In Vivo Functional Analysis of the Saccharomyces Cerevisiae SWI/SNF Complex: A Dissertation

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In vivo functional analysis of the *Saccharomyces cerevisiae* SWI/SNF complex

A Dissertation Presented

By

Loree Griffin Burns

Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences, Worcester
in partial fulfillment of the requirements for the degree of:

Doctor of Philosophy

July 1997

Biochemistry
IN VIVO FUNCTIONAL ANALYSIS OF THE SACCHAROMYCES CEREVISIAE SWI/SNF COMPLEX

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July 2, 1997
DEDICATION

for Gerry, with love and gratitude
ABSTRACT

Chromatin remodeling is crucial to transcriptional regulation in vivo and a number of protein complexes capable of altering genomic architecture in the budding yeast *Saccaromyces cerevisiae* have been identified. Among these, the SWI/SNF complex, a 2 MDa, eleven subunit protein assembly, has been the most extensively characterized. The SWI/SNF complex is required for the proper expression of a number of genes in yeast, although it is completely dispensable for the expression of others. Likewise, some, but not all, transcriptional activator proteins require SWI/SNF activity in order to function in vivo. The goal of this thesis work was to identify those components of the transcription process which dictate this dependence on SWI/SNF activity.

Using the well characterized UAS\textsubscript{GAL} system, we have determined that one of these components is the nucleosome state of activator binding sites within a promoter. We find that while SWI/SNF activity is not required for the GAL4 activator to bind to and activate transcription from nucleosome-free binding sites, the complex is required for GAL4 to bind and function at low affinity, nucleosomal binding sites in vivo. The SWI/SNF-dependence of these nucleosomal binding sites can be overcome by 1) replacing the low affinity sites with higher affinity, consensus GAL4 binding sequences, or 2) placing the low affinity sites into a nucleosome-free region. These results provide the first in vivo evidence that the SWI/SNF complex can regulate gene expression by modulating the DNA binding of a transcriptional activator protein.
To determine whether specific components of the GAL4 protein are necessary in order for the SWI/SNF complex to modulate binding to nucleosomal sites in our model system, we tested the SWI/SNF-dependent DNA binding of various derivative GAL4 proteins. We find that a functional activation domain is not required for SWI/SNF to modulate GAL4 binding in vivo. Interestingly, like the full length protein, GAL4 derivatives in which the activation domain has been mutated are able to partially occupy nucleosomal sites in the absence of SWI/SNF (binding in the absence of SWI/SNF is at least forty percent lower than in the presence of SWI/SNF), indicating the activation domain is also not required for SWI/SNF-independent DNA binding.

These results support a model in which the SWI/SNF-dependence of a gene reflects the nucleosomal context of its important regulatory sequences, e.g. binding sites for transcriptional regulatory proteins. Although nucleosomal promoter regions have been correlated with SWI/SNF-dependence in the past, there has of yet been no gene at which nucleosome location has correlated with a specific genetic function. In the final part of this thesis work, we initiated a search for an endogenous SWI/SNF-dependent gene for which the nucleosome state of activator binding sites could be determined.
TABLE OF CONTENTS

APPROVAL PAGE................................................................. ii
DEDICATION ........................................................................ iii
ABSTRACT........................................................................ iv
LIST OF TABLES...................................................................... viii
LIST OF FIGURES.................................................................... ix
ABBREVIATIONS USED......................................................... xi

CHAPTER 1: INTRODUCTION................................................... 1
  Transcription and its regulation
  The identification of the SWI/SNF complex
  SWI/SNF function in vivo
  Biochemical properties of the SWI/SNF complex
  Other chromatin remodeling complexes
  The GAL1,10 promoter
  The galactose utilization pathway of S. cerevisiae

CHAPTER 2: MATERIALS AND METHODS................................. 32
  Strain growth and maintenance
  Liquid β-galactosidase assays
  Filter β-galactosidase assays
  Nuclei preparation
  End-labeling of oligonucleotides
  Internal labeling of DNA fragments
  In vivo footprinting
  Indirect end-labeling
  RNA isolation
  Primer extension assays
  Denaturing gel electrophoresis
  Preparation of yeast lysates
  Western blotting
  Strain construction
  Plasmid construction

CHAPTER 3: THE YEAST SWI/SNF COMPLEX FACILITATES BINDING OF A TRANSCRIPTIONAL ACTIVATOR TO NUCLEOSOMAL SITES IN VIVO...... 50
  Background
CHAPTER 4: CHARACTERIZATION OF THE ROLE OF THE GAL4 ACTIVATION DOMAIN IN SWI/SNF TARGETING ................................................................. 82
  Background
  Results
  Discussion

CHAPTER 5: CHARACTERIZATION OF THE GAL- PHENOTYPE OF swi/snf MUTANTS .............................................................................................................. 106
  Background
  Results
  Discussion

CHAPTER 6: SUMMARY .................................................................................................................. 118

BIBLIOGRAPHY .................................................................................................................. 123

APPENDIX A: DETAILED PROTOCOLS .................................................................................. 135
  Isolation of yeast nuclei
  In vivo DMS footprinting
  Indirect end-labeling
  Nystatin permeabilization of spheroplasts

APPENDIX B: RELATED EXPERIMENTS .................................................................................. 151
LIST OF TABLES

Table 1-1: The subunits of the SWI/SNF complex.......................................................... 18

Table 1-2: Some SWI2/SNF2 ATPase family members and their preferred nucleic acid cofactors.............................................................................................................. 24

Table 2-1: Yeast strains used in this study.................................................................. 46

Table 2-2: Yeast strains constructed in this study .................................................. 48

Table 4-1: GAL4 expression plasmids used in this study........................................ 93

Table 5-1: Growth defects of swi/snf mutants.......................................................... 108

Table 5-2: Complementation studies......................................................................... 114
LIST OF FIGURES

Figure 1-1: Preinitiation complex assembly on a eukaryotic promoter..............4

Figure 1-2: The GAL1,10 promoter of Saccharomyces cerevisiae.........................27

Figure 3-1: GAL4 requires functional SWI/SNF complex to activate transcription from two low affinity binding sites ................................................................................ 53

Figure 3-2: GAL4 can bind its sites in the GAL1,10 UAS region in the presence or absence of SWI/SNF ........................................................................................................ 57

Figure 3-3: SWI/SNF is required for complete occupancy of two low affinity GAL4 binding sites in vivo........................................................................................................ 59

Figure 3-4: Phosphorimager analysis of GAL4 site 3 occupancy............................64

Figure 3-5: Nucleosome mapping of the GAL1,10 UAS region .........................67

Figure 3-6: Nucleosome mapping of the two low affinity site reporter locus......... 69

Figure 3-7: Nucleosome mapping of the two high affinity site reporter locus .... 74

Figure 3-8: Nucleosome mapping of the two low affinity site reporter containing a nucleosome positioning element................................................................. 76

Figure 3-9: Predicted nucleosome structure of SWI/SNF-dependent and SWI/SNF-independent promoters ................................................................................................. 79

Figure 4-1: Models for the stabilization of GAL4 binding in vivo.........................84

Figure 4-2: An activation domain is not required for GAL4 binding to low affinity, nucleosomal sites in vivo ................................................................................................. 86

Figure 4-3: Phosphorimager analysis of site occupancy by full length GAL4 protein and the truncated GAL4 DNA binding domain .................................................. 88

Figure 4-4: Western analysis of GAL4 derivative proteins .................................91

Figure 4-5: The GAL4 derivative CD19 is stable in a swi/snf strain ..................95

Figure 4-6: The activation domain mutant CD19 is able to partially bind nucleosomal GAL4 binding sites in the absence of swi/snf .............................. 98
Figure 4-7. Phosphorimager analysis of site occupancy by full length GAL4 and the activation domain mutant CD19 ................................................................. 100

Figure 5-1: *swi/snf* mutants display growth phenotypes............................................. 109

Figure 5-2: Analysis of *GAL* gene transcription in *SWI*⁺ and *swi⁻* yeast.............. 112
ABBREVIATIONS USED

4WJ ................................................................. four way junction

5-FOA ............................................................. 5-fluoroorotic acid

μCi ................................................................. microCurie

μl ................................................................. microliter

μM ................................................................. micromolar

A ................................................................. absorbance

BSA ............................................................... bovine serum albumin

bp ................................................................. basepairs

cpm ............................................................. counts per minute

Ci ................................................................. Curies

CTD ............................................................... carboxy-terminal domain

dATP ............................................................ deoxyadenosine triphosphate

dCTP ............................................................ deoxycytosine triphosphate

dGTP ............................................................ deoxyguanosine triphosphate

DMS .............................................................. dimethylsulfate

DNA ............................................................. deoxyribonucleic acid

dTTP ............................................................ deoxythymidine triphosphate

DTT .............................................................. dithiothreitol

e.g. ................................................................ exempli gratia (for example)

EM ................................................................. electron microscopy

ER ................................................................. estrogen receptor
et al..............................et alibi (and others)
ftz.................................................fushi tarazu
g....................................................gram
GR...............................................glucocorticoid receptor
GTF..............................................general transcription factor
HEPES..............................4- (2-hydroxyethyl) -1-piperazineethanesulfonic acid
i.e..................................................id est (that is)
Inr................................................initiator element
K......................................................thousand
kb...................................................kilo-basepairs
L.....................................................liter
\textit{LacZ}..............................\textit{\textbeta}-galactosidase gene
M....................................................molar
Mb................................................mega-basepairs
MDa........................................mega-Daltons
ml...............................................milliliter
mmol........................................millimoles
mM...............................................millimolar
MNase........................................micrococcal nuclease
ng................................................nanogram
nM...............................................nanomolar
OD............................................optical density
PIC..........................................................preinitiation complex
PIPS.........................................................piperazine - N, N' - bis [2-ethanesulfonic acid]
PK..................................................................proteinase K
PMSF..........................................................phenylmethylsulfonyl fluoride
RNAPII.........................................................RNA polymerase II
RNA....................................................................ribonucleic acid
RNase..........................................................ribonuclease
rpm..................................................................revolutions per minute
SDS..................................................................sodium dodecyl sulfate
SRB..............................................................suppressor of RNA polymerase b
TAF.............................................................TBP associated factor
TBP.............................................................TATA box binding protein
TCA..................................................................trichloroacetic acid
topoII...........................................................topoisomerase II
UAS..................................................................upstream activation sequence
w/v..................................................................weight per volume
X-gal.............................................................5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
CHAPTER I

INTRODUCTION

Transcription and its regulation

The nucleus of the budding yeast *Saccharomyces cerevisiae* houses 1400 kb of DNA and over 6500 open reading frames (Guthrie and Fink, 1991). If expressed at the appropriate time during the life of the cell and in amounts appropriate to specific environmental and developmental cues, these genes allow the yeast cell to grow and reproduce normally. Any interference in the expression of this genetic information, however, can have disastrous consequences for the cell. It is therefore not surprising that transcription, the process by which all eukaryotic cells access genetic information, is tightly regulated. The mechanisms of this regulation, and of the transcription process itself, are highly conserved among eukaryotes.

The past decade has seen an incredible amount of research in the field of eukaryotic gene expression and the fruits of these labors have illuminated the basic mechanisms of the transcription process for protein-coding (class II) genes (reviewed in Zawel and Reinberg, 1993 and Zawel and Reinberg, 1995). The most crucial genetic component of the class II gene is the open reading frame, which serves as the template for mRNA production by RNA polymerase II (RNAPII), the
enzyme responsible for transcribing these genes. In addition to an open reading frame, class II genes contain a number of regulatory sequences which both promote (promoter regions) and regulate (enhancer elements and silencer elements) their transcription. Minimally, a promoter region contains a TATA box and/or an initiator (Inr) element; these DNA sequences direct RNAPII and the rest of the transcription machinery to the proper site for transcription initiation. Enhancers (often called upstream activation sequences or simply UAS in lower eukaryotes) are genetic regulatory elements which function as binding sites for sequence-specific DNA binding proteins. Enhancers are often located far from the transcription start site and core promoter elements, yet the proteins which bind to these regions are able to profoundly affect the process of transcription. While core promoter elements are universal, the more distal enhancer or UAS elements are gene-specific. Together these regulatory sequences help the cell’s transcription machinery to express proper amounts of a given gene at the appropriate time in the life cycle of a cell.

In addition to RNAPII, the transcription apparatus consists of a dizzying array of both general transcription factors (GTFs) and regulatory proteins (activators, repressors, mediators and coactivators). The proper interplay of these proteins with each other and with the genetic regulatory components described above is crucial to proper gene expression. Furthermore, this myriad of protein-protein and protein-DNA interactions provides ample opportunity for the cell to tightly regulate transcription.
In vitro, basal gene expression typically requires a DNA template (coding sequence and a core promoter), RNAPII, the GTFs TATA binding protein (TBP), TFIIB, TFIIF, TFIIE, TFIIH, ribonucleotides and ATP (Zawel and Reinberg, 1995 and references therein). Careful dissection of this in vitro system has determined that transcription begins with the precisely choreographed assembly of the GTFs into a preinitiation complex (PIC) at the core promoter (Buratowski et al, 1989; see Figure 1-1A). PIC assembly is nucleated by the binding of TFIID (TBP and its associated TAFs) to the TATA box, which is typically located thirty basepairs upstream of the transcription start site (Buratowski et al, 1989; Kim et al, 1993). TFIIB enters the complex next by recognizing and binding to promoter-bound TBP (Buratowski et al, 1989; Nikolov et al, 1995). TFIIB has been implicated in start site selection (Pinto et al, 1992; Lagrange et al, 1996) and is thought to guide RNAPII to the proper promoter location via a direct interaction with one of RNAPII's eleven subunits (Li et al, 1994). TFIIF enters the complex at the same time as RNAPII, suggesting that it is also involved in RNAPII recruitment (Buratowski et al, 1989; Killeen et al, 1992). TFIIE and TFIIH appear to enter the complex after RNAPII and TFIIF and since transcription initiation can occur in their absence, these factors are thought to be involved in transcriptional elongation (reviewed in Drapkin and Reinberg, 1994). In support of this notion, TFIIH has a kinase activity capable of phosphorylating the carboxy-terminal domain (CTD) of RNAPII and it is the phosphorylated form of RNAPII which seems to be involved in elongation (Laybourn and Dahmus, 1990). Furthermore,
A.

[Diagram showing a sequence of interactions involving TBP, TFIIB, RNAPII, and other factors at the TATA and Inr sequences.]
Figure 1-1. Preinitiation complex assembly on a eukaryotic promoter. Panel A (previous page), Stepwise assembly pathway. Panel B, Holoenzyme recruitment pathway. Horizontal line depicts a typical eukaryotic promoter with TATA indicating the TATA element, Inr indicating the initiator element, bent arrow indicating the transcription start site and the closed boxes representing upstream activator binding sites. Shapes represent the indicated general transcription factors.
in vitro studies indicate TFIIE may modulate TFIIH activity (Hua et al, 1992; reviewed in Svejstrup et al, 1996). Finally, the GTF TFIIA deserves special comment. Although its role in PIC formation has historically been enigmatic, recent studies indicate TFIIA plays a primarily regulatory role in the initiation of transcription (reviewed in Roeder, 1996 and Nikolov and Burley, 1997). TFIIA recognizes the TBP-TATA element complex in vitro and is required for stable TBP binding at some (but not all) promoters in vivo. This variability in TFIIA requirement may reflect the intrinsic TBP-binding properties of individual promoters.

There are two notable exceptions to the PIC assembly pathway described above. First, the promoters of some class II genes do not contain a TATA element. For these genes, PIC nucleation appears to occur over the Inr element, which encompasses the transcription start site (Smale and Baltimore, 1989). It is believed that TBP as well as its associated factors (TAFs) are recruited to the PIC of TATA-less promoters by interactions with proteins which bind to the Inr (reviewed in Weis and Reinberg, 1992 and Roeder, 1996). Secondly, the isolation of stable RNAPII-containing complexes that contain many of the general transcription factors suggests PICs may exist preassembled in vivo and that for some genes this entire assembly may arrive at the promoter at once (Kim et al, 1994; Maldonado et al, 1996; see Figure 1-1B).

However the PIC comes to be assembled at the promoter, the end result of its presence in vitro is transcription initiation and basal gene expression. Since
the genome is compacted into a chromatin structure which is inhibitory to transcription (see below), and because the majority of transcription factors are likely to be limiting in the nuclear milieu, it is unlikely that “basal” expression exists in vivo. Instead, gene expression within the eukaryotic nucleus is thought to be absolutely dependent upon the activity of gene-specific activator proteins.

Activator proteins are typically modular: their two essential activities, promoter-binding and transcriptional activation, are conferred by distinct and separable domains (Keegan et al, 1986; Giniger and Ptashne, 1987). In addition to these functional domains, an activator protein requires promoter binding sites in order to properly activate the expression of a gene under its control. These binding sites can be found close to (proximal) or far from (distal) the core promoter. By associating with proximal promoter binding sites, the DNA binding domain will bring the activation domain into the general vicinity of the core promoter region, where the PIC is being assembled. Once there, the activation domain is thought to influence the rate and/or efficiency of PIC assembly, and therefore of gene expression, by interacting with one or more of the GTFs. These activator-GTF interactions are believed to stimulate transcription even when activator binding sites are located far from the core promoter; in these cases the DNA located between the distal activator binding sequences and the core promoter region provides a flexible tether which, when looped, can accommodate activator-PIC interactions (Ptashne, 1988; Ptashne and Gann, 1997).

Incidentally, activator-PIC interactions need not be direct; a number of
biochemical activities (reviewed in Koleske and Young, 1995) have been found to mediate transcriptional activation and may in fact serve to physically link enhancer-bound activators with promoter-bound GTFs.

Activators have been shown to interact with TFIID (the biochemical fraction containing TBP and its associated TAFs; Stringer et al. 1990), various TBP associated factors (TAFs; Goodrich et al., 1993; Weinzierl et al., 1993), TFIIB (Roberts et al., 1993; Choy et al., 1993) and TFIIH (Xiao et al., 1994). In some instances, these interactions have been shown to increase the rate and/or extent of PIC assembly, resulting in increased levels of transcription (Lin and Green, 1991; Lieberman and Berk, 1994). However, these experiments have utilized in vitro transcription reactions carried out with partially purified or recombinant transcription factors and naked DNA templates. In this synthetic situation, the DNA template is completely accessible to the proteins with which it is incubated. In contrast, DNA in vivo is tightly associated with cellular proteins to form a compacted, proteinaceous chromatin fiber in which the DNA helix is rather inaccessible. Careful experimentation has verified that the compaction of DNA into chromatin can impede the transcription process; in vitro, transcription from chromatin templates is markedly decreased when compared to transcription from naked DNA templates (Tsuda et al., 1986; Workman and Roeder, 1987; Lorch et al., 1987). Furthermore, this chromatin-mediated transcriptional repression can be overcome by activator proteins, suggesting that in addition to the so-called "true" activation described above, activators may also elevate levels of gene
expression by serving as “anti-repressors” of chromatin structure (reviewed in Paranjape et al, 1994).

Chromatin structure

The evolutionary advantage afforded the eukaryotic cell by the compaction of its genome into chromatin is evidenced by the rigid conservation of the structural components of chromatin. The histone proteins, which comprise the nucleosome core (see below) are among the most highly conserved proteins known; the gene encoding histone H4 is over 98% conserved between cows and peas (Wolffe, 1992). One obvious advantage to a compact chromatin structure is that a large amount of DNA can fit into a very small area. For example, the 3.3 billion basepairs of the human genome, equivalent to over two meters of DNA strand, is somehow packaged into a nucleus only 10 µm, or 10^-5 meters, in size (Wolffe, 1995). On the other hand, the formation of a compact chromatin structure is likely to occlude many of the protein-DNA interactions which, as described above, are required for proper transcriptional initiation.

The primary level of chromatin structure in vivo is the nucleosome. A nucleosome consists of 145 bp of DNA wrapped approximately twice around a core of eight histone proteins (two copies each of histones H2A, H2B, H3 and H4) to form a globular structure. This structure, aptly described as “beads-on-a-string”, is readily visible under the electron microscope (EM) when the chromatin fiber is
extended by low salt treatment. Interactions between adjacent nucleosomes results in still further compaction of the genome as the 10 nm “bead-on-a-string” structure folds into a thicker 30 nm fiber. The formation of higher order chromatin structures is likely to further occlude regulatory proteins from the DNA.

That chromatin structure plays a role in transcriptional regulation has been well established (reviewed in Paranjape et al, 1994). Early experiments in yeast found that misexpression of the genes encoding the core histone proteins could markedly alter gene expression (Clark-Adams et al, 1988; Han and Grunstein, 1988). Furthermore, in some cases these alterations in gene expression were shown to be accompanied by changes in the chromatin structure of the genes in question (Norris et al, 1988). Careful controls utilizing transcriptionally defective reporters have verified that these changes in chromatin architecture at activated genes are not due to the process of transcription itself (Hirschhorn et al, 1992), leaving unchallenged the intriguing possibility that the chromatin alterations are required for (although not necessarily the cause of) gene activation.

The problems posed to transcription by a compacted chromatin conformation are obvious. For example, the formation of a nucleosome directly over a TATA element could sterically impede TBP binding if the histone-DNA contacts within the nucleosome core precluded the necessary TBP-TATA box interactions. Indeed, nucleosomes have been shown to inhibit transcription initiation either by blocking access of the general transcription machinery to
promoter sequences or by hindering the binding of upstream activator proteins in vitro (reviewed in Workman and Buchman, 1993). Evidence for nucleosome repression of transcription has been found in vivo as well. In *Saccharomyces cerevisiae*, nucleosomes located over the UAS of the PHO5 gene under repressing conditions were found to be altered upon gene induction and these alterations are not dependent on transcription of PHO5 (Almer et al., 1986). In contrast, the UAS region of the divergently transcribed GAL1 and GAL10 genes (see below) was found to be constitutively nucleosome-free while nucleosomes positioned over promoter elements seem to be involved in transcriptional regulation (Fedor and Kornberg, 1989; Axelrod et al., 1993; see below). Clearly the regulation of gene expression by chromatin, like the other regulatory mechanisms discussed already, are gene-specific. Advances in the characterization of chromatin structures in genetically amenable systems like *Saccharomyces cerevisiae* has allowed researchers to begin to elucidate the mechanisms involved in chromatin-mediated transcriptional regulation. These studies have uncovered a number of proteins and biochemical activities, distinct from those already discussed, which appear to regulate gene expression at the level of chromatin structure.

In addition to interfering with transcription, the tight association of DNA with histones and non-histone proteins in vivo is likely to inhibit other DNA-mediated cellular processes. Possible examples include recombination, replication and DNA repair. A number of distinct multisubunit complexes that posses chromatin disruption activity have recently been identified and mounting
evidence suggests these complexes may be involved in mediating these other DNA-mediated cellular processes in the context of a repressive chromatin structure (reviewed in Peterson, 1996). The SWI/SNF complex of the budding yeast *Saccharomyces cerevisiae* appears to represent the yeast homologue of a widely conserved complex which regulates transcriptional activation. This complex is the focus of the research presented in this dissertation.

