THE MOLECULAR MECHANISMS GOVERNING THE GAL GENE SWITCH OF SACCHAROMYCES CEREVISIAE

DISSERTATION

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By

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ABSTRACT

Many insights into how genes are regulated in higher organisms including humans have stemmed from over four decades of studies on the *Saccharomyces cerevisiae* GAL gene switch. Basic mechanisms remain to be elucidated concerning the three principal GAL gene switch proteins, Gal3, Gal80 and Gal4. The transcriptional regulation of the GAL genes is repressed in absence of galactose via Gal80 inhibition of Gal4, a site-specific DNA binding transcriptional activator. Galactose binds to Gal3, converting it to a form that binds to Gal80 and releases Gal4 from the Gal80 inhibition. It was thought that such a mechanism, consistent with the data at the time, could explain the very rapid response to galactose that is a hallmark of this system. According to one previous working model, it was proposed that Gal80 shuttles rapidly between nucleus and strictly cytoplasmic Gal3 sequesters Gal80 in the cytoplasm in response to galactose. That model proposed dissociation of Gal80 from Gal4 in response to galactose. Another, competing model proposed that galactose triggers entry of Gal3 into the nucleus where it binds to Gal4-associated Gal80, causing rearrangement of the Gal80-Gal4 complex, but not gross dissociation of Gal80 from Gal4. Some previous work led to data against dissociation of Gal80 from Gal4, while other previous work provided evidence for dissociation.
Studies initiated in the course of this thesis work aimed at testing the above competing models and discovering how binding of Gal3 to Gal80 alters Gal80 so as to promote Gal4-mediated activation of GAL gene transcription. We first discovered that Gal3 is uniformly distributed between nucleus and cytoplasm. We found that Gal80 does not show rapid nucleo-cytoplasmic shuttling. And we showed by fluorescence recovery after photobleaching that Gal80 did not rapidly shuttle between the two compartments.

We also tested the notion that Gal3 binding to Gal80 interferes with Gal80 self-association, and we found that it does. All together, these results led to a new model, where we proposed the initial Gal3-Gal80 interaction occurs within the nucleus and destabilizes the Gal80 dimer or multimer associated with Gal4, causing Gal80 to dissociate from Gal4. We determined that Gal80-Gal4 complex at the promoters has a slow off-rate, and suggest that Gal3 interacts transiently with Gal4-associated Gal80 to promote its dissociation from Gal4.

During search for previously proposed NLS and NES sequences we discovered a phospholipid-binding surface of Gal80 (named the HALO fragment). We then discovered that Gal80 could associate with biological membranes extracted from yeast extracts, and that Gal4 could associate with membrane-bound Gal80. On the other hand, we found that Gal3 binding to Gal80 causes dissociation of Gal80 from membranes. This observation, together with the fact that in the published crystal structure for the Gal3-Gal80 complex the HALO fragment is entirely buried. That is not the case in the Gal4-Gal80 complex, as solved by the same group. Thus, the Gal3 binding to Gal80 would seemingly prevent membrane association of Gal80.
In fact, we present evidence for clusters of Gal80 within the nucleus, and such clusters dissipate in response to Gal3 binding to Gal80 (in galactose).

Based on observations in this thesis taken together with data from others, we proposed a broader model of the GAL gene switch that incorporates nuclear clustering of Gal80 multimers at the nuclear periphery as a mechanism for maintenance of tight Gal80-mediated repression of the GAL genes in the absence of galactose.
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I dedicate this work to the Human Fund
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<td>Nuclear Export Sequence</td>
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<tr>
<td>NLS</td>
<td>Nuclear Localization sequence</td>
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<tr>
<td>Gal4AD</td>
<td>Gal4 Activation Domain</td>
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<tr>
<td>AD</td>
<td>Activation Domain</td>
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<tr>
<td>Gal4DBD</td>
<td>Gal4 DNA Binding Domain</td>
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<tr>
<td>DBD</td>
<td>DNA Binding Domain</td>
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<tr>
<td>NPC</td>
<td>Nuclear Pore Complex</td>
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<tr>
<td>FRAP</td>
<td>Fluorescence Recovery After Photobleaching</td>
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<tr>
<td>FRET</td>
<td>Fluorescence Resonance Energy Transfer</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescence Protein</td>
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<td>YFP</td>
<td>Yellow Fluorescence Protein</td>
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<td>ATP</td>
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CHAPTER 1

INTRODUCTION

The fundamental understanding of transcription initially came from the investigations into the lactose metabolism of *Escherichia coli* (Jacob and Monod 1961, Monod 1978). These bacterial cells are evolved to turn on their genes that are involved in processing lactose only in presence of lactose, saving the energy required to express the genes in its absence. In eukaryotic *Saccharomyces cerevisiae*, the genes of the galactose metabolism behave in a similar manner. Thus, the molecular mechanism that collectively regulates these genes, the *GAL* gene switch, has served as the canonical system for probing eukaryotic transcription regulation for decades. Even though we have acquired a reliable body of knowledge about this system, the exact molecular pathways that govern the galactose-dependent transcription regulation remains elusive. Not surprisingly, there is still much debate
on how specific molecular interactions of the \textit{GAL} regulatory proteins lead to transcription of the \textit{GAL} genes.

In this work, I will first review current views of the scientific community on regulation of transcription activators, then introduce the \textit{S. cerevisiae GAL} gene switch as a model of conditional gene expression and regulation. Subsequently, I will present my data addressing some of the questions central to the molecular mechanisms governing this system. Finally, I will discuss all the data presented here and propose a new working model.

\textbf{1.1 Transcription Regulation}

Transcription is a biological process that synthesizes RNA molecules using the genes found on chromosomes as a template. In eukaryotes, the chromosomes are linear stretches of DNA tightly packed around protein complexes that consist of histone molecules. For transcription to start, the DNA is required to become “loose” for accessibility, and the main enzymatic complex undertaking the RNA synthesis needs to be recruited upstream of the genes to a DNA region called the promoter. These steps take place continuously for some genes that are generally referred to as the “housekeeping” genes. However, others, unlike the “housekeeping” genes, are only required under specific circumstances. The spatial and temporal regulation of such conditional transcription is very tightly controlled.

The eukaryotic cells accommodate immense amount of DNA in a relatively small nuclear space. The DNA wraps around histones to form nucleosomes (Kornberg and Lorch 1999). A combination of interactions between nucleosomes
packs the chromatin into several different levels of higher-order structures (Carruthers and Hansen 2000). The more densely packed the DNA, the less available it is for binding of transcription factors. Thus, the first step of eukaryotic transcription initiation addresses the issue of accessibility, which can directly affect transcriptional states of the genes. The nucleosome core is composed of four histones (H2A, H2B, H3 and H4), and the linker DNA between the nucleosomes binds to histone H1 to stabilize the interactions between nucleosomes (Wolffe and Hayes 1999). The extent of these interactions provides the impediment for recruitment of transcription factors. As chromatin-decondensing complexes require DNA-binding transcription factors to target specific regions of chromatin, and transcription factors require decondensed chromatin to bind the DNA, the situation appears paradoxical. The well-established idea is that the chromatin exists in ensembles of conformations, allowing binding of transcription factors to the DNA with low affinity. When these factors receive a signal to activate transcription, they recruit the chromatin remodeling complexes and bind to their specific DNA element more tightly (Tsai and Fondell 2004, Lee, K. C., and W. Lee Kraus 2001; Zhou, V. W., A. Goren, and B. E. Bernstein 2011, Flaus and Owen-Hughes 2011).

Three major classes of RNA polymerase carry out eukaryotic transcription. The work here focuses on regulation of the class II polymerases via DNA binding transcription activators. RNA pol II produces the heterogeneous nuclear RNAs that are the precursors to mRNAs coding for proteins. As pol II transcribes a broad range of genes, the promoters targeted by this polymerase show great variation. As well, the transcriptional regulation of these genes is modulated by a variety of multiple
cis-regulatory elements. The core promoter region comprise of the TATA box, TFIIB recognition site, the initiator and the downstream promoter element. Although it is relatively challenging to identify these elements in metazoans, the yeast gene core promoters conform to more conserved structures. These elements can be discerned solely by investigating the DNA sequence.

The enhancers and silencers are relatively short stretch of DNA that is targeted by DNA-binding proteins, called the trans-acting factors. In many cases, a group of genes are regulated by the same trans-acting factors, and slight variation among the cis-elements of these genes may be functional in varied regulation of the genes, i.e. the same trans-acting factor might affect two different genes at hundreds of fold different levels. While the enhancers and silencers can be found very close to the core promoters, it is not uncommon to find them several kilo bases away from the gene, or even on a different chromosome.

The insulators are the regulatory elements that create a boundary between neighboring genes. These sites are commonly required to distinctively regulate two different genes that are in close proximity of each other. They can either act to block the effect of the enhancers on the non-targeted gene, or they might create a boundary for the chromatin modeling complexes.

