific target sites in vivo when the basic patch is found on every nucleosome? Presently the only protein shown directly to recruit Isw2 complex to specific promoters is the dual repressor/activator protein Ume6p. Testing localization of Isw2ci in vivo, Gelbart and Tsukiyama have shown that a functional Ume6p binding site (URS1) is

necessary for specific localization of Isw2 complex to the *REC104* promoter (Gelbart and Tsukiyama manuscript in preparation). That data, combined with the data presented here, suggests that specific association of Isw2 complex requires both recruitment by a sequence-specific DNA binding protein and a functional H4 basic patch. Perhaps the strength of binding to either Ume6p or the H4 basic patch alone is not sufficient to prevent dissociation of Isw2 complex from chromatin, whereas binding to both simultaneously reduces the dissociation rate. It will be of considerable interest to test this possibility in vitro.

Experimental Procedures

Yeast strains , Plasmids and Growth Conditions

All yeast strains were derived from W1588-4c, which is congenic to W303 (Zhao et al. 1998). Strains expressing Isw2p with three tandem copies of the FLAG tag at its C-terminus were constructed as described previously (Goldmark et al. 2000). The histone tail deletions and point mutations were constructed by site-directed-mutagenesis, sequenced and subcloned into pRS414. The H4 tail deletions used in this study were provided by Jay Vary. For expression of H3-H4 basic patch mutant tetramer, the R17A R19A mutation was made by site directed mutagenesis in a pET vector containing a version of yeast H4 in which codon usage was optimized for bacterial expression (Gelbart et al. 2001). This mutant H4 gene, along with wild type yeast H3 were then cloned sequentially into the pET-DUET-1 vector.

Nuclease Digestions and Southern Hybridizations

DNaseI digestions and indirect end labeling experiments were performed as described previously (Fazzio and Tsukiyama 2003), except cells were grown in YEPD (2% Bacto peptone 1% Bacto yeast extract 2% dextrose) to $OD_{660} = 0.7$, at which time cells were harvested and processed. In addition, Zymolyase was used at 10 mg/ml.

Chromatin Immunoprecipitation

Chromatin immunuprecipitation experiments were performed as described previously (Fazzio and Tsukiyama 2003).

Core Nucleosome Assembly and Binding Assays

Wild type core nucleosomes containing recombinant yeast histones were assembled on the *X. borealis* 5S nucleosome positioning sequence (Hayes et al. 1990) by salt dialysis as described previously (Gelbart et al. 2001; Vary et al. 2004), with a fraction of the DNA radioactively labeled for the purpose of detection.

To express soluble H3-H4 tetramer, the H3-H4 basic patch mutant expression plasmid was transformed into BL21-CodonPlus(DE3)-RIL cells, which were grown in 2L of 2X YT broth (1.6% Bacto Tryptone, 1% Yeast Extract, 0.5% NaCl) with ampicillin (100 μg/ml) and chloramphenicol (25 μg/ml) at 37 °C to OD_{600} = 0.5. IPTG was added to 0.5 mM and growth continued for three hours. Cells were pelleted and washed once in Buffer H containing 100 mM KCl (Buffer $H_{0.1}$) (Tsukiyama et al. 1999) and pelleted again. The cell pellet was resuspended in a roughly equal volume of Buffer $H_{0.1}$ and sonicated using a Branson sonicator several times at setting 5-6, until the optical density was reduced > 10 fold. The extract was spun in a Beckman ultracentrifuge at 35,000 rpm for 30 minutes, and the supernatant, containing soluble H3-mutant H4 tetramer was flash frozen on liquid nitrogen and stored at –80 °C until purification.

To purify soluble tetramer, one third of the extract was thawed and centrifuged at 4000 rpm in a Beckman J-6 centrifuge to pellet insoluble material. The supernatant was added to 1.5 ml of Q-Sepharose that had been washed twice with Buffer $H_{0.1}$ to "preclear" the extract. After binding at 4 °C for 30 minutes on a rotating wheel, the beads were centrifuged at 800 rpm for 2 minutes in a Beckman J-6 centrifuge and the unbound material was added to 1.5 ml of SP-Sepharose that had been washed twice with Buffer $H_{0.1}$. Extract was allowed to bind for 30 minutes at 4 °C on a rotating wheel and the beads were pelleted as above. Beads were washed twice with Buffer $H_{0.1}$ and transferred to a disposable column (Poly-Prep Chromatography Columns, Bio Rad). The

column was washed once with 5 ml of Buffer H $H_{0.5}$ (500 mM KCl) and protein was eluted 5 times with 1.5 ml of Buffer $H_{1.0}$ (1 M KCl). Core nucleosomes containing the H4 basic patch mutant were assembled as above for wild type H4, except that H3-mutant H4 tetramer and wild type H2A-H2B dimer (obtained previously (Gelbart et al. 2001)) was substituted for wild type histone octamer. Both the wild type and mutant core nucleosomes were heated at 55 °C overnight to obtain a single correctly assembled and positioned species and the resulting aggregate (which was considerable) was filtered out using 0.45 μm microcentrifuge filters (Ultrafree-MC, Microcon). Core nucleosomes were stored at 4 °C on ice.

Nucleosome binding assays were performed in 30 μl reactions containing Buffer $H_{0.05}$ (50 mM KCl). We incubated core nucleosomes for 30 minutes at room temperature with various amounts of wild type or catalytically inactive Isw2 complex (containing a lysine to arginine substitution in the conserved GKT motif (Goldmark et al. 2000; Gelbart and Tsukiyama manuscript in preparation)). Approximately 2 μg of anti-Myc (9E10) or anti-FLAG (M2) antibodies were added for supershift experiments. Complexes were resolved on 5% native polyacrylamide gels, using 0.25X TBE as the running buffer.

Chapter V: Conclusions and Future Questions

Summary of Thesis Work

At the time I started working on this project, the biochemical activities of many different ISWI complexes had been characterized and a number of groups were beginning to focus on understanding their enzymology. However, very little was known at that time about the in vivo functions of ISWI complexes. In contrast, the in vivo functions of Swi/Snf complexes had been studied since yeast *SWI2/SNF2* was discovered genetically in the 1980's (Neigeborn and Carlson 1984; Breeden and Nasmyth 1987), before the biochemical discovery of ATP-dependent chromatin remodeling in the early 90's (Tsukiyama et al. 1994) that gave the field a mechanistic framework around which to interpret their data. At the time, Swi/Snf complex was thought to be involved primarily in activation of transcription and had been found to disrupt chromatin structure in vivo at the promoter of one well characterized target gene, *SUC2*, during the process of activating its transcription (Hirschhorn et al. 1992). As a result of these data, and the finding that NURF complex functions with GAGA factor to disrupt chromatin and facilitate GAGA binding to nucleosomal DNA (Tsukiyama et al. 1995; Tsukiyama and Wu 1995), ATP-dependent chromatin remodeling factors were widely believed to function in disruption of chromatin structure to "clear a path" for transcription factors to bind DNA (this despite the discovery of an ATP-dependent chromatin assembly factor, ACF, in 1997 (Ito et al. 1997)).

With this in mind I decided to focus initially on characterizing the in vivo functions of yeast Isw2 complex, with the hope that this work would provide information about the in vivo functions of ISWI complexes in general, as well as broaden our understanding of how chromatin structure is regulated. Before I joined the lab, my advisor Toshio Tsukiyama had performed preliminary whole-genome expression analysis of *isw1*, *isw2* and *isw1 isw2* mutant yeast cells to get a handle on the genes directly or indirectly misregulated in these mutants, which should provide some hints

about their functions. While the quality of these data is unclear, as this experiment was performed in the early days of microarray technology, he found that a number of early meiotic genes (EMGs) were slightly derepressed in an isw2 mutant. This led to the experiments described in Chapter I, in which we found that Isw2 complex functions in a parallel pathway with the Sin3/Rpd3 histone HDAC complex to repress EMGs. This was the first example of an ATP-dependent chromatin remodeling factor functioning in a parallel pathway with an HDAC complex to repress a common set of target genes. While these two complexes function independently to repress transcription, we found that they were recruited to target promoters by the same sequence-specific DNA binding protein, Ume6p. We were unable to determine whether Isw2 and Sin3-Rpd3 complexes were bound to Ume6p at the same time, forming a large "dual-repressor" complex, or Ume6p recruits each complex at different times. We found that Isw2 complex functions at one early meiotic gene, *REC104*, to create compact, nuclease-inaccessible chromatin structure near the promoter. (Analysis of chromatin structure at two other early meiotic genes, *SPO13* and *HOP1* revealed similar, but less prominent changes in chromatin structure in *isw2* mutant cells (data not shown)). Based on this data we proposed that early meiotic genes were repressed during mitotic growth by two chromatin-based mechanisms: compact positioning of nucleosomes and deacetylation of their histone tails.