**The identification of the SWI/SNF complex**

The identification of genes that encode subunits of the SWI/SNF complex resulted from the convergence of two independent genetic screens in *S. cerevisiae*: one for products involved in the expression of the *HO* gene (SWIItch genes), and the other for gene products involved in *SUC2* expression (Sucrose Non-Fermenter genes). In addition to a number of genes specifically involved in *HO* expression, the former screen identified mutations in three genes (*SWI1*, *SWI2* and *SWI3*) which were required not only for the expression of *HO*, but also for the expression of a number of other yeast genes (Peterson and Herskowitz, 1992; Stern et al, 1984). Likewise, a subset of the genes identified in the *SUC2* screen (*SNF2*, *SNF5* and *SNF6*) seemed to play a global role in transcriptional activation rather than a specific role in *SUC2* expression (Neigeborn and Carlson, 1984; reviewed in Winston and Carlson, 1992). The functionally related *SWI* and *SNF* gene sets
were found to overlap at the molecular level when cloning and sequencing revealed that \textit{SNF2} and \textit{SWI2} were the same gene (Laurent et al, 1991).

Strikingly, most of the SWI/SNF-dependent genes are inducible, i.e., the expression is tightly regulated by the growth conditions and developmental state of the cell. Furthermore, the regulation of many of these genes involves a known gene-specific transcriptional activator protein. These data suggested that the SWI/SNF gene products might function as global activators of transcription by modulating the activity of activator proteins. In fact, a number of transcriptional activators were found to require SWI/SNF genes in order to function in yeast, such as GAL4 and LexA fusion proteins that contain the activation domain of GAL4 or the activation domain of the \textit{Drosophila} activator bicoid (Peterson and Herskowitz, 1992; Laurent and Carlson, 1992). Of particular interest was the SWI-dependence of the \textit{Drosophila} fushi tarazu (ftz) protein and the mammalian glucocorticoid (GR) and estrogen (ER) receptors (Yoshinaga et al, 1992); the SWI-dependence of these heterologous activators strongly suggested that SWI/SNF function is conserved across evolution.

Since deletion of the \textit{SWI1}, \textit{SWI2}, \textit{SWI3}, \textit{SNF5} and \textit{SNF6} genes independently or in various combinations resulted in identical phenotypes, and since these five genes were required for the expression of an identical set of yeast genes, it seemed likely that these products functioned together, perhaps in a complex. The Peterson and Kornberg laboratories have independently purified this complex and in so doing have verified that the five SWI/SNF gene products
isolated genetically do exist in a large multi-component complex in vivo (Cairns et al, 1994; Peterson et al, 1994). In addition to the subunits encoded by these five known genes, this 2 MDa assembly contains six additional polypeptides (see below).

**SWI/SNF function in vivo**

A clue as to how the SWI/SNF gene products function as global activators of gene expression came from suppressor analyses of *swi/snf* mutants. In these studies, mutations in genes that encode chromatin components were found to suppress the transcriptional defects caused by the loss of SWI/SNF genes (reviewed in Winston and Carlson, 1992; Wolfe, 1994; Peterson and Tamkun, 1995). These suppressors restored the ability of *swi* mutants to properly express SWI/SNF-dependent genes, including *HO* and *SUC2*, and thereby conferred a SWI-INdependent (SIN) phenotype. Mutations in *HHT1* or *HHT2* (encoding histone H3) and *HHF1* or *HHF2* (encoding histone H4) as well as in the *SIN1* gene (encoding an HMG1-like protein which is thought to be a non-histone chromatin component) were found to alleviate the growth and transcriptional defects of *swi1*, *swi2* and *swi3* mutants (Kruger and Herskowitz, 1991; Kruger et al, 1995). In addition, deletion of one of the two *HTA1-HTB1* gene clusters (encoding histones H2A and H2B; Hirschhorn et al, 1992) or mutations in *HHT1* (Prelich and Winston, 1993) were able to suppress the defects in *SUC2* expression of
swi2/snfl, snf5 or snf6 mutants. These genetic interactions suggest that the SWI/SNF gene products function to activate transcription by relieving the transcriptional repression caused by chromatin structure in vivo.

If the SWI/SNF complex functions by alleviating chromatin-mediated transcriptional repression, why does expression of only a subset of genes depend on its activity? For example, although many inducible genes require SWI/SNF function in order to be expressed, other genes, like GAL1, are only slightly affected by the loss of SWI/SNF (Peterson and Herskowitz, 1992). One possibility is that SWI/SNF activity is only required when the chromatin structure of a particular promoter is inhibitory to transcription. For example, a strongly positioned nucleosome located over a TATA box might impede the binding of TBP and thereby inhibit transcription of the adjacent gene. This model predicts that critical promoter elements of SWI/SNF-dependent genes exist within positioned nucleosomes in vivo. The chromatin structure of two SWI-dependent genes, SUC2 and ADH2, have been analyzed. In both cases, positioned nucleosomes appear to play a crucial role in gene expression. The expression of both genes is regulated by carbon source; there is no ADH2 or SUC2 expression when cells are grown in glucose. In this repressed state, nucleosomes are positioned over the TATA box and UAS region of the SUC2 promoter (Hirschhorn et al, 1992). Upon induction, these positioned nucleosomes are disrupted and the gene is actively transcribed. Nucleosome disruption does not require transcription, but does require SWI/SNF activity (Hirschhorn et al, 1992). In the case of ADH2, the promoter is also covered
by an array of positioned nucleosomes when cells are grown in glucose (Verdone et al, 1996). These precisely positioned nucleosomes cover the RNA initiation sites and the TATA box of the ADH2 gene, and are destabilized upon gene induction. It is not known whether the destabilization of these nucleosomes requires SWI/SNF activity. If so, the notion that SWI/SNF function involves chromatin disruption in the vicinity of nucleosomal protein binding sites becomes even stronger.

It is unlikely that the presence of positioned nucleosomes is the sole determinant of SWI/SNF-dependence, because a number of known SWI/SNF-independent genes contain positioned nucleosomes over or near crucial promoter elements. For example, the SWI/SNF-independent PHO5 promoter has a precise nucleosome structure, including a nucleosome positioned over the TATA box and UAS2 (one of two binding sites for the transcriptional activator PHO4; Almer et al, 1986). Similarly, nucleosomes are precisely positioned over the divergent GAL1,10 promoter such that the GAL10 TATA box and the GAL1 transcription start site are nucleosomal (Fedor et al, 1988; Fedor and Kornberg, 1989). In these two cases, gene expression is not strongly dependent on SWI/SNF activity, despite the presence of these positioned nucleosomes (Peterson and Herskowitz, 1992). Thus, SWI/SNF-dependent genes may have a more specific chromatin architecture or other intrinsic properties which render gene expression dependent on SWI/SNF activity. In the studies described in this dissertation, we have utilized the well characterized GAL regulatory system (see below) to directly test this hypothesis.
Biochemical properties of the SWI/SNF complex

Two groups have purified the 2 MDa SWI/SNF complex from *S. cerevisiae* (Cairns et al, 1994; Cote et al, 1994). In addition to the subunits encoded by the five genes isolated genetically, *SWI1, SWI2/SNF2, SWI3, SNF5* and *SNF6*, the complex contains six additional polypeptides (see Table 1-1). One of these, SNF11, was isolated in a two-hybrid screen for proteins that interact with the SWI2/SNF2 protein (Treich, et al, 1995). Another subunit, ANC1, was originally isolated in a genetic screen for proteins involved in actin cytoskeletal function (Welch et al, 1993). In addition to being a component of the SWI/SNF complex, the ANC1 protein is a subunit of the yeast TFIIF (Henry et al, 1994) and TFIID (Cairns et al, 1996c) complexes. The 73 kDa subunit, SWP73, has recently been cloned and sequenced; deletions of and some mutations in the gene encoding this protein result in transcription defects similar to those seen in other *swi/snf* mutants (Cairns et al, 1996a). The genes encoding the other three subunits, *SWP82, SWP61* and *SWP59*, have not yet been reported.

The sequence of the SWI2/SNF2 gene, encoding the largest subunit of the complex, contains seven sequence motifs that are characteristic of members of the DEAD/H superfamily of nucleic acid-stimulated ATPases and helicases (Carlson and Laurent, 1994). Therefore, it is not surprising that one of the biochemical properties displayed by the SWI/SNF complex in vitro is a DNA-stimulated ATPase activity (Cote et al, 1994). Neither the SWI2/SNF2 protein alone, nor the
<table>
<thead>
<tr>
<th>Subunit</th>
<th>Size (kDa)</th>
<th>Other names</th>
<th>Human homolog</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>SWI2</td>
<td>194</td>
<td>SNF2, GAM1, TYE3, RIC1</td>
<td>hbrm, BRG1</td>
<td>Laurent et al, 1991a</td>
</tr>
<tr>
<td>SWI1</td>
<td>165</td>
<td>ADR6, GAM3</td>
<td>?</td>
<td>Peterson and Herskowitz, 1992b</td>
</tr>
<tr>
<td>SWI3</td>
<td>91</td>
<td>TYE2</td>
<td>BAF155, BAF170</td>
<td>Peterson and Herskowitz, 1992c</td>
</tr>
<tr>
<td>SNF5</td>
<td>103</td>
<td>TYE4</td>
<td>INI1, BAF47</td>
<td>Muchardt et al, 1995c,d</td>
</tr>
<tr>
<td>SWP82</td>
<td>82</td>
<td></td>
<td>?</td>
<td>Cairns et al, 1994e</td>
</tr>
<tr>
<td>SWP73</td>
<td>73</td>
<td></td>
<td>BAF60</td>
<td>Wang et al, 1996f</td>
</tr>
<tr>
<td>SWP61</td>
<td>61</td>
<td></td>
<td>?</td>
<td>Cairns et al, 1994e</td>
</tr>
<tr>
<td>SWP59</td>
<td>59</td>
<td></td>
<td>?</td>
<td>Cairns et al, 1994e</td>
</tr>
<tr>
<td>SNF6</td>
<td>38</td>
<td></td>
<td>?</td>
<td>Estruch and Carlson, 1990</td>
</tr>
<tr>
<td>SWP29</td>
<td>27</td>
<td>ANC1, TFG3, yTAFii30</td>
<td>?</td>
<td>Welch et al, 1993f,h</td>
</tr>
<tr>
<td>SNF11</td>
<td>19</td>
<td></td>
<td>?</td>
<td>Treich et al, 1995</td>
</tr>
</tbody>
</table>

Table 1-1. The subunits of the SWI/SNF complex. The eleven polypeptides which comprise the yeast SWI/SNF complex are listed with their size, aliases and known human homologs. a, see also Khuvari et al, 1993 and Muchardt and Yaniv, 1993; b, see also Taguchi and Young, 1987; c, see also Wang et al, 1996; d, see also Laurent et al, 1990 and Kalpana et al, 1994; e, see also Cote et al, 1994; f, see also Cairns et al, 1996a; g, see also Cote et al, 1994; h, see also Henry et al, 1994.
pured SWI/SNF complex has detectable DNA helicase or DNA tracking activity in vitro (Cote et al, 1994; Laurent et al, 1993; Quinn et al, 1996). As with other members of the DEAD/H superfamily of ATPases, the ATPase activity of the SWI/SNF complex requires a nucleic acid cofactor (Cote et al, 1994; Cairns et al, 1994). In particular, the ATPase activity of the complex is stimulated over 30-fold by double-stranded, single-stranded or nucleosomal DNA, but not by RNA (Cote et al, 1994). Interestingly, the presence of synthetic four way junction (4WJ) DNA stimulates the ATPase activity of the complex to levels similar to those seen in the presence of plasmid DNA (Quinn et al, 1996).

The ATPase activity of the SWI/SNF complex is crucial to its function; a mutation in the putative nucleotide binding loop of SWI2 obliterates the ATPase activity of the complex in vitro and yeast strains carrying such mutant alleles of SWI2 display the growth and transcriptional defects of a swi2 deletion mutant (Laurent et al, 1993; Khuvari et al, 1993). Together with the genetic suppression data (see above), these results suggest that the SWI/SNF complex might use the energy of ATP hydrolysis to disrupt chromatin structure.

Consistent with this view, Cote et al (1994) have shown that purified SWI/SNF is capable of disrupting the rotational positioning of DNA within a mononucleosome in an ATP-dependent manner. Furthermore, this disruption of rotational positioning coincides with an increased affinity of the GAL4-AH activator for a nucleosomal binding site. In fact, the affinity of GAL4-AH for a nucleosomal site in the presence of SWI/SNF and ATP is only about three-fold
lower than for naked DNA. Like the disruption of rotational positioning, the ability of the SWI/SNF complex to modulate activator binding in this in vitro assay is completely dependent on ATP hydrolysis (Cote et al, 1994).

These experiments suggest that the SWI/SNF complex is able to use the energy of ATP hydrolysis to modulate transcription factor binding to nucleosomal templates, although the biochemical mechanism by which this occurs is as yet unknown.

Other chromatin remodeling complexes

Since the cloning of the SWI2/SNF2 gene, a number of genes have been identified that contain ATPase domains which are more homologous to that of the SWI2/SNF2 protein than to other members of the nucleic acid-stimulated ATPase and helicase family (Carlson and Laurent, 1994). These genes have recently been analyzed phylogenetically and arranged into eight subfamilies which appear to represent functionally and evolutionarily distinct classes of proteins (Eisen et al, 1995).

The SWI2/SNF2 subfamily contains S. cerevisiae SWI2/SNF2 as well as another yeast gene which was identified based on its homology to SWI2/SNF2, called STH1 (Laurent et al, 1992). Recently, Cairns et al (1996b) have purified a novel 15-subunit complex from S. cerevisiae which contains the STH1 protein as one of its subunits. In addition, this abundant complex, called RSC (for its ability
to Remodel the Structure of Chromatin), also contains two other SWI/SNF-related subunits: RSC6 is homologous to SWP73 and RSC8 is homologous to SWI3. Like the SWI/SNF complex, RSC complex purified from yeast displays a DNA-dependent ATPase activity and is able to alter nucleosome structure in vitro. Unlike the SWI/SNF complex, however, the genes encoding the subunits of the RSC complex are essential for yeast viability.

Other members of the SWI2/SNF2 subfamily include the human genes *BRG1* (Khuvare et al, 1993; Chiba et al, 1994) and *hBRM* (Chiba et al, 1994; Muchardt and Yaniv, 1993) and the *Drosophila* gene *brahma* (Tamkun et al, 1992). The *brg1* protein was first isolated from HeLa cells as part of a multimeric protein complex which, like RSC, has many of the same biochemical properties as the yeast SWI/SNF complex (Khuvare et al, 1993; Kwon et al, 1994; Cairns et al, 1996b). *hBRM* is also present in a high molecular weight complex in human cells and these two complexes are thought to represent human variants of the yeast SWI/SNF and RSC complexes (Wang et al, 1996). It has further been speculated that the *brahma* protein is the catalytic subunit of the *Drosophila* version of the SWI/SNF complex as *brahma* can be isolated in a high molecular weight complex with the product of the *SNR1* gene, a homologue of the yeast *SNF5* (Dingwall et al, 1995; Muchardt et al, 1995). Domain swapping experiments suggest that the members of the SWI2/SNF2 subfamily function in ways similar to that of the SWI2/SNF2 protein. In these experiments, the ATPase domain of SWI2/SNF2 was replaced with the corresponding ATPase domain of either *brg1*, *hBRM* or
STH1. In all cases the chimeric proteins were able to restore proper growth and
transcriptional function in a swi2 mutant (Laurent et al, 1993; Khuvari et al,
1993; Elfring et al, 1994). In contrast, replacement of the ATPase domain of
SWI2/SNF2 with that of ISWI (a member of a distinct subfamily; see below) was
not able to complement a swi2 mutant (Elfring et al, 1994).

The subfamily which is most closely related to the SWI2/SNF2 subfamily
includes the human hSNF2L gene and the Drosophila ISWI gene (this subfamily
is referred to as the hSNF2L subfamily). These genes cannot complement the
transcriptional defects of a swi2- strain in domain swap experiments, suggesting
their roles are functionally distinct from that of SWI2/SNF2 (Elfring et al, 1994;
Okabe et al, 1992). However, recent evidence has suggested that the members of
this subfamily may also play a role in chromatin remodeling. Tsukiyama and Wu
(1995) have recently reported the purification of an ATP-dependent nucleosome
remodeling factor (NURF) from Drosophila embryo extracts. This complex
facilitates, in an ATP-dependent manner, the disruption and redistribution of
nucleosomes by the GAGA transcription factor in vitro. The NURF complex is
composed of four subunits; the 140 kDa subunit is encoded by the ISWI gene
(Tsukiyama et al, 1995). Although clearly related to the yeast SWI/SNF complex,
the NURF complex has unique cofactor requirements and biochemical
characteristics which suggest that it may disrupt chromatin through a distinct
biochemical mechanism. For example, unlike the yeast SWI/SNF complex, the
ATPase activity of NURF complex is not stimulated by the presence of double-
stranded plasmid DNA, but is stimulated five-fold in the presence of nucleosomal DNA (Tsukiyama and Wu, 1995).

The Becker laboratory has also identified an ATP-dependent chromatin remodeling activity in Drosophila extracts (Varga-Weisz et al, 1995; Wall et al, 1995). Molecular analysis of Chromatin Accessibility Complex (CHRAC) has identified two of its five subunits as ISWI and topoisomerase II (topo II; Varga-Weisz et al, In Press). The presence of ISWI in both the CHRAC and NURF complexes lends further support to the notion that members of the SWI2/SNF2 ATPase superfamily are modular components of the cell's chromatin remodeling machinery.

The list of proteins that belong to the SWI2/SNF2 superfamily of ATPases is growing steadily (Carlson and Laurent, 1994; Eisen et al, 1995). A number of these, including the recombination genes RAD16 and RAD54, are involved in cellular processes other than transcription. These results have led to the speculation that numerous chromatin reconfiguration machines, each driven by a distinct SWI2/SNF2-like ATPase motor, may exist in vivo to allow distinct classes of DNA binding proteins access to the DNA at the appropriate time (Peterson, 1996). Auble et al (1994) proposed that the different SWI2/SNF2-like proteins have unique functions because they are targeted to different protein-DNA complexes (see Table 1-2). For example, the yeast SWI/SNF complex is targeted to DNA bound histones, RAD16/RAD54 to repair enzymes bound to sites of DNA damage, and MOT1, a TBP associated factor (TAF), to TBP-bound DNA.
Table 1-2. Some SWI2/SNF2 ATPase family members and their preferred nucleic acid cofactors. The cofactor requirement for each of the SWI2/SNF2-like proteins listed has been determined and may dictate the specificity of complex activity. DS, double-stranded; SS, single-stranded

<table>
<thead>
<tr>
<th>Family member</th>
<th>Preferred cofactor</th>
</tr>
</thead>
<tbody>
<tr>
<td>SWI2/SNF2 (SWI/SNF)</td>
<td>Synthetic four-way junctions (strand crossover) DS or SS plasmid DNA Nucleosomal DNA (arrays)</td>
</tr>
<tr>
<td>ISWI (NURF)</td>
<td>Nucleosomal DNA</td>
</tr>
<tr>
<td>MOT1</td>
<td>TBP-DNA complex</td>
</tr>
</tbody>
</table>
However, the differences in cofactor requirements between some of these ATPases (see above) may point to another explanation for their specificity. Each enzyme may require a unique structured DNA to stimulate its ATPase activity, which may dictate its role in chromatin regulation; the yeast SWI2/SNF2 complex uses DNA strand crossovers as a cofactor for its ATPase activity, NURF ATPase activity is stimulated by nucleosomal DNA, RAD16/RAD54 ATPase activities may require damaged DNA as a cofactor and MOT1 might recognize the bent DNA structure formed upon TBP binding to DNA. Cofactor specificity would limit the catalytic activity of each complex to the appropriate site in the genome.

Testing these hypotheses awaits the purification of these distinct SWI2/SNF2-like ATPase complexes.

The identification of multiple chromatin remodeling complexes and the apparent conservation of these complexes from yeasts to mammals underscores the importance of this level of transcriptional regulation. In order to begin a comprehensive analysis of the role of one chromatin remodeling complex (the yeast SWI/SNF complex) in transcriptional regulation, we utilized the well characterized GAL gene system of *S. cerevisiae*.

**The GAL1,10 promoter**

The UAS region which controls expression of the divergently expressed *GAL1* and *GAL10* genes of *S. cerevisiae* has been well characterized at a molecular
level (West et al., 1984). This region contains four binding sites for the transcriptional activator GAL4 as well as a site for the abundant cellular protein GRF2 (Fedor et al., 1988; Chasman et al., 1990). Nucleosome mapping experiments have clearly demonstrated that this region adopts a precise chromatin architecture in vivo: using indirect end-labeling, Fedor and Kornberg (1989) have found that the 150 bp region including and immediately surrounding the four GAL4 binding sites is nucleosome-free under all growth conditions (See Figure 1-2). This nucleosome-free region is flanked by precisely positioned nucleosomes, one between the TATA element and transcription start site of the GAL1 gene and the other in the region encompassing the GAL10 TATA element. These strongly positioned nucleosomes serve as a boundary between the constitutively nucleosome-free GAL4 binding sequences and two surrounding nucleosome arrays (see Figure 1-2). This architecture is believed to be dependent upon the protein GRF2, which recognizes a DNA sequence overlapping sites 1 and 2 of the GAL1,10 UAS (Fedor et al., 1988) and may be involved in setting up and/or maintaining the nucleosome structure of this region.

Peterson et al have found that transcriptional activation by GAL4 on a LacZ reporter gene whose expression is driven by the GAL1,10 UAS region is SWI/SNF-independent, i.e. in a swi1Δ strain, GAL4 can activate the same high levels of transcription as it does in a SWI+ strain. Derivatives of this reporter, however, display varying SWI/SNF-dependencies (Craig L. Peterson and Ira Herskowitz, unpublished observations), suggesting that the determinants of
Figure 1-2. The *GAL1,10* promoter of *Saccharomyces cerevisiae*. Schematic representation of the divergent GAL1,10 promoter demonstrating the nucleosome-free GAL4 binding sites (shaded boxes) and the precisely positioned nucleosomes (ovals). Figure is adapted from Fedor and Kornberg, 1989 and is not drawn to scale.
SWI/SNF-dependent gene expression lie within the promoter region itself.

Utilizing the \textit{GAL} regulatory system described below, we set out to identify these determinants.

The galactose utilization pathway of \textit{S. cerevisiae}

There are nine genes that are necessary for yeast cells to properly utilize galactose as a carbon source (reviewed in Johnston, 1987 and Lohr et al, 1995). The structural genes \textit{GAL1} (encoding a kinase), \textit{GAL2} (encoding a permease), \textit{GAL7} (encoding a transferase), \textit{GAL10} (encoding an epimerase), \textit{MEL1} (encoding a galactosidase) and \textit{GAL5} (encoding a mutase) allow the cell to take up galactose from its surroundings and process it into glucose-6-phosphate, a substrate for glycolysis. The expression of these genes is entirely restricted to those instances when galactose is present in the media and glucose, the preferred carbon source, is absent. This tight regulation involves the products of the remaining three \textit{GAL} genes, \textit{GAL4} (encoding a transcriptional activator), \textit{GAL80} (encoding a transcriptional inhibitor) and \textit{GAL3} (encoding a molecule required for rapid induction).