**1.2 Recruitment of RNA polymerase II to the promoter**

At a specific time, only a subset of genes of an organism is actively transcribed. For example, of the approximately 30 thousand human genes only a fraction of them are expressed depending on the cell type and the developmental
stage of the entire body. Such specificity is mainly due to the fact that the eukaryotic transcription initiation is a multi-step assembly of several protein complexes at the core promoter and enhancers, and these steps are tightly regulated at multiple points. Once a gene is destined to transcription, general and gene-specific transcription factors are recruited to the regulatory elements of the gene. While most of the general transcription factors target the core promoter, the gene-specific factors target the enhancer and silencer regions. In addition, a large number of co-regulators that do not bind to DNA directly are involved in transcription initiation via interactions with DNA-bound transcription factors.

The enhancers were initially identified as upstream cis-elements of the core promoters. Later studies, however, showed that the enhancers differed from the other core promoter elements in the correlation between the positioning of the elements and their transcription activity. The enhancers needed not to be in close proximity of the transcription start site (Gillies et al. 1983). These cis-regulatory elements, like other core promoter components, carry out their function via proteins binding to the conserved sequences. The transcription activators targeting the enhancers are so-called gene specific as the enhancer DNA sequence is seldom found in the genome of millions of bases. Upon binding, the transcription activators can interact with chromatin remodeling complexes, components of the pre-initiation complex (PIC) and/or the RNA pol II complex to stabilize transcription machinery at the promoters of their target genes.

The sequence-specific transcription activators come in diverse structures. Generally, they consist of two independent domains for activation and DNA binding
functions. The activation domains (AD) are relatively challenging to categorize. They are usually made up of a malleable structure, since they constitute a binding surface for multiple transcription factors and co-regulators. In general they contain a stretch of acidic residues. The DNA binding domains (DBD), on the other hand, conform to more recognizable structures. The seven major types of DBD are helix-turn-helix, zinc finger, leucine zipper, winged helix, winged helix turn helix, helix loop helix, and HMG-box (Lilley, 1995).

Similarly to enhancers, the silencers are cis-regulatory elements that can act on a gene over long distances, but in an inhibitory manner. The mating genes of yeast (MAT) provide a good example of silencer elements, which apparently acts on the chromatin structure and limit the accessibility of the target genes to the transcription machinery (Lynch and Rusche 2009).

1.3 Signal transduction and transcription initiation

The conditional gene expression mechanisms are diverse and very specific to each stimulus. A common hallmark of every single gene switch is the rapid transcriptional response derived from the stimuli; therefore, the transcriptional regulators need to be present within the cell at the time the signal becomes available to the cell. This indicates that a structural change has to take place with respect to the transcriptional regulators. Such an alteration can involve an enzymatic activity modifying the transcription factors at the post-transcriptional level. Alternatively, the signal transduction mechanism can modify the quaternary structure of protein complexes that consist of the regulators. In this section, I will
summarize some of the prominent transcriptional switch mechanisms, while
demonstrating different ways of signal transduction.

Eukaryotic cells have three major types of cell-surface receptors that are
categorized by their mechanism of action. A cell-surface receptor can be a ligand-
gated channel, which is usually observed in the neurons, working to modify the
membrane permeability for specific ions. A second class of receptors interacts with
trimeric GTP-binding proteins (G-proteins) at their cytoplasmic domains. Binding of
the specific ligands at their extra-cellular domains activates the G-proteins within
the cell. Subsequently, the G-proteins can modify the function of specific regulators,
including transcription factors. A third class of receptors containing single-span
transmembrane domains can carry out an enzymatic function via their own
cytoplasmic domains. This group of receptors includes the extensively studied
receptor tyrosine kinases, receptor tyrosine phosphatases, receptor
serine/threonine kinases, receptor guanyl cyclases, and histidine kinases.
Downstream targets of all of the above receptors can directly act on transcription by
modulating the activity of transcription regulators in response to the non-
internalized signaling molecules. For example, the Ras/MAPK pathway activated by
epidermal growth factor receptor (EGFR) phosphorylates c-myc and c-fos, both of
which directly act as transcription factors in both positive and negative manner. All
of the above receptors generate a secondary signal within the cytoplasm in response
to the primary signal detected at the extra-cellular matrix. These secondary signals
and other primary signals that can actually diffuse into the cell are the basis of the
intra-cellular signaling acting on transcription factors in the nucleus.
The environmental stimuli enter the cell in two different ways. As I mentioned above, the signal transduction through the cytoplasm can be initiated via production of a secondary signal by interaction of the cell surface receptors and their specific ligands, or it can involve internalization of the primary signal into the cytoplasm. Either way, once the cytoplasmic pathways are activated, the message needs to be transferred to the nucleus and to the promoters of the target genes. The cells achieve this step via several distinct molecular mechanisms. For example, phosphorylation of c-myc affects its DNA binding domain activity and results in enhanced recruitment of c-myc to its specific promoters (Benassi et al. 2006, Mol. Cell). In another prominent receptor-dependent signaling pathway, phosphorylation of the IkB inhibitor protein causes dissociation of IkB from NF-kB heterodimers, which translocates to the nucleus and binds to its target gene promoters. In this system, a post-translational modification of a binding partner to the transcription factor not only regulates its transcription activation function by steric hinderance, but it also contributes to regulation by compartmental sequestration. Another novel molecular mechanism includes proteolytic cleavage of the transcription factor. For example, the Notch receptor activation leads to proteolysis of its cytoplasmic domain, which translocates to the nucleus to interact with other DNA binding proteins and activate transcription.

In addition to cell-surface receptor mediated signaling cascades, small signaling molecules can enter the cell and directly act on transcription factors in the cytoplasm or nucleus. One big class of such transcription factors is the nuclear receptor superfamily that binds to steroid ligands and translocate to the nucleus.
These molecules directly bind to the promoters when activated, and recruit the transcriptional machinery to the genes. For example, the glucocorticoid receptor protein (GR) resides in the cytoplasm bound to the heat shock protein, Hsp90, prior to the glucocorticoid ligand entering the cell. The ligand binding results in dissociation of Hsp90 from GR. The ligand-bound receptor dimers are, then, imported into the nucleus, and the bind to DNA.

1.4 The GAL gene switch

The brewers yeast, *Saccharomyces cerevisiae*, has been a useful tool for the mankind long before the emergence of the scientific method. Soon after early biologists discovered the cell, *S. cerevisiae* has become a scientific tool to investigate basic biological processes, because of their abundance and non-hazardous nature. Since yeasts have been cultured for its fermentative properties over thousands of years, the domesticated species were usually the ones that were well adapted to utilize the carbon sources for alcohol production. *S. cerevisiae* is well-evolved to metabolize glucose as a primary carbon source. It can also utilize other less abundant sugars, such as galactose, maltose, sucrose... etc. However, the pathways for metabolizing these molecules are directly repressed in presence of glucose. The genes that are required to process the carbon sources other than glucose are also tightly inhibited in absence of glucose, if the specific carbon source is not present in the cell.
The *S. cerevisiae* galactose metabolism genes are collectively regulated at the transcriptional level. The *GAL* gene switch can be considered under three different states: (i) Glucose repressed state. In this state, glucose-dependent inhibitory mechanisms act on the promoters of *GAL* genes directly, reducing the availability of transcription factors and accessibility of the promoters. (ii) Uninduced state. This state is different than the glucose-repressed state. When there isn’t high enough level of glucose in the cell, the glucose-dependent inhibition pathways do not act on the genes. If, however, galactose is also absent, transcription is still inhibited via to *GAL* gene specific mechanism. The transcription inhibitor, Gal80, binds to the transcription activator, Gal4, masking its activation domain. (iii) Induced state. When the cells encounter galactose in absence of glucose, the binding of the sensor protein, Gal3, to galactose initiates an intricate interplay between the *GAL* regulatory proteins. Subsequently, the transcription activator becomes available to recruit transcription machinery to the promoters.

1.4.1 The GAL gene structure

There are 12 genes under the *GAL* gene switch regulation, including the Leloir pathway and *GAL* regulatory genes. Although the same regulatory mechanism acts on all the *GAL* genes, the basal and induced level of gene expression for each gene is unique. This feature is often attributed to the fact that there is varied number of Gal4 binding sites, called the upstream activation sequence (UAS\textsubscript{GAL}), at the promoter of each gene. Moreover, UAS\textsubscript{GAL} sequence is slightly different at each
promoter, suggesting varied binding affinity of Gal4 to each site might also be contributing to the differences in expression levels (Lohr et al. 1995).

The GAL genes have the prototypical gene structure. The promoter consists of an easily identified TATA box in close proximity of the transcription start site, and the enhancer UAS\text{GAL} region within a few hundred bases upstream. Although a search of the yeast genome identifies over 200 locations as putative Gal4 binding sites (Li and Johnston 2001), it is probably only the ones found in the promoters of GAL genes that are functionally relevant. One possible regulation to determine Gal4 binding specificity to the correct binding sites is the nucleosome placement at the non-specific sites to impair binding site accessibility. Indeed, it is shown that the nucleosome density at the GAL promoters is reduced compared to non-specific sites (Bash and Lohr 2001).

