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Identification of cellular factors required for intracellular receptor signaling and transcriptional activation

Author
Levinson, Randy Scott

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IDENTIFICATION OF CELLULAR FACTORS REQUIRED FOR

INTRACELLULAR RECEPTOR SIGNALING AND TRANSCRIPTIONAL ACTIVATION

by

RANDY SCOTT LEVINSON

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

BIOCHEMISTRY &

MOLECULAR BIOLOGY

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco

Lord, I'm free. I'm free at last...

- quoted from an old Negro spiritual

Acknowledgments

What can I say? My time here at UCSF has been a period of great growth, both scientifically and personally. I came here with a fair amount of experience in science, but with a lot of hubris. I leave here with a great deal of experience in science, but with a lot of humility. And I think I am a better person for it. I want to thank several people who I think have been instrumental in my growth as a scientist and as a person. First, I would like to thank my parents for being so supportive over the years, though they still do not understand what I do or why, if I was going to spend so many years in school, I did not go to medical school.

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Abstract

Identification of cellular factors required for intracellular nuclear receptor signaling and transcriptional activation

Randy Scott Levinson

Glucocorticoids are important physiological signaling molecules. The primary mediator of glucocorticoid signaling is the glucocorticoid receptor (GR), which regulates distinct target genes in different cell types. The factors that determine which genes GR regulates, and how it regulates them, are of utmost interest. Thus, an unbiased genetic screen in *Saccharomyces cerevisiae* was performed to identify factors that interact with GR. The screen revealed *SWP73*, a gene encoding a novel member of the yeast Swi/Snf complex, a large, multiprotein complex believed to be involved in reorganizing chromatin and derepressing transcription.

The activities of several transcriptional activators were compared in a wild type strain (W303-1A), Wswp73-1, or in strains harboring genomic deletions of either *SWP73* (Wswp73Δ) or *SNF2* (Wsnf2Δ). Typically, a rank order of activity was observed as W303-1A > Wswp73-1 >> Wswp73Δ > Wsnf2Δ. However, the rank order was influenced by context, such as the nature of the regulatory factor and its DNA response element. The activators Yap1p and the fusion protein GR-LexA both showed a strong dependence on Snf2p but no dependence on Swp73p. The presence of swp73-1p, though,

caused both activators to behave as they did in the absence of Snf2p. Also, a version of GR that harbors the mutation K461A displayed a reversal of the typical rank order when tested at the response element plfG3. Deletion of Swp73p yields a complex similar in size to the wild type complex, whereas deletion of Snf2p results in a much smaller complex. Thus, Swp73p and Snf2p have common, but also distinct, functions in the Swi/Snf complex.

Suggesting a mechanism for specificity of Swi/Snf action, GR co-immunoprecipitates with Swi3p, Swp73p, and Snf6p, and, likely, the whole Swi/Snf complex. GR's interaction with Swi/Snf may be conserved in evolution. Thus, BAF60, the human homolog of Swp73p, may interact with GR and play a role in determining cell type specificity of GR genetic regulation.

Analysis of Swp73p has shown that chromatin remodeling can be highly specific and depends on an interplay between remodeling complexes, regulators, and DNA elements. Also, the results of the unbiased genetic screen argue that an expansion might yield additional interesting co-factors.

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Introduction

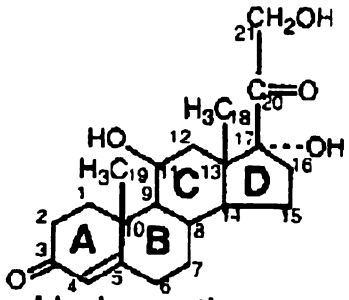
Steroid hormones represent a large class of signaling molecules. Indeed, many systemic activities in higher organisms are influenced by steroid hormones in a classic endocrine fashion (reviewed in McDonnell et al. 1993). These hormones fall into further subclasses, which include the sex steriods (for example, estradiol, testosterone, progesterone, and androgen), the glucocorticoids and the mineralicorticoids (for example, cortisone, hydrocortisone, and aldosterone, see Figure 1), the retinoids (for example, retinoic acid), and some vitamins (for example, vitamin D). Steroid hormones exert their systemic effects by binding and activating a class of proteins termed steroid hormone receptors (reviewed in Baniahmad and Tsai 1993; Truss and Beato 1993). These steroid hormone receptors mediate the signal propagated by their cognate hormones by up-regulating and down-regulating the transcription of a set of target genes in the appropriate cell types (Yamamoto and Alberts 1976).

Glucocorticoids were initially identified by the control they exert over gluconeogenesis (hence their name) and thus cellular metabolism. The hormone signal is propagated through the hypothalamic-pituitary-adrenal (HPA) axis, and indeed the effect of glucocorticoids on carbohydrate metabolism is just one component in the hormone's primary role as the mediator of the stress response (Ottaviani and Franceschi 1996). When an animal is stressed, serum levels of both epinephrine and cortisol can rise dramatically (Munck et al. 1984). These hormones enable an animal to focus its energy toward the muscles to ready the animal for the "flight-or-fight" response. While epinephrine leads to a rapid response and is relatively short-lived, cortisol leads to a much slower response but can stay in the blood

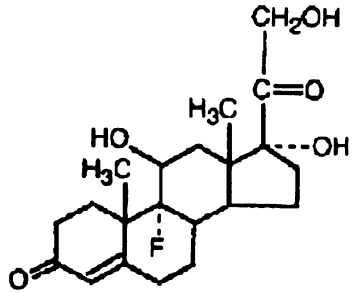
Figure 1. Schematic diagram illustrating the chemical structures of various glucocorticoids.

The chemical structures of various glucocorticoids are diagrammed. The numbering of the carbon atoms that comprise the steroid hormone is illustrated in the case of hydrocortisone, along with the letters A-D indicating the basic ring structures of the steroidal backbone.

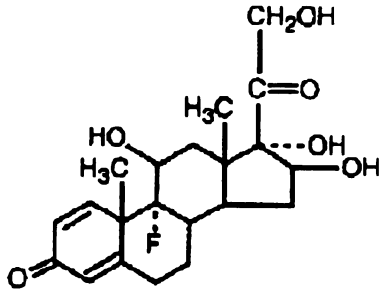
Structures of Various Glucocorticoids



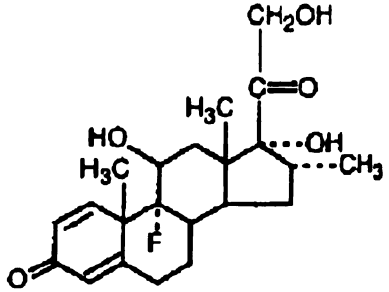
Hydrocortisone



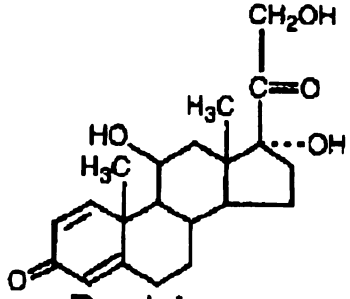
Fludrocortisone



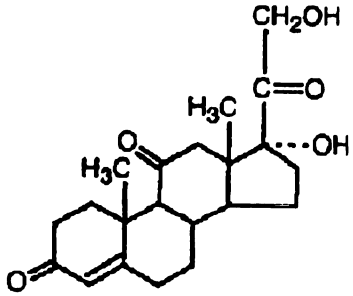
Triamcinolone



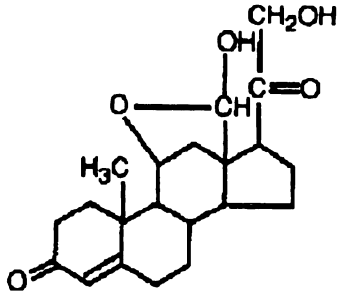
Dexamethasone



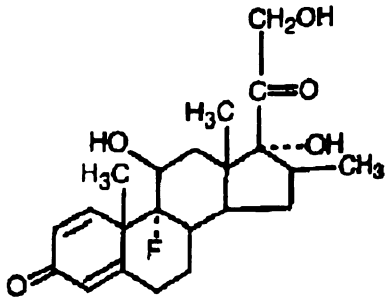
Prednisone



Cortisone



Aldosterone,
hemiacetal derivative



Betamethasone

stream for prolonged periods. Therefore, chronic stress can have deleterious effects on an animal. Continued exposure to high levels of cortisol in the serum can lead to a shifting of energy away from the muscle stores and toward fat stores, especially in the abdomen. This reshaping of the body can eventually lead to cardiovascular disease and diabetes. Also, conditions, such as Cushing's syndrome, that lead to hypercorticism result in a similar redistribution of fat, as well as hypertension (Whitworth et al. 1997).

It should be pointed out, however, that the actions of glucocorticoids are widespread and go far beyond mediating the stress reponse. Indeed, they play important roles in the normal activities of practically every major system in the body. For example, they can initiate anti-inflammation and appear to play a role in thymocyte selection (Ashwell et al. 1996). The mechanism by which glucocorticoids suppress inflammation is complicated and multi-leveled (Daynes et al. 1995). It appears that immune suppression is achieved by inhibiting the production of intercellular factors required for immune cell proliferation and intracellular adhesion molecules required for leukocyte localization.

Glucocorticoids also play a role in kidney function by affecting fluid and electrolyte balance through their permissive effects on tubular function and actions that maintain proper glomerular filtration rates (Muto 1995). Likewise, glucocorticoids affect the cardiovascular system by enhancing vascular reactivity to other vasoreactive substances. For example, glucocorticoids increase the expression of adrenergic receptors in the vascular wall which can lead to hypotension (Schimmer and Parker 1996). Conversely, as mentioned above for Cushing's syndrome, excessive glucocorticoids in the blood can lead to hypertension (Whitworth et al. 1997).

Glucocorticoids also control surfactant expression in the developing lung during embryogenesis (Asabe et al. 1996). Of particular note, a homozygous genetic knockout of the gene encoding the glucocorticoid receptor (the protein that mediates the glucocorticoid response) in mice leads to the death of the organism shortly after birth due to a lack of respiration (Schmid et al. 1995).

Glucocorticoids also play a role in skeletal muscle maintenance. While the mechanism is unknown, it is commonly observed that muscle wasting occurs in situations that lead to prolonged excess serum levels of glucocorticoids, such as chronic stress and certain medical conditions (such as Cushing's syndrome or chronic glucocorticoid therapy) (Rooyackers and Nair 1997).

Many emotional and behavioral changes are seen in patients receiving glucocorticoid therapies (Wolkowitz et al. 1997). Of note, mood elevation usually occurs, as well as insomnia, restlessness, and increased motor activity. More severe alterations in behavior can include anxiousness, depression, and overt psychoses (Wolkowitz et al. 1997). The exact reasons for these occurances are unknown, but chronic stress (and thus elevated serum levels of glucocorticoids) can lead to dramatic changes in the intracellular morphology of neurons in the brain. Glucocorticoids have been observed to regulate cell birth and cell death in the CA3 region of the hippocampus (Sapolsky et al. 1990), and therefore they may play a role in learning and memory (Wolkowitz et al. 1997). Indeed, glucocorticoids appear to be therapeutic in Alzheimer's patients (Aisen 1996; Breitner 1996). Interestingly, it has been recently suggested that glucocorticoids and other steroid hormones are produced in local regions of the brain (termed neurosteroids) and may therefore regulate neuronal excitability (Mellon 1994).

Considering the diverse spectrum of glucocorticoid effects, it is not surprising they are among the most widely prescribed therapeutics. A representative list of medical conditions that are treated with glucocorticoids includes adrenal insufficiencies, most autoimmune diseases, inflammatory dermatoses, occular inflammation, certain infectious diseases, asthma, allergic diseases, certain renal diseases, acute lymphocytic leukemia and lymphomas, inflammatory bowel disease, cerebral edema, thrombocytopenia, organ transplantation, Duchenne muscular dystophy, and spinal cord injuries (Schimmer and Parker 1996). This wide range of treatments involving glucocorticoids clearly indicates that an increased knowledge of the underlying molecular mechanisms by which glucocorticoids mediate physiological effects is important.

The primary target of glucocorticoids in their molecular signaling pathway is the glucocorticoid receptor (GR). GR is a transcription factor and mediates the hormonal signal by influencing the rate of transcription from a set of target genes (Yamamoto and Alberts 1976). Since transcription is an important cellular process, much of the cell's energy is devoted to its regulation. Thus, transcription factors represent one of the most important classes of proteins in the cell. For example, information from the yeast sequencing project resulted in an estimation that ~7% of the gene products of *Saccharomyces cerevisiae* that are currently assigned function (which represents ~60% of the proteome) play a role in transcription (Goffeau et al. 1996). While mammalian cells may not devote as high a percentage of their genome to encoding transcription factors as does yeast, nonetheless the importance of understanding more fully the mechanisms of transcriptional regulation in all organisms is critical to our understanding of biology in general. Thus, another reason to study the actions of GR is its important role

as a model system for the study of transcription. Fortunately, great progress has been made in dissecting the molecular pathway used by GR to propagate its hormonal signal. Figure 2 displays a simple schematic diagram illustrating how glucocorticoids actuate the hormone signal in a "typical" cell type. Many questions remain to be answered, and it is the goal of my thesis project to both develop and use a method to dissect further the intracellular pathway that mediates the cellular response to glucocorticoid signaling.

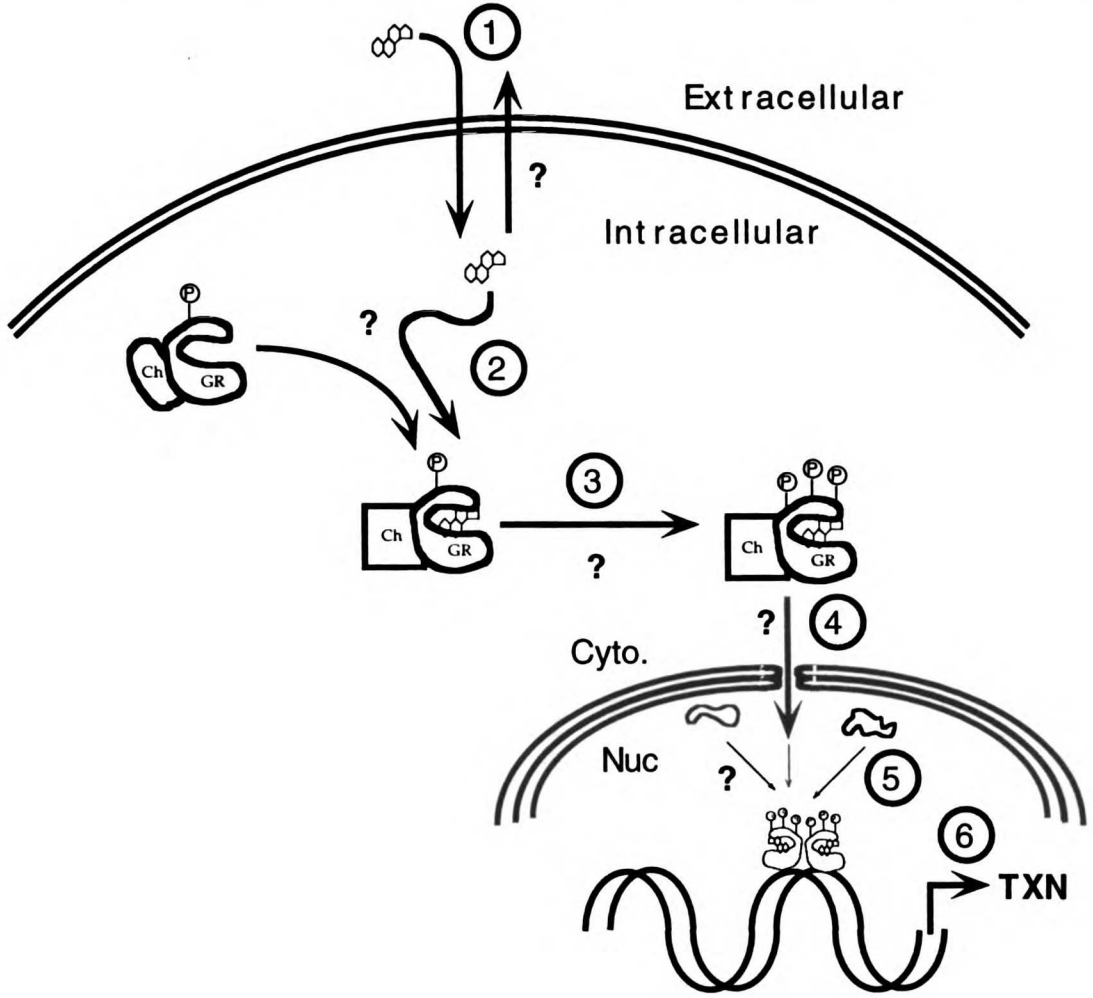
I decided to develop as simple a system as possible to study the actions of GR. Previous results had already shown that GR activates transcription from a synthetic promoter in *S. cerevisiae* (Schena and Yamamoto 1988). So I decided to perform an unbiased genetic screen in yeast to identify mutations in the yeast genome that prevent GR from activating transcription from a test promoter. I will discuss in more detail both the rationale and the development of this genetic screen in the next chapter. For now, though, it suffices to point out that some of the advantages of performing a genetic screen in yeast, as opposed to performing a similar screen in mammalian cells, are that yeast has a small, haploid genome that is completely sequenced, a short generation time that does not involve complicated growth conditions, and a well-characterized genetic system. My hope was that information gleaned from studying GR function in yeast would be applicable to understanding how GR functions in higher organisms.

The next chapter is devoted to describing the development of my yeast screen. In the meantime, I first explain what is known about GR's molecular signaling pathway as outlined in Figure 2 and at each step point out some of the questions that remain; thus, identifying some of the issues that potentially could be addressed in my genetic screen.

Figure 2. The molecular pathway by which glucocorticoids actuate their hormone response.

The steps by which glucocorticoids achieve a cellular response are shown. The steps are as follows: 1) the steroid hormone (illustrated by the steroid chemical structure) crosses the cell membrane and 2) binds the glucocorticoid receptor (indicated by the label GR), which is associated with a protein chaperone complex (indicated by the label Ch). After hormone binding, certain members of the chaperone complex are released (smooth polygon → square). Also, in step 3) the hormone-bound molecule of GR is phosphorylated further and then 4) translocates through the nuclear pore. It should be noted that it is unknown if hormone-dependent phosphorylation occurs before or after nuclear translocation. Once in the nucleus, GR can interact with other nuclear factors (as indicated by the arrows converging on the DNA) and bind DNA, resulting in step 6) where the transcription of the downstream linked gene is modulated. The question marks indicate that many questions remain to be answered regarding the exact mechanism at each step.

The Molecular Pathway by Which the Hormone Signal is Actuated



First, the steroid hormone crosses the cell membrane. At this point, we do not know if the hormone is actively transported across the cell membrane or simply diffuses across. There is some evidence, however, for a mechanism that actively pumps certain steroid hormones out of the cell in yeast (Kralli et al. 1995; Kralli et al. 1996). Presently, similar proteins have not been identified in mammalian cells, but if they exist, they may regulate the persistence time of the hormone signal. The result in yeast is one example of how information that was obtained by studying GR signaling in yeast could potentially lead to informative results in mammalian cells.

Second, the hormone receptor is bound in a molecular complex known as the glucocorticoid aporeceptor complex. This complex consists of GR, which is the binding moiety of the complex (a schematic diagram illustrating the various functional domains of GR is presented in Figure 3), and several heat-shock proteins and associated factors, which include HSP90, HSP70, p23, p60 and one of three immunophilins (FKBP52, FKBP59, or CYP40) (Picard et al. 1990b; Bohen et al. 1995). These associated factors are believed to keep GR in a hormone-binding competent state by acting as a molecular chaperone complex (Bohen et al. 1995). Once GR binds its cognate hormone, certain members of the aporeceptor are released and the remaining complex is available for translocation to the nucleus. Many questions in this step of the pathway remain to be answered. The order of addition by which the complex is built up and the mechanism by which it facilitates GR's ability to bind hormone are still being addressed. Also, the intermediates of the complex that exist both before and after hormone binding have yet to be fully determined. Interestingly, homologs of the chaperone factors listed above exist in yeast. Whether they perform functions identical to their mammalian

counterparts, at least with regard to GR function, remains to be determined, but the yeast system is presently being used to study the questions just listed.

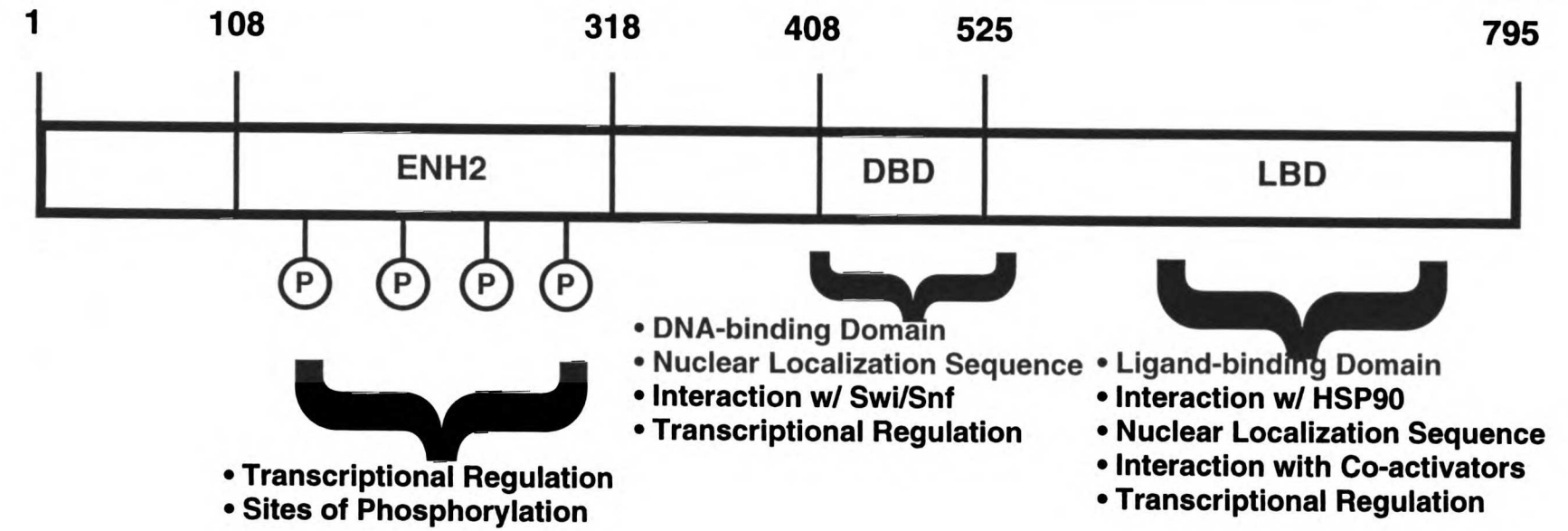
Third, upon binding to its cognate hormone and shedding certain members of the chaperone complex, the GR-hormone complex translocates through the nuclear pore and into the nucleus. GR contains two nuclear localization sequences (NLSs - see Figure 3) (Picard et al. 1990a). One of these (NL2) is in the hormone-binding domain, but is poorly defined. The other NLS (NL1) is upstream of the hormone-binding domain and is well defined. It falls into the well-represented class of bipartite NLSs seen in several nuclear proteins (Dingwall and Laskey 1991). While great advances have been made in the last couple of years in the biochemical analysis of nuclear transport, it is still poorly understood exactly how proteins are transported through the nuclear pore. Unfortunately, GR is no exception. A complete description of this transport process would be a great advance in understanding not only an aspect of GR function but an aspect of the function of several nuclear proteins.

Fourth, GR is a phosphoprotein (reviewed in Orti et al. 1992) and is phosphorylated in the absence of bound hormone. Upon hormone binding, GR is phosphorylated further, either in the cytoplasm or the nucleus or both. The role of phosphorylation in GR function is presently unknown, but it is believed that GR is phosphorylated by both a CDK kinase and a MAP kinase (Krstic et al. 1997). If so, then phosphorylation may play a role in regulating GR's activity in different points in the cell cycle. Based on the position of the phosphorylation sites in the transcriptional regulation domain of GR and the differential phosphorylation in the absence or presence of bound hormone, phosphorylation of GR may play a further role in GR function, such as

Figure 3. The functional domains of the rat glucocorticoid receptor.

The functional protein domains of the rat glucocorticoid receptor (rGR) are illustrated. The amino acid positions that mark the boundaries of each domain are indicated above the schematic. Full-length rGR is 795 amino acids in length. The enh2 domain, which contains a transcriptional regulatory domain and the major sites of phosphorylation, is bounded by amino acid positions 108 - 318. The presence of phosphorylated serine/threonine residues is indicated by the encircled P's, but no attempt to indicate the exact sites and the number of sites is made. The DNA-binding domain (indicated by the label DBD) is bounded by amino acid positions 408 - 525. In addition to the two Zn-fingers that comprise the DNA-binding domain, a bipartite nuclear localization sequence exists in this region, as well as an interaction domain with the yeast Swi/Snf complex. The ligand-binding domain (indicated by the label LBD) is bounded by amino acid positions 525 - 795. This region also contains an interaction domain with HSP90, a second nuclear localization sequence, an interaction surface with co-activators (e.g. GRIP1), and a transcriptional regulatory domain.

The Functional Domains of the Rat Glucocorticoid Receptor



regulating its ability to bind certain promoters or interacting with certain protein partners during the signaling pathway.

Fifth, after GR translocates to the nucleus, its DNA-binding domain (see Figure 3) can recognize specific sequences of DNA (termed glucocorticoid response elements) and bind these sequences, typically as a homodimer. It should be pointed out that GR can also influence the transcription rate of genes without binding to DNA, apparently by interacting with other transcription factors that in turn bind to sequences linked to these genes. Thus, GR can exert a positive or negative influence on the transcription of nearby linked genes, depending upon the context of the response element with regard to its promoter and the activity of other factors in the cell nucleus (Miner et al. 1991; Diamond et al. 1990). One of the questions that remains to be answered is the identity of the proteins that GR interacts with in the nucleus. The identities of a few transcription factors that interact with GR are known (Diamond et al. 1990; Chavez and Beato 1997; Wissink et al. 1997), but potentially there could be several more.

One set of nuclear factors that can influence the activity of GR is chromatin and the complex cellular machinery that regulates it. A detailed explanation of chromatin and its regulating factors will be offered in the Introduction to Chapter 3. Briefly, chromatin acts as the nuclear infrastructure by which DNA is packaged within the confines of the cell nucleus (Kornberg 1974). Because of this packaging, chromatin is known to have strong repressive effects on transcription (Clark-Adams et al. 1988; Kim et al. 1988). Cellular machinery has arisen in evolution to counteract these repressive effects of chromatin (Kingston et al. 1996). Many questions remain as to how chromatin is selectively assembled onto particular sites of DNA and what exact mechanisms are used to regulate this chromatin formation so that

it does not interfere with transcription or DNA replication. Thus, another level of regulation of GR function may be in its ability to interact with this chromatin-regulating machinery which in turn could enable GR to overcome the barriers set upon its activity by the existence of chromatin. Interestingly, GR's interaction with chromatin and at least one of its regulating complexes exists even in yeast. Thus, the notion that what we learn about GR function in yeast can be applied in mammalian cells holds true in this case. The provocative result indicating that GR interacts with yeast host-cell machinery will be discussed in more detail in Chapters 3-5.

The picture that emerges from research over the last 20 years is that the molecular pathways that exist to mediate glucocorticoid-controlled cellular responses are quite complex and potentially could be regulated at several steps. Each step in GR's functional pathway, from hormone-binding to nuclear translocation to interaction with nuclear factors off or on DNA, could possibly be further illuminated by the identification of mutants in my genetic screen. Thus, it is my belief that an unbiased genetic screen could lead to great advances in our knowledge of how GR functions to integrate the hormone signal into the appropriate cellular response. This knowledge in turn could allow us to understand on a more basic, fundamental level how other transcription factors (including the other members of the steroid hormone receptor superfamily) influence gene activity. Likewise, a clearer understanding of the molecular pathways that mediate the glucocorticoid signal eventually may lead to the design of more efficacious therapeutics.

Chapter 1

The Development of an Unbiased Genetic Screen in Yeast to Identify Intracellular Factors Required for Glucocorticoid Receptor-dependent Transcriptional Activation

Abstract

In order to identify some of the cellular factors that interact with the glucocorticoid receptor (GR), an unbiased genetic screen in *Saccharomyces cerevisiae* was developed. A yeast strain expressing GR and harboring an appropriate reporter gene was mutagenized and 4800 survivors were assayed for GR-dependent transcriptional activation. Four mutants displayed recessive, single-gene lesions that abrogated normal GR function. One of these mutant strains (W525), which also displayed a linked Ts^- growth defect at 37°C, was analyzed further.

Introduction

Identification of the intracellular factors that are required for GR function is essential to obtain a clearer picture of how GR mediates the hormone signal. Traditional biochemical analysis has already allowed us to identify some of these factors, such as those described in the Introduction (see also Figure 2). A general genetic screen would be of further assistance in the identification of such factors because literally thousands of mutants could be generated quickly and easily. If the read out of such a screen is simple enough, then these mutants could be quickly analysed and the interesting ones would be kept for further study. Given that GR functions in a hormone-dependent manner in the simple organism *Saccharomyces cerevisiae,* it seemed a logical choice to set up a genetic screen using yeast.

S. cerevisiae, a species of brewer's yeast, has a small, haploid genome (that is now completely sequenced), easy and quick growth conditions, an extensively studied genetic system, the ability to generate numerous mutants

quickly and easily, and typically a convenient method to characterize these mutants. Given that GR appears to function in a very similar manner in yeast as compared to mammalian cells suggests that many steps in GR's signaling pathway are conserved between these systems. Therefore, intracellular factors required for GR function that are identifed in yeast might have direct homologs in higher organisms. Thus, in some ways, especially considering that the yeast genome only encodes for roughly six thousand gene products (Goffeau 1996), we could consider the yeast system as a sort of *in vivo* test tube for studying transcription.

While yeast has been extensively studied for many decades, it was not until the late 1980's that mammalian proteins were expressed in this organism to see if they would function. One of the first proteins tested was GR (Schena and Yamamoto 1988). Yeast transformed with DNA plasmids that express rat GR (referred to as N795) and carry a *lacZ* reporter gene linked to three GREs from the tyrosine aminotransferase gene (TAT3-GRE) display stable GR expression and hormone-dependent β-galactosidase (the product of *lacZ*) expression. Strains of yeast expressing constitutive forms of GR (such as N556, which lacks the carboxy-terminal ligand-binding domain) and the same TAT3-GRE-*lacZ* reporter gene also display β-galactosidase expression in the absence of hormone, as observed in mammalian cells. This result indicates that the transcriptional activation domain in the amino-terminus of GR (enh2 - see Figure 3 in the Introduction) is also sufficient for activating gene expression in yeast from a TAT3-GRE-linked reporter gene, as it is in mammalian cells. This last result further points to the relative conservation of the GR signaling pathway in these two cell types.

Exploiting the conservation of GR activity in yeast from a TAT3-GRE-linked reporter, I have developed a general genetic screen to study GR

function. I report in this chapter the development of such a screen and its initial results.

Materials and methods

Yeast strains and techniques

The yeast strains used in this chapter are described in Table 1-1. Standard yeast techniques, such as yeast transformation, sporulation, tetrad dissection, and preparation of yeast media, are as previously reported (Gietz et al. 1992; Sherman 1991; Sherman and Hicks 1991). All incubations of yeast cultures were at 30°C, unless otherwise noted.

Plasmids

The GR expression plasmid pTCA-GN795 consists of the sequence from pGN795 (Yoshinaga et al. 1992) between the *Hin*dIII and *Ngo*MI sites, which contain the *GPD* promoter, the GR coding sequence, and the *PGK* terminator sequence, cloned into the same sites of pRS314 (Sikorski and Heiter 1989). The reporter gene plasmid, pHCA-G3Z was constructed by moving the TAT3-GRE-*lac*Z reporter gene from pΔS26x (Yoshinaga et al. 1992) into pRS313.