Most early meiotic genes are not highly derepressed in *isw2* mutant cells in the presence of wild type Sin3-Rpd3 complex, as the two chromatin remodeling factors are partially redundant for their repression function. Therefore, it was somewhat fortunate that a handful of early meiotic genes were identified using whole genome expression analysis of an *isw2* mutant. We therefore considered the possibility that a significant number of Isw2-target genes were similarly masked by Sin3-Rpd3 complex, and that these targets might be revealed if we knocked out *ISW2* in the presence of a *sin3* or *rpd3* mutation. As described in Chapter II, we found that this was indeed the case and that Isw2 and Sin3-Rpd3 complexes collaborate to repress transcription (directly or indirectly) of about 5% of yeast genes. Unfortunately, genes repressed by these two

complexes fell into a large number of functional categories, making it difficult to pinpoint the precise regulatory pathways in which these factors are involved. Nevertheless, these data demonstrated that Isw2 complex mainly functions as a transcriptional repressor and that yeast cells have evolved some level of functional redundancy, since most of the Isw2 targets are not significantly derepressed unless both the Isw2 and Sin3/Rpd3 complexes are removed.

In addition, we examined the chromatin structure of genes targeted by Isw2 complex independently of Ume6p. We found that Isw2 complex functions similarly at every target gene we examined, locally modifying chromatin structure to make it more compact (and less nuclease accessible). For the *SUC2* gene, the result of Isw2-dependent nuclease-inaccessible chromatin structure is an increased level of basal transcription. While this seems odd at first glance since nuclease-inaccessible chromatin structure is generally associated with activation of transcription, this result might be explained by the fact that Isw2 complex appears to reduce the accessibility of a binding site for the Mig1 transcriptional repressor protein located upstream of the *SUC2* gene. Together, these results indicate that Isw2 complex functions to create compact chromatin structure at its target sites in vivo, and this, in turn, (usually) leads to repression of transcription.

Having gone to some lengths to identify the in vivo targets of Isw2-dependent chromatin remodeling, we felt we were in a relatively unique position to ask the question: How does Isw2 complex remodel chromatin to create compact chromatin structure at its physiological target sites in vivo? As detailed above, a considerable body of information has accumulated about the in vitro activities of ATP-dependent chromatin remodeling factors in general and ISWI complexes in particular, revealing chromatin disruption, nucleosome sliding, loading and spacing activities. However, the experiments done to characterize these activities necessarily make use of simplified systems: recombinant histones, nucleosome positioning sequences and purified proteins are often used. It was therefore unclear if and how these activities identified in vitro were relevant to the mechanisms used by ISWI complexes to remodel chromatin in the

more complex chromatin environment found in vivo. On the other hand, the methods we and others generally use to examine chromatin structure in vivo have almost the opposite set of strengths and weaknesses. By examining chromatin structure before and after induction of a target gene or comparing chromatin structure of wild type cells to that of mutant cells, we see two different chromatin *states* representing chromatin structure before and after chromatin remodeling. However, to gain insight into the mechanism used to remodel chromatin structure, we need to examine the *path* of chromatin remodeling. We therefore reasoned that if we were able to trap chromatin structure intermediates as the Isw2-dependent chromatin remodeling reaction progresses, these intermediates might provide insight into the mechanism of chromatin remodeling. As described in Chapter III, we created an inducible allele of the *ISW2* gene and examined chromatin structure during a time course of *ISW2* induction. We found that Isw2 complex slides nucleosomes toward the promoters of both the *REC104* and *POT1* genes, as revealed by the directional migration of nuclease accessibility at these loci. As previously shown in vitro (Kassabov et al. 2002), nucleosome sliding by Isw2 complex in vivo occurs without general disruption of the structure of the nucleosome. Interestingly, Isw2 complex slides one nucleosome upstream of the *REC104* gene into a position adjacent to, or possibly overlapping the Ume6-binding site URS1. This finding led us to propose the model that Isw2 complex slides nucleosomes toward its site of recruitment, possibly by forcing linker DNA in between the two into the nucleosome. As a "bulge" of DNA proceeds through the nucleosome and out the other side, the net result is nucleosome sliding (Figure 3.6).

One prediction of this model is that Isw2 complex should have separate binding sites for the histone octamer and DNA, in order to generate the necessary force to push DNA into the nucleosome during the nucleosome sliding process. In Chapter IV, we sought to identify the binding site on the histone octamer by identifying the regions on each of the four histone N-terminal tails required for Isw2-dependent chromatin remodeling in vivo. We found that the histone H4 basic patch was the only portion of any histone tail necessary for Isw2 complex to remodel chromatin. Considering that

Isw2 complex was previously shown to bind nucleosome arrays and free DNA equally well, we were surprised to find that Isw2 complex is required for specific association of Isw2 complex with its target sites on chromatin in vivo. Furthermore, we found that Isw2 complex binds directly to core nucleosomes in vitro, and this interaction depends on the H4 basic patch. These data strongly suggest that Isw2 binds directly to the H4 basic patch, and support a role for the basic patch as the "handle" on the nucleosome to which Isw2 complex binds bduring the nucleosome sliding process.

Future Questions

In total, the work presented in this dissertation has revealed that Isw2 complex functions as a transcriptional repressor in vivo. Interestingly, over this same period of time, a similar conclusion has started to emerge for *Drosophila* ISWI (Deuring et al. 2000; Corona et al. 2002), although which ISWI complexes are responsible for this repressive function is not fully understood. As we begin to learn the specific chromatin targets of ISWI complexes in *Drosophila* and human cells, it will be of interest to determine whether these complexes also function to create compact chromatin structure locally at discrete sites, or if they have evolved to regulate broader chromatin domains that are more prevalent in higher eukaryotes. In addition, my thesis work indicates that Isw2 complex functions by a nucleosome sliding mechanism at its target sites in vivo. However, this does not necessarily apply to all ISWI complexes. For example, recent evidence supports a role for *Drosophila* ACF and/or CHRAC complex in chromatin assembly in vivo (Fyodorov et al. 2004), consistent with the known biochemical activities for ACF and CHRAC complexes in vitro (Ito et al. 1997; Varga-Weisz et al. 1997; Ito et al. 1999; Fyodorov and Kadonaga 2002b; Fyodorov and Kadonaga 2002a; Kukimoto et al. 2004). It will be important to determine which ISWI complexes function to slide nucleosomes into repressive configurations and which function in other processes such as chromatin assembly, before generalizing about the in vivo functions of ISWI complexes. Collectively, these data suggest that ISWI complexes may be involved

in several different aspects of chromatin regulation fundamental to gene regulation and maintenance of genomic integrity.

Regarding yeast Isw2 complex, two of the more interesting questions extending from my thesis research are how and why does Isw2 complex remodel only a small number of nucleosomes (usually two to three) at each of its chromatin targets in vivo? One possibility is illustrated in figure 5.1. In this case, Isw2 complex is recruited to a target gene by interaction with a sequence-specific DNA-binding protein (Ume6p, for example). Isw2 complex then forces linker DNA between this site and the next nucleosome into the nucleosome, resulting in sliding of the nucleosome toward the site of Isw2 recruitment. This nucleosome is moved until a critical limit is reached: Isw2 complex cannot position two nucleosomes closer together than X base pairs of DNA (Figure 5.1). At this point, Isw2 complex moves on to the next nucleosome, which now has a long linker between it and the nucleosome that just moved away from it, so the process repeats. The reason that nucleosome sliding doesn't continue for more than a few nucleosomes is that the minimum spacing limit (X base pairs) reduces the distance that Isw2 complex has available for sliding with each nucleosome it remodels beyond its site of recruitment. When it no longer has room to slide nucleosomes, Isw2 complex dissociates from chromatin. While it would be very difficult to test this model in vivo, it may be possible to set up an in vitro system with nucleosomes of different spacing to test this hypothesis.