In a wildtype yeast cell, the \textit{GAL} genes can exist in three regulatory states, depending on the carbon source. When cells are grown with glucose as the sole carbon source, expression of all of the \textit{GAL} genes except \textit{GAL80} is repressed. Under these repressing conditions the genes encoding the transcriptional activator
GAL4 and the inducer molecule GAL3 are repressed by global transcription repression mechanisms involving the MIG1 protein (Johnston et al., 1994). In addition, any residual GAL4 protein present in the cell is prevented from activating transcription of the other GAL genes by repressive mechanisms involving inhibitory DNA elements located near the UAS_GAL region of these genes (URS element; Johnston et al., 1994).

In the absence of glucose, the global (MIG1-mediated) and specific (URS-mediated) repression mechanisms just described are circumvented and the transcription state of the GAL genes now depends on the presence of the inducer galactose. For example, when cells are grown with glycerol as the sole source of carbon (therefore no glucose and no galactose), GAL4 protein is made and can bind its sites in UAS_GAL regions. However, this promoter-bound GAL4 protein is unable to activate transcription because of the presence of the inhibitor GAL80. GAL80 binds to and masks the C-terminal transactivation domain of GAL4 (Ma and Ptashne, 1987b; Lue et al., 1987), thereby inhibiting its ability to activate the transcription of downstream genes.

On the other hand, when cells are grown in the absence of glucose (therefore no glucose repression mechanisms are activated) and in the presence of galactose, the GAL1, GAL2, GAL7, GAL10 and MEL1 genes are induced 100 to 1000-fold within minutes. The ability of galactose to induce this robust expression requires the product of the GAL3 gene, which is thought to interact with GAL80 in such a way as to inhibit its ability to interact with the GAL4 activation domain (Suzuki-
Fujimoto et al, 1996). The unmasked, UAS-bound GAL4 is therefore able to induce high levels of expression of all the UAS_{GAL}-containing genes. Unlike the significant induction of expression seen for the other GAL genes (see above), GAL5, GAL3 and GAL80 expression are induced only 3-10-fold (reviewed in Lohr et al, 1995). This variability in GAL4 induction may reflect the number and intrinsic effectiveness of the GAL4 binding sites within individual UAS_{GAL} regions; endogenous and consensus GAL4 binding sequences have been found to differ in GAL4 binding affinity in vitro (Kang et al, 1993, Vashee et al, 1993) and in vivo (Giniger and Ptashne, 1988).

GAL4 transcriptional activation has been extensively characterized. The various UAS_{GAL} regions contain from one (in the case of the GAL80, GAL3 and MEL1) to four (in the case of the divergently transcribed GAL1 and GAL10 genes; see above) copies of the GAL4 binding site. These binding elements together define a consensus GAL4 binding sequence, 5' - CGG A(C/G)G AC(A/T) GTC (G/C)TC CG - 3', and a synthetic DNA that differs from this consensus at only two positions (5' - CGGAAGACTCTCCTCCG - 3') has been shown to bind GAL4 protein in vivo (Giniger et al, 1985; this study) and in vitro (Carey et al, 1989) with high affinity. Consensus or endogenous GAL4 binding sites, either singly or in combination, are able to confer galactose-dependent expression onto heterologous genes in vivo (West et al, 1984; Giniger et al, 1985; and for example Martin et al, 1990). In general, increased copy number correlates with increased levels of
transcriptional activation and cooperativity is thought to play a significant role in GAL4 activity.

Since GAL4-responsive promoters which require SWI/SNF activity have been identified (see previous section) and because the galactose utilization pathway is so well characterized, we chose the GAL system of *Saccharomyces cerevisiae* to investigate the relationship between transcriptional activation, the SWI/SNF complex and chromatin structure in vivo. In particular, we attempted to determine: 1) which elements, if any, of a GAL gene promoter dictate SWI/SNF-dependence, 2) which domains, if any, of the transcriptional activator protein GAL4 are involved in SWI/SNF regulation, and 3) if any endogenous SWI/SNF-dependent, GAL4-responsive genes exist in *Saccharomyces cerevisiae*. The results of these lines of inquiry are detailed in the following chapters.
CHAPTER II

MATERIALS AND METHODS

Strain growth and maintenance

Yeast strains were grown in YEP medium (10 g/L yeast extract and 20 g/L peptone) supplemented with 20 mg/L adenine and containing as a carbon source either 2% glucose or 2% galactose with 0.5 % sucrose. To select growth of transformed yeast strains, cells were grown in minimal medium (6.7 g/L yeast nitrogen base without amino acids) supplemented with the appropriate amino acids and as a carbon source either 2% glucose or 2% galactose with 0.5% sucrose. YEPD and YEPGal plates were made as described above and contained 20 g/L agar. Where appropriate, antimycin was added to a final concentration of 1 μg/μl.

Liquid β-galactosidase assays

β-galactosidase activity of strains harboring LacZ reporter constructs was monitored by liquid β-galactosidase assay as described (Stern et al, 1984). Strains were grown in the appropriate medium to an OD₆₀₀ of 0.5-0.9. One ml of this log phase culture was harvested by centrifugation and the pellet was washed in 1 ml of Z Buffer (8.53 g/L Na₂HPO₄, 5.5 g/L NaH₂PO₄,
0.75 g/L KCl, 0.246 g/L MgSO₄). Washed pellet was resuspended in 150 μl of Z Buffer and cells were treated with 50 μl chloroform and 20 μl of 0.1% SDS. After 30 seconds of vigorous vortexing, 700 μl of 0.1 mg/ml ONPG, 2.7 μl/ml β-mercaptoethanol was added and the sample was mixed by inversion. Samples were incubated at 30°C until a yellow color was visible. Reactions were stopped with 500 μl 1M Na₂CO₃. The absorbance (420 nm) of each sample was determined via spectrophotometry and Miller Units of β-galactosidase activity was determined with the following formula:

Units = (1000 × A₄₂₀) / (OD₆₀₀ × time × volume).

**Filter β-galactosidase assays**

Yeast cells were grown overnight on filter paper (Whatman 50) placed over the appropriate agar medium. In a sterile petri dish, a second filter paper (Whatman 3) was soaked in 0.03% X-gal (prepared from a stock of 2% X-Gal in dimethyl formamide) in Z Buffer (8.53 g/L Na₂HPO₄, 5.5 g/L NaH₂PO₄, 0.75 g/L KCl, 0.246 g/L MgSO₄, 2.7 μl/ml β-mercaptoethanol). Yeast cells were permeabilized on the Whatman 50 filter by immersion in liquid nitrogen for thirty seconds and the frozen filter was placed on top of the X-gal soaked filter. Care was taken to remove any air from between the two filters and plates were incubated at 37°C until a blue color became visible.
Nuclei preparation

Nuclei were prepared essentially as described (Shimuzu et al, 1991). Briefly, a one liter culture was grown in the appropriate medium to an OD$_{600}$ of 0.5-1.4. Cells were harvested by centrifugation and washed in S Buffer (1.4 M sorbitol, 40 mM HEPES pH 7.3, 0.5 mM MgCl$_2$, 10 mM β-mercaptoethanol, 1 mM PMSF). After washing, cells were resuspended in S Buffer and incubated for ten minutes at 30°C. Cells were collected by centrifugation and resuspended in a volume of S Buffer equal to four times the weight of the pellet. Cells were digested with 1 ml of 10 mg/ml 100T Zymolyase (Seikagaku Corporation) for 45 minutes at 30°C. Digestion was monitored under the microscope and was considered complete when >90% of the cells burst upon suspension in 0.1% SDS. Digestion was stopped by dilution with S Buffer lacking β-mercaptoethanol.

Spheroplasts were washed twice in S buffer lacking β-mercaptoethanol and disrupted by four passes through a Yamato LS-21 homogenizer (100 rpm) in F Buffer (18% w/v Ficoll 400, 20 mM PIPES pH 6.5, 0.5 mM MgCl$_2$, 1mM PMSF). Disrupted spheroplasts were carefully layered on top of GF Buffer (20% w/v glycerol, 7% w/v Ficoll 400, 20 mM PIPES pH 6.5, 0.5 mM MgCl$_2$, 1mM PMSF) and subjected to high speed centrifugation. The resulting pellet was completely resuspended in F Buffer by vortexing for five minutes at 4°C. Cellular debris was collected by low speed centrifugation and discarded.
Nuclei were isolated from the supernatant by high speed centrifugation.

Nuclear pellet was resuspended in D buffer (10mM HEPES, pH 7.3, 0.5 mM MgCl₂, 0.05 mM CaCl₂) to make approximately 5 X 10⁸ cell equivalents/ml. These nuclei were frozen in liquid nitrogen in and stored at -80°C.

**End-labelling of oligonucleotides**

End-labelled oligonucleotides were prepared for use in the in vivo footprinting assay and the high resolution nucleosome mapping experiments. 100 ng of oligonucleotide was labelled in a 25 µl reaction containing 70 mM Tris-HCl pH 7.6, 10 mM MgCl₂, 5 mM dithiothreitol and 200 µCi ³²P-γ-ATP (Amersham; 6000 Ci/mmol). Ten units of T4 polynucleotide kinase (New England BioLabs) was added and the entire reaction was incubated at 37°C for 10-30 minutes. Labelled oligonucleotides were purified over a G-25 Sephadex (Pharmacia) spin column and incorporation of label measured on a scintillation counter. The specific activity of probes prepared using this protocol ranged between 5-10 X 10⁸ cpm/µg.

**Internal labelling of DNA fragments**

Body labelled DNA probes were prepared for use in indirect end-labelling analyses. Approximately 0.1-1 µg of agarose gel purified DNA fragment was incubated with random primers and heated to 100°C for two
minutes, followed by a brief cooling on ice. Random Priming Buffer (2.5X stock: 500 mM HEPES pH 6.6, 125 mM Tris pH 8.0, 12.5 mM MgCl2, 28 mM β-mercaptoethanol, 0.5 mM dTTP, 0.5 mM dGTP, 0.5 mM dATP), 100 μCi α 32P-dCTP (Amersham, 6000 Ci/mmol) and 5-10 units DNA polymerase I Klenow fragment (New England BioLabs) were then added and the entire reaction was incubated at room temperature for one hour. Labelled DNA was purified over a G-25 Sephadex (Pharmacia) spin column and incorporation of label measured in a scintillation counter.

In vivo footprinting

A 200 ml or 100 ml yeast culture was grown in the appropriate medium to an OD600 of 0.75 (minimal media) or 1.5-2 (rich media), respectively. Intact cells were treated with dimethyl sulfate and the methylated DNA was isolated essentially as described (Giniger et al, 1985), except final pellets were dissolved in water and quantitated on a spectrophotometer. DNA samples were then digested with HaeIII and analyzed via a cyclic primer extension reaction (Axelrod and Majors, 1989) using a labelled oligonucleotide. Primer extension products were extracted with chloroform:isoamyl alcohol (24:1), precipitated and electrophoresed on a 5% denaturing gel. The gel was dried and exposed to film for 12-48 hours. As a control for spurious primer extension products, DNA was also prepared from a
strain that was not treated with DMS. Footprinting at the wildtype GAL1,10 UAS was analyzed using an oligonucleotide with the following sequence: 5' - GAG CCC CAT TAT CTT AGC - 3'. This oligonucleotide anneals to a sequence located 60 bp upstream of GAL4 binding site 1. Footprinting at the integrated GAL1,10 UAS derivatives was analyzed using an oligonucleotide with the following sequence: 5' - CCG GCT CGT ATG TTG TGT GG - 3'. This oligonucleotide anneals to the unique pUC sequences located upstream of the GAL4 binding sites in these reporters. Footprinting at integrated GAL1,10 UAS derivatives containing the nucleosome positioning element (NPE) was analyzed using an oligonucleotides with the following sequence: 5' - CGG TTA GTA CTT AAT TCC - 3' (which anneals to basepairs 159 through 176 of the NPE element; numbering of Meersseman et al, 1991) or 5' - CCG GTT CTC GTC CGA TCA CCG - 3' (which anneals to basepairs 118 through 137 of the NPE element; numbering of Meersseman et al, 1991).

**Indirect end-labelling**

Nuclei were prepared as described above. To prepare Free DNA, 80µl 10% SDS and 30 µl 20 mg/ml Proteinase K were added to a 1 ml aliquot of nuclei (approximately 5 X 10⁹ cell equivalents) and the sample incubated at 37°C for two hours. Lysed nuclei were treated with 180 µl 5 M potassium acetate on ice for one hour, subjected to centrifugation and the supernatant
precipitated with isopropanol. This DNA sample was resuspended in water and reprecipitated with ethanol. The resultant Free DNA was resuspended in 1 ml Buffer D2 (10 mM HEPES pH 7.3, 5 mM MgCl₂, 2 mM CaCl₂). Free DNA and nuclei samples were digested with 0 to 50 Units of MNase for five minutes at 37°C. MNase reactions were stopped by the addition of SDS and Proteinase K. Samples were next treated with 5 M potassium acetate on ice for one hour, subjected to centrifugation and the supernatant precipitated with isopropanol. DNA was then digested with either ClaI (to map nucleosomes at the integrated GAL₁₁₀ UAS derivative reporters) or EcoRI (to map nucleosomes at the endogenous GAL₁₁₀ locus) in the presence of RNase. Digestions were extracted with buffered phenol and the aqueous layer precipitated with ethanol. Final pellets were electrophoresed on a 1.5% agarose gel overnight. DNA was transferred to a nylon membrane and blots were probed with an internally labelled 870 bp LacZ fragment (to map nucleosomes at the integrated GAL₁₁₀ UAS derivative reporters) or an internally labelled 550 bp fragment from the GAL₁ gene (to map nucleosomes at the endogenous GAL₁₁₀ locus).
RNA isolation

A saturated yeast culture was diluted to an OD\textsubscript{600} of 0.15 into 100 ml of the appropriate medium and allowed to grow to an OD\textsubscript{600} of 0.6 - 0.8. Cells were harvested by centrifugation, washed in 5 ml of sterile water and resuspended in 100 μl of RNA Prep Buffer (0.5 M NaCl, 0.2 M Tris pH 7.5, 0.01 M EDTA, 1% SDS). Resuspended cells were added to a tube containing 100 μl of phenol/chloroform/isoamyl alcohol (25:24:1) and approximately 0.2 g of glass beads. Samples were vortexed five times in thirty second intervals with incubations on ice between pulses. An additional 150 μl RNA Prep Buffer and 150 μl phenol/chloroform/isoamyl alcohol was added to each sample, followed by 15 seconds of mixing. Samples were then spun for 10 minutes at room temperature in a microcentrifuge. 150 μl of the aqueous phase was extracted with phenol/chloroform/isoamyl alcohol and precipitated with three volumes of ethanol at -20°C for at least one hour. RNA was collected by centrifugation and washed with 70% ethanol before resuspension in sterile, RNase-free water. RNA samples were quantitated on a spectrophotometer and integrity was monitored on a 1% TBE agarose gel. Samples were then stored at -20°C until needed.
Primer extension assays

Transcription levels were monitored using a modified version of a previously described primer extension protocol (McKnight and Kingsbury, 1982). Briefly, 10-20 μg of total yeast RNA and 1 ng of end-labelled oligonucleotide were boiled in 1.25 M KCl, 10 mM Tris pH 8.0, 1 mM EDTA for one minute. Reactions were then incubated for 30-60 minutes at 50°C. The annealed oligonucleotide primer was extended with 10 Units of AMV Reverse Transcriptase (Promega) in the presence of 2.5 mM deoxynucleotides (dATP, dCTP, dGTP, dTTP), 50 mM Tris-HCl pH 8.0, 5 mM MgCl₂, 100 μg/ml Actinomycin D, 10 mM DTT and 0.25 mM EDTA for 30 minutes at 42°C. Reactions were halted with 30 μl 5 M ammonium acetate pH 7.5 and precipitated with 180 μl ethanol at -20°C. Reaction products were collected by centrifugation, resuspended in 8 μl of loading dye (95% formamide, 20 mM EDTA, 0.05% Bromophenol Blue, 0.05% Xylene Cyanol) and electrophoresed on an 6% denaturing gel. After electrophoresis, the gel was dried and exposed to film. Sequences of the oligonucleotides used are listed below:

GAL1 5' - GCG CTA GAA TTG AAC TCA GG - 3'
GAL2 5' - GAT TGT GCG CTT AAA TGG G - 3'
GAL3 5' - CTC TGC CCA GGA GAC CTA GCG - 3'
GAL5 5' - GGC ACC TTT AGA ACC CTC TGG - 3'
GAL7 5' - GGT CTT TTA GCT CTG TGT GG - 3'
Denaturing gel electrophoresis

Denaturing gels of the appropriate percentage were prepared using National Diagnostics Sequagel® sequencing system according to the manufacturer’s instructions. Gel solutions were filtered immediately before polymerization.

Preparation of yeast lysates

Yeast cultures were grown to saturation overnight, diluted to an OD$_{600}$ of approximately 0.15 in the appropriate medium and then allowed to grow to an OD$_{600}$ of 0.5 - 0.9. Five OD units of culture were treated with 1/10 volume of 100% trichloroacetic acid (TCA). Cells were collected by centrifugation, resuspended in cold TCA buffer (20 mM Tris pH 8.0, 50 mM NH$_4$OAc, 2 mM EDTA) and disrupted by vigorous mixing in the presence of glass beads. Supernatant was transferred to a fresh tube and the beads washed with a fresh 1:1 mixture of 20% TCA: TCA Buffer. Wash buffer was added to supernatant and samples spun for five minutes in a microfuge on the highest
setting. Pellets were washed with cold acetone, dried and resuspended in TCA Resuspension Solution (3% SDS, 100 mM Tris, pH 11.0, 3 mM DTT). Finally, samples were heated to 65°C for five minutes, boiled for five minutes more and then cooled briefly on ice. Insoluble debris was collected by centrifugation and supernatants were frozen at -80°C until use.

Western blotting

Lysates prepared from yeast strains harboring GAL4 expression plasmids were electrophoresed on 10% SDS-containing, polyacrylamide gels and transferred to a nitrocellulose membrane (Schleicher & Schuell) by standard methods (Maniatis, 1989). Membranes were incubated with agitation in a solution of 10% powdered milk (prepared in TBST; 100 mM Tris pH 7.5, 0.9% NaCl, 0.1% Tween) for 30 minutes at room temperature to block non-specific binding sites on the membrane. Following two ten second rinses in TBST, the membrane was incubated with an antibody directed against the DNA binding domain of GAL4 (α-Gal4 DBD; Santa Cruz) that had been diluted 1:1000 in 2% milk/TBST. Best results were obtained when this incubation was allowed to continue for twelve hours with agitation. After washing, the membrane was incubated for 45 minutes with goat anti-mouse secondary antibody that had been diluted 1:10,000 in 2% milk/TBST. Finally,
the membrane was washed and antibody recognition was detected with the Lumi-Glo® chemiluminescence kit according to manufacturer's instructions.

Strain construction

Four sets of isogenic yeast strains were created for the studies described in Chapter 3 of this thesis (see Table 2-2). The four strains composing each of these sets were derived from the yeast strains CY388 (GAL4+, SWI1+), CY398 (gal4Δ, SWI1+), CY399 (GAL4+, swi1Δ) and CY400 (gal4Δ, swi1Δ; see Table 2-1). These founder strains contained a reporter construct integrated at the ura3-52 locus. In each case, the integration resulted in a tandem duplication of the URA3 gene (one wildtype and one ura3-52 allele) with intervening plasmid sequences. To select for cells in which this integrated reporter had been lost (via a genetic recombination event, i.e. “pop-out”), each strain was passaged twice on plates containing 5-FOA. The 5-FOA resistant strains created in this way were called CY524, CY525, CY526 and CY527, respectively.

These four new strains were then used to create the four strain sets mentioned above. In each case, a unique LacZ reporter construct was targetted for integration at the ura3-52 locus by digestion with ApaI. CY528, CY529, CY530 and CY531 were created by integrating the plasmid pLB7 (which contains a LacZ reporter driven by a GAL1,10 UAS derivative bearing
only two high affinity GAL4 binding sites) into CY524, CY525, CY526 and CY527, respectively. CY532, CY533, CY534 and CY535 were created by integrating the plasmid pLB8 (which contains a LacZ reporter driven by a GAL1,10 UAS derivative bearing only two low affinity GAL4 binding sites) into CY524, CY525, CY526 and CY527, respectively. Similarly, strains CY586, CY587, CY588 and CY589 were prepared by integrating the plasmid pLB16 (which is identical to pLB8 except for the addition of a nucleosome positioning element upstream of the GAL4 binding sites) and strains CY594, CY595, CY596 and CY597 were prepared by integrating the plasmid pLB15 (which is identical to pLB7 except for the addition of a nucleosome positioning element upstream of the GAL4 binding sites). The genotypes of these strains are summarized in Table 2-2. For details on the plasmids, see below.