Screening for GR activation mutants

The yeast strain WG3Z7C was cultured in 5 ml SD-his-trp media and grown until the culture was saturated. The culture was subcultured 1:5 in 5 ml fresh SD-his-trp media and grown to OD_{600} = 0.8. The cells were pelleted by

Table 1-1. *Yeast strains*

Strain		Relevant Genotype	Source
W303-1A	*MATa*,	*trp1-1 ura3-1 leu2-3,112 his3-11,15*	A. Tzagoloff
W303-1B	*MATα*,	*trp1-1 ura3-1 leu2-3,112 his3-11,15*	A. Tzagoloff
WG3Z7C[a]	*MATa*,	*trp1-1 ura3-1 leu2-3,112 his3-11,15* [pTCA-GN795] [pHCA-G3Z]	this study
WPCWL	*MAT a/α*	a result of a cross between WG3Z7C and W303-1B harboring pRS315 (a *CEN4*, *LEU2*-marked plasmid)	this study

[a] W303-1A strain background

centrifugation and resuspended in 1 ml PBS; 20 µl ethyl methanesulfonate (EMS; Sigma) was added, and the cell suspension was incubated for 60 min to give 50% killing. The EMS was neutralized by pelleting the cells, resuspending the pellet in 1 ml 1% sodium thiosulfate (w/v with dH_2O), and washing 3 more times in PBS. The cells were pelleted once more and resuspended in 5 ml YPD and allowed to grow 2 hr before being diluted and plated onto SD-his-trp plates to a density of 100-200 colonies/plate. The plates were incubated at room temperature until the colonies were 2-3 mm in size. The colonies were then replica plated via velvets onto SD-his-trp + 10 µM deoxycorticosterone and allowed to grow at 30°C for 20 hr. Plate β-galactosidase assays were performed on these plates by filter lifts and those colonies that remained white were picked from the original master plate and patched for further study onto a new SD-his-trp plate. Liquid β-galactosidase assays were performed as previously described (Garabedian 1993), except that hormone treatment was usually for six hr and sodium dodecylsulfate was not added to the cells during the permeabilization step.

Results

Strain construction

I chose to work with the wild-type haploid strain W303-1A because of the availabilty of several auxotrophic mutations in the strain (see Table 1-1), its high transformation efficiency, its good growth rate, and the fact that the strain reproducibly displayed high levels of GR-dependent expression from a GRE-linked reporter gene.

I transformed W303-1A with two plasmids: 1) pTCA-GN795, a centromeric expression plasmid directing the expression of full-length rat GR from the yeast glyceraldehyde-3-phosphate dehydrogenase (*GPD*) promoter; 2) pHCA-G3Z, a centromeric reporter plasmid containing the *lac Z* gene linked to three TAT-GREs upstream of the minimal yeast cytochrome C 1 (*CYC1*) promoter from yeast. The resulting strain containing these two plasmids was denoted WG3Z7C.

Mutagenesis and screening

As shown in Fig. 1-1, I mutagenized approximately 2 x 10^8 cells of the yeast strain WG3Z7C with ethane methylsulfonate, treated approximately 4800 survivors with 10 μM deoxycorticosterone (DOC) (Garabedian and Yamamoto, 1992), and assessed GR-dependent activation with β-galactosidase plate assays. Colonies with diminished levels of β-galactosidase activity in plate assays were subsequently tested for β-galactosidase levels in liquid cultures. Thirteen mutants were isolated that produced β-galactosidase activity at levels at least four-fold below those displayed by the wild type parent strain.

Initial characterization of mutants

Figure 1-2 displays the results of the β-galactosidase liquid assays for these thirteen mutants. To determine whether the mutants were recessive or dominant to wild type, I crossed the mutant strains to the isogenic parent strain W303-1B and tested the resulting heterozygous diploids for β-galactosidase activity in liquid culture assays (Figure 1-3). Eleven of these

Figure 1-1. The scheme used to identify mutations in yeast that abrogate normal glucocorticoid receptor function.

The yeast strain WG3Z7C (containing a rat glucocorticoid receptor (GR) expression plasmid and a TAT3-GRE reporter plasmid) displays blue colonies in the presence of hormone, which indicates the hormone-dependent activation of the TAT3-GRE-linked reporter gene. This strain was mutagenized with ethane methylsulfonate (EMS) and the survivors assayed for β-galactosidase production in the presence of hormone. Those survivors that carry mutations that do not abrogate GR function will still appear blue in the presence of hormone (indicated by a single asterisk within the cell). Survivors that carry mutations that abrogate GR function will appear white in the presence of hormone (indicated by two asterisks within the cell). The white colonies were picked for further study and mated to an unmutagenized parental strain that lacks both the GR expression and the TAT3-GRE-linked reporter plasmids. The resulting diploids were then tested for β-galactosidase in the presence of hormone to determine if the mutation resides in a yeast gene. The mutant strains that displayed mutations in yeast genes and were also recessive were picked for further study.

Scheme to Identify Mutations in Yeast that Abrogate GR Function

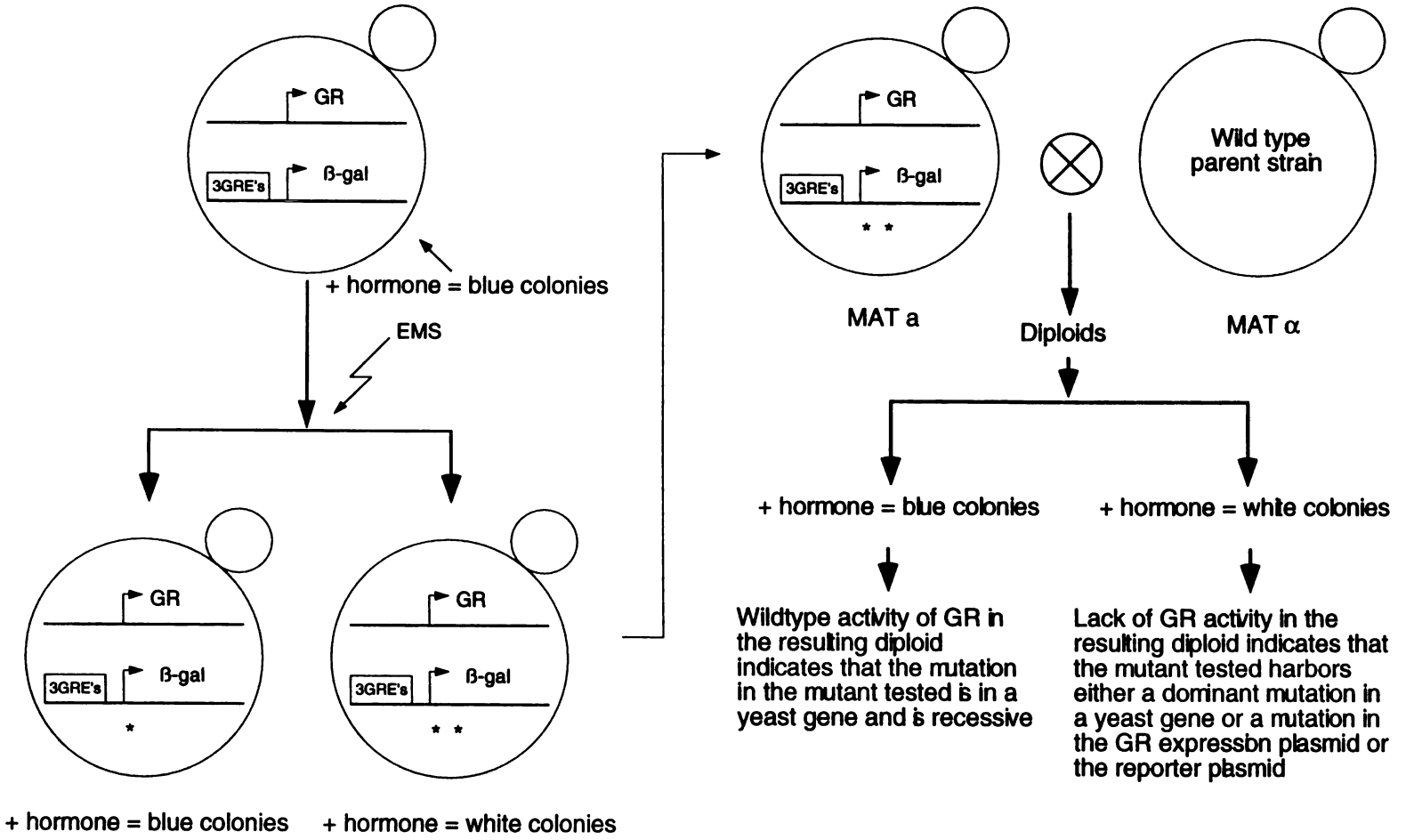


Figure 1-2. β-galactosidase activity of 13 mutants isolated in the genetic screen to isolate mutants defective in GR function.

The β-galactosidase activities of 13 mutant strains that displayed white or light blue color in the initial plate assay after mutagenesis were remeasured by liquid β-galactosidase assays, as described in Methods and materials. The strains tested were arbitrarily assigned a strain number and are indicated below each column. The activity of the unmutagenized, wild type strain (WG3Z7C) is indicated in the leftmost column. Mutagenized strains that displayed β-galactosidase activity at 25% of that displayed in wild type cells were considered mutant and chosen for further study. This level was arbitrarily chosen.

β-galactosidase Activity of 13 Mutants Defective in GR Function

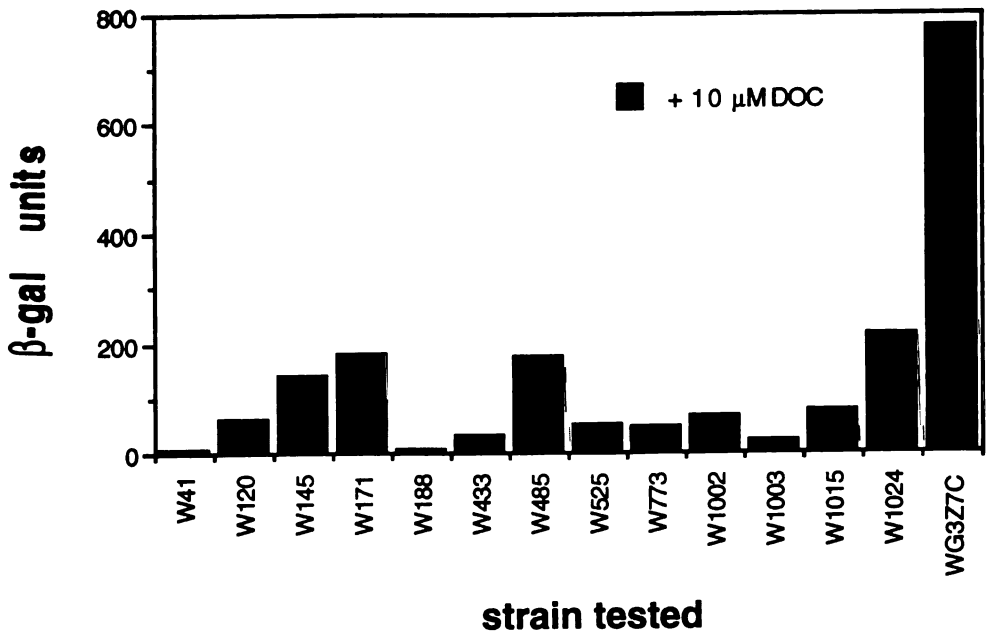
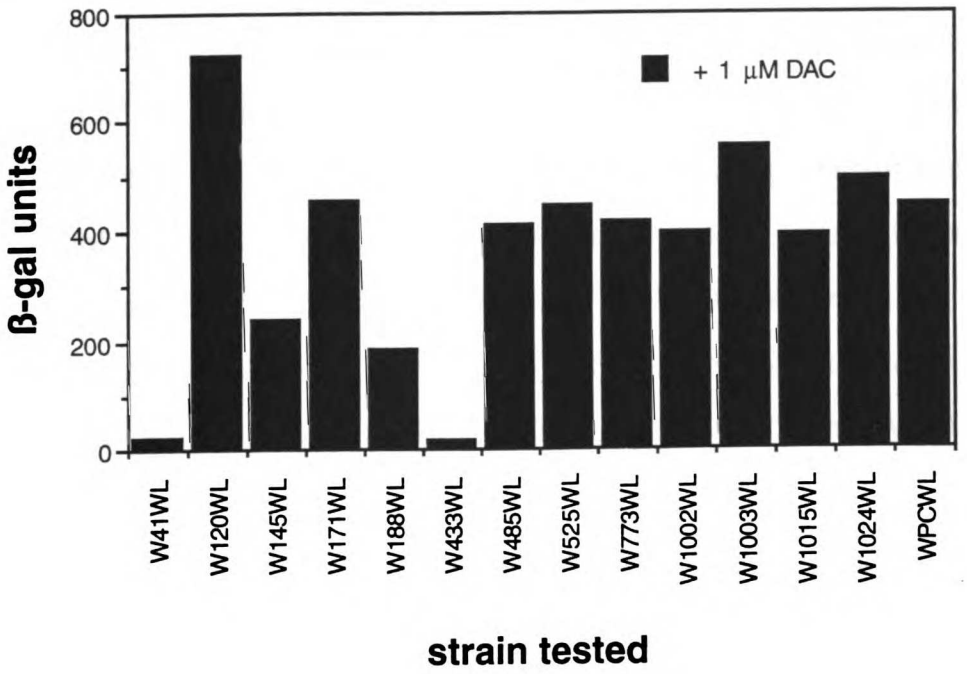


Figure 1-3. β-galactosidase activity of 13 heterozygous mutants.

The 13 haploid mutant strains isolated in the genetic screen were backcrossed to an unmutagenized wild type parent strain (W303-1B) lacking the GR expression vector and the TAT3-GRE linked reporter gene. The β-galactosidase activities of the resulting heterozygous diploids were assayed by liquid cultures, as described in the Methods and materials. Deacylcortivazol at a concentration of 1 μM was used as the homone agonist. Cells were treated for 24 hr at 30°C. The unmutagenized wild type strain (WG3Z7C) was also backcrossed to W303-1B to develop a control diploid strain and was labeled WPCWL.

β–galactosidase Activity of 13 Heterozygous Diploids



diploids displayed wild type β-galactosidase activity, indicating that they harbored recessive mutations that lead to a defect in GR function. The two additional diploids were not studied further because one harbored a mutation in the GR expression plasmid and the other a dominant mutation that abrogates GR function and additionally interferes with respiration as determined by a lack of growth on a nonfermentable carbon source (glycerol).

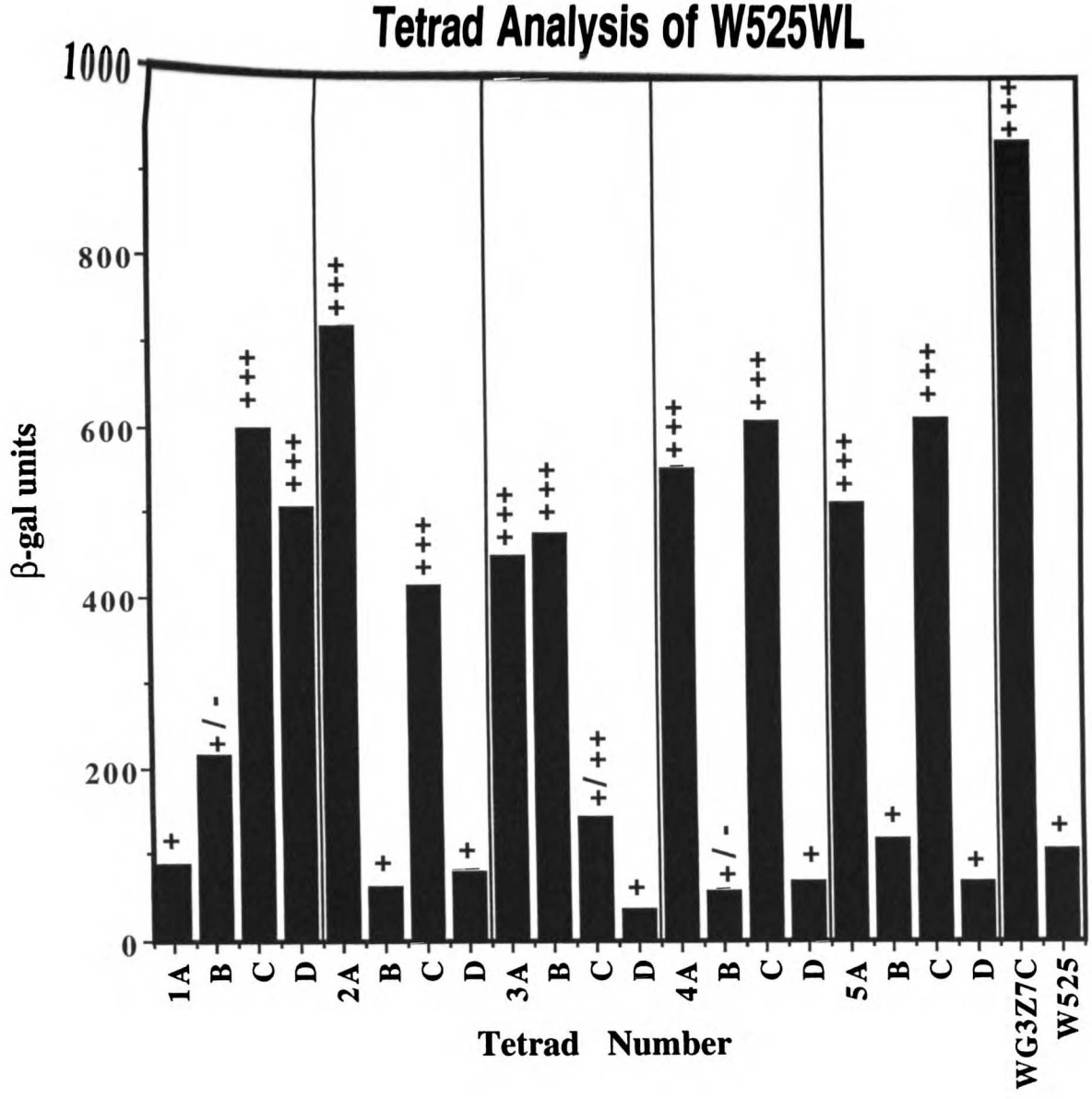
The observed defect in GR-dependent activation was not limited to a specific agonist, as all eleven of the recessive mutants displayed diminished levels of GR activation with 1 μM deacylcortivazol (DAC) (data not shown). However, only seven mutants produced β-galactosidase at levels at least four-fold below those observed with wild type cells for both hormones, and four of those seven displayed segregation patterns in subsequent tetrad analysis consistent with single gene mutations: W120, W188, W525, and W1002. W525 also displayed a temperature-sensitive growth phenotype (Ts^-) that was tightly linked to the lack of GR activation (see Figure 1-4). Figure 1-5 summarizes the initial results of the screen.

Discussion

The WG3Z7C strain expresses full-length GR from a constitutive promoter on a low-copy plasmid and carries a TAT3-GRE-linked *lacZ* reporter gene also on a low-copy plasmid. These low-copy plasmids kept GR activity in a range in which I could detect small, but perhaps significant, changes in its activity. Reporter gene activity was first monitored in a plate assay, then retested in liquid cultures to quantitate β-galactosidase activity. The threshold

Figure 1-4. Tetrad analysis of the heterozygous mutant strain W525WL

The heterozygous mutant strain W525WL was sporulated, and the resulting 4-spored tetrads were tested for β-galactosidase activity at 30°C in the presence of 10 μM DOC, as described in the Methods and materials. Also, each spore of each tetrad was tested for growth at 37°C on solid rich medium containing 2% glucose. β-galactosidase activities and growth at 37°C were also tested in parallel of the wild type strain WG3Z7C and the haploid mutant strain W525. The black bars represent the level of β-galactosidase produced by each strain and the level of growth at 37°C is indicated as follows: "+++" = wild type growth (Ts^+), while "+/-", "+", "+/++" = varying levels of poor growth (Ts^-). Note that poor growth always correlates with relatively low levels of β-galactosidase activity and that good growth always correlates with relatively high levels of β-galactosidase activity. These results indicate that the Ts^- growth defect is linked to the lack of GR transcriptional activation.



Results of Genetic Screen in Yeast to Identify Intracellular Factors Required for GR Activation

1) After mutagenesis, 4800 survivors were screened for reduced GR activation from a GRE-*lacZ* reporter gene

2) 13 mutants were identified that were at least 4-fold lower for β-gal activity as compared to the wild type parent

3) 4 of these mutants displayed a mutation in a single gene
   - W120
   - W188
   - W525, which also has a linked Ts-growth defect @ 37°C
   - W1002

of four-fold loss in activity, as compared to the unmutagenized wild-type parent, was established arbitrarily.

The mutant strains so identified were then assayed for a recessive or dominant phenotype by crossing them to an isogenic wild-type parent strain (W303-1B) that lacked the GR expression plasmid and the TAT3-GRE-linked reporter gene plasmid. The identified recessive mutants were then tested with a second agonist to determine whether the mutation was hormone-specific. All of the recessive mutants displayed at least a two-fold loss in β-galactosidase activity when assayed with either 10 μM DOC or 1 μM DAC, but only seven displayed at least a four-fold loss in β-galactosidase with both hormones. I chose to study further those that showed defects with both hormones. These mutants were then subjected to tetrad analysis to determine if the recessive mutation was due to a defect in a single gene. If so, then complementation by a genomic library could be performed and, in this way, the gene harboring the mutation could be identified.

Dominant mutations, while potentially interesting, are more difficult to pursue. The gene harboring the mutation cannot be cloned by complementation with a wild type genomic library. Instead a library must be developed from the mutant strain and transformed into a wild type strain. The resulting transformants are then screened for the mutant phenotype. The single dominant mutant recovered in my screen also displayed a dominant, lack-of-respiration phenotype as determined by a lack of growth on glycerol. As respiration is required for sporulation, tetrad analysis could not be performed on this strain. This mutant was not studied further.

The results of my screen are presented in Figure 1-5. Four strains displayed a single-gene mutant phenotype, and one (W525) also had a

Chapter 2

Genetic Analysis of Mutant Strain W525

Abstract

The mutant yeast strain W525 carries a Ts$^-$ growth defect associated with a lack of GR function. Complementation of the growth defect with a yeast genomic library revealed *YNR023w*. Contemporaneously, others had shown that mutations in this gene relieved the toxicity of E1A expression in yeast and that biochemical purification of the yeast Swi/Snf complex revealed the 73 kDa polypeptide in this preparation (Swp73p) was encoded by *YNR023w*. Hence the gene was renamed *SWP73*. The mutant allele of *SWP73* in W525 was shown to contain a single point mutation causing a glycine to aspartic acid change at amino acid position 323; thus, W525 was renamed Wswp73-1. In addition, an isogenic yeast strain was constructed that harbored a genomic deletion of *SWP73* (Wswp73Δ). Typically Wswp73-1 displayed intermediate phenotypes for growth relative to an isogenic wild type strain (W303-1A) and Wswp73Δ. Therefore, swp73-1p appears to retain partial functional capacity.

Introduction

Mutant strain W525 was picked for further characterization for two reasons. First, the strong Ts$^-$ growth defect in W525 was associated with the lack of GR function in this strain (see Figure 1-4). This phenotype would allow me to identify the gene harboring the mutation in this strain via a selection scheme rather than a screen, which is simpler and faster. A selection scheme can be carried out because after transforming the mutant

strain with a yeast genomic library, the resulting transformants can be plated on the appropriate selection plates and incubated at 37°C. Only those colonies that form at this non-permissive temperature are likely to carry a complementing plasmid from the library. The plasmid DNA from these colonies can then be isolated and sequenced to determine the genes that are present. After the appropriate cloning procedures, the gene harboring the mutation in the mutant strain can be identified.

Second, the Ts^- growth defect of W525 suggested that the mutation lies in a gene that is important for cell function and not just for GR activity. Thus, information gleaned from the examination of W525 potentially could have an impact on several fields of study in biology.

Materials and methods

Yeast strains and techniques

The yeast strains used in this chapter are described in Table 2-1. Standard yeast techniques, such as yeast transformation, sporulation, tetrad dissection, and preparation of yeast media, are as previously reported (Gietz et al. 1992; Sherman 1991; Sherman and Hicks 1991). All incubations of yeast cultures were at 30°C, unless otherwise noted.

Cloning of SWP73

Table 2-1. *Yeast strains*

Strain	Relevant Genotype	Source
W303	*MATa/α, trp1-1/trp1-1 ura3-1/ura3-1 leu2-3,112/leu2-3,112 his3-11,15/his3-11,15*	A. Tzagoloff
W303-1A	*MATa, trp1-1 ura3-1 leu2-3,112 his3-11,15*	A. Tzagoloff
W303-73Δ[a]	*MATa/α, trp1-1/trp1-1 ura3-1/ura3-1 leu2-3,112/leu2-3,112 his3-11,15/his3-11,15 swp73::LEU2/SWP73*	this study
Wswp73-1[b]	*MATa, trp1-1 ura3-1 leu2-3,112 his3-11,15 swp73-1*	this study
Wswp73Δ[c]	*MATa, trp1-1 ura3-1 leu2-3,112 his3-11,15 swp73::LEU2*	this study
YPH499	*MATa, trp1Δ1 ura3-52 leu2-Δ1 his3-Δ200*	P. Hieter
Yswp73Δ[d]	*MATa, trp1Δ1 ura3-52 leu2-Δ1 his3-Δ200 swp73::LEU2*	this study

[a]W303 strain background (Thomas and Rothstein 1989)
[b]W303-1A strain background
[c]Derived from tetrad dissection of a W303 derivative
[d]YPH499 background

The yeast strain Wswp73-1 was transformed with a YEp351-based yeast genomic library (a gift from Jeanne Hirsch; Engebrecht et al. 1990). After transformation, the plated cells (approximately 500 cells/plate) were incubated at 37°C for 5 days. Plasmid linkage was determined by growing the surviving colony in non-selective media until leucine auxotrophs were recovered. The recovered leucine auxotrophs were then grown at 37°C to determine if they displayed a growth defect. Genomic library DNA was recovered using the Magic Mini-prep kit (Promega) according to the manufacturer's instructions, except that the lysis buffer was adjusted to a final concentration of 3% sodium dodecylsulfate and 0.2 M NaOH. The DNA was recovered in 50 µl of water and 2 µl of this solution was used to transform bacteria via electroporation. After amplification in bacteria, the DNA was recovered and transformed into Wswp73-1. The transformed cells were fully complemented for their growth and GR activation defects.

DNA sequencing and sequence analysis

Double-stranded plasmid templates were prepared and sequenced using the Sequenase 2.0 kit (U.S. Biochemical). The sequencing reactions were performed according to the technique of Del Sal et al. (1989). Terminated fragments were labeled by incorporation of [a-^{35}S]dATP (NEN), resolved on 6% acrylamide gels, and autoradiographed on Kodak X-Omat AR film. Sequencing of the genomic *SWP73* clone was performed using primers that hybridize to the polylinker of YEp351. Sequences internal to the 4.5 kilobase genomic insert were determined by making a deletion of the sequence between the *Hin*dIII site of *SWP73* and the *Hin*dIII in the polylinker

of the genomic clone. This deletion allowed the use of the downstream primer to sequence the middle of the insert.

The predicted protein sequence of *SWP73* was analyzed and homology searches were performed using the GCG software package from the Genetics Computer Group (Madison, WI).

Allele mapping of Wswp73-1

The DNA plasmid pUCA-SWP73 was incubated with *Bam*HI and *Cla*I or with *Cla*I and *Hin*dIII. The resulting linear plasmids were transformed into Wswp73-1 and uracil prototrophs were selected for by growth on SD-ura plates. The resulting transformants were then tested for growth at 37°C on YPD plates. Only four of eight transformants using the *Bam*HI-*Cla*I digested DNA displayed a temperature sensitive growth defect (Ts^-), whereas all eight transformants using the *Cla*I-*Hin*dIII digested DNA were Ts^-. This result implied that the mutation in *SWP73* lay somewhere between the *Cla*I and *Hin*dIII sites. The following primers were synthesized to sequence the region between the *Cla*I and *Hin*dIII sites in the gap-repaired plasmid pUCA-swp73(AR): 5'-GGTCGATTATTGGACAAT-3' (SWP73-1), 5'-CAGCAGCCCCAATTTGAC-3' (SWP73-2), 5'-GTGAAATGGCAGTATGACCCG-3' (SWP73-5). Sequencing revealed a single guanine to adenine change at nucleotide position 968 of the *SWP73* open reading frame that results in a glycine codon to aspartic acid codon change at amino acid position 323.

Site-directed mutagenesis of SWP73

The following primers were synthesized and used to mutagenize pUCA-SWP73: 5'-GGAAAATATTGCATCATGTGATGT**ATC**GCTCTTCAATCC-3' (SWP73-D), 5'-GGAAAATATTGCATCATGTGATGT**TTT**GCTCTTCAATCC-3' (SWP73-K), 5'-GGAAAATATTGCATCATGTGATGT**AAG**GCTCTTCAATCC-3' (SWP73-L). The position of the mutant codons are highlighted in bold-face. Mutagenesis was performed using the Chameleon Double-Stranded, Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer's instructions. Sequencing, using the SWP73-5 primer, was done to confirm the mutagenesis. The plasmids recovered by mutagenesis were labeled: pUCA-swp73(G323D), pUCA-swp73(G323K), and pUCA-swp73(G323L).

Construction of SWP73 *deletion strain*

The coding sequence of the LEU2 gene from pUC8-LEU2 (Sikorski and Heiter 1989) was excised with *Bam*HI and *Hin*dIII and used to replace the intervening sequences between the *Bgl*II and *Hin*dIII sites in the genomic clone of *SWP73*. The resulting plasmid, pRL100, was digested with *Msc*I and *Bsp*EI and ~150 ng of the digested DNA was transformed into W303 by electroporation (Becker and Guarente 1991). Eight transformants were selected, genomic DNA was isolated (Hoffman 1993), and a Southern analysis was performed (Brown 1993). One of the eight transformed diploids showed a proper integration of the *swp73::LEU2* knockout gene, such that one

chromosomal copy of *SWP73* was replaced with *LEU2* and the other chromosomal copy was wild type. The proper integration event was later confirmed when the diploid was sporulated and tetrad dissected. In each of the five four spore tetrads analyzed the Leu^- phenotype co-segregated with the Ts^+ phenotype and the Leu^+ phenotype co-segregated with the Ts^- phenotype. In addition, ten 3-spore tetrads were analyzed and in five cases the spore that failed to grow would have been Leu^+ and in the other five cases the spore that failed to grow would have been Leu^-. An additional strain lacking *SWP73* (Yswp73Δ) in the S288C genetic background was prepared in the same manner by a one-step gene replacement with pRL100 and the haploid parent strain YPH499.

Results

Identification of the mutant gene responsible for the phenotype of W525

To identify the gene responsible for the mutant phenotype in W525, I complemented the Ts^- growth phenotype by transforming W525 with a high-copy yeast genomic DNA library and examined 20,000 transformants for growth at 37°C. A single colony was isolated that grew as well as the wild-type parent strain under these conditions. Liquid β-galactosidase assays on this isolated transformant revealed GR activity at levels similar to those observed with the wild-type parent strain. Complementation of both the Ts^- phenotype and the GR-dependent transcriptional defect was conferred by the plasmid

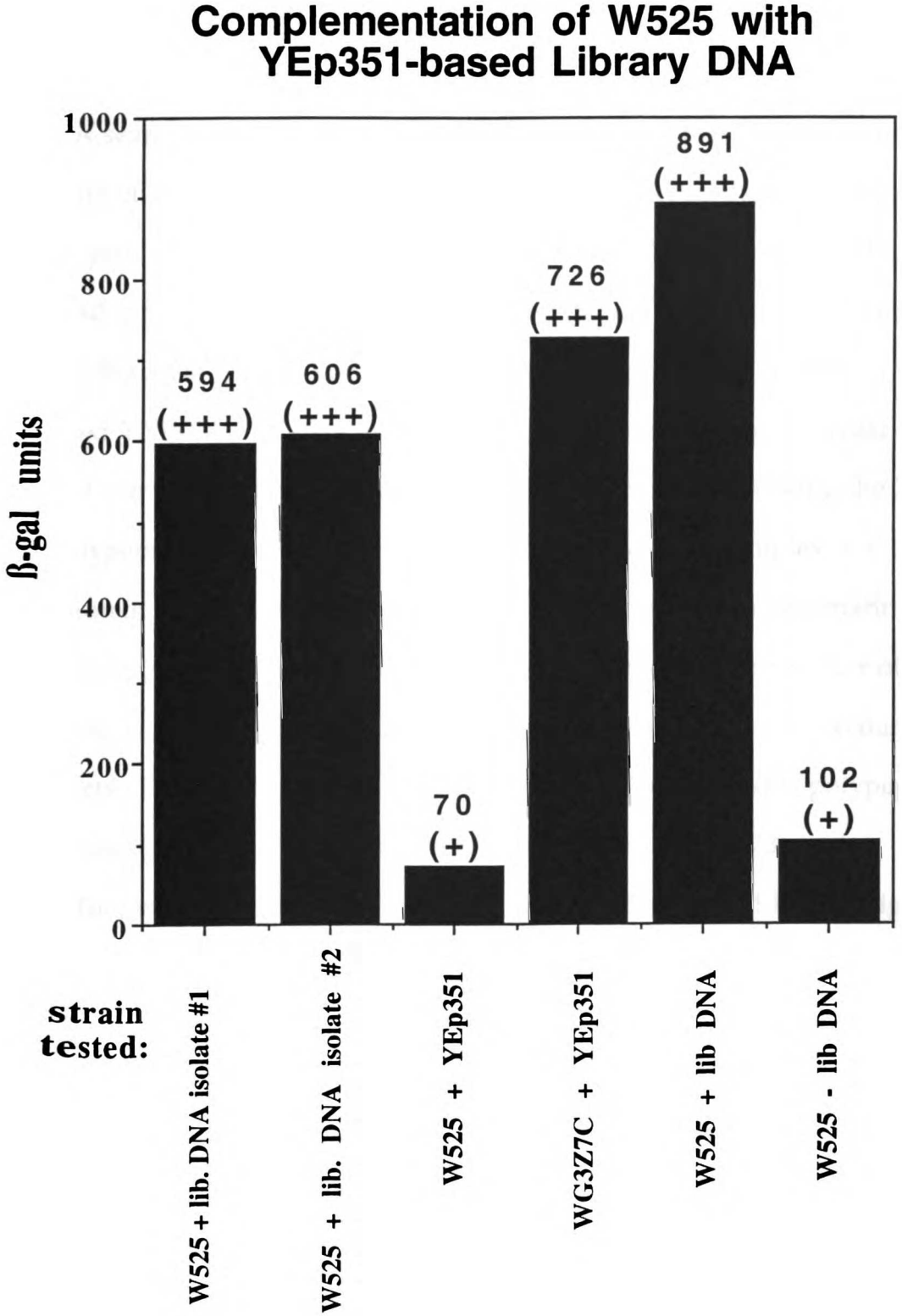
(see Material and methods). The results of this complementation are shown in Figure 2-1.