An additional question regards the directionality of chromatin remodeling by Isw2 complex: why does it only slide nucleosomes in one direction? Taking the *REC104* gene as an example, it is not clear why Isw2 complex only slides nucleosomes toward the Ume6p binding site from the upstream side. One possibility is that Isw2 is recruited in a specific orientation that directs the complex to remodel chromatin in one direction only. It would be interesting to reverse the orientation of the Ume6p binding site at the *REC104* gene to determine if it changes the direction of nucleosome

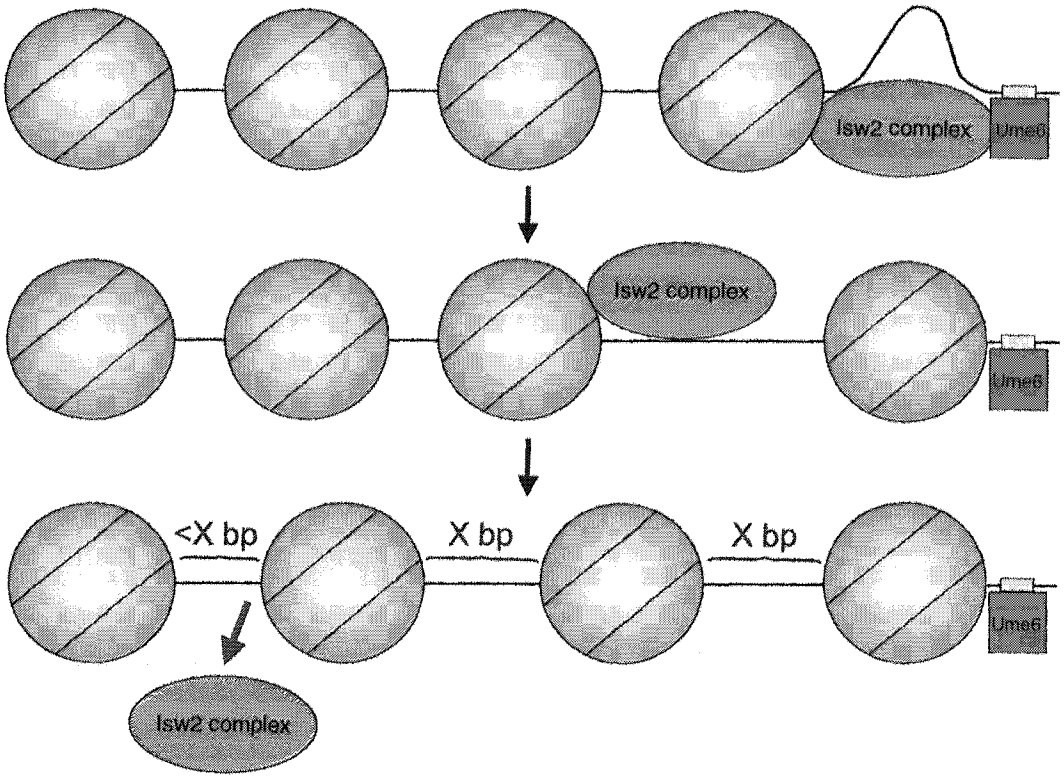


Figure 5.1. Model to explain how Isw2 complex acts locally to remodel chromatin. See text for details.

movement at this site. Other questions regarding Isw2 complex include: What are the functions of the small subunits in nucleosome sliding or Isw2 localization? What determines where Isw2 complex localizes on chromatin, besides interaction with DNA binding proteins? and Does Isw2 complex function in biological processes other than regulation of transcription? Experiments designed to address each of these questions are currently underway in the Tsukiyama laboratory.

Bibliography

Aach, J., W. Rindone, and G.M. Church. 2000. Systematic Management and Analysis of Yeast Gene Expression Data. *Genome Res* **10**: 431-445.

Aalfs, J.D., G.J. Narlikar, and R.E. Kingston. 2001. Functional differences between the human ATP-dependent nucleosome remodeling proteins BRG1 and SNF2H. *J Biol Chem* **276**: 34270-8.

Adkins, N.L., M. Watts, and P.T. Georgel. 2004. To the 30-nm chromatin fiber and beyond. *Biochim Biophys Acta* **1677**: 12-23.

Aihara, T., Y. Miyoshi, K. Koyama, M. Suzuki, E. Takahashi, M. Monden, and Y. Nakamura. 1998. Cloning and mapping of SMARCA5 encoding hSNF2H, a novel human homologue of Drosophila ISWI. *Cytogenet Cell Genet* **81**: 191-3.

Alani, E., L. Cao, and N. Kleckner. 1987. A method for gene disruption that allows repeated use of URA3 selection in the construction of multiply disrupted yeast strains. *Genetics* **116**: 541-5.

Alen, C., N.A. Kent, H.S. Jones, J. O'Sullivan, A. Aranda, and N.J. Proudfoot. 2002. A role for chromatin remodeling in transcriptional termination by RNA polymerase II. *Mol Cell* **10**: 1441-52.

Alland, L., R. Muhle, H. Hou, Jr., J. Potes, L. Chin, N. Schreiber-Agus, and R.A. DePinho. 1997. Role for N-CoR and histone deacetylase in Sin3-mediated transcriptional repression. *Nature* **387**: 49-55.

Allard, S., J.Y. Masson, and J. Cote. 2004. Chromatin remodeling and the maintenance of genome integrity. *Biochim Biophys Acta* **1677**: 158-64.

Allfrey, V.G., R. Faulkner, and A.E. Mirsky. 1964. Acetylation and Methylation of Histones and Their Possible Role in the Regulation of Rna Synthesis. *Proc Natl Acad Sci U S A* **51**: 786-94.

Almer, A. and W. Horz. 1986. Nuclease hypersensitive regions with adjacent positioned nucleosomes mark the gene boundaries of the PHO5/PHO3 locus in yeast. *Embo J* **5**: 2681-7.

Almer, A., H. Rudolph, A. Hinnen, and W. Horz. 1986. Removal of positioned nucleosomes from the yeast PHO5 promoter upon PHO5 induction releases additional upstream activating DNA elements. *Embo J* **5**: 2689-96.

Anderson, S.F., C.M. Steber, R.E. Esposito, and J.E. Coleman. 1995. UME6, a negative regulator of meiosis in Saccharomyces cerevisiae, contains a C-terminal Zn_2Cys_6 binuclear cluster that binds the URS1 DNA sequence in a zinc-dependent manner. *Protein Sci* **4**: 1832-43.

Aoyagi, S., G. Narlikar, C. Zheng, S. Sif, R.E. Kingston, and J.J. Hayes. 2002. Nucleosome remodeling by the human SWI/SNF complex requires transient global disruption of histone-DNA interactions. *Mol Cell Biol* **22**: 3653-62.

Badenhorst, P., M. Voas, I. Rebay, and C. Wu. 2002. Biological functions of the ISWI chromatin remodeling complex NURF. *Genes Dev* **16**: 3186-98.

Bannister, A.J., P. Zegerman, J.F. Partridge, E.A. Miska, J.O. Thomas, R.C. Allshire, and T. Kouzarides. 2001. Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain. *Nature* **410**: 120-4.

Barak, O., M.A. Lazzaro, W.S. Lane, D.W. Speicher, D.J. Picketts, and R. Shiekhattar. 2003. Isolation of human NURF: a regulator of Engrailed gene expression. *Embo J* **22**: 6089-100.

Bazett-Jones, D.P., J. Cote, C.C. Landel, C.L. Peterson, and J.L. Workman. 1999. The SWI/SNF complex creates loop domains in DNA and polynucleosome arrays and can disrupt DNA-histone contacts within these domains. *Mol Cell Biol* **19**: 1470-8.

Becker, P.B. and W. Horz. 2002. ATP-dependent nucleosome remodeling. *Annu Rev Biochem* **71**: 247-73.

Belotserkovskaya, R. and S.L. Berger. 1999. Interplay between chromatin modifying and remodeling complexes in transcriptional regulation. *Crit Rev Eukaryot Gene Expr* **9**: 221-30.

Berger, S.L. 2002. Histone modifications in transcriptional regulation. *Curr Opin Genet Dev* **12**: 142-8.

Bernstein, B.E., J.K. Tong, and S.L. Schreiber. 2000. Genomewide studies of histone deacetylase function in yeast. *Proc Natl Acad Sci U S A* **97**: 13708-13.

Biggar, S.R. and G.R. Crabtree. 1999. Continuous and widespread roles for the Swi-Snf complex in transcription. *Embo J* **18**: 2254-64.

Birchler, J.A., M. Pal-Bhadra, and U. Bhadra. 2003. Dosage dependent gene regulation and the compensation of the X chromosome in Drosophila males. *Genetica* **117**: 179-90.