After the strains described above were prepared, it was discovered that in the GAL4 deletion strain of each strain set (gal4Δ; CY525, CY529, CY533, CY587 and CY595), the reporter gene had been integrated at a site other than ura3-52, most likely the GAL1,10 locus. This is presumably due to the fact that the founding GAL4 deletion strain, CY398, did not contain an intact ura3-52 locus for integration.
Plasmid constructions

Plasmids pLB7 and pLB8 were constructed by inserting the 194 bp SmaI-PvuII fragment of pUC18 between the URA3 gene and the GAL4 binding sites of the plasmids pEG44 and pEG28, respectively. Plasmid pEG44 contains two synthetic, consensus GAL4 binding sites positioned 150 bp upstream of the GAL1 TATA element. These two sites are separated by 32 bp (center to center distance). Plasmid pEG28 contains GAL4 binding sites 3 and 4 positioned 190 bp upstream of the GAL1 TATA element, as they are in the endogenous GAL1,10 locus. Sites 3 and 4 are separated by 62 bp (center to center distance). Plasmids pLB15 and pLB16 were constructed by inserting a 182 bp PCR fragment encoding the strong nucleosome positioning element from the 5S RNA genes of the sea urchin Lytechinus variegatus immediately upstream of the GAL4 binding sites in pLB7 and pLB8, respectively. This fragment was amplified from pICN5S182 (gift from Jerry Workman) using the oligonucleotides 5' - CCACGAATAACTTCCAGGG - 3' and 5' - CCCCGAGGAATTAAGTAC - 3'.
Table 2-1. Yeast strains used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>CY257</td>
<td>MATa swi1Δ::LEU2 lys2-801 ade2-101 leu2-Δ1 his3-Δ200 ura3-Δ99</td>
</tr>
<tr>
<td>CY296</td>
<td>MATa gal4Δ::LEU2 lys2-801 ade2-101 leu2-Δ1 his3-Δ200 ura3-Δ99</td>
</tr>
<tr>
<td>CY297</td>
<td>MATa gal4Δ::LEU2 swi1Δ::LEU2 lys2-801 ade2-101 leu2-Δ1 his3-Δ200 ura3-Δ99</td>
</tr>
<tr>
<td>CY340</td>
<td>MATa lys2-Δ99 ade2-101 leu2-Δ1 his3-Δ200 ura3-Δ99 trp1-Δ99</td>
</tr>
<tr>
<td>CY341</td>
<td>MATa lys2-Δ99 ade2-101 leu2-Δ1 his3-Δ200 ura3-Δ99</td>
</tr>
<tr>
<td>CY348</td>
<td>swi1Δ::LEU2 gal80Δ ura3-52 URA3::pRY171 (GAL1,10)</td>
</tr>
<tr>
<td>CY349</td>
<td>gal80Δ ura3-52 URA3::pRY171 (GAL1,10)</td>
</tr>
<tr>
<td>CY353</td>
<td>MATa lys2-801 ade2-101 leu2-Δ1 his3-Δ200 ura3-52 URA3::pRY171 (GAL1,10)</td>
</tr>
<tr>
<td>CY366</td>
<td>MATa swi1Δ::LEU2 lys2-Δ1 ade2-101 leu2-Δ1 his3-Δ200 ura3-52 URA3::pRY171 (GAL1,10)</td>
</tr>
<tr>
<td>CY388</td>
<td>MATa lys2-801 ade2-101 leu2-Δ1 his3-Δ200 ura3-52 URA3::pEG44 (2 hi)</td>
</tr>
<tr>
<td>CY398</td>
<td>MATa gal4Δ::LEU2 lys2-801 ade2-101 leu2-Δ1 his3-Δ200 ura3-52 URA3::pEG44 (2 hi)</td>
</tr>
<tr>
<td>CY399</td>
<td>MATa swi1Δ::LEU2 lys2-801 ade2-101 leu2-Δ1 his3-Δ200 ura3-52 URA3::pEG44 (2 hi)</td>
</tr>
<tr>
<td>Strain</td>
<td>Gene Mutations</td>
</tr>
<tr>
<td>--------</td>
<td>----------------</td>
</tr>
<tr>
<td>CY400</td>
<td>MATa gal4Δ::LEU2 swi1Δ::LEU2 lys2-801 ade2-101 leu2-Δ1 his3-Δ200 ura3-52 URA3::pEG44 (2 hi)</td>
</tr>
<tr>
<td>CY401</td>
<td>MATa lys2-801 ade2-101 leu2-Δ1 his3-Δ200 ura3-52 URA3::pEG28 (2 lo)</td>
</tr>
<tr>
<td>CY422</td>
<td>MATa swi1Δ::LEU2 lys2-801 ade2-101 leu2-Δ1 his3-Δ200 ura3-52 URA3::pEG28 (2 lo)</td>
</tr>
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Table 2-2. Yeast strains constructed in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genetic Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>CY524</td>
<td>MATα lys2-801 ade2-101 leu2-Δ1 his3-Δ200 ura3-52</td>
</tr>
<tr>
<td>CY525</td>
<td>MATα gal4Δ::LEU2 lys2-801 ade2-101 leu2-Δ1 his3-Δ200 ura3-52</td>
</tr>
<tr>
<td>CY526</td>
<td>MATα swi1Δ::LEU2 lys2-801 ade2-101 leu2-Δ1 his3-Δ200 ura3-52</td>
</tr>
<tr>
<td>CY527</td>
<td>MATα gal4Δ::LEU2 swi1Δ::LEU2 lys2-801 ade2-101 leu2-Δ1 his3-Δ200 ura3-52</td>
</tr>
<tr>
<td>CY528</td>
<td>Same as CY524, but contains URA3::pLB7 (2hi-pUC)</td>
</tr>
<tr>
<td>CY529</td>
<td>Same as CY525, but contains URA3::pLB7 (2hi-pUC)</td>
</tr>
<tr>
<td>CY530</td>
<td>Same as CY526, but contains URA3::pLB7 (2hi-pUC)</td>
</tr>
<tr>
<td>CY531</td>
<td>Same as CY527, but contains URA3::pLB7 (2hi-pUC)</td>
</tr>
<tr>
<td>CY532</td>
<td>Same as CY524, but contains URA3::pLB8 (2lo-pUC)</td>
</tr>
<tr>
<td>CY533</td>
<td>Same as CY525, but contains URA3::pLB8 (2lo-pUC)</td>
</tr>
<tr>
<td>CY534</td>
<td>Same as CY526, but contains URA3::pLB8 (2lo-pUC)</td>
</tr>
<tr>
<td>CY535</td>
<td>Same as CY527, but contains URA3::pLB8 (2lo-pUC)</td>
</tr>
<tr>
<td>CY586</td>
<td>Same as CY524, but contains URA3::pLB16 (2 lo-NPE)</td>
</tr>
<tr>
<td>CY587</td>
<td>Same as CY525, but contains URA3::pLB16 (2 lo-NPE)</td>
</tr>
<tr>
<td>CY588</td>
<td>Same as CY526, but contains URA3::pLB16 (2 lo-NPE)</td>
</tr>
<tr>
<td>CY589</td>
<td>Same as CY527, but contains URA3::pLB16 (2 lo-NPE)</td>
</tr>
<tr>
<td>CY594</td>
<td>Same as CY524, but contains URA3::pLB15 (2 hi-NPE)</td>
</tr>
<tr>
<td>CY595</td>
<td>Same as CY525, but contains \textit{URA3::pLB15} (2 hi-NPE)</td>
</tr>
<tr>
<td>CY596</td>
<td>Same as CY526, but contains \textit{URA3::pLB15} (2 hi-NPE)</td>
</tr>
<tr>
<td>CY597</td>
<td>Same as CY527, but contains \textit{URA3::pLB15} (2 hi-NPE)</td>
</tr>
</tbody>
</table>
CHAPTER III

THE YEAST SWI/SNF COMPLEX FACILITATES BINDING OF A TRANSCRIPTIONAL ACTIVATOR TO NUCLEOSOMAL SITES IN VIVO

Background

Giniger et al (1985) were the first to demonstrate that the GAL4 protein is capable of binding to the four recognition sites found in the GAL1,10 UAS region in vivo. These authors also showed that GAL4 could recognize a synthetic 17 bp consensus sequence in vivo and that this sequence, when linked to a heterologous reporter gene, could confer high levels of galactose inducibility (Giniger et al, 1985). In subsequent studies, Giniger and Ptashne (1988) demonstrated that GAL4 binding can be cooperative in vivo; these authors also suggested that this cooperativity might explain the phenomenon of transcriptional synergy at the GAL1,10 locus. In addition to providing the early characterization of a genetic system which has since become an indispensable tool for the study of eukaryotic transcriptional regulation, these early studies served as a foundation for the experiments described in the following pages. First, the in vivo footprinting assay
developed in this study is based on that used in these earlier studies. In addition, the GAL1,10 UAS derivatives they constructed and characterized provided a crucial starting point for the derivative promoters studied here.

Selleck and Majors (1987) have also monitored GAL4 binding in vivo. These authors used a photofootprinting assay (some details of which were also incorporated into the assay described in this thesis) to verify GAL4 binding to the GAL1,10 UAS. In subsequent analyses, Axelrod et al (1993) found that GAL4 binding could induce the disruption of a repressing nucleosome located in the GAL1 promoter. This data complements earlier studies on the role of nucleosome positioning in transcriptional regulation at the GAL1,10 locus (Fedor and Kornberg, 1989) and suggests that in addition to interacting with targets in or near the PIC, transcriptional activator proteins may also interact with chromatin components.

Since the SWI/SNF complex is believed to be involved in transcriptional regulation at the level of chromatin structure (see Chapter 1), we wondered if this complex played a role in the regulation of the GAL1,10 promoter. Peterson and Herskowitz (1992) have shown that transcription of GAL1 is decreased 2- to 6-fold in swi/snf mutants. Although this effect is substantially smaller than the SWI/SNF effects seen at other genes (ADH2 expression is decreased 10- to 20-fold and INO1 expression is decreased 30-fold; Peterson and Herskowitz, 1992), it does suggest that SWI/SNF may be involved in regulating the GAL1,10 promoter.
As described in Chapter 1, biochemical experiments have shown that yeast or human SWI/SNF complex can stimulate the binding of an activator protein to a nucleosomal binding site (Cote et al., 1994; Kwon et al., 1994). Therefore, we began our studies of SWI/SNF function at the GAL1,10 promoter by exploring its effect on transcriptional activation and DNA binding by the GAL4 protein. These experiments utilized LacZ reporter genes whose expression was controlled by derivatives of the GAL1,10 UAS region; these UAS derivatives differ in the affinity of the GAL4 binding sites as well as the nucleosomal context of these sites.

Results

Isogenic SWI+ and swi- reporter strains were constructed as described in Chapter 2 and are shown schematically in Figure 3-1. Strains CY353 (SWI+) and CY366 (swi1-) contain a GAL1,10 UAS reporter consisting of four GAL4 binding sites upstream of the LacZ gene (reporter a; Guarente et al., 1982). Strains CY401 (SWI+) and CY422 (swi1-) contain a derivative of this reporter that contains only two of these low affinity GAL4 binding sites (sites 3 and 4; reporter b); strains CY532 (SWI+) and CY534 (swi1-) contain a version of reporter b that contains 192 bp of additional plasmid sequence 20 bp upstream of GAL4 site 3 (reporter c); strains CY528 (SWI+) and CY530 (swi1-) contain a reporter which contains two
<table>
<thead>
<tr>
<th></th>
<th>pLEXA</th>
<th>pGAL4</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a)</td>
<td>[1 2 3 4] 1| 3| 4| 1| GAL1-LacZ</td>
<td>SWI(^{+}) 7218 | swi(^{-}) 4691 (65%) | n.d. | n.d.</td>
</tr>
<tr>
<td>(b)</td>
<td>[3 4] 1| 3| 4| 1| GAL1-LacZ</td>
<td>390 | 16 (4.1%) | 1118 | 51 (4.5%)</td>
</tr>
<tr>
<td>(c)</td>
<td>pUC [3 4] 1| 3| 4| 1| GAL1-LacZ</td>
<td>658 | 30 (4.5%) | 2409 | 74 (3%)</td>
</tr>
<tr>
<td>(d)</td>
<td>pUC [17 17] 1| 3| 4| 1| GAL1-LacZ</td>
<td>2311 | 1726 (75%) | 2719 | 1616 (59%)</td>
</tr>
<tr>
<td>(e)</td>
<td>pUC [3 4] 1| 3| 4| 1| GAL1-LacZ</td>
<td>613 | 490 (80%) | n.d. | n.d.</td>
</tr>
</tbody>
</table>
Figure 3-1. GAL4 requires functional SWI/SNF complex to activate transcription from two low affinity binding sites. β-galactosidase assays were performed on isogenic SWI+ or swi- strains harboring the integrated GAL1-LacZ UAS reporter plasmid pRY171 (a), pEG28 (b), pLB8 (c), pLB7 (d) or pLB16 (e). GAL4 binding sites are denoted by small solid boxes; larger solid boxes represent the DNA sequences denoted. GAL4 sites numbered 1-4 represent sites from the GAL1,10 UAS region; GAL4 sites numbered 17 indicate synthetic, consensus GAL4 binding sites. Strains contained plasmids that overexpressed either the bacterial LexA protein (pEG202) or the full length GAL4 protein (pMA210). Numbers in parentheses show activities as a percentage of the wildtype levels. β-galactosidase activities of all reporters were < 1 unit in the absence of GAL4. Drawings are not to scale; n.d., not determined.
high affinity, consensus GAL4 binding sites in place of the two low affinity sites in reporter c (reporter d; Giniger and Ptashne, 1988). β-galactosidase assays were performed on these various strains to monitor GAL4 transcriptional activity in the presence of either endogenous concentrations of GAL4 (column labeled pLEXA) or under conditions in which GAL4 is overexpressed (column labeled pGAL4).

In the absence of functional SWI/SNF complex, transcriptional activation by GAL4 from the wildtype GAL1,10 UAS region is reduced only 1.5-fold compared to the activity in the respective wildtype strain (Figure 3-1, reporter a). This result is consistent with previous studies in which it was found that GAL1 expression was only weakly affected by swi/snff mutations (Peterson and Herskowitz, 1992). In contrast, GAL4 activity on the two low affinity GAL4 binding site reporters is SWI/SNF-dependent; transcriptional activity is reduced 22- to 32-fold in the swill- strain as compared to the wildtype strain (Figure 3-1, reporters b and c). When these two low affinity binding sites were replaced with high affinity, consensus GAL4 binding sites, transcriptional activation is only reduced 1.3-fold in the swill- strain as compared to the wild-type strain (Figure 3-1, reporter d).

Strains harboring the two low affinity site constructs were also distinct from those carrying the two high affinity site construct in that LacZ expression was increased 2.5 to 3.5-fold when GAL4 protein was overexpressed (Figure 3-1 and data not shown). The simplest interpretation of this result is that physiological levels of GAL4 are not sufficient to fully occupy the two low affinity
sites (see Giniger and Ptashne, 1988 and see below). Importantly, overexpression of GAL4 does not overcome the SWI/SNF dependence of this reporter (Figure 3-1, reporters b and c). It should be noted that the swi1 mutation does not affect expression of GAL4 from the overexpression plasmid (Peterson and Herskowitz, 1992; also see Chapter 5).

To directly assess the ability of GAL4 to bind its sites in the different reporter strains in the presence or absence of SWI/SNF, we used an in vivo DMS footprinting assay. GAL4 protects guanine residues at each end of its 17 base-pair recognition site both in vivo (Giniger et al, 1985) and in vitro (Carey et al, 1989). As shown in Figure 3-2, these guanine residues within the four GAL4 binding sites of the GAL1,10 UAS are protected from DMS methylation in the presence of GAL4 (lane 3), but are accessible to methylation in an isogenic gal4- strain (lane 2). Introduction of GAL4 expression plasmids into the gal4- strain restores protection of these guanines (lanes 6 and 8), while introduction of a plasmid expressing the bacterial LexA protein does not (lane 7). In the absence of SWI/SNF activity, protection of all four sites is retained (lane 4). Furthermore, as shown in Figure 3-3A, GAL4 also protects the guanine residues at each of the two high affinity binding sites of reporter d in the presence (lane 4) and absence (lane 5) of SWI/SNF. These results are consistent with our functional studies (Figure 3-1) in which SWI/SNF is not required for the activity of GAL4 from either the wildtype GAL1,10 UAS or the two high affinity site reporter (Figure 3-1, reporters a and d).
Figure 3-2. GAL4 can bind its sites in the GAL1,10 UAS region in the presence or absence of SWI/SNF. In vivo DMS footprinting analysis of the GAL1,10 UAS region was performed on strains CY341 (SWI+ GAL4+; lane 3), CY257 (swi1- GAL4+; lane 4), CY296 (SWI+ gal4-; lane 2), CY296 containing GAL4 expression plasmids (pMA210 or pSD15; lanes 6 and 8), and CY296 containing a LexA expression plasmid (pEG202; lane 7). Lane 1 contains a genomic guanine sequencing ladder. Boxes to the left of the figure denote the GAL4 binding sites of the GAL1,10 UAS and asterisks denote guanine residues shown previously to be protected by GAL4 binding.
Figure 3-3. SWI/SNF is required for complete occupancy of two low affinity GAL4 binding sites in vivo. In vivo DMS footprinting was performed on strains that harbor the two high affinity site reporter (Panel A): CY529 (gal4− SWI+; lane 3), CY528 (GAL4+ SWI+; lane 4) and CY530 (GAL4+ swi1−; lane 5), or strains that harbor the two low affinity site reporter (Panel B): CY533 (gal4− SWI+; lane 2), CY532 (GAL4+ SWI+; lane 3), CY534 (GAL4+ swi1−; lane 4), CY532 harboring a GAL4 expression plasmid (lane 5), and CY534 harboring a GAL4 expression plasmid (lane 6). Arrow denotes the control bands, G449 and G450 by the numbering of Yocum et al, 1984, to which phosphorimager data was normalized (see Figure 3-4). As a control, GAL4 binding in the samples shown in Panel B, lanes 2 and 6 were analyzed for GAL4 binding at the GAL1,10 UAS locus (Panel C). Symbols as described in Figure 3-2.
Figure 3-3B shows an in vivo footprinting analysis of the two low affinity site reporter in strains CY532 (SWI+) and CY534 (swi1-). We detect little protection of the two low affinity sites in our footprinting assay, even in a SWI+ strain (lane 3). As discussed above, this probably reflects the low occupancy of these sites at physiological GAL4 levels, because when GAL4 is overexpressed in this SWI+ strain protection is restored (lane 5). Overexpression of GAL4 in the absence of a functional SWI/SNF complex, however, does not restore complete protection of either low affinity binding site (lane 6). This is most apparent at GAL4 site 3, where strong protection is observed in the SWI+ strain (lane 5), but only weak protection in the swi1- strain (lane 6). Quantitation of these results by phosphorimager analysis indicates that when GAL4 is overexpressed in the absence of SWI/SNF activity, protection of the relevant guanine residue in site 3 is decreased 40% as compared to that seen in a wildtype strain (Figure 3-4).

Because all the strains analyzed in this study contain an unaltered copy of the endogenous GAL1,10 UAS region at its normal location in the genome (in addition to the reporter locus integrated at the URA3 locus), we were able to carry out an important internal control. The ability of GAL4 to bind to its sites within the GAL1,10 locus was analyzed in the same swi- sample which showed poor protection of the low affinity sites at the reporter locus (Figure 3-3B, lane 6). As shown in Figure 3-3C, we observed complete occupancy of the four GAL4 sites at the endogenous GAL1,10 locus in this sample. Thus, the lack of complete
B.

<table>
<thead>
<tr>
<th></th>
<th>gal4-</th>
<th>SWI+</th>
<th>swi-</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) G449/G450 control</td>
<td>335</td>
<td>122</td>
<td>146</td>
</tr>
<tr>
<td>(2) G438/G439 site 3</td>
<td>399</td>
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<tr>
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Figure 3-4. Phosphorimager analysis of GAL4 site 3 occupancy. (Panel A; previous page). The gel shown in Figure 3-3B was scanned on a phosphorimager and the traces for lanes 2 (gal4), 5 (SWI+ pMA210) and 6 (swi- pMA210) are shown. Top to bottom on the gel is depicted as left to right in the traces. Small arrows denote the peak representing the control doublet G449/G450 (see legend to Figure 3-3B) and large arrows denote the peaks representing the doublet in site 3 (G438/G439). (Panel B). Lines (1) and (2) show the numerical values for the area under the peaks designated in Panel A. For each sample, the values for the control doublet were represented as a fraction of the gal4- control doublet value of 335; this normalization factor is listed in line (3). This factor was then used to adjust the values for the site 3 doublet; the new values are shown line (4). Line (5) shows the occupancy of site 3 in a swi- strain as a percentage of the occupancy of this site in the gal4- strain (assigned a value of 0% occupancy) and in the SWI+ strain (assigned a value of 100%) after background had been subtracted.
occupancy at the two low affinity sites in this strain indicates that the SWI/SNF complex modulates GAL4 binding in vivo.

To assess whether the chromatin structure of the GAL4 binding sites was related to their SWI/SNF-dependence, a strong nucleosome positioning element (NPE) was inserted directly upstream of the two low affinity binding sites in strains CY532 (SWI+) and CY534 (swi1). This 182 bp NPE has been shown previously to translationally and rotationally position a nucleosome in vivo in yeast (Pederson and Fidrych, 1994; see also Figure 3-8). Insertion of the NPE had a dramatic effect on the SWI/SNF dependence of GAL4 function. While GAL4 transcriptional activity at the original two low affinity site reporter was decreased over 20-fold in the absence of SWI/SNF (Figure 3-1, reporter b), there was less than a 1.5-fold decrease when the nucleosome positioning element was inserted (Figure 3-1, reporter e). This effect is not simply due to insertion of foreign DNA sequences upstream of the GAL4 binding sites because insertion of 192 bp of pUC plasmid sequences at an identical position did not alter the SWI/SNF dependence of GAL4 activity (Figure 1, reporter c).

These results suggest that the SWI/SNF-dependence of GAL4 can be modulated by nucleosome positioning. To confirm this possibility, we analyzed the chromatin structure of some of the reporters tested in Figure 3-1. As shown previously by Fedor and colleagues, the four GAL4 binding sites at the GAL1,10 locus are located in a constitutively nucleosome-free region that is flanked by arrays of positioned nucleosomes (Fedor and Kornberg, 1989; see also Figure 3-5).
Figure 3-5. Nucleosome mapping of the *GALI,10* UAS region. Free DNA and nuclei from the two low affinity site *SWT*+ reporter strain (CY532) were treated with increasing amounts of MNase and nucleosome positioning at the *GALI,10* locus was analyzed by indirect end-labelling. MNase treatments were as follows: 1 Unit/ml, lane 1; 5 Units/ml, lanes 2 and 3; 25 Units/ml, lane 4. Schematic to the right of each panel depicts mapped locus, black rectangles represent *GAL4* binding sites, shaded boxes represent coding sequence of the indicated gene, and shaded ovals represent the predicted position of nucleosomes. Primers used to amplify the probe fragment by PCR are indicated by black bars. Migration of DNA size standards are noted to the left of each panel.
Figure 3-6. Nucleosome mapping of the two low affinity site reporter locus. Free DNA and nuclei from the two low affinity site SWI+ reporter strain (CY532) were treated with increasing amounts of MNase and nucleosome positioning at the reporter locus was analyzed by indirect end-labelling. MNase were as follows: 0 Units/ml, lanes 1 and 7; 0.005 Units/ml, lanes 2 and 8; 0.05 Units/ml, lanes 3 and 9; 0.5 Units/ml, lanes 4 and 10; 5 Units/ml, lanes 5 and 11; 50 Units/ml, lanes 6 and 12. Restriction sites used to generate the probe fragment are indicated on the map. Symbols as described in Figure 3-5.
The precise nucleosome positioning of this region is believed to require the GRF2 protein, which binds to a sequence that overlaps GAL4 binding sites 1 and 2 (Fedor et al, 1988). The GAL1,10 UAS derivatives that we have analyzed in this study (Figure 3-1, reporters b-e) lack this GRF2 binding site and therefore may not maintain nucleosome positioning in the region surrounding the GAL4 binding sites. To test this idea directly, we analyzed the chromatin structure of the two low affinity site reporters in the SWI+ strains CY532 (without NPE; reporter c in Figure 3-1) and CY586 (with NPE; reporter e in Figure 3-1) and compared these structures to that of the GAL1,10 locus in the same strains.

