UBIQUITIN, THE PROTEASOME, AND DYNAMICS AT THE PROTEIN / DNA

INTERFACE

APPROVED BY SUPERVISORY COMMITTEE

Thomas Kodadek, Ph.D.

George DeMartino, Ph.D.

Kathlynn Brown, Ph.D.

Margaret Phillips, Ph.D.

DEDICATION

Thank you to everyone who helped me through this; my Mom and Dad, Janice and Lawrence Nalley; my brother, Todd Nalley; my wife Anissa. Also thanks to all of my friends who have been there for me throughout; Tim, Fernando, Jackie, and all of the rest of the Kodadek lab members past and present. THANK YOU.

UBIQUITIN, THE PROTEASOME, AND DYNAMICS AT THE PROTEIN / DNA INTERFACE

by

KIP A. NALLEY

DISSERTATION

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

April 2006

UBIQUITIN, THE PROTEASOME, AND DYNAMICS AT THE PROTEIN / DNA INTERFACE

Publication No. _____

Kip A. Nalley, Ph.D.

The University of Texas Southwestern Medical Center at Dallas, 2006

Supervising Professor: Thomas Kodadek, Ph.D.

Recently it has been discovered that a mutant species of Gal4, that contains a three amino acid change in a surface loop of the DNA binding domain, does not occupy the GAL 1/10 promoter under Gal4 inducing conditions as measured by Chromatin Immunoprecipitation (ChIP) assays. However, this protein, Gap71, occupies the promoter similarly to Gal4 under non-inducing (poised) conditions. Additionally this protein was found to be poorly ubiquitylated *in vitro* under conditions where Gal4 is ubiquitylated. In order to determine the mechanisms involved in the protein destabilization I have examined the properties of the individual mutations that comprise Gap71. These experiments have

revealed that serine 22 is a site of phosphorylation of the Gal4 DBD and that lysine 23 is essential for S22 phosphorylation, possibly acting as part of the kinase recognition site. Mutation of either residue blocks Gal4 DBD phosphorylation, its subsequent ubiquitylation and compromises the ability of the activator to bind promoter DNA in vivo. These data represent the first report of an essential phosphorylation event for this paradigmatic transcription factor.

In addition, experiments were done to directly measure the dynamics of the Gal4 / DNA complex. To measure the dynamics I have exploited the system developed by Dr. D. Picard and others using the Gal4 DNA binding domain fused to the estrogen receptor ligand binding domain. Each of these constructs has been shown to be inactive until the addition of estradiol, when they are released and bind the Gal4 UAS. These constructs allow me to temporally control the appearance of a large quantity protein that is able to compete with the endogenous Gal4 for the UAS sites in the genome. Under non-inducing conditions, the results are consistent with a rapidly exchanging complex. However, upon induction, the Gal4-promoter complexes “lock in” and exhibit long half-lives of one hour or more. Furthermore, pharmacological inhibition of proteasome-mediated proteolysis had little or no effect of Gal4-mediated gene expression. These studies show that proteasome-mediated turnover is not a general requirement for transactivator function and, when considered in the context of previous studies, that different transactivator-promoter complexes can have widely different lifetimes.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER ONE

UBIQUITIN, THE PROTEASOME, AND TRANSCRIPTION

Throughout this dissertation I will describe the characterization of novel link between the dynamics of transcription factor / DNA binding and the proteasome. In this chapter I will introduce the pathways involved, the ubiquitin – proteasome pathway particularly as it relates to transcription, and regulation of transcription of the galactose responsive genes. In addition, I will present published and unpublished data from the Johnston and Kodadek laboratories that link a non – proteolytic activity of the proteasome with transcription.

The Ubiquitin – Proteasome Protein Degradation Pathway

The 26S proteasome is a large protein complex that is responsible for the majority of non – lysosomal protein degradation in cells.(Coux, Tanaka et al. 1996; Baumeister, Walz et al. 1998) This protein is found in eukaryotic cells from yeast to humans and some archeabacteria and eubacteria. The proteasome requires ATP to assemble efficiently and degrade ubiquitylated protein substrates.(Chu-Ping, Vu et al. 1994) The proteasome can be separated into two distinct subcomplexes the 20S core complex and the 19S regulatory complex also known as PA700 (Figure 1-1).(Glickman, Rubin et al. 1998) The 20S consists of two copies each of 14 individual subunits assembled in four rings of seven proteins each. The 20S complex contains at least three proteolytic activities. A chymotrypsin – like, a trypsin – like, and a peptidyl – glutamyl – peptide hydrolyzing activity.(Baumeister, Walz et al. 1998) The crystal structure of the 20S core from *Thermoplasma acidolphilum* show a narrow channel of about 13 angstroms that allows entry into the catalytic sites of the

proteasome.(Lowe, Stock et al. 1995) Due to the narrow size of the catalytic pore it is believed that protein substrates must be unfolded to allow access to the catalytic sites. This is thought to be accomplished by the 19S complex. The 19S can be further separated into the base and the lid sub – complexes. Separation of the 19S base and lid was first described biochemically from cells where Rpn10 was deleted. Rpn10 is thought to be the hinge between the base and lid and stabilizes the complete 19S complex. Deletion of Rpn10 allows the lid and base to be separated by a salt gradient.(Glickman, Rubin et al. 1998) The base of the proteasome is a heterohexameric ring containing the six ATPase subunits including Sug1 / Rpt6 and Sug2 / Rpt4 and also two large, non – ATPase subunits Rpn1 and Rpn2. The requirement of ATP for the degradation of ubiquitylated substrates is thought to occur through unwinding of the substrate by the ATPase subunits and feeding the polypeptide chain into the 20S core.(Glickman, Rubin et al. 1998) Consistent with this finding the 19S complex and more specifically the base of the proteasome was found to have a chaperone – like activity. The base of the proteasome was found to recognize the unfolded state of the citrate synthase enzyme. Ubiquitylation was not required for this binding activity. Following incubation with the base complex the levels of native citrate synthase increased and the levels of unfolded enzyme decreased suggesting a refolding activity is present in the base complex.(Braun, Glickman et al. 1999) This activity was also demonstrated using the entire 19S complex. Again misfolded proteins were recognized and bound by the 19S in a ubiquitin – independent manner.(Strickland, Hakala et al. 2000) This chaperone – like activity of the 19S and the base complex may be linked to the effects seen with the base complex in transcription. This will be discussed further throughout this thesis. The lid

complex contains at least 12 proteins including Rpn3-9, Rpn11, and Rpn12. Only a few activities of the lid complex proteins have been described. Rpn11 has been shown to have deubiquitylase (DUB) activity.(Verma, Aravind et al. 2002) When Rpn9 is deleted the proteasome are complexes lack Rpn10 and sediment at a lighter molecular weight than the wild – type 26S proteasome.(Takeuchi, Fujimuro et al. 1999) In addition the cells are temperature sensitive for growth and show defects in the proper regulation of cell cycle proteasome substrates.(Takeuchi and Toh-e 2001) This implies that Rpn9 is important for proper assembly of the proteasome in addition to any other activities it may possess.

The other component of the ubiquitin – proteasome pathway is the ubiquitylation system shown also in Figure 1-1. Ubiquitin is a small 76 amino acid globular protein first isolated from thymus tissue and was later found to be present in all tissues and most eukaryotic organisms. Ubiquitin is highly conserved with the differences between yeast and mammalian only three amino acids. Ubiquitin is synthesized as precursor proteins that are then proteolytically processed to free the individual ubiquitin molecules. Each of the ubiquitin molecules contains a glycine at the C – terminus that is required for conjugation. Ubiquitylation of proteins is a coordinated process involving at least three separate steps. First the ubiquitin is conjugated through a thioester linkage to the ubiquitin activating enzyme (E1) in an ATP dependent manner. Next the ubiquitin molecule is transferred by thioester exchange to a ubiquitin conjugating enzyme (E2). Members of the E2 family of proteins have been shown to be important in many cellular processes including cell cycle progression, stress response, DNA repair, and transcription. It is possible for the ubiquitin to be transferred to a substrate at this point with additional factors localizing the E2 to a

complex containing the substrate protein. These ancillary factors include the viral E6 oncoprotein associated factor (E6-AP) which is involved in the ubiquitylation and regulation of the p53 tumor suppressor.(McCance 2005) The ubiquitin can also be transferred to the substrate via thioester to another class of proteins the ubiquitin ligases (E3).(Hershko and Ciechanover 1998; Robinson and Ardley 2004) This series of transfers from E1 to E2 to E3 may impart specificity to a system to make sure substrates are properly ubiquitylated only when necessary. After the first ubiquitin is conjugated to the substrate protein the mono – ubiquitylation may remain or additional ubiquitins may be added to form a polyubiquitin chain. The presence of monoubiquitin on a substrate does not seem to involve degradation as monoubiquitylated histones or other proteins do not seem to be destabilized. The role of monoubiquitin is unknown although it appears it may act as a signal for further modifications. Monoubiquitylation of histone H2B at lysine 123 precedes methylation of histone H3 at lysine 4.(Sun and Allis 2002) In another example, ubiquitylation of IKappaB kinase - beta (IKK-beta) is a requirement for proper phosphorylation and regulates the kinase activity of the enzyme *in vivo*.(Carter, Pennington et al. 2005) The most well characterized system where monoubiquitin is involved is receptor endocytosis. In this system monoubiquitylation of membrane – bound receptors targets the proteins for lysosomal degradation. This trafficking of the receptor proteins is coordinated by the phosphorylation and ubiquitylation of the receptor followed by a series of protein / protein interactions to internalize the receptor and target the protein to the lysosome (reviewed in (Hicke and Dunn 2003; d'Azzo, Bongiovanni et al. 2005; Urbe 2005)

Ubiquitin contains seven lysine residues and several of these are known to capable of forming polyubiquitin chains. K48 linked chains are most often associated with proteasomal degradation of the protein substrate. K63 linked chains have been shown to be important for the internalization of the membrane proteins. In addition, K6, K11 and K29 linked polyubiquitin chains have been described with different properties and cellular effects for each.(Dubiel and Gordon 1999)

The Proteasome, Ubiquitin, and Transcription

Proteolytic degradation of transcription factors has been described as a major mechanism regulating the activation potential of transcriptional activators.(Maratani and Tansey 2003; Lipford, Smith et al. 2005) Much of this occurs through the actions of the ubiquitin proteasome pathway.(Conaway, Brower et al. 2002) Important evidence of this mechanism was the finding that in many activators, including Gcn4, myc, Hif1-α, jun, fos, and p53, the domains that were required for activation of transcription were also responsible to the proteolytic turnover of the protein.(Salghetti, Muratani et al. 2000) When the activation domain was removed or mutated the level of the protein increased but activation decreased. Further evidence was demonstrated by fusing one or multiple VP16 activation domains to the Gal4 DNA binding domain. As the number of VP16 domains fused to the protein increased the activation activity increased but the half – life of the protein decreased.(Salghetti, Muratani et al. 2000) Addition of the proteasome inhibitor MG132 stabilized the fusion proteins but also decreased the levels of transcription. This relationship between proteolytic turnover and activation has also been described in several other systems. One of the best characterized of these systems utilizes Gcn4, the transcriptional regulator

responsible for the expression of many of the amino acid biosynthesis pathway genes.(Hinnebusch 1986) Gcn4 levels change dramatically under conditions of amino acid starvation.(Hinnebusch 2005) This is accomplished by increasing the amounts of transcription and translation of the GCN4 gene as well as altering the degradation rate of the protein.(Kornitzer, Raboy et al. 1994) Gcn4 is ubiquitylated by a Skp/cullin/F-Box (SCF) complex.(Meimoun, Holtzman et al. 2000) SCF complexes are a form of ubiquitin ligase where the complex brings together an E2 protein by binding to the Cul1 and Rbx1 proteins and the substrate is bound by a specific F box protein. Gcn4 is bound by the Cdc4 F-box protein and is ubiquitylated by the Cdc34p E2.(Irniger and Braus 2003) This type of complex has been characterized with many different types of F-box proteins and therefore there may be many different SCF complexes present in cells at any given type. In addition there are several other different complexes including the Cul2/SOCS box adaptor complexes and the Cul3/BTB domain adaptor complexes.(Willems, Schwab et al. 2004) Each of these imparts specificity to the ubiquitin ligase activity. However, it has also been shown in yeast that many of the F-box containing proteins have effects on many cellular processes. For instance, the Met30 protein has been shown to interact with the transcription factor Met4 and alter the expression of the *MET* genes.(Patton, Peyraud et al. 2000; Rouillon, Barbey et al. 2000) Met30 also has been shown to be involved in multiple points in the cell cycle as well as sensitivity to cadmium and other heavy metals.(Flick, Ouni et al. 2004; Barbey, Baudouin-Cornu et al. 2005; Su, Flick et al. 2005; Yen, Su et al. 2005) The Cdc4 protein was originally discovered by analysis of mutations that affect cell cycle progression. Cdc4 temperature sensitive mutant strains were blocked at the G1 / S transition at the restrictive temperature.

This was also replicated with other members of the SCF^{CDC4} complex.(Meimoun, Holtzman et al. 2000) SCF activity is present throughout the cell cycle and therefore there must be additional controls to prevent ubiquitylation at improper times. For many substrates this occurs by phosphorylation events. Gcn4 is phosphorylated by the Srb10 protein kinase and the Pho85 protein kinase. Phosphorylation of Gcn4 by Srb10 occurs in the nucleus of the cell and is believed to occur concurrently with transcriptional activation. Phosphorylation of Gcn4 by Srb10 was also shown to be necessary for ubiquitylation of the Gcn4 protein in an in vitro ubiquitylation assay.(Chi, Huddleston et al. 2001) This multi – substrate phosphorylation of the CTD, mediator, and transcriptional activators by Srb10 is potentially necessary for regulation of many transcription factors. Gcn4 phosphorylation by the Pho85 kinase occurs through a separate pathway. Pho85 is a cyclin – dependent kinase involved in cell cycle progression and nutrient metabolism. Pho85 exists in complex with the Pcl family of proteins. It has been hypothesized that the Pho85 – Pcl complex monitors growth conditions. One specific Pcl protein Pcl-5 appears to regulate Pho85 phosphorylation and subsequent degradation of Gcn4. Expression of the Pcl-5 protein is regulated by Gcn4 mediated transcription forming a feedback loop that is regulated by the Pcl-5 protein levels.(Shemer, Meimoun et al. 2002)

In addition, experiments from several laboratories have demonstrated a non – proteolytic role for ubiquitin in transcriptional activation. Briefly, fusion proteins containing the LexA DNA binding domain and the VP16 activation domain are rapidly degraded and are present at low levels in yeast cells.(Salghetti, Caudy et al. 2001) When the Met30 E3 ligase is deleted from these cells, the levels of the fusion protein increase but the transcriptional

activation potential of the protein decreases. Transcription activity can be restored by genetic fusion of a single ubiquitin molecule to the N – terminus of the expressed protein. This Ubiquitin-LexA-VP16 protein is now a competent activator and is present at high levels. This led the authors to the hypothesis that ubiquitylation is not only required for degradation by the proteasome but also as a "licensing" event to promote full activity.(Salghetti, Caudy et al. 2001)

This requirement of monoubiquitylation has also been shown in another system. Bres et al. demonstrated that monoubiquitylation of the HIV encoded transactivator TAT required ubiquitylation by the Hdm2 protein. Similar to the experiments done with the LexA-VP16 protein, requirement for the Hdm2 protein was alleviated when ubiquitin was genetically fused to the TAT protein.(Bres, Kiernan et al. 2003) A potential explanation for the increased transcriptional activity of ubiquitylated activators is that ubiquitin enhances interactions with positive transcriptional regulators. This has been demonstrated by the observation that the ubiquitylated VP16-containing activators have increased interaction with the positive transcription elongation factor b (P-TEFb).(Kurosu and Peterlin 2004) Ubiquitylated activators were shown to increase the ratio of full – length transcripts to initiated transcripts. In addition, the ubiquitin modification is able to restore activity to activators where mutation in the VP16 activation domain has decreased activity.(Kurosu and Peterlin 2004) Another recent example of non – proteolytic effects of ubiquitylation is the regulation of the transcription factor Met4. As mentioned previously the Met4 transcription factor is ubiquitylated by the SCF^{Met30} complex. Depending on the growth conditions of the yeast the Met4 can have widely different fates. In minimal medium the Met4 protein is

polyubiquitylated and rapidly degraded upon the addition of excess methionine. However, when the same cells are grown in rich medium the protein is still polyubiquitylated but remains stable. Under these conditions the Met4 protein is not recruited the *MET* genes but is recruited to the *SAM* genes which are responsible for production of S-adenosylmethionine.(Kuras, Rouillon et al. 2002) This differential regulation and fates of proteins that appear to have similar ubiquitylation patterns exemplifies additional layers of complexity that determine the fate of any individual protein. Although there is not much information about the non – proteolytic processes involving polyubiquitylated substrates it appears to be an important mode of regulation. Other proteins also display differential regulation even within the same population of cells. Although the majority of the c-Myc protein is rapidly turned over there exists a population that is quite stable. Switching from the rapidly degraded pool to the stable pool is regulated during mitosis by interaction with the protein Max.(Tworkowski, Salghetti et al. 2002)

Another system that has been extensively studied with respect to the effects of ubiquitylation and the proteasome are the nuclear receptors (NR). Members of the nuclear receptors include the steroid receptors: estrogen receptor (ER), glucocorticoid receptor (GR), androgen receptor (AR), progestin receptor (PR) and mineralcorticoid receptor (MR). There are several other classes including the receptors for thyroid hormone (TR), retinoids (the RXR / RAR family), vitamin D (VDR), and a host of orphan receptors with no known ligands. The steroid receptors typically consist of several domains, including at least one activation domain and a ligand binding domain (LBD). The nuclear hormone receptors are an attractive model system for measuring protein dynamics because they are tightly regulated

by the presence or absence of hormones or other agonists and antagonists.(McKenna, Xu et al. 1999) The steroid receptors are sequestered in an inactive state by the heat shock proteins Hsp70 and / or Hsp90. This interaction is mediated through the LBD and is relieved after the addition of hormone, upon which the protein undergoes a conformational change and is released from the heat shock proteins. At this point the receptor binds DNA and may activate transcription. Antagonists of the receptors often allow release from the heat shock proteins and translocation to the nucleus but do not allow the protein to adopt the proper confirmation recruit the necessary factors to activate transcription. This regulation of protein localization through the addition of hormone has been used in many instances, including the experiments in this thesis, to regulate chimeric proteins containing the LBD of a hormone receptor and domains from exogenous proteins including Gal4.(Picard 2000)

Estrogen receptor, androgen receptor, and glucocorticoid receptor have all been shown to interact with and require proteasome at different stages of the transcription cycle. Estrogen receptor is a fairly long-lived protein with a half – life greater than three hours in the unliganded state and less than one hour with ligand bound.(Welshons, Grady et al. 1993) Degradation in both cases occurs through proteasome-mediated proteolysis.(El Khissiin and Leclercq 1999; Nawaz, Lonard et al. 1999) Sug1, in particular, was found to interact with vitamin D receptor, pregnane X receptor, retinoid X receptor, thyroid hormone receptor, and estrogen receptors α and β in a ligand dependent manner.(vom Baur, Zechel et al. 1996; Masuyama and MacDonald 1998; Masuyama, Hiramatsu et al. 2000) Overexpression of Sug1 was found to decrease the transcriptional efficiency and increase degradation of ER-α and ER-β.(Masuyama and Hiramatsu 2004) The mechanism of the effects of Sug1

overexpression is not well understood and may involve complex interplay between Sug1 binding proteins, the proteasome, and the ubiquitylation pathway. Another study has shown that overexpression of Sug1 decreases transcription mediated by a VP16-containing fusion protein but also decreases the degradation of the fusion protein. Fusion of a monoubiquitin to the N-terminus of the activator enhances these inhibitory effects.(Zhu, Yao et al. 2004)

Gal4 and Metabolism in Saccharomyces Cerevisiae

The budding yeast *Saccharomyces cerevisiae* is able to utilize different sugars and other organic compounds as sources of carbon and energy.(Barnett 1976) Differential regulation of the genes necessary to metabolize each carbon source is controlled by one or more regulatory genes. Gal4 is a transcription factor that is the master regulator of galactose metabolism in *Saccharomyces cerevisiae*. (Reviewed in (Johnston 1987) Gal4 protein binds to specific upstream activator sequences (UAS_{GAL}) on galactose responsive promoters.(Carey, Kakidani et al. 1989) Transcription of genes regulated by Gal4 is a tightly regulated process with different protein complexes present on the promoter under differing growth conditions (Figure 1-2). When the yeast is grown on glucose as the sole carbon source the expression of the Gal4 protein is low and, in addition, the upstream activator sequences on the Gal4 responsive genes are bound by repressor proteins such as Mig1 and Nrg1. When the growth medium is switched to a non – fermentable source such as raffinose, ethanol, glycerol, or lactic acid the expression of the *GAL4* gene is increased, expression of the repressor genes is decreased, and Gal4 occupies the UAS_{GAL} on the galactose responsive genes.(Alberti, Lodi et al. 2003) However, this represents a primed, but not induced, state,

since under these conditions the transcriptional repressor Gal80 binds to the activation domain of Gal4 and prevents it from interacting with coactivators or other target proteins .(Torchia, Hamilton et al. 1984; Johnston 1987) When galactose is added to the cells Gal80 repression is relieved and the repressor is translocated out of the nucleus and sequestered by the Gal3 protein.(Ma and Ptashne 1987; Sil, Alam et al. 1999).

Gal4 is a dimeric protein comprised of several domains. The N – terminal region of the protein (residues 1-63) is a Cys_6 zinc finger DNA binding domain (DBD).(Yano and Fukasawa 1997) The Cys_6-Zn_2 family of DNA – binding proteins is characterized by a binuclear zinc cluster bound by six cysteine residues.(Kraulis, Raine et al. 1992) This domain appears to be unique to fungi and includes the proteins Ppr1, Lac9, Gal4, and Hap1.(Pan and Coleman 1990) This domain has been co-crystallized with its cognate DNA site. The structure shows the zinc fingers, the leucine zipper structure and the coordinated metal ions (purple) (Figure 1-3A).(Johnston and Dover 1987). Residues 147-841 form the regulatory domain of the Gal4 protein. Very little is known about the function of this region of the protein. When nearly the entire domain (residues 148-728) is removed the protein retains only 5% of wild – type activity as measured by beta – galactosidase (β-gal) reporter gene assays. However, another deletion of the same domain (residues 210-716) retains more than 50% of wild – type activity.(Marmorstein, Carey et al. 1992) Therefore, there are important regulatory features contained within the regulatory domain but as yet they have not been mapped precisely or studied more extensively.

The C – terminal portion of the protein (residues 841-881) is the activation domain (AD).(Ding and Johnston 1997) In general, activation domains are thought to function by

making contacts with many of the proteins required for transcribing genes, the basal transcription factors .(Ma and Ptashne 1987) The Gal4 activation domain is a member of a family of activation domains denoted as acidic activation domains (AAD). Other members of this family are the activation domains of VP16, Gcn4, and many other transcriptional activators.(Triezenberg 1995) AADs have very little homology to each other yet appear to be interchangeable as suggested by the fact that many different chimeric DNA binding domain / activation domain combinations often exhibit substantial activity.(Sadowski, Ma et al. 1988) The activation domains appear to be unstructured in solution although it is not known whether they adopt a more rigid structure when bound to the general transcription machinery.(Titz, Thomas et al. 2006) The activation domain of Gal4 is required for activated transcription of the galactose responsive genes. The activation domain has been mapped by mutation to contain two separate regions. First, residues 857 – 875 are necessary for interaction with the Gal80 protein and subsequent negative regulation of Gal4-mediated transcription.(Triezenberg 1995) Residues 842-856 are necessary for transcription and possibly play an additional role in the regulation of transcription by Gal4 by interactions with portions of the proteasome as described below.(Ma and Ptashne 1987)

The proteasomal subunits Sug1 and Sug2 can suppress a mutation of Gal4

One mutation of Gal4 that has been particularly interesting was described by Matsumoto et al. as *gal4-62* which was later termed $gal4^{D}$.(Salmeron, Leuther et al. 1990) The $gal4^{D}$ mutation is a truncation mutant that encodes the first 855 amino acids, which includes only the first 14 amino acids of the activation domain.(Matsumoto, Adachi et al. 1980) This mutation is deficient for growth on galactose and retains only a small amount of

the activation potential of wild – type Gal4. See Figure 1-3B. Gal4^{D} can recover nearly wild – type Gal4 activity in several ways that appear distinct but are related. Overexpression of the *gal4^{D}* product from a 2 micron plasmid recovers the majority of the activation potential. If the last residues of Gal4^{D} are changed from Phe-Gly-Ile-Thr-Thr to Phe-Met-Val-Asn the protein now activates nearly as well as wild – type Gal4. This protein product, called Gal4^{Dc}, does not recover the ability to bind Gal80 but is a competent activator and yeast expressing Gal4^{Dc} recover the ability to grow on galactose. Also, two, and only two extragenic suppressors of the *gal4^{D}* mutation were found: *sug1-1* and *sug2-1*.(Swaffield, Bromberg et al. 1992) Sug1 and Sug2 are two ATPase subunits of the base of the 19S proteasome. The proteins were found to bind the Gal4 activation domain *in vitro*. This protein / protein interaction is antagonized by the addition of ATP. Interestingly, Sug1 and Sug2 interact similarly with the Gal4^{D} activation domain fragment but, in contrast to the intact activation domain, the interaction is not antagonized by the addition of ATP. When the experiment was repeated with *sug1-1* and *sug2-1* proteins, the interaction with the truncated AD derived from Gal4^{D} did show ATP dependence. In cells with wild – type Sug1 and Sug2 Gal4^{D} does not occupy the *GAL* gene promoters when expressed at levels similar to wild – type Gal4. This seems to indicate that at least part of the defect in Gal4^{D} is the ability to occupy UAS_{GAL} which is curious given that the DNA-binding domain of this protein is wild-type. In cells with either the *sug1-1* mutant or the *sug2-1* mutant Gal4^{D} recovers the ability to occupy promoters and subsequently activate the *GAL* genes. (Gonzalez, Delahodde, and Johnston)

These data led to a model where Gal4 occupancy is regulated by interactions between the Gal4 activation domain and the proteasomal ATPases Sug1 and Sug2 (Figure 1-4). Briefly, wild – type Sug proteins are able to destabilize the Gal4 protein from the DNA. Under normal conditions ATP can bind to the proteasomal ATPases, release the Gal4 activation domain, and the Gal4 is allowed to recycle back to the promoter and activate transcription. However, in the case of $Gal4^{D}$ the ATP effect is reduced and the protein stays bound to the Sug1 and Sug2 and sequestered from the DNA. This sequestering of the protein can then be relieved by using the sug1-1 or sug2-1 mutant proteins, restoring ATP dependence and allowing for efficient binding of the protein to the promoter DNA thus activating transcription.

Although the model presented above is very appealing, there were many holes to fill. First, and foremost, it was necessary to show that the interactions seen *in vitro* were also present in living cells. To this end Sug1 and Sug2 were shown localize to the promoters of actively transcribing *GAL* genes. This was demonstrated by ChIP assay on the *GAL 1/10* promoter following addition of galactose. In these experiments 20S and 19S lid components were not seen on the promoter. The localization requires the presence of Gal4 on the promoter. Although this is not direct evidence of the Gal4 / Sug interaction, the co-localization is in agreement with *in vitro* binding experiments where the Sug proteins were found to be recruited independently of the 20S catalytic core and the lid complex of the proteasome.(Matsumoto, Adachi et al. 1980)

Although the ChIP assays demonstrated the localization of the proteasomal ATPases to the promoters of active genes, the biological activity of complex was still unknown. In

one set of in vitro transcription experiments, the 19S complex was shown to be required for efficient elongation.(Gonzalez, Delahodde et al. 2002) In nuclear extracts that had been immunodepleted of the 26S proteasome, less full – length transcripts accumulated compared to mock-depleted extracts. Inhibition of proteolysis by chemical inhibitors or mutants in the 20S catalytic core had no effect on efficient elongation. When yeast 19S proteasome or mammalian PA700 were added back to the depleted nuclear extracts the production of full – length transcripts was restored. This suggested that the Sug proteins were involved in the process of transcriptional elongation. Cells with a temperature – sensitive mutation, *sug1-20,* showed decreased tolerance for 6-aza-uracil, a compound known to be toxic to cells with defects in efficient transcriptional elongation. Additionally, it was shown that the 19S subunits also co – immunoprecipitate with Cdc68, a known elongation factor.(Ferdous, Gonzalez et al. 2001; Ferdous, Kodadek et al. 2002) Sug1 has also been shown to interact with the XPB subunit of the transcription and DNA repair factor TFIIH which is also required for efficient transcript elongation.(Ferdous, Gonzalez et al. 2001)

These effects of the 19S subunits on elongation provided strong evidence that the proteasomal ATPases were intimately involved in transcription. However, it was still unknown whether the ATPases were capable of altering the DNA binding ability of the transcriptional activators as was proposed in the model (Figure 1-4). To test the activity of the 19S proteasome a protein destabilization assay was developed by Dr. Anwarul Ferdous in the Johnston laboratory. In this experiment, GST-Gal4-VP16 protein was incubated with immobilized DNA containing five consensus Gal4 binding sites and subsequently exposed to 19S or 26S protein and competitor DNA containing a Gal4 binding site. The amount of the

GST-Gal4-VP16 protein remaining on the DNA after pelleting the DNA was greatly reduced in the presence of the proteasome (lanes 2 and 4, Figure 1-5A). This destabilization was inhibited by the addition of antibodies against Sug1 (lanes 3 and 6) but not the proteasome inhibitor lactacystin (lane 5). This activity of the proteasome required both the presence of the VP16 activation domain (Figure 1-5B compare lanes 2 and 4) as well as ATP (Figure 1-5C).