Bochar, D.A., J. Savard, W. Wang, D.W. Lafleur, P. Moore, C.t. J, and R. Shiekhattar. 2000a. A family of chromatin remodeling factors related to Williams syndrome transcription factor. *Proc Natl Acad Sci U S A* **97**: 1038-1043.

Bochar, D.A., L. Wang, H. Beniya, A. Kinev, Y. Xue, W.S. Lane, W. Wang, F. Kashanchi, and R. Shiekhattar. 2000b. BRCA1 is associated with a human SWI/SNF-related complex: linking chromatin remodeling to breast cancer. *Cell* **102**: 257-65.

Bowdish, K.S. and A.P. Mitchell. 1993. Bipartite structure of an early meiotic upstream activation sequence from Saccharomyces cerevisiae. *Mol Cell Biol* **13**: 2172-81.

Bowdish, K.S., H.E. Yuan, and A.P. Mitchell. 1995. Positive control of yeast meiotic genes by the negative regulator UME6. *Mol Cell Biol* **15**: 2955-61.

Bozhenok, L., P.A. Wade, and P. Varga-Weisz. 2002. WSTF-ISWI chromatin remodeling complex targets heterochromatic replication foci. *Embo J* **21**: 2231-41.

Breeden, L. and K. Nasmyth. 1987. Cell cycle control of the yeast HO gene: cis- and trans-acting regulators. *Cell* **48**: 389-97.

Brownell, J.E., J. Zhou, T. Ranalli, R. Kobayashi, D.G. Edmondson, S.Y. Roth, and C.D. Allis. 1996. Tetrahymena histone acetyltransferase A: a homolog to yeast Gcn5p linking histone acetylation to gene activation. *Cell* **84**: 843-51.

Bruno, M., A. Flaus, C. Stockdale, C. Rencurel, H. Ferreira, and T. Owen-Hughes. 2003. Histone H2A/H2B dimer exchange by ATP-dependent chromatin remodeling activities. *Mol Cell* **12**: 1599-606.

Bu, Y. and M.C. Schmidt. 1998. Identification of cis-acting elements in the SUC2 promoter of Saccharomyces cerevisiae required for activation of transcription. *Nucleic Acids Res* **26**: 1002-9.

Bussemaker, H.J., H. Li, and E.D. Siggia. 2001. Regulatory element detection using correlation with expression. *Nat Genet* **27**: 167-71.

Cairns, B.R. 1998. Chromatin remodeling machines: similar motors, ulterior motives. *Trends Biochem Sci* **23**: 20-5.

Carmen, A.A., S.E. Rundlett, and M. Grunstein. 1996. HDA1 and HDA3 are components of a yeast histone deacetylase (HDA) complex. *J Biol Chem* **271**: 15837-44.

Carrozza, M.J., R.T. Utley, J.L. Workman, and J. Cote. 2003. The diverse functions of histone acetyltransferase complexes. *Trends Genet* **19**: 321-9.

Cavalli, G. and F. Thoma. 1993. Chromatin transitions during activation and repression of galactose-regulated genes in yeast. *Embo J* **12**: 4603-13.

Chen, G., J. Fernandez, S. Mische, and A.J. Courey. 1999. A functional interaction between the histone deacetylase Rpd3 and the corepressor groucho in Drosophila development. *Genes Dev* **13**: 2218-30.

Chu, S., J. DeRisi, M. Eisen, J. Mulholland, D. Botstein, P.O. Brown, and I. Herskowitz. 1998. The transcriptional program of sporulation in budding yeast [published erratum appears in Science 1998 Nov 20;282(5393):1421]. *Science* **282**: 699-705.

Clapier, C.R., G. Langst, D.F. Corona, P.B. Becker, and K.P. Nightingale. 2001. Critical role for the histone H4 N terminus in nucleosome remodeling by ISWI. *Mol Cell Biol* **21**: 875-83.

Clapier, C.R., K.P. Nightingale, and P.B. Becker. 2002. A critical epitope for substrate recognition by the nucleosome remodeling ATPase ISWI. *Nucleic Acids Res* **30**: 649-55.

Collins, N., R.A. Poot, I. Kukimoto, C. Garcia-Jimenez, G. Dellaire, and P.D. Varga-Weisz. 2002. An ACF1-ISWI chromatin-remodeling complex is required for DNA replication through heterochromatin. *Nat Genet* **32**: 627-32.

Corona, D.F., C.R. Clapier, P.B. Becker, and J.W. Tamkun. 2002. Modulation of ISWI function by site-specific histone acetylation. *EMBO Rep* **3**: 242-7.

Corona, D.F., G. Langst, C.R. Clapier, E.J. Bonte, S. Ferrari, J.W. Tamkun, and P.B. Becker. 1999. ISWI is an ATP-dependent nucleosome remodeling factor. *Mol Cell* **3**: 239-45.

de La Serna, I.L., K.A. Carlson, D.A. Hill, C.J. Guidi, R.O. Stephenson, S. Sif, R.E. Kingston, and A.N. Imbalzano. 2000. Mammalian SWI-SNF complexes contribute to activation of the hsp70 gene. *Mol Cell Biol* **20**: 2839-51.

de la Serna, I.L., K.A. Carlson, and A.N. Imbalzano. 2001. Mammalian SWI/SNF complexes promote MyoD-mediated muscle differentiation. *Nat Genet* **27**: 187-90.

DeRisi, J.L., V.R. Iyer, and P.O. Brown. 1997. Exploring the metabolic and genetic control of gene expression on a genomic scale. *Science* **278**: 680-6.

Deuring, R., L. Fanti, J.A. Armstrong, M. Sarte, O. Papoulas, M. Prestel, G. Daubresse, M. Verardo, S.L. Moseley, M. Berloco, T. Tsukiyama, C. Wu, S. Pimpinelli, and J.W. Tamkun. 2000. The ISWI chromatin-remodeling protein is required for gene expression and the maintenance of higher order chromatin structure in vivo. *Mol Cell* **5**: 355-65.

Dhalluin, C., J.E. Carlson, L. Zeng, C. He, A.K. Aggarwal, and M.M. Zhou. 1999. Structure and ligand of a histone acetyltransferase bromodomain. *Nature* **399**: 491-6.

Dudley, A.M., C. Rougeulle, and F. Winston. 1999. The Spt components of SAGA facilitate TBP binding to a promoter at a post-activator-binding step in vivo. *Genes Dev* **13**: 2940-5.

Eisen, J.A., K.S. Sweder, and P.C. Hanawalt. 1995. Evolution of the SNF2 family of proteins: subfamilies with distinct sequences and functions. *Nucl. Acids Res.* **23**: 2715-2723.

Fan, H.Y., X. He, R.E. Kingston, and G.J. Narlikar. 2003. Distinct strategies to make nucleosomal DNA accessible. *Mol Cell* **11**: 1311-22.

Fazzio, T.G., C. Kooperberg, J.P. Goldmark, C. Neal, R. Basom, J. Delrow, and T. Tsukiyama. 2001. Widespread collaboration of Isw2 and Sin3-Rpd3 chromatin remodeling complexes in transcriptional repression. *Mol Cell Biol* **21**: 6450-60.

Fazzio, T.G. and T. Tsukiyama. 2003. Chromatin remodeling in vivo: evidence for a nucleosome sliding mechanism. *Mol Cell* **12**: 1333-40.

Fyodorov, D.V., M.D. Blower, G.H. Karpen, and J.T. Kadonaga. 2004. Acf1 confers unique activities to ACF/CHRAC and promotes the formation rather than disruption of chromatin in vivo. *Genes Dev* **18**: 170-83.

Fyodorov, D.V. and J.T. Kadonaga. 2002a. Binding of Acf1 to DNA involves a WAC motif and is important for ACF-mediated chromatin assembly. *Mol Cell Biol* **22**: 6344-53.

-. 2002b. Dynamics of ATP-dependent chromatin assembly by ACF. *Nature* **418**: 897-900.

Gelbart, M.E., T. Rechsteiner, T.J. Richmond, and T. Tsukiyama. 2001. Interactions of Isw2 chromatin remodeling complex with nucleosomal arrays: analyses using recombinant yeast histones and immobilized templates. *Mol Cell Biol* **21**: 2098-2106.

Gelbart, M.E. and T. Tsukiyama. manuscript in preparation.

Goldmark, J.P., T.G. Fazzio, P.W. Estep, G.M. Church, and T. Tsukiyama. 2000. The Isw2 chromatin remodeling complex represses early meiotic genes upon recruitment by Ume6p. *Cell* **103**: 423-33.