I isolated the complementing plasmid and partially sequenced the genomic library insert. A search of the Protein Information Resource (PIR) database revealed that the DNA insert contained a previously identified open reading frame, which was denoted *YNR023w* in the yeast genome sequencing project. *YNR023w* encodes a gene product that complements the Tpy1$^-$ mutant phenotype, which is defective for growth on pyruvic acid (a non-fermentable carbon source) but still allows growth on ethanol (another non-fermentable carbon source). Thus, Tpy1$^-$ is not a true petite phenotype. Rather, it was hypothesized that the Tpy1$^-$ mutant phenotype may reflect a defect in pyruvate transport across the mitochondrial membrane. How GR function might be affected by a pyruvate transport defect was unknown. Unfortunately, attempts to contact D.E. Griffiths (Univ. of Warwick, Coventry, UK), who deposited *YNR023w* into GenBank, proved futile. Subsequent discussions with Christopher Wills (UC, San Diego), who originally isolated and described the Tpy1$^-$ mutant phenotype (Wills et al. 1986), were not illuminating.

Contemporaneous with my work, *YNR023w* was also recloned by Mary Miller and M. Mitchell Smith (Univ. of VA). Miller and Smith had discovered that the expression in yeast of the adenovirus transcriptional activator, E1A was toxic or growth inhibitory, depending on the yeast strain in which it is expressed (Miller et al. 1995). Their screen for mutations that relieved this toxicity identified *YNR023w* (Miller et al. 1996). Thus, the hypothesis of a defect in pyruvate transport aside, *YNR023w* had been isolated

Figure 2-1. Complementation of W525 with YEp351-based library DNA.

The mutant strain W525 was transformed with a *LEU2*-marked yeast, genomic DNA library and plated at 37°C to select for complementation of the Ts^- growth phenotype. A single transformant was isolated that grew indistinguishably from wild type. The plasmid DNA harbored in this strain was isolated and two independent isolates of the *LEU2*-marked library plasmid were retransformed into W525. These transformants were tested for β-galactosidase production in the presence of 10 μM DOC and growth on solid, rich media + 2% glucose at 37°C. The levels of β-galactosidase units produced are indicated above each column and the level of growth at 37°C is indicated in parentheses, where "+++" = good growth and "+" = poor growth. The strain originally isolated after transformation with the genomic DNA library and selection at 37°C is denoted "W525 + lib DNA". The transformants tested after retransformation with the two independent isolates of the library DNA recovered from the strain denoted "W525 + lib DNA" are denoted, respectively, as "W525 + lib. DNA isolate #1" and "W525 + lib. DNA isolate #2". As a control, the mutant strain W525 and the wild type strain WG3Z7C were transformed with YEp351 (the parental vector of the genomic DNA library) and denoted as "W525 + YEp351" and "WG3Z7C + YEp351", respectively. The mutant strain W525 without a library plasmid or YEp351 is denoted as "W525 - lib DNA"



in two different screens looking for defects in the activity of two different transcriptional activators.

A search of the PIR database revealed a mouse open reading frame (Johnston et al. 1989) with significant sequence identity to the predicted protein product of *YNR023w*. Leslie Kozak (Jackson Laboratory, Bar Harbor, ME), had informed me that he did not know the function of the mouse gene, but that Brad Cairns and Roger Kornberg (Stanford) had also recently identified *YNR023w*. Cairns and Kornberg were investigating the yeast Swi/Snf complex and had identified *YNR023w* by microsequencing the 73-kDa polypeptide from the isolated complex. The Swi/Snf complex is a multiprotein complex that is hypothesized to be involved in chromatin remodeling (see the Introduction of Chapter 3). If the protein product of *YNR023w* is indeed in the Swi/Snf complex, this situation might account for the effects of the W525 mutation on GR function. As the 73-kDa polypeptide was denoted Swp73p, the *YNR023w* gene was renamed *SWP73*.

To ensure that the complementation of W525 observed by the high-copy yeast genomic library DNA was not the result of an elevated dosage of Swp73p, I subcloned the *SWP73* gene into a centromeric vector, creating pUCA-SWP73. Strain W525 harboring pUCA-SWP73 did not display the growth defects observed with strain W525 harboring a control plasmid (pRS316), demonstrating that the level of Swp73p produced with a centromeric plasmid is sufficient for complementation (see Figure 2-1).

Mutations at amino acid position 323 of SWP73 confer a Ts- phenotype

To establish further whether a mutation in the *SWP73* gene was involved in the mutant phenotype of W525, I mapped the allele of *swp73* in W525 by gap repair (Rothstein 1991). Recovery and sequencing of the gap-repaired plasmid, pUCA-swp73(AR), revealed a single base pair mutation resulting in the substitution of aspartic acid for glycine at amino acid position 323 (Swp73p is 566 amino acids in length). To determine whether the G323D change is the cause of the phenotype in W525, I prepared this mutation by performing site-directed mutagenesis on pUCA-SWP73. The resulting plasmid [pUCA-swp73(G323D)] failed to support the growth of W525 at 37°C and GR function was diminished. Additional mutations at this position (G323K and G323L) behaved identically to G323D, indicating that the mutant phenotype is most likely due to a change in the size of the amino acid residue at position 323 rather than a change in charge. With the mapping of the allele, denoted *swp73-1*, and the confirmation that a mutation in *SWP73* was involved in the mutant phenotype of W525, the mutant strain was renamed Wswp73-1.

Phenotypes displayed by swp73Δ strains are similar to those displayed by Wswp73-1, but of greater penetrance

To examine the null phenotype of *SWP73*, I performed a single-step gene replacement with the *swp73::LEU2* disruption plasmid pRL100 in the diploid strain W303 (designated W303-73Δ; see Table 2-1). Tetrad analysis of the resulting heterozygous diploid revealed that *SWP73* is not an essential

gene. However, in each of the tetrads tested, the two *swp73::LEU2* displayed a Ts^- growth phenotype at 37°C and displayed little or no GR-dependent activation (Figure 2-2). The *swp73Δ::LEU2* strain (designated Wswp73Δ) derived from W303-73Δ also had a longer generation time than did W303-1A at 24°C (4.3 hrs and 2.0 hrs for the two strains, respectively) and at 30°C (5.3 hrs and 1.6 hrs, respectively). The Ts^- phenotype was not limited to W303-derived swp73 mutants, as Yswp73Δ (YPH499 parent, S288C background) was also Ts^-.

Wswp73-1 cells displayed an intermediate phenotype that resembled the deletion mutant (Wswp73Δ) more than the wild-type strain (W303-1A), requiring seven days to form small colonies at 37°C. At 24°C and 30°C, however, Wswp73-1 closely resembled W303-1A (they have similar generation times). The greater growth capacity of Wswp73-1 cells at intermediate temperatures may indicate that swp73-1p is partially functional in these cells. However, immunoblot analysis revealed that the level of swp73-1p in Wswp73-1 cells was about 10-fold less than that of wild-type protein in Wswp73-1 cells harboring pUCA-SWP73 or in W303-1A, raising the possibility that a reduced amount of protein might account for the mutant phenotype.

To test whether the phenotypic difference between Wswp73-1 and wild-type cells was attributable entirely to the single amino acid substitution in the *swp73-1* allele, Wswp73Δ was transformed with pUCA-SWP73 and with one of the three mutant derivatives (G323D, G323K, G323L). The growth of these transformants then was examined at 37°C (see Figure 2-3). Each of the plasmids bearing site-directed mutations in *SWP73*, as well as the plasmid

Figure 2-2. Tetrad analysis of W303-73Δ.

Swp73p is required for growth at 37°C and for transcriptional activation by the glucocorticoid receptor. Two four-spore tetrads derived from sporulation of the heterozygous *swp73::LEU2/SWP73* strain, W303-73Δ, were dissected, and the phenotypes of the resulting eight haploid strains were determined. The four spores derived from tetrad 1 were designated 1A, 1B, 1C, and 1D and those from tetrad 2 designated 2A, 2B, 2C, and 2D. To assess transcriptional activation by the glucocorticoid receptor, the strains were transformed with pTCA-GN795 (which directs expression of full-length GR) and pHCA-G3Z (a centromeric plasmid with three glucocorticoid response elements upstream of a *CYC1-lacZ* fusion gene). Cultures were treated with 10 μM deoxycorticosterone for 6 hr, and β-galactosidase assays were performed as described. The units determined are indicated above each column. All strains were tested for growth at 37°C on solid rich medium containing 2% glucose, and for leucine auxotrophy at 30°C on solid minimal medium containing 2% glucose but lacking leucine. In all strains derived from these tetrads the Leu^+ and Ts^- phenotypes segregated with the defect in GR-mediated transcriptional activation.

Tetrad Analysis of W303-73Δ

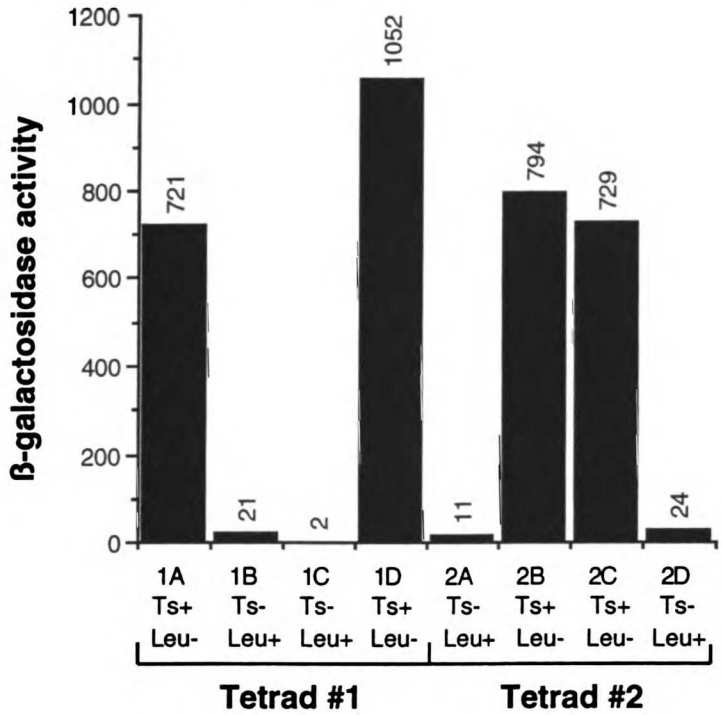
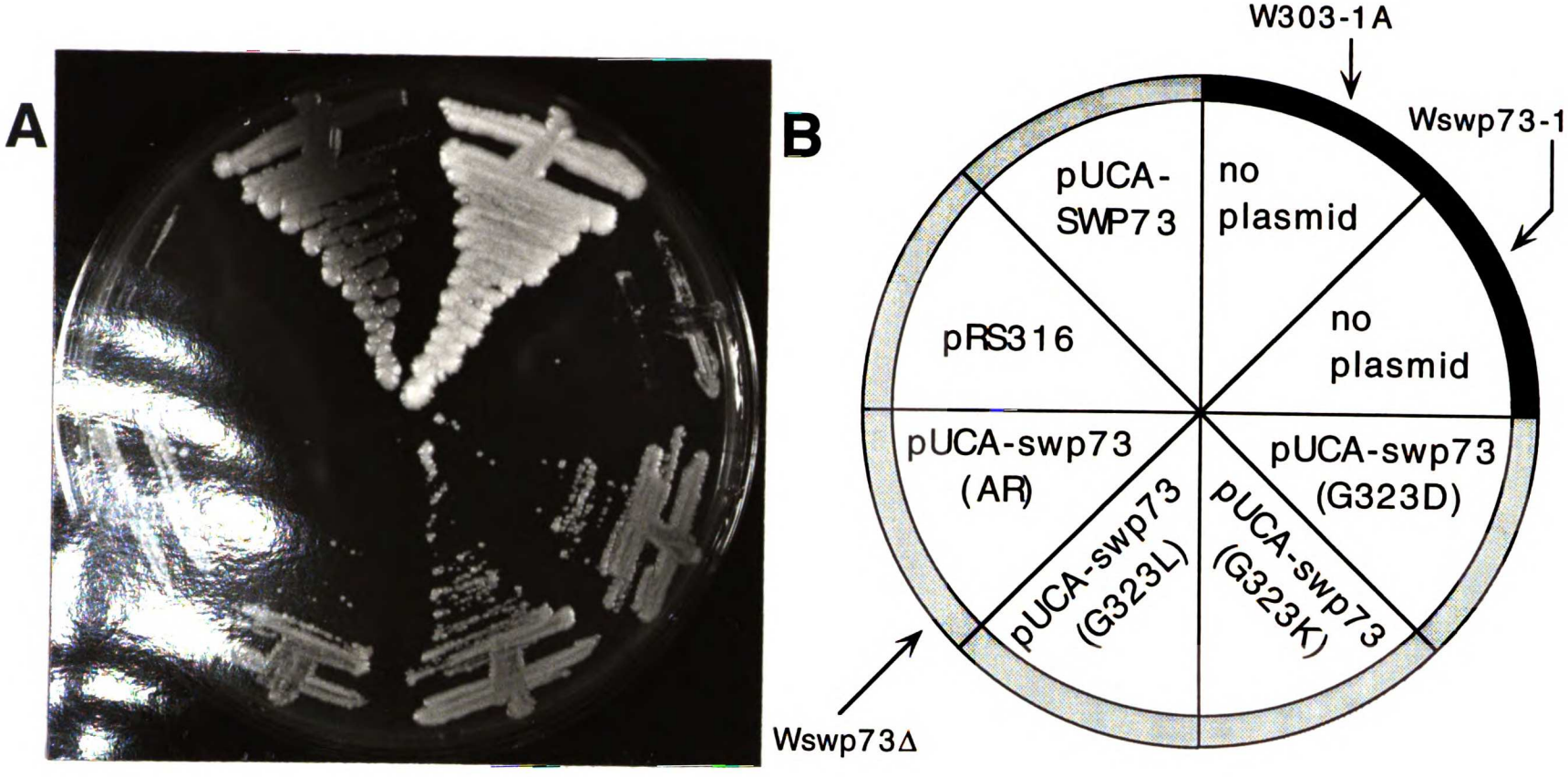


Figure 2-3. Growth ability of strains at 37°C.

The growth ability of various strains were tested on solid, rich media at 37°C. *(A)* Growth ability on YEPD at 37°C. *(B)* Schematic diagram illustrating which strains were tested. The strains tested are indicated with an arrow. The black arc surrounds W303-1A, the dark gray arc surrounds Wswp73-1, and the light gray arc surrounds Wswp73Δ. The plasmids that they harbored, if any, are indicated inside the diagram. The plasmid pUCA-SWP73 expressed wild type Swp73p. As described in Methods and materials, the plasmids pUCA-swp73(G323D), pUCA-swp73(G323K), and pUCA-swp73(G323L) expressed the corresponding site-directed mutant of swp73p, while pUCA-swp73(AR) was the plasmid recovered by allele rescue from Wswp73-1. The plasmid pRS316 was the parent vector for the other plasmids tested and acted as a control vector in this experiment.


A
B
W303-1A
Wswp73-1
pUCA-
SWP73
no
plasmid
pRS316
no
plasmid
pUCA-swp73
(AR)
pUCA-swp73
(G323D)
pUCA-swp73
(G323L)
pUCA-swp73
(G323K)
Wswp73Δ

recovered by the allele rescue from Wswp73-1, was able to complement partially the Ts$^-$ phenotype of Wswp73Δ, resulting in a growth behavior like that of Wswp73-1, including similar growth rates at 24°C and 30°C. Wswp73Δ harboring the mutant plasmids grew somewhat better the Wswp73-1 at 37°C but not nearly as well as Wswp73Δ harboring pUCA-SWP73 or the wild-type strain (see Figure 2-3). Immunoblot analysis showed that the three mutant proteins were present at levels approximately two- to threefold higher than that of swp73-1p in Wswp73-1 cells (presumably because centromeric plasmids are typically present at one to three copies per cell). Therefore, phenotypes of Wswp73-1 may be attributable to a combination of a reduced protein level and a diminished functional capacity of swp73-1p. This hypothesis is supported further by data indicating that Wswp73Δ harboring a high-copy plasmid expressing swp73(G323D)p grows at levels nearly indistinguishable from W303-1A at 37°C (data not shown).

Discussion

It is remarkable and fortunate that *SWP73* was isolated three times within the span of a month by three independent investigators studying three very different questions. The fact that *SWP73* was isolated biochemically complemented my genetic results, and, conversely, my genetic results give *in vivo* significance to the biochemical identification of *SWP73*. In addition, the genetic isolation of another member of Swi/Snf that effects GR function gives further support to the initial analysis that mutations in SWI1, SWI2, and

SWI3 effect GR function (Yoshinaga et al. 1992 and discussed in the Introduction to Chapter 3).

The identification of *SWP73* in the genetic screen involving E1A toxicity also complemented my functional analysis with regard to GR, since two very different transcriptional activators were both affected by mutations in *SWP73*. And considering the potentially different natures by which GR and E1A play a role in regulating transcription, further credence is given to a possible global role for Swp73p in the function of the yeast cell. This last point is firmly supported by the existence of the Ts^- growth phenotype when either the *swp73-1* and *swp73Δ* alleles are present in the haploid genome.

Chapter 3

Differential Effects of *swp73* Mutations on Various Transcriptional Activators

Abstract

The Swi/Snf complex is believed to be involved in chromatin remodeling and transcriptional derepression. It is known that Swi/Snf is required for the function of various transcriptional activators. To assess the role of Swp73p in Swi/Snf function the activities of multiple activators were compared in the wild type W303-1A strain, as well Wswp73-1, Wswp73Δ, and Wsnf2Δ. In general, the rank order in activity in these strains was: W303-1A > Wswp73-1 >> Wswp73Δ > Wsnf2Δ. However, the rank order was dependent on the activator and promoter contexts. Both Yap1p and GR-LexA showed a strong dependence on Snf2p expression, but no dependence on Swp73p expression. For these two activators, *swp73-1* displayed a gain-of-function phenotype in which regulator activity was practically identical to that seen in the absence of Snf2p. These results suggest that Swp73p and Snf2p have common, but also distinct, roles in the Swi/Snf complex.

Introduction

As was stated in the last chapter, Swp73p is a member of the yeast Swi/Snf complex, which is hypothesized to be involved in chromatin rearrangement. So what exactly is chromatin? First, a paradox in eucaryotic cell biology needs to be stated. Using human DNA as an example, if the DNA content in all the chromosomes where stretched out end-to-end it would be about a meter long. And since the average human cell is on the order of microns this dilemma presents a million-fold packaging problem. Of course,

this problem is not unique to human cells, but is faced by all eucaryotes. Eucaryotes have solved this problem by packaging their DNA content into protein:DNA complexes that are collectively referred to as chromatin. This solution to the packaging dilemma is illustrated in Figure 3-1.

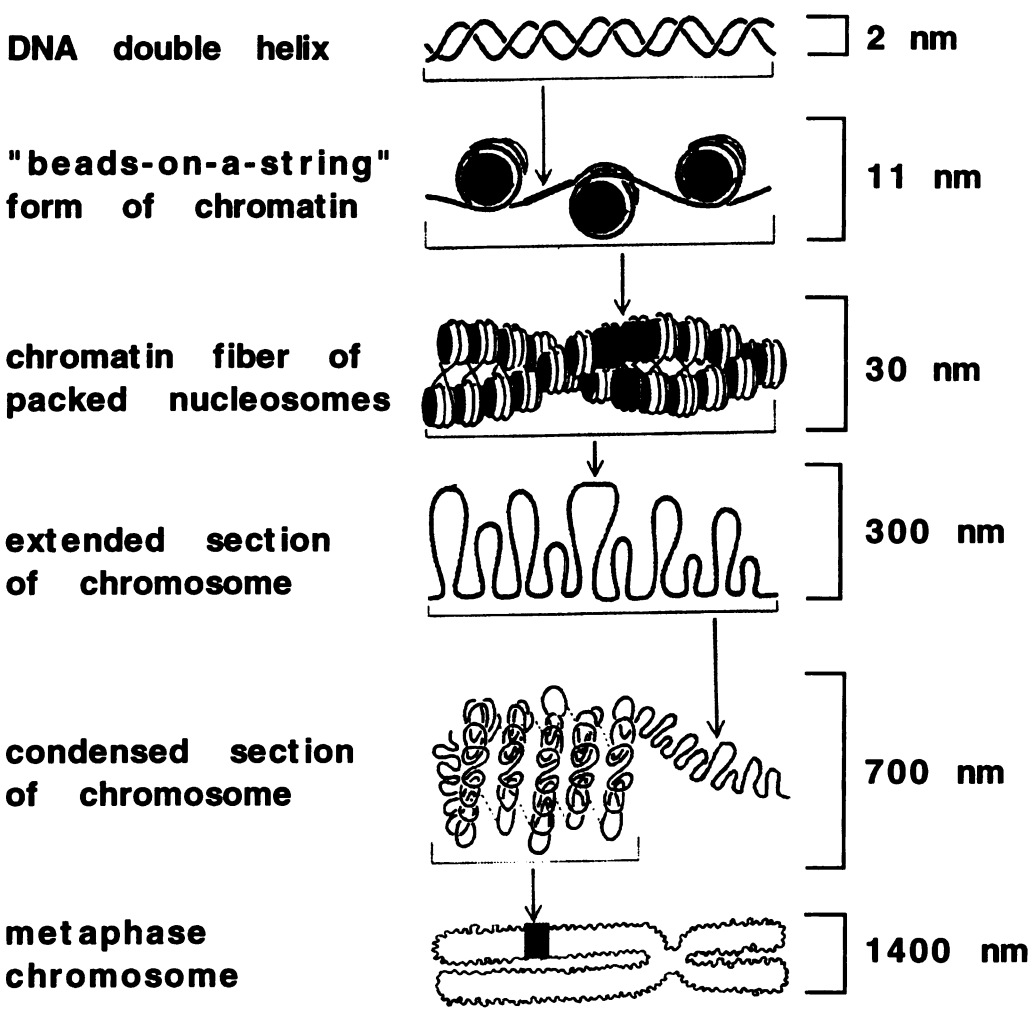
The base unit of chromatin is the nucleosome, which consist of the core histones H2A, H2B, H3, and H4 assembled into an octomer containing dimers of each protein (Kornberg 1974; Eickbush and Moudrianakis 1978). With a periodicity of ~200 bp, the DNA is wound around nucleosomes to form what appears in electron micrographs to be "beads-on-a-string". Nucleosomal DNA is then compacted further by higher order folding until chromosomes are formed, and the full content of the organism's DNA fits readily into the cell nucleus. But this is where the paradox comes into play. If the DNA is packaged too tightly into chromatin then it becomes inaccesible to other proteins that regulate and perform the transcription of the genes residing within the DNA. Indeed, a role for chromatin in the repression of transcription in yeast has been firmly established; histone depletion and histone modification experiments in yeast have shown that defects in chromatin components cause defects in transcriptional repression (Clark-Adams et al. 1988; Kim et al. 1988).

To overcome this paradox, the prevailing view is that specialized machinery has evolved that "remodels" chromatin structure and, thus, allows transcription to occur (for review, see Kingston et al. 1996). Specific alterations in chromatin structure have, indeed, been shown to accompany the activation of many transcribed genes. For example, positioned nucleosomes on the yeast *PHO5* and *SUC2* promoters are disrupted under

Figure 3-1. The structure of chromatin.

Ilustrated is the various levels of DNA packaging in a cell nucleus. Starting with the DNA double helix, occuping a space of ~2 nm, the DNA is wound around nucleosomes to form a "bead-on-a-string", which can be visualized in electron micrographs. This structure occupies a space of 11 nm. From these initial chromatin structures higher order chromatin fibers are formed that occupy a space of 30 nm. The chromatin fibers in turn are formed into chromosomes that form structures from 300 nm to 1400 nm. In this way, copious amounts of genetic material in the form of DNA can be stored within a cell's nucleus. Each arrow in the figure points to a condensed view of the preceding illustration. This figure was adapted from "Molecular Biology of the Cell", 1st ed. (eds. Alberts et al.) 1983.

A Schematic of Chromatin Structure



derepressing growth conditions (Almer et al. 1986; Fascher et al. 1990; Hirschhorn et al. 1992). Importantly, these disruptions require neither DNA replication nor transcription from the promoter to occur (Schmid et al. 1992; Hirschhorn et al. 1992). These results suggest that repressive chromatin structures must be remodeled before transcription can occur and further support the notion that specialized factors exist which remodel chromatin independently from transcription.

Many experiments performed *in vitro* support the idea that certain DNA-binding proteins display an altered (typically lower) apparent affinity for their binding sites when their target DNA is packaged into a nucleosome (Pina et al. 1990; Taylor et al. 1991). This reduced affinity is believed to be a contributing factor in the repression of transcription. For example, promoters assembled into chromatin, and subsequently incubated with RNA polymerase II transcription components, are often refractory to transcription, whereas naked DNA templates are robust substrates (Knezetic and Luse 1986; Workman and Roeder 1987; Knezetic et al. 1988). Addition of transcriptional activators during chromatin assembly enables transcriptional activation at levels observed with naked DNA templates, showing that transcriptional activators assist in overcoming nucleosomal repression *in vitro* (Workman and Roeder 1987; Cronston et al. 1990; Workman et al. 1990, 1991).

Transcriptional activators, however, do not tell the whole story with regard to relieving chromatin repression. Several factors have been described that may collaborate with transcription factors to perturb nucleosomes and reorganize chromatin. One such factor, Swi/Snf complex, was purified to homogeneity from extracts of *S. cerevisiae*, and shown to contain eleven

polypeptides, including the DNA-dependent ATPase Snf2p (also called Swi2p) (Cairns et al. 1994; Côté et al. 1994). Purified Swi/Snf complex can perturb nucleosomes and assist the binding of Gal4p-derivatives to nucleosomal DNA (Côté et al. 1994). A complex of similar biochemical properties to Swi/Snf has been extensively purified and characterized from human cells (Kwon et al. 1994; Imbalzano et al. 1994; Wang et al. 1996).

The RSC (remodel the structure of chromatin) complex also has been identified (Cairns et al. 1996), a second chromatin remodeling complex in yeast. It contains 14-15 polypeptides, is estimated to be 1 MDa in size, is estimated to be 10-fold more abundant then the Swi/Snf complex, and has a potent chromatin remodeling activity. Based on amino acid sequence identity, several members of the Swi/Snf complex have closely related homologs that exist in the RSC complex. However, the genes encoding these homologs in RSC are required for viability (Laurent et al. 1992; Cairns et al. 1996b; Treich and Carlson 1997). Thus, it has been difficult to obtain information *in vivo* as to which promoters are RSC-dependent for their activity.

In *Drosophila* genetic analysis has revealed the brm complex, which appears to have a similar composition to Swi/Snf (Tamkun et al. 1992; Dingwall et al. 1995). In addition, biochemical studies revealed the NURF complex (NUcleosome Remodeling Factor), which is approximately 1 MDa in size and contains ISWI, a Snf2/Swi2p homolog (Elfring et al. 1994). It co-operates with the transcription factor GAGA to create a nuclease-hypersensitive site on a nucleosome array prepared *in vitro* (Tsukiyama et al. 1994; Tsukiyama and Wu 1996). Finally, another protein complex that also

contains ISWI, ACF (ATP-utilizing chromatin assembly and remodeling factor), has been purified from *Drosophila*. In addition to mediating promoter-specific chromatin remodeling by Gal4-VP16, ACF, in concert with a core histone chaperone (such as NAP-1 or CAF-1), also plays a role in chromatin assembly (Ito et al, 1997).

Returning to yeast Swi/Snf, genetic studies from several labs identified six of the eleven genes encoding the members of the complex: *SWI1/ADR6*, *SWI2/SNF2*, *SWI3*, *SNF5*, *SNF6*, and *SNF11*. All except *SNF11* are required for proper control of the same set of yeast genes (O'Hara et al. 1988; Estruch and Carlson 1990; Laurent et al. 1990; Laurent et al. 1991; Peterson and Herskowitz 1992; Treich et al. 1995). Most of these components were identified in two seperate genetic screens designed to yield either *swi* mutants (Stern et al. 1984; Breeden and Nasmyth 1987), which are defective in the expression of HO (an endonuclease required for mating-type switching), or *snf* mutants (Neigeborn and Carlson 1984), which are unable to ferment sucrose anaerobically. Null mutations in any of the *SWI* or *SNF* genes listed above confer similar phenotypes, and studies from several labs suggest these phenotypes result from defects in the transcriptional activation of genes whose products participate in these processes. For example, each *swi* and *snf* mutant activates neither the *HO* nor the *SUC2* promoters. Genetic supression analyses indicate null mutations in *SWI* or *SNF* are suppressed similarily by mutations in various genes, including those for histones.

Biochemical isolation of the Swi/Snf complex from yeast yielded a complex containing six gene products from the *SWI* and *SNF* genes mentioned above and five additional polypeptides, termed Swp82p, Swp73p,

Swp61p, Swp59p, and Swp29p (for Swi/Snf-associated protein; Cairns et al. 1994; Côté et al. 1994; Peterson et al. 1994). The isolated complex perturbs nucleosome structure *in vitro*, as judged from accessibility of the nucleosomal DNA to DNase I and to sequence-specific binding proteins (Côté et al. 1994)

Unfortunately, one of the most important aspects of Swi/Snf function remains unresolved; how is the complex targeted to particular promoters? Swi/Snf complex lacks specific DNA-binding activity. There is some evidence that transcriptional activators themselves may target the complex by binding the complex directly and recruiting it to promoters containing their cognate sites. Support for this model was provided through experiments with GR in yeast. Transcriptional activation by GR is greatly potentiated by members of the Swi/Snf complex, and a physical association between GR derivatives and the Swi/Snf complex member Swi3p has been demonstrated by co-immunoprecipitation (Yoshinaga et al. 1992). The co-immunoprecipitation between GR and Swi3p requires the presence of Swi1p and Swi2p, which gives further evidence that a complex is formed involving the *SWI* and *SNF* gene products. In addition, the co-immunoprecipitation between GR and Swi3p also gives biological significance to a role for Swi/Snf in GR function. Finally, experiments indicate that activation by GR in mammalian cells involves a related human Swi/Snf complex, and the perturbation of chromatin structure (Muchardt and Yaniv, 1993). Taken together, the mechanism of transcriptional enhancement by GR may involve a chromatin transition that involves a physical association between GR and Swi/Snf-like complexes in both yeast and mammalian cells.

In collaboration with Brad Cairns, I decided to test several transcriptional activators in the *swp73* strains to determine if these mutant strains would behave like the strains harboring mutations in the genes encoding the other complex members.