If the 19S and 26S proteasome are able to destabilize Gal4 is there a mechanism to stabilize the Gal4 / DNA interaction to counteract this activity? To answer this question Gal4 (DBD)-VP16 was bound to the immobilized DNA as before and treated with HeLa nuclear extract and ATP. After treatment, the Gal4 protein was shown to have a reduced mobility by SDS-PAGE (Figure 1-6A compares lanes 1 and 3). The protein was found to be ubiquitylated by western blotting with anti – ubiquitin antibodies (Figure 1-6A lane 6). Since the VP16 AD lacks lysine residues, the ubiquitylation must be occurring within the 147 amino acids of the Gal4 DNA binding domain. Ubiquitylation of the Gal4 (DBD) required the VP16 activation domain (Figure 1-6B). This suggests a model where the activation domain recruits a ubiquitin ligase to ubiquitylate the Gal4 DBD. To test if the ubiquitylated DBD stabilized the DNA binding, the destabilization assay using purified 19S complex was performed on DNA – bound GST-Gal4-VP16 that had been previously treated with HeLa nuclear extract to ubiquitylate the protein. The GST-Gal4-VP16 protein that had been ubiquitylated by the addition of nuclear extract and ATP was bound stably to the DNA in the presence of 19S whereas protein that had not been modified was removed from the DNA (Figure 1-6C lane 3). The protein that had been treated with nuclear extract but not ATP, an

essential cofactor for ubiquitylation, remained susceptible to destabilization by the 19S (Figure 1-6C lane 4).

Although there has been a multitude of work done to determine the causes and effects of differences in dynamics the question remains how a given activator behaves at a specific location in living cells. A model showing the modifications of transcription and the potential consequences for regulating gene transcription is shown in figure 7. In this thesis I will describe experiments we have used to determine some of the important features of this model including post – translational modifications and dynamic exchange of the protein / DNA complex. In chapter II I examine how post – translational modifications including phosphorylation and ubiquitylation of Gal4 protein can alter the DNA occupancy levels, protein levels, and transcriptional activity in different carbon sources. In chapter III I will discuss a new methodology to measure the dynamics of transcription factors at specific sites along the DNA and whether proteasome mediated proteolysis is required for transcriptional activity of Gal4. In chapter IV I will discuss how alterations in the assay described in chapter III affect the dynamics of Gal4 binding. And, in chapter V I will discuss how each of these has allowed us to form a model for Gal4 activation dynamics and how this can be used to help explain differences between activators.

Figure 1-1

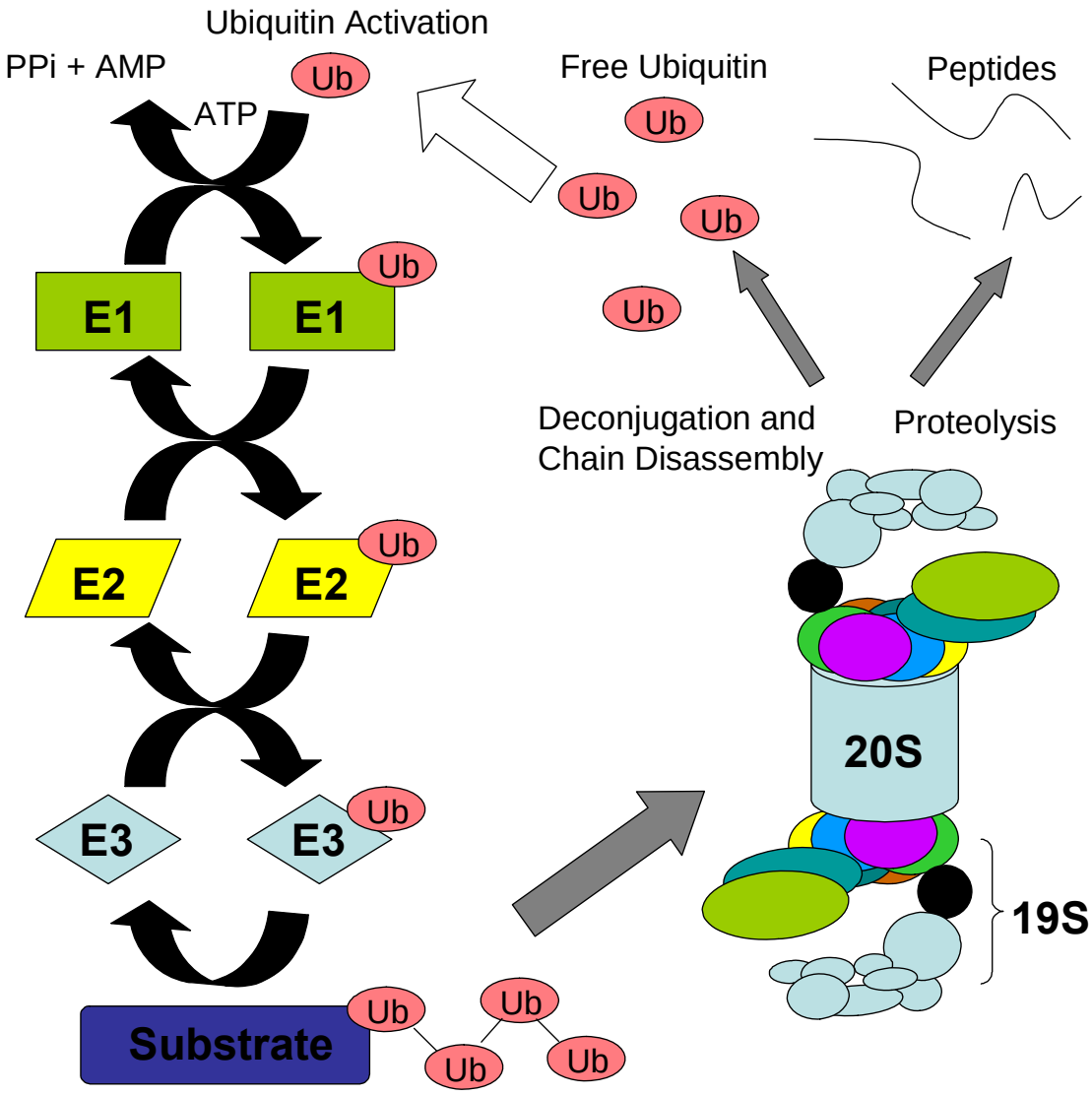


Figure 1-1

Schematic of the ubiquitin / proteasome (UPP) protein degradation pathway. Ubiquitin is bound to the E1 ubiquitin activating enzyme in an ATP dependent step. The ubiquitin molecule is then transferred to an E2 protein and subsequently transferred to the substrate through an E3 ligase. Polyubiquitylated protein is then targeted to the 26S proteasome and degraded. Ubiquitin is removed from the substrate and recycled. Peptide fragments from the proteolyzed protein are released from the proteasome.

Figure 1-2

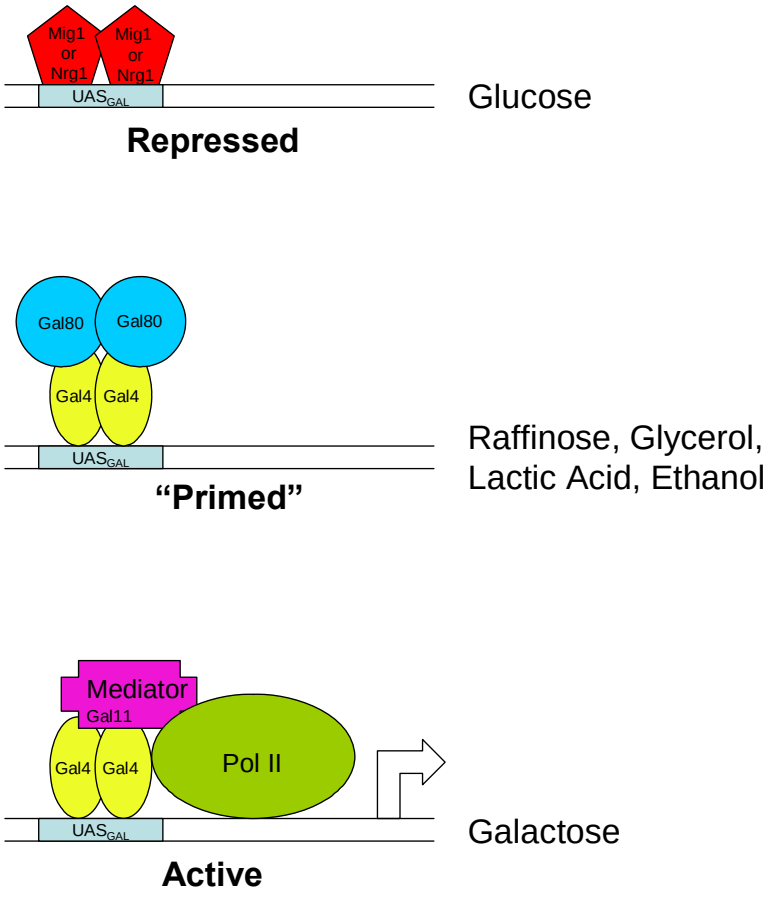


Figure 1-2

Schematic showing the state of Gal4 binding and transcriptional activation at Gal4 responsive promoters. Under repressed conditions Gal4 is not present and the promoters are bound by supressor proteins to prevent activation of the genes. Under the non – induced state Gal4 is bound to the promoter but activation is suppressed by Gal80 binding to the activation domain of Gal4. When the cells are grown on galactose Gal80 suppression is relieved and Gal4 recruits mediator through interactions with Gal11 and general transcription factors including Pol II to the promoter, activating transcription of the galactose responsive genes.

Figure 1-3

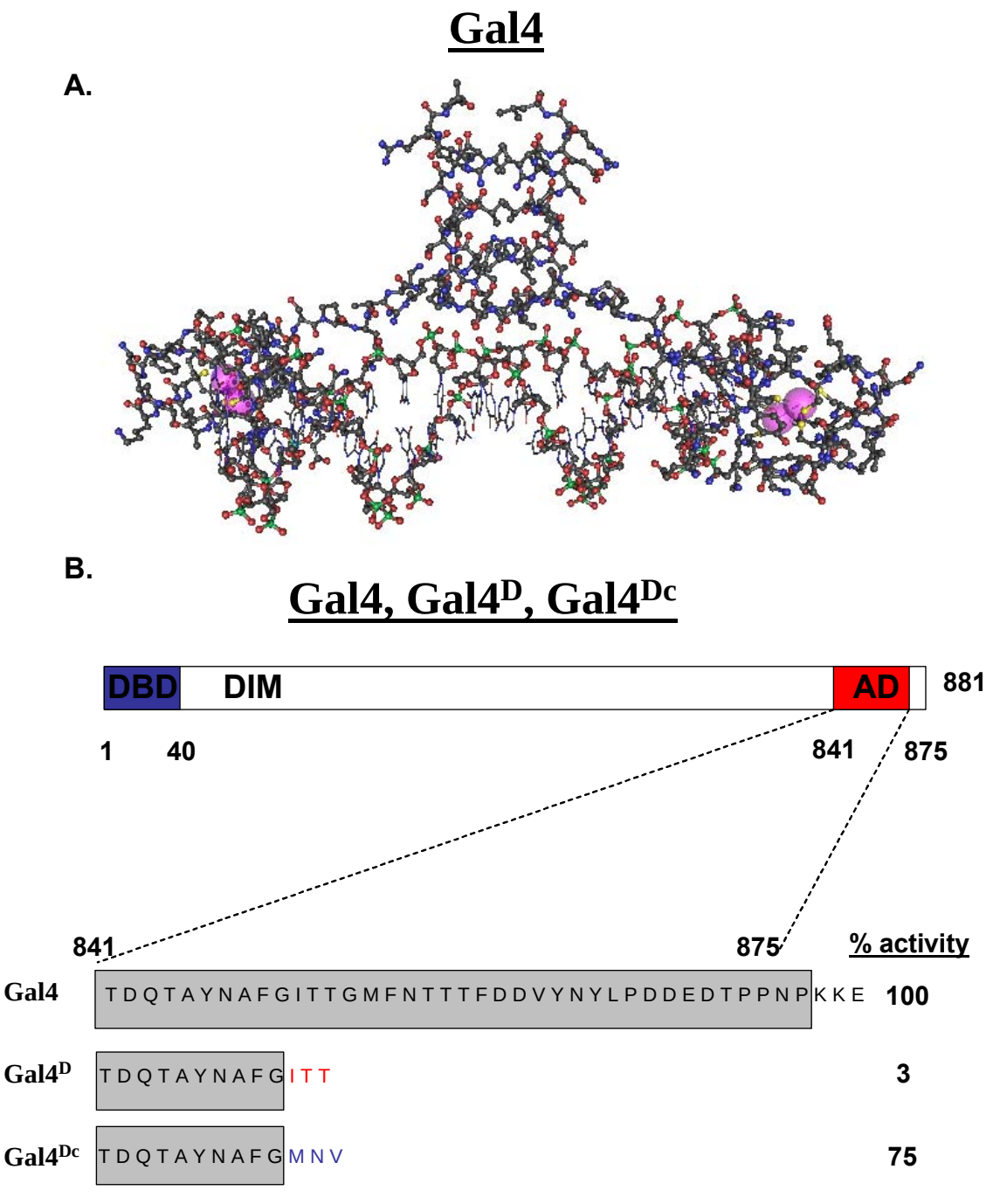

Gal4
A.
B.
Gal4, Gal4D, Gal4Dc
DBD
DIM
AD
881
1
40
841
875
841
875
% activity
Gal4
T D Q T A Y N A F G I T T G M F N T T T F D D V Y N Y L P D D E D T P P N P K K E
100
Gal4D
T D Q T A Y N A F G I T T
3
Gal4Dc
T D Q T A Y N A F G M N V
75

Figure 1-3

Structure and architecture of the Gal4 protein. A. Crystal structure of the Gal4 DNA binding domain (DBD) dimer bound to the consensus UAS_{Gal4} DNA. Visible features include; the leucine zipper interactions at the dimer interface, Cysteine-coordinated metal ions (purple), and the DNA / protein interface. B. Organization of the Gal4 protein showing the major domains as well as the amino acid sequences of the Gal4 activation domain, and the $Gal4^{D}$, and $Gal4^{Dc}$ mutants. Transcription activity of each of the mutants (normalized to wild – type Gal4) is shown next to the corresponding sequence.

Figure 1-4

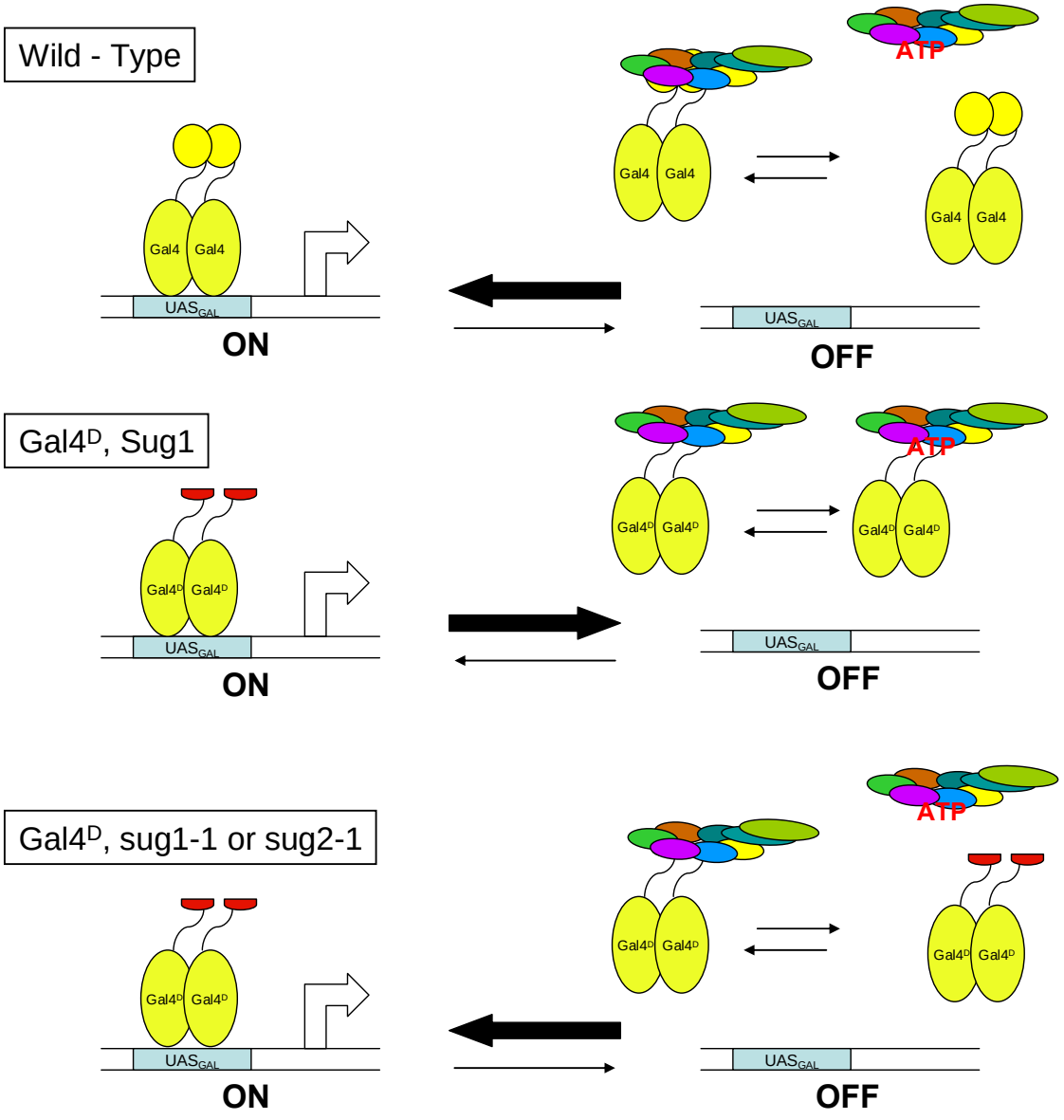

Wild - Type
Gal4
Gal4
UAS$_{GAL}$
ON
ATP
Gal4
Gal4
Gal4
Gal4
UAS$_{GAL}$
OFF
Gal4^D, Sug1
Gal4^D
Gal4^D
UAS$_{GAL}$
ON
ATP
Gal4^D
Gal4^D
Gal4^D
Gal4^D
UAS$_{GAL}$
OFF
Gal4^D, sug1-1 or sug2-1
Gal4^D
Gal4^D
UAS$_{GAL}$
ON
ATP
Gal4^D
Gal4^D
Gal4^D
Gal4^D
UAS$_{GAL}$
OFF

Figure 1-4

Model for the role of the proteasomal ATPases limiting activity of the $Gal4^{D}$ protein. Gal4 and $Gal4^{D}$ are each capable of binding to the UAS_{GAL} of the galactose regulated genes (left side). The proteasomal ATPases can bind to the activation of the protein and remove the protein from the DNA (right side). Wild – type Gal4 can be released from the wild –type Sug1 and Sug2 ATPases in the presence of ATP whereas $Gal4^{D}$ is stably bound to Sug1 and / or Sug2. $Gal4^{D}$ is released from the ATPases in an ATP dependent manner when sug1-1 or sug2-1 is present. ATP independent binding to the proteasomal ATPases does not allow for sufficient occupancy of $Gal4^{D}$ on the promoter and substantially decreases the transcription activity (middle panel). Restoration of the ATP dependent release of the $Gal4^{D}$ protein restores the occupancy of the protein and transcriptional activity (bottom panel).

Figure 1-5

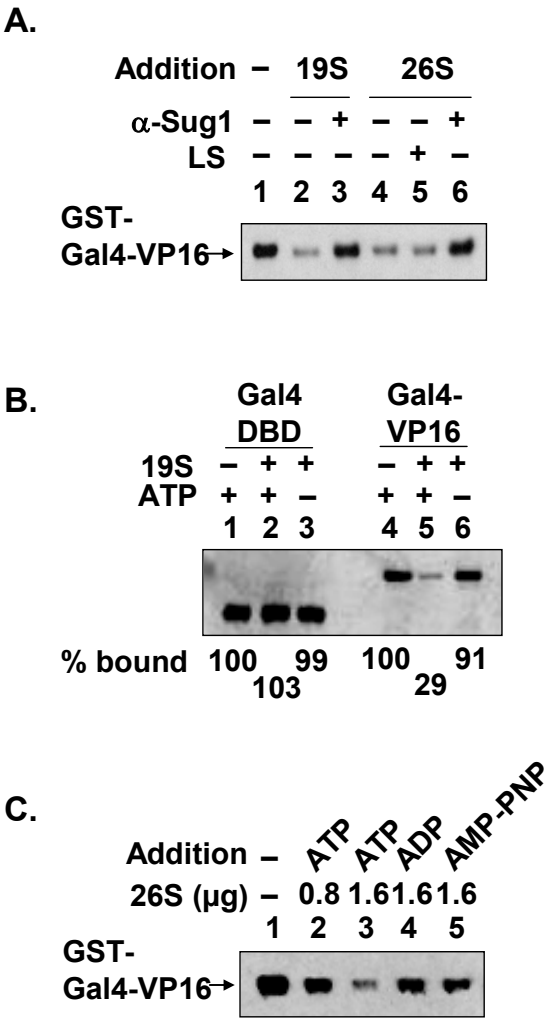
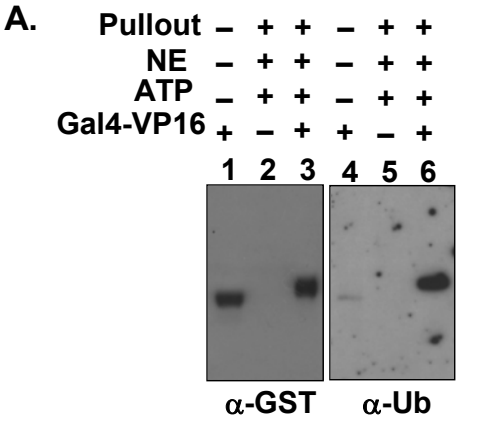
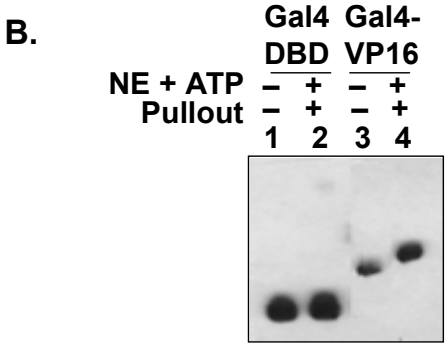

A.
Addition – 19S 26S
α-Sug1 – – + – – +
LS – – – – + –
1 2 3 4 5 6
GST-
Gal4-VP16
B.
Gal4 DBD
Gal4-VP16
19S – + + – + +
ATP + + – + + –
1 2 3 4 5 6
% bound 100 99 100 91
103 29
C.
Addition – ATP ATP ADP AMP-PNP
26S (μg) – 0.8 1.6 1.6 1.6
1 2 3 4 5
GST-
Gal4-VP16

Figure 1-5

Destabilization of activator-DNA complex by proteasome is not dependent on its proteolytic activity but does require and activation domain and ATP. A. Destabilization of activator-DNA complex in the presence of competitor DNA by proteasome is independent of proteolysis. Destabilization reactions were performed with a ten-fold excess of competitor DNA (containing five Gal4 binding sites only) added. Preincubation of 19S and 26S with anti-Sug1 antibodies and 200 μM of the proteasome inhibitor lactacystin (LS) are indicated. B. Isolation and destabilization of the indicated protein-DNA complex by 19S was carried out in the presence or absence of ATP as described in panel A. Percent protein retained on DNA is indicated. C**.** ATP hydrolysis is required for efficient destabilization of activator-DNA complex by 26S proteasome. Destabilization of activator-DNA complex by 26S proteasome was analyzed in the presence of 10 fold excess of specific competitor DNA as described in panel A, except that 500 μM of ATP and indicated ATP analogs were added. Experiments performed by Dr. Anwarul Ferdous, Johnston Lab.

Figure 1-6


A.
Pullout – + + – + +
NE – + + – + +
ATP – + + – + +
Gal4-VP16 + – + + – +
1 2 3 4 5 6
α-GST
α-Ub


B.
Gal4 DBD
Gal4-VP16
NE + ATP – + – +
Pullout – + – +
1 2 3 4

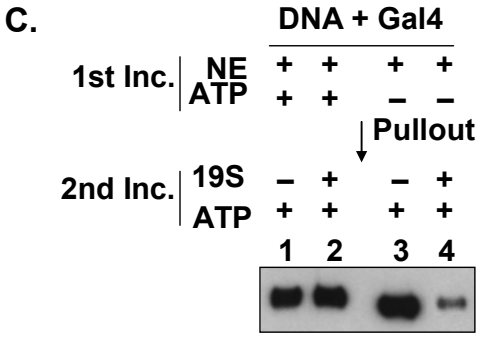

C.
DNA + Gal4
1st Inc.
NE + + + +
ATP + + – –
Pullout
2nd Inc.
19S – + – +
ATP + + + +
1 2 3 4

Figure 1-6

Ubiquitination of Gal4-VP16 in NE impedes activator-DNA complex destabilization by 19S. A. DNA-bound Gal4-VP16 is ubiquitinated. HeLa NE and immobilized DNA were incubated in the presence (+) or absence (–) of Gal4-VP16 and ATP for 30 min at 30 °C. DNA-bound activator was isolated and analyzed first with anti-GST antibodies and then re-probed with anti-ubiquitin antibodies. B. Activation domain is necessary for ubiquitination of the DNA-bound GST-Gal4-VP16. Immobilized DNA and GST-Gal4DBD or GST-Gal4-VP16 (Gal4-VP16) were incubated with (+) or without (–) HeLa NE and ATP. After 30 min incubation at 30 °C, DNA-bound protein was isolated (+ pullout) and separated on an SDS gel along with the respective purified proteins (– pullout) and detected with anti-Gal4DBD antibodies. C. Effect of ubiquitination on activator-DNA complex destabilization. Gal4-VP16, DNA and NE were first incubated (1st Inc.) with (+) or without (–) ATP as in panel A. Activator–DNA complexes were isolated, washed and analyzed for 19S-dependent destabilization as described in Figure 1-5A. Experiments performed by Dr. Anwarul Ferdous, Johnston Lab.

Figure 1-7

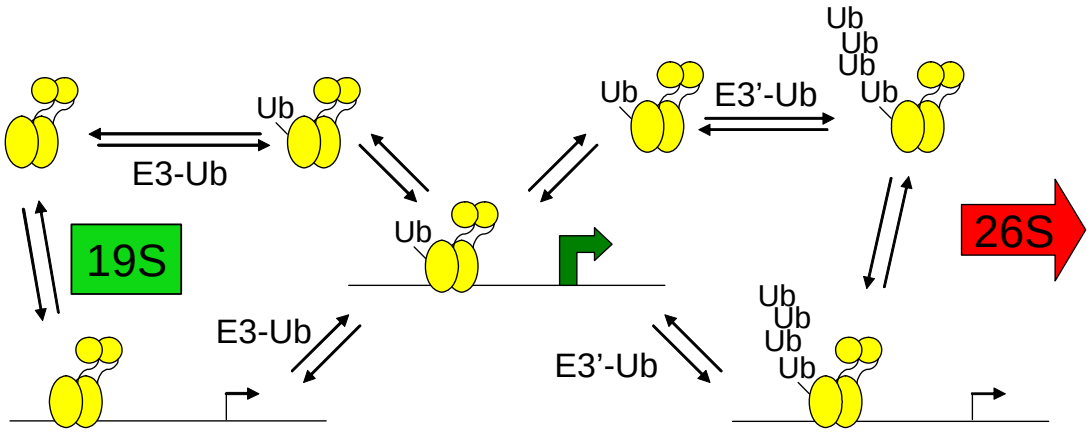


Figure 1-7

Model for the dynamics of mono-ubiquitylation, poly-ubiquitylation, DNA-binding, destabilization by the 19S proteasome, and degradation by the 26S proteasome.

CHAPTER TWO

POST-TRANSLATIONAL MODIFICATIONS OF GAL4 AND THE EFFECTS ON PROTEIN STABILITY

SUMMARY

Given the data implicating a role for the proteasome in the occupancy defects of the $Gal4^{D}$ protein, it was hypothesized that the proteasomal ATPases might have a role in removing activators from DNA. Gap71 and individual point mutations representing the three amino acid differences (S22D, K23Q, and K25F) as well as the S22A and K23R mutations were used to elucidate a possible mechanistic rationale for the instability of the protein / DNA complex *in vivo*. Gap71, Gal4 (S22A), and Gal4 (K23Q) were each found to have a defect in the ability to occupy Gal4 binding sites under inducing conditions. Gal4 (S22D) retained some activity and occupancy *in vivo*. Given these data, it is possible that S22 is normally a site pf phosphorylation and that the negatively charged aspartic acid is acting as a phosphomimetic, thus supporting partial activity. Indeed, we have found that in vitro, serine 22 and lysine 23 are both required for phosphorylation and ubiquitylation of the Gal4 DNA binding domain. In addition, the mutant proteins that were not ubiquitylated were hypersensitive to a "stripping" activity of the proteasome. These experiments suggest a positive role for phosphorylation and ubiquitylation by protecting the protein from negative regulation by the ATPases of the proteasome.

This work was a collaborative effort between me, who did the mutagenesis and full – length plasmid construction, activity assays, growth assays, and protein destruction assay (figures 1A, 1B, 2A, 2B, and 9B), Dr. Anwarul Ferdous, who performed all of the in vitro phophorylation, ubiquitylation, and "stripping" assays (figures 3-6), Dr. Devanjan Sikder who performed the ChIP assays for the full – length proteins (figures 1C, 1D, 2C, 2D, 8, and 9A), and Melissa O'Neal who did the ChIP assays and activity assays with Gal4-VP16 *in vivo* (figure 7).