1.4.2 The GAL gene switch regulatory proteins.

The three major players in the GAL gene switch mechanism, as I mentioned above, are Gal4, Gal80, and Gal3. The identification of these genes goes back to the era prior to emergence of modern molecular techniques and genome sequencing. During that period, geneticists were trying to identify the molecular elements that characterize gene expression by investigating specific mutants. The first published genetic model appeared in a 1966 manuscript by Douglas and Hawthorne, describing three distinct genetic loci (GA\textsubscript{4}, i, and C) involved in regulation of GAL genes. Today, we know that both GA\textsubscript{4} and C mutations, which they referred to as distinct elements, map to the Gal4 and i to the Gal80 genes. The Gal3 gene was not
characterized as a regulatory element till later, even though the mutations at this locus were long known to cause a delay in expression of GAL genes (Winge and Robert 1948). Below, I will first summarize what we know about the genes, then I will introduce the working model, which I started testing in the beginning of my work.

1.4.2.1 The transcriptional activator Gal4p

Gal4 is arguably the most extensively studied eukaryotic transcription activator. The 881-amino-acid Gal4 protein is comprised of the N-terminal DNA binding domain (DBD), a dimerization domain, a middle linker region, and an activation domain at the C-terminus (Gal4AD). The DBD is not a member of the zinc finger family (Pan and Coleman 1990, Gardner 1991), but it resembles one by forming a binuclear cluster via its cysteine (Cys) rich peptide complexed with two zinc(II) molecules (Pan and Coleman 1990, Marmorstein et al. 1992). The Gal4 dimers with a very high affinity bind to a palindromic consensus binding site of 17 base pairs (UASGAL). The Zn-Cys domains make contacts with the conserved trinucleotide sequences on each end of the binding site, whereas the linker and the dimerization domains make contacts with the phosphate backbone according to the crystal structure.

The acidic activation domain spanning the residues 761-881 is the prominent Gal4 region that interacts with the transcriptional machinery under inducing conditions (Brent and Ptashne 1985, Ma and Ptashne 1987). This region directly interacts with the Tra1 protein in the chromatin remodeling SAGA complex
and Gal11 of the mediator complex (Jeong et al 2001, Bhaumik et al. 2004). Other mentionable interaction partners of Gal4AD include TBP, SWI/SNF, TFIIB, Srb10 and the 19S proteasome subunits Sug1 and Sug2 after galactose activation (Melcher and Johnston 1995, Wu et al. 1996, Yudkovsky et al. 1999, Brown et al. 2001, Chang et al. 2001, Ansari et al. 2002). These interactions bring about a step-wise recruitment of the transcription co-factors to the GAL genes, and at least recruitment of SAGA, the chromatin-remodeling complex, depends on other DNA elements of the GAL promoter other than UAS_{GAL}, suggesting the Gal4-activated transcription is very specific to consensus sequences at the promoter regions (Larschan and Winston 2001).

Gal4 undergoes post-translational modifications upon galactose induction. It is found in three distinct phosphoforms (Mylin et al. 1989, Mylin et al. 1990, Sadowski et al. 1991). The nature and function of each phosphoform is not very clear, but mutational analysis suggests that the phosphorylation is a critical event for Gal4 activity. It has been suggested that the Srb10 subunit of the mediator complex is responsible for phosphorylating Gal4 Ser699 residue (Hirst et al. 1999), suggesting this event occurs early after galactose induction and prior to RNA pol II recruitment. Additionally, recent investigations demonstrated that Gal4 is also ubiquitinylated via two F-box protein dependent mechanisms, Grr1 and Dsg1 (Muratani et al. 2005). While Grr1-dependent pathway appears to inhibit Gal4 activity under non-inducing condition by diverting the Gal4 molecules to degradation, CHIP experiments demonstrate that Dsg1 is recruited to the activated GAL gene promoter. This indicates that Gal4-DNA complex is destabilized upon
induction. In fact, the authors of that study proposed a Gal4-cyle system, and suggest that the Dsg1-mediated removal of $\text{UAS}_{\text{Gal}}$-associated Gal4 molecules is the molecular trigger that destabilizes PIC closed complex in favor of open complex formation.

1.4.2.2 The transcriptional inhibitor Gal80p

In absence of galactose, the GAL gene expression is not needed for obvious reasons. The cells achieve inhibition of $\text{GAL}$ genes by controlling the Gal4 activity directly with the transcription inhibitor, Gal80. The interaction between Gal4 and Gal80 under non-inducing conditions results in masking of Gal4AD and prevents recruitment of the transcription machinery. The Gal80 protein is a relatively small globular protein of 435 amino acids. Deletion studies indicate that Gal80 requires the Gal4 residues 851-874 for effective binding (Nogi et al. 1977, Johnston et al. 1987, Ma and Ptashne 1987). When galactose is introduced to the system, Gal80 interacts with the sensor protein, Gal3, to relieve its inhibitory function on Gal4 activity. In recent years, the crystal structure of Gal80 in complex with Gal4AD and Gal3 has been reported (Kumar et al. 2008, Lavy et al. 2012). As it was predicted previously (Pilauri et al. 2005), crystal structure of Gal80 in complex with Gal4AD was also bound to a nicotinamide adenine dinucleotide (NAD) ligand at a specific pocket near the Gal4AD binding site (Kumar et al. 2008). After further investigation, the authors realized that nicotinamide adenine dinucleotide phosphate (NADP) binding competes with Gal80-Gal4 interaction in vitro; however, the in vivo implication of Gal80-NADP interaction has not been solidified to date.
Gal80 dimerizes with high affinity (approximately 5 nM, Melcher et al. 2001). A genetic selection for Gal80 mutants that are defective in Gal3-binding, demonstrated that the mutants also lost Gal4 binding in every case if the mutant failed to self associate (Pilauri et al. 2005). While these studies supported the notion that Gal80 self-association interactions are required for binding to and inhibition of Gal4, it is still not clear what oligomeric form of Gal80 binds to Gal4 in vivo. On the other hand, the gel filtration analysis of Gal80-Gal3 complex indicates heterodimer (Timson et al. 2002). These observations prompt a Gal3-dependent destabilization of Gal80 oligomers as a molecular mechanism for disrupting Gal4-Gal80 complex and relieving transcriptional inhibition.

**1.4.2.3 The galactose sensor Gal3p**

Gal3 is a 520 amino acid protein that has a 74% identity with the galactokinase, Gal1 gene, which can also act as an inducer with a relatively lesser affinity for Gal80. Despite a substantial similarity between Gal1 and Gal3, the latter has lost the galactokinase activity. Interestingly, insertion of two amino acids in Gal3 can give rise to a Gal3 derivative that has acquired galactokinase activity (Platt et al. 2000). One critical difference between Gal1 and Gal3 lies in the basal level expressions. In absence of galactose, Gal3 is expressed at relatively higher level. Gal1, on the other hand, is very tightly repressed. So, at the initial early induction Gal3 acts as the sole galactose sensor, and it is responsible for normally rapid \( GAL \) gene switch in response to galactose.
As a typical member of the galactokinase, homoserine kinase, mevalonate kinase, and phosphomevalonate kinase (GHMP) family (Bork et al. 1993), Gal3 has a phosphate-binding domain. Presence of ATP in this pocket appears to enhance the galactose-mediated interaction between Gal3 and Gal80. Recently, the crystal structure of Gal3 and Gal80 has been published, showing Gal3 in complex with Gal80, ATP, galactose and Mg$^{2+}$ (Lavy et al. 2012). According to this structure, two Gal3 monomers bind to a Gal80 dimer to form a heterotetrameric complex (Lavy et al. 2012). This result is in direct contrast to earlier results from gel filtration assays, showing a heterodimer as the most prominent Gal3-Gal80 complex in solution (Timson et al. 2002).

1.4.3 The changing face of the GAL gene switch

The earliest GAL gene switch model by Douglas and Hawthorne consisted of three regulatory elements: i, C, and GA$_4$. Recessive mutation in the GA$_4$ locus abolished galactose dependent expression of the enzymes in the Leloir pathway. The authors correctly surmised that GA$_4$ produced a positive regulatory factor for the GAL genes. Later, GA$_4$ region is shown to code for the Gal4 protein (Laughon and Gesteland 1982). Douglas and Hawthorne also showed that recessive mutations in the “i” locus and dominant mutations in the C locus produced constitutively expressed GAL genes. Accordingly, they proposed i coded for a transcriptional inhibitor that targeted a cis-element at the C locus to regulate GA$_4$ expression. In absence of galactose, the product of i would be bound to C element to repress GA$_4$
expression; therefore, *GAL* genes would be shut off. Galactose binding to the transcription inhibitor would cause its dissociation from C, allowing GA₄ expression, which in turn would induce *GAL* genes. Not surprisingly, this model was in clear resemblance to the *lac* operon, the more prominent genetic regulation model at the time (Jacob and Monod 1961).