Nuclei and free DNA were prepared from the SWI+ strain CY532, which contains the parental two low affinity site reporter (reporter c in Figure 3-1) and samples were analyzed by MNase digestion and indirect end-labeling. First, we analyzed the chromatin structure at the GAL1 locus of this strain (Figure 3-5). Comparison of the MNase cleavage pattern in free and chromatin DNA samples reveals a repeating pattern of MNase protections, each approximately 140 bp in size and flanked by MNase hypersensitive sites. These results are essentially identical to a previous study (Fedor and Kornberg, 1988) and are consistent with an array of positioned nucleosomes downstream of the nucleosome-free GAL4 binding sites.

A similar analysis of the two low affinity site reporter locus in this same strain yielded very different results (Figure 3-6). In the region upstream of the two GAL4 binding sites, the MNase digestion pattern in the chromatin sample is
similar to the pattern of digestion in the free DNA, although we do reproducibly observe several preferred cleavage sites in free DNA that appear to be enhanced in the chromatin sample. Importantly, the MNase cleavage sites directly upstream and adjacent to the GAL4 binding sites are cleaved with equal efficiency in the free and chromatin DNA samples. A region of approximately 80 bp, which contains the GAL4 binding sites, is not cleaved efficiently in either the free or chromatin samples; this is due to the inherent sequence specificity of MNase I and is not due to a positioned nucleosome (Fedor and Kornberg, 1988). In addition, this protected region is not flanked by hypersensitive sites. Directly downstream of the GAL4 binding sites, however, a 140 bp region of MNase resistance which is flanked by MNase hypersensitive sites is observed, suggesting the presence of a positioned nucleosome. The location of this putative nucleosome corresponds to the positioned nucleosome located between the GAL4 sites and the GAL1 TATA box of the endogenous GAL1,10 locus that has been identified by others (Fedor and Kornberg, 1988; Axelrod et al, 1993; see also Figure 3-5). Thus, although one nucleosome appears to be positioned at the GAL1,10 UAS derivative in this strain (reporter c), the majority of nucleosome positioning has been lost.

Nucleosome mapping of the SWI+ strain CY528, which harbors the two high affinity site reporter derivative (reporter d in Figure 3-1), revealed a pattern very similar to that seen with the two low affinity site reporter strain just described. Specifically, 1) in the region upstream of the GAL4 binding sites, the MNase digestion pattern in the chromatin sample is similar to the pattern of digestion
observed in the free DNA, 2) as expected, the GAL4 binding sites themselves are resistant to MNase cleavage, and 3) a protected region the size expected of a nucleosome was observed between the GAL4 sites and the downstream GAL1 TATA box (Figure 3-7). Together with the results presented in Figure 3-6, these results suggest that the removal of the presumptive nucleosome positioning elements of the GAL1,10 UAS region (i.e. the GRF2 binding sites) can alter nucleosome positioning over this region in vivo. In the absence of a positioned array of nucleosomes, these GAL4 binding sites are unlikely to reside in a constitutively nucleosome-free region.

MNase digestions and indirect end-labeling were also used to confirm that the NPE in strain CY586 (see Figure 3-1, reporter e) positioned a nucleosome upstream of the two low affinity GAL4 binding sites (Figure 3-8). Comparison of the free and chromatin DNA samples reveals an MNase resistant region of about 250 bp (including the NPE sequences as well as the MNase-resistant GAL4 binding site sequences), consistent with positioning of a nucleosome over the NPE sequence. In addition, the putative positioned nucleosome located between the GAL4 binding sites and the GAL1 TATA box that was detected at the GAL1,10 locus (Figure 3-5), at the parental two low affinity site reporter (reporter c; see Figure 3-6), and at the two high affinity site reporter (reporter d; see Figure 3-7) is also detected at the NPE-containing reporter locus of CY586 (reporter e; see Figure 3-8).
Figure 3-7. Nucleosome mapping of the two high affinity site reporter locus. Free DNA and nuclei from the two high affinity site SWI+ reporter strain (CY528) were treated with increasing amounts of MNase and nucleosome positioning at the reporter loci was analyzed by indirect end-labelling. MNase treatment was as follows: 0 U/ml, lanes 1 and 6; 0.005 U/ml, lanes 2 and 7; 0.05 U/ml, lane 8; 0.5 U/ml, lanes 3 and 9; 5 U/ml, lanes 4 and 10; 50 U/ml, lanes 5 and 11. In this map, the two high affinity GAL4 binding sites are depicted as open boxes. Migration of DNA size standards are noted to the right.
Figure 3-8. Nucleosome mapping of the two low affinity site reporter containing a nucleosome positioning element. Free DNA and nuclei from the NPE-containing, two low affinity site $SWT^+$ reporter strain (CY586) were treated with increasing amounts of MNase and nucleosome positioning at the reporter locus was analyzed by indirect end-labelling. MNase treatment was as follows: 0.5 Units/ml, lanes 1 and 3; 5 Units/ml, lanes 2 and 4. Restriction sites used to generate the probe fragment are indicated on the map accompanying each figure. Symbols as described in Figure 3-5.
In results reported elsewhere, Burns and Peterson (1997) have used a restriction enzyme accessibility assay to monitor the nucleosome state of the GAL4 binding sites in some of the reporters described above (reporters a, c and e). This data is in good agreement with the indirect end-labeling experiments described above and verify that in reporter c, the two low affinity GAL4 binding sites are nucleosomal (as evidenced by their inaccessibility to restriction enzyme digestion in the accessibility assay). In this same assay, the four GAL4 binding sites of the endogenous GAL1,10 UAS region as well as the two low affinity sites in the NPE-containing reporter e are completely accessible to enzyme cleavage, indicating that these regions are free of nucleosomes (See Appendix B).

Discussion

The results described above suggest that the SWI/SNF-dependence of a transcriptional activator protein is not an innate feature of the activator protein per se, but rather reflects the chromosomal context of the activator binding sites. When the activator binding sites are encompassed in a nucleosome, as in the two low affinity site reporter used in this study, SWI/SNF function is necessary for the activator protein to bind to and activate transcription from those sites (see Figure 3-9, top). On the other hand, when binding sites are nucleosome-free, as is the case at the GAL1,10 UAS region or at the NPE-containing two low affinity site reporter, then SWI/SNF function is dispensable for GAL4 activity. In this case,
Figure 3-9. Predicted nucleosome structure of SWI/SNF-dependent and SWI/SNF-independent promoters. Schematics depicting the predicted nucleosome structure of the two low affinity site reporter construct and the same construct containing a nucleosome positioning element directly upstream of the GAL4 binding sites. Closed ovals represent positioned nucleosomes; dashed ovals represent randomly located nucleosomes. Nucleosomes located randomly over the GAL4 binding sites of the former cause the expression of this reporter gene to require SWI/SNF activity. In the NPE-containing construct, on the other hand, the GAL4 binding sites are nucleosome-free and therefore expression of this reporter is SWI/SNF-independent.
GAL4 can bind to its sites and activate transcription in the absence of SWI/SNF activity (see Figure 3-9, bottom). Furthermore, SWI/SNF dependence does not appear to correlate with the level of transcriptional activation since we have found both weak and strong promoters whose activity is dependent upon SWI/SNF (see Figure 3-1). Thus, based on the results presented here, we predict that the subset of genes whose expression requires SWI/SNF will have activator binding sites that are encompassed in nucleosomes; genes that do not require SWI/SNF will have such sites positioned between nucleosomes. This positioning may be determined by DNA sequence, as in the NPE-containing reporter, or by abundant DNA binding proteins like GRF2, as in the case of the intact GAL1,10 reporter.

Our results also require that binding site affinity be incorporated into the definition of SWI/SNF-dependence. While GAL4 requires SWI/SNF in order to access low affinity, nucleosomal binding sites, high affinity, nucleosomal sites are occupied even in the absence of SWI/SNF. The ability of higher affinity binding sites to alleviate SWI/SNF-dependence provides strong evidence that the SWI/SNF complex modulates transactivation at the level of DNA binding. Furthermore, these results suggest that GAL4 binding to high affinity nucleosomal sites can occur in vivo via a SWI/SNF independent mechanism. These results are consistent with in vitro studies of Workman and Kingston (1992) who found that GAL4 can bind to a nucleosomal site to form a tripartite transcription factor-histone-DNA complex.
As noted above, we detect a consistent level of GAL4 binding to the low affinity sites in the swi7 strain (Figure 3-3B, compare lanes 5 and 6); phosphorimager analysis indicates site 3 may be occupied at 60% of the levels seen in a SWI+ strain (Figure 3-4). The fact that this level of occupancy results in negligible levels of transcription (3% of wildtype levels, see Figure 3-1, line b) may indicate a role for the SWI/SNF complex in transcriptional activation beyond the modulation of activator binding. In light of recent reports suggesting that the SWI/SNF complex associates with the RNA polymerase II holoenzyme (Wilson et al, 1996; but see also Cairns et al, 1996b), it is interesting to consider the possibility that an activator-SWI/SNF interaction is involved in preinitiation complex assembly. On the other hand, our in vivo footprinting procedure does not allow us to determine the extent to which site 3 and site 4 are simultaneously occupied, nor can we distinguish whether the 60% occupancy in a swi/snf strain reflects the stable binding of GAL4 in 60% of the cells, or reflects weak, unstable binding in 100% of the cells. Therefore, our results may support an additional role for the SWI/SNF complex in facilitating the cooperative binding of GAL4 or in enhancing the stability of GAL4 binding to low affinity, nucleosomal sites in vivo.

Finally, it is important to note that although the protected region identified (by indirect end-labeling analysis; see Figures 3-5, 3-6, 3-7 and 3-8) between site four and the GAL1 TATA box of all the reporters analyzed in this chapter is consistent with a positioned nucleosome, these results may also indicate the binding of a sequence-specific DNA binding protein to this region in vivo.
CHAPTER IV

CHARACTERIZATION OF THE ROLE OF THE GAL4 ACTIVATION DOMAIN
IN SWI/SNF TARGETING

Background

The 888 residue GAL4 protein has been extensively characterized and its modularity well documented (Brent and Ptashne, 1985; Keegan et al, 1986). The N-terminal 147 amino acids encode the DNA binding and dimerization activities of the protein (Keegan et al, 1986; Carey et al, 1989). This domain functions independently of the rest of the protein and has been used to target a diverse array of transcription factors and activation domains to promoter regions bearing GAL4 sequence binding elements in vivo and in vitro (for example see Barberis et al, 1995; Chatterjee and Struhl, 1995). Two separable activation domains have been identified within the GAL4 protein, although it is the region of the C-terminus between residues 768 and 881 which is thought to be the physiologically relevant transcriptional activating region (Ma and Ptashne, 1987a). This domain is the target of the inhibitor GAL80 (see above) and a number of studies have identified residues which appear to be crucial for transactivation (Lue et al, 1987; Leuthner et al, 1993). The volume of studies characterizing the structure and function of the GAL4 protein has resulted in the availability of a library of GAL4
expression plasmids which encode GAL4 derivatives varying in stability, DNA binding ability and transcriptional activity.

As discussed in detail in Chapters 2 and 3, we have constructed a set of isogenic yeast strains harboring a *LacZ* reporter gene whose expression is dependent upon the presence of 1) the transcriptional activator GAL4 and 2) a functional SWI/SNF complex. In the absence of SWI/SNF, *LacZ* expression from this two low affinity site reporter is decreased over 20-fold and GAL4 binding to one of the two low affinity sites is decreased by 40% (see Chapter 3). In the experiments described below, we have used this reporter gene and the library of available GAL4 expression plasmids to dissect the functional components of GAL4 which play a role in the SWI/SNF-dependent transcriptional activation.

Results

Although GAL4 binding to the two low affinity site reporter is decreased substantially in the *swi1* strain, we do detect a consistent level of GAL4 binding to these sites (see Chapter 3, Figure 3-3B); in fact, phosphorimager analysis indicates site 3 may be occupied at 60% of the levels seen in a SWI+ strain (Chapter 3, Figure 3-4). One possible explanation for this finding is that GAL4 binding to nucleosomal sites is stabilized by two independent pathways (Figure 4-1). In the first pathway, the SWI/SNF complex facilitates the interaction between the activator and the DNA in the context of underlying nucleosomes. In the second, interactions between the activation domain of GAL4 and its target(s) in
Figure 4-1. Alternative models for the stabilization of GAL4 binding in vivo. Schematic depiction of a eukaryotic promoter. Shaded boxes represent binding sites for transcriptional activator proteins, bent arrow depicts transcription start site, dashed circles represent nucleosomes. In pathway (1), chromatin remodeling machines like the SWI/SNF complex stabilize activator binding to nucleosomal sites. In pathway (2), interactions between the activation domain of the activator protein and components of the general transcription apparatus stabilize activator binding. These models are not mutually exclusive.
the preinitiation complex is able to stabilize its binding to the sites. In order to address this possibility, we tested the ability of a GAL4 derivative lacking an activation domain (pMA424) to bind to the two low affinity site reporter in the $SWI^+$ yeast strain CY533.

As shown in Figure 4-2, the guanine residues within the two low affinity GAL4 binding sites in this strain are protected when full length GAL4 protein is overexpressed (lane 2), but not when an irrelevant bacterial protein is overexpressed (lane 4). When the GAL4 DNA binding domain alone is overexpressed, the relevant guanines are still protected (lane 3). Phosphorimager analysis verifies that the protection observed for the DNA binding domain alone is similar to that of the full length protein (Figure 4-3). As expected, both full length GAL4 and the DNA binding domain alone are able to bind to all four GAL4 sites found at the endogenous $GAL1,10$ locus (Figure 4-2B). These results indicate that the GAL4 activation domain is not required in order for SWI/SNF to modulate GAL4 binding in vivo. In the context of the model presented in Figure 4-1, these results suggest that the two pathways for stabilizing GAL4 binding to nucleosomal sites are redundant; in the absence of either pathway, DNA binding continues to be stabilized by the other.

A prediction of this model is that the inactivation of both pathways would lead to a loss of GAL4 binding to the nucleosomal sites and therefore a loss of protection in our in vivo footprinting assay. To test this prediction, we set out to measure the ability of the GAL4 DNA binding domain to bind the two low affinity
Figure 4-2. An activation domain is not required for GAL4 binding to low affinity, nucleosomal sites in vivo. In vivo DMS footprinting was performed on strains harboring the two low affinity site reporter and the GAL4 expression plasmids pMA210 (Panel A, lane 2; Panel B, lane 3), pMA424 (Panel A, lane 3; Panel B, lane 2) or the negative control plasmid pEG202 (Panel A, lane 4; Panel B, lane 1). In Panel A protection was analyzed at the two low affinity site reporter locus. In Panel B protection was analyzed at the endogenous GAL1,10 locus. Lanes marked G represent genomic guanine sequencing ladders for the respective loci. Schematics to the side of each panel depict the respective loci; open boxes denote GAL4 binding sites and asterisks denote guanine residues shown previously to be protected by GAL4 binding.
Figure 4-3. Phosphorimager analysis of site occupancy by full length GAL4 protein and the truncated GAL4 DNA binding domain. (Panel A; previous page). The gel shown in Figure 4-2A was scanned on a phosphorimager and the traces for lanes 2 (pMA210), 3 (pMA424) and 4 (pEG202) are shown. Top to bottom on the gel is depicted as left to right in the traces. Small arrows denote the peak representing the control doublet G449/G450 (numbering of Yocum et al, 1984) and large arrows denote the peaks representing the doublet in site 3 (G438/G439). (Panel B). Lines (1) and (2) show the numerical values for the area under the peaks designated in Panel A. For each sample, the values for the control doublet were represented as a fraction of the EG202 control value of 832; this normalization factor is listed in line (3). This factor was then used to adjust the values for the site 3 doublet; the new values are shown line (4). Line(5) shows the occupancy of site 3 in each strain as a percentage of the occupancy of this site in the gal4- strain (assigned a value of 0% occupancy) and in the SWI+ strain (assigned a value of 100%) after subtraction of background.
GAL4 binding sites in our reporter in the absence of functional SWI/SNF complex. Unfortunately, many fusion proteins and protein derivatives, including the GAL4 DNA binding domain derivative used in these studies, are notoriously unstable in swi/snf mutants (C. Peterson, unpublished results and Figure 4-4). In order to overcome this setback, we set out to identify transactivation-defective GAL4 derivatives which were stable in a swi/snf mutant.

Table 4-1 lists the GAL4 expression plasmids tested in these studies. Each expression plasmid was transformed into the isogenic strains CY296 (SWI+) and CY297 (swi-); in both of these strains the chromosomal GAL4 gene has been deleted (gal4Δ). GAL4 levels in lysates prepared from these transformants was determined by Western blotting with an antibody that recognizes the DNA binding domain of GAL4. As shown in Figure 4-4, full length GAL4 protein (pMA210) was detected in lysates from both SWI+ and swi- cells (lanes 1 and 2); the apparent increase in GAL4 levels in the swi- sample shown in Figure 4-4 is probably not genuine as it is not seen in other experiments (data not shown and see Figure 4-5). Lysates prepared from SWI+ and swi- strains transformed with the GAL4 expression plasmids pGG23 (lanes 5 and 6), pGG23 D199Y (lanes 13 and 14), pCD14XX (lanes 11 and 12) and the control plasmid pEG202 (lanes 3 and 4) contained no detectable GAL4 protein. The absence of detectable GAL4, however, does not suggest a priori that no GAL4 protein is produced in these cells, as in some instances the GAL4 derivative is able to activate transcription of reporter genes (for example pGG23: see Table 4-1 and see text below).
Figure 4-4. Western analysis of GAL4 derivative proteins. Lysates prepared from the yeast strains CY296 (SWI+) and CY297 (swiI-) transformed with the indicated GAL4 expression plasmids were analyzed by Western blot (see Chapter 2). Numbers to the right denote the migration of protein size standards. NOTE: The samples in lanes 9, 11 and 13 were prepared from a swi- strain, contrary to the labels in the figure. Likewise, the samples in lane 10, 12 and 14 were prepared from a SWI+ strain. Lanes 1 through 8 and 15 through 18 are labeled properly.
### Table 4-1. GAL4 expression plasmids used in this study.

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<th>Reference</th>
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</tr>
<tr>
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<td>Ma and Ptashne, 1987b</td>
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<tr>
<td>pCP175 (pEG50)</td>
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<td>n.d.: 467</td>
<td>Ma and Ptashne, 1987b</td>
</tr>
</tbody>
</table>

(¹) pEG202 is a negative control plasmid encoding the bacterial protein LEXA.

(b) β-galactosidase expression was measured in yeast strains harboring the indicated expression plasmid and either the two low affinity site LacZ reporter (2lo) or a GAL1,10 UAS LacZ reporter (UAS<sub>GAL</sub>). The former were performed in this study, the latter were reported in the reference indicated. n.d., not determined.
Only three of the GAL4 expression plasmids tested in this study produced detectable GAL4 derivatives: lysates prepared from cells transformed with pCD19XX (lane 7), pMA424 (lane 9) and pCP175 (lane 15) contained GAL4 proteins with approximate molecular weights of 43 kDa (677 amino acids), 17 kDa (147 amino acids) and 17.5 kDa (162 amino acids), respectively. Of these, the GAL4 derivatives produced in the pMA424 and pCP175 transformants were unstable in the swi- strain (lanes 10 and 16, respectively). On the other hand, the GAL4 derivative produced in the pCD19XX transformant was present in the swiI- strain (lane 8), indicating it was stable in the swiI- strain. This derivative (hereafter referred to as CD19) was chosen for further analysis.

To verify that the CD19 protein was defective in transactivation, liquid β-galactosidase assays were performed. The strain CY533, which harbors the two low affinity site reporter, was transformed with the plasmid pCD19 and liquid β-galactosidase assays were performed on three individual transformants. As shown in Table 4-1 (column labeled 2lo), the overexpression of full-length GAL4 in this strain led to high levels of transactivation, however, very little activity was seen when CD19 was overexpressed. Interestingly, the GAL4 derivative encoded by the plasmid pGG23, which was completely undetectable by a Western blot of a cell lysate, was able to activate reporter gene expression to low levels (see Table 4-1 and Figure 4-4, lane 4). Western blots were used to verify the stability of CD19 in this new strain background and the results are shown in Figure 4-5. Similar to the full length GAL4 protein (lanes 1 and 2), CD19 protein levels in the swi
Figure 4-5. The GAL4 derivative CD19 is stable in a swi/snf strain. Western analysis was performed on yeast strains CY533 (SWI+) and CY535 (swi1) that had been transformed with the GAL4 expression plasmids pMA210 (lanes 1 and 2), pCD19XX (lanes 3 and 4), or the control plasmid pEG202 (lanes 5 and 6). These strains carry the two low affinity site reporter and a deletion of the endogenous GAL4 gene. Numbers to the right indicate migration of protein size standards.
mutant are essentially unchanged in the isogenic SWI+ strain (lanes 3 and 4). As expected, lysates from strains harboring the control plasmid pEG202, which encodes a bacterial protein unrelated to GAL4, contained no detectable GAL4 protein. These results identify a CD19 as a transcriptionally defective yet inherently stable GAL4 derivative. We next used this derivative to test the model presented in Figure 4-1 and described above.

The ability of CD19 to bind to the GAL4 sites in the two low affinity site reporter in the presence and absence of SWI/SNF was monitored by in vivo DMS footprinting. As shown in Figure 4-6, both CD19 and full length GAL4 protein (MA210) were able to occupy low affinity GAL4 binding sites in the presence of SWI/SNF (Panel A, lanes 3 and 4). In the swi- strain, however, neither protein was able to fully occupy these sites (Panel A, lanes 6 and 7). In agreement with the results presented in Chapter 3, we observe a decrease in protection by the full length GAL4 protein in the swi- strain as compared to the SWI+ control. In the experiment shown, GAL4 occupancy was decreased by over 60% (Figure 4-7). A similar decrease (53%) was seen in CD19 binding to the site 3 (Figure 4-7).

Discussion

Workman and Kingston (1992) have shown that the transcriptional activator GAL4 can bind to its sites in a nucleosomal DNA template to form a tripartite DNA/histone/GAL4 complex. These results indicate that GAL4 may
The activation domain mutant CD19 is able to partially bind nucleosomal GAL4 binding sites in the absence of swi/snf. In vivo DMS footprinting analysis was performed on strain CY533 (gal4Δ SWI+) that had been transformed with the GAL4 expression plasmids pMA10 (lane 2), pCD19 (lane 3) or the control plasmid pEG202 (lane 4); the same analysis was carried out on the strain CY535 (gal4Δ swi) that had been transformed with the same plasmids (lanes 5, 6 and 7, respectively). GAL4 binding to the two low affinity site reporter locus in these strains is shown. Lane 1 contains a genomic guanine sequencing ladder. Symbols as described in the legend to Figure 4-2.
A.

SWI+ pEG202

SWI+ pMA210

SWI+ pCD19
Figure 4-7. Phosphorimager analysis of site occupancy by full length GAL4 and the activation domain mutant CD19. (Panels A and B, previous pages) The gel shown in Figure 4-6 was scanned on a phosphorimager and the traces for lanes 2 (pEG202, Panel A), 3 (pMA424, Panel A), 4 (pCD19, Panel A), 5 (pEG202, Panel B), 6 (pMA424, Panel B) and 7 (pCD19, Panel B) are shown. See legend to Figure 4-3 for details.