INTRODUCTION

Gal4 is the transcription factor that regulates the ability of the budding yeast, *Saccharomyces cereviciae,* to grow on galactose as the sole carbon and energy source. Gal4 binds to specific upstream activator sequences (UAS_{GAL}) in the promoters of the genes necessary for the yeast to metabolize galactose as well as other sites throughout the yeast genome.(Weeda, Rossingnol et al. 1997) A protein constructed by replacing a surface loop from Gal4 with the analogous loop from the homologous yeast activator Ppr1 resulted in a mutant form of Gal4 dubbed Gap71 that was unable to activate transcription of the galactose responsive genes. This protein, however, retained the ability to bind the UAS_{GAL} consensus sequence *in vitro*.(Carey, Kakidani et al. 1989) The crystal structure of the relevant loop highlighting the mutated residues is shown in figure 2-1A. This dichotomy between the ability to activate *in vivo* and the ability to bind DNA *in vitro* suggested additional factors were required to fully activate transcription in yeast. In fact, when Gap71 was tested for the ability to bind to promoters *in vivo* using the chromatin immunoprecipitation (ChIP) assay (Figure 2-1B), it was found that Gap71 was able to bind to the *GAL 1/10* promoter under non – inducing conditions (raffinose or glycerol / lactic acid as the carbon source) at levels equivalent to wild – type Gal4 (lanes 1 and 3). When galactose was added to the medium Gal4 remained stably bound to the DNA but Gap71 was no longer present on the promoter (lanes 2 and 4). (Dr .Devanjan Sikder, Kodadek Lab) Therefore, Gal4 appears to contain a sequence within this loop that allows it to remain bound to promoters after addition of galactose.One of the reasons for this could be alterations in the post – translational

modifications of the protein. This seemed likely due to the fact that the residues changed from Gal4 to Gap71 were two lysines and a serine, all potential sites for modification. Post – translational modifications of transcriptional activators is an important way cells regulate the activation of gene transcription. (For examples see: (Li and Johnston 2001) Although many of the sites of modifications have been mapped there are still questions about how the modifications affect a given protein's ability to modulate transcription. Gal4 has been shown to be phosphorylated in at least five positions, four of which have been mapped near the C-terminal activation domain of the protein.(Corton and Johnston 1989; Corton, Moreno et al. 1998; Freiman and Tjian 2003) However, these phosphorylation events do not appear to be critical for the activity of Gal4, since activation of the GAL genes is affected modestly or not at all by mutating these resiudes to alanine. (Sadowski, Neidbala et al. 1991) However, the phosphorylation of Gal4 near the N-terminal DNA binding domain has not been studied.

Many transcription factors have also been found to be ubiquitylated and this addition of the ubiquitin can have both positive and negative roles in transcription.(Mylin 1990; Sadowski, Neidbala et al. 1991; Hirst, Kobor et al. 1999) The addition of polyubiquitin, consisting of multiple ubiquitin molecules in a chain, has been extensively studied and is thought to target proteins to the proteasome for degradation.(Muratani, Kung et al. 2005) However, this may not be the only role as polyubiquitylation of Met4 has been shown to affect the ability of the protein to activate transcription in a non – proteolytic manner.(Tansey 2001) Monoubiquitylation, or the attachment of a single ubiquitin molecule to a protein, has recently been studied in more detail. The monoubiquitin has been shown to be necessary for full activity of a number of activators.(Conaway, Brower et al. 2002; Lipford and Deshaies

2003) Although the exact role of the monoubiquitin modification in transcription is not known there is some indication that ubiquitin increases the affinity for the elongation factor P-TEFb and therefore increases the amounts of full – length transcripts derived from the ubiquitylated transcription factor.(Maratani and Tansey 2003) Monoubiquitin has also been shown to be important for receptor sorting (Thrower, Hoffman et al. 2000) and is also necessary for the IkB kinase IKKbeta to achieve full activity.(Flick, Ouni et al. 2004)

In this chapter the first phosphorylation event with a direct effect on Gal4 transcriptional activity is described. Additionally, evidence is presented that Gal4 is also ubiquitylated and that the phophorylation and ubiquitylation of the DNA binding domain affects the ability of the protein to occupy the UAS_{GAL} sequence *in vivo*.

RESULTS

Serine 22 and Lysine 23 are required for activity and occupancy

To determine the effects of the Gap71 mutations (S22D, K23Q, K25F) on the ability to activate transcription *in vivo,* plasmids expressing Gal4 or the mutant proteins at near wild – type levels were transfected into *Δgal4* yeast strain containing the α-galactosidase (*MEL1*) gene. Expression of the *MEL1* gene is dependent on the transcriptional activation activity of the Gal4 gene. Thirty minutes after induction with galactose, α-galactosidase (α-gal) activity was measured from yeast whole cell extracts. As seen in Figure 2-2A the induced α-gal activity supported by Gap71 and Gal4 (K23Q) was roughly equivalent to background, while Gal4 (S22D) was about 20% of wild – type Gal4, and Gal4 (K25F) was equivalent to wild – type Gal4. Since the negative charge of the aspartic acid mutation can substitute for a

phosphoserine the activity of the S22A mutation was examined. Consistent with the hypothesis that the partial activity of the S22D mutant was dependent on the negative charge, the Gal4 (S22A) mutant protein had only background levels of α-gal activity. Each of the mutant Gal4 containing strains was also tested for the ability to grow on galactose. Since growth on galactose requires transcriptional activation mediated through Gal4 growth on galactose as the sole carbon source can be used to confirm the results from the reporter gene assay. Growth on galactose directly correlated with the results of the α-gal assay with wild – type and the K25F mutant showing good growth, K23Q and S22A showing little to no growth, and S22D showing a punctate growth pattern (Figure 2-2B). The spotty growth in the S22D strain suggests that only a portion of the cells containing the mutant express enough of the *GAL* gene products to survive. Since Gap71 was shown previously to have an occupancy defect on galactose, each of the mutants was tested for the ability to occupy the *GAL 1/10* promoter *in vivo* by ChIP assay. As seen in figure 2-3A and quantified in figure 2-3B, each of the mutants occupied the promoter at equivalent levels when the cells were grown under non – inducing conditions with raffinose as the carbon source. Recall that under these conditions, the activation domain is masked by Gal80. However, when galactose was added to the cells there were marked differences in occupancy that correlate with the ability of each protein to activate transcription.

If the defect in activation potential of the mutant proteins was caused by deficiencies in occupying the promoter, overexpression of the protein may overcome the defect. To test this each of the mutant Gal4 proteins, Gap71, and wild – type Gal4 were all expressed from the native Gal4 promoter on a two-micron plasmid. This results in an approximately 25-fold

increase in the level of Gal4 relative to wild-type cells (unpublished observations). When the mutant proteins were overexpressed the pattern of expression was similar with the exception of Gal4 (S22D) and Gal4 (S22A) which recovered nearly full activity as compared to wild – type. Figure 2-4A. Although Gap71 and Gal4 (K23Q) have reduced activity as compared to the overexpressed wild – type Gal4 the mutant strains had recovered enough activity to restore the ability to grow on galactose as seen in Figure 2-4B. Overexpression of all of the proteins was sufficient to restore full occupancy of the proteins on the *GAL 1/10* promoter after the addition of galactose (Figures 2-5A and 2-5B). The reduced activity, but not reduced occupancy, of Gap71 and Gal4 (K23Q) indicate that there may be effects of the mutation on the ability of the protein to activate transcription in addition to effects on their ability to occupy the promoter.

S22 and K23 are Required for Phosphorylation and Ubiquitylation *In Vitro*

Since it was suspected that the S22D mutation recapitulated a phosphorylation event at that position, we sought to determine whether the Gal4 DNA binding domain could be phosphorylated *in vitro*. Each of the mutations was inserted into an E. Coli expression vector that expressed GST fused to the Gal4 DNA binding domain with or without the VP16 activation domain (GST-Gal4 DBD-VP16 and GST-Gal4 DBD respectively) shown in figure 2-6A. Each of the proteins was expressed and purified on glutathione sepharose beads to greater that 95% purity as seen on a coomassie stained gel (Figure 2-6B). To test the ability of the proteins to be phosphorylated *in vitro* HeLa nuclear extract, γ-^{32}P ATP, and GST-Gal4 DBD or GST-Gal4 DBD-VP16 were incubated together for 20 minutes in the presence or

absence of DNA containing five Gal4 binding sites. After incubation the protein was isolated by purification on glutathione sepharose, SDS – PAGE and then the protein was transferred to a membrane. As shown in figure 2-6C, a western blot using anti – GST antibodies showed GST and the Gal4 DBD containing proteins were isolated at similar levels. The presence of phosphorylated protein was measured by exposing the membrane to a phosphorimager screen to detect the conjugation of the radioactive phosphate. As indicated in the middle panel the GST-Gal4 DBD-containing proteins were phosphorylated whereas GST protein was not. GST-Gal4 DBD was phosphorylated in a DNA dependent manner. GST-Gal4 DBD-VP16 was phosphorylated in the presence and absence of DNA possibly reflecting a phosphorylation event mediatiated through the VP16 activation domain that is separable from the phosphorylation of the Gal4 DBD. The lower panel of figure 2-6C shows the total phosphorylated protein in the assay.

GST-Gal4 DBD or GST-Gal4 DBD with either the S22D or the K23Q mutation was incubated with HeLa nuclear extract and DNA as before. As seen in figure 2-6D the wild – type protein was phosphorylated almost five-fold more efficiently in the presence of DNA. The mutant proteins were phosphorylated to a similar extent in the absence of DNA. This inhibition of the specific phosphorylation event by mutation implies that S22 may be the site of phosphorylation as was hypothesized. Additionally, K23 may be part of the recognition sequence for the kinase to bind and phosphorylate Gal4.

S22 and K23 are Required for Efficient Ubiquitylation of Gal4

Since phosphorylation of serine or threonine residues often precedes ubiquitylation of nearby lysine side chains, we tested whether mutation of S22 would affect the ubiquitylation of GST-Gal4 DBD-VP16. Experimentally GST-Gal4 DBD-VP16 and each of the mutants were bound to immobilized DNA containing five Gal4 binding sites. The DNA was then isolated and washed to remove the unbound protein. A portion of this DNA was then denatured and SDS-PAGE and western blotting was used to determine the amount of protein bound to the DNA. As seen in the bottom panel of figure 2-7 each of the proteins was retained on the DNA to a similar extent showing no significant differences in the ability of each protein to recognize and bind the DNA. This is similar to the *in vivo* results described above for the full – length mutant proteins under non – inducing conditions where no differences in binding were detected. After binding the protein to the DNA the complex was then exposed to HeLa nuclear extract that had been pre – treated with anti-TRIP1 antibodies. The antibodies prevent the "stripping" of the protein from the DNA mediated by the proteasomal ATPases as was described previously. In addition to nuclear extract the proteins were incubated with ATP and His_6-tagged ubiquitin. After incubation the proteins were denatured and purified by metal affinity chromatography to isolated His_6-tagged proteins. Under these conditions only the proteins that had been covalently modified with the His_6-tagged ubiquitin would be retained. Western blotting with anti-Gal4 DBD antibodies was used to detect ubiquitylated Gal4 or mutant proteins. As shown in the top panel of figure 2-7 wild – type Gal4 and Gal4 (K25F) were ubiquitylated in this assay. Gal4 (S22D) was ubiquitylated to a lesser extent consistent with the hypothesis that the aspartic acid can substitute for the phosphorylated residue. Gap71, Gal4 (S22A), and Gal4 (K23Q) were not

ubiquitylated in this assay to any significant extent. Signal was not present in the assay either in the absence of His6-tagged ubiquitin or when no Gal4 DBD containing protein was added.

Mutations that effect ubiqtuitylation also effect destabilization by the proteasome

Since Gap71 and the S22A and K23Q mutations do not allow the protein to be ubiquitylated we tested resistance of each of the mutations to destabilization by the proteasome present in the HeLa nuclear extract. Each of the mutant GST fusion proteins was bound to the immobilized DNA, washed and then incubated with nuclear extract and ATP. The protein remaining on the DNA was measured by western blotting and is shown in figure 2-8A. Consistant with the ubiquitylation assay the wild – type and Gal4 (K25F) remained bound to the DNA and showed reduced mobility indicative of the post – translational modifications. Gap71 was efficiently removed from the DNA in this assay as was the majority of the Gal4 (K23Q) protein. Gal4 (S22D) was retained at an intermediate level. All of the retained protein showed the reduced mobility compared to the input protein. To confirm that this was an ATP and proteasome mediated destabilization GST-Gap71-VP16 was incubated with nuclear extract that had been preincubated with either hexokinase (HK) to deplete the ATP from the extract, anti-TRIP1 antibodies to block the interaction with the proteasome, or proteasome inhibitor to block the protease action of the proteasome. As seen in Figure 2-8B, depleting ATP (lane 2) or antibody binding to the proteasomeal ATPase (lane 4) completely inhibited the destabilization of Gap71. Proteasome inhibitors and non – ATPase antibodies had no effect on the destabilization (lanes 5 and 6). To confirm that

ubiquitin was the important modification a fusion protein was constructed to encode for ubiquitin at the N-terminus of the Gap71-VP16 protein (Ub-Gap71-VP16). Gap71-VP16 and Ub-Gap71-VP16 were bound to DNA and exposed to nuclear extract and ATP as before. As seen in figure 2-8C the ubiquitin fusion to the Gap71-VP16 protein significantly protected the protein from the destabilization activity (compare lanes 2 and 4). These experiments demonstrate that there is a proteasome dependent destabilization activity with purified proteasome and in nuclear extract and that phosphorylation and ubiquitylation block this activity.

Gal4 (K23Q)-VP16 does not occupy the *GAL 1/10* promoter *in vivo*

To make sure the result we had seen in vitro with HeLa nuclear extract were relevant in yeast cells we expressed Gal4-VP16 and Gal4 (K23Q) in a Δgal4 yeast strain. Since the VP16 activation domain causes proteasomal degradation in yeast, we expressed the fusion proteins from the ADH1 promoter to ensure sufficient protein levels.(Salghetti, Caudy et al. 2001; Bres, Kiernan et al. 2003) As measured by ChIP assay the Gal4-VP16 protein occupies the *GAL 1/10* promoter but very little Gal4 (K23Q)-VP16 is present. Figure 2-9A. This result is supported by measuring the α-gal activity from lysates prepared from each of these strains. As evident in figure 2-9B the Gal4-VP16 protein results in significant α-gal activity whereas the Gal4 (K23Q)-VP16 activity is greatly reduced and barely above background. These results confirm that there is a protein occupancy defect in the K23Q mutant DNA binding domain and that the biochemical assays done with the nuclear extract may represent activities that are present *in vivo*.

Ubiquitin is present on the *GAL 1/10* promoter dependent on active forms of Gal4

Next we wanted to determine whether we could detect the presence of ubiquitin on a Gal4 responsive promoter with each of the mutations. Using anti-ubiquitin antibodies in a ChIP assay it was found that ubiquitin is present on the *GAL 1/10* promoter with wild – type and each of the mutant proteins when the cells were grown on raffinose. Figure 2-10A top panel. When galactose was added to the media the ubiquitin ChIP signal decreased for the mutants that were defective in transcriptional activity and remained high for wild – type and the K25F mutant. S22D was again at an intermediate level. The ChIP data was quantified and the results shown in figure 2-10B. When the same constructs were expressed from a multi – copy plasmid, increasing the expression level, the ubiquitin ChIP signal remained constant throughout. Figure 2-10C. The presence of the ChIP signal correlates strongly with the presence of the Gal4 protein on the promoter (see figures 2-3 and 2-4). However, this is not a direct indication of ubiquitiylation of Gal4 since other proteins bound to the *GAL 1/10* promoter could be ubiquitylated and therefore be responsible for the ChIP signal.

Destabilization of Gap71 *in vivo* is not due to proteolysis of the protein

To confirm that the destabilization of Gap71 in vivo was not due to degradation of the protein the time course of removal from the GAL 1/10 promoter after addition of galactose was measured by ChIP assay and compared to the change in protein levels after the addition of galactose. By ChIP assay in figure 2-11A the disappearance of the Gap71 protein occurs with a half – life of approximately 2.5 minutes and is completely gone by 10 minutes. When

the protein levels of Gap71 are measured by immunoprecipitating the protein and then western blotting the protein can be seen out to at least 120 minutes and has an approximate half – life of one hour (Figure 2-11B). Therefore, it appears that the Gap71 protein is first removed from the DNA and then subsequently degraded.

Conservative mutations of K23 do not significantly affect activity

To answer the question whether K23 was the site of ubiquitylation I made the conservative mutation of lysine to arginine. Arginine retains the positive charge of the lysine yet is unable to be ubiquitylated by the ubiquitin conjugation system. As seen in figure 2-12, when the activity of the protein was analyzed by α-gal assay the K23R mutation seemed to have little effect on the ability to activate transcription. This suggests that K23 is not the site of ubiquitylation, but part of a recognition sequence for the ubiquitin ligase to bind and ubiquitylate the DNA binding domain at a different lysine residue.

DISCUSSION

A previously described mutant form of the Gal4 protein, called Gap71, was found to have an interesting phenotype. Although the protein binds the UAS_{GAL} sequence *in vitro*, the protein was found to be absent from promoters with the UAS_{GAL} sequences *in vivo*.(Kurosu and Peterlin 2004) Further analysis of the behavior of Gap71 showed that the protein was present on the promoters under non – inducing conditions when the activation domain is bound by the repressor protein Gal80. (see figure 2-3A lane2) When the mutations were broken down into the individual point mutations that comprise the difference between Gap71

and Gal4 (S22D, K23Q, and K25F) the proteins with the individual mutations showed differences in the ability to activate transcription and occupy the *GAL 1/10* promoter on galactose. K23Q appeared to have the same phenotype as the Gap71 protein, K25F had little to no effect on the activity, and S22D retained approximately 10% of the wild – type activity. Since a serine to aspartic acid mutation can possibly substitute for a phosphorylated reside the S22A mutation was also studied and found to be similar to Gap71 and K23Q.

With this evidence that S22 may be phosphorylated we set out to determine if we could reproduce the phosphorylation *in vitro*. Indeed, the Gal4 DNA binding domain could be phosphorylated by HeLa nuclear extract in a DNA dependent manner. The phosphorylation of S22 is also dependant on Lysine 23. This dependence seems to indicate a recognition sequence for a yet unknown Gal4 kinase in the loop between the zinc fingers in the Gal4 DBD. With the known links between phosphorylation and ubiquitylation(Salghetti, Muratani et al. 2000; Hicke and Dunn 2003; Carter, Pennington et al. 2005) we set out to determine if Gal4 is ubiquitylated *in vitro*. As with the phosphorylation, Gal4 DBD-VP16 is not ubiquitylated when the S22A or K23Q mutations are present and some portion is ubiquitylated with the S22D mutation. No effect was seen with the K25F mutation. However, when the K23R mutation was examined very little effect was seen on the activity of the protein as opposed to the K23Q mutant which abolished activity. Further studies are necessary to determine whether the K23R mutation affects occupancy *in vivo* and phosphorylation or ubiquitylation *in vitro*.

Some clues to the biological significance of the mutations may be evident from in vitro assays measuring the effects of a proteasome – mediated “stripping” activity acting on

Gal4. In the presence of 19S proteasome and ATP Gal4 DBD-VP16 is efficiently removed from DNA containing the UAS_{GAL} consensus sites. This activity is also present in HeLa nuclear extract and the activity can be blocked by preincubating the extract with antibodies raised against the mammalian Sug1 protein, Trip1. The ability of the 19S to destabilize Gal4 DBD-VP16 correlates with the ability of the protein to be phosphorylated and ubiquitylated. To test whether the post – translational modification would stabilize the protein a genetic fusion of ubiquitin to the DNA bind domain of Gap71 was used. When the Gap71-VP17 protein had ubiquitin attached it was resistant to the destabilization by the proteasome. Therefore, ubiquitylation is the modification that protects the protein from the 19S as the Ub-Gap71-VP16 fusion protein cannot be phosphorylated.

When the ubiquitin on the *GAL 1/10* promoter was determined by ChIP assay for each of the mutant proteins the signal for ubiquitin on the promoter correlated with the presence of the transcription factor. Although this does not directly measure the presence of an ubiquitylated transcription factor it does co-localize ubiquitin and the transcription factor on the promoter. However, low expression levels of the Gal4 protein have thus far prevented direct measurements of ubiquitylated, full – length protein. With the involvement of ubiquitylation in protein degradation we determined whether the removal of the Gap71 from the promoter was a proteolytic or non – proteolytic process. When the time courses for the protein disappearing from the DNA measured by ChIP assay were compared change in protein levels after addition of galactose the timing was widely different. Although the Gap71 protein levels decrease over time the protein half – life is greater than one hour whereas the protein is completely removed from the DNA within 10 minutes.

The removal of the mutated proteins from DNA *in vivo* and *in vitro* may have wide implications for mechanisms of gene regulation. The directed removal of proteins from DNA can be used by cells to coordinate gene transcription by removing either defective transcription factors as well as transcription factors which are no longer needed. Although this is often thought of as a proteolytic process we have shown a non – proteolytic role of the proteasome in removing proteins from DNA. How and when this activity is present *in vivo* is still to be determined and will be studied in the future.

MATERIALS AND METHODS

Yeast

Single copy pSB32 Gal4 or multi-copy YEp351 Gal4 were transformed into Sc 726 (w303a), *MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 S10$_3$-SUG1 gal4::HIS3*, to create Sc857 and Sc840. Gap71 and specific mutant of Gal4 were generated by PCR mediated site-directed mutagenesis of pSB32 S10$_3$-Gal4 using the following oligo pairs:

Oligo Pair	Forward	Reverse
S22A	GCTCAAGTGCGCCAAAGAAAAACCGAAGTGCGCC	GGCGCACTTCGGTTTTTCTTTGGCGCACTTGAGC
S22D	GCTCAAGTGCGACAAAGAAAAACCGAAGTGCGCC	GGCGCACTTCGGTTTTTCTTTGTCGCACTT GAGC
K23Q	GCTCAAGTGCTCCCAAGAAAAACCGAAGTGCGCC	GGCGCACTTCGGTTTTTCTTGGGAGCACTTGAGC
K23R	GCTCAAGTGCTCCCGAGAAAAACCGAAGTGCGCC	GGCGCACTTCGGTTTTTCTCGGGAGCACTTGAGC
K25F	GCTCAAGTGCTCCAAAGAATTTCCGAAGTGCGCC	GGCGCACTTCGGAAATTCTTTGGAGCACTTGAGC

Mutants were then cloned into the YEp351 plasmid to generate the corresponding multi-copy plasmids. The mutations were confirmed by sequencing (data not shown) and then transformed into Sc 726 and Sc244, MATa Δgal4 ura3-52 leu2 MEL1, for expression of Gap71 and other mutant Gal4 proteins.

Yeast strains expressing pHCA Gal4(1-93)-VP16 or pHCA Gal4(K23Q)-VP16 are under the ADH promoter in Sc 311(YJ0Z), Δgal4 Δgal80 ura3-52 leu2-3,112 his3 ade1 MEL1 GAL1::lacZ fusion (Salghetti, Caudy et al. 2001). pHCA Gal4(1-93)-VP16 was a generous gift from Dr. D. Picard (Corton, Moreno et al. 1998). pHCA Gal4(K23Q)-VP16 was constructed using PCR-mediated site-directed mutagenesis as described above.

Construction of expression vectors and purification of recombinant proteins

GST-Gap71-VP16 was generated by site directed mutagenesis of the pGEXCS-Gal4 (1-141)-VP16 (Brooks and Gu 2003) using the Quickchange system (Stratagene). Expression vectors for K23Q, K25F, S22D and S22A mutant Gal4-VP16 were generated by PCR mediated site-directed mutagenesis as described before. Plasmids pGEX-Gal4(S22D)-VP16 and pGEX-Gal4(K23Q)-VP16 were cut with Eco R1, purified from agarose gel and then re-ligated to generate pGEX-Gal4(S22D)DBD and pGEX-Gal4(K23Q)DBD, expressing Gal4 DNA binding domain with S22D and K23Q mutation. GST-Ub-Gap71-VP16 was constructed by in frame insertion of a PCR generated DNA fragment from pQE-Ub into Nco I site of GST-Gap71-VP16 where Gly^{76} (GGG) was changed to Ala (GCC). Expression and purification of each protein from *E. coli* was done as before and purity was checked by SDS-gel electrophoresis. His6-tagged ubiquitin was purified as described previously (Carter, Pennington et al. 2005).

Preparation of immobilized DNA templates

Biotinylated DNA template containing five tandem repeats of Gal4 binding sites binding sites upstream of core promoter (TATA) sequence was generated by PCR methods using pG5E4T-550 C_2AT (Mitsui and Sharp 1999) and universal primers where the primer at

the 3′-end of the each template (i.e. 3′-end of the G-less cassette (C_2AT)) was biotinylated. The PCR products were purified by QIAquick column (Qiagen) and then immobilized on Dynabeads M-280 (Dynal) according to manufacturer's instructions. Unbiotinylated primers were used to PCR amplify a DNA fragment that contained only the five Gal4 binding sites and was used as competitor DNA.

Preparation of HeLa nuclear extract

A pellet derived from a five liter culture of S3 HeLa cells was purchased from the National Cell Culture Center. HeLa Nuclear extract (NE) was prepared according to the Dignam method (Leuther and Johnston 1992). Protein concentration was determined with the Bradford method using bovine serum albumin (BSA) as a standard.

Antibodies and Western blotting

Mouse monoclonal anti-GST, anti-Gal4DBD-HRP and polyclonal anti-(His)5 antibodies were from Santa Cruz. Anti-T7 HRP antibody was from Novagen. Polyclonal anti-Gal4 antibody was isolated from a rabbit immunized with a C-terminal or amino-terminal fragment of the protein. Mouse polyclonal anti-ubiquitin antibody was generated by genetic immunization as described (Louvion, Havaux-Copf et al. 1993). Western blotting was performed as described (Ferdous, Gonzalez et al. 2001).

Analysis of protein phosphorylation

A reaction mixture (30 µl) in transcription buffer containing GST (300 ng), GST fusion proteins (100 ng), HeLa NE (40 µg), 100 µM cold ATP, 1 µl of [γ-^{32}P]-ATP (3000 Ci/mmol, ICN) and 0.2 µM/µl of okadaic acid was incubated for 20 minute at 30 °C. An aliquot (5 µl) was removed to analyze the overall kinase reaction and the recombinant proteins in the

remaining reaction (25 µl) were purified on glutathione-sepharose beads in PBS-500. The beads were dissolved in protein dye, boiled for 5 minutes and then loaded on a 10% SDS-polyacrylamaide gel. Proteins were transferred to PVDF membrane and protein phosphorylation was analyzed and quantitated as described (Ferdous et al., 2001). The membrane was then probed with anti-GST or anti-DBD antibodies.

Isolation of activator-DNA complex and analysis of its destabilization by 19S and 26S

Immobilized-DNA (~100–150 ng), salmon sperm DNA (2 µg), 20 µg of bovine serum albumin (BSA) and indicated activator proteins (100 ng/reaction) in transcription (TXN) buffer (20µl) (Ferdous et al., 2002) were incubated for 30 min at 30 °C. Activator-DNA complexes were isolated by magnetic particle concentrator, washed and then analyzed by western blotting. Activator–DNA complexes were also isolated after 30 min incubation with or without ATP in hexokinase-treated (all lanes without ATP/NTP) or untreated HeLa NE (40 µg) and the bound proteins were analyzed by western blotting. For destabilization assays, activator–DNA complexes were aliquot into different tubes, pre-coated with BSA and incubated in the presence or absence of HeLa NE or highly purified yeast 19S (500 ng), 26S (0.8–1.6 µg) (Ferdous et al., 2001), ATP (500 µM), antibodies and 10-fold excess of competitor DNA containing five Gal4 binding sites. Amount of DNA-bound activators after 15-30 min incubation at 30 °C were analyzed as above. Preincubation of NE or purified 19S and 26S with buffer, antibodies, hexokinase (Sigma), lactacystin and MG132 (Calbiochem) for 15 min at 30 °C is indicated. In some reactions, Gal4-VP16 and DNA were first incubated in hexokinase-treated or untreated HeLa NE with or without ATP and activator-

DNA complexes were then isolated to analyze 19S-mediated destabilization in the presence of ATP and excess competitor DNA.

Analysis of activator ubiquitylation *in vitro*

GST fusion activators were pre-incubated with the immobilized DNA to isolate the activator-DNA complex. An aliquot (5 µl) was removed to analyze the protein levels bound to the immobilized DNA. Ubiquitylation of DNA-bound wild-type Gal4-VP16 or mutant Gal4-VP16 was then carried out for 30 minute at 30 °C in the presence or absence of His6-tagged ubiquitin (Ub) Proteins modified with tagged ubiquitin were affinity-purified with Talon beads (Clontech) under denaturing conditions (50 mM sodium phosphate, pH 7.0, 6 M urea, 300 mM NaCl and 0.1% NP40). Ubiquitylated activators were detected by Western blotting.

Chromatin Immunoprecipitation (ChIP) assay

Promoter occupancy of WT and mutant versions of Gal4p and Gal4-VP16 on *GAL1* promoter was analyzed by ChIP assay as described (Ferdous, Gonzalez et al. 2001). Triple T7-tagged WT Gal4, Gap71 and other mutant Gal4s, expressed from a single or multi-copy plasmid were introduced into w303a *gal4::HIS3*. Cells grown in raffinose containing medium at 30 °C were treated with galactose for 30 minutes to induce the *GAL* genes. On the other hand, yeast strains, expressing WT and mutant Gal4-VP16 were grown in raffinose containing medium at 30 °C to an OD_{600} of 0.6 and formaldehyde was then added. Immunoprecipitations were carried out using anti-Gal4 carboxy terminal antibody for full-length Gal4 and anti–Gal4 amino terminal antibody for Gal4-VP16. Promoter occupancy of

wild-type and mutant Gal4 was analyzed by amplifying the DNA fragment corresponding to upstream three Gal4 sites was described (Ferdous, Kodadek et al. 2002).