Gregory, P.D. and W. Horz. 1999. Mapping chromatin structure in yeast. *Methods Enzymol* **304**: 365-76.

Gregory, P.D., A. Schmid, M. Zavari, M. Munsterkotter, and W. Horz. 1999. Chromatin remodelling at the PHO8 promoter requires SWI-SNF and SAGA at a step subsequent to activator binding. *Embo J* **18**: 6407-14.

Grunstein, M. 1997. Histone acetylation in chromatin structure and transcription. *Nature* **389**: 349-52.

Guarente, L. 2000. Sir2 links chromatin silencing, metabolism, and aging. *Genes Dev* **14**: 1021-6.

Guldener, U., S. Heck, T. Fielder, J. Beinhauer, and J.H. Hegemann. 1996. A new efficient gene disruption cassette for repeated use in budding yeast. *Nucleic Acids Res* **24**: 2519-24.

Guschin, D., T.M. Geiman, N. Kikyo, D.J. Tremethick, A.P. Wolffe, and P.A. Wade. 2000. Multiple ISWI ATPase complexes from xenopus laevis. Functional conservation of an ACF/CHRAC homolog. *J Biol Chem* **275**: 35248-55.

Hakimi, M.A., D.A. Bochar, J.A. Schmiesing, Y. Dong, O.G. Barak, D.W. Speicher, K. Yokomori, and R. Shiekhattar. 2002. A chromatin remodelling complex that loads cohesin onto human chromosomes. *Nature* **418**: 994-8.

Hamiche, A., R. Sandaltzopoulos, D.A. Gdula, and C. Wu. 1999. ATP-dependent histone octamer sliding mediated by the chromatin remodeling complex NURF. *Cell* **97**: 833-42.

Hassig, C.A., T.C. Fleischer, A.N. Billin, S.L. Schreiber, and D.E. Ayer. 1997. Histone deacetylase activity is required for full transcriptional repression by mSin3A. *Cell* **89**: 341-7.

Havas, K., A. Flaus, M. Phelan, R. Kingston, P.A. Wade, D.M. Lilley, and T. Owen-Hughes. 2000. Generation of superhelical torsion by ATP-dependent chromatin remodeling activities. *Cell* **103**: 1133-42.

Havas, K., I. Whitehouse, and T. Owen-Hughes. 2001. ATP-dependent chromatin remodeling activities. *Cell Mol Life Sci* **58**: 673-82.

Hayes, J.J., T.D. Tullius, and A.P. Wolffe. 1990. The structure of DNA in a nucleosome. *Proc Natl Acad Sci U S A* **87**: 7405-9.

Heinzel, T., R.M. Lavinsky, T.M. Mullen, M. Soderstrom, C.D. Laherty, J. Torchia, W.M. Yang, G. Brard, S.D. Ngo, J.R. Davie, E. Seto, R.N. Eisenman, D.W. Rose, C.K. Glass, and M.G. Rosenfeld. 1997. A complex containing N-CoR, mSin3 and histone deacetylase mediates transcriptional repression. *Nature* **387**: 43-8.

Hieter, P., C. Mann, M. Snyder, and R.W. Davis. 1985. Mitotic stability of yeast chromosomes: a colony color assay that measures nondisjunction and chromosome loss. *Cell* **40**: 381-92.

Hirschhorn, J.N., S.A. Brown, C.D. Clark, and F. Winston. 1992. Evidence that SNF2/SWI2 and SNF5 activate transcription in yeast by altering chromatin structure. *Genes Dev* **6**: 2288-98.

Holstege, F.C., E.G. Jennings, J.J. Wyrick, T.I. Lee, C.J. Hengartner, M.R. Green, T.R. Golub, E.S. Lander, and R.A. Young. 1998. Dissecting the regulatory circuitry of a eukaryotic genome. *Cell* **95**: 717-28.

Hughes, T.R., M.J. Marton, A.R. Jones, C.J. Roberts, R. Stoughton, C.D. Armour, H.A. Bennett, E. Coffey, H. Dai, Y.D. He, M.J. Kidd, A.M. King, M.R. Meyer, D. Slade, P.Y. Lum, S.B. Stepaniants, D.D. Shoemaker, D. Gachotte, K. Chakraburtty, J. Simon, M. Bard, and S.H. Friend. 2000a. Functional discovery via a compendium of expression profiles. *Cell* **102**: 109-26.

Hughes, T.R., C.J. Roberts, H. Dai, A.R. Jones, M.R. Meyer, D. Slade, J. Burchard, S. Dow, T.R. Ward, M.J. Kidd, S.H. Friend, and M.J. Marton. 2000b. Widespread aneuploidy revealed by DNA microarray expression profiling. *Nat Genet* **25**: 333-7.

Imai, S., C.M. Armstrong, M. Kaeberlein, and L. Guarente. 2000. Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. *Nature* **403**: 795-800.

Ito, T., M. Bulger, R. Kobayashi, and J.T. Kadonaga. 1996. Drosophila NAP-1 is a core histone chaperone that functions in ATP-facilitated assembly of regularly spaced nucleosomal arrays. *Mol Cell Biol* **16**: 3112-24.

Ito, T., M. Bulger, M.J. Pazin, R. Kobayashi, and J.T. Kadonaga. 1997. ACF, an ISWI-containing and ATP-utilizing chromatin assembly and remodeling factor. *Cell* **90**: 145-55.

Ito, T., M.E. Levenstein, D.V. Fyodorov, A.K. Kutach, R. Kobayashi, and J.T. Kadonaga. 1999. ACF consists of two subunits, Acf1 and ISWI, that function cooperatively in the ATP-dependent catalysis of chromatin assembly. *Genes Dev* **13**: 1529-39.

Jackson, J.C. and J.M. Lopes. 1996. The yeast UME6 gene is required for both negative and positive transcriptional regulation of phospholipid biosynthetic gene expression. *Nucleic Acids Res* **24**: 1322-9.

James, T.C., J.C. Eissenberg, C. Craig, V. Dietrich, A. Hobson, and S.C. Elgin. 1989. Distribution patterns of HP1, a heterochromatin-associated nonhistone chromosomal protein of Drosophila. *Eur J Cell Biol* **50**: 170-80.

Johnson, N.L. and S. Kotz. 1970. *Continuous Univariate Distributions*. Houghton-Mifflin, Boston.

Kadonaga, J.T. 1998. Eukaryotic transcription: an interlaced network of transcription factors and chromatin-modifying machines. *Cell* **92**: 307-13.

Kadosh, D. and K. Struhl. 1997. Repression by Ume6 involves recruitment of a complex containing Sin3 corepressor and Rpd3 histone deacetylase to target promoters. *Cell* **89**: 365-71.

-. 1998a. Histone deacetylase activity of Rpd3 is important for transcriptional repression in vivo. *Genes Dev* **12**: 797-805.

-. 1998b. Targeted recruitment of the Sin3-Rpd3 histone deacetylase complex generates a highly localized domain of repressed chromatin in vivo. *Mol Cell Biol* **18**: 5121-7.

Kassabov, S.R., N.M. Henry, M. Zofall, T. Tsukiyama, and B. Bartholomew. 2002. High-resolution mapping of changes in histone-DNA contacts of nucleosomes remodeled by ISW2. *Mol Cell Biol* **22**: 7524-34.

Kasten, M.M., S. Dorland, and D.J. Stillman. 1997. A large protein complex containing the yeast Sin3p and Rpd3p transcriptional regulators. *Mol Cell Biol* **17**: 4852-8.

Kent, N.A., N. Karabetsou, P.K. Politis, and J. Mellor. 2001. In vivo chromatin remodeling by yeast ISWI homologs Isw1p and Isw2p. *Genes Dev* **15**: 619-26.

Khavari, P.A., C.L. Peterson, J.W. Tamkun, D.B. Mendel, and G.R. Crabtree. 1993. BRG1 contains a conserved domain of the SWI2/SNF2 family necessary for normal mitotic growth and transcription. *Nature* **366**: 170-4.

Kikyo, N., P.A. Wade, D. Guschin, H. Ge, and A.P. Wolffe. 2000. Active remodeling of somatic nuclei in egg cytoplasm by the nucleosomal ATPase ISWI. *Science* **289**: 2360-2.

Kingston, R.E. and G.J. Narlikar. 1999. ATP-dependent remodeling and acetylation as regulators of chromatin fluidity. *Genes Dev* **13**: 2339-52.