<table>
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<th>swi-</th>
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<td>pMA210</td>
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<tr>
<td>(4) Adjusted values</td>
<td>717</td>
<td>278</td>
</tr>
<tr>
<td>(5) % occupancy</td>
<td>(0%)</td>
<td>(100%)</td>
</tr>
</tbody>
</table>
recognize its sites in vivo even when they are encompassed in a nucleosomal structure. The fact that overexpression of GAL4 protein in vivo leads to increased occupancy of nucleosomal sites may indicate that in the presence of nucleosomes, GAL4 binds its sites weakly and has a high dissociation rate. Increased local concentrations of GAL4 protein can partially compensate for this high off rate and is demonstrated in our footprinting assay by increased protection (see Chapter 3).

Importantly, we find that the ability of increased GAL4 levels to support occupancy of nucleosomal sites requires the SWI/SNF complex. This result indicates that the SWI/SNF complex can further stabilize GAL4 binding, although the mechanisms by which SWI/SNF accomplishes this stabilization remains unclear. Based on the biochemical and genetic results summarized in Chapter 1, this stabilization may involve alteration of histone-DNA contacts within the underlying nucleosome, resulting in an increased affinity of GAL4 for its site (e.g. decrease in the dissociation rate).

The data presented in this chapter indicate that the SWI/SNF complex can also modulate the binding of a truncated GAL4 derivative consisting solely of the DNA binding domain. These results indicate that the mechanism by which the SWI/SNF complex stabilizes binding to nucleosomal sites does not require the activation domain. Furthermore, these results leave unanswered the question of how the mere one hundred copies of the SWI/SNF complex which are thought to exist within the typical yeast cell are targeted to the sites in the genome at which its function is required (Dingwall et al, 1994; Cote et al, 1994; Cairns et al 1994).
Even in the absence of SWI/SNF complex, we detect some binding to the nucleosomal GAL4 sites in our two low affinity site reporter. As shown in Figure 4-1, one possible model for this result is that GAL4 binding to nucleosomal sites is stabilized by at least two different pathways. In the first, the SWI/SNF complex modulates binding via an unknown mechanism. In the second, interactions between the activation domain of GAL4 and its downstream targets (the general transcription machinery) leads to more stable GAL4 binding. If this model is correct, our previous results suggest that these two pathways are not mutually exclusive and that in the absence of one, the other continues to stabilize binding (we see substantial residual GAL4 binding in a swi/snf mutant; see Chapter 3). In the studies presented here, we used a GAL4 activation domain mutant, CD19, to test this model further.

We find that in the presence of SWI/SNF, CD19 is able to bind to nucleosomal sites as well as the full length GAL4 protein does. If the model presented above is correct, CD19 binding in this case is solely the result of SWI/SNF modulation of binding, since interactions between the bound activator and its downstream targets are disrupted in this activation domain mutant. On the other hand, the activation domain of the full length protein is able to interact with downstream targets and as a result, its binding to nucleosomal sites is stabilized by both SWI/SNF and activator-PIC interactions. If this is true, then one would predict that in the absence of SWI/SNF, full length protein would retain the binding conferred by interactions between its activation domain and its
downstream targets; CD19, however, would be unable to bind to the nucleosomal sites at all as both means of stabilization have been disrupted. This is not the observed result. Instead, we find that CD19 is able to bind the nucleosomal sites in our two low affinity site reporter almost as well as the full length GAL4 protein (Figures 4-6 and 4-7). These results suggest that the residual GAL4 binding seen in a swi/snf strain is not due to the stabilizing effect of interactions between the activation domain and downstream transcription machinery and that this residual SWI/SNF-independent GAL4 binding to low affinity, nucleosomal binding sites reflects intrinsic binding properties of the DNA binding domain.
CHAPTER V
CHARACTERIZATION OF THE GAL\textsuperscript{\textprime} PHENOTYPE OF \textit{swi/snf} MUTANTS

Background

Yeast strains bearing mutations in genes encoding components of the SWI/SNF complex display a slow growth phenotype on all types of growth media. This slow growth is exacerbated on media containing galactose as the sole source of carbon as well as on media lacking inositol; on these medias \textit{swi/snf} mutants cannot grow at all (Neigeborn and Carlson, 1984, Peterson, et al 1992). These phenotypes have been attributed to reduced transcription of the genes involved in galactose and inositol utilization in \textit{swi/snf} mutants, in part because the defects can be suppressed by the same chromatin mutations (e.g. deletion of the HTA1-HTB1 gene set) that suppress the characterized transcriptional defects in \textit{swi/snf} mutants (Hirschhorn et al 1992).

In the experiments described below, an attempt was made to identify genes in the galactose utilization pathway (\textit{GAL} genes) which are dependent upon SWI/SNF activity for expression. If the inability of \textit{swi/snf} mutants to grow on galactose media is indeed due to misexpression of one of the \textit{GAL} genes, we expected to uncover an endogenous, SWI/SNF-dependent, GAL4-responsive promoter analogous to the synthetic SWI/SNF-dependent two low affinity site
reporter we have previously characterized (see Chapters 3 and 4). The GAL4 binding sites of such a gene might reside within a region of positioned nucleosomes such that GAL4 cannot occupy the sites in the absence of SWI/SNF. The correlation of SWI/SNF-dependence with the nucleosomal state of promoter binding sites in the promoter region of an endogenous, SWI/SNF-dependent GAL gene, would provide strong support for our model.

Results

As shown in Table 5-1, swi1 cells display a growth defect on both glucose- and galactose-containing media. This growth defect is well documented (Neigeborn and Carlson, 1984; Peterson et al, 1991) and is identical to that observed in other swi/snf mutants (including swi2, swi3, snf5, snf6 and swp73). Hirschhorn et al (1992) have shown that this growth defect is exacerbated on galactose plates containing antimycin. In the presence of this electron transport chain inhibitor, yeast cells are forced to rely on glycolysis to provide the energy for cell growth, as the energy normally afforded by oxidative metabolism is disrupted. In the experiment shown in Figure 5-1, a swi1Δ strain (CY257) and its isogenic SWI* counterpart (CY340) were streaked onto agar plates containing the drug antimycin. While the SWI* cells grow at similar rates on glucose-containing (panel A) and galactose-containing (panel B) media in the presence of antimycin, the swi1Δ cells are unable to grow at all on galactose plates containing antimycin (panel B). The swiΔ cells do grow on glucose-containing media in the presence of
<table>
<thead>
<tr>
<th>Growth</th>
<th>Media</th>
<th>\textit{SWI}^+</th>
<th>\textit{swi}^-</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>YPD</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>YPD + antimycin</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>YPGal</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>YPGal + antimycin</td>
<td>++</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 5-1. Growth defects of \textit{swi}/\textit{snf} mutants. Growth on the indicated media is designated by "+" symbols, no growth is designated by "-" symbols.
A. Glucose + antimycin

B. Galactose + antimycin

SWI+  swi1Δ
SWI+  swi1Δ
Figure 5-1. *swi/snf* mutants display growth phenotypes on antimycin-containing media. SWI+ and swi- yeast strains were streaked onto plates containing the drug antimycin and either glucose (Panel A) or galactose (Panel B) as the sole carbon source. Schematics to the right indicate the genotypes of the strains shown in the plates on the left.
antimycin (panel A). This sensitivity to antimycin under anaerobic conditions has been used by others to define the Gal- phenotype of \textit{swi/snf} mutants (Hirschhorn et al, 1992; Cairns et al, 1996a).

In order to identify the gene(s) responsible for the Gal- phenotype of the \textit{swi1} strain CY257, expression of each of the known \textit{GAL} gene family members was monitored in \textit{SWI+} and \textit{swi} cells grown under inducing conditions. Primer extension analysis (Figure 5-2) was performed on an equal amount of total RNA prepared from the yeast strains CY340 (\textit{SWI+}) and CY257 (\textit{swi1}) using primers specific for the coding sequence of \textit{GAL1} (lanes 3 and 4), \textit{GAL2} (lane 5 and 6), \textit{GAL7} (lanes 7 and 8), \textit{GAL10} (lanes 9 and 10), \textit{GAL80} (lanes 11 and 12), \textit{MEL1} (lanes 13 and 14), \textit{GAL3} (lanes 15 and 16), and \textit{GAL5} (lanes 17 and 18). We find that the levels of \textit{GAL1}, \textit{GAL2}, \textit{GAL7}, \textit{GAL10}, \textit{GAL80} and \textit{MEL1} transcripts are not substantially altered in the \textit{swi1} strain when compared to the levels detected in the respective \textit{SWI+} strains. Likewise, transcripts from the \textit{CLN3} gene, which encodes a mitotic cyclin that is not involved in galactose utilization, are unaltered in the \textit{swi1} mutant. However, we do detect a consistent decrease in the level of transcription of the \textit{GAL3} and \textit{GAL5} genes (compare lane 15 to lane 16 and lane 17 to lane 18).

To verify that the GAL- phenotype displayed by \textit{swi/snf} mutants is related to the misexpression of either \textit{GAL3} or \textit{GAL5}, complementation analyses were carried out (Table 5-2). In these experiments the \textit{SWI+} (CY340) and \textit{swi1} (CY257) strains described above were transformed with plasmids directing the expression
Figure 5-2. Analysis of GAL gene transcription in SWI+ and swi- yeast. Primer extension reactions were performed on total RNA isolated from the SWI+ yeast strain CY340 and the swi- strain CY257 using primers designed to measure the level of transcripts for each of the indicated genes.
Table 5-2. Complementation studies. (a-e) SWI⁺ and swi⁻ yeast strains transformed with the indicated plasmid were streaked onto glucose or galactose medium containing antimycin. (f) SWI⁺ and swi⁻ yeast strains were constructed in which the chromosomal GAL80 gene was deleted. These strains were streaked as above. Growth is indicated by “+” symbols, no growth is indicated by “−” symbols.
of either GAL5 (line 2; pTI-3B, gift of D.M. Bedwell, University of Alabama, Birmingham), GAL3 (line 4; pGAL3HA, gift of Toshio Fukasawa, Kazusa DNA Research Institute, Japan) or the corresponding control plasmids pRS426 (line 3) and pVT102UHA (line 5), respectively. As expected, the SWI+ transformants were able to grow on both glucose- and galactose-containing media in the presence of antimycin (Table 5-2). On the other hand, none of the swiI transformants was able to grow on galactose containing media in the presence of antimycin (Table 5-2), although they could grow on glucose-containing media in the presence of antimycin. Overexpression of GAL3 seemed to partially suppress the growth defect of the swi- strain (Table 5-2, line 2), although it did not suppress antimycin sensitivity.

GAL3 encodes an inducer molecule which is believed to interact with the GAL80 protein in the presence of galactose (i.e. inducing conditions). This interaction is thought to alter the interaction between GAL80 and the transcriptional activator GAL4 such that the latter can now activate the expression of downstream genes (Suzuki-Fujimoto et al, 1996). If the Gal defect of swi/snf mutants is indeed due to the inability of these cells to express GAL3 properly, then it is possible that this defect would be overcome by a corresponding decrease in the expression of GAL80. To test this idea, strains CY349 (gal80Δ) and CY348 (gal80Δ, swi1Δ) were streaked onto glucose and galactose plates containing antimycin and growth after two days at 30°C was monitored. As
shown in Table 5-2, the deletion of the GAL80 gene did not suppress the Gal phenotype of the swi1 strain.

Discussion

The results described above suggest that the Gal defect observed in swi/snf mutants is not simply the result of a transcriptional defect of a single member of the GAL gene family. While the expression of two GAL genes, GAL3 and GAL5, was significantly altered in a swi1 strain, the presence of the respective overexpression plasmid was not able to suppress the Gal defect. It is possible, however, that the Gal phenotype is due to the combined misexpression of both GAL3 and GAL5. This model remains to be tested.

The expression plasmid used in the GAL3 complementation experiment, pGAL3HA, consists of the entire GAL3 coding sequence cloned into an ADH2 expression cassette and has been shown to result in the constitutive overexpression of HA-tagged GAL3 protein (Suzuki-Fujimoto et al, 1996). However, in its endogenous context, the expression of the ADH2 gene is known to require SWI/SNF, suggesting this promoter is SWI/SNF-dependent. Therefore, the pGAL3HA plasmid may not express the levels of GAL3 that are necessary to overcome the Gal defect. In addition, the construct used in the GAL5 complementation experiment is a genomic GAL5 clone; the expression of GAL5 in this plasmid is controlled by the endogenous GAL5 promoter. Therefore, expression of GAL5 from this plasmid will be subject to the same constraints as
the endogenous \textit{GAL5} gene, including SWI/SNF-dependence. It should be noted, however, that both \textit{pGAL3HA} and \textit{pTI-3B} are high copy number constructs and therefore the transformants tested in Table 5-2 should have a copy number 10-50 times higher than the nontransformed strain.

We consistently observed small (less than two-fold) decreases in the expression of all the other \textit{GAL} genes in \textit{swi1} mutants (see Figure 5-2). It is possible that the Gal defect results from the combination of these small transcriptional defects, or from the cumulative defects in the expression of a unique combination of \textit{GAL} genes. In either event, our results do indicate that a single gene is not responsible for the Gal defect observed in \textit{swi/snf} mutants.
CHAPTER VI
SUMMARY

The yeast SWI/SNF complex is emerging as a paradigm for a growing class of chromatin remodeling assemblies that appear to allow a spectrum of DNA binding proteins access to the eukaryotic genome. Biochemical experiments utilizing SWI/SNF purified from yeast indicate the complex can modulate the ability of DNA binding proteins to access nucleosomal binding sites (Cote et al, 1994; see also Kwon et al, 1994; Imbalzano et al, 1994). Furthermore, the complex can induce a persistent alteration specifically in that nucleosome within an array which occludes a protein-DNA interaction (Owen-Hughes et al, 1996). More recent results have suggested that the complex may recognize the region of the nucleosome where the DNA strand crosses over itself to form a four-way junction-like structure (Quinn et al, 1996). This idea is supported by crosslinking experiments which demonstrate that certain subunits of the purified complex can interact closely with specific regions of a nucleosome in vitro and that the specificity of these interactions is sensitive to the presence of ATP (B. Bartholemew and C.L. Peterson, unpublished observations). All of this biochemistry is evidence that the mechanism of SWI/SNF function involves direct
physical contact with nucleosomes, presumably followed by alterations in histone-DNA contacts within the nucleosome such that the DNA is more accessible.

Despite rapid progress in understanding the function of purified SWI/SNF complex in vitro, many intriguing questions regarding the function of the complex in vivo remain unanswered. For example, what dictates the SWI/SNF-dependence of gene expression? How is this relatively low abundance complex targeted to specific sites in the genome? The work presented in this dissertation addresses these questions by establishing the molecular basis for SWI/SNF function at the yeast GAL1,10 promoter. We were able to change this well characterized, SWI/SNF-independent promoter into one completely dependent upon the SWI/SNF complex for proper expression. We find that the SWI/SNF-dependence of GAL1,10 derivative promoters correlates with the nucleosome state of activator binding sites in the UAS region, indicating that the role of the SWI/SNF complex at these promoters is to modulate activator binding to nucleosomal binding sites. These results complement the biochemical experiments described above and provide the first evidence that the SWI/SNF complex can modulate activator binding in vivo.

It is not clear whether the rules for SWI/SNF-dependence established here are applicable to all yeast promoters. Existing data argues that they are not, as yeast promoters that contain nucleosomal activator binding sites but are expressed independently of SWI/SNF have been characterized (e.g. PHO5; see previous discussion). Instead, SWI/SNF-dependence may reflect a combination of
nucleosome structure and the intrinsic binding properties of a given activator protein. For example, activators like GAL4, which are unable to stably occupy nucleosomal sites in vivo, will require SWI/SNF activity. In contrast, other activators may bind nucleosomal sites in vivo without SWI/SNF modulation. Furthermore, the SWI/SNF-dependence of a particular activator may vary depending on the individual promoter binding site. For example, we find SWI/SNF is required for complete occupancy of the two low affinity nucleosomal GAL4 sites used in our studies but not for binding to other nucleosomal sites which differ in binding sequence.

Our studies also address the targeting question. We find that the ability of SWI/SNF to modulate activator binding does not require a functional activation domain. These results suggest that either the SWI/SNF complex is targeted to its site of action solely through the DNA binding domain of the activator, or that it is not targeted at all. In the former scenario, SWI/SNF could recognize some specific feature of the DNA binding domain or, alternatively, the complex might recognize proteins which are loosely associated with their binding sites within the context of nucleosomal DNA. On the other hand, it is possible that SWI/SNF interacts with the genome continuously, in a random fashion, such that targeting is not necessary. In this situation, coincidental occupation of a nucleosomal promoter region by SWI/SNF and an activator protein would lead to productive activator binding (and eventually to transactivation). This latter model seems unfavorable in light of the low abundance of SWI/SNF in yeast (estimated 100 copies per
nucleus). Interestingly, the relative abundance of SWI/SNF complex in human cells is much higher than in yeast.

Finally, there has been much controversy recently over the observation of Wilson et al (1996) that the yeast SWI/SNF complex is a component SRB-containing mediator complex and therefore of the RNAPII holoenzyme itself. These authors theorize that holoenzyme recruitment, which has been shown to result in transcriptional activation (reviewed in Ptashne and Gann, 1997) would bring SWI/SNF to the promoter where it could then alter chromatin structure. However, Cairns et al (1996b) were unable to detect an association between SWI/SNF and holoenzyme complexes.

The results presented in Chapter IV of this thesis address this issue. Our results indicate that a GAL4 derivative lacking an activation domain (GAL4 DNA binding domain construct MA424) remains sensitive to SWI/SNF activity, suggesting that this complex is able to reach the promoter independently of holoenzyme. Furthermore, while we do see some co-purification of SRB subunits with SWI/SNF complex in our preparations, the amounts represent less than 20% of the SRB subunits in a yeast lysate (L. Boyer and C.L. Peterson, unpublished observations). Taken together, these data suggest that the SWI/SNF complex is physically and functionally independent of holoenzyme. It remains possible that the complexes are able to interact in vivo, either directly or indirectly, and that this association results in small amounts of co-purification.
Finally, recent studies from a number of groups have verified the importance of protein acetylation/deacetylation in transcription (reviewed in Pazin and Kadonaga, 1997). Interestingly, some of the gene products found to be involved in histone acetylation also interact genetically with the SWI/SNF complex (K. Pollard and C.L. Peterson), suggesting that SWI/SNF may play a direct or regulatory role in this aspect of transcription regulation. The strains and techniques developed in this thesis will allow a careful analysis of the role of these other factors in transcription factor binding, transactivation and SWI/SNF regulation in vivo.
BIBLIOGRAPHY


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APPENDIX A

DETAILED PROTOCOLS

Isolation of yeast nuclei

The following protocol was obtained from Randall H. Morse (Molecular Genetic Program, Wadsworth Center, New York State Department of Health and SUNY School of Public Health, Albany, NY 12201-2002) and is based on a procedure published in Shimizu et al (1991) EMBO J. 10, 3033-3041.

1. Grow 1 L of cells to OD600 of 0.5-1.4
   (The procedure can be scaled up, e.g. grow 4 L of cells and multiply all reagents used by 4.)

2. Harvest cells by centrifugation for fifteen minutes at 4K rpm in a J6 rotor.

3. Wash pelleted cells in 50 ml of S Buffer. Spin as above for five minutes.

4. Incubate cells in 25 mL S Buffer at 30°C for ten minutes. This can be done in a shaking incubator (20 rpm) or in a standing incubator. Spin cells as above for five minutes.

5. Drain pellet and weigh. Add volume of S Buffer equal to four times the weight of the pellet (for example, 10 ml to a 2.5 g pellet).

6. Digest cells with 1 ml 10 mg/ml 100T Zymolyase.

7. Incubate 30-90 minutes at 30°C with gentle shaking (60 rpm) until cells are well spheroplasted. This can be monitored by lysing the cells in 0.1% SDS under a microscope; if cells are well digested >90% of them should lyse under these conditions. I have found that 45 minutes is usually sufficient.

   Remaining steps should be carried out in the cold using chilled buffers.

8. Dilute sample to 30 ml with S Buffer. Spin five minutes at 4K.

9. Wash spheroplasts twice more in cold S Buffer, about 30 mL each time. The supernatant from these washes will be progressively cloudy.
10. After the second wash, resuspend the pellet in 19 ml F Buffer. Mechanically disrupt the spheroplasts by passing through a homogenizer. I found four passes through a Yamata LH-21 homogenizer set at 100 rpm provided sufficient disruption. Disruption can be monitored under a microscope. Dilute the sample enough to see individual cells -- there should be a lot of smallish debris visible and very few intact, live cells.

11. Layer homogenized sample onto 19 ml of GF Buffer in an Oak Ridge tube. Spin 13.5K rpm (21,625 X g) in a JA20 rotor for 30 minutes at 4°C. Note: You will need glycerol in a balance tube to achieve sufficient density to balance!

12. Discard supernatant. Resuspend pellet in 20 ml of F Buffer. Mechanically resuspend pellet with a wooden stick to dislodge it from the bottom of the tube. Vortex for five minutes at 4°C to thoroughly resuspend the pellet.

13. Spin sample for fifteen minutes at 4K rpm in J6 rotor at 4°C.

14. Transfer supernatant to a fresh tube. Spin at 13.5K rpm (21,625 X g) in a JA20 rotor for 25 minutes at 4°C to isolate nuclei. Nuclei should be a white pellet, fairly large and homogeneous looking.

15. Discard supernatant and drain nuclei pellet.

16. Resuspend nuclei pellet in 4 ml of D buffer (to make a final concentration of approximately 6 X 10^9 cell equivalents/ml). Quick freeze 1 ml aliquots in eppendorf tubes in liquid nitrogen and store at -80°C.
Solutions

S Buffer
- 1.4 M Sorbitol
- 40 mM HEPES pH 7.3
- 0.5 mM MgCl₂
- 10 mM β-mercaptoethanol (added fresh)
- 1 mM PMSF (added fresh)

F Buffer
- 18% w/v Ficoll 400
- 20 mM PIPES pH 6.5
- 0.5 mM MgCl₂
- 1 mM PMSF (added fresh)

GF Buffer
- 20% w/v glycerol
- 7% w/v Ficoll 400
- 20 mM PIPES pH 6.5
- 0.5 mM MgCl₂
- 1 mM PMSF (added fresh)

D Buffer
- 10 mM HEPES pH 7.3
- 0.5 mM MgCl₂
- 0.05 mM CaCl₂
In vivo DMS footprinting


I. PREPARATION OF METHYLATED GENOMIC DNA

1. Grow 100 ml yeast culture in appropriate rich medium to an OD600 of 1.5-2, corresponding to a density of 5 x 10^7 cells/ml. Cells grown in minimal medium should be grown to an OD600 between 0.5 and 0.75. Minimal cultures grown over 0.75 are saturated and consistently demonstrate poor GAL4 footprinting.