Analysis of Gap71 protein levels

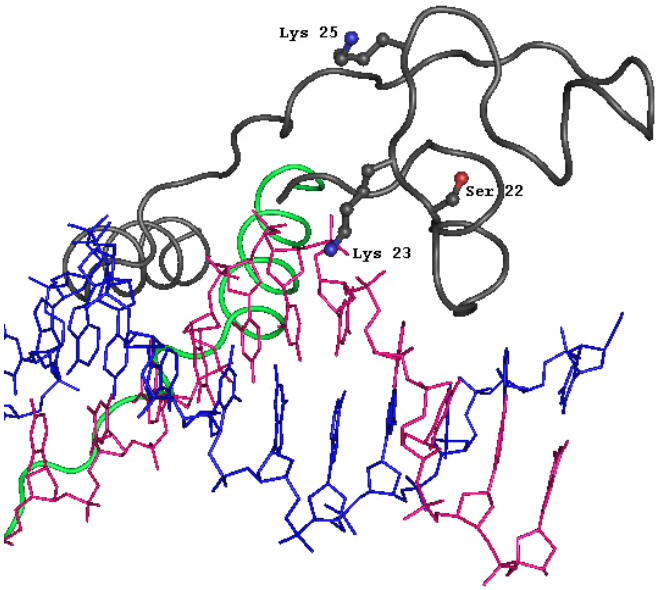
Yeast cells expressing $S10_3$-tagged Gap71 were grown to an OD600 of 0.6 on selective media containing raffinose as the carbon source. Galactose was then added to a final concentration of 2%. Aliquots were removed prior to and at specific time points after the addition of galactose. Cells were pelleted and lysates prepared by disruption of the yeast with zirconium – silica beads. Gap71 was immunoprecipitated with anti-Gal4 C-terminal antibodies from 1.2mg of extract. Immunoprecipitated protein was run on SDS-PAGE and transferred to nitrocellulose. Western blots were performed with anti-T7 antibodies (Novagen).

Reporter gene expression assay

Before adding formaldehyde in yeast cultures for ChIP assays, a 50 mL aliquot was removed for an activity assay. Cells were disrupted by vortexing with zirconia / silica beads at 4° C in citric acid buffer (31mM citric acid, 39mM KH_2PO_4, pH 4.0). Protein concentration was measured by Coomassie Plus protein assay (Pierce, Rockford, IL). Equal protein amounts were added to each assay. Transcriptional activity of WT and mutant activators was analyzed by α-galactosidase assay as described (Dignam, Lebovitz et al. 1983).

Figure 2-1

A. **Gal4 S22-K25 Loop Region**



B.

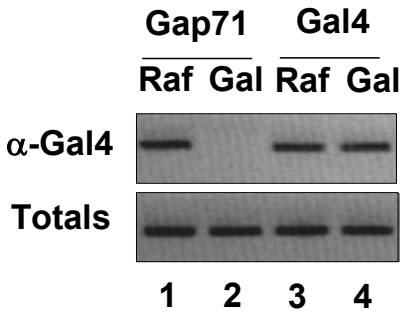
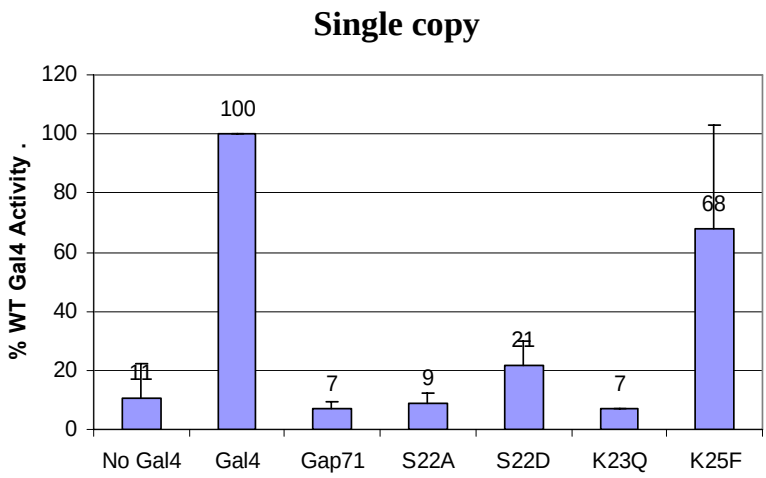


Figure 2-1

Characteristics of the Gap71 mutation. A. Expanded view of the Gal4 loop region mutated in Gap71. Residues serine 22, lysine 23, and lysine 25 that are mutated in Gap71 are highlighted. B. Chromatin immunoprecipitation (ChIP) assay showing the occupancy defect of Gap71 under inducing conditions.

Figure 2-2

A.


Single copy
% WT Gal4 Activity .
120
100
80
60
40
20
0
11
100
7
9
21
7
68
No Gal4
Gal4
Gap71
S22A
S22D
K23Q
K25F

B.

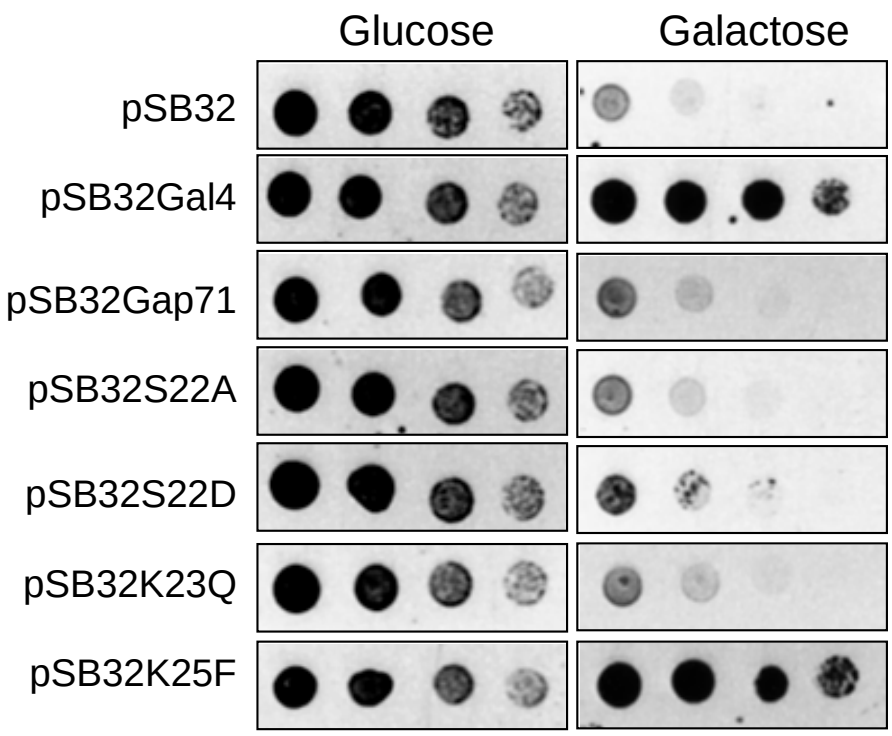

Glucose
Galactose
pSB32
pSB32Gal4
pSB32Gap71
pSB32S22A
pSB32S22D
pSB32K23Q
pSB32K25F

Figure 2-2

Mutations in the Gal4 DNA binding domain affect gene activation and the ability to grow on galactose. A. Activity of the α-galactosidase (*MEL1*) gene product in Δgal4 cells expressing the Gal4 mutants from a single – copy (*CEN*) plasmid. B. Growth assay shows strong growth defects on galactose for yeast expressing the mutant Gal4 proteins from a single – copy plasmid.

Figure 2-3

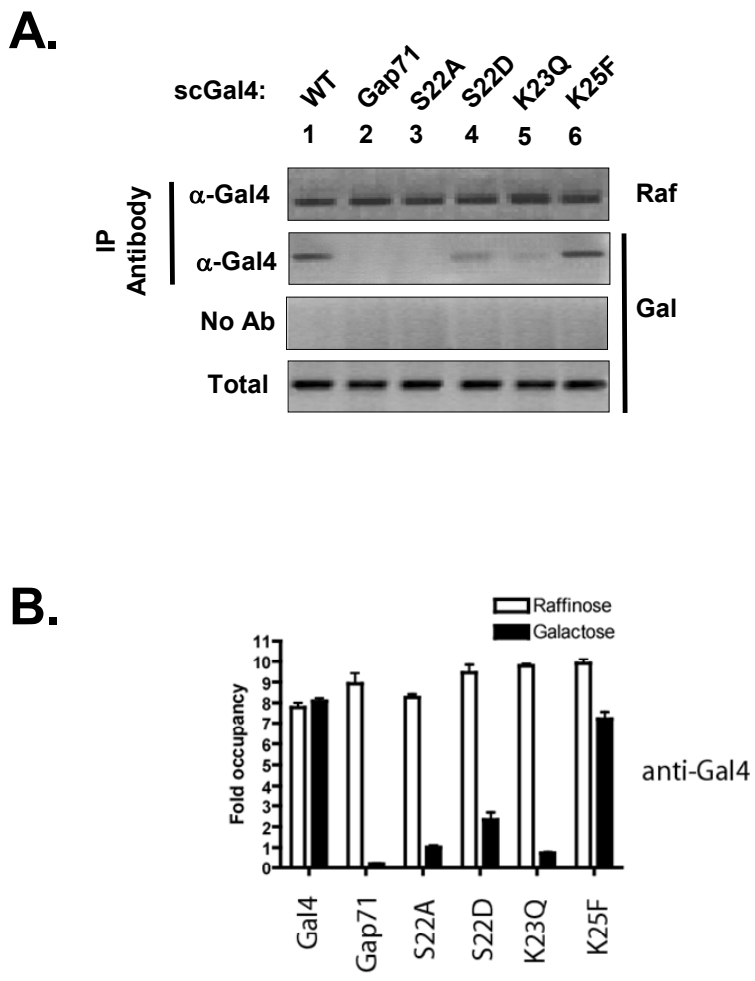


Figure 2-3

Transcription defect in the Gal4 mutants expressed from a single – copy plasmid is due to reduced occupancy in the presence of galactose. Precipitated *GAL 1/10* promoter DNA from the chromatin immunoprecipitation assay under inducing or non – inducing conditions as indicated measured by conventional PCR (A) and quantitative PCR (B)

Figure 2-4

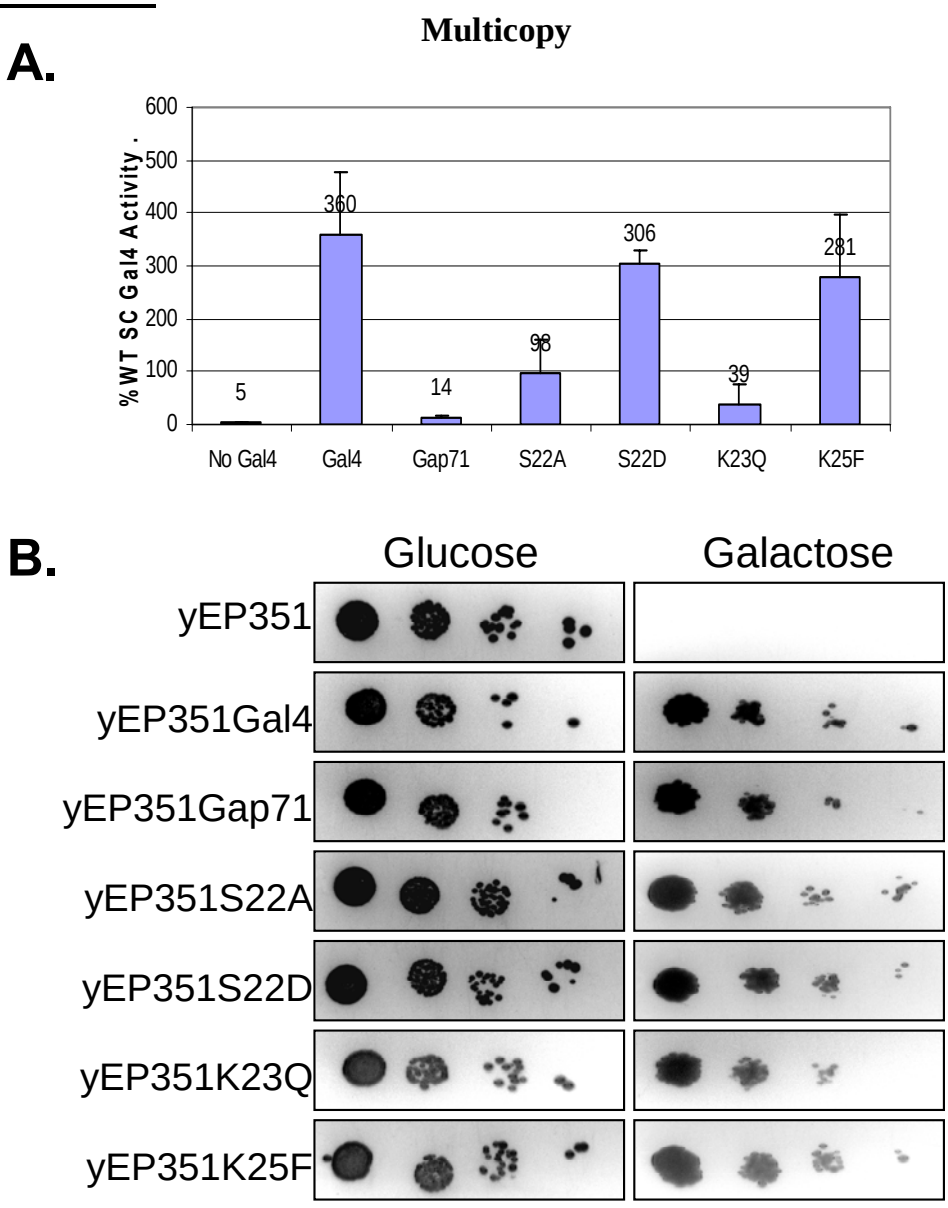

A.
Multicopy
% WT SC Gal4 Activity .
600
500
400
300
200
100
0
5
360
14
98
306
39
281
No Gal4
Gal4
Gap71
S22A
S22D
K23Q
K25F
B.
Glucose
Galactose
yEP351
yEP351Gal4
yEP351Gap71
yEP351S22A
yEP351S22D
yEP351K23Q
yEP351K25F

Figure 2-4

Overexpression of the Gal4 mutants restores some transcriptional activity and the ability to grow on galactose. A. Activity of the α-galactosidase (*MEL1*) gene product in *Δgal4* cells expressing the Gal4 mutants from a multi - copy (2μ) plasmid. B. Growth assay shows the ability of the yeast expressing the mutant Gal4 proteins from a multi – copy plasmid to grow on galactose.

Figure 2-5

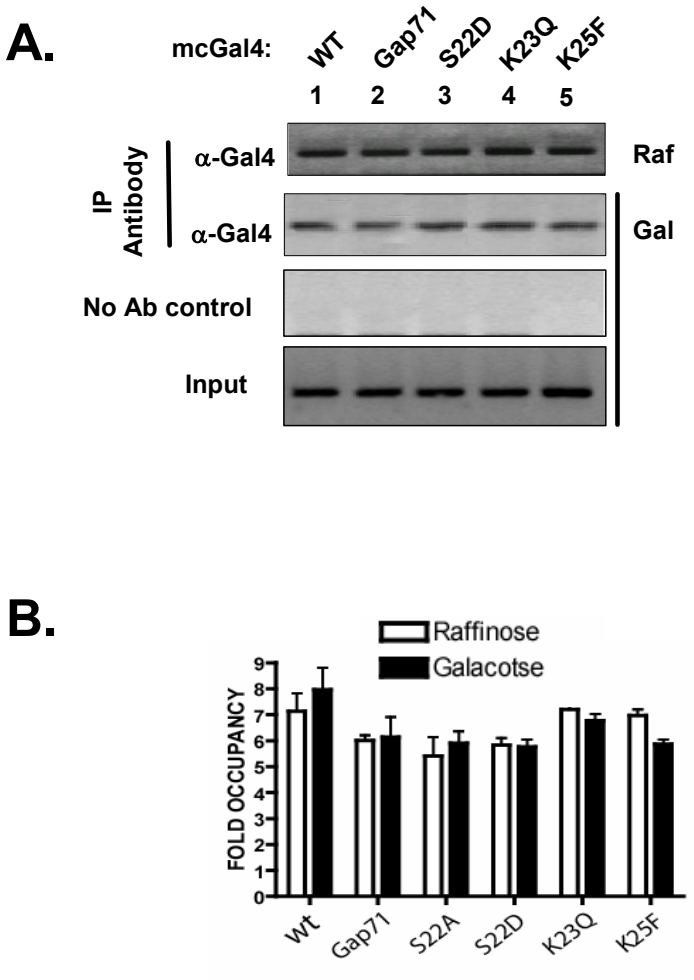


Figure 2-5

Expression of the Gal4 mutants from multi – copy plasmids restores occupancy of the protein when grown on galactose. Precipitated *GAL 1/10* promoter DNA from the chromatin immunoprecipitation assay under inducing or non – inducing conditions as indicated measured by conventional PCR (A) and quantitative PCR (B)

Figure 2-6

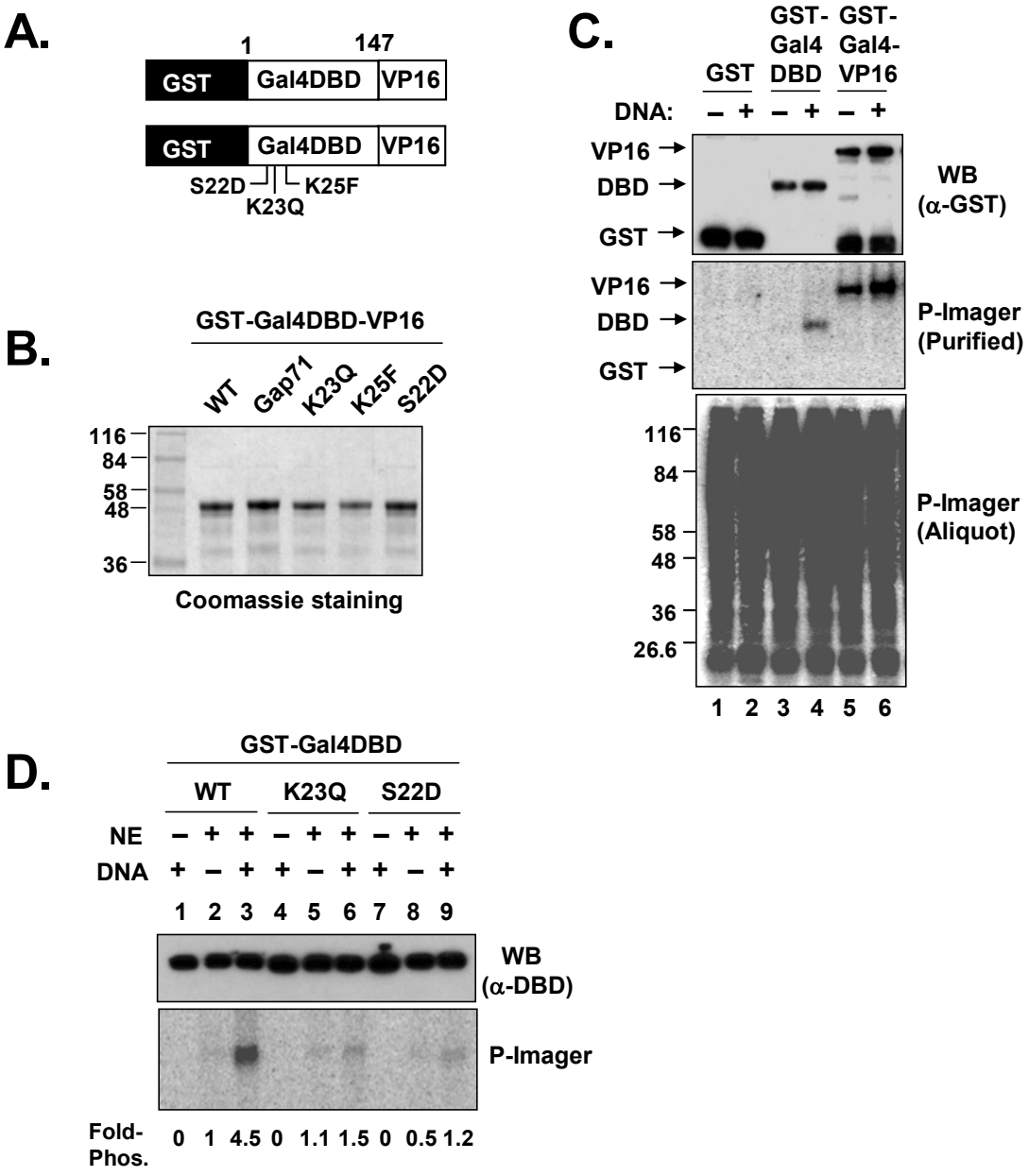

A.
1
147
GST
Gal4DBD
VP16
GST
Gal4DBD
VP16
S22D
K23Q
K25F
B.
GST-Gal4DBD-VP16
WT
Gap71
K23Q
K25F
S22D
116
84
58
48
36
Coomassie staining
C.
GST
GST-Gal4 DBD
GST-Gal4-VP16
DNA: – + – + – +
VP16
DBD
GST
WB (α-GST)
VP16
DBD
GST
P-Imager (Purified)
116
84
58
48
36
26.6
P-Imager (Aliquot)
1 2 3 4 5 6
D.
GST-Gal4DBD
WT
K23Q
S22D
NE – + + – + + – + +
DNA + – + + – + + – +
1 2 3 4 5 6 7 8 9
WB (α-DBD)
P-Imager
Fold-Phos.
0 1 4.5 0 1.1 1.5 0 0.5 1.2

Figure 2-6

The Gal4 DNA binding domain (DBD) is phosphorylated *in vitro*. A. Schematic representations of the general structure of the GST-Gal4 DBD-VP16 derivatives employed for these experiments. B. Coomassie-stained gel showing the relative purity of each of the GST-Gal4-VP16 proteins expressed in, and purified from, *E. coli*. Numbers on the left indicate the molecular masses (in kDa) of the protein standards. C. Phosphorylation of GST, GST-Gal4 DBD and GST-Gal4-VP16 in HeLa NE. The indicated GST fusion proteins were incubated with NE and γ-^{32}P-ATP in the presence (+) or absence (–) of DNA containing Gal4 binding sites. An aliquot (5 µl) was removed and processed for phosphorimager analysis, which showed extensive protein phosphorylation (bottom panel). GST fusion proteins were purified on glutathione-agarose, separated on a SDS-PAGE gel and then transferred to PVDF membrane for western blotting with anti-GST (top panel) and phosphorimager analysis (middle panel). D. Effect of K23Q and S22D on DNA-stimulated Gal4 phosphorylation. Phosphorylation of wild-type GST-Gal4-DBD and the K23Q and S22D mutants was analyzed as described in panel A in the presence (+) and absence (–) of NE and DNA.

Figure 2-7

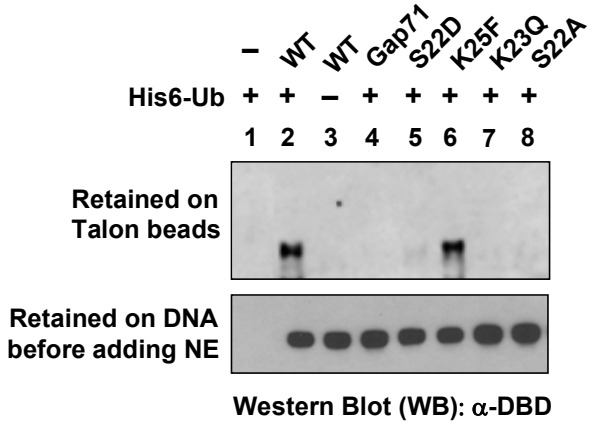
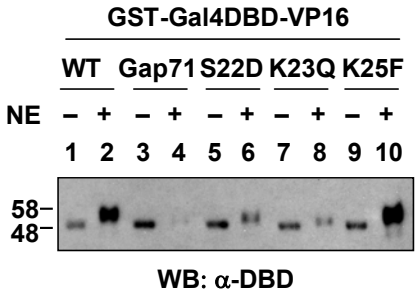


Figure 2-7

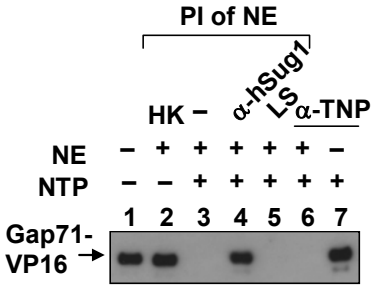
Mutations in the Gal4 DNA binding domain that affect the occupancy *in vivo* also affect the ability of the protein to be ubiquitylated *in vitro*. Top panel: Retention of GST-Gal4-VP16 derivatives on Talon (immobilized metal ion) beads under denaturing conditions. In each experiment, the Gal4 derivative indicated was incubated first with immobilized DNA containing five Gal4 binding sites, the beads were pelleted and washed briefly. The level of protein retained on the beads in each case is shown in the bottom panel. The beads were then incubated in the presence or absence of His6-ubiquitin with ATP and a HeLa nuclear extract that had been pre-incubated with anti-Trip1 antibody. The proteins were then denatured and incubated with Talon beads, conditions under which only His6-ubiquitylated proteins are retained. The bottom panel shows a Western blot revealing the level of GST-Gal4-VP16 protein retained on the Talon beads prior to NE addition in each case.

Figure 2-8

A.



B.



C.

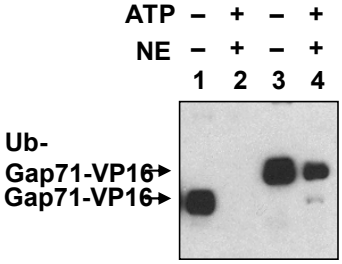


Figure 2-8

Gal4 mutant proteins are sensitive to destabilization by the proteasome. A. GST Gal4DBD-VP16 constructs were isolated as immobilized activator-DNA complexes and an approximately equivalent amount of each activator-DNA complex (compare lanes without NE) was incubated with NE and ATP for 30 minute at 30 °C. The material retained on the beads was re-isolated and analyzed by SDS-PAGE and Western blotting using an anti-Gal4 DBD antibody. B. ATP-dependent destabilization of preformed activator–DNA complex by 19S. Preformed GST-Gap71-VP16–DNA complexes were incubated in the presence (+) or absence (–) of NE, NTP and anti-TNP (negative control) antibodies. Preincubation (PI) of NE with buffer (–), hexokinase (HK), lactacystin (LS) and antibodies against Trip1 (hSug1) or TNP is indicated. The amount of DNA-bound activator after 30 min incubation in the absence of competitor DNA was analyzed. C. Genetic fusion of ubiquitin restores DNA binding of Gap71-VP16 in NE. DNA-binding activity of the indicated proteins without GST tag was analyzed in the presence (+) or absence (–) of NE and ATP. DNA-bound protein was detected by western blotting with anti-Gal4 DBD antibodies.

Figure 2-9

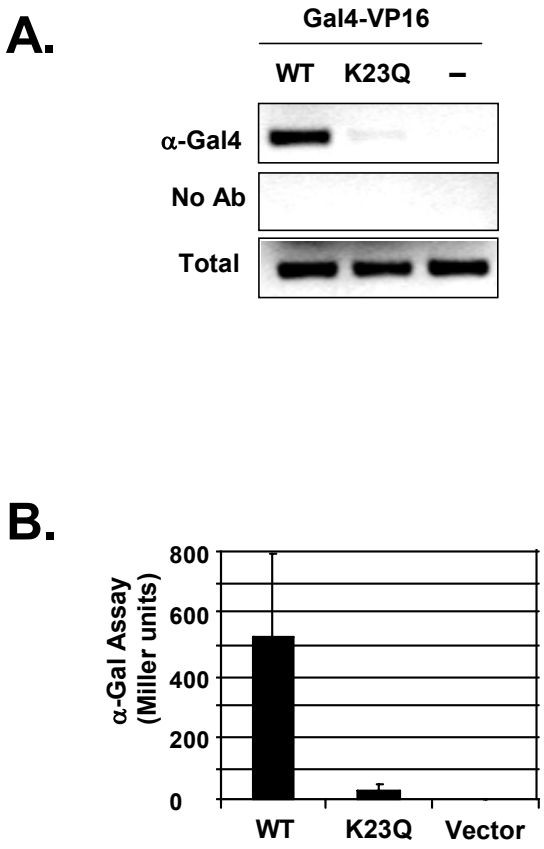


Figure 2-9

The K23Q mutation in the Gal4-VP16 affects promoter binding as well as transcriptional activity. A. Promoter binding activities of Gal4-VP16 and Gal4-VP16 K23Q *in vivo* as monitored by the chromatin precipitation assay using an anti-Gal4 N-terminal antibody. Gal4-VP16 and K23Q Gal4-VP16 were expressed at high levels (single - copy plasmid with the ADH1 promoter in a *Δgal4 Δgal80* yeast strain. B. Activities of wild-type Gal4-VP16 and the K23Q mutant *in vivo* as determined by α-galactosidase activity assay.