Methods and materials

Yeast strains

The yeast strains used in this chapter (Table 3-1) were manipulated by standard techniques (Gietz et al. 1992; Sherman 1991; Sherman and Hicks 1991). All cultures were grown at 30°C unless otherwise indicated. A haploid strain lacking the *SNF2* gene, Wsnf2Δ (YBC402), was obtained from tetrad dissection of the heterozygous *snf2Δ/SNF2* strain YBC400, which was prepared by transformation of W303 with *Bam*HI-digested pKOSNF2L. Replacement of one of the *SNF2* loci with the *LEU2* gene by γ-transformation (Sikorski and Hieter, 1989) was confirmed by Southern blot analysis. Sporulation yielded tetrads with two wild type and two slow-growing spores. In all eight four-spore tetrads tested, the slow growth phenotype segregated with Snf^- and Leu^+ phenotypes. To characterize further the isolated haploid *snf2Δ::LEU2* strain (Wsnf2Δ), it was transformed with YCp50-SNF2. All eight transformants tested displayed Snf^+ phenotypes and grew as well as wild type cells.

Table 3-1. *Yeast strains*

Strain	Relevant Genotype	Source
W303-1A	*MATa, trp1-1 ura3-1 leu2-3,112 his3-11,15*	A. Tzagoloff
W303-73Δ[a]	*MATa/α, trp1-1/trp1-1 ura3-1/ura3-1 leu2-3,112/leu2-3,112 his3-11,15/his3-11,15 swp73::LEU2/SWP73*	this study
Wswp73-1[b]	*MATa, trp1-1 ura3-1 leu2-3,112 his3-11,15 swp73-1*	this study
Wswp73Δ[c]	*MATa, trp1-1 ura3-1 leu2-3,112 his3-11,15 swp73::LEU2*	this study
Wsnf2Δ[c]	*MATa, trp1-1 ura3-1 leu2-3,112 his3-11,15 swi2::LEU2*	this study
MCY2064	*MATa, ura3-52 his3-Δ200 lys2-801 ade2-101 snf5-Δ2*	B. Laurent
YPH499	*MATa, trp1Δ1 ura3-52 leu2-Δ1 his3-Δ200*	P. Hieter
Yswp73Δ[d]	*MATa, trp1Δ1 ura3-52 leu2-Δ1 his3-Δ200 swp73::LEU2*	this study

[a]W303 strain background (Thomas and Rothstein 1989).
[b]W303-1A strain background.
[c]Derived from tetrad dissection of a W303 derivative.
[d]YPH499 background.

Plasmids

Expression plasmids for LexA-fusion proteins, derivatives of pSH2-1 (*HIS3*, 2μ origin, expresses LexA (1-87) from the *ADH1* promoter; gift of R. Brent), were as follows: pLexA-Snf2, pLexA-Snf5, and pLexA-Snf6 (Laurent et al. 1990, 1991) gifts of B. Laurent; pLexA-Gal4 (pSH17-4), gift of R. Brent (Hanes and Brent 1988); and pG-NLX (*TRP1*, 2μ origin, expresses the first 452 amino acids of the rat glucocorticoid receptor fused to amino acids 1-87 of LexA from the yeast *GPD* promoter; Yoshinaga et al. 1992).

For preparation of pLexA-SWP73.6XHIS, which expresses LexA (1-87) fused to full-length Swp73p with six histidine residues at the carboxy-terminus, a 1.8 kb DNA was amplified from genomic DNA by polymerase chain reaction (PCR) with the following oligonucleotide primers: 5′-CCCGAATTCACCATGGCCAAAGTAATGAAACCCAGCAAC-3′ and 5′-CCCAGATCTCTAATGGTGATGGTGATGGTGATGGTGCATTCGTCCATTT GATAATATGAC-3′. The PCR product was digested with *Eco*RI and *Bgl*II and inserted between the *Eco*RI and *Bam*HI sites of pSH2-1.

The GR expression plasmids pGN795 and pGN556, and the 3GRE-*lacZ* reporter plasmid, pΔS26x, were as described (Yoshinaga et al. 1992). The GR expression plasmid pTCA-GN795 consists of the sequence from pGN795 between the *Hin*dIII and *Ngo*MI sites, which contains the *GPD* promoter, the full-length GR coding sequence, and the *PGK* terminator sequence, inserted between the same sites of pRS314 (*TRP1, CEN4*)(Sikorski and Heiter 1989). Construction of pTCA-GN556 was accomplished in the same way except that pGN556 was used as the source of the *Hin*dIII/*Ngo*MI DNA fragment. The

GR reporter pHCA-G3Z was constructed by moving the 3GRE-*lacZ* reporter fragment from pΔS26x into pRS313 (*HIS3, CEN4*) (Sikorski and Heiter 1989). The GR expression plasmid pGN556-K461A and the reporter plasmid plfG_3-β-gal were as described (Starr et al. 1996). The RARβ expression plasmid, pG-RARβ, and reporter plasmid, pΔS-DR5, were as described (Chen et al. 1993).

The Yap1p reporter pUCΔS.TRE2 was constructed by inserting the *Sal*I/*Bam*HI fragment of pSM38 (Moye-Rowley et al. 1989), containing two SV40 AP-1 recognition elements and a minimal *CYC1* promoter, between the *Xho* I and *Bam*HI sites of pUCΔSS (Picard et al. 1990). The Gal4p reporter p121-Δ10 (West, Jr. et al. 1984) contains Gal4p-binding site 3 and 4 upstream of *GAL1-lacZ*. The Gal4p reporter pSV14 (Giniger et al. 1985) contains a single, near consensus, synthetic 17-mer Gal4p-binding site upstream of *CYC1-lacZ* . The Swi5p/Pho2p reporter plasmid was *HO*(46)-*CYC1-lacZ* (M632) (Brazas and Stillman 1993). For preparation of pUCA-SWP73, the region of the genomic clone of *SWP73* from the *Sal*I site in the upstream promoter (located at nucleotide position -219) to the *Xba*I site in the 3' untranslated region (located 50 nucleotides downstream of the stop codon) was inserted between the *Xho*I and *Xba*I sites of pRS316 (*URA3, CEN4*)(Sikorski and Heiter 1989).

The *SNF2* disruption plasmid, pKOSNF2L, designed to replace 92% of the *SNF2* coding region with the *LEU2* gene, was prepared from pRS305 and two PCR fragments of the *SNF2* gene. The first fragment contained 600 bp of the 5'-noncoding region from nucleotides 220 to 820 (*SNF2* initiator codon starts at nucleotide 890), except that the *Sac*II site at nucleotide 228 was

destroyed. This fragment was prepared from plasmid YCP50-SNF2 and the following oligonucleotide primers: 5'-CGCCGGATCCATGCGGCTGCCCTAAATACACACATA-3' and 5'-GCGCCCCCGCGGCAGAATAGCTGACCTTCCCGATGA-3'. The second PCR fragment contained 722 bp of the 3'-coding and noncoding region from nucleotides 5288 to 6000 (*SNF2* termination codon starts at position 5587), and was prepared with the following oligonucleotide primers: 5'-GCGCCCCTGCAGCCACCAGCCCTTGAATCAAGTCCA-3' and 5'-GCGCCCGGATCCCGCTTCTGTCATGCTCGAGTCCGCT-3'. The first PCR fragment was digested with *Sac*II and *Bam*HI, the second fragment was digested with *Pst*I and *Bam*HI, and pRS305 was digested with *Pst*I and *Sac*II. A three-piece ligation with the digestion products yielded pKOSNF2L.

Enzyme assays

Transcriptional activation by LexA-fusion proteins *in vivo* was quantified with β-galactosidase assays using either the reporter plasmid pRS1840 (2μ origin, *URA3*) (gift of R. Brent) or pΔSLex (2μ origin, *URA3*), each of which contains a single high-affinity binding site for the *E. coli* repressor LexA. Activation by Swi5p/Pho2p was assayed *in vivo* using the reporter plasmid HO-CYC1-lacZ. Strains containing a reporter plasmid (alone or in combination with plasmids expressing LexA fusion proteins) were grown in 100 ml of synthetic medium containing the appropriate amino acids and 2% glucose to an optical density at 600 nm of 0.5-0.8. The cell suspension was centrifuged at 5,000 x g for 10 min, washed with sterile water, harvested,

and suspended in 0.5 ml Buffer A containing 400 mM sodium chloride. The cells were disrupted by beating with glass beads for 9 min at 4°C. The extract was centrifuged at 13,000 x g for 10 min, and the supernatent was centrifuged again at 13,000 x g for 10 min. β-galactosidase activities, determined as described by Miller (1972), and are given in units/μg of protein in the whole-cell extract.

Transcriptional activation by all other proteins was quantified by liquid β-galactosidase assays as described (Garabedian 1993), except that hormone treatment (when indicated) was for 6 h, and the addition of SDS was eliminated from the permeabilization step.

Invertase assays were performed according to the method of Goldstein and Lampen (1975), as modified by Celenza and Carlson (1984). Liquid acid phosphatase assays were performed as described (O'Neill et al. 1996), except that 1 M sodium carbonate was used to stop the reaction. Acid phosphatase units are defined as $1000(A_{420}/A_{600} \cdot v \cdot t)$, where t=time (in min) and v=the cell fraction used (t=10 and v=0.1 for these experiments).

Western analysis of N556-K461A

Crude whole-cell extracts were prepared by using cells harvested at an OD_{600} 0.5-0.8 after growth in synthetic minimal media supplemented with 2% glucose and 1X amino acids lacking tryptophan. The cells were lysed by glass beads (0.5 mm) in extraction buffer [40 mM Hepes, pH 7.3; 350 mM NaCl; 0.1% Tween-20; 10% glycerol (v/v); 1 mM phenylmethylsulfonyl fluoride (PMSF);

and 1x protease inhibitor cocktail (Boehringer Mannheim, Indianapolis, IN - cat. no. 1 836 153)] with 5 pulses, each 50 sec in duration, at maximum speed on a BioSpec Products (Bartlesville, OK) beadbeater. The cell debris was removed by centrifugation and the resulting supernatent was centrifuged in a microfuge for 15 min at top speed. The resulting supernatent from the second spin was used for Western analysis.

Approximately 25 μg of total protein extract was electrophoresed on a 7.5% SDS-PAGE gel and transferred to an Immobilon-P membrane (Millipore, Bedford, MA - cat # IPVH 000 10) by semi-dry electrophoresis. After transfer the membrane was incubated in PBS + 0.05% Tween-20 (PBST) containing 10% (w/v) non-fat dry milk for 3-4 hr at 4°C. The membrane was then removed and incubated at 4°C for 16 hr in PBST containing 2% non-fat dry milk and the monoclonal antibody A250 (α-GR - Okret et al. 1984; tissue culture supernantent diluted 1:150). After extensive washing in PBS, the membrane was incubated for 1 hr at RT in PBST containing 2% non-fat dry milk and goat anti-mouse secondary antibody conjugated to horseradish peroxidase (diluted 1:3000). After extensive washing in PBS, the membrane was developed using enhanced chemiluminescent substrate (Pierce, Rockford IL. - cat # 34080). Exposure time was 30 sec.

Results

swp73Δ *strains display a Snf⁻ phenotype*

One of the hallmark phenotypes of strains carrying deletions in the previously identified members of the Swi/Snf complex (*swi1Δ, swi2Δ, swi3Δ, snf5Δ, snf6Δ*) is a defect in growth on sucrose or raffinose under anaerobic conditions (Snf⁻) To investigate whether deletion of the *SWP73* gene has similar consequences, *swp73Δ* strains were grown on rich medium containing sucrose or raffinose and antimycin A, an inhibitor of electron transport, whose presence simulates anaerobic conditions (Neigeborn et al. 1986). Yswp73Δ and Wswp73Δ required five or seven days to form colonies, respectively, compared to two days for isogenic wild type strains. The Wswp73-1 strain displayed an intermediate phenotype, resembling the wild type parent more than the deletion strain, requiring three to four days to form colonies.

Since diminished levels of invertase (product of the *SUC2* gene, whose expression depends on the Swi/Snf complex) may account for the Snf⁻ phenotype in other *swi/snf* mutants, invertase assays were performed on wild type (W303-1A), Wswp73-1 and Wswp73Δ strains. Although the invertase level was only reduced approximately two-fold in the Wswp73-1 strain, the Wswp73Δ strain lacked appreciable invertase activity (Table 3-2). We conclude that *swp73* mutants, like other *swi/snf* mutants, display a Snf⁻ phenotype related to the production of invertase.

Table 3-2. *Effect of* swp73 *mutations on the levels of secreted invertase*

	Secreted invertase activity (μmole glucose/min/mg)		
		derepressed	
Strain	repressed	2 hr	3 hr
W303-1A (wild type)	40	833	831
Wswp73-1	13	274	556
Wswp73Δ	21	44	55

The levels of secreted invertase were measured in the strains indicated under repressing conditions and after derepressing in the presence of low levels of glucose for 2 or 3 hours.

Certain activators that require Swi2/Snf2p also require Swp73p

Another hallmark phenotype of *swi* and *snf* mutants is a defect in the transcriptional activation of genes linked to an *HO* promoter. The transcription factors involved in *HO* activation are Swi5p and Pho2p (also known as Grf10p), which function as a heterodimer. A 46-bp region from the *HO* promoter and regulatory region enables Swi5p- and Pho2p-dependent transcriptional activation when placed upstream of the *CYC1* promoter (Brazas and Stillman 1993). We compared the activity of this region of the *HO* promoter in our *swp73* mutant strains, as well as a *snf2* mutant strain since *SNF2* was originally isolated in a screen looking for a loss of activity from the *HO* promoter. We found that transcriptional activation with the Swi5p/Pho2p-binding element was dependent on Swi2/Snf2p and on Swp73p as well, since only 6 or 13 units of β-galactosidase activity were obtained with Wsnf2Δ or Wswp73Δ strains, respectively, compared with 118 units of activity with the wild type W303-1A strain (Table 3-3). Wswp73-1 displayed an intermediate phenotype, yielding 42 units of activity. A requirement for Swi/Snf complex to mediate activation by Swi5p/Pho2p may therefore underlie the defect in *HO* expression originally observed with *swi/snf* mutants.

Swi2/Snf2p is reported to potentiate transcriptional activation by various activator proteins including full-length GR (N795), a constitutive (hormone-independent) GR derivative (N556), and Gal4p (Yoshinaga et al. 1992; Laurent and Carlson 1992; Peterson and Herskowitz 1992). Swp73p is also required by GR (N795) and GR (N556), as little transcription was induced

Table 3-3. *Activation by various transcriptional activators assessed with a* CYC1 *promoter reporter plasmid*

Activator expressed	Reporter Plasmid	β-galactosidase activity (units)			
		W303-1A	Wswp73-1	Wswp73Δ	Wsnf2Δ
Swi5p/Pho2p (endogenous)	*HO-CYC1-lacZ*	118[a]	42[a]	13[a]	6[a]
Full length GR (N795)	pHCA-G3Z	824	86	84	9
Constitutive GR (N556)[b]	pHCA-G3Z	1167	921	125	20
Constitutive GR (N556)[c]	pΔS26x	2716	1150	180	ND
Yap1p (endogenous)	pΔS-TRE2	996	79	951	124
RARß	pΔS-DR5	303	24	5	ND
Gal4p (endogenous)	pSV14	4794	1029	589	ND
Gal4p (endogenous)	p121-Δ10	1577	24	<1	ND

The indicated activators were expressed, and β-galactosidase assays performed. See Materials and methods for the identity of reporter plasmids. The values reported are the average of at least four transformants done in duplicate. Standard errors were <20%. ND: not determined.
[a]Determined in units/mg (see Materials and methods).
[b] Centromeric expression plasmid pTCA-GN556.
[c] 2μ origin expression plasmid pGN556.

by these activators in the Wswp73Δ strain (Table 3-3). Activation by endogenous Gal4p was also dependent on Swp73p, showing 4- and 8-fold reduction in Wswp73-1 and Wswp73Δ strains, respectively, when assessed with a reporter plasmid containing a single near consensus binding site for Gal4p (pSV14; Giniger et al. 1985). This dependence was even stronger (60- and 1500-fold reduction in Wswp73-1 and Wswp73Δ strains, respectively) when assessed with a reporter plasmid containing two lower affinity binding sites for Gal4p (p121-Δ10; West, Jr. et al. 1984). Retinoic acid receptor β was similarly dependent on Swp73p.

An activator that does not require Swi2/Snf2p or Swp73p

Activation of *PHO5* transcription by Pho4p is not dependent on Swi2/Snf2p (Schneider 1995). To test dependence on Swp73p, activity of the endogeneous *PHO5* promoter was assessed in Wsnf2Δ, Wswp73Δ, Wswp73-1, and wild type (W303-1A) strains. Growth on low phosphate medium to induce *PHO5* transcription and liquid assays of the *PHO5* gene product, acid phosphatase, gave 496 units for strain Wsnf2Δ, 512 for Wswp73Δ, 206 for Wswp73-1, and 542 for W303-1A. We assume that the two-fold lower activity for Wswp73-1 is not significant, and therefore Swp73p, like Swi2p/Snf2p, is not required for Pho4p activation of the *PHO5* promoter.

LexA-Swi/Snf fusion proteins require Swp73p to activate transcription, and LexA-Swp73p requires Swi/Snf

In addition to the intact activators that we tested, we also tested various chimeric proteins. Namely, fusion of the DNA-binding domain (amino acids 1-87) of the bacterial repressor LexA to Swi2/Snf2p, Snf5p, Snf6p, Snf11p, Gal4p, or the activation domain of the rat glucocorticoid receptor (amino acids 1-452) creates chimeric proteins that activate transcription of a promoter under control of a single *lexA* operator (Hanes and Brent 1989; Laurent et al. 1990, 1991; Laurent and Carlson 1992; Yoshinaga et al. 1992; Treich et al. 1995). The fusions to members of the Swi/Snf complex generally require the other complex members for full activation (Laurent and Carlson 1992). This last result provides further evidence that the Swi/Snf proteins function as a complex. Thus, when an individual member of the complex is fused to a specific DNA-binding protein and thereby targeted to a specific promoter, the rest of the complex, in turn, is targeted to the same promoter because of its association with the chimeric protein. The result of this complex-derived specific targeting is a molecular bypass, which allows the study of Swi/Snf function without regard for its hypothesized association with transcriptional activators.

This approach was extended to our Wswp73Δ and Wswp73-1 strains, with the use of a β-galactosidase reporter plasmid containing a *lexA* operator upstream of a minimal *GAL1* promoter. LexA-Snf2p and LexA-Snf5p fusion proteins, potent activators in the wild type strain W303-1A, were ineffective in either the Wswp73Δ, or Wswp73-1 backgrounds (Table 3-4).

Transcriptional activation by LexA-Snf6p was reduced two- or ten-fold in Wswp73Δ or Wswp73-1 strains, respectively. We conclude that Swp73p is

Table 3-4. *Activation by LexA-activator fusion proteins assessed with one* lexA *operator and the* GAL1 *promoter*

	β-galactosidase activity (units/mg)			
Activator expressed	W303-1A	Wswp73Δ	Wswp73-1	Wsnf2Δ
LexA-1-87 (control)	10	8	ND	ND
LexA -Snf2p	3787	49	55	5066
LexA -Snf5p	3295	10	21	ND
LexA -Snf6p	13584	5396	1306	456
LexA -Swp73p	904	708	ND	25
LexA -Gal4p	2691	2317	3784	141
LexA -GR(1-452)	1185	1129	27	16

The indicated LexA-fusion proteins were expressed, and β-galactosidase assays performed as described in the Materials and Methods. Transformants also contained the reporter pRS1840, which contains one concensus binding site for LexA upstream of the *GAL1* promoter fused to the β-galactosidase gene. The values reported are the average of at least four transformants done in duplicate. Standard errors were <20%. ND: not determined.

required for transcriptional activation involving other members of the Swi/Snf complex.

To ask, conversely, whether other members of the Swi/Snf complex are required for function of Swp73p, a LexA-Swp73p fusion was tested in various *swi/snf* mutant backgrounds. This fusion protein proved to be a strong activator, driving production of 904 units of β-galactosidase activity in wild type cells. In contrast, the fusion protein caused production of only 5 or 25 units of activity in *snf5Δ* (S288C background) and *Wsnf2Δ* (W303 background) strains, respectively. The fusion protein could also substitute for wild type Swp73p, as expression of LexA-Swp73 in either Yswp73Δ or Wswp73Δ strains conferred full Ts^+ and Snf^+ phenotypes. These findings indicate that Swp73p is a full functional member of the Swi/Snf complex.

Certain activators and promoter contexts that require Swi2/Snf2p do not require Swp73p

Activation by LexA-Gal4p requires Swi2/Snf2p, Snf5p, and Snf6p when assessed with a reporter plasmid containing a minimal *GAL1* promoter (and one *lexA* operator), but is independent of these Swi/Snf components when assessed with a minimal *CYC1* promoter and one *lexA* operator (Laurent and Carlson 1992). LexA-GR (amino acids 1-452 of GR fused to the LexA DNA-binding domain) behaved similarly to LexA-Gal4p with a *lexA-GAL1* promoter, activating transcription in a wild type strain but not in the isogenic Wsnf2Δ strain (Table 3-4). By contrast with the requirement for Swi2/Snf2p,

Swp73p was dispensable, as the levels of transcription observed in the Wswp73Δ strain were indistinguishable from those in the wild type strain. The lack of Swp73p dependence was not promoter-specific, as shown by experiments with a reporter plasmid containing the *CYC1* promoter (and one *lexA* operator). Activation by LexA-GR or LexA-Gal4p was reduced only two- or three-fold, respectively, in Wswp73Δ cells compared to wild type cells (Table 3-5). These findings raise the possibility that Swp73p performs roles in the Swi/Snf complex distinct from those of Swi2/Snf2p.

Activator dependence of swp73-1p function

As already mentioned, Wswp73-1 cells behave more like Wswp73Δ cells than wild type with regard to the Ts^- phenotype, whereas they resemble wild type cells more closely with regard to the Snf^- phenotype. Evidently some functions of the Swi/Snf complex are more affected by the *swp73-1* mutation than others. In keeping with this variation, we observed differences in potency among activators in Wswp73-1 cells. LexA-Snf2p, LexA-Snf5p, full-length glucocorticoid receptor, and RARβ were ineffective in Wswp73-1 cells (much as in Wswp73Δ cells), whereas LexA-Gal4p retained full activity (see Tables 3-3 and 3-4). Other activators, such as a constitutive version of GR (N556), endogenous Gal4p (assessed with pSV14), and Swi5p/Pho2p showed an intermediate dependence. An unexpected finding was that two activators, LexA-GR (assessed with pRS1840) and Yap1p, were more effective in Wswp73Δ cells than in Wswp73-1 cells (see Tables 3-4 and 3-

Table 3-5. *Activation by LexA-Gal4p or LexA-GR fusion proteins assessed with one* lexA *operator and the* CYC1 *promoter*

	β-galactosidase activity (units/mg)		
Activator expressed	W303-1A	Wswp73-1	Wswp73Δ
LexA-1-87 (control)	27	ND	ND
LexA-Gal4p	11704	11222	3800
LexA-GR(1-452)	10886	6722	5206

The indicated LexA-fusion proteins were expressed, and β-galactosidase assays performed as described in the Materials and methods. Transformants also contained the reporter pΔSLEX, which contains one concensus binding site for LexA upstream of the *CYC1* promoter fused to the β-galactosidase gene. The values reported are the average of at least four transformants done in duplicate. Standard errors were <20%. ND: not determined.

3). This behavior did not reflect variation in abundance of the activators, as Wswp73Δ and Wswp73-1 transformants contained approximately the same amount of LexA-GR as did wild type cells (data not shown). The diminished effectiveness of LexA-GR in Wswp73-1 cells was promoter-dependent, since little loss in activity was seen with the *lexA-CYC1* promoter (Table 3-5). Apparently, swp73-1p can either facilitate or interfere with transcriptional activation, depending on the cellular conditions and promoter context.

The K461A mutation in GR yields a transcriptional activator that is inhibited by Swi/Snf

An unexpected result was obtained when the activity of GR containing the K461A mutation was tested in the mutant strains. A lysine to alanine substitution at amino acid position 461 of rat GR (which is in the DNA-binding domain) leads to the unusual phenotype of transcriptional activation from GRE contexts that normally confer repression (Starr et al. 1996). Thus, the question arises as to what determines whether GR is an activator or a repressor. Is it something inherent about the structure of GR itself, its interaction with co-factors, the promoter element it binds to, or a combination of the above? Thus, it is conceivable that Swi/Snf plays a role in determining whether GR is an activator or a repressor. Therefore, the activity of GR-N556(K461A) was tested from a reporter plasmid harboring a trimerized composite GRE from the proliferin gene (plfG3; described in Diamond et al. 1990), which binds both GR and AP-1, and at which GR is normally a repressor while GR(K461A) is an activator. The results of GR-

N556(K461A) activity from plfG3 in wild type cells and *swp73* and *snf2* cells are in Figure 3-2. An average of 138 units of β-galactosidase activity were measured in wild type strain W303-1A, whereas an average of 219, 550, and 545 units of β-galactosidase activity were measured in the mutant strains Wswp73-1, Wswp73Δ, and Wsnf2Δ, respectively. It appears that the presence of wild-type Swp73p or Swi2/Snf2p actually interferes with GR-N556(K461A) transcriptional activation from the plfG3 promoter element. This increased activity in the mutant strains is specific to GR-K461A at plfG3. When GR-N795(K461A) was tested at TAT3-GRE (pΔS-26X) there was an approximate 5-fold loss in activity observed in the Wswp73-1, Wswp73Δ, and Wsnf2Δ strains as compared to W303-1A (M. Cronin, pers. comm.). The corollary to this experiment (wild-type GR tested at plfG3) was inconclusive because the units of β-galactosidase produced was so low in all the strains tested (<10 units each).

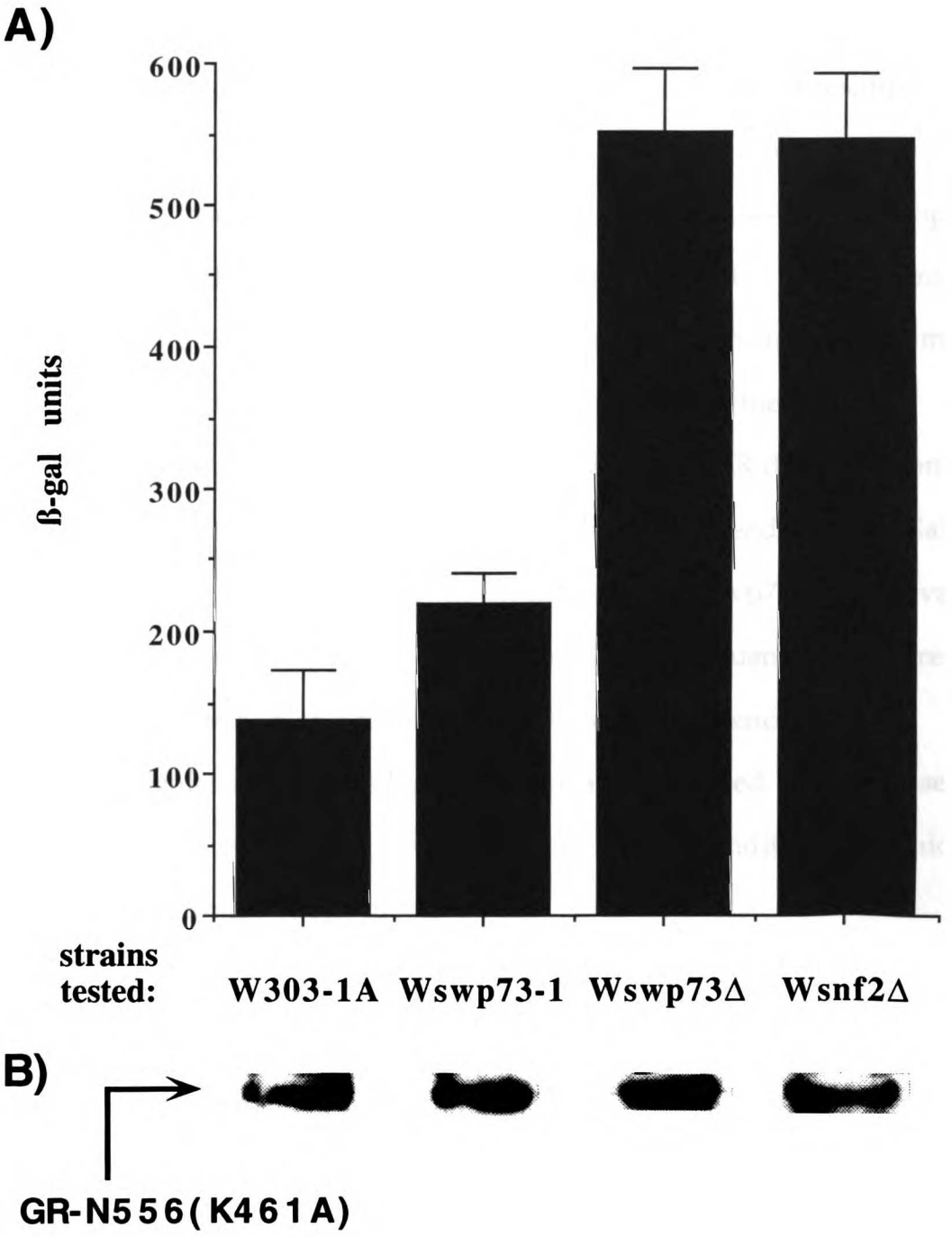
Discussion

The activities of several transcriptional activators, both intact activators and those created by gene fusions, were tested in *swp73* mutant strains and these activities were compared to the activities determined in a *snf2* strain, as well as the parental wild type strain. Although the behavior of Swp73p largely parallels that of previously documented members of the Swi/Snf complex (in particular Swi2/Snf2p), some distinctions were noted. For

Figure 3-2. Activity and expression level of GR-N556(K461A) from a plfG3-β-gal reporter gene.

The activity of GR-N556(K461A) from a plfG3-β-gal reporter gene was tested. *(A)* The production of β-galactosidase was tested in wild type cells versus three mutant strains. Five independent transformants harboring pGN556-K461A and $plfG_3$-β-gal were tested in wild type W303-1A cells and mutant strains Wswp73-1, Wswp73Δ, and Wsnf2Δ. Each strain tested is indicated below each column. The standard deviation from the mean are indicated by the error bars. *(B)* Western analysis of transformants used in panel (A). The two transformants out of five that were closest to the mean in each set of strains tested in panel (A) were tested for GR-N556(K461A) expression by Western analysis as described in the Methods and materials. A representative band from the Western analysis for each set of transformants is presented here and the strain that they represent is indicated above each band.

Activity of GR-N556(K461A) from plfG_3-β-gal



example, deletion of *SWP73* led to a modest defect in LexA-Snf6p activity, but deletion of *SWI2/SNF2* led to a dramatic reduction in LexA-Snf6p activity (see Table 3-3). This differential dependence of LexA-Snf6p on Swi2/Snf2p versus Swp73p may reflect on the function of the residual complex that remains after deletion of either *SWP73* or *SWI2/SNF2*. Thus, LexA-Snf6p may be able to assemble into a complex with a high amount of residual function (<3-fold loss in activity vs wt) even in the absence of Swp73p, whereas in the absence of Swi2/Snf2p LexA-Snf6p assembles into a complex with only marginal activity (~30-fold loss in activity vs wt). The data from the examination of the residual complexes that remain in the various mutant strains is presented in Chapter 4 and will be discussed further therein.

Similarly, activation by LexA-Gal4p and by LexA-GR depend strongly on Swi2/Snf2p, yet neither requires Swp73p. In contrast endogenous Gal4p and full-length GR depend upon both Swi2/Snf2p and Swp73p for activation. Whether these differential effects of Swp73p are a consequence of differential expression of the fusion and full length proteins or differences in the activator molecules themselves has not yet been determined. In any case it is clear that Swp73p is relatively unimportant in certain conditions in which Swi2/Snf2p is strongly required.

The Ts$^-$ phenotype of *swp73Δ* strains has not been reported for other *swi* and *snf* mutants, and may represent an additional functional difference between Swp73p and other members of Swi/Snf complex. I find that *swi1Δ* and *swi3Δ* strains (derived from the wild type yeast strain S288C) are only slightly sensitive to elevated temperature, while a congenic *swi2Δ* strain also grows at 37°C, albeit slowly. Swp73p may perform a function at 37°C that is

independent of Swi/Snf complex. Alternatively, Swi/Snf subcomplexes have been reported in strains lacking certain components of the complex (Peterson et al. 1994), and Swp73p might be an obligate member of a subcomplex that functions at 37°C. The lesser penetrance of the Ts^- phenotype in Wswp73-1 may be due to a lesser impairment of function by the mutation compared with deletion, or to instability of swp73-1p (present at about 10% of the level of Swp73p in wild type cells).