The Douglas and Hawthorne model of *GAL* gene switch was first reinforced by discovery of super-repressor mutations at the transcriptional inhibitor region, “i” (Douglas and Hawthorne 1972). However, later investigations of the system revealed that the positive regulator, Gal4, was constitutively expressed in the cells (Matsumoto et al. 1978, Perlman and Hopper 1979), refuting Douglas and Hawthorne model. Today, we know that the i locus was indeed coding for a negative regulator, Gal80; however, it did not bind to the DNA directly, and the C locus was mapped within the Gal4 coding region (Matsumoto et al. 1980), mutation of which caused a defect in Gal4-Gal80 interaction to express *GAL* genes constitutively.

The galactose sensor, Gal3, was not a part of the early *GAL* gene switch models, even though mutations in that locus were long known to cause a profound delay in galactose response (Winge and Roberts 1948). Gal3 was first introduced into the model as an enzyme that converted galactose to an inducer molecule (Broach 1979); yet another resemblance to the *lac* operon. It later became clear that Gal3 did not need any enzymatic activity to turn on the system. Over-expression of the protein in absence of galactose was sufficient to trigger the *GAL* switch, indicating galactose rather modified Gal3 protein to carry out its function more efficiently (Bhat and Hopper 1992). Finally, it was shown that the protein-protein

The exact molecular mechanism that linked Gal3-Gal80 interaction and blocking of Gal80’s inhibitory function was at the center of much debate during the last two decades. It is intriguing, at least in my opinion, that this issue has not been resolved to this day. There are two major schools of thought on the matter: to dissociate or not to dissociate (as Shakespeare would say, if he worked on the GAL system). The first evidence in favor of non-dissociation model showed that a Gal80 molecule fused to a strong activation domain from the VP16 transcription activator did not dissociate from a mutant Gal4 protein bound to a reporter gene promoter in response to galactose-activated Gal3 (Leuther and Johnston 1992). In-vitro visualization of a Gal3 mutant, which does not require galactose-activation, in complex with Gal80 and Gal4 in a gel mobility assay also supported the non-dissociation model (Platt and Reece 1998). More recently, fluorescence resonance energy transfer (FRET) and microscopic co-localization experiments (Bhaumik et al. 2004, Abramczycyk et al. 2012) yielded observations in support of the same notion. This model featured Gal3 forming a ternary structure with the Gal80-Gal4 complex at the promoter to cause a conformational change or re-localization of Gal80 on Gal4. Consequently, Gal4 activation domain would become available to the transcription machinery without Gal80 leaving Gal4.

In the mean time, other data were emerging that contradicted the non-dissociation model. First, Sil et al. showed a reduced interaction between Gal4 and Gal80 in presence of Gal3, galactose and ATP, using immunoprecipitation assays.
The same study also provided in-vivo two-hybrid experiments in favor of the dissociation model (Sil et al. 1999). Later, chromatin immunoprecipitation experiments demonstrated reduced association of Gal80 molecules with the GAL promoters in galactose, suggesting Gal3-Gal80 interaction caused destabilization of Gal80-Gal4 complex (Peng and Hopper 2000). Most recently, it has been shown that Gal80 molecules, which were concentrated at a genomic locus consisting of 8 chimeric GAL genes, dissipated upon galactose-induction in live cells (Jiang et al. 2009). All together, these observations supported a GAL gene switch that required dissociation of Gal80 from Gal4 in order to unmask the Gal4 activation domain.

A newly uncovered feature of GAL gene regulation that was not directly related to dissociation vs. non-dissociation controversy emerged in the last decade. The GAL1 gene locus was shown to associate with the nuclear periphery in the presence of galactose more frequently relatively to glucose (Casolari et al. 2004, Drubin et al. 2006, Brickner et al. 2007). At first, a transcriptional memory function was attributed to this relocalization of GAL genes to the nuclear pore complex (Brickner et al. 2007, Brickner 2010). Later, however, it was shown that the transcriptional memory was a function of Gal1 protein (Zacharioudakis et al. 2007). Moreover, it is recently shown that the GAL1 locus is also found at the nuclear periphery predominantly, if the cells were grown in non-repressing and non-inducing carbon sources such as raffinose or glycerol, instead of glucose (Green et al. 2012).
1.5 Focus of this work

The \textit{GAL} gene switch is one of the most extensively studied molecular mechanisms governing any biological process. Despite so much having been done and published about it, it still remains very heavily debated between different groups, as it can be inferred from the historical summary above. At the time I started working on this system at the J. Hopper lab, we were studying under a working model based on the previous works from our lab and others. Briefly, we envisioned a molecular system, in which Gal4 was bound to the promoter as a dimer in presence or absence of galactose. Gal3 had not been detected in the nucleus, so it was viewed as a strictly cytoplasmic protein. It was long proposed that Gal80 possessed two distinct nuclear localization signals, and was observed in both cytoplasm and nucleus; therefore, we believed Gal80 was a nucleo-cytoplasmic shuttling protein. In 2002, Peng and Hopper proposed that Gal3-Gal80 interaction occurred in the cytoplasm, rapidly sequestering Gal80 and leading to dissociation of Gal80-Gal4 complex at the promoters. Moreover, a linked equilibrium between Gal3-Gal80 and Gal80-Gal4 interactions was hypothesized in 2005 (Pilauri et al. 2005). This notion arose from genetic observations that lead us to believe the quaternary structure of Gal80 that interacts with Gal3 was different than that interacting with Gal4.

Needless to say, many features of our working model needed to be tested. My first project was on identifying a nuclear export sequence on the Gal80 structure. At the same time, Dr. Fenglei Jiang published her results supporting the dissociation model, and reported presence of Gal3 in the nucleus. After this work, it became
imperative to test the Gal80 shuttling and cytoplasmic sequestration hypotheses (Peng and Hopper 2002). This journey, which will be the central topic of the next chapter, took me to unforeseen places to uncover new insights about the \textit{GAL} gene switch.

During my six years in the J. Hopper lab, I have also encountered somewhat serendipitous observations, which I will be presenting in chapter 3. These studies indicated novel interactions between Gal80 and cellular membranes. Although I have not personally identified an in vivo significance of these interactions, recent publications showing a role of phosphoinositides in \textit{GAL} regulation (Han and Emr 2011) and a negative regulatory feedback loop at the nuclear periphery (Green et al. 2012) suggest a function for Gal80-membrane interaction. Finally, I will discuss my perspectives on the \textit{GAL} gene switch in chapter 5 in the light of all the data I will be presenting in this thesis, and will propose a new working model in conclusion.

Next, together with Christine Schaffer, we set to test the linked equilibrium hypothesis between Gal3-Gal80 and Gal80-Gal4 interactions (Pilauri et al. 2005). Our findings in this body of work helped us understand how the Gal3 and Gal4 competed for Gal80 binding, even though crystal structure studies indicated that Gal3 and Gal4 activation domain do not share binding surfaces on Gal80 (Lavy et al. 2012). I will present these data in chapter 4.
CHAPTER 2

THE RAPID GAL GENE SWITCH OF S. CEREVISIAE DEPENDS ON NUCLEAR GAL3, NOT NUCLEO-CYTOPLASMIC TRAFFICKING OF GAL3 AND GAL80

Egriboz, Onur; Jiang, Fenglei; Hopper, James E.

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2.1 ABSTRACT

The yeast transcriptional activator Gal4 localizes to UASgal sites even in the absence of galactose but cannot activate transcription due to an association with the Gal80 protein. By four minutes after galactose addition Gal4 activated gene transcription ensues. It is well established that this rapid induction arises through a galactose-triggered association between the Gal80 and Gal3 proteins that decreases the association of Gal80 and Gal4. Mechanistically how this happens remains unclear. Strikingly different hypotheses prevail concerning the possible roles of nucleocytoplasmic distribution and trafficking of Gal3 and Gal80 and where in the cell the initial Gal3-Gal80 association occurs. Here we tested two conflicting hypotheses by evaluating the sub-cellular distribution and dynamics of Gal3 and Gal80 with reference to induction kinetics. We determined that the rates of nucleocytoplasmic trafficking for both Gal80 and Gal3 are slow relative to the rate of induction. We find that depletion of the nuclear pool of Gal3 slows the induction kinetics. Thus, nuclear Gal3 is critical for rapid induction. FRAP experiments provided data suggesting that the Gal80-Gal4 complex exhibits kinetic stability in the absence of galactose. Finally, we detect Gal3 at the UASgal only if Gal80 is covalently linked to the DNA binding domain. Taken altogether these new findings lead us to propose that a transient interaction of Gal3 with Gal4-associated Gal80 could explain the rapid response of this system. This notion could also explain earlier observations.
2.2 INTRODUCTION

A hallmark of the GAL gene switch is its rapid induction response. A de novo increase in GAL1 and GAL10 mRNAs is observed within 4-6 minutes of galactose addition to cells cultured at 30°C (Yarger et al. 1984). This rapid induction of transcription stems in part from the fact that a round of protein synthesis subsequent to galactose addition is not required for target gene activation (Perlman and Hopper 1979). The rapid response correlates well with the documented rapid recruitment of RNA Pol II to the GAL1 promoter that begins by about approximately 2-4 minutes after galactose addition (Bryant and Ptashne, 2003). Because Gal4-mediated recruitment of SAGA and Mediator complexes precede recruitment Pol II (Bryant and Ptashne, 2003), it is apparent that galactose-activated Gal3 begins to relieve Gal80 inhibition of Gal4AD by 1-3 minutes following galactose addition. Accordingly, the mechanisms by which the galactose-activated Gal3 brings about Gal80 dissociation from Gal4 must be fast-acting.