Figure 2-10

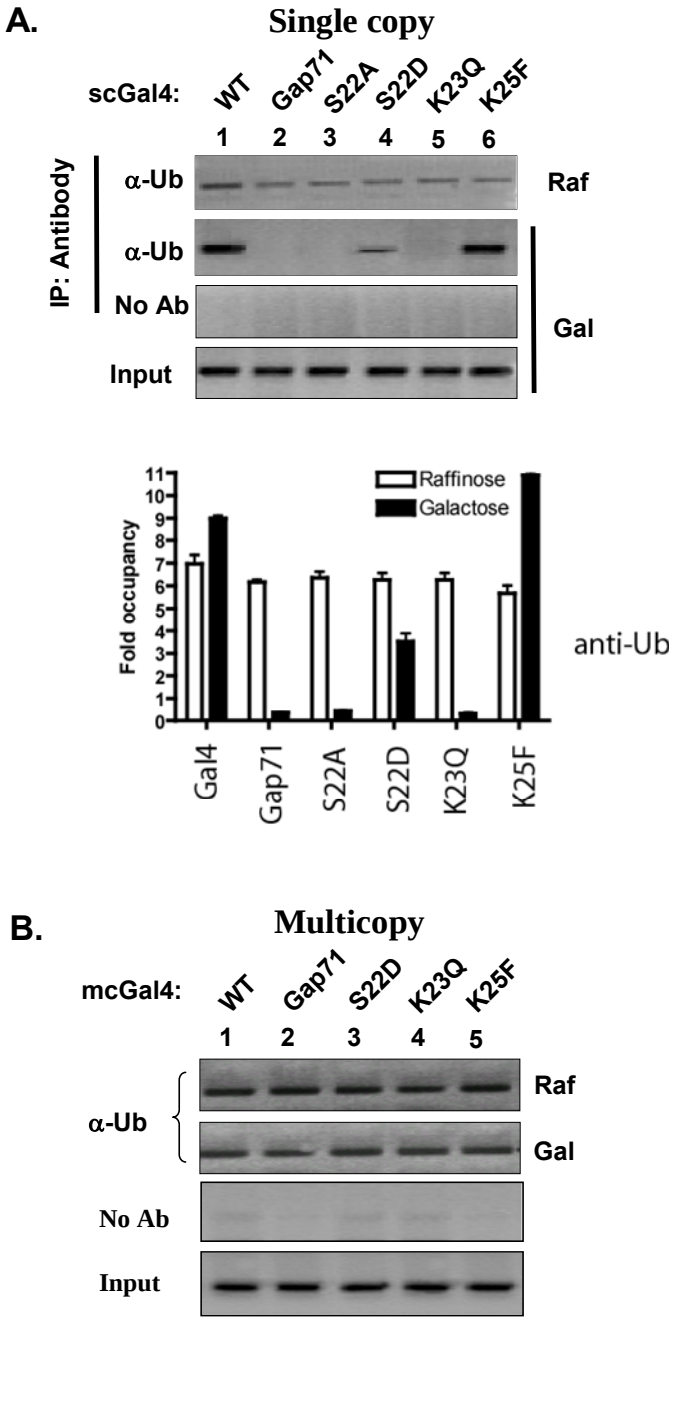

A.
Single copy
scGal4:
WT
Gap71
S22A
S22D
K23Q
K25F
1 2 3 4 5 6
IP: Antibody
α-Ub
α-Ub
No Ab
Input
Raf
Gal
Raffinose
Galactose
Fold occupancy
anti-Ub
Gal4
Gap71
S22A
S22D
K23Q
K25F
B.
Multicopy
mcGal4:
WT
Gap71
S22D
K23Q
K25F
1 2 3 4 5
α-Ub
No Ab
Input
Raf
Gal

Figure 2-10

Ubiquitin is not present on the *GAL 1/10* promoter in strains expressing mutant Gal4 proteins. ChIP assays were conducted using an anti-Ubiquitin antibody in the strains indicated, where the Gal4 derivatives were expressed from a single- (A.) or multi-copy (B.) plasmid in yeast grown under inducing or non-inducing conditions.

Figure 2-11

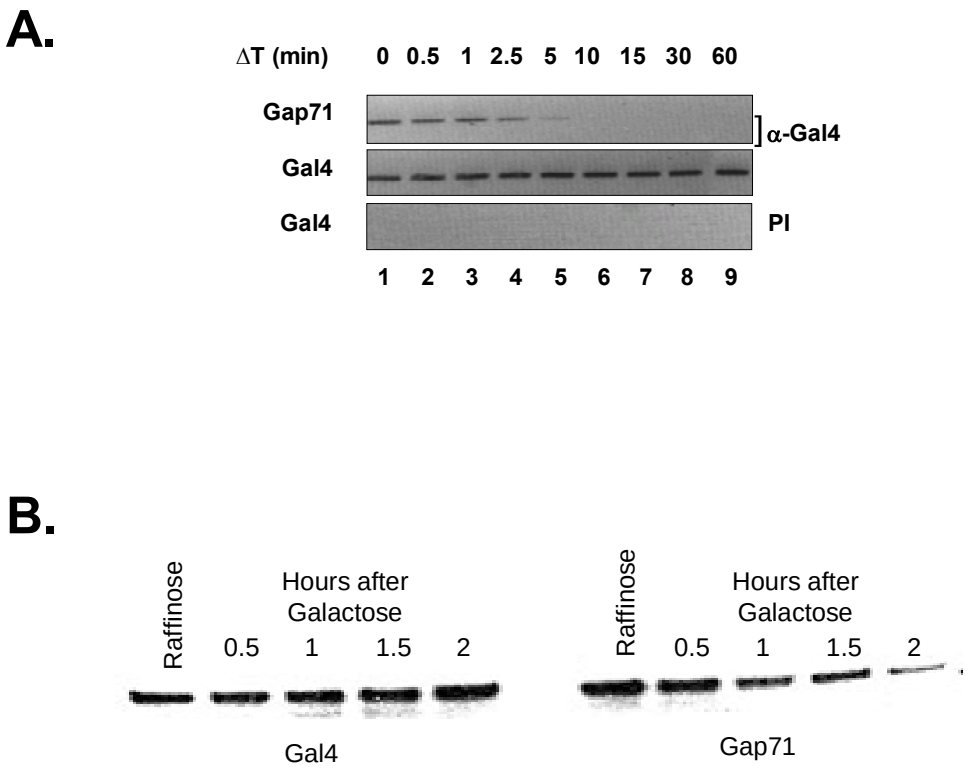


Figure 2-11

Removal of the Gap71 protein from the promoter is a non – proteolytic process. A. ChIP assay with anti-Gal4 antibodies from strains expressing Gap71 from a single – copy plasmid. B. Protein levels for the Gap4 and Gap71 protein after the addition of galactose. Cell extracts from Δgal4 cells expressing $S10_3$ tagged Gal4 or Gap71 were made by disruption with zirconium / silica beads. Gal4 or Gap71 protein was immunoprecipitated with anti – Gal4 antibodies, separated by SDS – PAGE, transferred to nitrocellulose and western blotted with anti-T7 HRP antibodies.

Figure 2-12

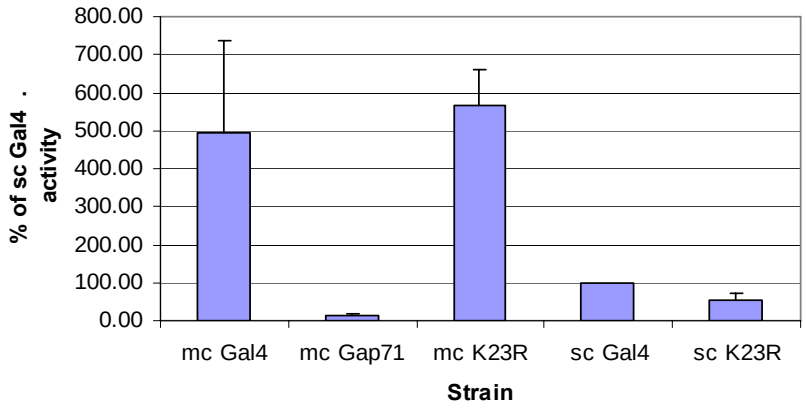


Figure 2-12

Gal4 (K23R) retains much of the activity of the wild – type Gal4 protein. α-galactosidase activity from cells expressing the wild – type Gal4, Gap71, or Gal4 (K23R) proteins from single – copy (sc) or multi – copy (mc) plasmids.

CHAPTER THREE

COMPETITIVE CHROMATIN IMMUNOPRECIPITATION ASSAY

SUMMARY

As more data arises highlighting the importance of dynamic exchange of transcription factors new technologies are needed to facilitate the study of these dynamics. To this end I have developed a new method for studying the dynamics at the transcription factor / DNA interface. Using chromatin immunoprecipitation assays to measure the exchange between the native activator and a large excess of competitor protein the stability of the transcription factor can be determined. Using this competitive ChIP assay the dynamics of the Gal4 protein was studied. Surprisingly, the Gal4 protein was shown to readily exchange when the protein was repressed but was quite stable after activation. Since this type of stabilized activator had not been described to date I tested the requirement for proteasome – mediated proteolysis in the Gal4 mediated gene activation. Consistent with the stable protein levels, the activation of the Gal4 responsive genes was not affected by the proteasome inhibitor MG132. This is the first demonstration of a transcriptional activator that is stabilized after activation. In addition, this assay represents a significant advancement in the study of transcription factor dynamics. It allows for the study of the dynamics of native proteins at natural levels on promoters throughout the genome.

INTRODUCTION

Most eukaryotic genes require the action of one or more activator proteins in order to be transcribed efficiently. These sequence-specific DNA-binding proteins recognize promoter regions in the vicinity of the target gene and subsequently recruit the transcriptional machinery and chromatin remodeling and modification complexes to activate transcription. Intensive research over many years has revealed that a common mechanism by which genes are turned on in a controlled fashion is to restrict access of the activator to the target promoter until the appropriate signal is received. This can be accomplished by a variety of mechanisms, the most common being nuclear exclusion. However, much less is known about how transcription of these inducible genes is turned off when appropriate. In particular, once an activator becomes established on a promoter, how is it removed? Does this reflect the intrinsic dissociation rate of the protein-DNA complex or are active, energy-dependent mechanisms employed to turn off gene expression?

One common mechanism that has been described is turnover of the activated transcription factor by the 26S proteasome.(reviewed in (Ferdous, Gonzalez et al. 2001; Gonzalez, Delahodde et al. 2002; Chambers and Johnston 2003) Although the protein turnover due to proteolysis appears to be an important part of the transcription process recent data has revealed that the activator / DNA interaction is a much more dynamic process. The Gannon laboratory has done a nearly comprehensive study to examine the major complexes that exist at the pS2 estrogen receptor responsive promoter. To accomplish this they used a Re-ChIP method where the initial immunoprecipitations are eluted and reprecipitated with

another antibody. From this experiment they were able to detect six distinct complexes associated with ER-α. In all of the complexes TBP and TFIIA appeared to be constant. However, histone acetyl transferases (HATs), histone methyl transferases (HMTs), Mediator components, Elongator component Elp1, members of the SWI / SNF family of proteins, and general transcription factors were all present in only one or a few of the complexes on the pS2 promoter. The presence of these different complexes all on the same promoter suggested the transcriptional complexes may be quite variable in the process of initiating transcription.(Gonzalez, Delahodde et al. 2002) To address the dynamics of the system the Gannon laboratory tracked estrogen receptor and the other components after release from α-amanitin inhibition of transcription by kinetic ChIP assay. At various time points after the release the cells were cross – linked with formaldehyde and ChIP assays were performed with antibodies to a multitude of proteins known to be involved in transcription. Each of the transcriptional cycles lasted approximately 40-50 minutes. The first unproductive cycle resulted in the recruitment of ER, members of the SWI / SNF family and GTFs including TBP and TFIIA but not Pol II. Each successive cycle afterwards included the appearance of Pol II approximately 10 minutes after the appearance of ER and phosphorylated Pol II approximately 10 minutes later. Other transcription factors cycle on and off with the ER levels on the promoter. HATs and HMTs appear during the initial stages of ER loading, TAFp130 and TAFp250 and mediator components appear concurrently with ER, and Elongator components appear concurrently with phosphorylated Pol II. Some histone modifications also oscillate with the receptor with the exception being dimethylation of histone H4 which seems to persist over the course of several cycles. This multiple cycle

appearance correlates with the binding of the GTFs prevalent in the most complexes on the promoter TBP, and TFIIA. Other factors were recruited to the promoter when ER levels were decreasing. These factors included SWI / SNF proteins, histone deacetylases (HDACs), heat shock proteins, and a component of the proteasome Trip1 or the mammalian homolog of Sug1.(Johnston and Hopper 1982) Similar oscillations of transcription factors were observed *in vitro* with the glucocorticoid receptor. Initial experiments revealed GR binding to the MMTV promoter was relieved by the addition of SWI / SNF and ATP.(Tansey 2001) In later experiments UV mediated cross – linking was used to immobilize GR and the SWI / SNF complex on chromatinized DNA templates. In an ATP dependent manner GR levels oscillated in a regular pattern with an oscillation rate of approximately five minutes and a residence time of approximately two minutes. When GR levels were increased the amounts of the SWI / SNF component Brg1 were decreased. The oscillations of GR and SWI / SNF were dependent on each other as well as ATP. In addition, the levels of histones H2A and H2B cross – linked to the DNA also oscillated with similar frequencies as GR.(Maratani and Tansey 2003)

Although the experiments measuring protein dynamics have given tremendous insight into the magnitude of protein complex exchange in cells still little is known about the dynamics of any individual protein molecule. Cross – linking by kinetic ChIP or UV is a powerful tool but it only allows one to look at the bulk distribution of proteins bound to the DNA. Individual protein molecules could be exchanging much more rapidly and the dynamic behaviors observed could either be the true rate of protein exchange or the accumulation and dispersal of proteins over time. To address these questions several

laboratories have been working on new techniques. One of the more powerful techniques is the use of fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP) methodologies. FRAP experiments measure the recovery of a fluorescent signal after bleaching a specific region of a cell with directed laser light. Rapidly diffusing or exchanging molecules will recover fluorescent signal much more quickly than slowly exchanging molecules. FLIP experiments use two separate cells, one as a reference and one as the "experimental" cell. Portions of the "experimental" cell are photobleached repeatedly in a region distal from the region to be measured. As more fluorophores are bleached the fluorescence of the measured region relative to the reference is decreased. This decrease in fluorescence is due to the exchange of fluorescence molecules from the measured region to the bleached region. FLIP experiments are performed in an attempt to make sure that the bleaching of the molecule of interest does not fundamentally change the properties of the molecules in the measured region.

The dynamics of some histone subunits measured by FRAP have been described by several groups. When a heterochromatic region or euchromatic region of the nucleus was photobleached the H1-GFP was able to completely recover fluorescence in 220 minutes or 300 minutes respectively. If the experiment was repeated with H1 that lacked either the globular domain or the C – terminal domain the fluorescence recovery occurred in less than 10 seconds. Similar FRAP experiments were also performed by another group using the major human histone subtype H1.1-GFP. In this case the fluorescence recovery time was roughly 10 minutes. It was shown that the mobility of the Histone H1.1 is not an ATP dependent process as depletion of cellular ATP did not significantly affect the recovery

time.(Conaway, Brower et al. 2002) Another GFP tagged histone subunit, Histone H2B, did not recover fluorescence significantly even after 30 minutes post – photobleach. Separate studies revealed that there was a small pool of H2B (~6%) that exchanged within several minutes although two large pools exchanged much more slowly with exchange half – lives of 130 minutes or 8.5 hours. Additionally it was found that the H3 and H4 subunits did not exchange even after several hours.(Metivier, Penot et al. 2003) The relative stability of the majority of the H2B subunits may highlight differences between the *in vitro* experiments described above and the behavior of the majority of histones in the cell. Histones near the promoters of "active" genes or near other specific sites represented by the 6% of rapidly exchanging H2B subunits *in vivo* may be similar to the H2A and H2B subunits that exchange rapidly *in vitro*.

Glucocorticoid receptor was also found to have different dynamics *in vivo* versus *in vitro*. FRAP and FLIP experiments were done using GFP tagged glucocorticoid receptor (GFP-GR) to measure the dynamics of the nuclear receptor *in vivo*. Experiments were performed using a tandem array of the mouse mammary turmor virus / Harvey viral ras (MMTV/v-Ha-ras) reporter. This array contains about 200 copies of the long terminal repeat and includes approximately 800-1200 binding sites for GR. The MMTV array allows visualization of GFP tagged nuclear receptors by binding sufficient GR molecules for substantial fluorescence signal.(Metivier, Penot et al. 2003) In both FRAP and FLIP experiments the dexamethasome activated GFP-GR was found to exchange very rapidly with complete recovery of fluorescence in approximately 20 seconds.(Fletcher, Xiao et al. 2002) In addition the recovery time of the GR interacting protein labeled with GFP (GFP-GRIP1)

was also 20 s. Recovery time of Pol II on this same promoter was found to be significantly slower with a recovery time of 13 minutes.(Nagaich, D.A. et al. 2004) These experiments demonstrate that the oscillations seen *in vivo* and *in vitro* may indeed be representing net changes in the amounts of protein bound at the promoters and does not necessarily represent the exchange rate of any given protein.

Until now most transcriptional activators on the promoters were thought to be rapidly exchanged by proteolytic or non – proteolytic means. However, recent examples of slowly exchanging transcriptional activators, including the data presented in this thesis, have been described. The Lis laboratory has shown that the transcriptional activator responsible for activation of genes after heat shock, HSF, is a slowly exchanging protein in drosophila melangaster. In drosophila salivary glands GFP-Pol II and GFP-HSF can be visualized in the polytene cells using multiple photon microscopy. Prior to heat shock GFP-HSF is mainly excluded from the chromosomal region. Using FRAP it was found that the HSF bound to chromosomal DNA exchanged rapidly with a half – recovery time of approximately 15 seconds. After heat shock the GFP-HSF is redistributed throughout the chromosomal DNA and is visible in multiple loci throughout the polytene nucleus. When the dynamics of the GFP-HSF protein were measured by FRAP at these loci the HSF was found to be quite stable with a half – recovery time greater than 6 minutes (Lis Laboratory, 2006 Submitted) This data and the data presented in this thesis are the first known examples of transcriptional activators that are not only stably bound to the DNA but also switch between a more labile and a stable form.

I this chapter I describe the development of a simple and potentially general assay for monitoring the dynamics of activator-promoter interactions in living cells and utilize it to probe directly the dynamics of Gal4-promoter interactions in yeast. I have found that Gal4-promoter complexes are quite stable under inducing conditions, with a half-life of between 30-120 minutes, depending on the nature of the promoter. We also show that Gal4-mediated gene expression is completely insensitive to proteasome inhibitors. These data show that the activity of Gal4 is not tied in an obligatory fashion to rapid, proteasome-mediated recycling of Gal4-DNA complexes.

RESULTS

To study the dynamics of Gal4–promoter complexes in living yeast cells we developed the competition assay shown schematically in Figure 3-1. The methodology relies on the fact that fusion proteins containing the ligand binding domain (LBD) of nuclear hormone receptors such as ER-α are inactive due to high affinity interactions with Hsp90 (Gong, Ni et al. 2002; Kimura 2005). When the inducing steroid is added to cells however, the complex dissociates and allows the fusion protein to migrate to the nucleus if it contains the appropriate localization sequence. We expressed a Myc-Gal4-ER LBD-VP16-FLAG fusion protein from a plasmid containing the *ADH1* promoter (Walker, Htun et al. 1999), providing a high concentration of the protein in the cytoplasm. Upon addition of β-estradiol, it was envisioned that rapid nuclear translocation would set up a situation where dissociation of native Gal4 from a promoter site would result in irreversible loss of the complex due to competition with the large excess of the LBD-containing fusion protein over the native activator. Using antibodies that recognize native Gal4 or the fusion protein uniquely, one

could then employ time-resolved ChIP assays to monitor the net rate of transcription factor exchange following steroid addition (Figure 3-1).

Myc-Gal4-ER-VP16-FLAG binds specifically and rapidly to unoccupied promoters

The time resolution of this system will be defined by the time required to build up a high concentration of the engineered transcription factor following addition of estradiol to the cells. This is known to be rapid (McNally, Müller et al. 2000), but it was important that we measure specifically the rate at which the incoming competitor protein associates with the promoter of interest in the absence of competing native transactivator. To do so, a *Δgal4* strain was transformed with a plasmid expressing Myc-Gal4-ER LBD-VP16-FLAG under the control of the *ADH1* promoter. As can be seen in Figure 3-2A, ChIP analysis using an anti-Myc polyclonal antibody failed to reveal the presence of the engineered protein on the *GAL1/10* promoter prior to addition of steroid, as expected. Upon treatment with β-estradiol the Myc-Gal4-ER LBD-VP16-FLAG protein could be detected on the promoter by ChIP using anti-Myc antibody within five minutes. The signal reaches a maximum by 15 minutes after steroid addition.

As another important calibration experiment, we analyzed the specificity of the interaction between the fusion protein and the *GAL 1/10* promoter by assessing the ability of the protein to bind to the UAS in the promoter of the Put3-regulated *PUT2* gene (Becker, Baumann et al. 2003). These sites differ from consensus *GAL* UASs only in that the essential 5'-CGG sites at each end of the pseudosymmetic sites are separated by 10 base pairs rather than eleven (Fankhauser, Briand et al. 1994). As shown in figure 3-2A, the anti-Myc antibody did not precipitate the *PUT2* promoter even after the addition of estradiol. As

another test, we examined the level of transcription of the *GAL1* and *PUT2* genes before and after the addition of estradiol. As shown in Figure 3-2B, a robust increase in *GAL1*, but not *PUT2*, transcription was seen after steroid addition, consistent with Myc-Gal4-ER LBD-VP16-FLAG association with the *GAL1/10*, but not the *PUT2*, promoter. We conclude that the competitor protein evinces the expected DNA-binding specificity and does not bind spuriously to non-cognate promoters even though it is present at very high levels.

Activated Gal4 exchanges slowly with the competitor protein

Having validated that the competitor fusion protein behaves as expected, we proceeded to employ this system to monitor the rate of Gal4 loss from a native promoter under both inducing and non-inducing conditions. Gal4, unlike many other transactivaors, binds to its cognate promoters even in the absence of an activating signal, but is prevented from driving transcription due to masking of the activation domain by high affinity binding of the specific repressor Gal80 (Picard 2000). Yeast expressing Myc-Gal4-ER LBD-VP16-FLAG were grown on media containing glycerol and lactic acid as the carbon source (non-inducing media), then induced with galactose. As expected, the Gal4-promoter complexes can be precipitated with an anti-Gal4 polyclonal antibody raised against the C-terminus of the Gal4 protein prior to the addition of β-estradiol (Figure 3-3A). The same antibody was then used to assess occupancy of the promoters by native Gal4 at various times following addition of estradiol. As shown in Figures 3-3A and B, the signal due to the native Gal4 protein bound to the *GAL1/10* promoter DNA decreased only modestly over the course of one hour after the addition of steroid, despite the presence of a large excess of the Myc-Gal4-ER LBD-VP16-FLAG competitor protein in the nucleus. This signal was clearly due to

continued Gal4 occupancy, since in a Δ*gal4* strain, no precipitation of the *GAL1/10* promoter was detected using this antibody (Figure 3-2A). Correspondingly, there was only a small amount of the competitor fusion protein bound to the *GAL1/10* promoter, as evidenced by the weak signal obtained using the anti-Myc antibody (Figures 3-3A and B). This indicates that the native Gal4-promoter complex is quite stable on the *GAL1/10* promoter, with a half-life of approximately one hour. Reporter gene experiments confirmed that over this period massive induction of *GAL* gene expression occurred.

The *GAL1/10* promoter contains three strong and one weak Gal4 binding sites. Therefore, we also examined the stability of the Gal4-Gal3 promoter complex, since the *GAL3* promoter contains only a single activator binding site. As shown in Figure 3-3A and B, this complex was also quite stable, though some dissociation occurred over the course of an hour. This indicates that the stable complex on the *GAL1/10* promoter cannot be ascribed simply to cooperative binding and instead argues that even a single activator dimer (Louvion, Havaux-Copf et al. 1993) is bound stably to a promoter site under inducing conditions.

Gal4 exchanges rapidly under non – inducing conditions

We then repeated these experiments under non-inducing conditions (glycerol/lactic acid media). As can be seen in Figures 3-3C and D, the results were very different than those observed in galactose-containing media. The ChIP signals representing native Gal4 occupancy of both the *GAL1/10* and *GAL3* promoters decayed rapidly, with a half-life of approximately five minutes or less after the addition of β-estradiol. There was also a corresponding buildup of the signal due to the Myc-Gal4-ER LBD-VP16-FLAG-promoter

complex on each promoter with a similar time course (Figure 3-3B). As expected, no signal on the orthogonal *PUT2* promoter was observed for either native Gal4 or the incoming competitor protein (Figure 3-3B).

The lability of the Gal4-promoter complexes under non-inducing conditions was confirmed by measuring the rate of transcriptional induction after the addition of β-estradiol. In glycerol and lactic acid-containing media, native Gal4 does not drive transcription due to Gal80-mediated repression. However, since Myc-Gal4-ER LBD-VP16-FLAG lacks the native Gal4 activation domain and therefore does not bind Gal80, it will act as a constitutive activator once bound to *GAL* promoters. As seen in Figure 3-4A, the induction of transcription of the *GAL1* gene after addition of estradiol to the cells was rapid and correlated well with the rate of binding detected by ChIP. The activity of the native Gal4 was also confirmed in cells expressing the competitor protein by measuring the induction of the *GAL1* gene after the addition of galactose to the cells (Figure 3-4B). Thus, we conclude that the Gal4-promoter complexes are far more labile under non-inducing conditions than is the case when the activator is driving transcription. Indeed, the act of transcriptional activation appears to "lock in" the Gal4-promoter complex.

Given this result, we next examined the stability of the transcribing Gal4-promoter complexes over longer time periods. In these experiments, we employed $S10_3$-tagged native Gal4 expressed in a Δ*gal4* strain from a *CEN* plasmid equipped with the native Gal4 promoter, providing approximately wild-type-like levels of the protein. This was done because the aliquot of C-terminal anti-Gal4 antibody that had been employed for the previous experiments was exhausted in the course of these studies and antibodies obtained from

subsequent bleeds did not perform as cleanly in ChIP assays. However, a new bleed from rabbits immunized with a S10-tagged C-terminal Gal4 fragment worked well with $S10_3$-tagged Gal4 and so were employed for subsequent experiments. As shown in Figure 3-5, the ChIP signal representing $S10_3$-Gal4 occupancy of *GAL1/10* was stable for approximately two hours in the galactose-containing media, after which it declined with a half-life of approximately two hours. Note that the doubling time of this strain was measured to be approximately 160 minutes. This experiment, using a different antibody, confirms that the Gal4-promoter complexes are long-lived under inducing conditions.

Proteasome inhibition does not significantly affect Gal4 activation

The results presented in Figures 3-3 and 3-5 are difficult to reconcile with a mandatory coupling of proteasome-mediated recycling of Gal4-promoter promoter complexes with transcription activity (Wehrman, Casipit et al. 2005). Therefore, we tested directly the sensitivity of Gal4-mediated activation of *GAL1* gene expression to the potent proteasome inhibitor MG-132 in two strains that allow this compound to enter into the cell. As shown in Figure 3-6A and B, both the levels and kinetics of induction of *GAL1* expression (shown normalized to *ACT1* expression) were identical in the presence and absence of MG-132 in the two distinct proteasome inhibitor sensitive strains. The same result was obtained when Gal4-induced α-galactosidase (the product of the *MEL1* gene) activity was measured in these cells, demonstrating that RNA processing steps downstream of transcription were also unaffected by the inhibitor. (Figure 3-6C) Proteasome inhibition was confirmed by immunoblotting to

detect the increase of poly-ubiquitylated proteins upon treatment with MG-132 (Figure 3-7A) and peptidase assay (Figure 3-7B).

DISCUSSION

In summary, I have developed a simple ChIP-based method with which to monitor the dynamics of transcription factor-DNA complexes in living cells with a minimum time resolution of a few minutes. I have applied the system to monitor Gal4-DNA complexes in yeast and have demonstrated that the activator forms an extremely stable promoter complex when engaged in activating the transcription of target genes. This is in stark contrast to previous studies of nuclear hormone receptor-DNA complexes in mammalian cells that supported a "hit and run" mechanism in which DNA-bound activators exchange rapidly with the free pool (Saddiqui and Brandriss 1989). Our finding is important in demonstrating that frequent activator dissociation from a promoter is not a general requirement for driving high-level transcription. Clearly, a single Gal4 dimer can drive many rounds of reinitiation without dissociating from the promoter or being turned over proteolytically. On the other hand, rapid replacement of native Gal4 with Myc-Gal4-ER LBD-VP16-FLAG was observed in cells under non-inducing conditions, indicating that these native Gal4-promoter complexes are more labile. One potential explanation for this striking difference is that the activator-DNA association may be stabilized by cooperative binding to promoters with the general transcription machinery, which activators are thought to bind directly. Some evidence for this model has been presented previously (Vashee, Xu et al. 1993). Finally, it is important to point out that the apparently rapid dissociation of Gal4 from promoter sites under non-inducing conditions is unlikely to represent simple dissociation of the complex, since

biochemical studies of Gal4-DNA complexes *in vitro* have suggested significantly longer lifetimes (Carey, Kakidani et al. 1989; Lohr, Venkov et al. 1995). Thus, we suspect that there exists in cells some active mechanism by which these complexes are disrupted. Further research will be required to elucidate the detailed mechanism.

MATERIALS AND METHODS

Plasmid Construction and Protein Expression

The DNA binding domain (1-93) of Gal4 or the DNA binding domain (1-100) of Put3, ER ligand binding domain (282-595), and VP16 activation domain (424-490) were amplified by PCR and cloned into PRS313 with the *ADH1* promoter. N-terminal Myc tag and C-Terminal FLAG tag were inserted by PCR to create PRS313 ADH1 Myc-Gal4ERVP16-FLAG and PRS313 ADH1 Myc-Put3ERVP16-FLAG. *Saccharomyces cereviciae* strain BY4741 (*GAL4*) or BY4741 (*Δgal4*) was transformed with PRS313 ADH1 Myc-Gal4ERVP16-FLAG and grown on selective (SC His^-) media. Individual colonies were picked and expression of Myc-Gal4ERVP16-FLAG protein was confirmed by western blot. HA-Gal4ERVP16-FLAG was constructed by excision of the Myc-Gal4 (1-93) from PRS313 Myc-Gal4ERVP16-FLAG and replacement with HA-Gal4 (1-93). PRS313 HA-Gal4ERVP16-FLAG and PSB32 $S10_3$-Gal4 were co-transfected into BY4741 *(Δgal4*) and confirmed by growth on galactose and western blot.