Four classes of transcriptional activators may be defined on the basis of their behavior in Wswp73-1 and Wswp73Δ cells: (I) those that were equally affected in both mutant strains; (II) those that were equally unaffected in both mutant strains; (III) those that were more negatively affected in Wswp73Δ; and (IV) those that were more negatively affected in Wswp73-1. Perhaps most notable are the class IV activators, which function normally in the *swp73Δ* strain but are severely defective in the *swp73-1* background. One interpretation is that Swi/Snf complexes formed in the absence of Swp73p differ from those formed in the presence of swp73-1p. According to this idea, the complexes lacking Swp73p are as active with class IV activators as the wild type Swi/Snf complex, whereas the complex containing the mutant swp73-1p is inactive with these activators. In this cellular context, swp73-1p appears to be an inhibitor of Swi/Snf action and may reflect the formation of nonproductive complexes. In addition, the effects of the *swp73-1* mutation and the *SWP73* deletion differ in different promoter settings: for example, LexA-GR activates the *lexA-CYC1* promoter equally in the two genetic backgrounds, but displays lower activity on the *lexA-GAL1* promoter in *swp73-1* than in *swp73Δ* (Tables 3-3 and 3-4). Thus, it appears that the

phenotypic effects of *SWP73* mutations are highly dependent upon both activator and promoter contexts.

Exploring this theme a little further, different versions of GR that were tested fall into different classes. N795 (the full-length version of GR that is hormone dependent) falls into class I, N556 (a constitutive version of GR) falls into class III, and LexA-GR (another constitutive version of GR) falls into class IV. A possible explanation for these results is that the DNA-binding and hormone-binding domains of GR may act as a molecular brake on GR transcriptional activation activites and Swp73p oppose such inhibition. By this view, then, deletion of the hormone-binding domain would relieve GR, in part, of its dependence on Swp73p activity, and fusion of the GR N-terminus to a heterologous DNA-binding domain may lead to full independence from Swp73p. Further experiments exploring these possibilites should prove to be interesting.

Finally, a very interesting result was observed when the mutant GR, GR-N556(K461A), was tested for its transcriptional activity from the plfG3 response element. It appeared that the presence of wild-type Swp73p or Swi2/Snf2p actually inhibited GR(K461A) transcriptional activation. As was mentioned earlier, GR(K461A) acts as an activator at plfG3 in situations in which wild-type GR acts as a repressor. However, due to the point mutation in the DNA-binding domain, GR(K461A) acts as weak activator from TAT3-GRE. The prevailing theory is that the K461A mutation places GR in a conformation that leads to a misinterpretation of the repression signal at plfG3, which in turn, leads to transcriptional activation instead of repression (Starr et al. 1996) . Thus, if the K461A mutation is causing GR to undergo a

unique conformational change at plfG3 then perhaps interaction with the Swi/Snf complex inhibits this conformational change and/or it interferes with the function of GR(K461A) upon this change. Either way, mutations in Swi/Snf that abrogate its function may then relieve this possible inhibition and allow GR(K461A) to activate transcription from plfG3 even more potently. However, at TAT3-GREs GR(K461A) is Swi/Snf-dependent indicating that the possible interference by the Swi/Snf complex on GR-K461A activity is promoter specific. Whether this interference is promoter:activator specific (i.e. only GR(K461A) at plfG3) remains to be seen.

Chapter 4

Biochemical Analyses of Swi/Snf Assembly
and Swi/Snf Association with GR

Abstract

Biochemical experiments were performed to characterize Swi/Snf complex formation and GR:Swi/Snf interactions in wild type and various mutant yeast strains. Superose-6 chromatography indicated that deletion of Swp73p yields a complex similar in size to the wild type complex, whereas deletion of Snf2p results in a much smaller complex. Thus, the observed functional rank order (*SWP73/SNF2* > *swp73-1* >> *swp73Δ* > *snf2Δ*) may be explained by the residual activities of the Swi/Snf subcomplexes that assemble upon mutation or deletion of Swp73p or Swi2/Snf2p. Co-immunoprecipation experiments indicated that purified GR derivatives physically interact with Swi3p, Swp73p, and Snf6p, and thus most likely with the whole Swi/Snf complex. This interaction was maintained in several GR mutants with lesions in the enh2 region or the DNA-binding domain. Likewise, GR derivatives retained their interactions with Swi3p in extracts from the mutant strain Wswp73-1. These results suggest that Swi/Snf may be targeted to specific genes by physical interactions with transcriptional regulators, but the members of the complex required for these interactions can be different according to which regulator is present, as well as the nature of the DNA target site.

Introduction

Most activators tested in my collaborative studies with Brad Cairns displayed a rank order of activity in which the wild type strain (W303-1A)

displayed potent activity, Wswp73-1 displayed slightly diminished activity, Wswp73Δ displayed much weaker activity, and Wsnf2Δ displayed even weaker activity (i.e. W303-1A > Wswp73-1 >> Wswp73Δ > Wsnf2Δ). However, important differences were observed. Most dramatically, GR-N556(K461A), when tested from the plfG3 element, displayed a complete reversal of the rank order listed above. Also, Yap1p and GR-LexA both showed a strong dependence on the expression of Swi2/Snf2p for potent transcriptional activation, but no dependence on the expression of Swp73p. Interestingly, the presence of swp73-1p, in turn, caused Yap1 and GR-LexA to behave as they did in the absence of Swi2/Snf2p. Thus, these genetic studies indicated that Swp73p and Snf2p have overlapping functions, but also separate and distinct functions in certain contexts. In other words, the Swi/Snf complex is not a monolithic entity, but rather its individual components can be ascribed certain common, but also distinct, functions.

It is these important differences, though, that allow us to determine the distinct function of the individual components of the complex. Biochemical studies to date indicate that Swi/Snf complexes elute from gel filtration columns at positions predicted for globular proteins of approximately 2 MDa (Peterson et al. 1994; Cairns et al. 1994); deleting certain individual members of the complex (in particular, Swi1p, Swi2p, Swi3p, Snf5p, and Snf6p) results in subcomplexes that elute at half the apparent molecular weight of the wild type complex (Peterson et al. 1994). These subcomplexes may be partially functional, or they may be metastable but nonfunctional. Perhaps, then, the typical rank order of activity that was observed in my genetic studies, as well as the noted differences in behavior by Yap1p and GR-LexA, may be at least

partially explained by possible differential effects on complex formation upon deletion of Swp73p or Snf2p.

The reversal of the typical rank order when GR-N556(K461A) was tested at plfG3 is remarkable. One plausible explanation is that GR(K461A) interacts with Swi/Snf much in the same way that wild type GR is believed to do (Yoshinaga et al. 1992). However, when complex formation is perturbed by mutations or deletions of *SWP73* or *SWI2/SNF2*, GR(K461A) may interact with another chromatin remodeling complex (RSC, for example) that results in more potent transcriptional activation from plfG3. Presently, the behavior of GR(K461A) interaction with members of Swi/Snf is unknown.
Thus, in this chapter I present biochemical analyses that examine both the role of complex formation and GR:Swi/Snf interaction on activator function in wild type and mutant yeast strains.

Methods and materials

Yeast strains

The yeast strains used in this chapter (Table 4-1) were manipulated by standard techniques (Gietz et al. 1992; Sherman 1991; Sherman and Hicks 1991). All cultures were grown at 30°C.

Table 4-1. *Yeast strains*

Strain	Relevant Genotype	Source
W303-1A	*MATa, trp1-1 ura3-1 leu2-3,112 his3-11,15*	A. Tzagoloff
Wswp73-1[a]	*MATa, trp1-1 ura3-1 leu2-3,112 his3-11,15 swp73-1*	this study
Wswp73Δ[b]	*MATa, trp1-1 ura3-1 leu2-3,112 his3-11,15 swp73::LEU2*	this study
Wsnf2Δ[b]	*MATa, trp1-1 ura3-1 leu2-3,112 his3-11,15 swi2::LEU2*	this study

[a]W303-1A strain background.
[b]Derived from tetrad dissection of a W303 derivative.

Preparation of extracts and fractionation

Crude whole-cell extracts were prepared from cells harvested at OD_{600} 0.5-0.8, lysed by glass beads (0.5 mm) in extraction buffer [40 mM Hepes, pH 7.3; 350 mM NaCl; 0.1% Tween-20; 10% glycerol (v/v); 1 mM phenylmethylsulfonyl fluoride (PMSF); and 1x protease inhibitor cocktail (Boehringer Mannheim, Indianapolis, IN - cat. no. 1 836 153)] with 5 pulses, 50 sec each in duration, at maximum speed on a BioSpec Products (Bartlesville, OK) beadbeater. The extracts were clarified at 100,000 x *g* for 45 min in a TL-100.3 rotor. Protein concentrations of ~20 mg/ml were typically achieved. An aliquot (0.2 ml; 3-4 mg) was loaded onto a fast protein liquid chromatography Superose-6 (Pharmacia, Uppsala, Sweden) gel-filtration column (0.25 ml/min, equilibrated in extraction buffer), and 0.5 ml fractions were collected. The relevant fractions were precipitated in trichloroacetic acid and resuspended in 50 µl of 1x SDS sample buffer, pH 11. Aliquots of each precipitated fraction was analyzed by Western blot (see below). For W303-1A, one-tenth of the fraction precipitates (5 µl) were loaded on the SDS PAGE gel used for Western blot analysis, while similarly one-fifth of each fraction (10 µl) from the extracts of the mutant strains was used. Size standardization of the Superose-6 column was achieved by running a sample of Blue Dextran (2 MDa) over the column and then in a separate run a mixture of known size standards (all from Sigma kit # MW-GF-1000; also see the legend to Figure 4-1).

Antibodies

The antibodies used in this study include rabbit polyclonal α-Swi3p (Peterson and Herskowitz 1992), rabbit polyclonal α-Snf6p, α-Swp73p, and α-Rsc6p (all gifts from B. Cairns, Harvard Univ.), rabbit polyclonal α-Tup1p and α-Ssn6p (both gifts from Rebecca Smith, UCSF), and mouse monoclonal BuGR2 (α-GR; Gametchu and Harrison 1984).

The α-Swi3p, α-Snf6p, and α-Swp73p antibodies were pre-absorbed to fixed, spheroplasted cells of yeast strains that contained a genomic deletion of either *SWI3*, *SNF6*, or *SWP73*, respectively. In each case, the cells were fixed by adding formaldehyde to a final concentration of 5% to a 50 ml saturated culture grown in YPD media. The cells were incubated with shaking at RT for 1 hr. The fixed cells were washed extensively with water, resuspended in 1 ml of zymolyase solution (0.05 mg/ml of zymolase 100-T in 1.2 M sorbitol, 0.1 M potassium phosphate (pH 7.5), 28.6 mM β-mercaptoethanol), and incubated 40 min at 37°C. After spheroplasting, the cells were pelleted, washed extensively with PBS, and resuspended in 0.5 ml of PBS. A 100 µl aliquot of the ascites of each antibody to be pre-absorbed was mixed with 100 µl of the fixed, spheroplasted cells of the appropriate deletion strain. The mixture was incubated at 4°C with rocking for 1 hr. The mixture was pelleted, the supernatent removed to a fresh tube, and again 100 µl of the appropriate fixed, spheroplasted cells was added. After another 1 hr incubation at 4°C, the

process was repeated 3 times. The final supernatent was kept and used for future use.

Co-immunoprecipitations

The BuGR2 antibody was bound to magnetic beads covalently linked to goat anti-mouse IgG (Dynal, Lake Success, NY - cat # M-450) by incubation of the beads and antibodies in PBS + 0.1% BSA at 4°C. After an overnight incubation, the beads were collected magnetically and washed 4 times with low-salt buffer (40 mM Tris-Acetate, pH 7.9; 100 mM KOAc; 10% glycerol; 0.1% Tween-20). After washing, the beads were resuspended in the original bead volume with low-salt buffer.

The bacterially expressed and purified derivatives of rat GR (rGR) were obtained as described by Freedman et al. (1989; for the X556 rGR derivatives) and by Starr et al. (1996; for the EX525-HMK and EX525(K461A)-HMK derivatives of rGR). EX525(30IIB)-HMK (see Iñiguez-Lluhí et al. 1997 for a description of the 30IIB mutation) was obtained in a similar manner to EX525-HMK (Starr et al. 1996; Iñiguez-Lluhí, unpubl. results). In addition to the rGR protein sequences, the EX525-HMK derivatives contained a 5 amino-acid heart muscle kinase site (Arg-Arg-Ala-Ser-Val) fused at their carboxy termini (Starr et al. 1996).

Typical co-immunprecipitation reactions involved mixing 1 µl of yeast extract (~20 µg), prepared as described above for the Superose-6 chromatography experiments, with 1 µl of purified GR derivatives (~40 ng) in

18 µl of low-salt buffer. The reaction was incubated at 4°C for 45 min and then 15 µl of BuGR2-conjugated magnetic beads were added and the reaction was incubated at 4°C for another 2 hr. The beads were collected magnetically, the supernatent removed, and 12 µl of 4x SDS sample buffer was added to the supernatent. The pellet was washed 4 times with low-salt buffer and then resuspended in 30 µl of low-salt buffer + 10 µl of 4X SDS sample buffer. A quarter of the supernatent and the pellet was used for Western blot analysis.

Western analysis

Typically the proteins to be analyzed were electrophoresed on 7.5% SDS-PAGE. The proteins from the resulting gel were transfered to an Immobilon-P membrane (Millipore, Bedford, MA - cat # IPVH 000 10) by semi-dry electrophoresis. After transfer the membrane was incubated in PBS + 0.05% Tween-20 (PBST) containing 10% (w/v) non-fat dry milk for 3-4 hr at 4°C. The membrane was then removed and incubated at 4°C for 16 hr in PBST containing 2% non-fat dry milk and the appropriate primary antibody. After extensive washing in PBS, the membrane was incubated for 1 hr at RT in PBST containing 2% non-fat dry milk and the appropriate secondary antibody conjugated to horseradish peroxidase (diluted 1:3000). After extensive washing in PBS, the membrane was developed using enhanced chemiluminescent substrate (Pierce, Rockford IL. - cat # 34080). Exposure times were typically 15 sec to 5 min.

Results

Deletion of Swp73p and Swi2/Snf2p yield distinct Swi/Snf subcomplexes

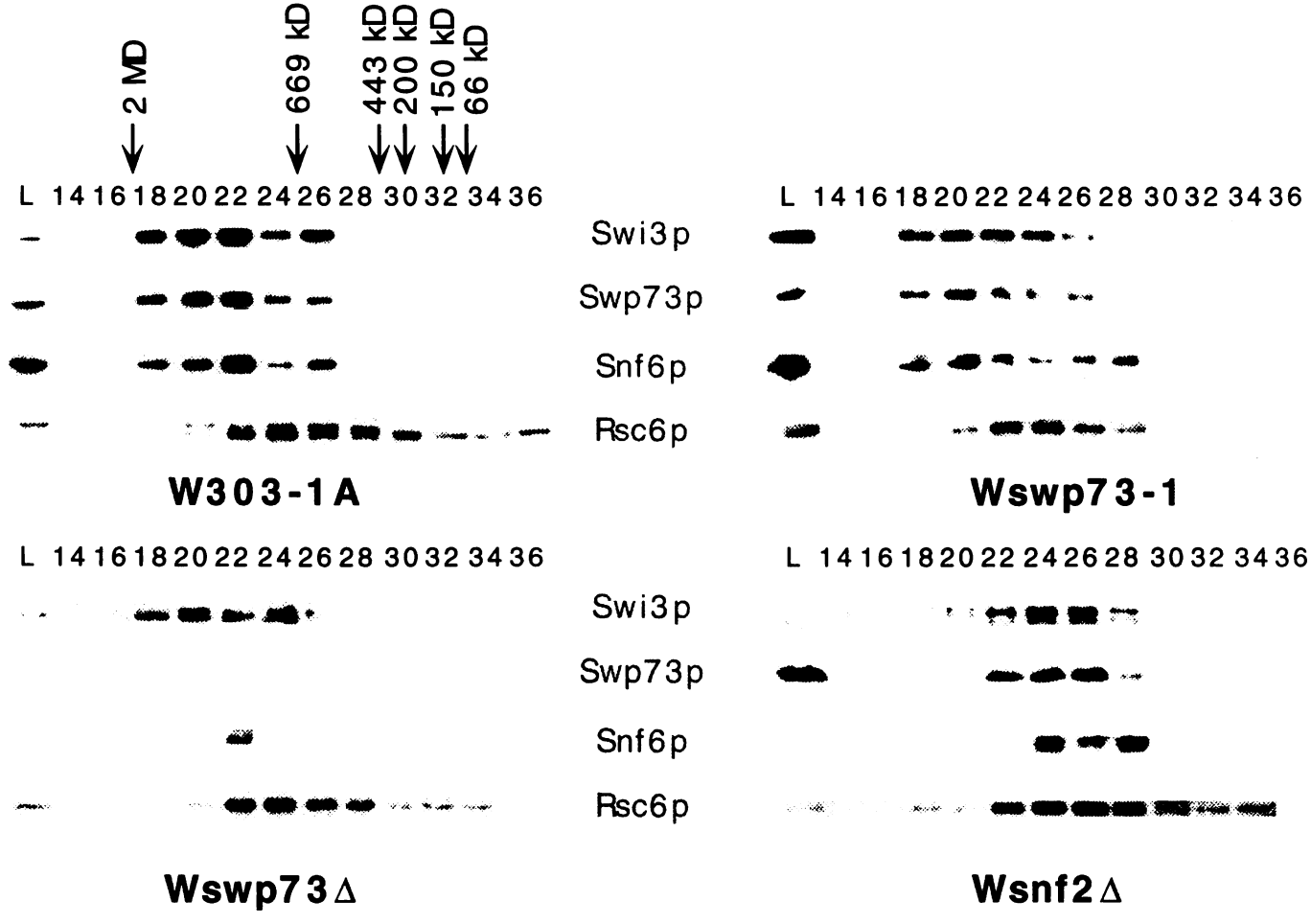
Previous experiments that examined complex formation of wild type Swi/Snf or those strains missing individual components of the complex (*swi1Δ, swi2Δ, swi3Δ, snfΔ, and snf6Δ*) involved chromatography of whole cell yeast extracts over a Superose-6 gel filtration column (Peterson et al. 1994). As mentioned in the Introduction of this chapter, deletion of the individual components of Swi/Snf indicated above result in subcomplexes that are roughly half the apparent molecular weight of wild type complexes (1 MDa versus 2 MDa) (Peterson et al. 1994), which may explain the reduced transcriptional activator activity observed in these strains. Thus, to understand more fully the genetic results of my activator studies I examined the formation of Swi/Snf complex in W303-1A, Wswp73-1, Wswp73Δ, and Wsnf2Δ by Superose-6 chromatography as described by Peterson et al. (1994) (also described in Methods and materials). The results of this chromatography are displayed in Figure 4-1. In the case of extracts from wild type W303-1A cells, Swi3p, Swp73p, and Snf6p co-elute in a peak fraction (#20) corresponding to a molecular complex of apparent molecular weight of ~1.6 MDa. This apparent molecular weight is smaller than the ~2 MDa reported for the wild type complex by Peterson et al. (1994), but conclusive calibrations at these fraction sizes on a Superose-6 column are difficult to achieve with commercially available molecular weight standards. Even so, the results with extracts from Wsnf2Δ parallel the prior results very well. Removel of

Figure 4-1. Chromatography of yeast whole cell extract from wild type and various mutant strains.

Whole cell yeast extract was prepared and chromatographed over a Superose-6 column as described in Methods and materials. Aliquots of the relevant eluted fractions were analyzed by Western blot. The fractions used in each blot are indicated. The yeast strain used in each extraction is indicated below each blot. The proteins analyzed for the blots (Swi3p, Swp73p, Snf6p, and Rsc6p) are indicated next to each row of bands. Note that the relative positions of the bands are not indicative of their molecular weight. For blots of samples from W303-1A, exposure times typically were 15-30 sec, while for samples from Wswp73-1 the times were 30-60 sec. For Wswp73Δ and Wsnf2Δ the times were 3-5 min.

"L" = an aliquot of the extract sample loaded onto the Superose-6 column. For W303-1A and Wswp73-1, "L" = 20 μg of total protein extract. For Wswp73Δ, "L" = 100 μg of total protein extract, while for Wsnf2Δ "L" = 75 μg of total protein extract. The peak fractions in which the size standards eluted (see Methods and materials) are indicated by arrows in the blot for W303-1A extract. The following peak elutions were observed: Blue Dextran (2 MDa) - fraction 17, thyroglobulin (669 kD) - fraction 25, apoferritin (443 kD) - fraction 29, β-amylase (200 kD) - fraction 30, alcohol dehydrogenase (150 kD) - fraction 32, and bovine serum albumin (66 kD) - fraction 33.

Superose-6 Chromatography of Yeast Whole Cell Extract from Wild Type Cells and Various *swi* Mutant Strains



Swi2/Snf2p from this strain results in a subcomplex of Swi/Snf that displays a co-elution of Swi3p, Swp73p, and Snf6p in a peak fraction of apparent molecular weight ~0.8 MDa. Thus, deletion of Swi2/Snf2p yields a substantially different residual Swi/Snf complex consistent with the findings of Peterson et al. (1994). As a control, I examined complexes containing Rsc6p, a homolog of Swp73p that resides in a second chromatin remodeling complex in yeast (see the Introduction to Chapter 3). Rsc6p displayed an identical fractionation profile in all four strains tested.

Interestingly, extracts from Wswp73-1 and Wswp73Δ strains did not display this 2-fold reduction in apparent molecular weight. In Wswp73-1 extracts, Swi3p, Swp73p, and Snf6p were assembled into similar to wild type Swi/Snf (peak elution in fraction #20). The results were less clear for extracts from Wswp73Δ because Swp73p elution was absent and Snf6p gave only a single band (in fraction #22, which corresponds to an apparent size of ~1.2 MDa). Swi3p, though, appeared to assemble into a complex similar to wild type Swi/Snf. Thus, it appears that Swp73p, unlike Swi2/Snf2p, is not required for assembly of the Swi/Snf complex, but is required for proper function.

Levels of Swi3p are reduced in swp73 *and* snf2 *mutants*

While it appears that Swp73p is not required for assembly of Swi/Snf, it may still be required for its accumulation. Thus, the rank order that was typically observed in my genetic analyses might be partially explained by a loss of accumulation of the residual Swi/Snf complex that remains after mutation

or removal of some of its component members. The levels of the Swi/Snf complex was determined by measuring the steady-state accumulation of Swi3p in both whole cell extracts and in the peak elutions from the Superose-6 fractionation of extracts from the wild type and three mutant strains. The results are presented in Figure 4-2. Samples of whole cell extract and the peak Superose-6 elutions from W303-1A, Wswp73-1, Wspw73Δ, and Wsnf2Δ were analyzed by Western blot. It appears that in total extract Swi3p levels are down ~5-fold in the Wswp73-1 strain, though there appears to be ~20-30-fold less fully-assembled Swi3p in the peak elution of Swi/Snf in these same extracts. Likewise, compared to W303-1A total Swi3p levels are down ~300-fold in Wswp73Δ and down ~100-fold in Wsnf2Δ. This loss in accumulation of Swi3p is certainly a partial explanation of my genetic results (see Discussion).

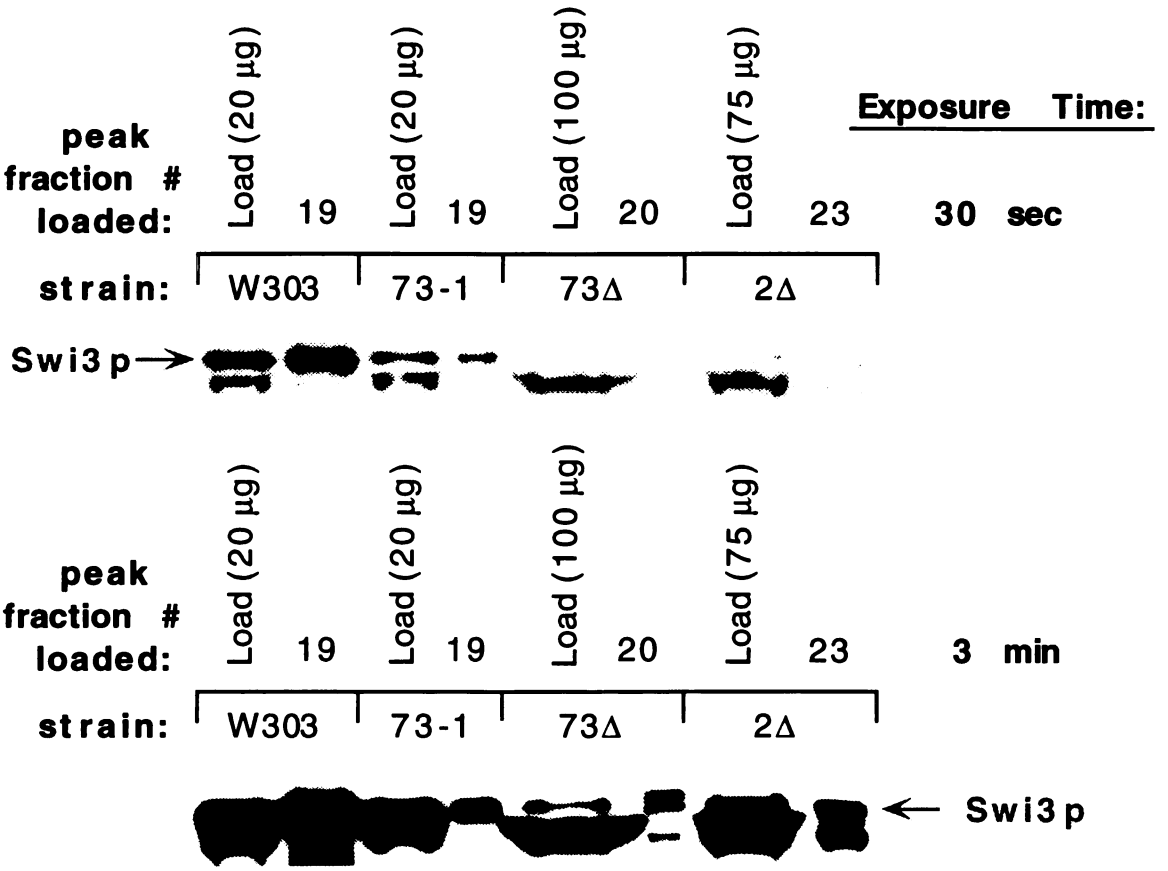
Derivatives of GR interact with the Swi/Snf complex

As mentioned in the Introduction of this chapter, one explanation for the GR-N556(K461A) results is that this derivative may interact with an alternative chromatin remodeling complex in the absence of fully functional Swi/Snf that in turn leads to more potent transcriptional activation at the plfG3 element. To examine this notion, I monitored the interactions of GR derivatives with various Swi/Snf complexes. It is known that GR will co-immunoprecipitate (co-ip) Swi3p and that this co-ip requires the presence of Swi1p and Swi2p (Yoshinaga et al. 1992). It is not known, though, whether GR is interacting with assembled Swi/Snf complexes or only with the free

Figure 4-2. Relative levels of Swi3p in wild type and various mutant strains.

Samples from the Superose-6 fractionation were re-examined on a separate gel in order to determine the relative levels of Swi3p in each of the strains used for fractionation. The strain from which the extract was derived is indicated above each set of bands. The first band in each set is an aliquot of the load used on the column (the amount of total protein analyzed on the gel is indicated in parentheses), while the second band in each set represents an aliquot of the fraction (the number of which is indicated) that displayed the peak elution of Swi3p for each strain tested. The blot was probed for Swi3p and exposed for either 30 sec or 3 min.

Relative Amounts of Total and Assembled Swi3p Produced in Wild Type Cells and Various *Swi* Mutants



pools of Swi3p, and, in the absence of Swi1p and Swi2p, Swi3p pools may be so diminished (assuming that Swi1p, as with Swi2p and Swp73p - see Figure 4-2, also plays a role in Swi3p accumulation) that GR has nothing to interact with. To test this notion I repeated the immunoprecipation of bacterially expressed and purified derivatives of GR mixed with yeast whole cell lysates and probed the pellets of these immunoprecipitations for other members of the Swi/Snf complex. The results of these co-ips are presented in Figure 4-3. Figure 4-3A shows that a purified derivative of GR (EX525-HMK GR) containing a fusion of the enh2 region (amino acids 107-318; Godowski et al. 1988; Hollenberg and Evans 1988) with the DNA-binding domain (DBD) (amino acids 408-525; Freedman 1988) will co-ip Swi3p, Swp73p, and Snf6p. A similar GR derivative, but containing the K461A mutation [EX525(K461A)-HMK GR], co-ips these three proteins identically. This co-ip between GR and Swi/Snf is dependent on the presence of GR since yeast extract that was not mixed with these purified derivatives of GR displayed negligible levels of Swi3p, Swp73p, and Snf6p in the pellet fraction.

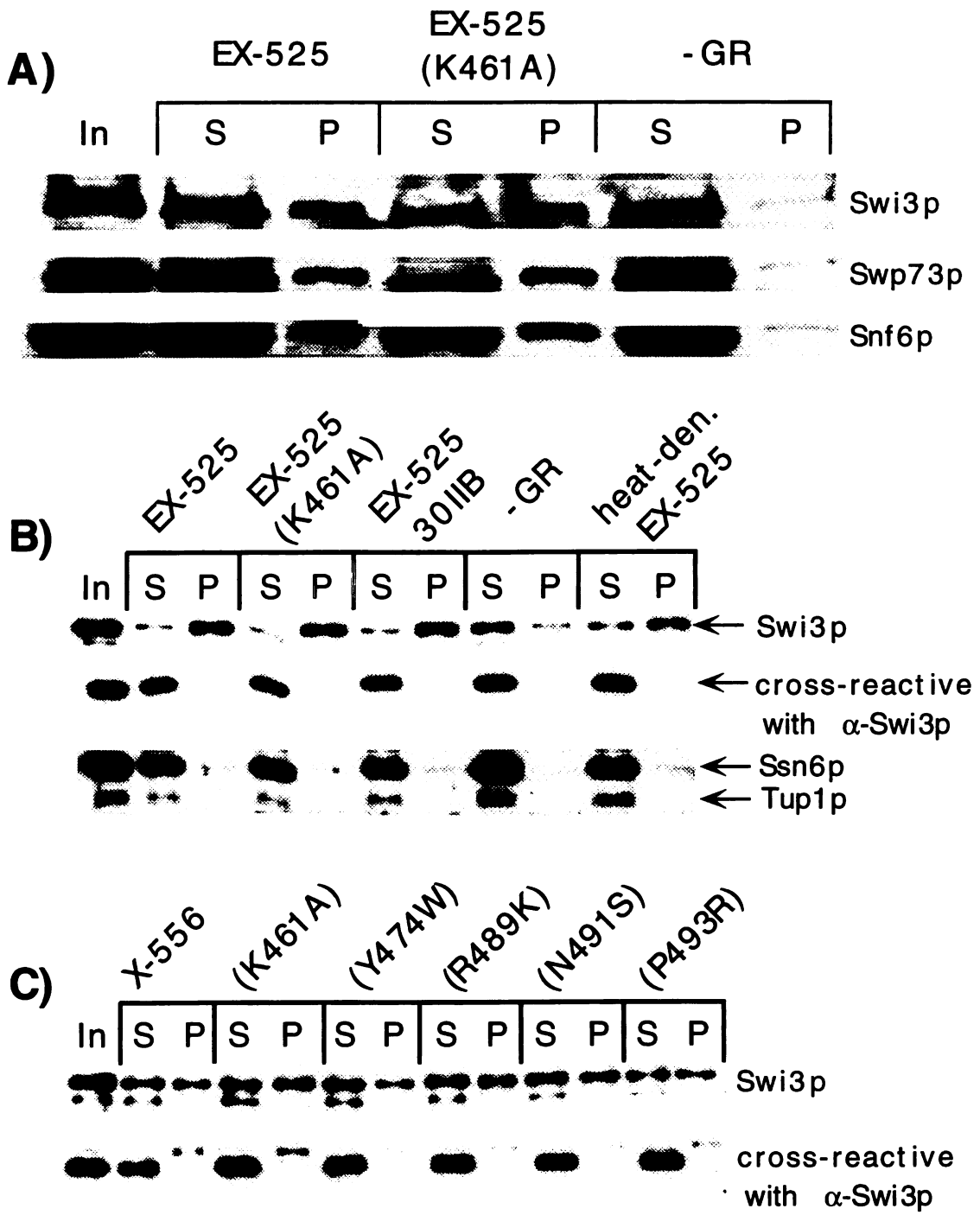
As shown in Figure 4-3B, the co-ip between EX525-HMK GR and Swi3p can be optimized (see Methods and materials and the legend to Figure 4-3) so that nearly 100% of the Swi3p is in the pellet fraction, whereas neither a cross-reactive band to the α-Swi3p antibody nor Ssn6p or Tup1p is in the pellet fraction. This high efficiency of co-ip between GR and Swi3p is also true of the same derivative of GR harboring either the K461A mutation or the 30IIB mutation (a 3-amino acid mutant in the enh2 region of GR that abrogates transcriptional activation; Iñiguez-Lluhí et al. 1997). Interstingly, if EX525-

Figure 4-3. Co-immunoprecipitation of various rat GR (rGR) derivatives and members of the Swi/Snf complex.