Knoepfler, P.S. and R.N. Eisenman. 1999. Sin meets NuRD and other tails of repression. *Cell* **99**: 447-50.

Kooperberg, C., T.G. Fazzio, J.J. Delrow, and T. Tsukiyama. 2002. Improved background correction for spotted DNA microarrays. *J Comput Biol* **9**: 55-66.

Kornberg, R.D. and Y. Lorch. 1999a. Chromatin-modifying and -remodeling complexes. *Curr Opin Genet Dev* **9**: 148-51.

-. 1999b. Twenty-five years of the nucleosome, fundamental particle of the eukaryote chromosome. *Cell* **98**: 285-94.

Kukimoto, I., S. Elderkin, M. Grimaldi, T. Oelgeschlager, and P.D. Varga-Weisz. 2004. The histone-fold protein complex CHRAC-15/17 enhances nucleosome sliding and assembly mediated by ACF. *Mol Cell* **13**: 265-77.

Kuo, M.H. and C.D. Allis. 1998. Roles of histone acetyltransferases and deacetylases in gene regulation. *Bioessays* **20**: 615-26.

Kupiec, M., B. Byers, R.E. Esposito, and A.P. Mitchel. 1997. Meiosis and sporulation in *Saccharomyces cerevisiae*. In *The molecular and cellular biology of the yeast Saccharomyces cerevisiae* (ed. J.R. Pringle, J.R. Broach, and E.W. Jones), pp. 889-1036. Cold Spring Harbor Laboratory Press, Plainview.

Lachner, M., D. O'Carroll, S. Rea, K. Mechtler, and T. Jenuwein. 2001. Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. *Nature* **410**: 116-20.

Laherty, C.D., W.M. Yang, J.M. Sun, J.R. Davie, E. Seto, and R.N. Eisenman. 1997. Histone deacetylases associated with the mSin3 corepressor mediate mad transcriptional repression. *Cell* **89**: 349-56.

Landry, J., A. Sutton, S.T. Tafrov, R.C. Heller, J. Stebbins, L. Pillus, and R. Sternglanz. 2000. The silencing protein SIR2 and its homologs are NAD-dependent protein deacetylases. *Proc Natl Acad Sci U S A* **97**: 5807-11.

Langst, G. and P.B. Becker. 2001a. ISWI induces nucleosome sliding on nicked DNA. *Mol Cell* **8**: 1085-92.

-. 2001b. Nucleosome mobilization and positioning by ISWI-containing chromatin-remodeling factors. *J Cell Sci* **114**: 2561-8.

Langst, G., E.J. Bonte, D.F. Corona, and P.B. Becker. 1999. Nucleosome movement by CHRAC and ISWI without disruption or trans- displacement of the histone octamer. *Cell* **97**: 843-52.

Laurent, B.C., I. Treich, and M. Carlson. 1993. The yeast SNF2/SWI2 protein has DNA-stimulated ATPase activity required for transcriptional activation. *Genes Dev* **7**: 583-91.

LeRoy, G., A. Loyola, W.S. Lane, and D. Reinberg. 2000. Purification and Characterization of a Human Factor That Assembles and Remodels Chromatin. *J Biol Chem* **275**: 14787-14790.

LeRoy, G., G. Orphanides, W.S. Lane, and D. Reinberg. 1998. Requirement of RSF and FACT for transcription of chromatin templates in vitro. *Science* **282**: 1900-4.

Lohr, D. 1984. Organization of the GAL1-GAL10 intergenic control region chromatin. *Nucleic Acids Res* **12**: 8457-74.

Lomvardas, S. and D. Thanos. 2001. Nucleosome sliding via TBP DNA binding in vivo. *Cell* **106**: 685-96.

Longtine, M.S., A. McKenzie, 3rd, D.J. Demarini, N.G. Shah, A. Wach, A. Brachat, P. Philippsen, and J.R. Pringle. 1998. Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae. *Yeast* **14**: 953-61.

Lorch, Y., B.R. Cairns, M. Zhang, and R.D. Kornberg. 1998. Activated RSC-nucleosome complex and persistently altered form of the nucleosome. *Cell* **94**: 29-34.

Lorch, Y., M. Zhang, and R.D. Kornberg. 1999. Histone octamer transfer by a chromatin-remodeling complex. *Cell* **96**: 389-92.

-. 2001. RSC unravels the nucleosome. *Mol Cell* **7**: 89-95.

Loyola, A., G. LeRoy, Y.H. Wang, and D. Reinberg. 2001. Reconstitution of recombinant chromatin establishes a requirement for histone-tail modifications during chromatin assembly and transcription. *Genes Dev* **15**: 2837-51.

Luger, K., A.W. Mader, R.K. Richmond, D.F. Sargent, and T.J. Richmond. 1997. Crystal structure of the nucleosome core particle at 2.8 A resolution [see comments]. *Nature* **389**: 251-60.

Lusser, A. and J.T. Kadonaga. 2003. Chromatin remodeling by ATP-dependent molecular machines. *Bioessays* **25**: 1192-200.

Maldonado, E., M. Hampsey, and D. Reinberg. 1999. Repression: targeting the heart of the matter. *Cell* **99**: 455-8.

Martens, J.A. and F. Winston. 2002. Evidence that Swi/Snf directly represses transcription in S. cerevisiae. *Genes Dev* **16**: 2231-6.

McConnell, A.D., M.E. Gelbart, and T. Tsukiyama. 2004. Histone fold protein dls1p is required for isw2-dependent chromatin remodeling in vivo. *Mol Cell Biol* **24**: 2605-13.

Mitchell, A.P. 1994. Control of meiotic gene expression in Saccharomyces cerevisiae. *Microbiol Rev* **58**: 56-70.

Mizuguchi, G., X. Shen, J. Landry, W.H. Wu, S. Sen, and C. Wu. 2004. ATP-driven exchange of histone H2AZ variant catalyzed by SWR1 chromatin remodeling complex. *Science* **303**: 343-8.

Mizuguchi, G., T. Tsukiyama, J. Wisniewski, and C. Wu. 1997. Role of nucleosome remodeling factor NURF in transcriptional activation of chromatin. *Molec Cell* **1**: 141-150.

Moreau, J.L., M. Lee, N. Mahachi, J. Vary, J. Mellor, T. Tsukiyama, and C.R. Goding. 2003. Regulated displacement of TBP from the PHO8 promoter in vivo requires Cbf1 and the Isw1 chromatin remodeling complex. *Mol Cell* **11**: 1609-20.

Morillon, A., N. Karabetsou, J. O'Sullivan, N. Kent, N. Proudfoot, and J. Mellor. 2003a. Isw1 chromatin remodeling ATPase coordinates transcription elongation and termination by RNA polymerase II. *Cell* **115**: 425-35.

Morillon, A., J. O'Sullivan, A. Azad, N. Proudfoot, and J. Mellor. 2003b. Regulation of elongating RNA polymerase II by forkhead transcription factors in yeast. *Science* **300**: 492-5.

Nagy, L., H.Y. Kao, D. Chakravarti, R.J. Lin, C.A. Hassig, D.E. Ayer, S.L. Schreiber, and R.M. Evans. 1997. Nuclear receptor repression mediated by a complex containing SMRT, mSin3A, and histone deacetylase. *Cell* **89**: 373-80.

Narlikar, G.J., H.Y. Fan, and R.E. Kingston. 2002. Cooperation between complexes that regulate chromatin structure and transcription. *Cell* **108**: 475-87.

Narlikar, G.J., M.L. Phelan, and R.E. Kingston. 2001. Generation and interconversion of multiple distinct nucleosomal states as a mechanism for catalyzing chromatin fluidity. *Mol Cell* **8**: 1219-30.

Neely, K.E. and J.L. Workman. 2002a. The complexity of chromatin remodeling and its links to cancer. *Biochim Biophys Acta* **1603**: 19-29.

-. 2002b. Histone acetylation and chromatin remodeling: which comes first? *Mol Genet Metab* **76**: 1-5.

Neigeborn, L. and M. Carlson. 1984. Genes affecting the regulation of SUC2 gene expression by glucose repression in Saccharomyces cerevisiae. *Genetics* **108**: 845-58.

Ng, H.H., Q. Feng, H. Wang, H. Erdjument-Bromage, P. Tempst, Y. Zhang, and K. Struhl. 2002. Lysine methylation within the globular domain of histone H3 by Dot1 is important for telomeric silencing and Sir protein association. *Genes Dev* **16**: 1518-27.