Proteasome Inhibited Activation Assay

Cells with the *ise1* mutation (JN284)(Muratani, Kung et al. 2005) or deleted for *PDR5* (*Δpdr5*) were grown to an O.D. of 0.5-0.7 on raffinose containing medium and treated

with MG132 (100μM final from 10mM stock in DMSO) or DMSO only for 30 minutes prior to addition of galactose. Galactose was added to a final concentration of 2%. Cells were harvested by centrifugation at each time point and frozen in liquid N_2. RNA was extracted by acidic phenol / chloroform extraction and kept at -80 C until used. Total cell extract for western blotting was extracted by boiling cell pellets in 2X SDS loading buffer for 10 minutes and then centrifuged for 5 minutes at room temperature. Supernatants were loaded onto an SDS – PAGE gel and western blotted with anti-Ubiquitin antibody (Boston Biochem #A-100).

In addition, lysates were prepared by disruption of the cells with zirconium / silica beads in PBS with protease inhibitors. Approximately 50 μg of extract was then tested for alpha – galactosidase activity as described. (McNally, Müller et al. 2000)

Chromatin Immunoprecipitation Assays

Cross-linked cells were centrifuged 5 minutes 3000g in a Sorvall RT7 centrifuge with a RTH-750 swing bucket rotor. Cells were washed with PBS and centrifuged as before. Cell pellets were frozen in liquid nitrogen and stored at -80 C. Cells were thawed and then converted to spheroblasts by digesting with zymolyase (0.4 mg/ml of 20T, Sekagaku) for 30 min at 28°C. Spheroplasts were washed and sonicated in lysis buffer (1% SDS, 10mM EDTA, 50mM TRIS pH8.1). To generate a mean DNA size of 0.4-1.0 kb, samples were sonicated on ice with two 15s pulses followed by three 10s pulses at constant power and an output setting of 20 watts. Sonicated spheroplasts were clarified by centrifugation (15 min, 13000x g). 550μL of IP buffer (0.01% SDS, 1.1% Triton X100, 1.2 mM EDTA, 16.7 mM TRIS pH 8.1, 167 mM NaCl) was added to 450μL of the chromatin supernatant. 100μL of

the clarified supernatant was diluted to 1 ml in immunoprecipitation buffer and was used in IPs as described below.

IPs were performed by adding antiserum to 1ml chromatin lysate, mixing on a nutator at 4°C overnight, then adding 30 μl of immobilized Protein A (Pierce) and continuing the incubation for an additional 2 hours. Following reversal of formaldehyde-induced crosslinks, input and immunoprecipitated DNAs were analyzed by PCR.

DNA amplification

DNA regions were amplified using the following primers: *GAL1* promoter TGTGGAAATGTAAAGAGCCCC; CTTTATTGTTCGGAGCAGTGC, *PUT2* promoter CCGACATCAGAAGAAACACAC; GATTTGAGGCACCTTGCTG, *GAL3* promoter TATGTGTTGCAGGCGGTCAAT; AAGATTTCGTGTTCATGCAGA, Non-transcribed region GTGGAAGGTGACGATAATACG; CGGACATCCTAAATCTTTGGG.

DNA was amplified by PCR for (94°C 45s, 50°C 45s, 72°C 1min, 28 cycles). Products were separated by agarose gel and detected with ethidium bromide.

qPCR

Quantitative PCR of precipitated chromatin was performed using an iCycler Thermal Cycler and the IQ SYBR Green Supermix, 2x mix containing 100 mM KCl, 40 mM Tris-HCl, pH 8.4, 0.4 mM each dNTP, 50 U/ml iTaq DNA polymerase, 6 mM MgCl2, SYBR Green I, 20 nM fluorescein, stabilizers (Biorad, Hercules, CA). Relative enrichment of specific DNA was calculated by comparing products derived from primers against the *GAL1* promoter and a non-transcribed region of chromosomal DNA in the precipitated samples and the total DNA.

RT-PCR

Total RNA was isolated from 10 mL of cells OD_{600} 0.6-0.8 after addition of β-estradiol or galactose. Cells were centrifuged 5 minutes 3000g in a Sorvall RT7 centrifuge with a RTH-750 swing bucket rotor. Cells were washed with PBS and centrifuged as before. Cell pellets were frozen in liquid nitrogen and stored at -80 C. Cell pellets were resuspended in 400 μL water and 400 μL water – saturated phenol was added and vortexed 1 min. The mixture was incubated at 65°C for 45 minutes. The aqueous layer was removed and extracted with water – saturated phenol followed by chloroform. The RNA was precipitated by adding 40 μL 3M NaOAc pH 5.3 and 1 mL 95% EtOH. RNA quantity was measured by measuring OD_{260}. One μg of Total RNA was used to make cDNA using the Stratascript first strand cDNA synthesis kit (Stratagene, La Jolla, CA) and oligo dT. cDNA was amplified by PCR using primers for the *GAL1* gene (CTCTGTTTGCGGTGAGGAAG; ACCTTTATTCGTGCTCGATCC) or the *PUT2* gene (GCAAGTCCAATTTCTGGTGG; CAGTTAAGGCGTATTGACTCG) (94°C 45s, 50°C 45s, 72°C 1min, 23 cycles) or quantified by qPCR. PCR products were separated by agarose gel and detected with ethidium bromide staining. qPCR was performed as described above and normalized by comparing *GAL1* to *ACT1* (primers: CCTACGTTGGTGATGAAGCT; GTCAGTCAAATCTCTAACCGG) transcript levels.

Figure 3-1

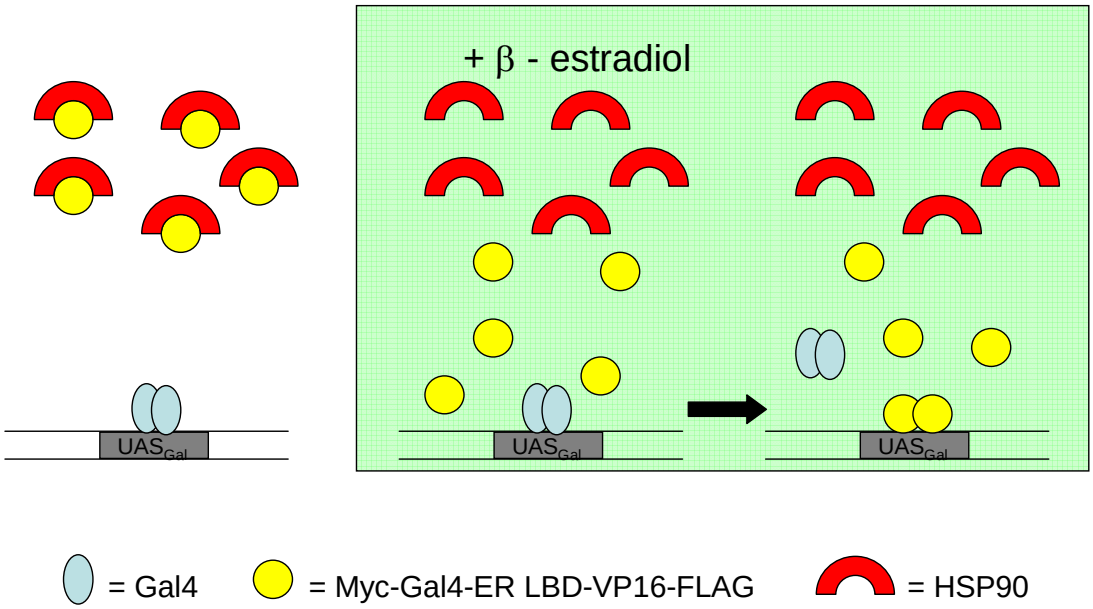


Figure 3-1

Experimental Scheme for competition assay. Cells express wild – type levels of Gal4 bound to chromosomal DNA and overexpress Myc-Gal4-ER LBD-VP16-FLAG which is sequestered in an inactive state by chaperones such as Hsp90 (First Panel). Upon addition of β-estradiol the ER LBD containing fusion protein is released from the Hsp90 (Second Panel). As the native Gal4 dissociates from the DNA it is quickly replaced with the fusion protein due to the large excess of the fusion protein (Third Panel). Chromatin immunoprecipitation is used to detect the level of proteins bound to DNA.

Figure 3-2

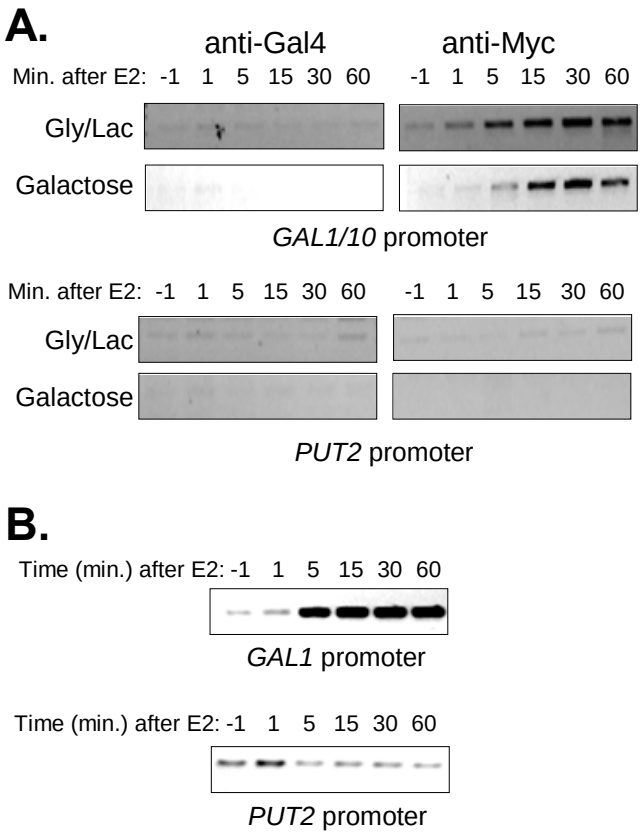


Figure 3-2

Myc-Gal4-ER LBD-VP16-FLAG associates rapidly and specifically with Gal4 activated promoters and can activate transcription of the *GAL1* gene. (A) Chromatin immunoprecipitation (ChIP) assays performed on cells deleted for Gal4 (Δgal4) and expressing Myc-Gal4-ER LBD-VP16-FLAG fusion protein. Cells were grown in minimal medium with glycerol / lactic acid or galactose as the carbon source. β-estradiol (E_2) was added at T=0. Fusion protein bound to chromatin was detected by precipitating DNA using anti-myc agarose and amplifying the DNA regions by PCR. (B) Transcript from the *GAL1* gene but not the *PUT2* gene is detectable within 5 minutes after adding β-estradiol. Total RNA was prepared from cells and RT-PCR was performed.

Figure 3-3

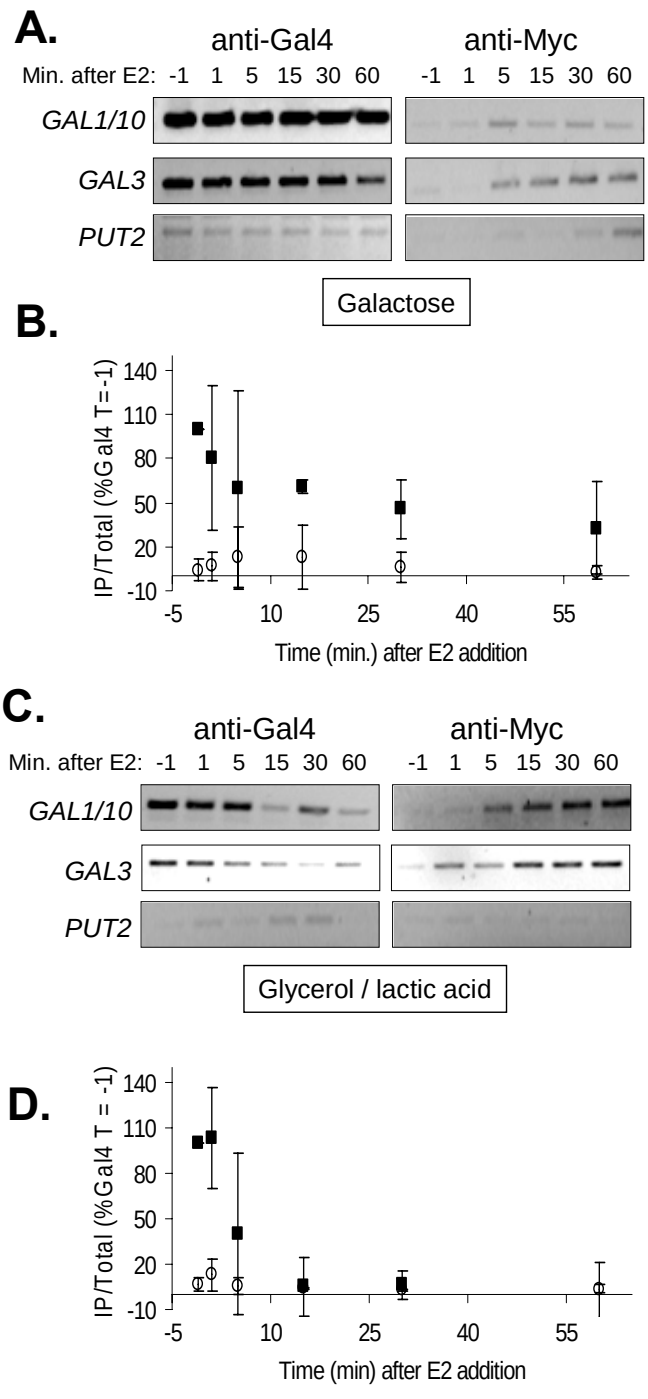

A.
anti-Gal4
anti-Myc
Min. after E2: -1 1 5 15 30 60 -1 1 5 15 30 60
GAL1/10
GAL3
PUT2
Galactose
B.
IP/Total (%Gal4 T=-1)
140
110
80
50
20
-10
-5
10
25
40
55
Time (min.) after E2 addition
C.
anti-Gal4
anti-Myc
Min. after E2: -1 1 5 15 30 60 -1 1 5 15 30 60
GAL1/10
GAL3
PUT2
Glycerol / lactic acid
D.
IP/Total (%Gal4 T = -1)
140
110
80
50
20
-10
-5
10
25
40
55
Time (min) after E2 addition

Figure 3-3

Competition assay reveals the Gal4 protein / DNA complex is stable when activating transcription. ChIP assays performed on cells expressing endogenous Gal4 and overexpressing myc-Gal4-ER LBD-VP16-FLAG. Cells were grown in minimal medium with galactose (A) or glycerol / lactic acid (C) as the carbon source. β-estradiol (E_2) was added at T=0. Gal4 protein was precipitated with anti-Gal4 C-terminal antibodies. Fusion protein was precipitated with anti-myc antibodies. DNA precipitated and total DNA were amplified by PCR. (B and D) Quantitation by qPCR of the DNA precipitated by anti-Gal4 antibodies (black squares) or the no antibody control (open circles) under inducing and non-inducing conditions respectively.

Figure 3-4

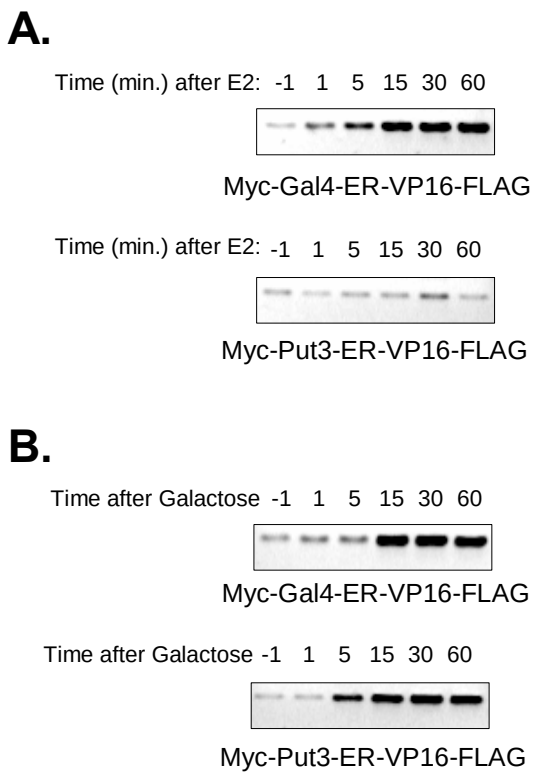


Figure 3-4

Specific DNA / protein interactions are necessary to induce transcription with ER LBD containing fusion proteins. (A) β-estradiol can induce transcription of the *GAL1* gene only in cells expressing competitor protein containing the Gal4 DNA binding domain and not the related Put3 DNA binding domain. (B) Addition of galactose to cells expressing wild – type Gal4 and overexpressing Myc-Gal4-ER LBD-VP16-FLAG or Myc-Put3-ER LBD-VP16-FLAG can induce transcription of the *GAL1* gene demonstrating the presence of functional Gal4 protein

Figure 3-5

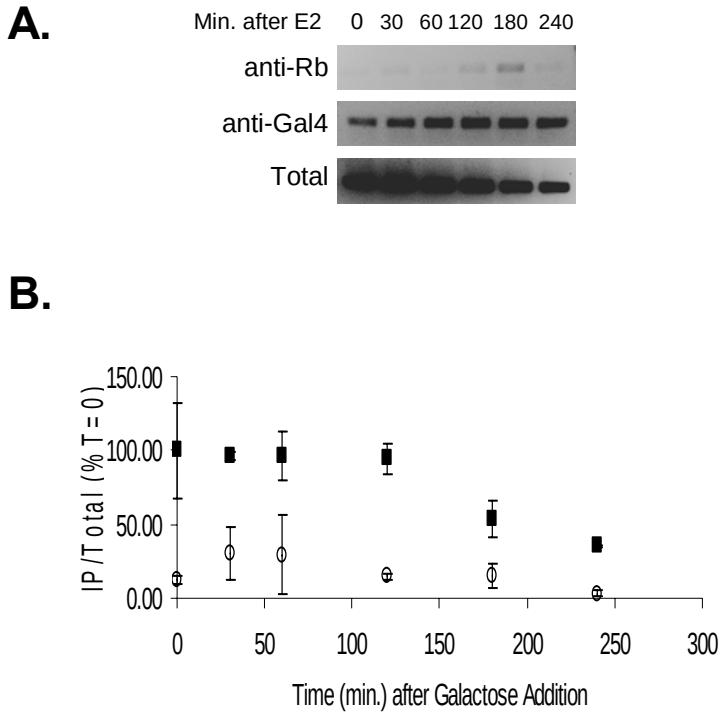


Figure 3-5

Competition ChIP assay with induced Gal4 over 4 hours shows distinct populations of Gal4. Cells grown on galactose were treated with β – estradiol at T = 0 and cells were removed and crosslinked at the indicated time points. ChIP assays were perfomed with anti – Rabbit (anti – Rb, open circles) and α – Gal4 (black squares) antibodies. Total DNA and precipitated DNA were measured by conventional (A) and qPCR (B).

Figure 3-6

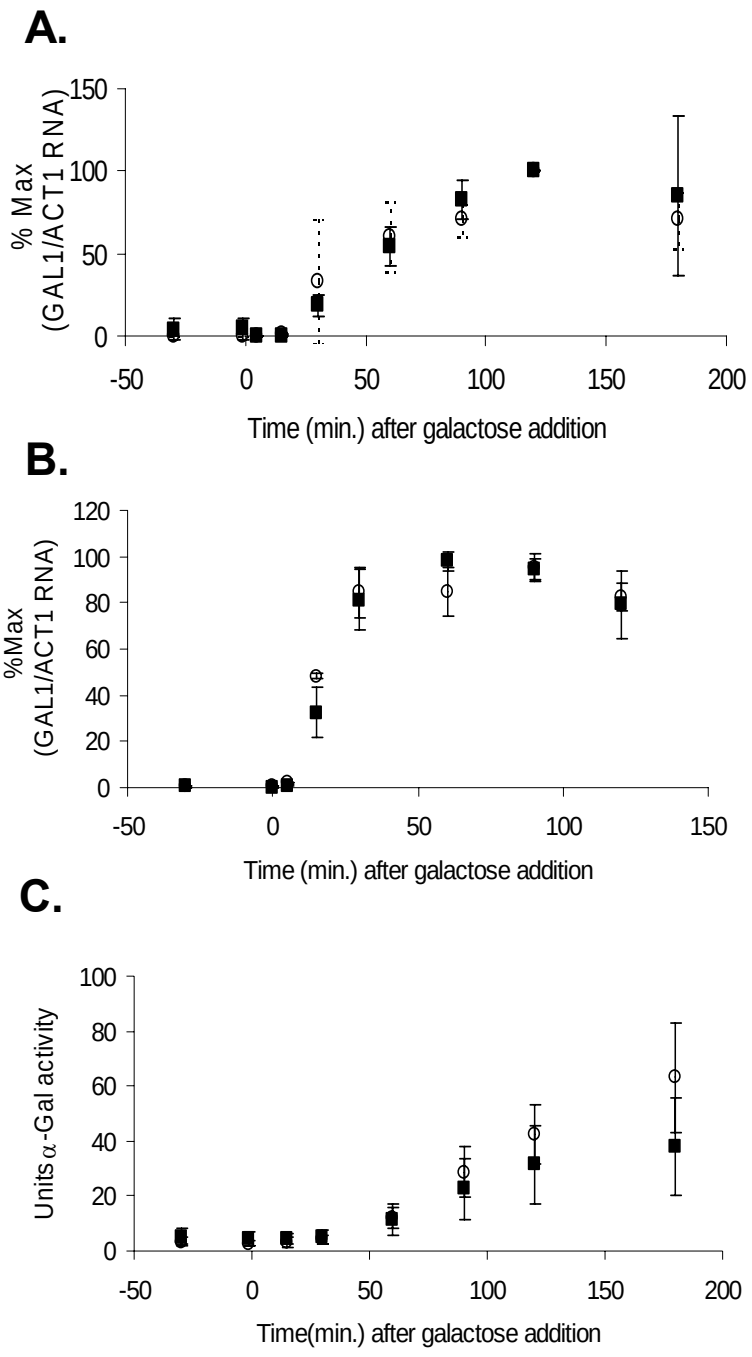

A.
% Max
(GAL1/ACT1 RNA)
150
100
50
0
-50
0
50
100
150
200
Time (min.) after galactose addition
B.
%Max
(GAL1/ACT1 RNA)
120
100
80
60
40
20
0
-50
0
50
100
150
Time (min.) after galactose addition
C.
Units α-Gal activity
100
80
60
40
20
0
-50
0
50
100
150
200
Time(min.) after galactose addition

Figure 3-6

Inhibition of Proteasome does not effect Gal4 activated transcription. MG132 in DMSO or DMSO only was added 30 minutes before addition of galactose to inhibitor – sensitive *ise1* or *Δpdr5* cells. Either *ise1* (A) or *Δpdr5* (B) cells for RNA and protein samples were taken before addition of inhibitor (T=-30), before the addition of galactose (T=-1) and at 5, 15, 30, 60, 90, 120 and 180 minutes after the addition of galactose. Relative transcription of the *GAL1* gene was measured by RT-PCR. C. Protein lysates were prepared from the *ise1* cells at the -30, -1, 15, 30, 60, 90, 120 and 180 minute time points. Lysate was then used to measure the units of alpha – galactosidase activity at each time point.

Figure 3-7

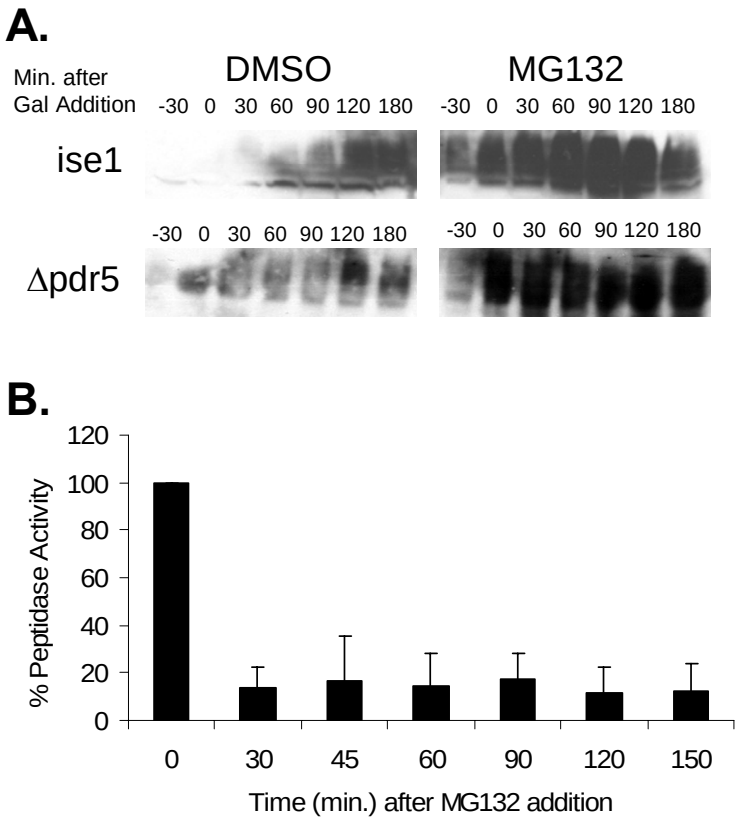


Figure 3-7

MG132 inhibited cells were lysed in SDS buffer and the extracts were separated by SDS – PAGE. (A) Western blots were performed with anti – ubiquitin antibodies to detect the accumulation of poly-ubiquitylated proteins in the proteasome inhibited cells. (B) Proteasome inhibition was also measured by peptidase assay in cell extracts from MG132 treated *ise1* cells. Protein lysates were prepared from the *ise1* cells at the -30, -1, 15, 30, 60, 90, 120 and 180 minute time points.

CHAPTER FOUR

MECHANISMS OF PROTEIN INSTABILITY

SUMMARY

Since Gal4 has been shown to have different exchange kinetics under induced and non – induced conditions (chapter 3) it is important to determine the mechanism of the exchange process. Possibilities include increased proteolysis of the Gal4 protein under non – inducing conditions compared to the induced protein, non – proteolytic exchange of the Gal4 protein mediated by unknown proteins, or diffusion of the Gal4 protein on and off the DNA. Throughout this chapter I will demonstrate that the degradation rate of Gal4 is slow relative to the kinetics of exchange seen in chapter 3. However, in two independent experiments when the degradation rate of the Gal4 protein bound to DNA was measured by ChIP assay there appeared to be distinct pools of the Gal4 protein. In addition, proteolytic turnover of Gap71 does not explain the occupancy defects seen in chapter 2 supporting the notion that the proteasomal ATPases may be responsible for the occupancy defect seen on galactose *in vivo*.

INTRODUCTION

The Gal4 / DNA complex itself has been shown to be long – lived in the absence of the 19S proteasome (see chapter 2 and (Vashee, Xu et al. 1993; Vashee and Kodadek 1995). Regulation of transcriptional activation requires the proteolytic turnover of the activator proteins in some systems. This turnover often uses the ubiquitin / proteasome system to regulate the activity of the activator protein.(Lee and Goldberg 1996; Melcher, Sharma et al. 2000; Melcher and Xu 2001) Yeast activators such as Gcn4 and Met4, for example, are ubiquitylated and degraded rapidly. This degradation regulates the activity of the protein.(Vashee, Xu et al. 1993; Melcher and Xu 2001) The role of this proteolytic turnover in the maintenance and regulation of transcriptional activity is just now being uncovered.(Maratani and Tansey 2003) The 26S proteasome is a large multiprotein complex that is responsible for most non-lysosomal proteolysis (Tansey 2001; Conaway, Brower et al. 2002) Proteolysis by the proteasome occurs in the 20S "core" subcomplex and contains three distinct proteolytic activities: trypsin – like, chymotrypsin – like, and peptidyl – glutamyl hydrolyzing.(Kornitzer, Raboy et al. 1994) Proteolysis usually also requires the activity of the 19S regulatory sub-complex that is thought to unfold the substrate protein and feed the polypeptide chain into the narrow pore in the 20S.(Kuras, Rouillon et al. 2002; Lipford and Deshaies 2003) This unfolding activity is thought to reside within the six ATPase subunits at the base of the 19S. This chaperone – like activity has been demonstrated *in vitro* with the regulatory complex binding and refolding misfolded proteins.(Coux, Tanaka et al. 1996; Baumeister, Walz et al. 1998)

The increased protein lability under non – inducing conditions may be due to the chaperone activity of the 19S proteasome or other molecular chaperones in the nucleus. Under non – inducing conditions the Gal4 activation domain is bound by the Gal80 repressor and the ATPases are not recruited to the promoter.(Lowe, Stock et al. 1995; Baumeister, Walz et al. 1998) It was demonstrated previously that the VP16 activation domain could recruit the ATPases of the proteasome as well as the Gal4 activation domain. Therefore, it is possible that the competitor protein myc-Gal4 DBD-ER-VP16 may bring the proteasomal ATPases or other chaperones in proximity to the Gal4 protein, thus catalyzing the destabilization.

In this chapter I will examine the half – life of the Gal4 protein under inducing and non – inducing conditions to determine if proteolysis is responsible for the decreased stability of the Gal4 protein under non – inducing conditions. Also I will measure the half – lives of the Gap71 and $Gal4^{D}$ proteins to determine the relative contribution of proteolysis to the occupancy defect observed. In addition the requirement for the VP16 activation domain on the competitor protein will be tested to determine if the domain recruits a destabilizing factor to the promoter.