2. Pellet at 4K for 7 minutes.

3. Resuspend in 1.5 ml of the same growth medium. Incubate at room temperature for five minutes.

4. Add 2µl DMS and vortex well. Incubate two minutes at room temperature.

Note: Use only fresh DMS (less than 4 months old)
Also, DMS is nasty stuff. Have a beaker with 100 ml 5N NaOH (20g NaOH pellets/100 ml dH2O) ready to inactivate DMS tips and waste.

5. Quench reaction with 40 ml of ice cold TEN.

6. Pellet at 4K for 7 minutes.

7. Wash pellet in 1 ml SCED. Spin as above.

8. Resuspend pellet in 1 ml SCED. Transfer to a 1.5 ml Eppendorf tube and add 100 µl 10 mg/ml 100 T Zymolyase (Seikagaku Corporation; DO NOT USE LESS PURE THAN 100T!). Invert tubes to mix well.

9. Incubate at 37°C for 25 minutes. Monitor spheroplasting under microscope. When spheroplasting is complete, you should see >90% lysis when water or 0.1% SDS is added to spheroplasts.

11. Resuspend spheroplasts in 1 ml of 50mM Tris pH 8.0, 20 mM EDTA. Resuspension into a homogeneous solution will require careful repeated pipetting. Transfer half of the suspension to a second Eppendorf tube.

12 Lyse spheroplasts by adding 50 µl 10% SDS to each tube. Incubate at 37°C for 30 minutes.

13. Add 3 µl 20 mg/ml Proteinase K, mix by inversion and incubate 30 minutes more at 37°C.

14. Add 200 µl 5M potassium acetate pH 8.0 to each sample and incubate on ice for at least one hour.

15. Spin samples 5 minutes at room temperature. Transfer supernatant to fresh tubes, spin again for 5 minutes at room temperature, transfer supernatant to another fresh tube and precipitate with 700 µl isopropanol.

16. Pellet nucleic acid in microfuge (3-5 min), rinse pellets with 70% ethanol and air dry approximately 10 minutes.

17. Resuspend pellets in 300 µl TE. Add 30 µg RNAse (DNAse-free) to each sample and incubate 37°C for one hour. Pellets may not resuspend readily, but will go into solution after incubation at 37°C with a little intermittent vortexing.

18. Combine samples into one tube. Spin for two minutes on highest setting to remove insoluble debris. Transfer supernatant to a fresh tube.

19. Precipitate DNA by adding 5.5 µl 1M spermidine, pH 4.0. Flick the tube after spermidine addition. A white, threadlike precipitate should form. This may require another 5.5 µl aliquot of spermidine (final spermidine concentration should be 3-9 mM). Incubate on ice for at least fifteen minutes.

Note: Precipitation of DNA by spermidine is critical to the primer extension assay later. Use a 1M stock of spermidine, pH 4. THE PH OF THE SPERMIDINE IS CRITICAL!

19. Pellet DNA by centrifugation for two minutes. It is a good idea to check the efficiency of the spermidine precipitation on an agarose gel. I typically see 80-90% of the DNA in the pellet.
20. Resuspend the spermidine pellet in 300 μl 0.1M Tris pH 8.0. Incubate at 37°C for two hours with some vortexing. It will take almost this entire incubation for the pellet to dissolve.

21. Add 165 μl 7.5M ammonium acetate and 1 ml ethanol. Chill on dry ice for 15 minutes. Spin, wash and dry pellet.

22. Resuspend pellet in 100 μl dH20. Addition of salt will help the dissolution process, so add the salt required of the digestion reaction and incubate at 37°C for a few minutes until the pellet dissolves readily upon vortexing. I typically set up a 200 μl digestion reaction using the entire sample.

Note: The digestion of the genomic DNA into smaller fragments will facilitate the primer extension reaction. Choose an enzyme which cleaves outside the region you are footprinting, including the primer binding site.

23. Extract the digestion with phenol/chloroform/isoamyl alcohol and precipitate with 1/10 volume 3M sodium acetate pH 5.2 and 2.5 volumes of ethanol.

24. Spin, wash and dry pellet. Resuspend in 15 μl dH20; this will give approximately 1μg/μl.

NOTE: I have been able to get very clean, reproducible footprints without using a piperidine cleavage step. (In fact, I have had trouble with high background when I use piperidine.) Therefore, proceed directly to the primer extension step.

II. PRIMER EXTENSION

1. Set up primer extension reactions in 0.5 ml eppendorf tubes:

   2.5-5 μl methylated, restricted DNA (from step I)
   5 μl 500 μM dNTP Mix
   5 μl 5X Taq Buffer
   3 μl end-labelled oligonucleotide (3ng)
   0.5 μl (2.5 Units) Sequencing grade Taq Polymerase* (Promega)
   dH2O to make a 25 μl reaction

*Note: This is the only polymerase that has worked for me.
2. To make a guanine ladder, use the same amount of UNMETHYLATED DNA (isolated exactly as described above, simply omit DMS treatment) and instead of using the 500 μM dNTP mix, use 5μl of 5X G Mix in the primer extension reaction.

3. Overlay samples with 50μl mineral oil and cycle for two minutes each at 95°C/(T_m + 2°C)/70°C 19 times.

Note: To determine appropriate T_m, use the formula below:

\[ T_m = 81.5 + 1.6 \cdot (\log M) + 0.41(\%GC) - 500/n \]

M = molarity of salt in buffer, here 0.059 M
n = length of oligonucleotide

5. When cycling is finished, add 75 μl dH2O to each sample and extract with 100 μl chloroform/isoamyl alcohol. Precipitate with 5 μl 3M sodium acetate pH 5.2 and 165 μl ethanol.

6. Collect sample by centrifugation, wash pellet with 70% ethanol and air dry 5-10 minutes.

Note: Do not overdry pellet or it will be harder to resuspend!

7. Resuspend pellet in 5 μl Loading Buffer. Vortex well and check resuspension (<20% of the counts remain in the tube when the sample liquid is removed).

8. Boil samples 5 minutes, remove to ice, and load entire sample on a denaturing gel. Use a 0.4 mm thick gel to increase resolution. With this thickness, low percentage gel can be used, I usually pour a 5% gel.

III. PREPARING A SEQUENCING LADDER

While the primer extension reactions are cycling, I usually prepare sequencing markers. This ladder is not necessary if you make a genomic G ladder when you do the primer extension reaction (see above). This is a modification of the Sequenase® protocol and uses the same reagents.

1. Add 1 μl (1μg) plasmid DNA and 19 μl dH2O to an eppendorf tube.

2. Add 2 μl 2N NaOH and incubate 5 minutes at room temperature.
3. Precipitate with 3 μl 3M sodium acetate pH 5.2 and 75 μl ethanol.

4. Chill on dry ice 10 minutes, spin, wash and dry pellet.

5. Resuspend pellet in 10 μl 2X Sequenase Buffer. Add 3 μl end-labelled oligo and 7 μl dH₂O.

6. Boil 1 minute and incubate at 37°C for 10 minutes.

7. Spin briefly. Aliquot 4.5 μl to each of four tubes containing 3.2 μl ddNTP mix. Add 1 μl of diluted Sequenase® (1:8 in Enzyme Dilution Buffer) to each tube and incubate at 37°C for 5 minutes.

8. Ethanol precipitate with 1 μl 3M sodium acetate, pH 5.2 and 30 μl ethanol. Chill on dry ice and spin. Wash and dry pellet and resuspend as above for primer extension samples.
Solutions I

TEN  
10 mM Tris pH 8.0  
1 mM EDTA pH 8.0  
40 mM NaCl

SCED  
1 M Sorbitol  
100 mM Sodium Citrate pH 5.8  
10 mM EDTA  
2 mM DTT

Solutions II

5X Taq Buffer  
250 mM Kcl  
50 mM Tris pH 8.5  
12.5 mM MgCl$_2$  
850 ug/ml BSA

5X G Mix  
1 mM ddCTP  
25 µM dCTP  
0.5 mM dATP  
0.5 mM dTTP  
0.5 mM dGTP

Loading Buffer  
95% formamide  
20 mM EDTA  
0.05% Bromophenol Blue  
0.05% Xylene Cyanol FF

Solutions III

5X Sequenase Buffer  
200 mM Tris-HCl, pH 7.5  
100 mM MgCl$_2$  
250 mM NaCl

Enzyme Dilution Buffer  
10 mM Tris-HCl, pH 7.5  
5 mM DTT  
0.5 mg/ml BSA
Indirect end-labelling

I. PREPARATION OF FREE DNA

1. Thaw two frozen 1 ml aliquots of nuclei at room temperature.

2. Add 80 µl 10% SDS and 30 µl 20 mg/ml Proteinase K to each.

3. Incubate at 37°C for two hours.

4. Add 180 µl 5 M potassium acetate and incubate on ice for one hour.

5. Spin samples at room temperature in microfuge for 10 minutes. Transfer supernatant to a fresh tube, repeat if necessary (sometimes it is impossible to avoid the pellet after the first spin).

6. Precipitate supernatant with an equal volume of isopropanol.

7. Resuspend the washed pellet in 300 µl water and reprecipitate.

Note: Do NOT overdry the pellet or it will be impossible to resuspend.

8. Resuspend the washed pellet in 1 ml of Buffer D2

Note: Free DNA must be resuspended in a buffer containing high cation concentrations to compensate for the loss of these ions due to coating of the phosphate backbone.

II. MNASE DIGESTION

1. Thaw 2 frozen 1 ml aliquots of nuclei at room temperature.

2. Aliquot 325 µl into six eppendorf tubes. Do the same for the Free DNA samples from above.

3. Dilute MNase stock to 5 Units/µl with Buffer D. Serial dilute this 5 Units/µl stock in Buffer D to make 0.5, 0.05 and 0.005 Units/µl.

4. Prewarm sample tubes at 37°C. Digest with MNase (0 to 50 Units) for five minutes at 37°C.
5. Stop reactions with 26 µl 10% SDS and 10 µl 20 mg/ml Proteinase K. Incubate samples at 37°C for two hours.

6. Add 60 µl 5 M potassium acetate, mix well and incubate samples for at least one hour on ice.

7. Spin samples in a microfuge for ten minutes at room temperature. Transfer supernatant to a fresh tube and precipitate with 350 µl isopropanol.

8. Spin, wash and dry pellets. Resuspend in 150 µl TE pH 8.0. Add 5 µg RNase and incubate at 37°C for 30-60 minutes.

9. Precipitate DNA with 2.25 µl 1 M spermidine pH 4.0. Incubate on ice for at least 15 minutes.

10. Pellet the DNA and resuspend in 150 µl 0.1M Tris pH 8.0. Incubate at 37°C until pellets are easily resuspended upon vortexing (10-30 minutes).

11. Precipitate DNA with 82.5 µl 7.5 M ammonium acetate and 500 µl ethanol.


III. SOUTHERN ANALYSIS

1. Electrophorese samples on a 1.5% agarose gel overnight (longest gel size).

2. Soak gel in 0.25% HCl.

3. Rinse gel in sterile water.

4. Soak gel in Southern Solution D for 30 minutes at room temperature with gentle agitation.

5. Rinse gel once in Southern Solution N and then soak in this solution for 15 minutes at room temperature with gentle agitation. Discard solution and soak gel in fresh Southern Solution N for 15 more minutes as above.

6. Transfer DNA to nylon membrane (Quiabrane®) by capillary action (see Maniatis). Transfer should be carried out in 20X SSC for at least 12 hours.

7. When transfer is complete, mark origin in pencil on membrane. Autocrosslink DNA to the membrane (Stratalinker®) and air dry 15 minutes
at room temperature. If necessary, membrane can be stored between two pieces of 3MM filter paper at 4°C.

8. Pre-hybridize membrane for 1-4 hours at 42°C in freshly prepared Prehybridization Solution.

9. Hybridize membrane for at least 12 hours at 42°C in 5 ml Hybridization Solution containing 1X10⁶ cpm/ml labelled DNA probe. Be sure to denature double stranded DNA probes by boiling for 5-10 minutes before use.

10. Wash membrane twice for five minutes at room temperature in 2 X SSC, 0.1 % SDS.

11. Wash membrane in 0.1X SSC, 0.1% SDS for 15 minutes at hybridization temperature (or higher if necessary). Repeat if necessary.
Solutions I

Buffer D2  10 mM HEPES pH 7.3  
5 mM MgCl₂  
2 mM CaCl₂

Solutions II

MNase stock  15 Units/ml in Digestion Buffer

Digestion Buffer

Buffer D  10 mM HEPES pH 7.3  
0.5 mM MgCl₂  
0.05 mM CaCl₂

TE pH 8.0  10 mM Tris-HCl pH 8.0  
1 mM EDTA pH 8.0

Solutions III

1.5% agarose gel  1.5% agarose in 1X TBE (0.089 M Tris base, 0.089 M boric acid, 20 mM EDTA pH 8.0)

Southern Solution D  0.4 N NaOH  
0.6 M NaCl

Southern Solution N  1.5 M NaCl  
0.5 M Tris-HCl pH 7.5

20X SSC  3 M NaCl  
0.3 M sodium citrate  
Adjust pH to 7.0 with 1 M HCl

Pre-hybridization Solution  6X SSC  
50% formamide  
5X Denhardt's Solution  
50 mM sodium phosphate pH 6.5  
100 µg/ml salmon sperm DNA  
0.5% SDS

Hybridization Solution  5X SSC  
50% formamide
1X Denhardt’s Solution
50 mM sodium phosphate pH 6.5
100 μg/ml salmon sperm DNA
0.1% SDS

100X Denhardt’s Solution
2% Ficoll 400
2% polyvinylpyrrolidone
2% BSA
Nystatin permeabilization of spheroplasts

This protocol is based on that published in Venditti and Camilloni (1994, Mol. Gen. Genet. 242: 100-104.) and that worked out by Monique Mukrit at UMMC.

1. Grow 200 ml of yeast culture in YEPD.

2. Harvest cells by centrifugation for ten minutes at 4K rpm in a J6 rotor.

3. Resuspend cell pellet in 20 ml Pre-spheroplasting Buffer and add 86 µl β-mercaptoethanol to make a final concentration of 60 mM.

4. Incubate cells at room temperature for ten minutes.

5. Spin cells as above.

6. Resuspend pellet in 10 ml Spheroplasting Buffer and add 7.2 µl β-mercaptoethanol to make a final concentration of 10 mM.

7. Remove 20 µl of cell suspension, mix with 980 µl dH2O, and record OD600.

8. To the remainder, add 200 µl recombinant lyticase.

9. Incubate at 37°C until OD600 is decreased by 90% (usually 10-15 minutes).

10. Spin spheroplasts at 4K rpm for ten minutes in the cold.

11. Wash spheroplasts in 750 µl Nystatin Buffer.

12. Resuspend washed spheroplasts in 1.5 ml Nystatin Buffer.

13. Add 30 µl 10 mg/ml nystatin.


15. Permeabilized spheroplasts can now be digested as desired, i.e. with MNase (for titration or indirect end-labeling experiments) or with restriction enzymes (for accessibility assays).

16. To stop digestion, add 1/10 volume of SDS/EDTA Stop Solution to each sample.
### Solutions

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-spheroplasting Buffer</td>
<td>100 mM Tris-HCl, pH 7.8-8.0</td>
</tr>
<tr>
<td>Spheroplasting Buffer</td>
<td>0.7 M sorbitol</td>
</tr>
<tr>
<td></td>
<td>0.75% yeast extract</td>
</tr>
<tr>
<td></td>
<td>1.5% peptone</td>
</tr>
<tr>
<td></td>
<td>10 mM Tris-HCl, pH 7.5</td>
</tr>
<tr>
<td>Nystatin Buffer</td>
<td>50 mM NaCl</td>
</tr>
<tr>
<td></td>
<td>1.5 mM CaCl2</td>
</tr>
<tr>
<td></td>
<td>20 mM Tris-HCl, pH 8.0</td>
</tr>
<tr>
<td></td>
<td>1 M sorbitol</td>
</tr>
<tr>
<td>SDS/EDTA Stop Solution</td>
<td>10 % SDS</td>
</tr>
<tr>
<td></td>
<td>50 mM EDTA</td>
</tr>
</tbody>
</table>
The preprint included in this appendix contains the restriction enzyme accessibility assay experiments described in Chapter 3 (see p. 78). This manuscript is scheduled for publication in the August 1997 issue of *Molecular and Cellular Biology*. While the experiments in question were not performed by the author of this thesis, the results are directly relevant to the work presented herein.
The Yeast SWI-SNF Complex Facilitates Binding of a Transcriptional Activator to Nucleosomal Sites In Vivo

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Received 2 January 1997/Returned for modification 18 February 1997/Accepted 24 April 1997

The Saccharomyces cerevisiae SWI-SNF complex is a 2-MDa protein assembly that is required for the function of many transcriptional activators. Here we describe experiments on the role of the SWI-SNF complex in activation of transcription by the yeast activator GAL4. We find that while SWI-SNF activity is not required for the GAL4 activator to bind to and activate transcription from nucleosome-free binding sites, the complex is required for GAL4 to bind to and function at low-affinity, nucleosomal binding sites in vivo. This SWI-SNF dependence can be overcome by (i) replacing the low-affinity sites with higher-affinity, consensus GAL4 binding sequences or (ii) placing the low-affinity sites into a nucleosome-free region. These results define the criteria for the SWI-SNF dependence of gene expression and provide the first in vivo evidence that the SWI-SNF complex can regulate gene expression by modulating the DNA binding of an upstream activator protein.

The SWI-SNF complex is required for the expression of a number of diversely regulated genes in the yeast Saccharomyces cerevisiae. The results of genetic experiments indicate that the SWI-SNF requirement reflects the ability of the complex to antagonize chromatin-mediated transcriptional repression (26). In these studies, mutations in genes that encode chromatin components, including HHT1 (encoding histone H3), HHF1 (encoding histone H4), and SNN1 (encoding an HMG-1-like protein which is thought to be a chromatin component), were found to alleviate the transcriptional defects of swi mutants (14, 15). In addition, alteration of relative histone levels by deletion of one of the two endogenous HTA1-HTB1 gene clusters (encoding histones H2A and H2B) is also able to partially restore transcriptional activity to swi-snf cells (12). These results suggest that the SWI-SNF complex acts to promote the expression of genes within the context of a compact eukaryotic genome.

The primary level of chromatin architecture in vivo is the nucleosome. The results of in vivo and in vitro studies indicate that nucleosomes inhibit transcription by competing with transcription factors for occupancy of DNA binding sites (for a review, see reference 27). In vitro, nucleosomes can inhibit transcription initiation either by blocking access of the transcription machinery to promoter sequences or by hindering the binding of upstream activator proteins. It is not known at present which of these steps might be overcome by the SWI-SNF complex in vivo. The results of biochemical studies have shown that yeast or human SWI-SNF complex can stimulate the binding of an activator protein to a nucleosomal binding site (5, 16). Furthermore, the purified human SWI-SNF complex can facilitate the binding of the general transcription factor TATA box binding protein (TBP) to a positioned nucleosome (13). Therefore, the SWI-SNF complex may function at one or more discrete steps to facilitate gene expression in vivo.

In this study, we address the ability of the SWI-SNF complex to facilitate the binding of a transcriptional activator to upstream activation sites (UASs). To do this, we monitored the transcriptional activity and DNA binding of the yeast activator GAL4 to derivatives of the UAS of the divergently transcribed GAL1 and GAL10 genes. These UAS derivatives differ in the affinity of the GAL4 binding sites as well as the nucleosomal context of these sites. We find that the SWI-SNF complex is required for GAL4 to activate transcription from a promoter containing two low-affinity, nucleosomal binding sites in vivo. In addition, we show that the SWI-SNF dependence of this promoter reflects, in part, the ability of the complex to facilitate GAL4 binding to these nucleosomal sites.

MATERIALS AND METHODS

β-Galactosidase assays. Strains were grown in minimal medium containing 2% galactose, 0.5% sucrose, and all the amino acids except histidine to an optical density at 600 nm (OD600) of 0.5 to 0.9. Assays were performed (23) on three transformants, and Miller units (19) were averaged. Standard deviations were <25%. β-Galactosidase activity from all strains in the absence of GAL4 expression was <1 Miller unit.

In vivo footprinting. Intact cells were treated with dimethyl sulfate (DMS), and methylated DNA was isolated essentially as previously described (10), except final pellets were dissolved in water and quantitated with a spectrophotometer. DNA samples were then digested with HaeIII and analyzed via a cyclic primer extension reaction (1) using a labeled oligonucleotide. Primer extension products were extracted with chloroform-isooctyl alcohol (24:1), precipitated, and electrophoresed on a 5% National Diagnostics sequencing gel. The gels were dried and exposed to film for 12 to 48 h. For a control for spurious primer extension products, DNA from a strain that was not treated with DMS was also prepared. Footprinting at the wild-type GAL1,10 UAS (see Fig. 2) was analyzed by using an oligonucleotide with the following sequence: 5'-CCG GCT CTT TCG TCT GGC-3'. This oligonucleotide anneals to the unique pUC sequences located upstream of the GAL4 binding sites in the reporters (see below).

Indirect end labeling. Nuclei were prepared as described previously (22). To prepare free DNA, 80 μl of 10% sodium dodecyl sulfate (SDS) and 30 μl of 20-μg/ml proteinase K were added to a 1-ml aliquot of nuclei (approximately 5 × 106 cell equivalents), and the sample was incubated at 37°C for 2 h. Lyed nuclei were treated with 180 μl of 5 M potassium acetate on ice for 1 h and subjected to centrifugation, and the supernatant was precipitated with isopropanol. This DNA sample was resuspended in water and precipitated with ethanol. The resultant free DNA was resuspended in 1 ml of buffer D2 (10 mM HEPES [pH 7.3], 5 mM MgCl2, 2 mM CaCl2). Free DNA and nuclei samples (approximately 1.7 × 106 cell equivalents in 300-μl aliquots) were digested with 0 to 50 U of micrococcal nuclease (MNase) for 5 min at 37°C. Reactions were halted by adding 25 μl of 10% SDS and 10 μl of 20-μg/ml proteinase K and incubating the
mixtures at 37°C for 2 h. Samples were treated with 60 μl of 5 M potassium acetate on ice for 1 h and subjected to centrifugation, and the supernatant was precipitated with isopropanol. DNA was then digested with either CiaI (see Fig. 4A and B) or EcoRI (see Fig. 4C) in the presence of RNase. Digested fragments were extracted with buffered phenol, and the aqueous layer was precipitated with ethanol. Pellets were then electrophoresed on a 1.5% agarose gel. DNA was transferred to a nylon membrane, and blots were probed with an internally labeled 870-bp lacZ fragment (see Fig. 4A and B) or an internally labeled 550-bp fragment from the GAL1 gene (see Fig. 4C).