Park, H.D., R.M. Luche, and T.G. Cooper. 1992. The yeast UME6 gene product is required for transcriptional repression mediated by the CAR1 URS1 repressor binding site. *Nucleic Acids Res* **20**: 1909-15.

Peterson, C.L. 1998. SWI/SNF complex: dissection of a chromatin remodeling cycle. *Cold Spring Harb Symp Quant Biol* **63**: 545-52.

-. 2002. Chromatin remodeling: nucleosomes bulging at the seams. *Curr Biol* **12**: R245-7.

Peterson, C.L., W. Kruger, and I. Herskowitz. 1991. A functional interaction between the C-terminal domain of RNA polymerase II and the negative regulator SIN1. *Cell* **64**: 1135-43.

Phelan, M.L., G.R. Schnitzler, and R.E. Kingston. 2000. Octamer transfer and creation of stably remodeled nucleosomes by human SWI-SNF and its isolated ATPases. *Mol Cell Biol* **20**: 6380-9.

Pollard, K.J. and C.L. Peterson. 1997. Role for ADA/GCN5 products in antagonizing chromatin-mediated transcriptional repression. *Mol Cell Biol* **17**: 6212-22.

Poot, R.A., G. Dellaire, B.B. Hulsmann, M.A. Grimaldi, D.F. Corona, P.B. Becker, W.A. Bickmore, and P.D. Varga-Weisz. 2000. HuCHRAC, a human ISWI chromatin remodelling complex contains hACF1 and two novel histone-fold proteins. *Embo J* **19**: 3377-3387.

Roberts, S.M. and F. Winston. 1997. Essential functional interactions of SAGA, a Saccharomyces cerevisiae complex of Spt, Ada, and Gcn5 proteins, with the Snf/Swi and Srb/mediator complexes. *Genetics* **147**: 451-65.

Rothstein, R. 1991. Targeting, disruption, replacement, and allele rescue: integrative DNA transformation in yeast. *Methods Enzymol* **194**: 281-301.

Rubin-Bejerano, I., S. Mandel, K. Robzyk, and Y. Kassir. 1996. Induction of meiosis in Saccharomyces cerevisiae depends on conversion of the transcriptional represssor Ume6 to a positive regulator by its regulated association with the transcriptional activator Ime1. *Mol Cell Biol* **16**: 2518-26.

Rundlett, S.E., A.A. Carmen, R. Kobayashi, S. Bavykin, B.M. Turner, and M. Grunstein. 1996. HDA1 and RPD3 are members of distinct yeast histone deacetylase complexes that regulate silencing and transcription. *Proc Natl Acad Sci U S A* **93**: 14503-8.

Rundlett, S.E., A.A. Carmen, N. Suka, B.M. Turner, and M. Grunstein. 1998. Transcriptional repression by UME6 involves deacetylation of lysine 5 of histone H4 by RPD3. *Nature* **392**: 831-5.

Saha, A., J. Wittmeyer, and B.R. Cairns. 2002. Chromatin remodeling by RSC involves ATP-dependent DNA translocation. *Genes Dev* **16**: 2120-34.

Santos-Rosa, H., R. Schneider, B.E. Bernstein, N. Karabetsou, A. Morillon, C. Weise, S.L. Schreiber, J. Mellor, and T. Kouzarides. 2003. Methylation of histone H3 K4 mediates association of the Isw1p ATPase with chromatin. *Mol Cell* **12**: 1325-32.

Schmid, A., K.D. Fascher, and W. Horz. 1992. Nucleosome disruption at the yeast PHO5 promoter upon PHO5 induction occurs in the absence of DNA replication. *Cell* **71**: 853-64.

Shimizu, M., S.Y. Roth, C. Szent-Gyorgyi, and R.T. Simpson. 1991. Nucleosomes are positioned with base pair precision adjacent to the alpha 2 operator in Saccharomyces cerevisiae. *Embo J* **10**: 3033-41.

Smith, H.E., S.S. Su, L. Neigeborn, S.E. Driscoll, and A.P. Mitchell. 1990. Role of IME1 expression in regulation of meiosis in Saccharomyces cerevisiae. *Mol Cell Biol* **10**: 6103-13.

Smith, J.S., C.B. Brachmann, I. Celic, M.A. Kenna, S. Muhammad, V.J. Starai, J.L. Avalos, J.C. Escalante-Semerena, C. Grubmeyer, C. Wolberger, and J.D. Boeke. 2000. A phylogenetically conserved NAD+-dependent protein deacetylase activity in the sir2 protein family [In Process Citation]. *Proc Natl Acad Sci U S A* **97**: 6658-63 [MEDLINE record in process].

Spencer, F., S.L. Gerring, C. Connelly, and P. Hieter. 1990. Mitotic chromosome transmission fidelity mutants in Saccharomyces cerevisiae. *Genetics* **124**: 237-49.

Steber, C.M. and R.E. Esposito. 1995. UME6 is a central component of a developmental regulatory switch controlling meiosis-specific gene expression. *Proc Natl Acad Sci U S A* **92**: 12490-4.

Strahl, B.D. and C.D. Allis. 2000. The language of covalent histone modifications. *Nature* **403**: 41-5.

Strich, R., M.R. Slater, and R.E. Esposito. 1989. Identification of negative regulatory genes that govern the expression of early meiotic genes in yeast. *Proc Natl Acad Sci U S A* **86**: 10018-22.

Strich, R., R.T. Surosky, C. Steber, E. Dubois, F. Messenguy, and R.E. Esposito. 1994. UME6 is a key regulator of nitrogen repression and meiotic development. *Genes Dev* **8**: 796-810.

Strohner, R., A. Nemeth, P. Jansa, U. Hofmann-Rohrer, R. Santoro, G. Langst, and I. Grummt. 2001. NoRC--a novel member of mammalian ISWI-containing chromatin remodeling machines. *Embo J* **20**: 4892-900.

Strohner, R., A. Nemeth, K.P. Nightingale, I. Grummt, P.B. Becker, and G. Langst. 2004. Recruitment of the nucleolar remodeling complex NoRC establishes ribosomal DNA silencing in chromatin. *Mol Cell Biol* **24**: 1791-8.

Struhl, K. 1998. Histone acetylation and transcriptional regulatory mechanisms. *Genes Dev* **12**: 599-606.

-. 1999. Fundamentally different logic of gene regulation in eukaryotes and prokaryotes. *Cell* **98**: 1-4.

Sudarsanam, P., Y. Cao, L. Wu, B.C. Laurent, and F. Winston. 1999. The nucleosome remodeling complex, Snf/Swi, is required for the maintenance of transcription in vivo and is partially redundant with the histone acetyltransferase, Gcn5. *Embo J* **18**: 3101-6.

Sudarsanam, P., V.R. Iyer, P.O. Brown, and F. Winston. 2000. Whole-genome expression analysis of snf/swi mutants of saccharomyces cerevisiae [In Process Citation]. *Proc Natl Acad Sci U S A* **97**: 3364-9.

Suka, N., A.A. Carmen, S.E. Rundlett, and M. Grunstein. 1998. The regulation of gene activity by histones and the histone deacetylase RPD3. *Cold Spring Harb Symp Quant Biol* **63**: 391-9.

Suka, N., Y. Suka, A.A. Carmen, J. Wu, and M. Grunstein. 2001. Highly specific antibodies determine histone acetylation site usage in yeast heterochromatin and euchromatin. *Mol Cell* **8**: 473-9.

Sweet, D.H., Y.K. Jang, and G.B. Sancar. 1997. Role of UME6 in transcriptional regulation of a DNA repair gene in Saccharomyces cerevisiae. *Mol Cell Biol* **17**: 6223-35.

Tanny, J.C., G.J. Dowd, J. Huang, H. Hilz, and D. Moazed. 1999. An enzymatic activity in the yeast Sir2 protein that is essential for gene silencing. *Cell* **99**: 735-45.

Tavazoie, S., J.D. Hughes, M.J. Campbell, R.J. Cho, and G.M. Church. 1999. Systematic determination of genetic network architecture. *Nat Genet* **22**: 281-5.

Trachtulcov, P., I. Janatov, S.D. Kohlwein, and J. Hasek. 2000. Saccharomyces cerevisiae gene ISW2 encodes a microtubule-interacting protein required for premeiotic DNA replication. *Yeast* **16**: 35-47 [Record as supplied by publisher].

Trouche, D., C. Le Chalony, C. Muchardt, M. Yaniv, and T. Kouzarides. 1997. RB and hbrm cooperate to repress the activation functions of E2F1. *Proc Natl Acad Sci U S A* **94**: 11268-73.