The inherent mechanisms and molecular species involved in the process by which Gal3-Gal80 interaction overcomes Gal80 inhibition of Gal4 remain unclear and subject to conflicting hypotheses. In this work we addressed two unresolved questions: Where in the cell does Gal3 initially bind to Gal80 to trigger induction, the cytoplasm, the nucleus, or both? And, does nucleocytoplasmic trafficking of Gal3 and/or Gal80 play a role in the switch? There are two published hypotheses
concerning the first question. One suggests that Gal3 interacts with Gal80 exclusively in the cytoplasm and sequesters it away from nuclear Gal4 (Peng and Hopper 2002). A strikingly different hypothesis specifies that cytoplasmic Gal3 binds galactose and then moves into the nucleus to bind to Gal80 (Wightman et al. 2008). These two hypotheses specify not just different sub-cellular compartments for where the initial interaction of Gal3 and Gal80 occurs, but also different nucleocytoplasmic trafficking dynamics for these proteins.

Deciphering the mechanisms that overcome Gal80’s inhibition of Gal4 in live cells requires imaging the Gal3, Gal80 and Gal4 molecules, their associations, and their sub-cellular distributions and dynamics prior to and during the first few minutes of galactose addition. However, imaging these molecules expressed from their native promoters under such conditions has been hampered, as their levels in uninduced cells are very low (Wightman et al. 2008). Here we have employed spinning disc confocal microscopy (SDCM) to alleviate this problem and facilitate critical tests of competing hypotheses specifying GAL gene switch mechanisms. SDCM requires less excitation light to achieve an equivalent quality of signal compared to conventional confocal microscopy, and thus provides a high quality of spatial and temporal resolution with relatively low photobleaching. These features make SDCM optimally suited for FRAP (fluorescence recovery after photobleaching) analyses and tracking of small foci in live cells (Graf et al. 2005).

Using SDCM-based FRAP we tested nucleocytoplasmic exchange rates of Gal80 and Gal3 molecules, and dynamics of protein-protein interactions at the uninduced GAL1 promoter. Our FRAP experiments showed that neither Gal80 nor
Gal3 rapidly shuttle between nucleus and cytoplasm. These results challenge central mechanistic elements of two current hypotheses concerning the GAL gene switch, one of which stems from this lab. Moreover, we discovered that a marked reduction of Gal3 levels in the nucleus diminishes the rate of induction from GAL1 promoters. These results strongly suggest that the nuclear concentration of Gal3 is a critical feature of the GAL gene switch regardless of whether Gal3 interacts with Gal4-associated Gal80 or free Gal80. We also show that in absence of the galactose-induced Gal3-Gal80 interaction the Gal80-Gal4 complex at the promoter-associated UASGAL is kinetically stable relative to the induction rate. The results are discussed in light of the older models of the GAL gene switch and lead us to propose a new model that highlights the importance of nuclear Gal3.

2.3 MATERIALS AND METHODS

2.3.1 Strains:

Genotypes of all the strains used in this study are presented in table 1. MATα and MATα strains used in this study were derived from S. cerevisiae ScTEB652 (MATα ade1 ile leu2-3,112 ura3-52 trp1-HIII his3-Δ1 MEL1) and FY630 (MATα his4-917Δ lys2-173R2 leu2Δ1 ura3-52 trp1Δ63), respectively (Blank et al. 1997, Arndt et al. 1995). Details of strain construction will be provided upon request. The array strains were constructed by consecutive insertion of pFJ58x64 and pFJ57x8 (Jiang et al. 2009) into Sc652 (MATα strains) and FY650 (MATα strains). The GAL3, GAL80 and GAL4 genes were tagged in the genome with the green fluorescent protein
(GFP) ORF using insertion cassettes obtained from pFJ120N, pFJ109N and pFJ110N, respectively.

Table 1. Strains used in chapter 2.

<table>
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<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
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<tbody>
<tr>
<td>RLY2800</td>
<td>MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Fus3-GFP</td>
<td>Slaughter et al. 2007</td>
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<td>MS739</td>
<td>MATα ura3-52 leu2-3,112 ade2-101 kar1-1</td>
<td>Vallen et al. 1992</td>
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<td>Sc723</td>
<td>MATα ade1 ile2-3,112 ura3-52 trp1-HIII his3-1 P_{GAL1}-HIS3</td>
<td>Blank et al. 1997</td>
</tr>
<tr>
<td>Sc724</td>
<td>MATα ade1 ile2-3,112 ura3-52 trp1-HIII his3-1 P_{GAL1}-HIS3 gal3Δ</td>
<td>Blank et al. 1997</td>
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<td>Sc859</td>
<td>MATα ade1 ile2-3,112 ura3-52 trp1-HIII his3-1 GAL3-2GFP::NAT</td>
<td>Jiang et al. 2009</td>
</tr>
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<td>Sc862</td>
<td>MATα ade1 ile2-3,112 ura3-52 trp1-HIII his3-1 GAL80-2GFP::NAT</td>
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<td>Sc920</td>
<td>MATα ade1 ile2-3,112 ura3-52 trp1-HIII his3-1 LacOx64::LEU2 (P_{GAL1}-GST)x8::Kan' GAL80-2GFP::NAT</td>
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<td>Sc930</td>
<td>MATα ade1 ile2-3,112 ura3-52 trp1-HIII his3-1 LacOx64::LEU2 (P_{GAL1}-GST)x8::Kan' GAL4-2GFP::CaURA3</td>
<td>This study</td>
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<td>Sc932</td>
<td>MATα ade1 ile2-3,112 ura3-52 trp1-HIII his3-1 LacOx64::LEU2 (P_{GAL1}-GST)x8::Kan' GAL80-GFP::NAT gal3Δ:CaURA3 Gal80-2GFP::NAT</td>
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<td>Sc963</td>
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<td>Sc965</td>
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<td>Ds300</td>
<td>MATα/α ADE1/ade1 ile/ILE his3-Δ1/HIS3 HIS4/His4-917Δ LYS2/lys2-173R2 leu2-3,112/ leu2Δ1, ura3-52/ura3-52 trp1-HIII/trp1Δ63 LacOx64::LEU2 (P_{GAL1}-GST)x8::Kan' / LacOx64::LEU2 (P_{GAL1}-GST)x8::Kan' Gal80-2GFP::NAT/Gal80-2GFP::NAT</td>
<td>This study</td>
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<tr>
<td>Ds301</td>
<td>MATα/α ADE1/ade1 ile/ILE his3-Δ1/HIS3 HIS4/His4-917Δ LYS2/lys2-173R2 leu2-3,112/ leu2Δ1, ura3-52/ura3-52 trp1-HIII/trp1Δ63 LacOx64::LEU2 (P_{GAL1}-GST)x8::Kan' / LacOx64::LEU2 (P_{GAL1}-GST)x8::Kan' Gal4-2GFP::NAT/Gal4-2GFP::NAT</td>
<td>This study</td>
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</table>
2.3.2 Plasmids:

The plasmids used in this study are listed in Table 2. The NAT selection marker in plasmids that were used as a source of genomic insertion cassettes was isolated from Toda10 (a gift from Dr. Jian-Qiu Wu). pFJ04 was constructed by insertion of Smal/XhoI fragments of pGP15 (Peng and Hopper 2000) and pSA80N1 (Alam and Hopper, unpublished) into pRS416 (NE Biolabs). pFJ109N was constructed by simultaneous insertion of the NAT marker and a 2GFP cassette obtained from pKT209 (Sheff and Thron, 2004) into pFJ04 by PCR-based and other molecular methods. pFJ110N was constructed by insertion of the NAT marker and the 2GFP cassette into the GAL4 containing plasmid, pLM34 (Mylin and Hopper, unpublished). pFJ120N was constructed by insertion of the NAT cassette into pGP17 (Peng and Hopper 2000). Plasmids pOE180 (NES-Gal3) and pOE182 (mNES-Gal3) were constructed as follows. To introduce Rna1 NES and mutant NES (mNES; L326, I328) into Gal3, the region of RNA1 encoding residues 316-357 was isolated from pGEM-T Rna1(316-357) and pGEM-T Rna1(316-357AA) (Feng et al. 1999) by PCR with primers OE167 (5’-CTCGTCGACGTCGAAAAGGGAAATTTACCTGA-3’) and OE168 (5’-CTCGTCGACGTCTCTTCAAAAATCGTCAACCT-3’). The resulting PCR products were inserted into the AatII site of pAKS15 to yield pOE180 (NES-Ga3) and pOE182. A single GFP ORF was inserted at the 3’end of Gal3 in pOE180 and pOE182 to make pOE183 and pOE185, respectively. The P_{GAL1}-2GFP reporter plasmid, pOE33, was constructed by PCR-based and other molecular methods in pRS416 (NE biolabs). Construction of pFJ35 was described previously (Jiang et al. 2009). All PCRs for plasmid construction purposes were carried out with high-fidelity Pfu DNA
polymerase (Stratagene). The oligonucleotides were purchased from Integrated DNA Technologies, Inc.