Whole cell extracts from W303-1A (see Methods and materials) were incubated with bacterially expressed, purified derivatives of GR. GR was then immunoprecipated from these samples (see Methods and materials) and the supernatent and pellet fractions were analyzed by Western blot. *(A)* 200 μg samples of yeast extract from W303-1A were mixed with 1 μg of either bacterially expressed and purified EX-525 (the ENH2 region of rGR fused to the DNA-binding domain of rGR), EX-525(K461A) (the same as EX525 but harboring the lysine to alanine mutation at amino acid position 461), or no GR. These mixtures were immunoprecipitated (IPed) with a monospecific α-GR antibody. "In" = the input of the IP reaction. "S" = one-half of the supernatent of the IP reaction. "P" = the pellet of the IP reaction. The blot was probed for Swi3p, Swp73p, and Snf6p, as indicated. Note that the relative positions of the bands do not correspond to the relative molecular weights of the proteins analyzed. *(B)* the same experiment as in panel (A) was perfomed except that only 20 μg of yeast extract and only 40 ng of each derivative of rGR was used. "In" = 1/4 of the input of the IP reaction. "S" and "P" = 1/4 of the supernatent and pellet of the IP reaction, respectively. The labels of the rGR derivatives used are the same as in panel (A), except that "EX525-30IIB" contains a 3-amino acid mutation in the ENH2 region of rGR (Iniguez-Lluhi et al. 1997) and "heat-den. EX-525" = heat denatured EX-525 achieved by boiling a sample of EX-525 for 5 min. Also, 800 ng of heat-denatured EX-525

was used for the IP reaction. The blot was probed for Swi3p, Ssn6p, and Tup1p, as indicated. As a negative control, a single cross-reactive band to the α-Swi3p antibody is also displayed. Note that the relative positions of the bands do not correspond to the relative molecular weights of the proteins analyzed. *(C)* An identical experiment as in panel (B) was performed except that the derivatives of rGR used only contain the DNA-binding domain (amino acids 408 - 556). Mutant derivatives of X-556 were also used and the mutations that they harbor are indicated in parentheses above each set of bands. The blot was only probed with the α-Swi3p antibody.

Co-Immunoprecipitation of GR derivatives and Swi/Snf



HMK GR is denatured by boiling for 5 min it can still co-ip Swi3p, though the efficiency is diminished somewhat.

The region of purified GR that interacts with Swi3p can be delineated further using derivatives of GR that contain only the DBD (X556 GR - amino acids 408-556; Freedman et al. 1989). Figure 4-3C shows that wild type X556 GR, and various point mutants of this derivative, all interact with Swi3p, though the efficiency of co-ip is somewhat reduced compared to that observed with EX525-HMK GR.

I tested the co-ip between either EX525-HMK GR or EX525(K461A)-HMK GR and Swi3p in extracts from W303-1A, Wswp73-1, and Wswp73Δ (data not shown). Again, EX525-HMK GR and EX525(K461A)-HMK GR co-immunoprecipitated with Swi3p identically in W303-1A extracts. In Wswp73-1 extracts, the overall level of Swi3p is down somewhat as shown above, but the co-ips between these two derivatives of GR and Swi3p in extract from this mutant strain are identical. Unfortunately, Swi3p was not detected in the Wswp73Δ sample in this experiment.

Discussion

Results from my previous genetic experiments indicated that most of the transcriptional activators tested were slightly diminished in activity in Wswp73-1, and displayed a much further drop in activity in Wswp73Δ, and an even further decline in Wsnf2Δ. This rank order (i.e. W303-1A > Wswp73-1 >> Wswp73Δ > Wsnf2Δ) may be explained by the residual complexes that remain after mutation or deletion of Swp73p and Swi2/Snf2p.

Superose-6 fractionation experiments suggested that Swp73p, unlike Swi2/Snf2p, is not required for assembly of the complex but is required for function, at least in most contexts. However, Swp73p, like Swi2/Snf2p, is required for the steady-state accumulation of Swi3p, and perhaps other members of the complex. Thus, the rank order that is observed with most of the transcriptional activators tested may be explained by the fact that the presence of swp73-1p still allows complete complex formation to occur, but that this mutation results in a complex that is either slightly less functional and/or slightly less abundant than wild type complex (see Figures 4-1 and 4-2). As a result, the transcriptional activators typically displayed diminished activity in Wswp73-1 as compared to W303-1A.

The complete absence of Swp73p also still allows a high molecular weight complex to form that is very similar to wild type complex (at least as can be determined by Swi3p assembly), but again this residual complex may have even less function and/or be even less abundant than the swp73-1p-containing complex. These possibilities may explain why most of the transcriptional activators commonly displayed a strong drop-off in activity in Wswp73Δ as compared to Wswp73-1. Finally, deletion of Swi2/Snf2p from the complex results in a residual complex that is 2-fold reduced in apparent molecular weight and that also may be less abundant. These factors probably result in a complex that is fairly stable but nonfunctional, which probably explains why Wsnf2Δ is last in the rank order. Thus, it appears that the biochemical fractionation data has recapitulated the genetic data.

The rank order of activity, interestingly, is affected by regulator and DNA response element contexts. For example, when Yap1p was tested from

two SV40 AP-1 sites or when GR-LexA was tested from a single *lexA* site upstream of the *GAL1* minimal promoter (see Chapter 3 for details), a strong dependence on Swi2/Snf2p expression was observed, while a total lack of dependence on Swp73p was seen. However, the presence of swp73-1p resulted in activity that looked like the activity measured in the absence of Swi2/Snf2p. The differential dependence of Yap1 and GR-LexA on Swi2/Snf2p and Swp73p may also be explained by the residual complexes that remain upon mutation or deletion of these two members of Swi/Snf. The strong dependence of Yap1p and GR-LexA on Swi2/Snf2p may reflect the fact that removal of this protein results in a smaller, and probably nonfunctional, subcomplex of Swi/Snf. Their lack of dependence on Swp73p, in turn, may follow from the observation that this protein is not essential for complex assembly. While there may be less complex around and it is missing Swp73p, what is assembled may be enough to give near wild type transcriptional activation, at least for these two activators from the promoter elements tested.

The mutation *swp73-1* represents an interesting gain-of-function with regard to Yap1p and GR-LexA since these two activators are inhibited in their activity in the presence of this mutant protein. The Superose-6 fractionation experiments indicate that swp73-1p does not interfere with complex assembly, though the *swp73-1* mutation does reduce complex accumulation as judged by Swi3p levels (see Figure 4-2). the complex bearing this mutant protein appears to interact with Yap1p and GR-LexA, but this interaction seems to interfere with function.

Co-immunoprecipitation (co-ip) experiments with a purified derivative of GR (EX-525) indicated an interaction between GR and Swi3p,

Swp73p, Snf6p, and thus likely the whole Swi/Snf complex. Furthermore, additional co-ip experiments with the X556 derivatives of GR, along with various point mutants of this derivative, show that the DNA-binding domain is sufficient for a co-ip between GR and Swi3p. Based on these results and the GR-LexA fusion results, perhaps the minimal interaction domain of GR and Swi/Snf is somewhere between amino acids 408 and 452. The epitope of the α-GR antibody BuGR2 is between 408-416 (Ip et al. 1991), so most likely this region is not in the interaction domain because otherwise it would be masked from the antibody. Also, it seems likely that the Swi/Snf interaction would be conserved among the nuclear receptors, so a probable region for interaction is somewhere in the first Zn-finger since this is a region that falls between 440 and 452 and is of high homology, whereas the region between 416 and 440 is of lower homology. It should be noted that the efficiency of co-ip between X556 GR and Swi3p was diminished compared to EX525 GR. Thus, other regions of GR may potentiate this interaction, although a 3-amino acid lesion in the enh2 region of GR (the 30IIB mutant) did not interfere with this co-ip. Also, since mutant derivatives of GR that are diminished in their ability to bind DNA can still co-ip Swi3p, then perhaps the interaction between GR and Swi/Snf does not require DNA binding.

The reversal in the typical rank order that was observed when GR-N556(K461A) was tested at plfG3 is another interesting difference among the various transcriptional activators that have been compared. In this case, the distict complexes that form in the absence of Swp73p or in the absence of Swi2/Snf2p behave similarily with regard to GR(K461A) function. Perhaps in response to the low abundance of the mutant complexes GR(K461A) interacts

with another chromatin remodeling complex that leads to increased transcriptional activation. For example, RSC (a second chromatin remodeling complex in yeast) is much more abundant and much more potent in its remodeling activity than Swi/Snf (Cairns et al. 1996). Notably, however, the interaction between GR and Swi/Snf is not abrogated by the presence of the K461A mutation. An identical co-ip between either purified wild type GR or purified GR(K461A) was observed with Swi3p, Swp73p, and Snf6p in extracts from W303-1A. Likewise, these two derivatives of GR co-ip Swi3p identically in extracts from Wswp73-1. Results of the co-ip in extracts from Wswp73Δ, unfortunately, are still inconclusive due to the low levels of Swi3p in this strain. Even so, the idea that mutations in Swi/Snf that lead to diminished levels of assembled complex allow GR(K461A) to interact with a second chromatin remodeling complex seems to be less probable. Instead it appears that Swi/Snf negatively interferes with GR(K461A) function at the plfG3 element.

DNA can act as an allosteric effector of transcriptional activators (Lefstin et al. 1994). Perhaps, then, the behavior of GR(K461A) at plfG3 in mammalian cells (Starr et al. 1996) may be due to a different conformational shift induced in this mutant GR when bound at this response element as compared to wild type GR bound at the same response element. This putative allosteric effect on GR(K461A) induced by plfG3 may result in novel functions by GR that relieve the necessity of interaction with Swi/Snf-like compexes. Thus, if the interaction domain between GR and Swi/Snf is in the first Zn-finger of the DBD, then the negative interference of Swi/Snf on GR(K461A) function in yeast could be explained further. Possibly, wild type

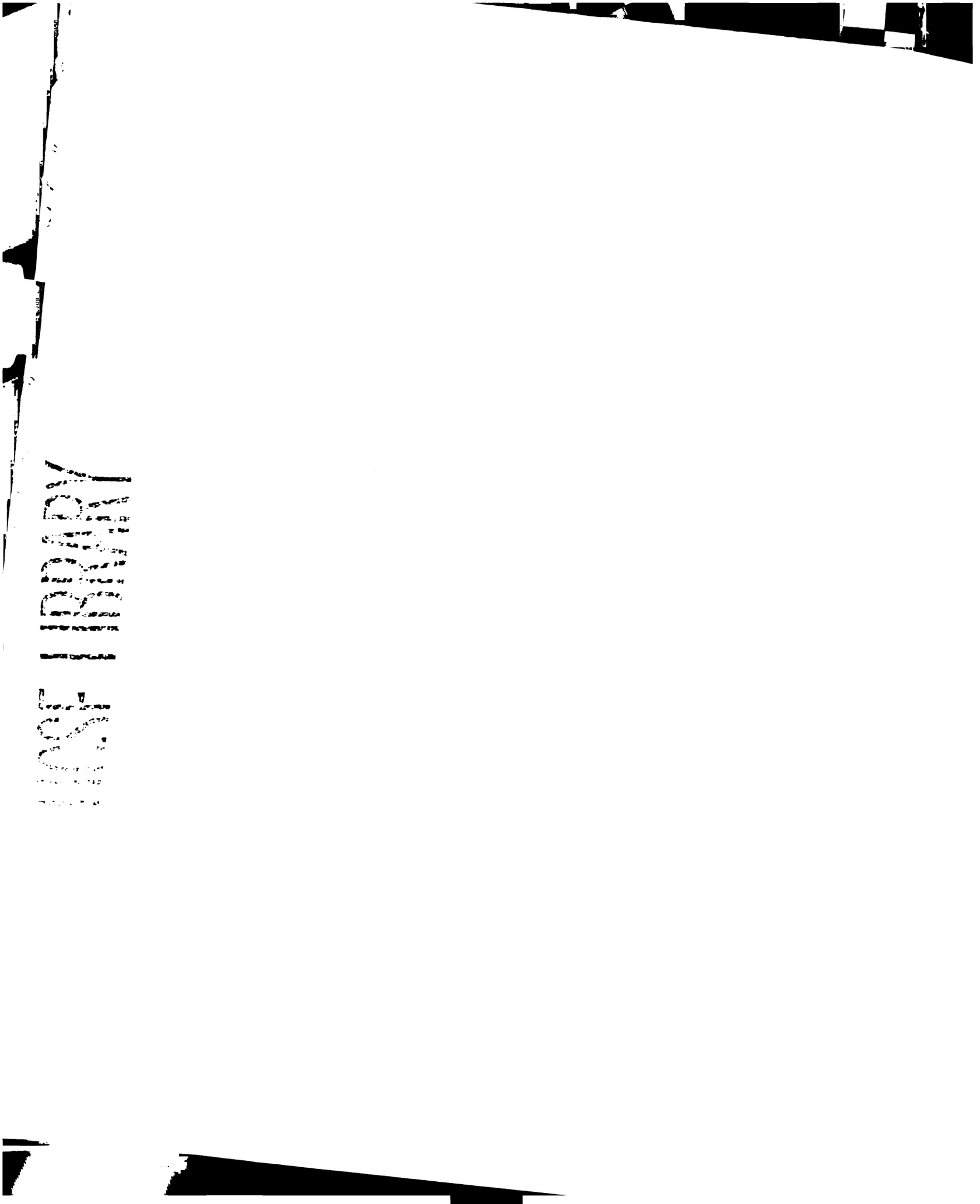
Swi/Snf interacts with GR(K461A) in a region of the DBD near the mutation in such a way that the complex may interfere with the conformational shift induced by plfG3 or interfere with the novel function gained by this conformational shift. Upon mutation of the Swi/Snf complex, this interaction with GR(K461A) is altered such that the interference of Swi/Snf on the potentiation of transcriptional activation by the K461A mutation is diminished. This alteration may be due to changes in the structure of Swi/Snf or by the resulting diminished levels of the complex.

It should be noted that the interference of Swi/Snf on the activity of GR(K461A) is context specific since GR(K461A) activity when tested from a simple GRE (TAT3-GRE; see Chapter 3 for details) is reduced in the mutant strains. Thus, so far, Swi/Snf interaction with GR(K461A) is only counter-productive when GR(K461A) activity is tested at plfG3. By this view, GR(K461A) either does not undergo the same sort of conformational change at a simple GRE or the response element is structured in such a way that Swi/Snf-interaction in a nearby region of the DBD does not interfere with function. These results point to the more general conclusion that not only can different members of the Swi/Snf complex have distinct functions, but that these distinct functions are activator- and response element-dependent.

So far, GR is the only transcriptional activator that has been shown to interaction physically with the Swi/Snf complex. While, as pointed out above, it is most likely that Swi/Snf is interacting with GR through its DBD, it is not known if this interaction is direct or indirect. For example, it has been suggested that Swi/Snf is a component of the RNA pol II holoenzyme (Wilson et al. 1996), but there is some controversy surrounding those results

(Cairns et al. 1996). Also, it is known that mutations in members of the Ada complex, which contains the histone acetyltransferase Gcn5p (Grant et al. 1997), have Swi$^-$ and Snf$^-$ phenotypes and it has been suggested that this complex physically interacts with Swi/Snf (C. Peterson, pers. comm.). Interestingly, the Ada complex was originally hypothesized to be an adaptor complex that physically linked transcriptional activators to the basal transcriptional machinery (Marcus et al. 1994). So it is conceivable that GR is interacting with Swi/Snf through the Ada complex. This possibility, though, is unlikely because the GR:Swi3p co-ip is not abolished in *ada2Δ*, *ada3Δ*, or *gcn5Δ* strains (C. Peterson, pers. comm.).

Needless to say, many more interesting experiments can be performed to examine more closely the relationship not only between GR and the Swi/Snf complex but many more activators, as well. A further discussion of these experiments will be given in Perspectives.



Chapter 5

Homologs of Swp73p

Abstract

All eucaryotes must contend with the repressive effects of chromatin and they appear to have evolved large multiprotein complexes that "remodel" chromatin structure. Swp73p has many homologs, including those in yeast, worms, mice, and man. These homologs probably represent a family of proteins with parallel functions in Swi/Snf-like complexes among different species. In addition, other predicted proteins exist that display more limited homology to the Swp73p family. These predicted proteins, along with the Swp73p family, all share a so-called YIKes motif that may represent a hydrophobic core region important for the function of these proteins. The G323D mutation in swp73-1p is only 10 amino acids upstream from this motif, perhaps explaining the phenotypic effects of this protein.

Introduction

The paradox involving the problem of compacting copious amounts of genetic material into a small space is obviously one that faced eucaryotes as soon as they appeared during evolution. Thus, it would not be surprising that the mechanisms that have evolved to deal with this problem also arose early on in eucaryotic development and would be seen in most eucaryotes studied. This scenario does appear to be the case, especially with regard to the Swi/Snf complex. Homologs of Swi/Snf and its members are seen throughout evolution.

Swi2/Snf2p represents a class of ATP-dependent proteins with several common sequence motifs (Laurent et al. 1993). The function of many of these Swi2/Snf2p homologs is unknown. But two of them appear in other complexes that are involved in chromatin remodeling. Sth1p is a member of the RSC complex in yeast (Cairns et al. 1996b) and I-SWI (which stands for Imitation-SWI) is a member of both NURF (Tsukiyama et al. 1995) and ACF (Ito et al. 1997) in *Drosophila*. Other Swi2/Snf2p homologs include *brm*, a member of the brm complex in *Drosophila*, which may also be involved in chromatin remodeling, and the human proteins, BRG1 (brm/Swi2p-related gene 1, also known as hSNF2α) and hbrm (human brm, also known as hSNFβ).

Drosophila brahma (brm) protein is involved in activation of homeotic genes of the Antennapedia and Bithorax complexes, and mutations in *brm* suppress mutations in *Polycomb*, a negative regulator of the homeotic genes (Tamkun et al. 1992). In addition, there are two murine versions of Swi2/Snf2p, called appropriately enough mbrm and brg1 (Muchardt and Yaniv 1993; Randazzo et al. 1994). *brg1* was cloned by sequence homology and its gene product is widely expressed in the developing mouse embryo, especially in the nervous system, and is found in adult tissue as well (Randazzo et al. 1994). It has been postulated that it plays a role in Hox gene regulation, which would correlate with brm's role as a regulator of homeotic genes in *Drosophila*. Interestingly, brg1 was identified in a two-hybrid screen for Rb-interacting proteins, and it interacts with Rb *in vitro* (Dunaief et al. 1994). Rb is a tumor suppressor protein that binds to various transcription

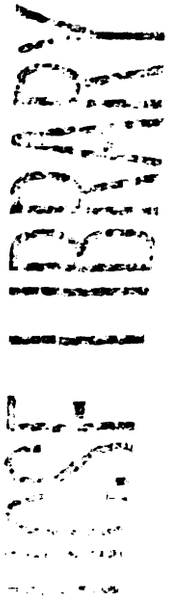
factors involved in cell-cycle progression or differentiation. Thus, it is intriguing to speculate that Rb might regulate brg1 function.

All the members of the Swi2/Snf2p family are large proteins (>1500 amino acids) and contain seven sequence motifs related to those found in DNA-stimulated ATPases and DNA helicases. What role these motifs play in the function of these proteins is unknown, but single amino acid changes in several of these motifs lead to an abrogation of Swi2/Snf2p function *in vivo*.

The two major sequence motifs of the Swi2/Snf2p family members are the DNA-stimulated ATPase domain and the bromodomain. The bromodomain appears in many proteins, but its function is unknown. In fact, the bromodomain can be deleted from *SWI2* without any apparent consequence on Swi/Snf function. The DNA-stimulated ATPase domain, however, appears to function as such. Purified Swi2/Snf2p exhibits weak ATPase activity that is stimulated 8-fold by double-stranded DNA (Laurent et al. 1993). A single amino acid change in the putative nucleotide-binding site abolishes this activity. Furthermore, purified Swi/Snf complex from *S. cerevisiae* has an even more potent ATPase activity (60 pmol per min per pmol of purified complex) that is stimulated 30- to 50-fold by double-stranded, single-stranded, or nucleosomal DNAs (Cairns et al. 1994; Côté et al. 1994). Purified complex containing a mutant Swi2/Snf2p, however, does not contain this activity. Unfortunately, the role of the DNA-stimulated ATPase activity in Swi/Snf function has not been identified firmly, but one may imagine that Swi/Snf may use the energy from ATP hydrolysis at a specific point(s) during its role in chromatin reorganization.

In addition to Swi2/Snf2p homologs, many of the other members of the complex have homologs as well. Swi3p is a homolog of Rsc8p, which is a member of the RSC complex in yeast. And Rsc8p has recently been shown to interact with the Swi2/Snf2p homolog, Sth1p (another member of the RSC complex), by co-immunoprecipitation and in the two-hybrid system (Treich and Carlson 1997). In addition, two human homologs of Swi3p, BAF155 and BAF170, have been identified (Wang et al. 1996). Also, ENL and AF9, two proteins that are involved in acute human leukemia (such as t(11; 19) leukemia and t(9; 11) leukemia, respectively (Rubnitz et al. 1994)), are similar in sequence to Swp29/Tfg3p (Cairns et al. 1996a; Welch and Drubin 1994). Finally, Snf5p-related proteins have been identified in *Drosophila* (snr1), humans (hSNF5/Ini1, BAF47), and *Caenorhabditis elegans*. The human Ini1 (integrase interactor 1) protein is very interesting because it was identified in a two-hybrid screen with the HIV-1 integrase, the enzyme responsible for integrating the viral DNA into the host genome, and purified Ini1 interacts with HIV-1 integrase *in vitro* (Kalpana et al. 1994). This interaction provides the exciting speculation that if Ini1 is a member of a Swi/Snf-like complex then its function might be exploited by the HIV-1 integrase to integrate the viral DNA into transcriptional active regions of the host genome.

Studying Swi/Snf activity in yeast has proven to be very interesting, but what makes it all the more interesting is that similar complexes appear to exist in higher organisms. As was mentioned earlier, the brm complex has been identified in *Drosophila* and a large complex has been isolated from HeLa cells (Kwon et al. 1994; Ostlund Farrants et al. 1997). The human complex disrupts nucleosomal structure and facilitates the binding of



transcription factors. Another complex was isolated from human YT cells and contains BRG1 (the homolog of Swi2/Snf2p), BAF47 (the homolog of Snf5p), and BAF155 and BAF170 (the homologs of Swi3p) (Wang et al. 1996). Likewise, clones of BAF155 and BAF170 were isolated from cDNA from Jurkat T-cells (Wang et al. 1996).

So far the only transcription factor that has shown a physical interaction with the Swi/Snf complex has been GR. GR has been shown to co-immunoprecipitate Swi3p, and antibodies to Swi3p have been shown to negatively interfere with GR-directed transcription in a *Drosophila* nuclear extract (Yoshinaga et al. 1992). Based on these results, and the remarkable conservation of GR activity in yeast, it seems likely that GR will interact with a Swi/Snf-like complex in mammalian cells. Indeed, tissue culture experiments have shown a stimulation of GR-dependent transcriptional activation in the presence of co-transfected hbrm (Muchardt and Yaniv 1993). Also, GR has been observed to stimulate nucleosomal disruption of a GRE-containing 95 bp DNA fragment using Swi/Snf complex purified from either HeLa cells or rat liver nuclei (Ostlund Farrants et al. 1997)

In this chapter, I examined the existence of homologs of Swp73p via computer database analysis in order to extend further the classification of the families of the various Swi/Snf members and to identify possible regions of Swp73p that are important for its function.

Methods and materials

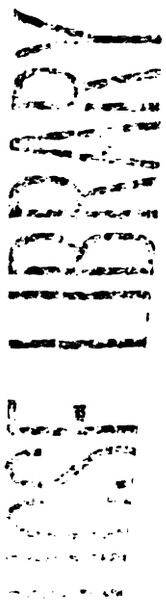
Sequence analyses

Homologs of Swp73p as of 7/1/1997 were identified with the program BLAST at the National Center for Biotechnology Information (Altschul et al. 1990). The following parameters were used: database, non-redundant; expect, 10; Cutoff, by default calculate using the expect values; matrix, blosum 62; word length, 3. Multiple sequence alignment was performed with a combination of the program 'PILEUP' (Genetics Computer Group, Madison, WI), the alignments derived from the BLAST output, and visual inspection. The BestFit program (Genetics Computer Group) was utilized to determine the percent identity and percent similarity for each of the pair-wise combinations presented in Table 5-1. The parameters used in the GCG programs were: gap creation penalty = 2.0 and gap continuation penalty = 0.1.

Results

The amino sequence of Swp73p is highly similar to the peptide sequence of many other proteins

The protein sequence of Swp73p was compared to other proteins in the NCBI database with the BLAST program, which identified significant similarity to the polypeptides encoded by one partial and ten full open



reading frames: *AgTHR4gns* (from *Ashbya gossypii*, a filamentous fungus), *YCR052w/RSC6* (from *S. cerevisiae*), *D15Kz1* (from *mus musculus*), ZK1128.5 and C18E3.2 (both from *Caenorhabditis elegans*), and BAF60a, BAF60b, and BAF60c (all from *Homo sapiens*), *YOR295w* and *YMR233w* (both from *S. cerevisiae*), and *T24G10.2* (from *C. elegans*) (Altmann-Jöhl and Philippsen 1996; Oliver et al. 1992; Johnston et al. 1989 and Wang et al. 1996; M. Burks, unpublished and R. Waterston, unpublished; Wang et al. 1996). As calculated by the program BestFit, Swp73p is 48% identical to AGTHR4GNS protein over 210 amino acids, 33% identical to Rsc6p, 30% identical to D15Kz1 protein, 26% identical to ZK1128.5 protein, 31% identical to C18E3.2, approximately 30% identical to each of the three BAF60 proteins, 23% identical to Yor295p, 26% identical to Ymr233p, and 23% identical to T24G10.2 (see Table 5-1).

Alignment of the twelve related protein sequences showed that the similarity among them extends throughout their length (Figure 5-1). Also a dendrogram illustrates the clustering of each of the peptide sequences into a family tree (Figure 5-2). The amino terminal portion of the protein encoded by *AgTHR4gns* is not yet available, but the extent of homology with Swp73p in the C-terminal region suggests that the two proteins are true homologs and that the C-terminal portion is important for function. The two *S. cerevisiae* proteins, while similar in sequence, could not substitute for one another, as neither high copy nor centromeric plasmids bearing *RSC6* could suppress the Ts^- phenotype of either Wswp73-1 or Wswp73Δ (data not shown). Nor could *SWP73*-containing plasmids rescue the lethal phenotype of *rsc6Δ* strains. Only the yeast protein Rsc6p contains a glycine residue at the position corresponding to that of the glycine to aspartic acid mutation in swp73-1p

Table 5-1. *The percent identity and similarity between pair-wise combinations of the polypeptides in the Swp73p family*

	AGTHR4GNS	Rsc6p	ZK1128.5	C18E3.2	D15Kz1	BAF60a	BAF60c	BAF60b	Yor295p	Ymr233p	T24G10.2
Swp73p	48/65	33/56	26/53	31/56	30/54	30/54	30/53	29/53	23/50	26/50	23/50
AGTHR4GNS		37/60	30/58	26/50	31/56	22/54	32/56	30/55	21/44	22/47	22/43
Rsc6p			27/52	24/49	26/48	28/52	27/52	25/48	28/50	30/55	22/47
ZK1128.5				69/82	56/73	57/74	54/74	52/73	29/48	29/50	22/44
C18E3.2					47/67	50/68	48/68	50/69	30/51	28/50	24/46
D15Kz1						99/100	72/85	68/81	35/58	33/52	21/44
BAF60a							73/85	68/81	33/53	34/55	20/43
BAF60c								69/81	23/49	32/52	21/43
BAF60b									27/47	29/52	19/46
Yor295p										38/57	26/51
Ymr233p											29/59

The numbers indicated above are the calculated percent identities and percent similarities, respectively, between the pair-wise combinations of the polypeptide sequences used in the alignment of Figure 5-1. The values were generated by using the program BestFit in the GCG software package (see Materials and methods for a further description).

Figure 5-1. An alignment of Swp73p and eleven other polypeptides.

Shown is the multiple sequence alignment of the predicted polypeptides encoded by *SWP73, AgTHR4gns, RSC6, ZK1128.5, C18E3.2, D15Kz1, BAF60a, BAF60c, BAF60b, YOR295w, YMR233w,* and *T24G10.2*. Only a partial open reading frame has been determined for *AgTHR4gns*. Therefore, the predicted polypeptide that is presented is only the carboxy-terminal sequence. Note that the carboxy termini of Swp73p and AGTHR4GNS are almost identical (78% identity and 91% similarity over 45 amino acids). For the other sequences the translated start codons have not been determined experimentally. Therefore, I have assumed that the first AUG codon downstream of the TATA box is the initiator. Positions of absolute identity in all the proteins (excluding gaps) are indicated with a bullet. Positions at which the majority of sequences (8 out 12) have a similar amino acid are indicated with an asterisk. The glycine that is converted to an aspartic acid in swp73-1p is indicated with a downward arrow. Gaps are shown with dashes. The region containing the "YIKes" motif is depicted with a darkly shaded box. Other regions that contain high levels of identity and similarity across species are depicted with lightly shaded boxes. Note that the tyrosine of the "YIKes" motif is absolutely conserved in all the sequences available.