Tsukiyama, T., P.B. Becker, and C. Wu. 1994. ATP-dependent nucleosome disruption at a heat-shock promoter mediated by binding of GAGA transcription factor. *Nature* **367**: 525-32.

Tsukiyama, T., C. Daniel, J. Tamkun, and C. Wu. 1995. ISWI, a member of the SWI2/SNF2 ATPase family, encodes the 140 kDa subunit of the nucleosome remodeling factor. *Cell* **83**: 1021-6.

Tsukiyama, T., J. Palmer, C.C. Landel, J. Shiloach, and C. Wu. 1999. Characterization of the imitation switch subfamily of ATP-dependent chromatin-remodeling factors in Saccharomyces cerevisiae. *Genes Dev* **13**: 686-97.

Tsukiyama, T. and C. Wu. 1995. Purification and properties of an ATP-dependent nucleosome remodeling factor. *Cell* **83**: 1011-20.

-. 1997. Chromatin remodeling and transcription. *Curr Opin Genet Dev* **7**: 182-91.

Tyler, J.K. 2002. Chromatin assembly. Cooperation between histone chaperones and ATP-dependent nucleosome remodeling machines. *Eur J Biochem* **269**: 2268-74.

Tyler, J.K. and J.T. Kadonaga. 1999. The "dark side" of chromatin remodeling: repressive effects on transcription. *Cell* **99**: 443-6.

van Holde, K.E. 1988. *Chromatin*. Springer-Verlag, New York.

van Leeuwen, F., P.R. Gafken, and D.E. Gottschling. 2002. Dot1p modulates silencing in yeast by methylation of the nucleosome core. *Cell* **109**: 745-56.

Varga-Weisz, P. 2001. ATP-dependent chromatin remodeling factors: nucleosome shufflers with many missions. *Oncogene* **20**: 3076-85.

Varga-Weisz, P.D., M. Wilm, E. Bonte, K. Dumas, M. Mann, and P.B. Becker. 1997. Chromatin-remodelling factor CHRAC contains the ATPases ISWI and topoisomerase II. *Nature* **388**: 598-602.

Vary, J. and T. Tsukiyama. unpublished data.

Vary, J.C., Jr., T.G. Fazzio, and T. Tsukiyama. 2004. Assembly of yeast chromatin using ISWI complexes. *Methods Enzymol* **375**: 88-102.

Vary, J.C., Jr., V.K. Gangaraju, J. Qin, C.C. Landel, C. Kooperberg, B. Bartholomew, and T. Tsukiyama. 2003. Yeast Isw1p forms two separable complexes in vivo. *Mol Cell Biol* **23**: 80-91.

Vidal, M. and R.F. Gaber. 1991. RPD3 encodes a second factor required to achieve maximum positive and negative transcriptional states in Saccharomyces cerevisiae. *Mol Cell Biol* **11**: 6317-27.

Vidal, M., R. Strich, R.E. Esposito, and R.F. Gaber. 1991. RPD1 (SIN3/UME4) is required for maximal activation and repression of diverse yeast genes. *Mol Cell Biol* **11**: 6306-16.

Wade, P.A. and A.P. Wolffe. 1999. Transcriptional regulation: SWItching circuitry. *Curr Biol* **9**: R221-4.

Whitehouse, I., A. Flaus, B.R. Cairns, M.F. White, J.L. Workman, and T. Owen-Hughes. 1999. Nucleosome mobilization catalysed by the yeast SWI/SNF complex. *Nature* **400**: 784-7.

Whitehouse, I., C. Stockdale, A. Flaus, M.D. Szczelkun, and T. Owen-Hughes. 2003. Evidence for DNA translocation by the ISWI chromatin-remodeling enzyme. *Mol Cell Biol* **23**: 1935-45.

Wodicka, L., H. Dong, M. Mittmann, M.H. Ho, and D.J. Lockhart. 1997. Genome-wide expression monitoring in Saccharomyces cerevisiae. *Nat Biotechnol* **15**: 1359-67.

Workman, J.L. and R.E. Kingston. 1998. Alteration of nucleosome structure as a mechanism of transcriptional regulation. *Annu Rev Biochem* **67**: 545-79.

Wu, C., P.B. Becker, and T. Tsukiyama. 2000. ATP-dependent chromatin remodeling by the ISWI complexes. In *Chromatin Structure and Gene Expression* (ed. S.C.R. Elgin and J.L. Workman), pp. 114-134. Oxford University Press, New York.

Wu, J. and M. Grunstein. 2000. 25 years after the nucleosome model: chromatin modifications. *Trends Biochem Sci* **25**: 619-623.

Wu, L. and F. Winston. 1997. Evidence that Snf-Swi controls chromatin structure over both the TATA and UAS regions of the SUC2 promoter in Saccharomyces cerevisiae. *Nucleic Acids Res* **25**: 4230-4.

Yasui, D., M. Miyano, S. Cai, P. Varga-Weisz, and T. Kohwi-Shigematsu. 2002. SATB1 targets chromatin remodelling to regulate genes over long distances. *Nature* **419**: 641-5.

Yoon, H.J. and J. Carbon. 1999. Participation of Bir1p, a member of the inhibitor of apoptosis family, in yeast chromosome segregation events. *Proc Natl Acad Sci U S A* **96**: 13208-13.

Zeng, L. and M.M. Zhou. 2002. Bromodomain: an acetyl-lysine binding domain. *FEBS Lett* **513**: 124-8.

Zhang, L., E.E. Eugeni, M.R. Parthun, and M.A. Freitas. 2003. Identification of novel histone post-translational modifications by peptide mass fingerprinting. *Chromosoma* **112**: 77-86.

Zhang, Y., R. Iratni, H. Erdjument-Bromage, P. Tempst, and D. Reinberg. 1997. Histone deacetylases and SAP18, a novel polypeptide, are components of a human Sin3 complex. *Cell* **89**: 357-64.

Zhao, X., E.G. Muller, and R. Rothstein. 1998. A suppressor of two essential checkpoint genes identifies a novel protein that negatively affects dNTP pools. *Mol Cell* **2**: 329-40.

Zhou, Y., R. Santoro, and I. Grummt. 2002. The chromatin remodeling complex NoRC targets HDAC1 to the ribosomal gene promoter and represses RNA polymerase I transcription. *Embo J* **21**: 4632-40.

VITA

Thomas G. Fazzio

Education: Ph.D. Molecular and Cellular Biology, May 2004
Fred Hutchinson Cancer Research Center and University of Washington
Seattle. Washington, USA
Thesis advisor: Toshio Tsukiyama, D.V.M., Ph.D.

B.S., *magna cum laude*, Biology, June 1997
University of Utah
Salt Lake City, Utah
Advisor: John R. Roth

Publications: Fazzio, T.G. and J.R. Roth. 1996. Evidence that the CysG protein catalyzes the first reaction specific to B12 synthesis in Salmonella typhimurium, insertion of cobalt. *J Bacteriol* **178**: 6952-9.

Goldmark, J.P., T.G. Fazzio, P.W. Estep, G.M. Church, and T. Tsukiyama. 2000. The Isw2 chromatin remodeling complex represses early meiotic genes upon recruitment by Ume6p. *Cell* **103**: 423-33.

Fazzio, T.G., C. Kooperberg, J.P. Goldmark, C. Neal, R. Basom, J. Delrow, and T. Tsukiyama. 2001. Widespread collaboration of Isw2 and Sin3-Rpd3 chromatin remodeling complexes in transcriptional repression. *Mol Cell Biol* **21**: 6450-60.

Kooperberg, C., T.G. Fazzio, J.J. Delrow, and T. Tsukiyama. 2002. Improved background correction for spotted DNA microarrays. *J Comput Biol* **9**: 55-66.

Carey, J.F., S.J. Schultz, L. Sisson, T.G. Fazzio, and J.J. Champoux. 2003. DNA relaxation by human topoisomerase I occurs in the closed clamp conformation of the protein. *Proc Natl Acad Sci U S A* **100**: 5640-5.

Fazzio, T.G. and T. Tsukiyama. 2003. Chromatin remodeling in vivo: evidence for a nucleosome sliding mechanism. *Mol Cell* **12**: 1333-40.

Vary, J.C., Jr., T.G. Fazzio, and T. Tsukiyama. 2004. Assembly of yeast chromatin using ISWI complexes. *Methods Enzymol* **375**: 88-102.