Table 2. Plasmids used in chapter 2.

<table>
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<th>Plasmid</th>
<th>Composition</th>
<th>Reference</th>
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<td>pAKS15</td>
<td>CEN ARS1 TRP1 P&lt;sub&gt;GAL3&lt;/sub&gt;-GAL3</td>
<td>Sil et al. 1999</td>
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<td>pOE33</td>
<td>CEN ARS1 URA3 P&lt;sub&gt;GAL1&lt;/sub&gt;-2GFP</td>
<td>This study</td>
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<td>pOE180</td>
<td>CEN ARS1 TRP1 P&lt;sub&gt;GAL3&lt;/sub&gt;-NES-GAL3</td>
<td>This study</td>
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<td>pOE182</td>
<td>CEN ARS1 TRP1 P&lt;sub&gt;GAL3&lt;/sub&gt;-mNES-GAL3</td>
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<td>pOE185</td>
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<td>pGP15Δ</td>
<td>CEN ARS1 URA3 P&lt;sub&gt;GAL80&lt;/sub&gt;-GAL80</td>
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<td>pGP17</td>
<td>CEN ARS1 TRP1 P&lt;sub&gt;GAL3&lt;/sub&gt;-GAL3-GFP</td>
<td>Peng and Hopper 2000</td>
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<td>pGP56GFP</td>
<td>CEN ARS1 TRP1 P&lt;sub&gt;GAL3&lt;/sub&gt;-myr-GAL3-GFP</td>
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<td>PGP57GFP</td>
<td>CEN ARS1 TRP1 P&lt;sub&gt;GAL3&lt;/sub&gt;-mom-GAL3-GFP</td>
<td>Peng and Hopper 2002</td>
</tr>
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<td>pFJ35</td>
<td>CEN ARS1 TRP1 P&lt;sub&gt;ADH2&lt;/sub&gt;-Htb2-mCherry</td>
<td>Jiang et al. 2009</td>
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<td>pFJ109N</td>
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<td>pFJ110N</td>
<td>CEN ARS1 TRP1 P&lt;sub&gt;GAL4&lt;/sub&gt;-GAL4-2GFP::NAT</td>
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<td>pPS1739</td>
<td>CEN AS1 URA3 P&lt;sub&gt;HOG1&lt;/sub&gt;-Hog1-GFP</td>
<td>Ferrigno et al. 1998</td>
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</table>
2.3.3 Microscopy:

All microscopy experiments was performed with a Nikon TE-2000U spinning disc confocal microscope that was equipped with a 100x /1.4 NA objective lens (Nikon, Melville, NY), 488-, 514-, and 568-nm argon ion lasers, and a charge-coupled device camera (ORCA-AG, Hamamatsu, Bridgewater, NJ). Live yeast cells were concentrated to appropriate cell density by centrifugation and immobilized on Superfrost slides (Fisher Scientific) coated with 2% gelatin mix. The experiments in which we monitored the effect of different media on a single cell were carried out using Y04C Cellasik Microfluidics plates (Cellasik Inc., CA) mounted onto the microscope stage.

The FRAP experiments testing the nucleocytoplasmic mobility of Gal3-GFP and Gal80-2GFP molecules were carried out with Sc859 and Sc862 cells, respectively. The cells were grown to mid-log phase in glycerol/lactic acid media. To produce sufficient levels of Gal80-2GFP signal, the Sc862 cells were induced with 2% galactose for 4 hours, and then shifted back to glycerol/lactic acid media for 4 hours in order to establish non-expressing conditions for GAL genes. It was previously demonstrated that Gal4-Gal80 interaction is reconstituted 2 hours after the cells were shifted from galactose to non-inducing conditions (Jiang et al. 2009). The nuclear or cytoplasmic regions of these cells were then bleached for 100 ms at 20% laser power to ablate the GFP signal in the target area. The cells were then monitored for signal recovery for indicated time periods. All fluorescence
quantifications of the acquired images were carried out with the software ImageJ (Wayne Rasband, NIH, USA).

The photobleaching experiments testing stability of Gal80:Gal4 and Gal4:DNA interactions were carried out with diploid cells (Ds300 and Ds301; table 1). A small sub-nuclear region overlapping one of the GFP spots and excluding the other spot was briefly bleached at 30% laser power for 100ms.

2.3.4 GFP reporter assay:

Sc724 cells expressing 2GFP from the native GAL1 promoter \( (P_{\text{GAL1}}) \) on pOE33 and wild-type Gal3 or NES-Gal3 or mNES-Gal3 from pAKS15, pOE180 or pOE182, respectively, were grown in 25 ml of glycerol/lactic acid media. Galactose was added (2% final) to induce synthesis of reporter GFP molecules. At indicated time points after induction, 3 ml of cells at OD\(_{600}\) = 0.5 were taken from the culture and cell pellets were collected. Protein extracts were prepared using the sodium hydroxide method, as described elsewhere (Kushnirov et al. 2000). Proteins were separated with sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis, and GFP was detected by Western blotting using anti-GFP (Invitrogen).
2.4 RESULTS

Two previous studies failed to detect Gal3 in the nucleus of uninduced cells (Peng and Hopper 2000, Wightman et al. 2008). The apparent lack of Gal3 in the nucleus of uninduced cells in conjunction with other data led to two strikingly different hypotheses concerning how Gal3 works. One hypothesis posits that Gal3 works primarily, if not exclusively, in the cytoplasm to bind to Gal80, which at the time was thought to shuttle between cytoplasm and nucleus (Peng and Hopper 2002). Accordingly, it was proposed that cytoplasmic Gal3-Gal80 interaction would sequester Gal80 in the cytoplasm to free Gal4 from Gal80’s inhibitory effect. In another study Gal3 could not be detected anywhere in the uninduced cells (Wightman et al. 2008). Those authors hypothesized that galactose-bound Gal3 protein moves from the cytoplasm into the nucleus (Wightman et al. 2008). These hypotheses are called into question by recent observations. First, making Gal3 predominantly nuclear by fusing it to a strong NLS does not alter the kinetics of induction (Jiang et al. 2009). Second, Gal3 expressed from its native gene is detectable within both the nucleus and cytoplasm of live yeast cells grown in the absence of galactose (uninduced cells) under similar conditions as in the previous works mentioned above (Jiang et al. 2009). Considering these new observations, we used SDCM to evaluate the sub-cellular dynamics of Gal3 and Gal80 and determine whether nuclear Gal3 plays a role in induction.
2.4.1 The cytoplasmic:nuclear ratio of Gal3 does not change in response to galactose:

In a recent study Gal3 was not detectable in uninduced cells but was readily detected in both cytoplasm and nucleus by 45 minutes following galactose addition (Wightman et al. 2008). Accordingly, those authors proposed that upon binding galactose, Gal3 translocates from the cytoplasm to the nucleus where it binds to Gal80. Here we used SDCM and a highly sensitive camera capable of detecting uninduced levels of wild type Gal3-GFP in living cells to evaluate whether Gal3 moves from cytoplasm to nucleus in the absence or presence of galactose. In one series of experiments we imaged Gal3-GFP nucleocytoplasmic distribution in cycloheximide treated Sc859 cells before and after galactose induction. Htb2-mCherry expressed from pFJ35 served as a nuclear marker. In glycerol/lactic acid media the Gal3-GFP was uniformly distributed between the two compartments, and this did not noticeably change throughout 45 minutes following galactose addition (Figure 1A). In contrast, our imaging set-up readily detected rapid sub-cellular redistribution of Hog1-GFP, which rapidly concentrated in the nucleus within 10 minutes after NaCl was added to the media (final; 500 mM) (Figure 1B) as was previously demonstrated (Ferrigno et al. 1998).
Figure 1. Gal3 distribution does not change in the course of early induction:

(A) Sc859 (Gal3-2GFP) cells expressing Htb2-mCherry from pFJ35 were grown to mid-log phase in gly/lac media, and were placed into the Cellasik Onyx microfluidics system. Prior to imaging, cells were treated with cycloheximide for 15 minutes to prevent galactose-induced increases in Gal3-GFP. Images were acquired before and 45 minutes after galactose induction. (B) Sc723 cells expressing Hog1-GFP from pPS1739 and Htb2-mCherry from pFJ35 were grown to mid-log phase and were placed into the Cellasik Onyx microfluidics system. Images were acquired before and 10 minutes after osmotic stress with 500 mM NaCl.
Figure 1:

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Gly/Lac

Galactose

B

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- NaCl

+ NaCl
In another series of experiments we utilized FRAP to evaluate whether Gal3 rapidly enters the nucleus. The nucleus of Sc859 cells as marked by Htb2-mCherry was photobleached briefly to eliminate the nuclear Gal3-GFP signal. Limited recovery of the GFP fluorescence in the nucleus was detectable beginning only after 450 seconds, and the compartments did not fully re-equlibrate by 10 minutes (Figure 2A). This result was obtained for both cells in glycerol-lactic acid and galactose media. As a positive control, we performed FRAP with cells expressing Fus3-GFP, which is a known nucleocytoplasmic shuttling protein (Blackwell et al. 2003). The GFP signal in the bleached nuclei of these cells exhibited apparently full re-equilibration within 15 seconds (Figure 2C). These results provide direct evidence that Gal3 does not exhibit rapid nuclear import and challenge the hypothesis that Gal3 moves from the cytoplasm into the nucleus in response to galactose.
Figure 2. *Gal3 does not move into the nucleus rapidly.* 

**(A,B)** Yeast strain Sc859 nuclei defined by the nuclear marker Htb2-mCherry from pFJ35, were bleached briefly. The recovery of GFP signal from Gal3-GFP was monitored up to 10 minutes, at which point only 19% re-equilibration was observed. 

**(C,D)** RLY2800 (Fus3-GFP) cells were grown to mid-log phase in gly/lac media. Distribution of Fus3-GFP was imaged, and the indicated nucleus was bleached. Nuclear:cytoplasmic ratio of the molecules completely re-equilibrated in less than a 20 seconds after bleaching.
Figure 2:

A

![Images of cellular processes](image)

B

![Graph showing nuclear/cytoplasmic ratio over time](image)

C

![Images of cellular processes](image)

D

![Graph showing nuclear/cytoplasmic ratio over time](image)
2.4.2 Diminishing the nuclear pool of Gal3 impairs the kinetics of induction:

In earlier work from this lab we were unable to detect Gal3 in the nucleus (Peng and Hopper 2000). Subsequently, it was found that fusing Gal3 to peptides for targeting to outer mitochondrial membranes (mom-Gal3) or vesicular membranes (myr-Gal3) outside the nucleus did not diminish maximal levels of induction (Peng and Hopper 2002). Taken together, those results led to the hypothesis that Gal3 acts in the cytoplasm and need not enter the nucleus to promote Gal4 activation (Peng and Hopper 2002). That hypothesis was also called into question by the recent detection of Gal3 in the nucleus of uninduced cells (Jiang et al. 2009). However, the possible relevance of the nuclear Gal3 to the regulatory mechanism has not been addressed to date.

Here, we directly addressed whether depletion of the nuclear Gal3 affects induction. For this test we fused Gal3-GFP to the strong nuclear export sequence (NES) consisting of residues 316-357 of Rna1 (Feng et al. 1999). The NES-Gal3-GFP was observed only in the cytoplasmic region (Figure 3A). This striking exclusion from the nucleus was, as expected, attenuated in control experiments with the L326A, L328A double mutant version of the NES (mNES) (figure 3B) previously shown to impair NES activity (Feng et al. 1999). We then assessed the galactose-induction activity of NES-Gal3 compared to wild type Gal3 and mNES-Gal3 using a galactose-inducible $P_{\text{GAL1}}$-2GFP reporter. Cells with NES-Gal3 showed impaired induction kinetics when compared to cells with wild type Gal3. Moreover, the effect of the NES tag was alleviated when the leucines residues were replaced by alanines
(Figure 4). These results indicate that a critical level of nuclear Gal3 is required for the normally very rapid GAL gene switch response to galactose.

The finding that some critical level of Gal3 within the nucleus is required for rapid induction challenges the previous hypothesis that Gal3 need not enter the nucleus to support induction (Peng and Hopper 2002). That hypothesis stemmed in part from results of studies using myr-and mom-tagged versions of Gal3 that highly localized Gal3 to vesicular membranes and mitochondrial outer membrane, respectively (Peng and Hopper 2002). Such tagged versions of Gal3 were shown to be similar to wild type Gal3 in supporting maximal levels of induction as determined at 6 hours following galactose addition.
Figure 3. Effect of different tags on Gal3 distribution. Sc724 (gal3Δ) cells expressing (A) NES-Gal3-GFP from pOE183 or (B) mNES-Gal3-GFP from pOE185 or (C) myr-Gal3-GFP from pGP56GFP or (D) mom-Gal3-GFP from pGP57GFP. All cells were grown to mid-log phase in gly/lac media. (E) The ratio of the nuclear:cytoplasmic signal exhibited by the indicated molecule tagged with GFP was determined by measuring signal density. 10 cells were evaluated for each molecular species, and the results averaged. The cells were induced for 4 hours in 2% galactose to enhance the GFP signal, and were shifted back to gly/lac for 4 hours, at which time image acquisition began. The nuclear regions were marked by Htb2-mCherry that was expressed from pFJ35. (F) Total cellular levels of the various Gal3 molecules in the whole cell extracts are demonstrated by the western blot.
Figure 3.:
Figure 4. Effect of depleting nuclear Gal3 on induction kinetics. Sc724 (gal3Δ) cells were transformed with pOE33 (Pgal1-2GFP), and either pAKS15 (Gal3WT), pOE180 (NES-Gal3), or pOE182 (mNES-Gal3). Cultures were grown to mid-log phase in glycerol/lactic acid media and induced for indicated times. The GFP produced at each time point was determined by a western blot. A non-specific band from the same blot served as a loading control.
Figure 4:
Although the myr-Gal3-GFP and mom-Gal3-GFP signals were seen in those previous experiments to be highly localized to the appropriate membranes outside the nucleus (Peng and Hopper 2002), the imaging sensitivity at the time did not permit detection of residual nuclear pools of these molecules. With our current greatly improved imaging capabilities afforded by SDCM and a more sensitive camera, we sought to determine the effectiveness of the myr, mom, NES and mNES tags in depleting the nuclear pool of Gal3. We found that all of these tagged forms of Gal3-GFP when compared to Gal3-GFP show reductions of the nuclear Gal3-GFP pool (figure 3). To better assess the relative effectiveness of the various tags in reducing nuclear levels of Gal3 we developed a novel sensitive assay for nuclear Gal3-GFP. This assay utilizes yeast containing a previously developed chromosomal UAS\textsubscript{GAL} array (Jiang et al. 2009) consisting of 32 Gal4 UAS\textsubscript{GAL} binding sites associated with 8 tandem GAL1 promoter-GST genes. The Gal3-GFP expressed in such cells formed a fluorescent spot at the site of the chromosomal UASgal array within the nucleus only in galactose-induced cells that also expressed Gal80 fused to the Gal4 DNA binding domain (DBD-Gal80) (figure 5A,B). When mNES-Gal3-GFP, myr-Gal3-GFP and mom-Gal3-GFP were expressed, each of these formed spots at the array (Figure 5C, 5D, 5F). In contrast, NES-Gal3-GFP did not (figure 5E). These results indicate that the myr- mom- and mNES tags are not as effective as NES in depleting the nuclear pool of Gal3, and thus provide a plausible explanation for the erroneous earlier proposal (Peng and Hopper 2002) that normal induction does not require Gal3 in the nucleus.
**Figure 5. Effect of mis-localization tags on the nuclear Gal3 pool.** In Sc932 (gal3Δ, [PGAL1-GSTx8]:[LacOx64]:LEU2) cells, (A) Gal80 or (B-F) DBD-Gal80 was expressed from pOE142 or pOE165, respectively. The indicated Gal3 species were expressed from (A,B) pGP17, (C) pGP56GFP, (D) pGP65, (E) pOE183, or (F) pOE185. (G) The schematic presentation of the array system. The cells were grown in gly/lac to mid-log phase, and placed into chambers of Cellasik Microfluidics plates. Synthetic drop-out media with 3% glycerol and 2% lactic acid as carbon source was fed through the chambers for 20 min. Images of cells were obtained before and at 1 hour after galactose addition. The same cells were monitored for Gal3-GFP spots, which co-localized with Lacl-mCherry expressed from pME9.
Figure 5:

A

Gal80
Gal3-GFP
LacI-mCherry

B

DBD-Gal80
Gal3-GFP
LacI-mCherry

C

DBD-Gal80
myr-Gal3-GFP
LacI-mCherry

(Continued)
Figure 5... continued

D

DBD-Gal80
mom-Gal3-GFP
LacI-mCherry

E

DBD-Gal80
NES-Gal3-GFP
LacI-mCherry

F

DBD-Gal80
mNES-Gal3-GFP
LacI-mCherry

(Continued)
Figure 5... continued
2.4.5 The exchange between nuclear and cytoplasmic pools of Gal80 is slow:

Earlier studies showed that either one of two distinct regions of Gal80, amino acids 1-321 and amino acids 341-423, were able to localize β-galactosidase to the yeast nucleus in long-term grown cultures (Nogi and Fukasawa, 1989). Those results suggested that Gal80 has two independent nuclear localization sequences (NLSs). More recent studies showed movement of NLS\textsuperscript{SV40}−Gal80-GFP molecules from one nucleus to the other within the binucleate heterokaryon zygote formed in a kar1-1 cross, suggesting the presence of an NES (Peng and Hopper 2000). These previous data led us to hypothesize that Gal80 shuttles between nucleus and cytoplasm. Thus, we surmised that galactose-activated Gal3, which was not detected in the nucleus at the time, sequestered Gal80 in the cytoplasm (Peng and Hopper 2002). The rapid shuttling of Gal80 molecules is one central feature of such a model, however, no evaluation of the kinetics of Gal80 nucleocytoplasmic trafficking has been reported.