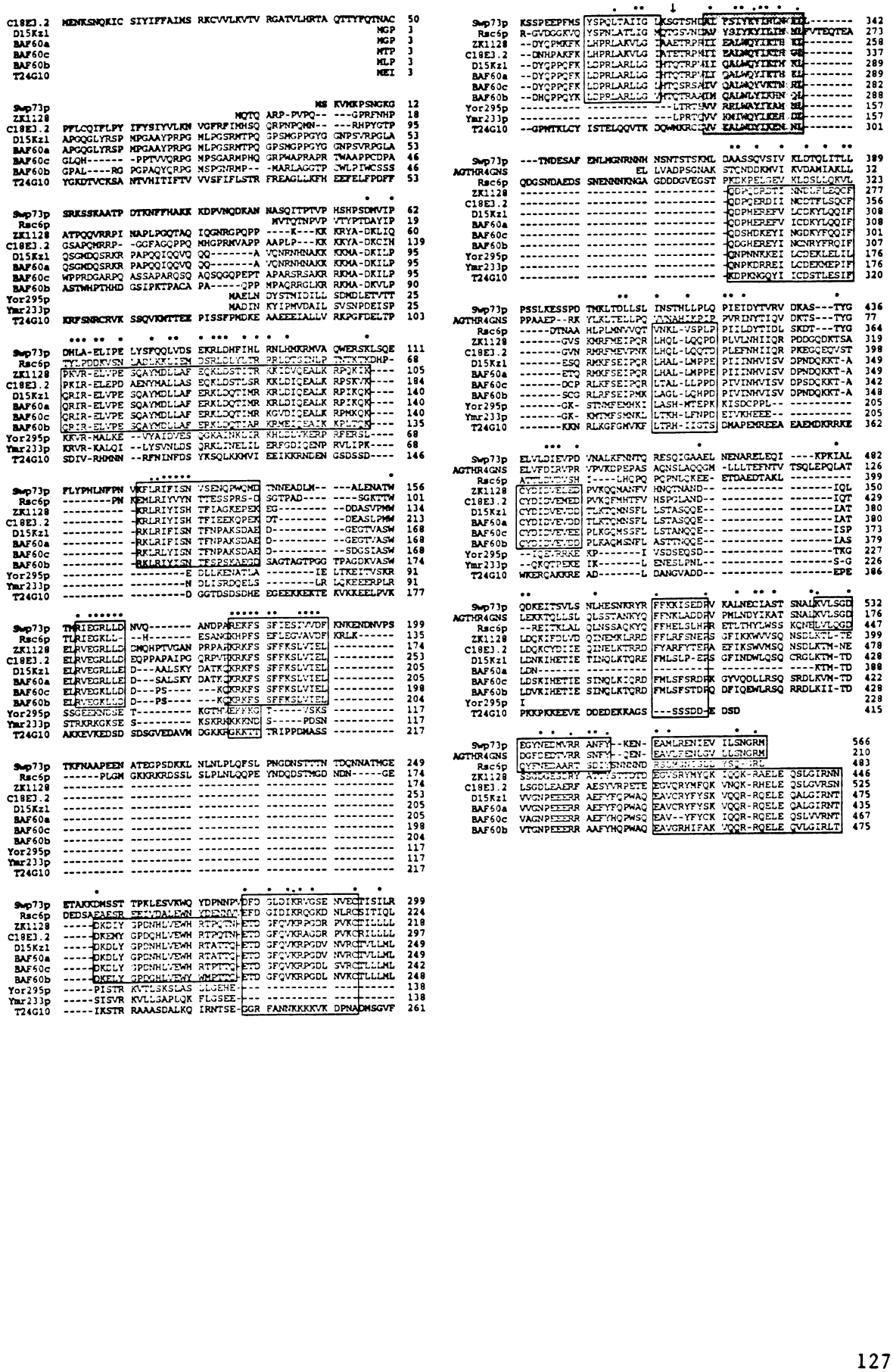
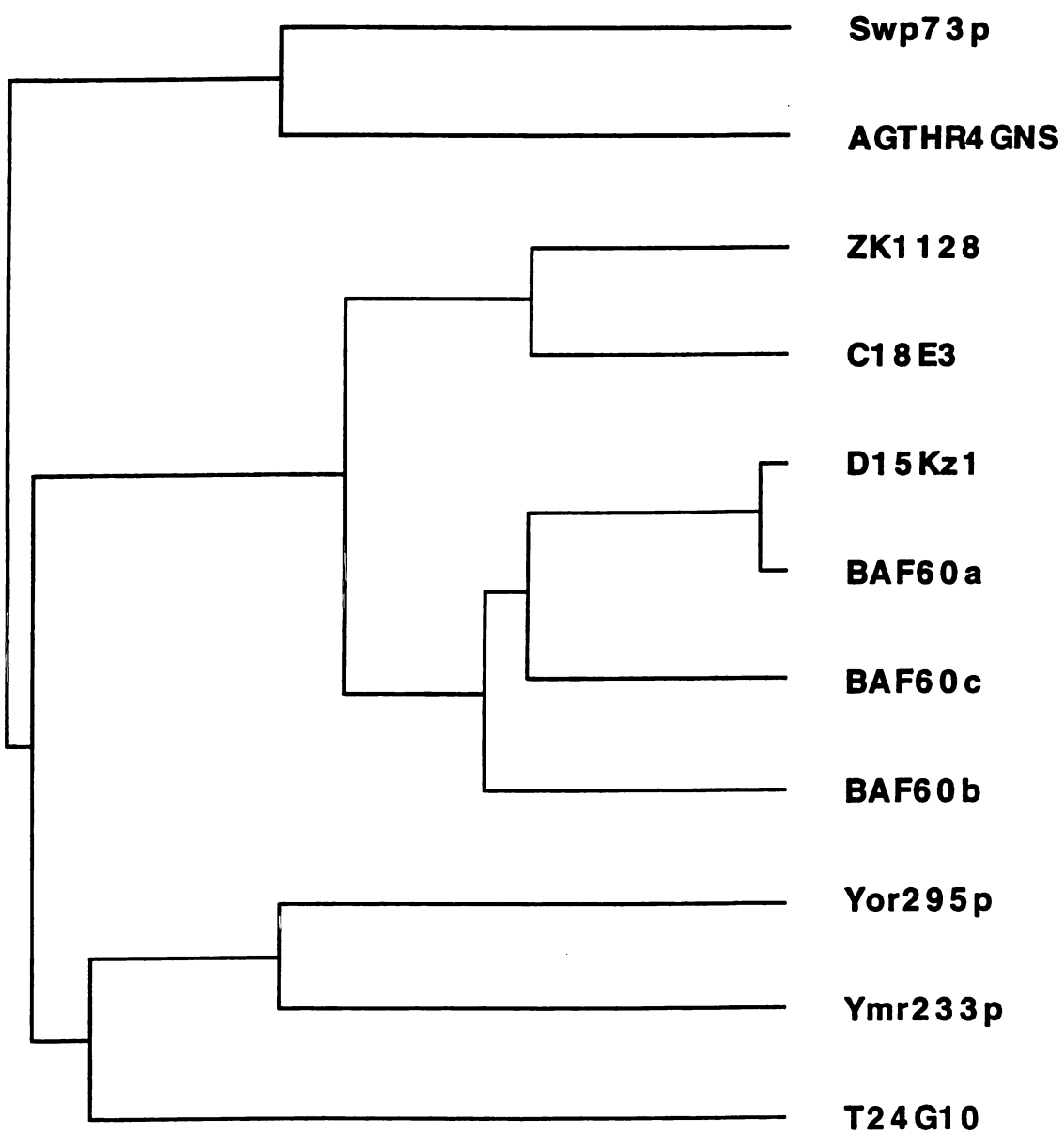


Figure 5-2. A dendrogram of the peptide sequences comprising the Swp73p protein family.

Shown is a dendrogram generated by the PileUp command from the GCG computer software package. PileUp created a multiple sequence alignment from the sequences in Figure 5-1 by using progressive, pairwise alignments. Before alignment, though, the sequences were first clustered by similarity to produce the dendrogram, or tree representation of clustering relationships. The clustering strategy that was used, as represented by the dendrogram, was the "unweighted pair-group method using arithmetic averages" (Sneath and Sokal 1973). The distances along the horizontal axis of the dendrogram is proportional to the differences between sequences, while the distances along the vertical axis has no significance.

Dendrogram of the Members of the Swp73p Protein Family



(position 323 in Swp73p). The two yeast proteins are more similar to one another than to the mouse or *C. elegans* proteins, but the similarity between the mouse and the *C. elegans* proteins, ZK1128.5 and CE18E3.2, are also high (56% and 47% identical, respectively). Likewise, the identity between the two *C. elegans* proteins, ZK1128.5 and CE18E3.2, is also very high at 69%. But what is truly striking is the identity of D15Kz1 to BAF60a. Except for a 40 amino acid insert near the C-terminus these two proteins are virtually identical. Thus, it is likely that these two proteins perform identical functions in their respective organisms and that all these proteins most likely belong to the same family.

Distinct proteins exist that share a conserved hydrophobic core region with members of the Swp73p protein family

Further probing of the NCBI database with sequences from ZK1128.5 yielded C18E3.2, which in turn yielded two more yeast proteins, Yor295p and Ymr233p, and an additional protein from *C. elegans*, T24G10.2. The functions of these additional proteins are not known, but they do exhibit fairly high identity to each other (see Table 5-1). The sequences of these three proteins were included in the alignment illustrated in Figure 5-1. While the alignment among these three proteins is fairly good, they do not show much of an alignment with the rest of the sequences. But there is a region near the mutation in *swp73-1* (highlighted by a downward arrow) that shows a very good alignment among all the sequences (except AGTHR4GNS because the sequence is unavailable), including these last three sequences. Approximately

10 amino acids downstream from the mutated glycine in *swp73-1*, there is a region of hydrophobic residues that are either absolutely or very highly conserved (the darkly shaded box in Figure 5-1). The tyrosine and the isoleucine at amino acid positions 335 and 336, respectively, in Swp73p represent the two most conserved amino acids in the whole family of proteins listed in the alignment. They are invariant in all the known sequences except for a very conservative substitution of valine for the isoleucine in BAF60c.

Discussion

BLAST searches of the NCBI database with Swp73p sequence yielded many proteins that may form a new protein family. Three additional yeast proteins were identified, along with three proteins from *C. elegans*, one protein from mouse, three proteins from humans, and a partial sequence from a filamentous fungus. The variety of organisms is striking and indicates the likelihood that Swi/Snf-like complexes arose early in eucaryotic evolution.

The only protein identified in the BLAST search that has a known function is Rsc6p. Rsc6p is a member of the RSC complex, an alternative chromatin-remodeling complex in yeast (Cairns et al. 1996b). Interestingly, deletions in *RSC6* are lethal (Cairns et al. 1996b), whereas deletions in *SWP73* are not (see Chapter 2). While Rsc6p and Swp73p are very similar in sequence

they are not redundant because neither one can complement the phenotypes arising from mutations in the other.

D15Kz1 was originally identified as an open reading frame 3.5 kilobases upstream of the glycerol-3-phosphate dehydrogenase gene. The gene has so far been only minimally described further, with preliminary results showing that the corresponding mRNA, although ubiquitous, is most highly expressed in the developing nervous system (Johnston et al. 1989), which is interesting considering the enriched expression pattern of brg1 (the mouse homolog of Swi2/Snf2p) in the nervous system of the developing embryo. D15Kz1 is nearly identical in sequence to the human protein BAF60a and thus they most likely perform similar functions. BAF60a, along with BAF60b and BAF60c, were cloned using cDNA from Jurkat T-cells after peptide sequencing of protein bands eluted from isolated Swi/Snf-like complexes from human YT cells. These complexes looked very similar in composition to yeast Swi/Snf complexes and contained two Swi3p-like proteins, BAF155 and BAF170, along with a Snf5p-like protein, BAF47, and a Swi2p-like protein, human BRG1.

The function of AGTHR4GNS, ZK1128.5, and C18E3.2 are still unknown. But the two *C. elegans* proteins show very high similarity to each other (69% identity and 82% similarity) and to two other yeast proteins, Yor295p and Ymr233p, along with a third protein of unknown function from *C. elegans*, T24G10.2. The function of Yor295p and Ymr233p is presently unknown. They are very small in size as compared to the rest of the family members (roughly half the size) and may represent an off-shoot of the family. There are two small proteins in the Swi/Snf complex, Snf11p and Swp29p,

but their sequences are known and they do not show any homology to Yor295p or Ymr233p.

While the overall identity of Yor295p, Ymr233p, and T24G10.2 to the rest of the family is not as high as the other proteins are to each other, there is a region of high conservation shared with the rest of the family. Namely, there appears to be a hydrophobic core approximately ten amino acids downstream of the mutated glycine residue in swp73-1p. A tyrosine and isoleucine residue are nearly absolutely conserved in the known sequences of all the family members (there is a conservative valine change for the isoleucine in BAF60c). The alignment, then, has presented us with a possible critical region for the proper function of Swp73p and the other family members and may even represent a novel sequence motif (which I affectionately refer to as the "YIKes" motif). If this region is, indeed, critical for Swp73p function, then perhaps the mutant phenotype of swp73-1p can be explained. It is possible that the glycine to aspartic acid change in swp73-1p causes a local conformational shift that destabilizes the nearby hydrophobic core and, thus, leads to either a loss in activity of swp73-1p or a reduction in its levels due to degradation.

A possible insight into the evolution of *SWP73* was also discovered. It is believed that chromosomes III and XIV in yeast have undergone an ancient interchromosomal duplication (Lalo et al. 1993, 1994; Wolfe and Shields 1997). It has been shown that *SWP73* is located on the right arm of chromosome XIV (M. Miller, personal communication, plus results from the yeast genome sequencing project), and my studies indicate that it resides about 12.5 cM from the centromere, which is similar to the position of *RSC6*

on the right arm of chromosome III. If *SWP73* and *RSC6* arose from an ancestrial gene through this interchromosomal duplication, it may go a long way in explaining how Rsc6p has similar, but very unique, functions to Swp73p.

A possible role in the evolution of *SWP73* by the aforementioned interchromosomal duplication is further supported by the isolation of *AgTHR4gns*. *AgTHR4gns* was originally identified as a partial open reading frame upstream of *AgTHR4*, which was cloned on the basis of its homology to *THR4* from *S. cerevisiae* (Altmann-Jöhl and Philippsen 1996). It was noted that loci in the vicinity of *AgTHR4* exhibit significant sequence identity and also an identical arrangement to loci in the vicinity of *THR4*, which resides on chromosome III of *S. cerevisiae*. On this basis, *AgTHR4gns* was thought to be the *A. gossypii* homolog of *S. cerevisiae RSC6*. The alignment in Figure 5-1 and the similarity data in Table 5-1 indicate that *AgTHR4gns* is more nearly identical in sequence to *SWP73*. This finding suggests that the interchromosomal duplication between chromosomes III and XIV occurred before the divergence of *A. gossypii* and *S. cerevisiae*. If so, then the prototype of *SWP73* originally resided near *THR4*, but following the interchromosomal duplication, it evolved to become *RSC6* and acquired cellular functions distinct from those of *SWP73*.

Swp73p appears to be the prototype of a family of proteins which are conserved across species. Some of the members most likely perform roles similar to Swp73p and at least one, Rsc6p, exists in a unique, but analogous, complex. The functions of the other members are unknown and they may play roles in the cell unique to themselves, but through a common sequence

Chapter 6

Cdc14p May Play a Role in GR Function

Abstract

GR-dependent transcriptional activation was tested in 3 yeast mutants that are blocked during M phase at elevated temperatures. One mutant strain, cdc14, was defective in GR function, even at permissive temperatures. Interestingly, one of the mutant strains isolated in the genetic screen to identify cellular factors required for GR function was partially complemented by a low-copy plasmid bearing *CDC14*. Cdc14p is a dual specificty protein phosphatase and thus may regulate, directly or indirectly, the phosphorylation state of GR, and thereby its function. Also, Cdc14p interacts genetically with Cdc15p, a kinase involved in cell cycle regulation. This interaction is independent of Cdc14p phosphatase activity. Thus, Cdc14p may affect GR function in ways other than through its phosphatase activity.

Introduction

All mitotic cells undergo a cell cycle. The cells grow for a period of time (referred to as the G1 phase) and then replicate their DNA (referred to as the S phase) in preparation for a cell division so that each resulting daughter cell receives a complete copy of the genome. S phase is followed by another round of growth (usually shorter than the G1 phase), which is referred to as the G2 phase. Finally, after this G2 phase the cells undergo mitosis, which is referred to as the M phase. During mitosis a copy of each genome is segregated to the two daughter cells and then cell division occurs.

The presence of the cell cycle is critical for all organisms. It presents the common mechanism by which all forms of life reproduce. In addition, it is

especially critical for multicellular organisms, such as ourselves, that arise from a single cell. All our tissues and organs, which involve billions of cells, have each arisen by a co-ordinated process from an initial cell. Needless to say, then, the cell cycle represents one of the most fundamental aspects of life and is probably one of the most regulated processes of the cell. Indeed, numerous proteins are devoted to the regulation of the cell cycle, many of which are still unidentified. And since all cells reproduce, the mechanisms involved are very well conserved, especially among the eucaryotes. Issues in develomental biology, immunology, and cancer have been greatly impacted by investigations of the cell cycle.

In *S. cerevisiae,* growth is rapid in appropriate media (generation times are typically two hours at 30°C in mid-log growth phase), and progression through the cell cycle can be monitored under a microscope. The hallmark of *S. cerevisiae* is the morphology of its cells during its asexual reproduction. Namely, a small bud appears during G1 that continues to grow through G1 and S phases. The morphology continues to change and becomes "dumbbell-shaped" when the cell has entered G2 and M phases. Eventually, the cells divide and the process repeats. The exact position of the cell cycle can be monitered further by staining the DNA and following its localization microscopically.

In classic experiments, Lee Hartwell and his colleagues categorized over fifty mutants in yeast that are defective in the cell division cycle (referred to as *cdc* mutants) at various points throughout (Culotti and Hartwell 1971; Hartwell et al. 1973). The eventual cloning of the genes harboring mutations in these *cdc* mutants, especially *CDC28,* lead to tremendous strides in the advancement of our ability to describe the molecular pathways involved in regulating the cell cycle. Similar proteins in higher organisms, including

humans, were soon identified that displayed not only similar sequences but were also functionally interchangeable with their yeast counterparts.

It is beyond the scope of this introduction to give a complete account of the molecular pathways involved in regulating the cell cycle (reviewed by Nasmyth 1996; Aldea et al. 1994; Futcher 1990). Suffice it to say that a complex signaling cascade exists that carefully regulates cell growth during G1 until the cell has decided to enter S phase. Once the cell has entered S phase it is committed to completing mitosis, but even so the rest of mitosis is still a very much regulated process. This point is illustrated by the existence of many *cdc* mutants whose growth arrest occurs outside of G1 and S phases. The signaling cascade that regulates the cell cycle consists of many kinases (which include levels upon levels of successive kinasing steps), phosphatases, and the cell's protein degradation machinery (Nasmyth 1996).

Are transcription factors regulated during the cell cycle? Many transcription factors are thought to be active during G1 when the cell is actively growing but before it has replicated its DNA. But what happens to the activity of transcription factors during the later phases of the cell cycle, such as G2 and M? In most higher organisms, the membrane surrounding the nucleus is broken down during mitosis, and is eventually restored once the process is complete. Also during mitosis the DNA is compacted into chromosomes so that they can be more easily segregated to the progeny cells. These factors might inhibit transcription. But for some transcription factors it is thought that active processes occur to down-regulate their activity. For example, Swi5p, a regulator of *HO* transcription in yeast (see the results of Chapter 3), is phosphorylated near its nuclear localization sequence during mitosis and thus prevented from entering the nucleus (yeast do not undergo nuclear membrane breakdown during mitosis) (Moll et al. 1991). Another

well-studied example is E2F, which regulates many of the genes active during S-phase. E2F is kept inactive by its association with the tumor suppressor protein, Rb (see the Introduction to Chapter 5), but upon entrance to S phase Rb is phosphorylated and loses its interaction with E2F (Shirodkar et al. 1992). E2F is then active and up-regulates many genes involved in S-phase progression. As was pointed out in Chapter 5, Rb interacts with brg1 (Dunaief et al. 1994), the murine homolog of Swi2/Snf2p. Thus, it is interesting to speculate that Rb may also regulate transcription factors by interacting with chromatin-remodeling factors in a cell-cycle-dependent fashion.

GR is now believed to be inactive during G2 phase (Hsu et al. 1992). But the mechanism of its repression is not known. I set out, in collaboration with Micheal Garabedian, to address this question by examining the activity of GR in yeast that have been blocked at various points in the cell cycle. Also, attempts to clone the gene harboring the mutation in one of my mutant strains recovered in my genetic screen may also bear on this question.

Methods and materials

Yeast strains and techniques

The yeast strains used in this chapter are described in Table 6-1. The plasmids pGPD-N795 and pGPD-N525/ER (see the next paragraph) were linearized with the restriction enzyme *Kpn*I and transformed into the appropriate strain. The transformants were grown on selective plates without leucine to select for proper integration. Standard yeast techniques, such as yeast transformation, sporulation, tetrad dissection, and preparation

Table 6-1. *Yeast strains*

Strain	Relevant Genotype	Source
IH2372	*MATa, ura3 leu2 his7 can1 cdc15-1*	I. Herskowitz
IH2373	*MATa, ura3 leu2 his7 can1 cdc14-1*	I. Herskowitz
IH2374	*MATa, ura3 leu2 his7 can1 cdc16-1*	I. Herskowitz
RL100[a]	*MATa, ura3 LEU2 his7 can1 cdc15-1 leu2::GR-N795* [pΔS-26X]	this study
RL101[a]	*MATa, ura3 LEU2 his7 can1 cdc15-1 leu2::GR-N525/ER* [pΔS-26X]	this study
RL200[b]	*MATa, ura3 LEU2 his7 can1 cdc14-1 leu2::GR-N795* [pΔS-26X]	this study
RL201[b]	*MATa, ura3 LEU2 his7 can1 cdc14-1 leu2::GR- N525/ER* [pΔS-26X]	this study
RL300[c]	*MATa, ura3 LEU2 his7 can1 cdc16-1 leu2::GR-N795* [pΔS-26X]	this study
RL301[c]	*MATa, ura3 LEU2 his7 can1 cdc16-1 leu2::GR-N525/ER* [pΔS-26X]	this study
BJ2168-GR[d]	*MATa, pep4-3 pcr1-407 prb-1122 trp1 ura3-52 leu2* [pG-N795] [pΔS-26X]	this study
JO68-GR[e]	*MATa, trp1Δ63 ura3-52 leu2 bar1::LEU2 his3Δ* [pG-N795] [pΔS-26X]	this study
W303-1A	*MATa, trp1-1 ura3-1 leu2-3,112 his3-11,15*	A. Tzagoloff
W303-1B	*MATα, trp1-1 ura3-1 leu2-3,112 his3-11,15*	A. Tzagoloff
WG3Z7C[f]	*MATa, trp1-1 ura3-1 leu2-3,112 his3-11,15* [pTCA-GN795] [pHCA-G3Z]	this study
W1002[g]	*MATa, trp1-1 ura3-1 leu2-3,112 his3-11,15* [pTCA-GN795] [pHCA-G3Z]	this study
W1002-3.16b[h]	*MATa, trp1-1 ura3-1 leu2-3,112 his3-11,15* [pTCA-GN795] [pHCA-G3Z]	this study

[a]Derived from IH2372.
[b]Derived from IH2373.
[c]Derived from IH2374.
[d]Derived from BJ2168 (gift of Beth Jones).
[e]Derived from JO68-7b (gift of Joe Ogas).
[f]W303-1A background.
[g]Derived from mutagenesis of WG3Z7C.
[h]Derived from three backcrosses between W1002 and W303-1A.

of yeast media, are as previously reported (Gietz et al. 1992; Sherman 1991; Sherman and Hicks 1991).

Plasmids

The 3GRE-*lacZ* reporter plasmid, pΔS26x, was as described (Yoshinaga et al. 1992). The expression plasmids pGPD-N795 (leu2) and pGPD-N525/ER (leu2) contain either full-length GR (N795) cloned in front of the yeast *GPD* promoter or similarly a GR/ER fusion (N525/ER) in which the hormone-binding domain of the estogen receptor replaces the hormone-binding domain of GR and is fused in frame following the DNA-binding domain of GR. These expression plasmids are marked with mutant versions of *LEU2* that can be restored to function by integration into the genome of yeast strains bearing enough sequence in their *leu2* alleles to allow homologous recombination.

The YEp351-based yeast genomic library was a gift from Jeanne Hirsch (Engebricht et al. 1990). The GR expression plasmid pTCA-GN795 consists of the sequence from pGN795 (Yoshinaga et al. 1992) between the *Hin*dIII and *Ngo*MI sites, which contains the *GPD* promoter, the full-length GR coding sequence, and the *PGK* terminator sequence, inserted between the same sites of pRS314 (*TRP1, CEN4*)(Sikorski and Heiter 1989). The GR reporter pHCA-G3Z was constructed by moving the 3GRE-*lacZ* reporter fragment from pΔS26x into pRS313 (*HIS3, CEN4*) (Sikorski and Heiter 1989).

pLCA-CDC14 was obtained by first cloning the ~2.5 kilobase *Xma* I/*Kpn* I fragment from the isolated yeast genomic clone containing *CDC14* into the *Ngo*MI/*Kpn*I sites of pRS316 (*URA3, CEN4*) (Sikorski and Heiter 1989). This clone was labeled pUCA-CDC14. Then pUCA-CDC14 was digested with

*Nhe*I/*Dra*III and the fragment containing *CDC14* was ligated into pRS315 (*LEU2, CEN4*)(Sikorski and Heiter 1989) to obtain pLCA-CDC14. Likewise, pLCA-CDC15 was created by ligating the *Dra*III/*Eag*I fragment containing *CDC15* from pUCA-CDC15 (a gift from Sue Jasperson) into the *Dra*III/*Eag*I sites of pRS315.

Enzyme assays

Liquid β-galactosidase assays were performed as described (Garabedian 1993). The length of hormone treatment varied and was as described in the Results.

DNA sequencing and sequence analysis

Double-stranded plasmid templates were prepared and sequenced using the Sequenase 2.0 kit (U.S. Biochemical). The sequencing reactions were performed according to the technique of Del Sal et al. (1989). Terminated fragments were labeled by incorporation of [a-^{35}S]dATP (NEN), resolved on 6% acrylamide gels, and autoradiographed on Kodak X-Omat AR film. Sequencing of the genomic *CDC14* clone was performed using primers that hybridize to the polylinker of YEp351. The determined sequence was used in the BLAST routine available through the Yeast Protein Database website of Proteome, Inc (http://www.proteome.com). The results indicated that the library plasmid contained a 5.5 kilobase insert from chromosome VI of *S. cerevisiae*. The complete sequence of the insert is available due to the yeast genome sequencing project and was obtained from GenBank. The open

reading frames in this region were identified by the signifiers obtained from GenBank.

Microscopic analysis of W1002 strains

To determine the morphology of W1002 and W1002-3.16b at non-permissive temperatures the cells were grown at 23°C for 5 hrs and then shifted to 37°C for 6 hrs. A small aliquot of cells (~3 µl) were deposited onto a multi-well slide pre-treated with polylysine. The cells were allowed to settle onto the slide for 10 minutes at 23°C and then the excess liquid was gently aspirated. A few drops of molten mounting solution (glycerol gelatin) containing the DNA stain DAPI (4,6-diamidino-2-phenylindole) at 1 µg/ml was placed on top of the cells and a coverslip was mounted over the slide. After allowing the mounting solution to harden for a few minutes the cells were viewed in a Zeiss Axioskop equipped for fluorescence. The point of arrest in the cell cycle was determined by both physical morphology and by nuclear staining as reported by Culotti and Hartwell (1971).

Results

The activity of GR in various cdc *mutants*

In order to determine if GR functions in the later points of the cell cycle we obtained yeast strains bearing mutations in *CDC14*, *CDC15*, and *CDC16*. These three strains are isogenic and bear temperature-sensitive mutations that block cell growth at similar points in the cell cycle (late M phase for *cdc14*

and *cdc15* and metaphase for *cdc16*). The three strains were transformed with linear plasmids expressing either full-length GR (GR-N795) or a fusion of GR and ER (the estrogen receptor) in which the ligand-binding domain of GR is replaced with the ligand-binding domain of ER (GR-N525/ER). These linear plasmids integrate into the *leu2* locus and restore *LEU2* activity. A high-copy reporter plasmid (pΔS26X) bearing a TAT3 GRE-*lacZ* gene was co-transformed with the expression plasmids.

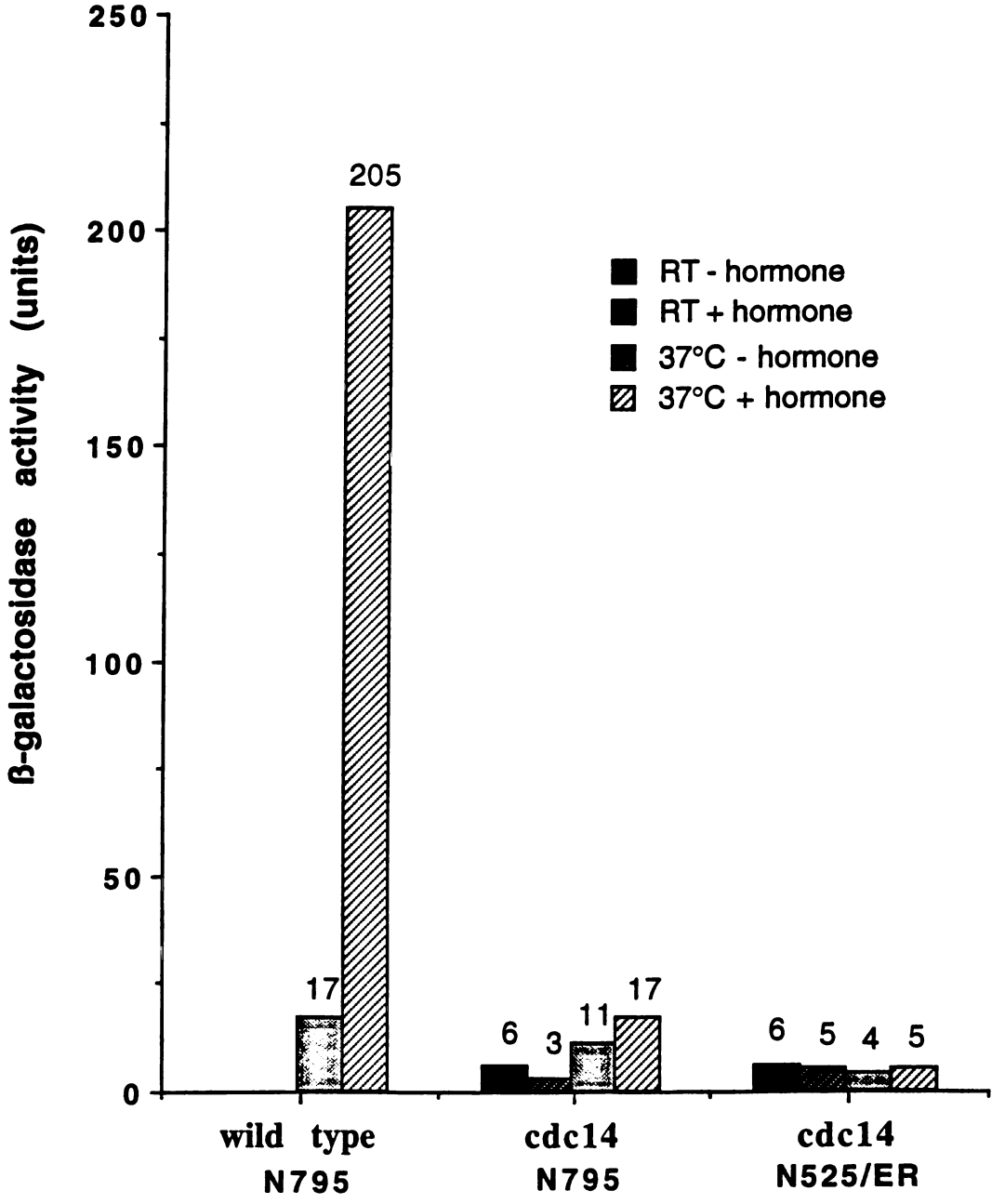
Transformants of the *cdc14* mutant and a non-isogenic wild-type strain (BJ2168-GR) were cultured overnight at room temperature. The following day the cultures were sub-cultured for 1 hr at either room temperature (for the cdc14 transformants) or at 30°C. The cultures were then split into two equal volumes and further cultered at either room temperature or at 37°C (the non-permissive temperature) for another 5.5 hours. Finally, both sets of cultures were again split into two equal cultures and further incubated at the appropriate temperature for 2.5 hours either in the absence or the presence of hormone - 10 μM deoxycorticosterone (DOC) for GR-N795 transformants or 1 μM β-estradiol for GR-N525/ER transformants. The results of these experiments are presented in Figure 6-1.

To determine whether the lack of GR activity in cdc14 was unique to a defect in this strain or due to a more general cause involving a block in cell cycle progression in M phase, the activity of GR was tested in the other *cdc*-bearing strains. Since a defect in GR transcriptional activity was observed in cdc14 even at room temperature, the activity of GR in these various strains was examined first by culturing the cells at room temperature. Cultures grown at room temperature of transformants of cdc14, cdc15, cdc16, BJ2168-GR, and JO68-GR (the last two strains are non-isogenic wild-type strains that display similar levels of GR-dependent transcription) were subcultured 1:10

Figure 6-1. GR-dependent transcriptional activation at room temperature and at 37°C in a *cdc14* strain.

The transcriptional activation activity of GR and a GR/ER fusion is inhibited at room temperature and at 37°C in a *cdc14* strain but not in a non-isogenic wild-type strain. Cultures were grown at room temperature (~23°C) for 1 hr and then split into two equal cultures. These cultures were then grown at either room temperature or 37°C for 5.5 hr. The cultures were subdivided further and were either treated with hormone (10 μM deoxycorticosterone for strains expressing GR-N795 or 1 μM β-estradiol for strains expressing GR-N525/ER) for 2.5 hr at the two temperatures or they were incubated without hormone for 2.5 hr at the two temperatures. Each culture was then assayed for β-galactosidase activity as described (see Materials and methods). The units determined are indicated above each column. The strains tested are indicated beneath each set of bars in the graph. The version of GR that they express is indicated beneath the strain label. The strain labeled "wild type" is BJ2168-GR (see Table 6-2). Only GR-N795 was tested in this strain and only at 37°C. The strain labeled "cdc14" and "N795" represents the activity of RL200, while the strain labeled "cdc14" and "N525/ER" represents the activity of RL201 (see Table 6-1). The black bar indicates the activity of the strain at room temperature with out hormone. The heavy stipled bar indicates the activity of the strain at room temperature with hormone. The gray-colored bar indicates the activity of the strain at 37°C without hormone. The light hatched bar indicates the activity of the strain at 37°C with hormone.

GR-dependent Transcriptional Activation at Room Temperature and at 37°C in a *cdc14* strain



and allowed to recover at room temperature for 11 hours. These cultures in turn were subcultured 1:3 and the appropriate hormone was added and the cultures were incubated further for 12 hours at room temperature. The levels of β-galactosidase were then determined and the results are presented in Figure 6-2. A very similar experiment was then performed except that the cultures were incubated at 30°C, the results of which are presented in Figure 6-3. Immunoblot analysis was performed to determine the expression levels of GR-N795 in cultures of *cdc14* and *cdc15* at room temperature and at 30°C. It appears that GR-N795 is expressed at similar levels in both strains at these two temperatures (data not shown). Also, there was no apparent loss in GR-N795 expression in the *cdc14* strain at 37°C as compared to 30°C (data not shown). So it appears that GR-N795 and GR-N525/ER are reduced in activity in the *cdc14* strain by about 10-fold at 30°C and at 37°C and by >30-fold at room temperature.