RESULTS

Gal4 is a long – lived protein under inducing and non – inducing conditions

To measure the half – life of the total Gal4 protein, $(S10)_3$-tagged Gal4 was expressed from the native *GAL4* promoter on the single copy *CEN* plasmid pSB32. Cells grown on raffinose or induced with galactose for two hours were treated with cycloheximide to block

new protein synthesis and then the cells were harvested at the indicated time points and whole cell extracts were prepared. Immunoprecipitation of the Gal4 protein from the extract was followed by SDS-PAGE and western blotting of the precipitant with anti-T7 antibodies to determine the levels of the protein. In figure 4-1A it can be seen that the protein half – life of the Gal4 protein is greater than 1 hour in cells grown on either raffinose or galactose. However, since this experiment measured the half life of the total Gal4 protein and included significant time in the whole cell extract I wanted to determine the half – life of the protein bound to the DNA. To do this the ChIP assay was employed to measure the amount of Gal4 bound to the *GAL 1/10* promoter. After treatment with cycloheximide the cells were cross-linked with formaldehyde. This has the dual effect of fixing the cells and freezing the Gal4 / DNA complex. After the ChIP assay the results are shown in figure 4-1B. It was found that the Gal4 ChIP signal does not seem to decrease much after an initial drop in either sugar source. This indicates that proteolysis cannot explain the susceptibility of the Gal4 protein to exchange by the myc-Gal4 DBD-ER-VP16 competitor under non – inducing conditions.

Mutant Gal4 proteins have decreased protein half – lives relative to Gal4.

The $Gal4^{D}$ and Gap71 mutant proteins have been shown in this thesis and elsewhere to interact with and be destabilized by the proteasomal ATPases. To further examine this effect I determined the protein half – life for each of the mutations compared to wild – type Gal4. Experiments were performed as above with raffinose as the sugar source. As seen in figure 4-2 the Gal4 protein is again shown to be quite stable with substantial protein visible out to four hours after cycloheximide treatment. The $Gal4^{D}$ protein is much more rapidly

degraded with the majority of the protein gone in less than one hour and the protein level is undetectable at two hours. Gap71 has an intermediate protein stability compared to Gal4 and $Gal4^{D}$ with protein detectable out to three hours and a half – life of approximately one hour.

Proteins lacking the VP16 activation domain exchange with Gal4 more slowly

To determine the effect of the VP16 activation domain on the ability of the competitor protein I first used the α-galactosidase assay to determine if the myc-Gal4 DBD-ER protein which is lacking the VP16 activation domain could compete with activated Gal4 and decrease activation of the Gal4 responsive *MEL1* gene. By comparing the activation kinetics when galactose is added to the kinetics when galactose and estradiol are added there is no significant difference in the amount of α-galactosidase produced (Figure 4-3A). Next I tested the ability of the myc-Gal4 DBD-ER protein to exchange with the Gal4 protein under inducing and non – inducing conditions. Under non – inducing conditions the exchange appears to be much slower than that of the VP16 containing competitor. Without the VP16 activation domain the exchange occurs with a 30 minute to one hour time scale compared to less than 15 minutes for complete exchange when the competitor contains VP16 (compare figure 4-3B with Chapter 3 figure 3C). Under induced conditions the competitor protein does appear on the promoter however, there is no discernible decrease in the amount of *GAL 1/10* promoter precipitated by the Gal4 antibodies. This potentially indicates the competitor protein binding to one or several of the four UAS_{GAL} sites on the *GAL 1/10* promoter without displacement of the rest of the Gal4 bound to the promoter.

Myc-Gal4 DBD-ER can limit Gal4 activity by exchange prior to activation

To confirm the results from the ChIP assay I examined the amount of residual Gal4 activity after induction of the myc-Gal4 DBD-ER competitor. Estradiol was added to cells expressing the competitor protein at T=0. Cells were then induced with galactose at the time points indicated in figure 4-3C. Cells were collected one hour after the induction with galactose, total RNA was isolated, and cDNA was made. The amount of *GAL1* transcript compared to the *ACT1* transcript is shown in figure 4-3C. The amount of transcription activity from the *GAL 1/10* promoter decreases rapidly with a 60% decrease after the first thirty minutes of estradiol treatment. After one hour 20% of the activity remains and remains out to three hours. This is very similar to the results seen in the competitive ChIP assay and the protein degradation ChIP assay.

DISCUSSION

With the results from the competitive ChIP assay in chapter 3 two possibilities for the mechanism of the Gal4 exchange under non – inducing conditions were tested. First the rate of protein degradation for the total Gal4 protein was measured using cycloheximide to block new protein synthesis. The degradation rate for uninduced wild – type Gal4 was quite slow compared to the exchange rate seen in chapter 3. When the degradation rates of the two mutant proteins $Gal4^{D}$ and Gap71 were measured it was found that the $Gal4^{D}$ protein was very unstable and Gap71 had an intermediate protein half – life. The instability of the $Gal4^{D}$ protein may not be an unexpected result since the $Gal4^{D}$ activation domain fragment has been shown to be stably bound to the wild – type proteasomal ATPases in the presence of ATP

whereas the intact Gal4 activation domain is only bound in the absence of ATP (Johnston lab unpublished data). It is interesting to note that the levels of the $Gal4^{D}$ protein at steady – state are equivalent to the wild – type Gal4 protein. This confirms results from the Johnston group(Adams, Crotchett et al. 1998) and may be due to increased synthesis of the $Gal4^{D}$ protein to compensate for the increased rate of turnover. The phenotype of the Gap71 mutation is not explained by increased protein degradation relative to wild – type and this does not conflict with the conclusions reached in chapter 2.

Although knowing the overall half – life of the Gal4 protein was informative it was more important to determine the half – life of the protein bound to the promoter DNA. To do this, the ChIP assay was performed after addition of cycloheximide to the cells to measure the amount of protein bound to the DNA after blocking protein synthesis. The results showed that there was a rapid decrease in the amount of protein bound to the DNA in the first 30 minutes followed by a steady pool of DNA – bound protein under induced and non – induced conditions. This is in contrast to the steady decrease seen for the bulk protein. This also contrasts with the rapid and complete exchange between the myc-Gal4 DBD-ER-VP16 competitor and wild – type Gal4 under non – inducing conditions seen in chapter 3. To test whether the VP16 activation domain on the competitor protein was influencing the exchange the ability of the myc-Gal4 DBD-ER competitor protein to compete with wild – type Gal4 was measured. As expected, the competitor lacking the VP16 activation domain did not compete with the Gal4 protein in the presence of galactose, similar to the results seen with the VP16 containing competitor. This was observed by the lack of any effect of the competitor on Gal4-mediated transcription after induction as well as no decrease in the levels

of induced Gal4 protein by ChIP assay. However, the myc-Gal4 DBD-ER competitor did compete effectively with the non – induced Gal4 protein. By ChIP assay there was a sharp decrease in the amount of Gal4 bound to the *GAL 1/10* promoter between 30 minutes and one hour. The levels then stabilized out to three hours. To confirm these data an activity assay was performed by measuring the residual amounts of Gal4 mediated transcription after the induction of the competitor protein. These results again showed a sharp decrease in the amount of Gal4 activity within 30 minutes after induction of the competitor followed by a residual 20% activity that remained out to three hours. These three experiments together suggest that there may be at least two separable pools of Gal4 protein bound to DNA under non – inducing conditions. One that is rapidly turned over in a proteolytic manner and another that is stable for many hours. Multiple forms of a protein with differing half - lives has been previously described for Gal4 as well as the activator c-myc by the Tansey group. (Braun, Glickman et al. 1999; Strickland, Hakala et al. 2000) The two pools of Gal4 are not evident when the turnover rate of the total protein is measured by conventional methods indicating the possibility of misinterpreting protein degradation rates for transcriptional activators. Again this demonstrates the utility of using *in vivo* competition assays to probe cellular dynamics.

MATERIALS AND METHODS

Protein half – life measurement

W303A cells (*MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 gal4::HIS3)* expressing $S10_3$Gal4, $S10_3$Gap71, or $S10_3Gal4^D$ from a single copy plasmid were grown to

an OD_{600} of 0.6 on minimal media supplemented with either raffinose or galactose. Cycloheximide was added to a final concentration of 100 μg / mL from a 100 mg / mL stock in DMSO. Cells were then collected at several time points by centrifugation, resuspended in YPER yeast lysis buffer (Pierce) and lysed by vortexing with zircon / silica beads. 1.2mg of protein extract was subjected to immunoprecipitation with 10μL rabbit anti-Gal4 serum from a total volume of 600 μL. Immunocomplexes were bound to protein A sepharose and pelleted by centrifugation. Pellets were washed three times with TBST. Precipitated proteins were eluted in 2X SDS load buffer separated by SDS PAGE. Proteins were transferred to a nitrocellulose membrane and western blots were performed with HRP conjugated anti-T7 antibodies (Novagen).

Protein half – life ChIP assays

Cells expressing wild – type Gal4 were grown to an OD_{600} of 0.5-0.6. Cycloheximide was added to a final concentration of 100 μg / mL from a 100mg / mL stock in DMSO. At each time points indicated 100ml aliquots were removed and cross-linked with 1% formaldehyde. Chromatin was immunoprecipitated as described below with a combination of anti-Gal4 C-terminal and anti-Gal4 N-terminal antibody bound to protein A agarose.

***In Vivo* Competition Assay**

Cells expressing myc-Gal4-ER-FLAG from the *ADH1* promoter as well as wild – type Gal4 were grown in selective media to an OD_{600} of 0.5-0.8. For activity assays 5 mL of cells were removed prior to the addition of 1% galactose and 1μM estradiol and 15, 30, 45, 60, and 90 minutes after the addition of galactose and estradiol. Cells were disrupted by vortexing with zirconia / silica beads at 4° C in citric acid buffer (31mM citric acid, 39mM

KH_2PO_4, pH 4.0). Protein concentration was measured by Coomassie Plus protein assay (Pierce, Rockford, IL). Equal protein amounts were added to each assay. Transcriptional activity was analyzed by α-galactosidase assay as described (Gonzalez, Delahodde et al. 2002) For chromatin immunoprecipitation assays 100ml aliquots were taken 1 minute before and 30, 60, 90, 120, and 180 minutes after the addition of estradiol and cross-linked in 1% formaldehyde. Chromatin was immunoprecipitated as described below with either anti – Gal4 C-terminal antibody bound to protein A agarose or anti – c-myc conjugated to agarose.

Residual Activity Assay

Cells expressing the myc-Gal4 DBD-ER-FLAG gene and wild – type Gal4 were treated with 1μM estradiol. Galactose was added prior estradiol addition or 30, 60, 90, 120, 150, and 180 minutes after estradiol addition. 1.5 mL of cells were collected one hour after the addition of galactose, pelleted by centrifugation, and frozen on liquid N_2. Gal4 activity was measured by measuring the *GAL1* gene RNA. RNA isolation and RT-PCR was performed as described below.

Chromatin Immunoprecipitation Assays

Cross-linked cells were centrifuged 5 minutes 3000g in a Sorvall RT7 centrifuge with a RTH-750 swing bucket rotor. Cells were washed with PBS and centrifuged as before. Cell pellets were frozen in liquid nitrogen and stored at -80 C. Cells were thawed and then converted to spheroblasts by digesting with zymolyase (0.4 mg/ml of 20T, Sekagaku) for 30 min at 28°C. Spheroplasts were washed and sonicated in lysis buffer (1% SDS, 10mM EDTA, 50mM TRIS pH8.1). To generate a mean DNA size of 0.4-1.0 kb, samples were sonicated on ice with two 15s pulses followed by three 10s pulses at constant power and an

output setting of 20 watts. Sonicated spheroplasts were clarified by centrifugation (15 min, 13000x g). 550μL of IP buffer (0.01% SDS, 1.1% Triton X100, 1.2 mM EDTA, 16.7 mM TRIS pH 8.1, 167 mM NaCl) was added to 450μL of the chromatin supernatant. 100μL of the clarified supernatant was diluted to 1 ml in immunoprecipitation buffer and was used in IPs as described below.

IPs were performed by adding antiserum to 1ml chromatin lysate, mixing on a nutator at 4°C overnight, then adding 30 μl of immobilized Protein A (Pierce) and continuing the incubation for an additional 2 hours. Following reversal of formaldehyde-induced crosslinks, input and immunoprecipitated DNAs were analyzed by PCR.

DNA amplification

DNA regions were amplified using the following primers: *GAL1* promoter TGTGGAAATGTAAAGAGCCCC; CTTTATTGTTCGGAGCAGTGC. DNA was amplified by PCR for (94°C 45s, 50°C 45s, 72°C 1min, 28 cycles). Products were separated by agarose gel and detected with ethidium bromide.

RT-PCR

Total RNA was isolated from 1.5 mL of cells OD_{600} 0.6-0.8 after addition of β-estradiol and / or galactose. Cells were harvested by centrifugation and the cell pellets were frozen in liquid nitrogen and stored at -80 C. Cell pellets were resuspended in 400 μL RNA lysis buffer and 400 μL water – saturated phenol was added and vortexed 1 min. The mixture was incubated at 65°C for 45 minutes. The aqueous layer was removed and extracted by chloroform. DNA was digested by addition of RQ1 RNAse free DNAse and the solution was extracted with phenol / chloroform / isoamyl alcohol followed by extraction

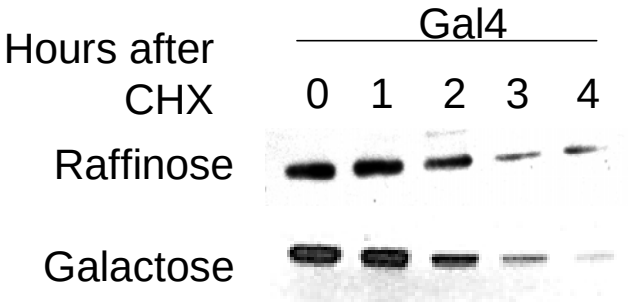
with chloroform. The RNA was precipitated by adding 40 μL 3M NaOAc pH 5.3 and 1 mL 95% EtOH. RNA quantity was measured by measuring OD_{260}. 500 ng of Total RNA was used to make cDNA using the Stratascript first strand cDNA synthesis kit (Stratagene, La Jolla, CA) and oligo dT. cDNA was amplified by PCR using primers for the *GAL1* gene (CTCTGTTTGCGGTGAGGAAG; ACCTTTATTCGTGCTCGATCC) (94°C 45s, 50°C 45s, 72°C 1min, 23 cycles) or quantified by qPCR. qPCR was performed as described below and normalized by comparing *GAL1* to *ACT1* (primers: CCTACGTTGGTGATGAAGCT; GTCAGTCAAATCTCTAACCGG) transcript levels.

qPCR

Quantitative PCR of precipitated chromatin was performed using an iCycler Thermal Cycler and the IQ SYBR Green Supermix, 2x mix containing 100 mM KCl, 40 mM Tris-HCl, pH 8.4, 0.4 mM each dNTP, 50 U/ml iTaq DNA polymerase, 6 mM MgCl2, SYBR Green I, 20 nM fluorescein, stabilizers (Biorad, Hercules, CA). Relative enrichment of specific DNA was calculated by comparing products derived from primers against the *GAL1* promoter and a non-transcribed region of chromosomal DNA in the precipitated samples and the total DNA.

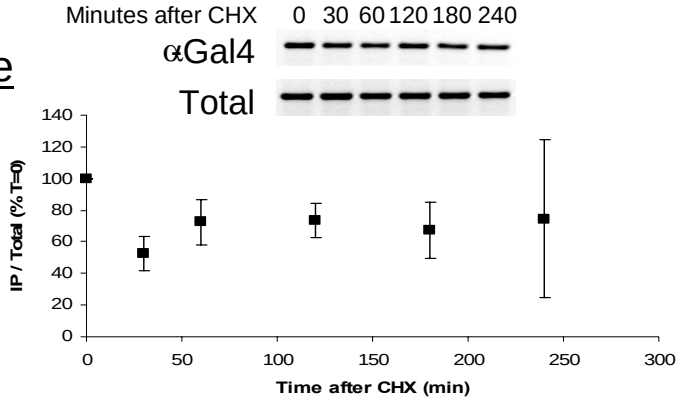
Figure 4-1

A.



B.

Raffinose



Galactose

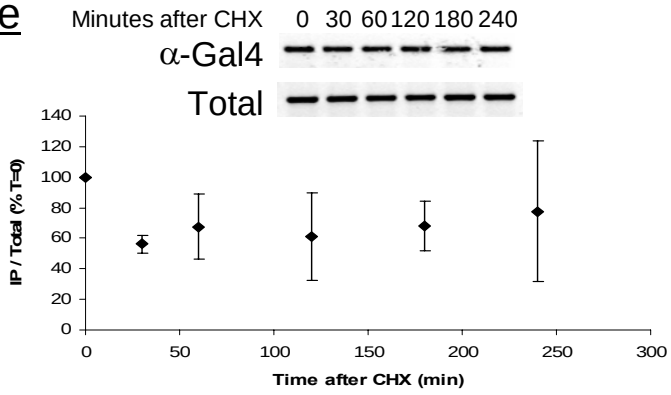


Figure 4-1

Gal4 protein has a long protein half – life. A. *⊿gal4* cells expressing $S10_3$Gal4 were grown in media with either raffinose or galactose as the carbon source. Cycloheximide (100μg / mL) was added and the cells were collected at the indicated time points. Cells were lysed and the Gal4 was immunoprecipitated with anti-Gal4 antibodies. Gal4 protein was detected by western blot using anti-T7 HRP antibodies. B. Cells with wild – type Gal4 were treated with cycloheximide as above and cross-linked with formaldehyde at the indicated time points. Amount of Gal4 bound to the DNA was measured by ChIP assay and quantitated by qPCR.

Figure 4-2

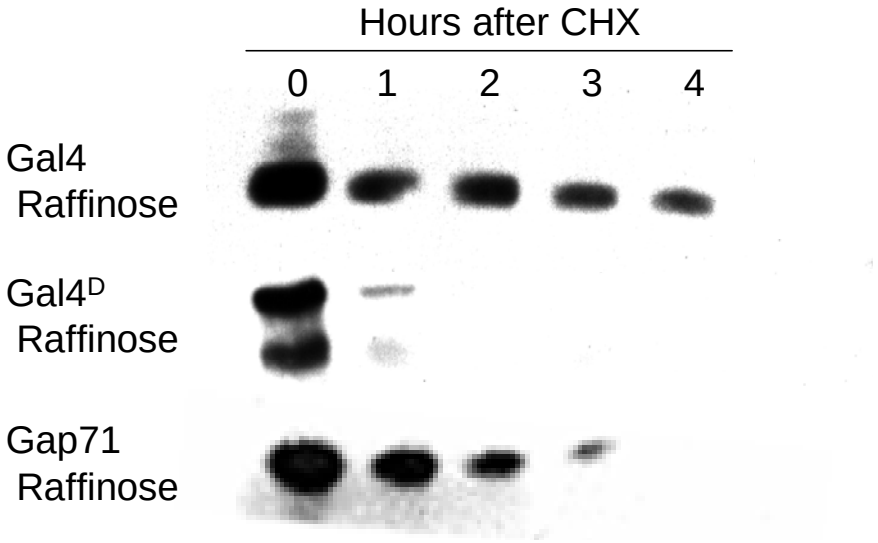


Figure 4-2

Gal4^{D} and Gap71 have reduced protein half – lives relative to wild – type Gal4. *⊿gal4* cells expressing S10$_3$Gal4, S10$_3$Gal4^{D}, or S10$_3$Gap71 were grown on raffinose – containing medium. Cycloheximide was added and the proteins were immunoprecipitated and detected as in figure 4-1A.

Figure 4-3

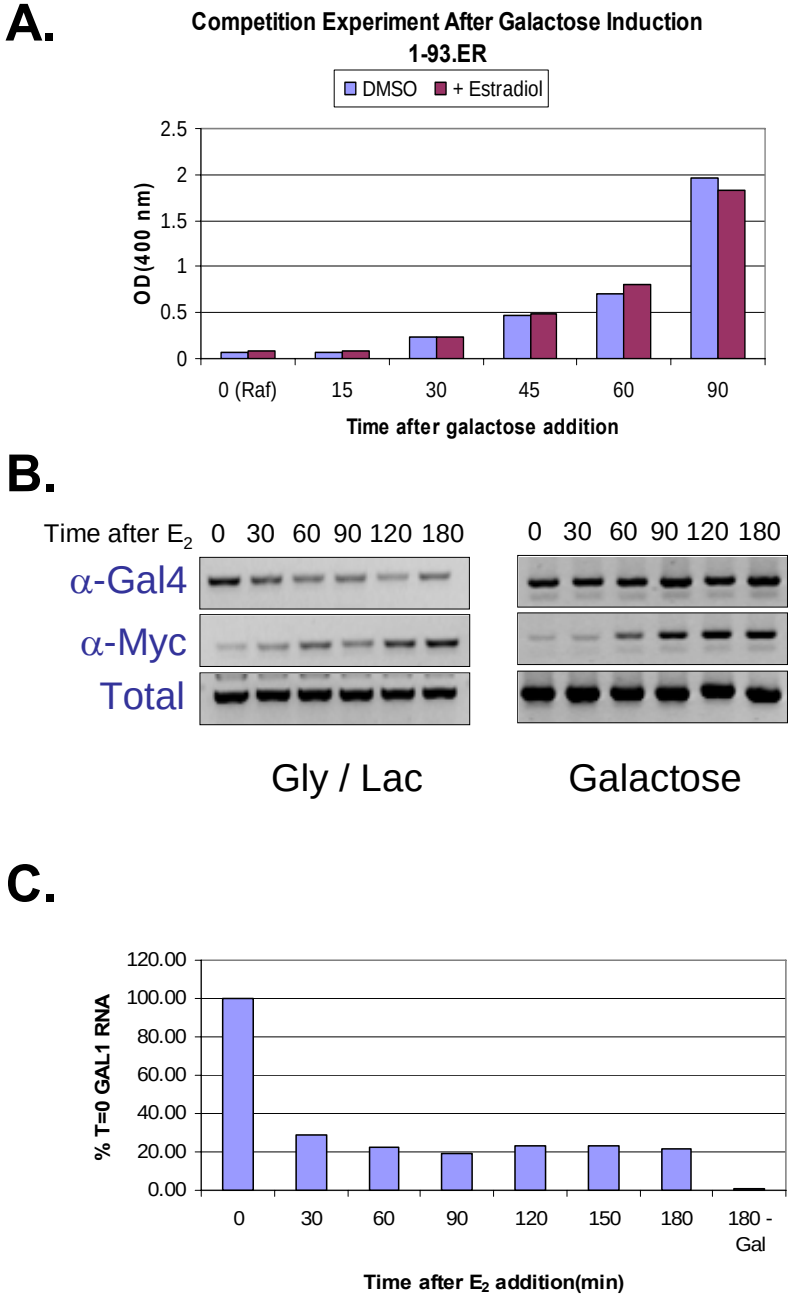

A.
Competition Experiment After Galactose Induction
1-93.ER
DMSO
+ Estradiol
OD(400 nm)
2.5
2
1.5
1
0.5
0
0 (Raf)
15
30
45
60
90
Time after galactose addition
B.
Time after E2
0 30 60 90 120 180
0 30 60 90 120 180
α-Gal4
α-Myc
Total
Gly / Lac
Galactose
C.
% T=0 GAL1 RNA
120.00
100.00
80.00
60.00
40.00
20.00
0.00
0
30
60
90
120
150
180
180 - Gal
Time after E2 addition(min)

Figure 4-3

Competitor protein lacking the VP16 activation domain do not compete efficiently for the *GAL 1/10* promoter. A. Cells expressing Gal4 and myc-Gal4-ER-FLAG were treated simultanteously with galactose and estradiol at T=0. Cells were collected at the indicated times and the activity of the α-galactosidase (*MEL1*) gene product was measured. B. The ability of the myc-Gal4-ER-FLAG protein to compete with wild – type Gal4 was measured by ChIP assay over a three hour time course. Limited exchange was detected by measuring both Gal4 levels with anti – Gal4 antibodies as well as the competitor protein with anti – myc agarose. C. Cells expressing Gal4 and myc-Gal4-ER-FLAG were pre-treated with estradiol for the times indicated and then galactose was added (galactose not added in the raf and –gal lanes). Cells were collected one hour after the addition of galactose and the level of induction of the *GAL1* RNA was measured by RT-PCR and normalized to *ACT1*.

CHAPTER FIVE

DISCUSSION AND BIOLOGICAL RELEVANCE

The data presented in this thesis represents significant advances in the understanding of transcriptional activation. The first is the discovery of mutations that selectively affect the occupancy of the activated Gal4 transcriptional activator in vivo under inducing, but not non-inducing, conditions. Nor do they affect the intrinsic DNA binding activity of the protein in vitro. The mutations mapped to a surface loop that does not make contact with the DNA and the mutations do not affect the binding of the protein under non – inducing conditions. Biochemical analyses revealed that the mutations prevented the phosphorylation and ubiquitylation of the Gal4 DNA binding domain *in vitro*. The link between phosphorylation and ubiquitylation has been established in other systems and is potentially a major mechanism for regulation of the post – translational modifications of activators.(Ma and Ptashne 1987; Russell and Johnston 2001) Protein-DNA complexes including the mutant proteins that could not be phosphorylated or ubiquitylated were also susceptible to destabilization by the 19S proteasome. These data provide evidence of a protective role for the mono-ubiquitylation of transcriptional activators. If an activator is ubiquitylated it remains bound to the DNA and can therefore recruit the rest of the transcriptional machinery. This may explain, at least in part, the mechanism underlying ubiquitin "licensing" that has been described previously by the Tansey group.(Tworkowski, Salghetti et al. 2002) This discovery also provides evidence of another activity of the proteasomal ATPases in removing

non – ubiquitylated activators from DNA. It is important to note that this activity is not a proteolytic activity but a chaperone – like activity involving only the base sub-complex of the proteasome.

The discovery of an activity capable of destabilizing Gal4-DNA complexes in vivo raises the more general question of how long the activator remains resident on promoters and how this lifetime is regulated, if at all. To probe this point, a novel assay was developed to measure the dynamics of the activator-DNA complex in living cells. The assay sets up a competition between native Gal4 and a large excess of a protein that contains the Gal4 DNA binding domain as well as the estrogen receptor ligand binding domain (LBD). The ligand binding domain sequesters the competitor protein in an inactive state until estradiol is added. Using this assay it was found that the Gal4 protein is much more labile in the non – induced state. After the addition of galactose the Gal4 protein-promoter complex becomes quite stable. This is in direct contrast to the "hit and run" mechanism that has been proposed for transcriptional activation in which the activator-DNA complex survives only a few (or even only one) round of transcription before being recycled.(Johnston and Hopper 1982; Muratani, Kung et al. 2005) Additionally it was shown that proteasome mediated proteolysis is not required for transcription of the Gal4 responsive genes. This separates the transcriptional activators into two distinct classes: activators that require turnover to activate and those which do not.

The development of the competition assay is a major advance for studying protein dynamics in living cells. It has been demonstrated that many proteins can be fused to a nuclear hormone receptor ligand binding domain to modulate activity.(Carter, Pennington et

al. 2005) Many of these LBD-containing fusion proteins could be used to compete with native proteins to measure protein dynamics. Fusion proteins containing DNA binding domains could be studied by chromatin immunoprecipitation (ChIP) assay as I have described for Gal4. Using this assay it would be possible to classify large numbers of transcription factors by the stability of the protein / DNA interface. It would also be informative to find the factors that influence the stability of the transcriptional activator. Very little is known about factors that may destabilize transcription factors independent of proteolysis. The competitive ChIP assay allows for the measurement of the dynamics at active and inactive promoters for specific transcription factors as well as general transcription factors. The ability to examine the dynamics of a specific protein at a specific promoter will uniquely allow for the study of general transcription factor dynamics in a highly regulated manner. Any protein that can be studied by ChIP assay could be a possible candidate for the competitive ChIP assay. The primary benefit of the competitive ChIP assay is the ability to study the dynamics of native proteins on native promoters. Aside from the overexpression of the competitor protein very little alteration of the native state of the cell is required. Since the majority of proteins are parts of multi-protein complexes the dynamics of the protein exchange within these complexes could also be studied. Techniques to study protein / protein interactions such as the protein crosslinking or fluorescence resonance energy transfer (FRET) could be used to determine when the competitor protein has entered into a complex or when a native protein has been removed. The ability to control the release of the competitor protein at a specific time makes this one of the most powerful systems developed to date to study protein dynamics in living cells.