**fpld restriction enzyme accessibility assay.** Permeabilized spheroplasts were prepared from exponentially growing cells (OD600 of 0.6, in glucose medium) by treatment with yeast lyticase (24). Briefly, cells were harvested, resuspended in 1/10 culture volume of preprotoplasting buffer (100 mM Tris (pH 8.0), 60 mM β-mercaptoethanol), and shaken for 10 min at room temperature. Cells were harvested and resuspended in 1/20 culture volume of spheroplasting buffer (0.7 M sorbitol, 0.75% yeast extract, 1.5% peptone, 10 mM MgCl2, 10 mM β-mercaptoethanol), lyticase was added, and cells were incubated at 37°C until the OD600 of cells diluted with water was decreased by 90% (30 to 40 min). Spheroplasts were harvested by centrifugation, washed once with nystatin buffer (50 mM NaCl, 1.5 mM CaCl2, 20 mM Tris [pH 8.0], 1 M sorbitol, 10 mM MgCl2), and resuspended in 1/500 culture volume of nystatin buffer. Nystatin was added to a final concentration of 15 μg/ml, and spheroplasts were incubated at 37°C for 5 min. HpaI (New England BioLabs) was added to the permeabilized spheroplasts at a concentration of 120 U/ml, and samples were incubated at 37°C for 0 to 30 min. Reactions were stopped by the addition of 1/5 volume of 100 mM Tris (pH 8.0), 1.5% peptone, and DNA was purified as described above (a negative control lacked HpaI). Purified DNA was digested with HindIII (New England BioLabs), electrophoresed on a 1.5% agarose gel, Southern blotted to a nylon membrane, and DNA was detected with the alkaline phosphatase method described previously (25).

### RESULTS

**SWI-SNF activity is required for transcriptional activation by GAL4 at some promoters.** Isogenic SWI and swir strains that contain similar single-copy integrated reporters were constructed and are shown schematically in Fig. 1A. Strains CY353 (SWI') and CY366 (swir') contain a GAL110 UAS reporter consisting of four GAL4 binding sites upstream of a GAL1-lacz fusion gene (reporter a). Strains CY401 (SWI') and CY422 (swir') contain a derivative of this reporter that contains only two of these low-affinity GAL4 binding sites (sites 3 and 4; reporter b); strains CY532 (SWI') and CY534 (swir') contain a version of reporter b that contains 192 bp of additional plasmid sequences 20 bp upstream of GAL4 site 3 (reporter c); strains CY528 (SWI') and CY530 (swir') contain a reporter which contains two high-affinity, consensus GAL4 binding sites in place of the two low-affinity sites (reporter d) (9). β-Galactosidase assays were performed on these strains to monitor GAL4 transcriptional activity either in the presence of endogenous concentrations of GAL4 (pLEXA columns) or under conditions in which GAL4 is overexpressed (pGAL4 columns) (Fig. 1A).

In the absence of functional SWI-SNF complex, transcriptional activation by GAL4 from the wild-type GAL110 UAS region is reduced only 1.5-fold from the activity in the respective wild-type strain (Fig. 1A, reporter a). This result is consistent with a previous study in which it was found that GAL4 expression was only weakly affected by swir mutation (21). In contrast, GAL4 activity on the two reporters with two low-affinity GAL4 binding sites is SWI-SNF dependent; transcriptional activity is reduced 22- to 32-fold in the swir' strains from that of the wild-type strain (Fig. 1A, reporters b and c). When these two low-affinity sites are replaced by two high-affinity, consensus GAL4 binding sites, transcriptional activation is reduced only 1.3-fold in the swir' strain from that of the wild-type strain (Fig. 1A, reporter d).

Strains harboring the two low-affinity site reporters were also distinct from those carrying the two high-affinity site reporters in that lacZ expression was increased 2.5- to 3.5-fold when GAL4 protein was overexpressed (Fig. 1A, reporters b and c; also data not shown). The simplest interpretation of this result is that physiological levels of GAL4 are not sufficient to fully occupy the two low-affinity sites (9; also see below). Importantly, overexpression of GAL4 does not overcome the SWI-SNF dependence of this reporter (Fig. 1A, reporters b and c). It should be noted that the swir mutation does not affect expression of GAL4 from this overexpression plasmid in this strain background (Fig. 1B) (20).

**GAL4-SNF activity is required for GAL4 occupancy of low-affinity binding sites in vivo.** To directly address the ability of GAL4 to bind to sites in the different reporter strains in the presence or absence of SWI-SNF, we used an in vivo DMS footprinting assay. GAL4 protects guanine residues at each end of its 17-bp

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>CY257</td>
<td>MATa swiΔ::LEU2 lys2-801 ade2-101 leu2-1Δ his3-Δ200 ura3-Δ99</td>
</tr>
<tr>
<td>CY296</td>
<td>MATa gal4Δ::LEU2 lys2-801 ade2-101 leu2-1Δ his3-Δ200 ura3-Δ99</td>
</tr>
<tr>
<td>CY297</td>
<td>MATa gal4Δ::LEU2 swi1 Δ::LEU2 lys2-801 ade2-101 leu2-1Δ his3-Δ200 ura3-Δ99</td>
</tr>
<tr>
<td>CY331</td>
<td>Same as CY524 but contains URA3::pRY171 (GAL110)</td>
</tr>
<tr>
<td>CY335</td>
<td>Same as CY524 but contains URA3::pRY171 (GAL110)</td>
</tr>
<tr>
<td>CY336</td>
<td>MATa swiΔ::LEU2 lys2-801 ade2-101 leu2-1Δ his3-Δ200 ura3-Δ99</td>
</tr>
<tr>
<td>CY401</td>
<td>Same as CY524 but contains URA3::pEG28 (2 low)</td>
</tr>
<tr>
<td>CY422</td>
<td>Same as CY526 but contains URA3::pEG28 (2 low)</td>
</tr>
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<td>MATa lys2-801 ade2-101 leu2-1Δ his3-Δ200 ura3-Δ99</td>
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<td>MATa swiΔ::LEU2 lys2-801 ade2-101 leu2-1Δ his3-Δ200 ura3-Δ99</td>
</tr>
<tr>
<td>CY528</td>
<td>Same as CY524 but contains URA3::pLB7 (2 high)</td>
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<td>CY530</td>
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</tr>
<tr>
<td>CY532</td>
<td>Same as CY524 but contains URA3::pLB8 (2 low; pUC spacer)</td>
</tr>
<tr>
<td>CY533</td>
<td>Same as CY526 but contains URA3::pLB8 (2 low; pUC spacer)</td>
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<td>CY586</td>
<td>Same as CY524 but contains URA3::pLB16 (2 low; NPE)</td>
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<tr>
<td>CY588</td>
<td>Same as CY526 but contains URA3::pLB16 (2 low; NPE)</td>
</tr>
</tbody>
</table>

* 2 low, two low-affinity GAL4 binding sites.
* 2 high, two high-affinity GAL4 binding sites.
Fig. 1. GAL4 requires functional SWI-SNF complex to activate transcription from two low-affinity binding sites. (A) β-Galactosidase assays were performed on isogenic SWT+ or swi− strains harboring the integrated GAL1-lacZ UAS reporter plasmids pRY171 (a), pEG28 (b), pLB8 (c), pLB7 (d), or pLB16 (e). GAL4 binding sites are denoted by the small black boxes. GAL4 sites numbered 1 to 4 indicate sites from the GAL1,10 UAS region; GAL4 sites numbered 17 indicate synthetic consensus GAL4 binding sites. Strains contained plasmids that overexpressed the bacterial LexA type DNA binding domain (pEG202) or the full-length GAL4 protein (pMA210). Numbers in parentheses show activities as percentages of the wild-type levels. β-Galactosidase activities of all reporters were <1 Miller unit in the absence of GAL4. Drawings are not to scale; n.d., not determined. (B) Western blot analysis of GAL4 protein in SWI− (CY296) and SWT+ (CY297) cells containing the GAL4 overexpression plasmid pMA210. Expression of GAL4 protein (indicated by the arrow) was identified by Western blotting using an antibody directed against the C terminus of GAL4 (21).

recognition site both in vivo (10) and in vitro (3). As shown in Fig. 2, these guanine residues within the four GAL4 binding sites of the GAL1,10 UAS are protected from DMS methylation in the presence of GAL4 (lane 3) but are accessible to methylation in an isogenic galB− strain (lane 2). Introduction of GAL4 expression plasmids into the galB− strain restores protection of these guanines (lanes 6 and 8), while introduction of a plasmid expressing the bacterial LexA protein does not (lane 7). In the absence of SWI-SNF activity, protection of all four sites is retained (lane 4). Furthermore, as shown in Fig. 3A, GAL4 also protects the guanine residues at each of the two high-affinity binding sites in the presence (lane 4) and absence (lane 5) of SWI-SNF. Similar results were obtained in at least six different experiments with five independent DNA preparations. These results are consistent with the results of our functional experiments (Fig. 1) in which the SWI-SNF is not required for the activity of GAL4 from either the wild-type GAL1,10 UAS or the two-high-affinity-site reporter.

Figure 3B shows the results of an in vivo footprinting analysis of the two-low-affinity-site reporter in strains CY532 (SWT+) and CY534 (swi−). We detect little protection of the two low-affinity sites in our footprinting assay, even in a SWT+ strain (lane 3). As discussed above, this probably reflects the low occupancy of these sites at physiological GAL4 levels, because when GAL4 is overexpressed in this SWT+ strain, protection is restored (lane 5). Overexpression of GAL4 in the absence of a functional SWI-SNF complex, however, does not restore complete protection of either low-affinity binding site (lane 6). This is most apparent at GAL4 site 3, where strong protection in the SWT+ strain (lane 5) and are only weak protection in the swi− strain (lane 6) are observed. Quantitation of these results by phosphorimager analysis indicates that protection of the relevant guanine residue in site 3 in the swi− strain is decreased (40%) from that seen in a wild-type strain. For an internal control, we also analyzed these samples for GAL4 binding at the endogenous GAL1,10 UAS locus. In all cases, we observe complete occupancy of the four GAL4 sites in the presence and absence of SWI-SNF (data not shown). The lack of complete occupancy at the two low-affinity sites in the swi− strain indicates that the SWI-SNF complex modulates GAL4 binding in vivo.

Introduction of an NPE into a SWI-SNF-dependent promoter region can suppress the requirement for SWI-SNF. The results of genetic and biochemical experiments have indicated that SWI-SNF complex facilitates activator function by disrupting chromatin structure. To assess whether the chromatin structure of the GAL4 binding sites was related to their SWI-SNF dependence, a strong NPE was inserted directly upstream of the two low-affinity binding sites in strains CY532 (SWT+) and CY534 (swi−). This 182-bp NPE sequence has been shown previously to translationally and rotationally position a nucleosome in vivo in yeast (20) (Fig. 4B). Insertion of the NPE had a dramatic effect on the SWI-SNF dependence of GAL4 function. While GAL4 transcriptional activity at the parental two-low-affinity-site reporter was decreased over 20-fold in the absence of SWI-SNF (Fig. 1A, reporters b and c), there was less than a 1.5-fold decrease when the NPE was inserted (Fig. 1A, reporter e). This effect of the NPE is not due...
simply to insertion of foreign DNA sequences directly upstream of the two low-affinity GAL4 binding sites, as insertion of 192 bp of pUC plasmid sequences at an identical position does not alter the SWI-SNF dependence of GAL4 activity (Fig. 1A, reporters b and c).

Nucleosomal context of GAL4 binding sites dictates SWI-SNF dependence. The results of functional experiments described above suggest that the SWI-SNF dependence of GAL4 can be modulated by nucleosome positioning. In the case of the \textit{GAL1,10} locus, the four GAL4 binding sites are maintained in a constitutive nucleosome-free region which is flanked on both sides by an array of positioned nucleosomes (6, 7) (Fig. 4). This precise nucleosome positioning at the \textit{GAL1,10} locus is believed to require the GRF2 protein which binds to a sequence that overlaps GAL binding sites 1 and 2 (4, 7). The \textit{GAL1,10} UAS derivatives that we have analyzed in this study (Fig. 1A, reporters b to e) lack this GRF2 binding site, and therefore, they may not maintain nucleosome positioning surrounding the GAL4 binding sites. To confirm this possibility, we analyzed the chromatin structure of the two low-affinity-site reporters (with and without an NPE) and compared these structures to that of the \textit{GAL1} locus in the same strains.

Nuclei and free DNA were prepared from the \textit{SWI+} strain CY532, which contains the parental two-low-affinity-site reporter (without an NPE), and samples were analyzed by MNase digestion and indirect end labeling. First, we analyzed the chromatin structure at the \textit{GAL1} locus in this reporter strain (Fig. 4C). Comparison of the MNase cleavage patterns for free and chromatin DNA samples reveals a repeating pattern of MNase protections (each about 140 bp in size) flanked on each side by MNase-hypersensitive sites. These results are essentially identical to a previous study (6) and are consistent with an array of positioned nucleosomes downstream of the nucleosome-free GAL4 binding sites. A similar analysis of the two-low-affinity-site reporter locus (without an NPE) yielded very different results (Fig. 4A). In the region upstream of the two GAL4 binding sites, the MNase digestion pattern of the chromatin sample is similar to the pattern of digestion of free DNA, although we do reproducibly observe several preferred cleavage sites in free DNA that appear to be enhanced in the chromatin sample. Importantly, the MNase cleavage sites directly upstream and adjacent to the GAL4 binding sites are cleaved with equal efficiency in the free and chromatin DNA samples. A region of about 80 bp that contains the GAL4 binding sites is not cleaved efficiently in either the free or chromatin DNA samples; this is due to the inherent sequence specificity of MNase I and is not due to a positioned nucleosome (6). In addition, this protected region is not flanked on both sides by hypersensitive sites. Directly downstream of the GAL4 binding sites, however, a 140-bp region of MNase resistance is detected, which is flanked on each side by MNase-hypersensitive sites, suggesting the presence of a positioned nucleosome. The location of this nucleosome corresponds to a positioned nucleosome mapped between the GAL4 sites and the GALI TATA box of the endogenous \textit{GAL1,10} locus (2, 6) (Fig. 4C and data not shown). Thus, although one nucleosome still appears to be positioned at this \textit{GAL1,10} UAS derivative, the majority of nucleosome positioning has been lost. Consequently, nucleosomes appear to be located randomly upstream of the two remaining GAL4 binding sites. In the absence of a positioned array of nucleosomes, the two low-affinity GAL4 binding sites are unlikely to reside in a constitutive nucleosome-free region. MNase digestions and indirect end labeling were also used to confirm that the NPE positioned a nucleosome upstream of the two low-affinity GAL4 binding sites (Fig. 4B). Comparison of the free and chromatin DNA samples reveals an MNase-protected region of about 250 bp which contains the NPE sequences as well as the MNase-resistant GAL4 binding site sequences. This result is consistent with the positioning of a nucleosome over the NPE sequence. In addition, the putative positioned nucleosome located directly downstream of the two low-affinity GAL4 binding sites is also detected at the NPE-containing reporter locus in CY586 (Fig. 4B).

These results are consistent with the model shown in Fig. 6. In the absence of the NPE, the two low-affinity GAL4 binding sites are encompassed by randomly positioned nucleosomes;
FIG. 3. SWI-SNF is required for complete occupancy of two low-affinity GAL4 binding sites in vivo. In vivo DMS footprinting was performed on strains that harbor the two-high-affinity-site reporter (A) or the two-low-affinity-site reporter (B). The strains used were as follows: for panel A, CY529 (gal4+ SWI+) (lane 3), CY528 (GAL4+ SWI+) (lane 4), and CY530 (GAL4+ swi1-) (lane 5); for panel B, CY533 (gal4- SWI+) (lane 2), CY532 (GAL4+ SWI+) (lane 3), CY534 (GAL4+ swi1-) (lane 4), CY532 harboring a GAL4 expression plasmid (lane 5), and CY534 harboring a GAL4 expression plasmid (lane 6). For quantitation of lanes 5 and 6 in panel B, phosphorimager data were normalized to the doublet band indicated by the arrowhead (G449 and G450 [numbering from reference 30]). Symbols and lane 1 are as described in the legend to Fig. 2.

Insertion of the NPE repositions the GAL4 binding sites into a nucleosome-free region between two positioned nucleosomes (see Fig. 6). To further test the possibility that insertion of the NPE changes the accessibility of the GAL4 binding sites, we investigated the ability of a restriction enzyme to cleave a site within GAL4 site 3 in permeabilized spheroplasts (Fig. 5). Spheroplasts were prepared from the SWI+ strains CY532 (which contains the parental two-low-affinity-site reporter) and CY586 (which harbors the NPE-containing two-low-affinity-site reporter). Spheroplasts were made permeable by treatment with nystatin (24) and incubated with HgaI restriction endonuclease for 15 to 30 min. Genomic DNA was then purified and digested to completion with HindIII, and the HgaI-HindIII cleavage products were analyzed by Southern blotting using a DNA probe from the GAL1 upstream region. This DNA probe will detect the HgaI-HindIII cleavage products derived from the GAL1.10 locus (1.6 kb) or the reporter locus (0.5 kb) in the same DNA sample (see the schematic in Fig. 5A). In the case of the GAL1.10 locus, we expected that the HgaI site within GAL4 site 3 would be highly accessible in permeabilized spheroplasts, since this GAL4 site is known to be nucleosome-free (6). In contrast, the model presented in Fig. 6 predicts that the HgaI site at the parental two-low-affinity-site reporter will be less accessible than the same site present at GAL1.10. Furthermore, the model predicts that insertion of the NPE will enhance the accessibility of the HgaI site at the reporter locus.

Figure 5B shows the phosphorimager quantitation of a typical HgaI accessibility assay. At 15 and 30 min of digestion, HgaI cleavage at the GAL1.10 locus was nearly complete (value set at 100%) (data not shown). However, in the case of the
DISCUSSION

In vitro experiments have demonstrated that the SWI-SNF complex can stimulate the binding of an activator protein to a nucleosomal binding site (5, 16). Here we have tested whether the complex exhibits this activity in vivo. We have shown that the SWI-SNF complex facilitates the binding of GAL4 to two low-affinity binding sites in vivo. Furthermore, we find that the inability of GAL4 to occupy these low-affinity sites in the absence of SWI-SNF activity can be overcome by (i) replacing the low-affinity binding sites with high-affinity GAL4 binding sites or (ii) placing the low-affinity binding sites into a nucleosome-free region. These results indicate that SWI-SNF is able to facilitate GAL4 binding in vivo, perhaps by helping GAL4 to
FIG. 5, An NPE leads to enhanced accessibility of a GAL4 binding site. (A) Schematic of the GAL1,10 and reporter loci. The black bar denotes the DNA fragment used as a probe in Southern blotting. The Hgal site is located at the 3' end of GAL4 site. +/− NPE, with or without an NPE. (B) Hgal accessibility assay. Hgal was added to nystatin-permeabilized spheroplasts prepared from SWI' strains harboring either the parental two-low-affinity-site reporter (+ NPE; CY532) or the NPE-containing two-low-affinity-site reporter (+ NPE; CY586). DNA digested in situ was purified, digested to completion with HindIII, and analyzed by Southern blotting using the DNA probe denoted in panel A. Phosphorimager quantitation of the Hgal-HindIII fragments is shown for the 15- and 30-min Hgal digestion time points. The yields of the 0.5-kb Hgal-HindIII fragments from the two different reporter loci are presented as a percentage of the 1.6-kb Hgal-HindIII fragment derived from the GAL1,10 locus which was set at 100% accessibility.

A. 

B.

A definition of SWI-SNF dependence. Our results suggest that the SWI-SNF dependence of a transcriptional activator protein is not an innate feature of the activator protein per se but rather reflects the chromosomal context of the activator binding sites. We propose that when the activator binding sites are encompassed in a nucleosome, as in the two-low-affinity-site reporter used in this study, SWI-SNF function is necessary for the activator protein to bind to and activate transcription from those sites (Fig. 6). On the other hand, when binding sites are nucleosome-free, as is the case at the GAL1,10 UAS region or at the NPE-containing two-low-affinity-site reporter, then SWI-SNF function is dispensable for GAL4 activity (Fig. 6). In this case, GAL4 can bind to its sites and activate transcription in the absence of SWI-SNF activity. Furthermore, SWI-SNF dependence does not appear to correlate with the level of transcriptional activation (i.e., promoter strength [Fig. 1A]). Thus, based on the results presented here, we predict that the subset of genes whose expression requires SWI-SNF will have activator binding sites that are encompassed in nucleosomes; genes that do not require SWI-SNF will have such sites positioned in the linker regions between nucleosomes or in other nucleosome-free regions. This positioning may be determined by DNA sequence, as in the NPE-containing reporter, or by abundant DNA binding proteins like GRF2, as in the case of the intact GAL1,10 reporter.

Our results also require that binding site affinity be incorporated into the definition of SWI-SNF dependence. While GAL4 requires SWI-SNF in order to access two low-affinity
binding sites, high-affinity sites are occupied even in the absence of SWI-SNF. The ability of higher-affinity binding sites to alleviate SWI-SNF dependence provides strong evidence that the SWI-SNF complex modulates transactivation at the level of DNA binding. Furthermore, if these high-affinity binding sites are encompassed by nucleosomes, then our results suggest that GAL4 binding to high-affinity, nucleosomal sites can occur in vivo via a SWI-SNF-independent mechanism. These results are consistent with those of Workman and Kingston (28), who found that GAL4 can bind to a nucleosomal site to form a tripartite transcription factor-histone-DNA complex.

Does the SWI-SNF complex play an additional role in transcriptional activation? As noted above, we detect a consistent level of GAL4 binding to the low-affinity sites in the swi7 strain (Fig. 3B, compare lanes 5 and 6); phosphorimager analysis indicates site 3 may be occupied at 60% of the level seen in a swi7 strain (Fig. 3C). The fact that this level of occupancy results in negligible levels of transcription (3% of wild-type level [Fig. 1A, reporter c]) may indicate a role for the SWI-SNF complex in transcriptional activation beyond the modulation of activator binding. In light of recent reports suggesting that the SWI-SNF complex may associate with the RNA polymerase II holoenzyme (25), it is interesting to consider the possibility that an activator-SWI-SNF interaction is involved in preinitiation complex assembly. On the other hand, our in vivo footprinting procedure does not allow us to determine the extent to which site 3 and site 4 are simultaneously occupied and we cannot distinguish whether the 60% occupancy in a swi7 strain reflects the stable binding of GAL4 in 60% of the cells or reflects weak, unstable binding in 100% of the cells. Therefore, our results may support an additional role for the SWI-SNF complex in facilitating the cooperative binding of GAL4 or in enhancing the stability of GAL4 binding to low-affinity, nucleosomal sites in vivo.

ACKNOWLEDGMENTS

We thank I. Herskowitz, M. Fedor, L. Apone, and members of the Peterson laboratory for comments on the manuscript; E. Giniger and J. Workman for plasmids; J. Axelrod and L. Wu for footprinting protocols; and L. Cerillo and C. Logic for guidance on phosphorimager analysis. We also acknowledge the initial observation that high-affinity GAL4 binding sites can alleviate the SWI-SNF requirement for GAL4 enhancement was made by C.L.P. while a postdoctoral fellow with I. Herskowitz.

This work was supported by NIH grant GM49650 and a Basil O’Connor Research Starter Scholar Award FY94-002 to C.L.P. C.L.P. is a scholar of the Leukemia Society of America.

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