A mutant isolated in the genetic screen is suppressed by CDC14

The mutant strain W1002 isolated in my genetic screen (see Chapter 1 for details) is both defective for GR-dependent activation and contains a very potent temperature-sensitive growth defect. Attempts to determine if the defects in GR activity and growth at 37°C were linked proved inconclusive (see Table 6-2). But due to the presence of such a strong Ts^- growth defect, I reasoned that perhaps a plasmid that was isolated that complemented the Ts^- phenotype might also complement the defect in GR activity.

The strain W1002-3.16b (a spore from the third back-cross of W1002 to the parental isogenic strain WG3Z7C) was transformed with a YEp351-based, *LEU2*-marked yeast genomic library (the same library that was used to clone

GR-dependent Transcriptional Activation at Room Temperature in Various *cdc* Mutant Strains

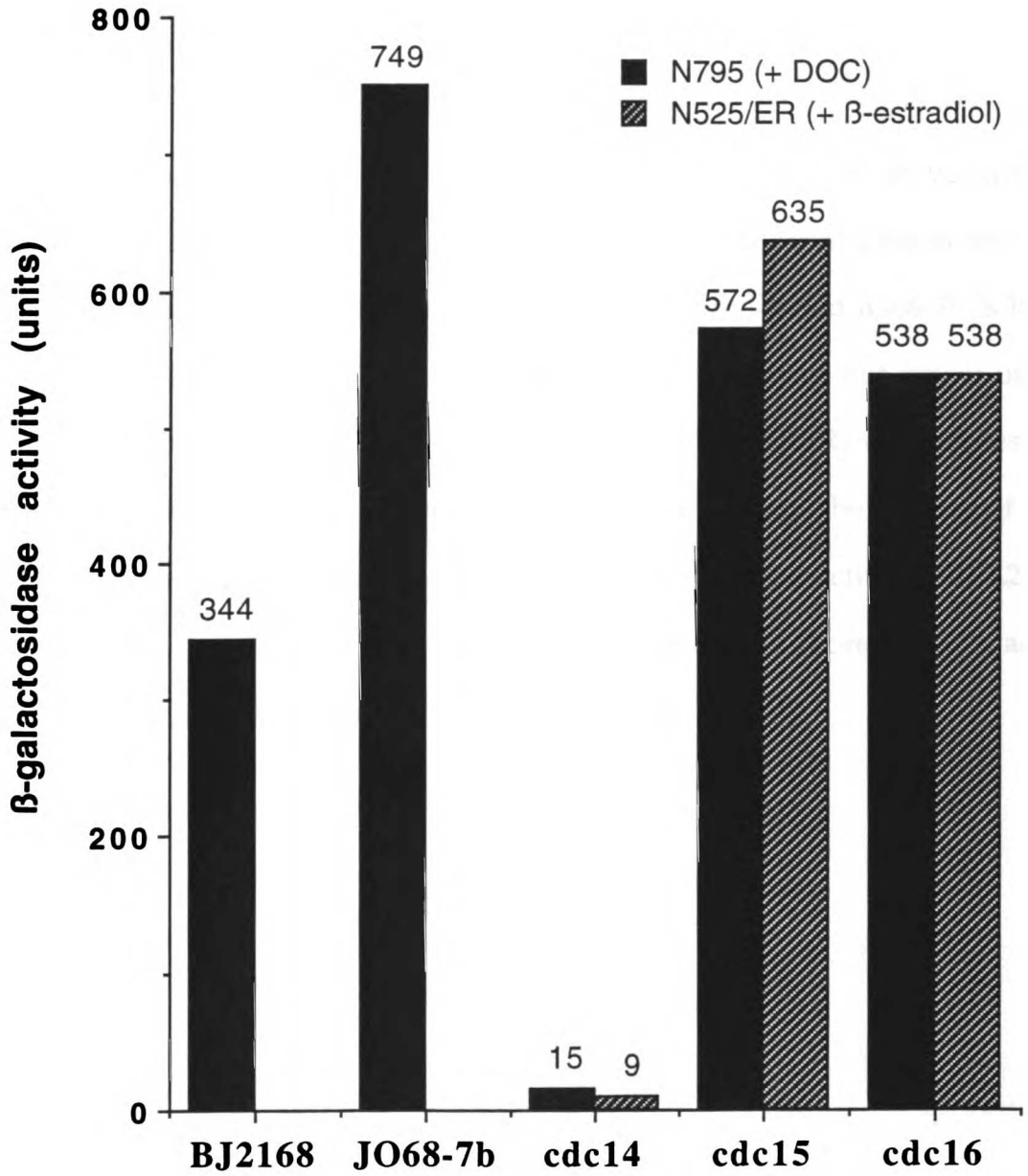


Figure 6-3. GR-dependent transcriptional activation at 30°C in various *cdc* mutant strains.

The transcriptional activation activity of GR and a GR/ER fusion is inhibited at 30°C in a *cdc14* strain but not in isogenic *cdc15* or *cdc16* strains. Cultures were treated with either 10 μM deoxycorticosterone or 1 μM β-estradiol for 11 hr at 30°C, and β-galactosidase assays were performed as described (see materials and methods). The units determined are indicated above each column. The strains tested are indicated beneath each set of bars in the graph. The strain labeled BJ2168 is BJ2168-GR and the strain labeled JO68-7b is JO68-GR (see Table 6-1). They are non-isogenic to the *cdc* strains but where used as wild-type control strains. The GR/ER fusion (GR-N525/ER) was not tested in these two strains. The black bar above "cdc14" represents the activity of RL200, while the stipled bar above "cdc14" represents the activity of RL201 (see Table 6-1). Likewise, the bars in the next set of data represents the activity of RL100, RL101, RL300, and RL301, respectively.

GR-dependent Transcriptional Activation at 30°C in Various *cdc* Mutant Strains

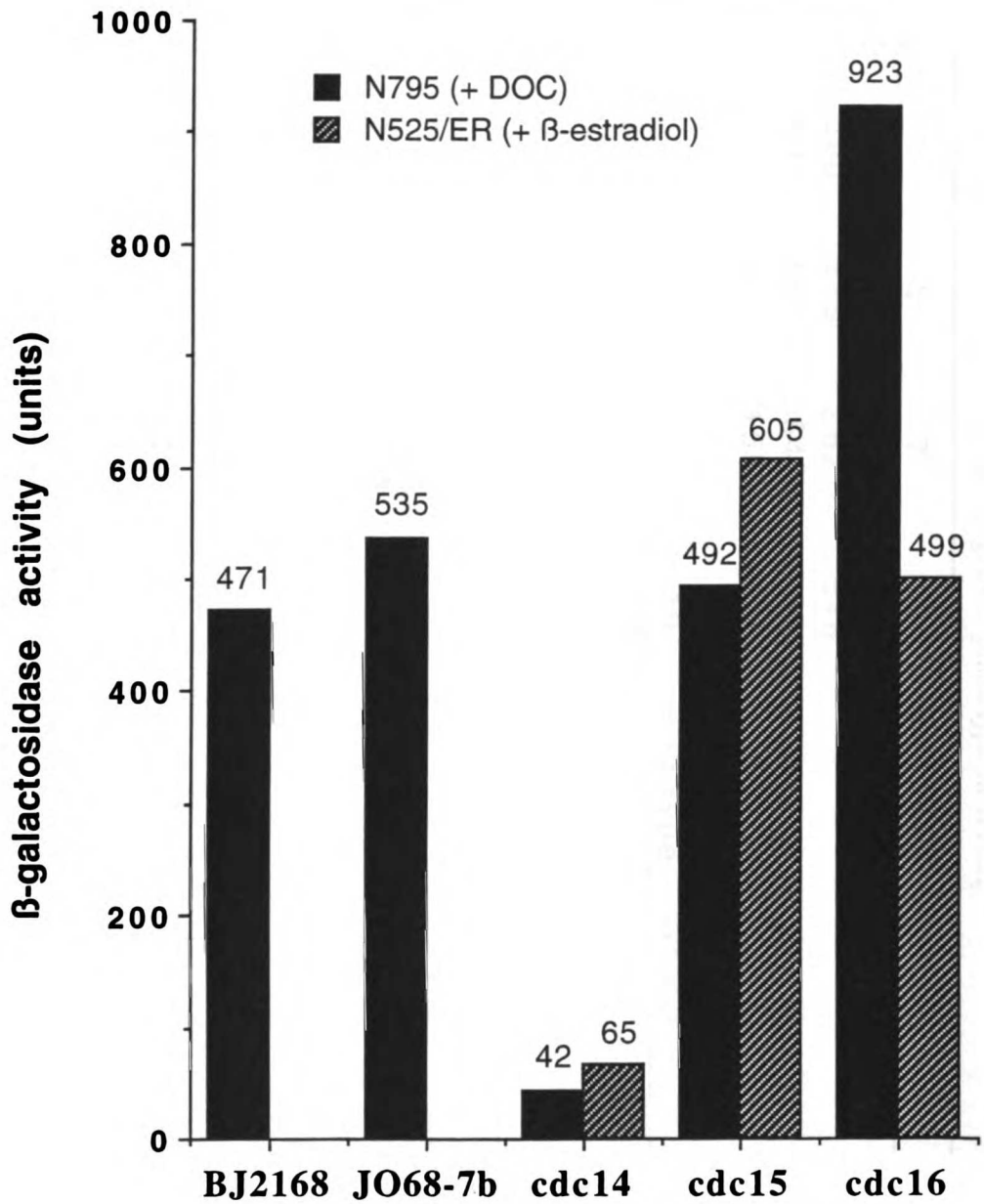


Table 6-2. *Genetic analysis of the GR mutant phenotype and the Ts⁻ growth phenotype in W1002*

Backcross#1:	1	2	5	7	8	wild-type	W1002
a	306	307	115*	366	136*	645	41*
b	73*	390	689	262*	578		
c	527	84*	96*	647	155*		
d	237*	110*	292	155*	520		

Backcross#2:	1	3	4	13	19	wild-type	W1002
a	104*	397	242*	210*	252	811	106*
b	362*	177*	998	484	322		
c	217	356	325	549	169*		
d	344	264*	204*	218*	174*		

Backcross#3:	1	2	4	6	8	16	wild-type	W1002
a	209*	426	288*	261	363	379	646	106*
b	262	258*	400	371*	331	200*		
c	70*	525	400	254	320*	617		
d	404	255*	120*	116*	180*	174*		

The tetrad tested is indicated by a number in the row above each solid line and the spore of each tetrad tested is indicated by a letter in the column to the left of each solid line. The values reported in the column beneath each tetrad number is the β-galactosidase activity in units. Those β-galactosidase activities that are marked with an asterisk indicate that the spore displayed a Ts⁻ growth defect at 37°C. Spore 8a from the first backcross was used to initiate the second backcross. Spore 1a from the second backcross was used to initiate the third backcross. In each backcross, WG3Z7C was tested for growth and β-galactosidase production as a positive control. The values are reported in the table. Also, the original mutant strain, W1002, was tested for growth and β-galactosidase production as a negative control. Note that in the third backcross an extra tetrad was tested. Many more tetrads were tested from each backcross for growth and in every case the Ts⁻ phenotype segregated 2:2.

SWP73). Approximately 20,000 transformants were examined for growth at 37°C on solid, rich media. Three colonies were isolated that grew indistinguishably from wild-type cells (W303-1A) under these conditions. I then attempted to determine whether the complementation of growth was plasmid linked. Growth in non-selective media allowed me to lose the library plasmid from two of the isolates but I was never able to lose the library plasmid from the third isolate. Growth was then re-examined at 37°C on solid, rich media for the two isolates that lost the library plasmids. Only one of these isolates now displayed the Ts^- growth defect indicating that the complementation of this isolate was, indeed, plasmid linked.

The library plasmid was recovered from the isolate that displayed a plasmid-linkage and sequenced. The determined sequence revealed that the library plasmid contained a 5.5 kb insert from chromosome VI of *S. cerevisiae*. There were only two complete open reading frames in the insert - *YFR023w* and *CDC14*. The function of the predicted protein product from *YFR023w* is unknown, whereas *CDC14* is important for cell cycle progression through late M phase.

To determine further whether W1002 was indeed defective in Cdc14p activity, I examined the morphology of W1002 after incubation at 37°C. Cultures of W1002 and W1002-3.16b were grown at 23°C for 5 hr and then shifted to 37°C for 6 hr. The cells were stained with DAPI to highlight the nuclear DNA and the morphology of the cells were examined microscopically. In both of these strains, >95% of the cells were arrested in late M phase. That is, the cells were dumbbell-shaped and there were two clearly distinguishable nuclei situated at the opposite poles of the two daughter cells. So it appears that W1002 harbors a mutation that blocks cell growth in late M phase when incubated at 37°C.

Since I had already seen a defect in GR activity in a *cdc14* strain, I then determined the activity of GR in W1002-3.16d harboring the library DNA or a control plasmid (YEp351). After treatment with 10 μM DOC for 6 hr, W303-1A harboring YEp351 displayed 561 units of β-galactosidase activity, whereas W1002-3.16b harboring YEp351 displayed 121 units of β-galactosidase activity. W1002-3.16b harboring the library plasmid DNA displayed 395 units of β-galactosidase activity, so there is a gain in GR activity but it is not quite equal to the levels in the wild-type strain.

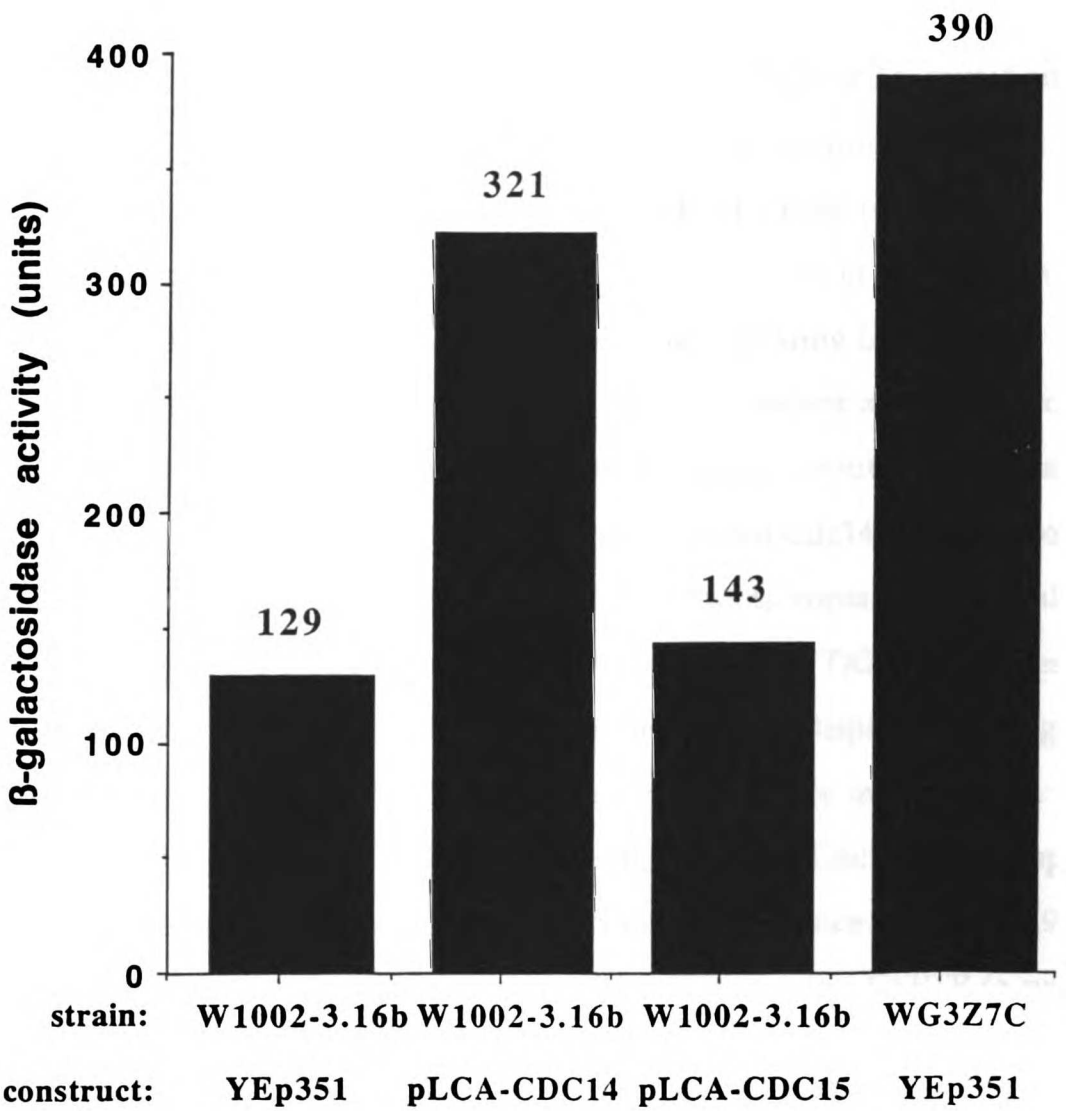
Since the library plasmid DNA was not able to complement completely the loss in GR activity in W1002-3.16b it was possible that *CDC14* represents a high-copy suppressor of this mutant phenotype in this strain. So *CDC14* was subcloned into a *LEU2*-marked, low-copy plasmid (pRS315) and labeled pLCA-CDC14. As a control, *CDC15* was also cloned into pRS315 and labeled pLCA-CDC15. W1002-3.16b was transformed with either pLCA-CDC14, pLCA-CDC15, or YEp351. Upon treatment with 10 μM DOC, the cells were assayed for β-galactosidase activity. The results (see Figure 6-4) reveal that a low-copy plasmid bearing *CDC14*, but not *CDC15*, will suppress the mutant phenotype of W1002-3.16b to the same extent as a high-copy plasmid bearing *CDC14*. Interestingly, the Ts^- growth defect of W1002-3.16b was not complemented by either pLCA.CDC14 or pLCA.CDC15. Thus, it appears that *CDC14* is a low-copy suppressor of the mutant GR phenotype in W1002-3.16b, but is a high-copy suppressor for the Ts^- growth defect in this strain.

The increase in GR activity in W1002-3.16b in the presence of a plasmid bearing CDC14 may be non-specific. Perhaps the presence of pLCA.CDC14 or the library DNA plasmid would also increase GR activity in a wild-type strain. To test this notion, I transformed W303-1A with either pLCA.CDC14, YEp351, or the library DNA plasmid. After 6 hr in the presence of 10 μM DOC, the

Figure 6-4. GR-dependent transcriptional activation in W1002-3.16b expressing Cdc14p or Cdc15p.

The expression of Cdc14p in W1002-3.16b results in a nearly complete suppression of the GR activation-deficiency phenotype whereas expression of Cdc15p has no effect. The strains tested are indicated beneath each bar in the graph. The expression plasmid that they harbored is also indicated. YEp351 acts as a control vector, while pLCA-CDC14 expresses Cdc14p and pLCA-CDC15 expresses Cdc15p. Cultures were treated with 10 μM deoxycorticosterone for 6 hr, and β-galactosidase assays were performed as described (see Materials and methods). The units determined are indicated above each column. Each value represents the average of at least four transformants and the deviation from the average for each transformant is <30%.

GR-dependent Transcriptional Activation in W1002-3.16b Expressing Cdc14p or Cdc15p



levels of β-galactosidase activity were determined and no discernable differences were seen among the three types of transformants.

Discussion

Cdc14p may play a role in GR function. The activity of GR was abrogated in a *cdc14* strain that is Ts^- for growth at 37°C, though the defect in GR function was evident at permissive temperatures, as well. The activity of GR, however, was not affected in isogenic strains harboring Ts^- mutations in either *CDC15* or *CDC16*, at least not at permissive temperatures. Finally, a mutant strain isolated in my genetic screen to identify defects in GR transcriptional activation was partially suppressed (~75%) in its defect in GR function by plasmids, either high-copy or low-copy, bearing *CDC14*.

Unfortunately, these results remain tentative, as not all the control experiments have been performed. But what is known about Cdc14p that might hint at a role in GR function? It is believed that Cdc14p belongs to the family of protein tyrosine phosphatases (PTP). Cdc14p contains the highly conserved, 11-amino acid HC motif [(I/V)**HC**XAGXXR(S/T)G] that is present in all members of the PTP family. Furthermore, Cdc14p displays a very good sequence alignment with the other members of the family in the region flanking the HC motif. Also, the amino-terminal half of Cdc14p also displays a very good sequence alignment with the complete sequence of BVP (a 19 kDa PTP encoded by the bacalovirus *Autographa californica*) and CEPTP (a protein of unknown function from *C. elegans*).

As was mentioned in the Introduction, GR is a phosphoprotein, thus Cdc14p may play a role in GR function by regulating its phosphorylation state,

especially with regard to the cell cycle. While GR is phosphorylated on serine and threonine residues, recent biochemical experiments show that Cdc14p does have dual-specificity phosphatase activity *in vitro* (Taylor et al. 1997) and, therefore, is capable of dephosphorylating phosphoserine and phosphothreonine residues. This dual specificity phosphatase activity was also seen in the case of BVP (Sheng and Charbonneau 1993). Interestingly, many dual specificity phosphatases are known to regulate mitogenic signal transduction and the cell cycle (Yuvaniyama et al. 1996 and references therein). Of further interest, double-stranded DNA increased the phosphatase activity of Cdc14p *in vitro* by 4-fold and Cdc14p could bind single- and double-stranded DNA-cellulose at up to 0.35 M NaCl. Thus, if Cdc14p is interacting with GR it could be at the level of DNA.

It is hypothesized that Cdc14p, BVP, and CEPTP form a subfamily in the PTP family that differs from the other members of the family in that they may have specialized function, mode of regulation, or substrate preference. The idea that Cdc14p might have a function other than its putative phosphatase activity is supported by recent genetic results. Lte1p activity is essential for the termination of M phase and Lte1p has homology with guanine nucleotide exchange factors for the Ras-type GTPases. Interestingly, mutations in *LTE1* lead to a cold-sensitive growth phenotype in which the cells arrest in late M phase at 11°C. A genetic screen was developed to find *lte1* strains that could grow at 11°C but not at 37°C upon further chemical mutagenesis. Mutant strains such identified were all shown to be dominant and to harbor specific mutations in *CDC15* (termed *cdc15-rlt* alleles) that mapped to kinase domains VI or VII, which are both important for Cdc15p function (Cdc15p is believed to be a MAP kinase kinase kinase). Moreover, a low-copy suppressor screen to identify genes that suppress the Ts^- phenotype of *cdc15-rlt1* cells yielded

CDC14. It should be noted that neither high-copy or low-copy plasmids containing *CDC14* could suppress the Ts$^-$ phenotype of a *cdc15-1* strain (the same allele of *cdc15* that I used in my studies). Although *cdc14* and *cdc15-1* strains both arrest at nearly identical points in the cell cycle this was the first time a genetic interaction between the two genes had been identified, although the interaction is allele specific. What was truly interesting, though, was that while alleles of *cdc14* harboring site-specific mutations in the conserved HC motif were unable to complement a *cdc14-1* strain, they could still complement the Ts$^-$ phenotype of various *cdc15-rlt* mutant strains. This result suggested that the PTPase activity of Cdc14p is required for the essential function of Cdc14p (deletions of *CDC14* are lethal) but is not required for its suppression of some mutant forms of *CDC15*. A possible dual role for Cdc14p function suggests that if Cdc14p is involved in GR function it may not be at the level of regulating its phosphorylation state but at some other point in its regulation. Obviously, further study is needed to show that Cdc14p is indeed involved in regulating GR function. But if a connection between Cdc14p and GR is established, perhaps many insights into both GR function and cell cycle regulation could be obtained.

Perspectives

Glucocorticoids are physiologically important signaling molecules that have wide spread systemic effects. The hormone signal is actuated by the glucocorticoid receptor (GR), an intracellular protein that regulates a diverse set of genes in many different cell types. GR binds DNA at selective target sites, termed glucocorticoid response elements, and can regulate transcription of nearby genes. But considering the vast panoply of cell types that respond to glucocorticoids and the multivaried physiological behaviors affected by these hormones, it is certain that there must be more to the molecular response pathway than the simple cholesterol-based hormone and the transcription factor that binds it. Undoubtedly, a complex array of cross-talk involving GR, kinases and phosphatases, other transcription factors, multiprotein complexes involved in regulating DNA structure, chaperone complexes, and so forth, are involved in determining which genes in which cell types are regulated by GR. Also, the mere nature of the DNA response element can have a large impact on GR function. Thus, our knowledge of the molecular mechanisms by which GR functions to regulate transcription in a cell-type specific manner is incomplete. It was the goal of my thesis to develop an unbiased genetic screen to identify cellular factors that interact with GR to regulate transcription. I performed this genetic screen in yeast, due to its amenable genetic system, with the hope that factors identified in yeast would have homologs in higher animals, including man.

A result of my screen was the identification of *SWP73* (Cairns et al. 1996c), a novel member of the yeast Swi/Snf complex that is involved in chromatin remodeling. Interestingly, characterization of Swp73p, Swi2/Snf2p, and their mutations revealed that their interaction with GR, as

well as other transcription factors, is regulator- and response element-specific. These findings suggest that transcription is a highly regulated process that depends heavily on the context of the cell type, transcriptional regulator, and the DNA element studied. Recent studies have also come to this conclusion (Guido et al. 1996; reviewed in Beato et al. 1996).

Further characterization of GR:Swi/Snf interaction would be interesting. For example, co-immunoprecipitation experiments with *in vivo* derivatives of GR would be illuminating because it would give further significance to the interaction between GR and Swi/Snf, as well as allowing the testing of a variety of different forms of GR that are not available as purified derivatives. Also, the co-ip studies could be extended to other transcriptional regulators, such as Yap1p and GR-LexA, the two regulators that did not display the typical functional rank order. Determining whether these two regulators still interact with the Swi/Snf complex in extracts from the various mutant strains would be of interest. Likewise, determining the strength of GR and GR(K461A) interaction with Swi/Snf in extracts from wild type or the various mutant strains by performing a salt titration of the co-ip might point to a difference for these two derivatives of GR and thus further explain the genetic results for these activators.

The yeast Swi/Snf complex, as well as its individual members, have numerous homologs in evolution. For example, Swp73p has three human homologs identified so far, BAF60a, BAF60b, and BAF60c (Wang et al. 1996). These homologs belong to large molecular weight complexes that appear very similar to yeast Swi/Snf. It would not be surprising that an interaction between GR (as well as the other nuclear receptors) and Swi/Snf is maintained in human cells. Indeed, it already has been shown that hbrm, a human homolog of Swi2/Snf2p, potentiates GR activity in mammalian cells

(Muchardt and Yaniv 1993) and that hbrm and BRG1 (a second human homolog of Swi2/Snf2p) interact with the ligand-binding domain of the estrogen receptor (Ichinose et al. 1997). Also, deletion of the two BRG1 alleles in F9 cells leads to an inviable phenotype and the heterozygous deletion affects retinoic acid-induced proliferation, but not differentiation (Sumi-Ichinose et al. 1997). Thus, perhaps one determinate of the cell-type specific regulation by nuclear receptors is a differential interaction with varying forms of Swi/Snf-like complexes in various cell types.

The known genes that are regulated by Swi/Snf, even in yeast, are limited. Recent advances in DNA array technology might allow a wide scale identification of these regulated genes. The wild type and mutant strains used in my screen would be good sources for material for such a screen. Likewise, experiments in genetically amenable higher organisms, such as *C. elegans*, would be of even further interest. ZK1128.5, the *C. elegans* homolog of Swp73p, could be knocked-out by homologous recombination and the resulting mutant tested by either standard subtractive hybridation techniques or using the DNA array technology, the results of which could be more easily compiled now that the entire *C. elegans* genome is nearly sequenced. In this way, the genes of higher organism that are dependent on Swi/Snf for their regulation could be identified. My a priori guess is that since brahma is a significant homolog of Swi2/Snf2p and is genetic suppressor of mutations in *Polycomb*, a negative regulator of the homeotic genes (Tamkun et al. 1992), then the HOX gene family may be a class of genes regulated by Swi/Snf-like complexes in higher organisms.

Other types of cellular factors must also interact with GR. One provocative result from my screen is that the dual specificty phosphatase, Cdc14p, appears to play a role in GR function. Interestingly, Cdc14p activity is

increased in the presense of DNA (Taylor et al. 1997) and thus may interact with GR at DNA. An expansion of the unbiased genetic screen would certainly be useful. Also, Snf$^-$ phenotype tests could be performed to rule out additional members of the Swi/Snf complex. In this way, cellular factors that interact with GR at steps other than chromatin remodeling could be identified.

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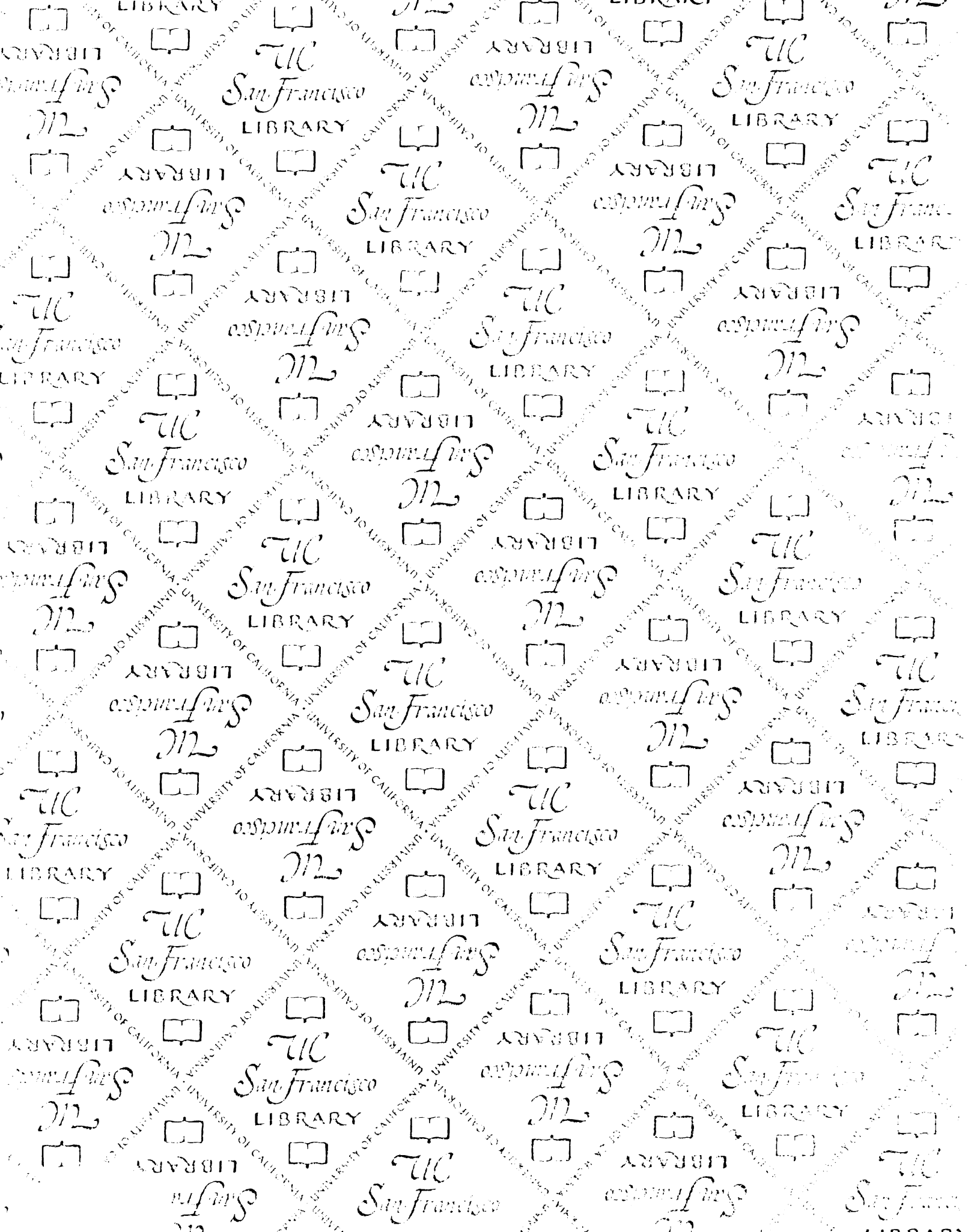
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