The combination of the *in vivo* effects of the Gal4 mutants, the *in vitro* ubiquitylation and destabilization of Gal4, and the differences between active and repressed Gal4 stability give a testable model for the changes that occur when Gal4 is activated. Details of this model are shown in figure 5-1. Uninduced Gal4 is bound by Gal80 and is susceptible to displacement and a portion of the protein is rapidly degraded. Whether the protein degradation occurs concurrent with removal from the DNA or after additional factors removed the Gal4 protein from the DNA remains to be studied. The degraded Gal4 is replaced by Gal4 which escapes degradation and new protein synthesis to keep Gal4 levels constant. After addition of galactose Gal80 is removed from the Gal4 activation domain and sequestered by the Gal3 protein.(Yang 2005) In addition there appears to be a necessary ubiquitylation of the Gal4 protein that protects the protein from destabilization by the base complex of the proteasome which associates with the Gal4 activated promoters after the addition of Galactose.(Salghetti, Caudy et al. 2001) If the activator protein cannot be ubiquitylated (such as Gap71) or if the activator associates irreversibly with the proteasome (such as $Gal4^{D}$) the proteasomal ATPases remove the activator from the promoter and there is no recruitment of the general transcription factors. If the DNA binding domain is ubiquitylated and phosphorylated the Gal4 activation domain recruits mediator through interaction with the Gal11 protein and other general transcription factors (GTFs) including TBP.(Tansey 2001; Lipford, Smith et al. 2005) The ubiquitin mediated resistance to destabilization by the proteasome as well as the interactions with the general transcription factors make the Gal4 / DNA very stable as measured by the competition assays in chapter 3. In fact cooperative binding of the Gal4 protein and TBP has been shown to stabilize the Gal4

/ DNA complex *in vivo*.(Picard 2000) Although this is a very attractive model for the regulation of the Gal4 protein by post – translational modifications there are many questions that remain to be answered. If the Gal4 protein exists in a stable and an unstable state under non – induced conditions it remains to be determined what are the differences in the protein that determine the stability of the complex. One possibility is the phosphorylation state of the protein. This could be readily tested by using mutant Gal4 proteins and measuring the half – life of the promoter bound protein by ChIP assay as described in chapter 4. Additionally it would be informative to monitor the phosphorylation state of the Gal4 protein throughout the transcription cycle. Using phosphoprotein specific antibodies that recognize the phosphorylated S22 residue the phosphorylation state of the protein bound to the DNA could be determined by ChIP assay. In conjunction with the phosphorylation state of Gal4 the ubiquitylation state of the protein under different conditions must be determined. Since the ubiquitylation of the Gal4 DNA binding domain *in vitro* requires the protein be bound to DNA, phosphorylation of S22, and the presence of an activation domain it is likely that the E3 ubiquitin ligase is recruited by the promoter bound Gal4. This may occur at several stages in the activation cycle; prior to activation with galactose when Gal80 is present, after the removal of Gal80 but prior to the recruitment of the GTFs, or after the recruitment of mediator and / or the GTFs. The DNA used for the *in vitro* ubiquitylation assays contained the promoter elements needed to initiate transcription. By using a modified form of this assay it should be possible to determine whether the E3 ligase is associated with the GTFs. This could be accomplished by altering the TBP binding site to prevent association by TFIID, using the Gal4 activation domain along with Gal80 protein to determine if Gal80

bound protein can be ubiquitylated. These experiments would be more informative if yeast nuclear extract could be used to phosphorylate and ubiquitylate Gal4. Nuclear extracts from appropriate yeast mutants could be used to clarify the link between transcriptional activation and post – translational modifications of the Gal4 DNA binding domain (Gal4 DBD). Additionally extracts from strains which are lacking known kinases or ubiquitin ligases could be used to find the enzymes responsible for the modifications of the Gal4 DBD.

In addition to the modifications of the Gal4 DBD, the role of the proteasome in destabilizing the activators *in vivo* needs to be addressed. Although there is co-localization of the proteasomal ATPases with the Gal4 protein, as measured by ChIP assay *in vivo,* and the Gap71 mutation is susceptible to proteasome – mediated destabilization *in vitro*, the link between the two effects is still tenuous. The most direct way to determine the protein(s) responsible for destabilization of the Gap71 protein *in vivo* would be to screen for mutants that restore the ability of Δgal4 cells expressing Gap71 protein to grow on galactose. This could be done in a biased manner by screening for mutants of the Sug1 or Sug2 proteins or in an unbiased manner by screening cells that have been randomly mutagenize

Figure 5-1

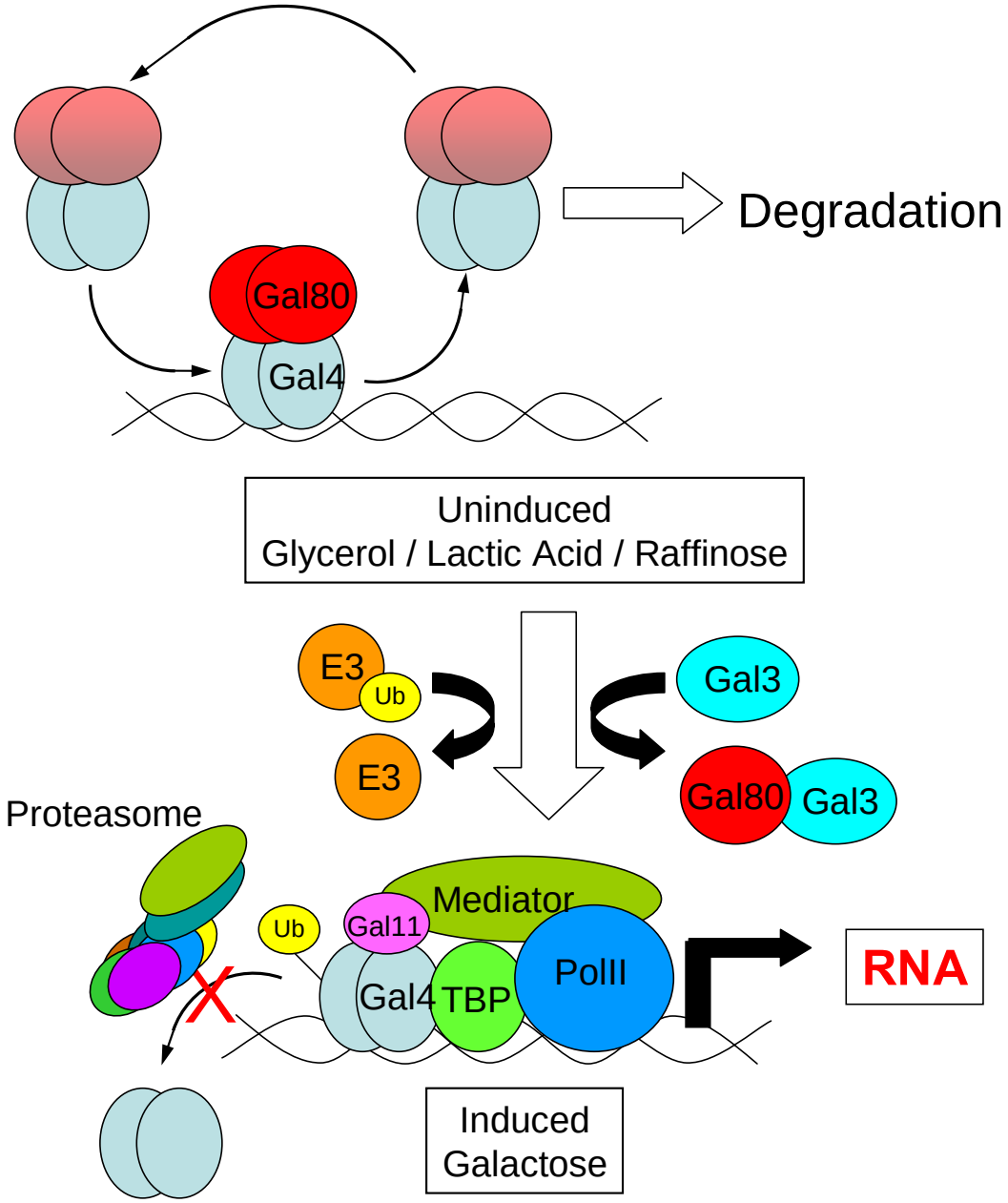

Degradation
Gal80
Gal4
Uninduced
Glycerol / Lactic Acid / Raffinose
E3
Ub
E3
Gal3
Gal80
Gal3
Proteasome
Mediator
Ub
Gal11
Gal4
TBP
PolII
RNA
Induced
Galactose

Figure 5-1

Model of the dynamic exchange of Gal4 protein *in vivo*. Gal4 bound to the promoter but uninduced is repressed by binding to the Gal80 protein. This protein can rapidly exchange with free activator. The status of the Gal80 binding to Gal4 away from the DNA is currently unknown. Upon DNA binding, Gal4 is ubiquitylated and protected from destabilization by the ATPases of the proteasome. Gal4 also can recruit the general transcription factors needed to activate transcription of the galactose responsive genes.

BIBLIOGRAPHY

Adams, G. M., B. Crotchett, et al. (1998). "Formation of the proteasome-PA700 complex directly correlates with activation of peptidase activity." Biochemistry **37**: 12927-12932.

Alberti, A., T. Lodi, et al. (2003). "MIG1-dependent and MIG1-independent regulation of GAL gene expression in Saccharomyces cerevisiae: role of Imp2p." Yeast **20**(13): 1085-96.

Barbey, R., P. Baudouin-Cornu, et al. (2005). "Inducible dissociation of SCF(Met30) ubiquitin ligase mediates a rapid transcriptional response to cadmium." Embo J **24**(3): 521-32.

Barnett, J. A. (1976). "The utilization of sugars by yeasts." Adv Carbohydr Chem Biochem **32**: 125-234.

Baumeister, W., J. Walz, et al. (1998). "The proteasome: Paradigm of a self-compartmentalizing protease." Cell **92**: 367-380.

Becker, M., C. T. Baumann, et al. (2003). "Dynamic behavior of transcription factors on a natural promoter in living cells." EMBO Reports **3**(12): 1188-1194.

Braun, B. C., M. Glickman, et al. (1999). "The base of the proteasome regulatory particle exhibits chaperone-like activity." Nature Cell Biol. **1**: 221-226.

Bres, V., R. E. Kiernan, et al. (2003). "A non-proteolytic role for ubiquitin in tat-mediated transactivation of the HIV-1 promoter." Nature Cell Biol. **5**: 754-761.

Brooks, C. L. and W. Gu (2003). "Ubiquitination, phosphorylation and acetylation: the molecular basis for p53 regulation." Curr Opin Cell Biol **15**: 164-71.

Carey, M., H. Kakidani, et al. (1989). "An amino-terminal fragment of GAL4 binds DNA as dimer." J. Mol. Biol. **209**: 423-432.

Carter, R. S., K. N. Pennington, et al. (2005). "Site-specific monoubiquitination of IkappaB kinase IKKbeta regulates its phosphorylation and persistent activation." J Biol Chem **280**(52): 43272-9.

Chambers, R. S. and S. A. Johnston (2003). "High-level generation of polyclonal antibodies by genetic immunization." Nature Biotech. **21**: 1088-1092.

Chi, Y., M. J. Huddleston, et al. (2001). "Negative regulation of Gcn4 and Msn2 transcription factors by Srb10 cyclin-dependent kinase." Genes & Dev. **15**: 1078-1092.

Chu-Ping, M., J. H. Vu, et al. (1994). "Identification, purification and characterization of a high molecular weight ATP-dependent activator (PA700) of the 20S proteasome." J. Biol. Chem. **269**: 3539-3547.

Conaway, R. C., C. S. Brower, et al. (2002). "Emerging roles of ubiquitin in transcriptional regulation." Science **296**: 1254-1258.

Corton, J. C. and S. A. Johnston (1989). "Altering DNA-binding specificity of GAL4 requires sequences adjacent to the zinc finger." Nature **340**: 724-727.

Corton, J. C., E. Moreno, et al. (1998). "Alterations in the GAL4 DNA-binding domain can affect transcriptoinal activation independent of DNA-binding." J. Biol. Chem. **273**: 13776-13780.

Coux, O., K. Tanaka, et al. (1996). "Structure and function of the 20S and 26S proteasomes." Ann. Rev. Biochem. **65**: 801-847.

d'Azzo, A., A. Bongiovanni, et al. (2005). "E3 ubiquitin ligases as regulators of membrane protein trafficking and degradation." Traffic **6**(6): 429-41.

Dignam, J. D., R. M. Lebovitz, et al. (1983). "Accurate transcription by RNA polymerase II in a soluble extract from isolated mammalian nuclei." Nuc. Acids Res. **11**: 1475-1489.

Ding, W. V. and S. A. Johnston (1997). "The DNA-binding and activation domains of Gal4p are sufficient for conveying its regulatory signals." Mol. Cell. Biol. **17**: 2538-2549.

Dubiel, W. and C. Gordon (1999). "Ubiquitin pathway: another link in the polyubiquitin chain?" Curr Biol **9**(15): R554-7.

El Khissiin, A. and G. Leclercq (1999). "Implication of proteasome in estrogen receptor degradation." FEBS Lett **448**(1): 160-6.

Fankhauser, C. P., P. A. Briand, et al. (1994). "The hormone binding domain of the mineralocorticoid receptor can regulate heterologous activities in cis." Biochemical & Biophysical Research Communications **200**(1): 195-201.

Ferdous, A., F. Gonzalez, et al. (2001). "The 19S regulatory particle of the proteasome is required for efficient transcription elongation by RNA polymerase II." Mol. Cell **7**: 981-991.

Ferdous, A., T. Kodadek, et al. (2002). "A nonprotelytic function of the 19S regulatory subunit of the 26S proteasome is required for efficient activated transcription by human RNA polymerase II." Biochemistry **41**: 12798-12805.

Ferdous, A., T. Kodadek, et al. (2002). "A nonproteolytic function of the 19S regulatory subunit of the 26S proteasome is required for efficient activated transcription by human RNA polymerase II." Biochemistry **41**(42): 12798-805.

Fletcher, T. M., N. Xiao, et al. (2002). "ATP-dependent mobilization of the glucocorticoid receptor during chromatin remodeling." Mol. Cell. Biol. **22**: 3255-3263.

Flick, K., I. Ouni, et al. (2004). "Proteolysis-independent regulation of the transcription factor Met4 by a single Lys 48-linked ubiquitin chain." Nat Cell Biol **6**(7): 634-41.

Freiman, R. N. and R. Tjian (2003). "Regulating the regulators: lysine modifications make their mark." Cell **112**: 11-17.

Glickman, M. H., D. M. Rubin, et al. (1998). "A subcomplex of the proteasome regulatory particle required for ubiquitin-conjugate degradation and related to the COP9-signalosome and eIF3." Cell **94**: 615-623.

Glickman, M. H., D. M. Rubin, et al. (1998). "The regulatory particle of the *Saccharomyces cerevisiae* proteasome." Mol. Cell. Biol. **18**: 3149-3162.

Gong, M., J. H. Ni, et al. (2002). "Increased exchange rate of histone H1 on chromatin by exogenous myogenin expression." Cell Res **12**(5-6): 395-400.

Gonzalez, F., A. Delahodde, et al. (2002). "Recruitment of a 19S proteasome subcomplex to an activated promoter." Science **296**: 548-550.

Hershko, A. and A. Ciechanover (1998). "The ubiquitin system." Ann. Rev. Biochem. **67**: 425-479.
Hicke, L. and R. Dunn (2003). "Regulation of membrane protein transport by ubiquitin and ubiquitin-binding proteins." Annu Rev Cell Dev Biol **19**: 141-72.
Hinnebusch, A. G. (1986). "The general control of amino acid biosynthetic genes in the yeast Saccharomyces cerevisiae." CRC Crit Rev Biochem **21**(3): 277-317.
Hinnebusch, A. G. (2005). "Translational regulation of GCN4 and the general amino acid control of yeast." Annu Rev Microbiol **59**: 407-50.
Hirst, M., M. S. Kobor, et al. (1999). "GAL4 is regulated by the RNA polymerase II holoenzyme-associated cyclin-dependent protein kinase SRB10/CDK8." Mol. Cell **3**: 673-678.
Irniger, S. and G. H. Braus (2003). "Controlling transcription by destruction: the regulation of yeast Gcn4p stability." Curr Genet **44**(1): 8-18.
Johnston, M. (1987). "A model fungal gene regulatory mechanism: The GAL genes of *Saccharomyces cerevisiae*." Microbiol. Rev. **51**: 458-476.
Johnston, M. and J. Dover (1987). "Mutations that inactivate a yeast transcriptional regulatory protein cluster in an evolutionarily conserved DNA binding domain." Proc Natl Acad Sci U S A **84**(8): 2401-5.
Johnston, S. A. and J. E. Hopper (1982). "Isolation of the yeast regulatory gene GAL4 and analysis of its dosage effects on the galactose/melibiose regulon." Proc. Natl. Acad. Sci. USA **79**: 6971-6975.
Kimura, H. (2005). "Histone dynamics in living cells revealed by photobleaching." DNA Repair (Amst) **4**(8): 939-50.
Kornitzer, D., B. Raboy, et al. (1994). "Regulated degradation of the transcription factor Gcn4." Embo J **13**: 6021-6030.
Kraulis, P. J., A. R. C. Raine, et al. (1992). "Structure of the DNA binding domain of zinc GAL4." Nature **356**: 448-450.
Kuras, L., A. Rouillon, et al. (2002). "Dual recognition of the Met4 transcription factor by ubiquitin-dependent degradation and inhibition of promoter recruitment." Mol. Cell **10**: 69-80.
Kurosu, T. and B. M. Peterlin (2004). "VP16 and ubiquitin: Binding of P-TEFb via its activation domain and ubiquitin facilitates elongation of transcription of target genes." Curr. Biol. **14**: 1112-1116.
Lee, D. H. and A. L. Goldberg (1996). "Selective inhibitors of the proteasome-dependent and vacuolar pathways of protein degradation in *Saccharomyces cerevisiae*." J. Biol. Chem. **271**(44): 27280-27284.
Leuther, K. K. and S. A. Johnston (1992). "Nondissociation of GAL4 and GAL80 in invo after galactose induction." Science **256**: 1333-1335.
Li, Q. and S. A. Johnston (2001). "Are all DNA binding and transcription regulation by an activator physiologically relevant?" Mol. Cell. Biol. **21**: 2467-2474.
Lipford, J. R. and R. J. Deshaies (2003). "Diverse roles for ubiquitin-dependent proteolysis in transcriptional activation." Nature Cell Biol. **5**: 845-850.
Lipford, J. R., G. T. Smith, et al. (2005). "A putative stimulatory role for activator turnover in gene expression." Nature **438**(7064): 113-6.

Lohr, D., P. Venkov, et al. (1995). "Transcriptional regulation in the yeast GAL gene family: a complex genetic network." FASEB J. **9**: 777-787.

Louvion, J. F., B. Havaux-Copf, et al. (1993). "Fusion of GAL4-VP16 to a steroid-binding domain provides a tool for gratuitous induction of galactose-responsive genes in yeast." Gene **131**(1): 129-34.

Lowe, J., D. Stock, et al. (1995). "Crystal structure of the 20S proteasome from the archaeon *Thermoplasma acidophilum* at 3.4 angstrom resolution." Science **28**: 533-539.

Ma, J. and M. Ptashne (1987). "The carboxy-terminal 30 amino acids of GAL4 are recognized by GAL80." Cell **50**(1): 137-42.

Ma, J. and M. Ptashne (1987). "The carboxy-terminal 30 amino acids of GAL4 are recognized by GAL80." Cell **50**: 137-142.

Ma, J. and M. Ptashne (1987). "Deletion analysis of GAL4 defines two transcriptional activating segments." Cell **48**: 847-853.

Maratani, M. and W. P. Tansey (2003). "How the ubiquitin-proteasome system controls transcription." Nature Reviews Mol. Cell Biol. **4**: 192-201.

Marmorstein, R., M. Carey, et al. (1992). "DNA recognition by GAL4: Structure of a protein-DNA complex." Nature **356**: 408-414.

Masuyama, H. and Y. Hiramatsu (2004). "Involvement of suppressor for Gal 1 in the ubiquitin/proteasome-mediated degradation of estrogen receptors." J Biol Chem **279**(13): 12020-6.

Masuyama, H., Y. Hiramatsu, et al. (2000). "Endocrine disrupting chemicals, phthalic acid and nonylphenol, activate Pregnane X receptor-mediated transcription." Mol Endocrinol **14**(3): 421-8.

Masuyama, H. and P. N. MacDonald (1998). "Proteasome-mediated degradation of the vitamin D receptor (VDR) and a putative role for SUG1 interaction with the AF-2 domain of VDR." J Cell Biochem **71**(3): 429-40.

Matsumoto, K., Y. Adachi, et al. (1980). "Need to get this title." J. Bacteriol. **141**: 508-527.

McCance, D. J. (2005). "Transcriptional regulation by human papillomaviruses." Curr Opin Genet Dev **15**(5): 515-9.

McKenna, N. J., J. Xu, et al. (1999). "Nuclear receptor coactivators: multiple enzymes, multiple complexes, multiple functions." J Steroid Biochem Mol Biol **69**(1-6): 3-12.

McNally, J. G., W. G. Müller, et al. (2000). "The glucocorticoid receptor: rapid exchange with regulatory sites in living cells." Science **287**: 1262-1265.

Meimoun, A., T. Holtzman, et al. (2000). "Degradation of the transcription factor Gcn4 requires the kinase Pho85 and the SCF(CDC4) ubiquitin-ligase complex." Mol Biol Cell **11**(3): 915-27.

Melcher, K., B. Sharma, et al. (2000). "Zero Background Yeast Reporter Plasmids." Gene **247**(1-2): 53-61.

Melcher, K. and H. E. Xu (2001). "Gal80-Gal80 interaction on adjacent Gal4p binding sites is required for complete GAL gene repression." EMBO J. **20**: 841-851.

Metivier, R., G. Penot, et al. (2003). "Estrogen receptor-alpha directs ordered, cyclical, and combinatorial recruitment of cofactors on a natural target promoter." Cell **115**(6): 751-63.

Mitsui, A. and P. A. Sharp (1999). "Ubiquitination of RNA polymerase II large subunit signaled by phosphorylation of carboxyl-terminal domain." Proc. Natl. Acad. Sci. USA **96**: 6054-6059.

Muratani, M., C. Kung, et al. (2005). "The F box protein Dsg1/Mdm30 is a transcriptional coactivator that stimulates Gal4 turnover and cotranscriptional mRNA processing." Cell **120**(6): 887-99.

Mylin, L. M., Johnston, M, Hopper J.E. (1990). "Phosphorylated forms of GAL4 are correlated with ability to activate transcription." Mol. Cell. Biol. **10**: 4623-4629.

Nagaich, A. K., W. D.A., et al. (2004). "Rapid Periodic Binding and Displacement of the Glucocorticoid Receptor during Chromatin Remodeling." Mol Cell **14**: 163-174.

Nawaz, Z., D. M. Lonard, et al. (1999). "Proteasome-dependent degradation of the human estrogen receptor." Proc. Natl. Acad. Sci. USA **96**: 1858-1862.

Pan, T. and J. E. Coleman (1990). "The DNA binding domain of GAL4 forms a binuclear metal ion complex." Biochemistry **29**: 3023-3029.

Patton, E. E., C. Peyraud, et al. (2000). "SCF(Met30)-mediated control of the transcriptional activator Met4 is required for the G(1)-S transition." Embo J **19**(7): 1613-24.

Picard, D. (2000). "Posttranslational regulation of proteins by fusions to steroid-binding domains." Methods in Enzymology **327**: 385-401.

Robinson, P. A. and H. C. Ardley (2004). "Ubiquitin-protein ligases." J Cell Sci **117**(Pt 22): 5191-4.

Rouillon, A., R. Barbey, et al. (2000). "Feedback-regulated degradation of the transcriptional activator Met4 is triggered by the SCF(Met30)complex." Embo J **19**(2): 282-94.

Russell, S. J. and S. A. Johnston (2001). "Proteolysis of Gal4 can not explain the transcriptional effects of proteasome ATPase mutations." J. Biol. Chem. **276**: 9825-9831.

Saddiqui, A. H. and M. C. Brandriss (1989). "The Saccharomyces cerevisiae PUT3 activator protein associates with proline-specific upstream activation sequences." Mol. Cell. Biol. **9**: 4706-472.

Sadowski, I., J. Ma, et al. (1988). "GAL4-VP16 is an unusually potent activator." Nature **335**: 563-564.

Sadowski, I., D. Neidbala, et al. (1991). "GAL4 is phosphorylated as a consequence of transcriptional activation." Proc Natl Acad Sci U S A **88**: 10510-10514.

Salghetti, S. E., A. A. Caudy, et al. (2001). "Regulation of transcriptional activation domain function by ubiquitin." Science **293**: 1651-1653.

Salghetti, S. E., M. Muratani, et al. (2000). "Functional overlap of sequences that activate transcription and signal
ubiquitin-mediated proteolysis." Proc. Natl. Acad. Sci. USA **97**: 3118-3123.

Salmeron, J. M., Jr., K. K. Leuther, et al. (1990). "GAL4 mutations that separate the transcriptional activation and GAL80-interactive functions of the yeast GAL4 protein." Genetics **125**(1): 21-7.

Shemer, R., A. Meimoun, et al. (2002). "Regulation of the transcription factor Gcn4 by Pho85 cyclin PCL5." Mol Cell Biol **22**(15): 5395-404.

Sil, A. K., S. Alam, et al. (1999). "The Gal3p-Gal80p-Gal4p transcription switch of yeast: Gal3p destabilizes the Gal80p-Gal4p complex in response to galactose and ATP." Mol. Cell. Biol. **19**: 7828-7840.

Strickland, E., K. Hakala, et al. (2000). "Recognition of misfolding proteins by PA700, the regulatory subcomplex of the 26S proteasome." J. Biol. Chem. **275**: 5565-5572.

Su, N. Y., K. Flick, et al. (2005). "The F-box protein Met30 is required for multiple steps in the budding yeast cell cycle." Mol Cell Biol **25**(10): 3875-85.

Sun, Z.-W. and C. D. Allis (2002). "Ubiquitination of histone H2B regulates H3 methylation and gene silencing in yeast." Nature **418**: 104-108.

Swaffield, J. C., J. Bromberg, et al. (1992). "Alterations in a yeast protein resembling HIV Tat-binding protein relieve the requirement for an acidic activation domain of GAL4." Nature **357**: 698-700.

Takeuchi, J., M. Fujimuro, et al. (1999). "Rpn9 is required for efficient assembly of the yeast 26S proteasome." Mol Cell Biol **19**(10): 6575-84.

Takeuchi, J. and A. Toh-e (2001). "Genetic dissection of the yeast 26S proteasome: cell cycle defects caused by the Deltarpn9 mutation." Biochimie **83**(3-4): 333-40.

Tansey, W. (2001). "Transcriptional activation: risky business." Genes & Dev. **15**: 1045-1050.

Thrower, J. S., L. Hoffman, et al. (2000). "Recognition of the polyubiquitin proteolytic signal." EMBO J. **19**: 94-102.

Titz, B., S. Thomas, et al. (2006). "Transcriptional activators in yeast." Nucleic Acids Res **34**(3): 955-67.

Torchia, T. E., R. W. Hamilton, et al. (1984). "Disruption of regulatory gene GAL80 in Saccharomyces cerevisiae: effects on carbon-controlled regulation of the galactose/melibiose pathway genes." Mol. Cell. Biol. **4**(8): 1521-1527.

Triezenberg, S. J. (1995). "Structure and function of transcriptional activation domains." Curr Opin Genet Dev **5**(2): 190-6.

Tworkowski, K. A., S. E. Salghetti, et al. (2002). "Stable and unstable pools of Myc protein exist in human cells." Oncogene **21**(55): 8515-20.

Urbe, S. (2005). "Ubiquitin and endocytic protein sorting." Essays Biochem **41**: 81-98.

Vashee, S. and T. Kodadek (1995). "The activation domain of GAL4 protein mediates cooperative promoter binding with general transcription factors *in vivo*." Proc. Natl. Acad. Sci. USA **92**: 10683-10687.

Vashee, S., H. Xu, et al. (1993). "How do Zn_2Cys_6 proteins distinguish between similar upstream activation sites? Comparison of the in vivo and in vitro DNA-binding specificities of the GAL4 protein." J. Biol. Chem. **268**: 24699-24706.

Verma, R., L. Aravind, et al. (2002). "Role of Rpn11 metalloprotease in deubiquitination and degradation by the 26S proteasome." Science **298**(5593): 611-5.

vom Baur, E., C. Zechel, et al. (1996). "Differential ligand-dependent interactions between the AF-2 activating domain of nuclear receptors and the putative transcriptional intermediary factors mSUG1 and TIF1." Embo J **15**(1): 110-24.

Walker, D., H. Htun, et al. (1999). "Using inducible vectors to study intracellular trafficking of GFP-tagged steroid/nuclear receptors in living cells." Methods **19**(3): 386-93.

Weeda, G., M. Rossingnol, et al. (1997). "The XPB subunit of repair/transcription factor TFIIH directly interacts with SUG1, a subunit of the 26S proteasome and putative transcription factor." Nuc. Acids Res. **25**: 2274-2283.

Wehrman, T. S., C. L. Casipit, et al. (2005). "Enzymatic detection of protein translocation." Nat Methods **2**(7): 521-7.

Welshons, W. V., L. H. Grady, et al. (1993). "Subcellular compartmentalization of MCF-7 estrogen receptor synthesis and degradation." Mol Cell Endocrinol **94**(2): 183-94.

Willems, A. R., M. Schwab, et al. (2004). "A hitchhiker's guide to the cullin ubiquitin ligases: SCF and its kin." Biochim Biophys Acta **1695**(1-3): 133-70.

Yang, X. J. (2005). "Multisite protein modification and intramolecular signaling." Oncogene **24**(10): 1653-62.

Yano, K.-I. and T. Fukasawa (1997). "Galactose-dependent reversible interaction of Gal3p with Gal80p in the induction pathway of Gal4p-activated genes of *Saccharomyces cerevisiae*." Proc. Natl. Acad. Sci. USA **94**: 1721-1726.

Yen, J. L., N. Y. Su, et al. (2005). "The yeast ubiquitin ligase SCFMet30 regulates heavy metal response." Mol Biol Cell **16**(4): 1872-82.

Zhu, Q., J. Yao, et al. (2004). "The ubiquitin-proteasome pathway is required for the function of the viral VP16 transcriptional activation domain." FEBS Lett **556**(1-3): 19-25.

VITAE

Kip Andrew Nalley was born in Rockford, IL on July 21, 1971, the son of Janice H. Nalley and Lawrence W. Nalley. Kip graduated from Auburn High School in June of 1989. He graduated *Cum Laude* from the University of Arkansas – Fayetteville in 1994 with a Bachelor's of Science in Chemistry, Biochemistry Option. After graduation he attended the University of California – San Diego and received the degree of Master of Science in Chemistry in 1997. After three years of employment at Idun Pharmaceuticals, he entered the graduate program at the University of Texas Southwestern Medical Center.

Permanent Address: 440 Elmcroft Blvd. #3410
Rockville, MD 20850