Here we used FRAP to directly assess the sub-cellular dynamics of Gal80 in Sc862 cell. We bleached Gal80-2GFP in the cytoplasm, and we observed only ~15% recovery by 10 minutes after bleaching (Figure 6B). When we bleached Gal80-2GFP in the nucleus we observed only ~8% recovery by 10 minutes after bleaching (Figure 6A). These results were in stark contrast to our FRAP results with Fus3-GFP, a known shuttling protein, that showed apparently full recovery by 15 seconds after nuclear bleaching (Figure 2C). We also used FRAP to test the kinetics of NLS-Gal80-GFP exchange between the two nuclei in a heterokaryon resulting from a kar1
cross performed identically to that in the work of Peng and Hopper 2000. In the present work, and as previously reported (Peng and Hopper 2000), the NLS-Gal80-GFP appeared within both nuclei of the heterokaryons, even though it was only expressed in one of the two mating haploids and its expression was terminated immediately prior to the kar1-1 cross (Figure 7). However, the FRAP analyses clearly showed that the NLS-Gal80-GFP in the unbleached nucleus did not re-equilibrate with the molecules within the bleached nucleus for up to 300 seconds post-bleach (Figure 7). Taken together, our FRAP results with the NLS-Gal80-GFP in heterokaryon zygotes and Gal80-2GFP in vegetative grown haploid cells reveal that Gal80 does not shuttle rapidly between the nucleus and cytoplasm.
Figure 6. Sub-cellular distribution and dynamics of Gal80. Sc862 (Gal80-2GFP) cells expressing Htb2-mCherry from pFJ35 were grown to mid-log phase in glycerol/lactic acid media. The cells were induced with galactose for 4-6 hours to enhance the GFP signal, and then shifted back to non-inducing media for 4 hours. The (A) nuclear or (B) cytoplasmic region was briefly bleached.
Figure 6:

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Figure 7. **NLS-Gal80-GFP does not shuttle rapidly in heterokaryons.** NLS-Gal80-GFP was expressed in wild-type MATα cells from a GAL1/CYC1 promoter. The expression was repressed with 2% glucose for 2h, after which the cells were mated with MATα kar1-1 cells. Binucleate heterokaryons appeared within 2 hours. The GFP signal was found in both nuclei of the heterokaryons. One of the two nuclei was bleached briefly, and the heterokaryon nuclei were monitored for 5 minutes.
Figure 7:
2.4.6 Assessment of the dynamics of the Gal80:Gal4:UAS\(_{\text{GAL}}\) and Gal4:UAS\(_{\text{GAL}}\) complexes:

Because Gal3 is in the nucleus prior to galactose addition (Figure 1, Jiang et al. 2009) and the nuclear pool of Gal3 is important for rapid induction kinetics (Figure 5), we wondered whether Gal3 might work by contacting Gal4-associated Gal80, as posited earlier in a non-dissociation model (Platt and Reece 1998). Although it is now clear that Gal80 does dissociate from Gal4, a galactose-triggered interaction of nuclear Gal3 with Gal4-associated Gal80 could be the event that triggers such dissociation. In such a case, rapid induction would be expected to be independent of the kinetic stability of the Gal80-Gal4 complex. If, on the other hand, Gal3 binds exclusively to free Gal80, we would expect that high kinetic stability (slow off-rate) for the Gal80:Gal4 complex at the UAS\(_{\text{GAL}}\) site would be rate-limiting for induction. Accordingly, we evaluated the kinetic stability of Gal80-Gal4 complexes at UAS\(_{\text{GAL}}\) sites using a novel genetic assay consisting of diploid cells that have two of the above-mentioned UAS\(_{\text{GAL}}\) chromosomal arrays (Jiang et al. 2009). Expression of Gal80-2GFP or Gal4-2GFP in these cells allowed us to visualize Gal4:Gal80 complexes as two separated nuclear spots, one for each array (Figure 8). We bleached one of the spots, and monitored the fluorescence recovery in the bleached array as well as loss of fluorescence in the unbleached array. The signal in the targeted spots of Gal4:Gal80-2GFP did not recover more than 10% within 10 minutes after bleaching. The lack of re-equilibration between the two spots within the same nucleus signifies very little dissociation of either the Gal4:Gal80-2GFP
complex from the DNA or the Gal80-2GFP from the Gal4:DNA complex within 10 minutes (Figure 8C). In contrast, our experiments with Gal4-2GFP:Gal80 showed that the Gal4-2GFP molecules re-equilibrated between the two arrays within 5 minutes after photobleaching when the cells were in galactose media (figure 8A), but not when they were in glycerol-lactic acid media (Figure 8B). These results indicate that the Gal4:Gal80 complex is kinetically stable.
Figure 8. **Dynamics of Gal4-DNA and Gal80-Gal4 interactions.** (A,B) Sc930 (MATa Gal4-2GFP [P\_GAL1-GSTx8]::[LacOx64]::LEU2) and (C) Sc920 (MATa Gal80-2GFP [P\_GAL1-GSTx8]::[LacOx64]::LEU2) strains were mated with Sc965 (MAT\(\alpha\) Gal4-2GFP [P\_GAL1-GSTx8]::[LacOx64]::LEU2) and Sc963 (MAT\(\alpha\) Gal80-2GFP [P\_GAL1-GSTx8]::[LacOx64]::LEU2), respectively. The resulting diploid cells containing two arrays were grown to mid-log phase in (A) galactose or (B,C) glycerol/lactic acid media. One of the GFP spots in the cells was briefly bleached, and the cells were monitored for 10 min. (A) The Gal4-2GFP signal in the bleached array of the cells grown with galactose recovered completely within 5 minutes, while no detectable re-equilibration was observed for (B) Gal4-2GFP and (C) Gal80-2GFP spots in cells grown in glycerol/lactic acid media.
Figure 8.

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2.5 DISCUSSION

It is well established and widely agreed that the GAL gene switch functions through a galactose triggered binding of Gal3 to Gal80 that relieves Gal80 inhibition of Gal4. Mechanistically just how this occurs is controversial (Platt and Reece 1998, Peng and Hopper 2002, Wightman et al. 2008, Jiang et al. 2009). Lack of agreement centers primarily on three issues: whether the molecular events that initiate induction involve the movement of Gal80 or Gal3 molecules between cytoplasm and nucleus; where in the cell the initial, induction-triggering, interaction between Gal3 and Gal80 occurs; and whether Gal3 binds to Gal4-associated Gal80, to exclusively free Gal80, or to both forms of Gal80. In order to address these issues we evaluated the kinetics of Gal80 and Gal3 movements between cytoplasm and nucleus and the dynamics of the Gal80:Gal4 complex.

2.5.1 Gal80-2GFP movement between sub-cellular compartments is relatively slow:

Our FRAP analyses involving Gal80-GFP in haploid cells and in heterokaryons revealed very slow kinetics for nucleocytoplasmic transport of Gal80-GFP molecules. This was in striking contrast to our results with Fus3-GFP, a protein known to exhibit rapid nucleocytoplasmic shuttling (Blackwell et al. 2003). These results, considered with respect to the rapid response of the GAL gene switch, challenge the hypothesis that nucleocytoplasmic shuttling of Gal80 is a central feature of the GAL gene switch mechanism (Peng and Hopper 2002). That hypothesis stemmed in large part from two studies. One previous study identified
two different fragments of Gal80, designated NLS I and NLS II, that independently localized the *E. coli* beta-galactosidase protein to the yeast nucleus (Nogi and Fukasawa 1989). However, in that study the sub-cellular localization was determined following several generations of growth. Consequently, those results could be explained by slow entry of the Gal80-β-galactosidase fusion protein into the nucleus. The other previous study determined that NLS<sub>SV40</sub>-Gal80-GFP moved from one nucleus (donor) to the other nucleus in a yeast heterokaryon, leading to the conclusion that Gal80 has an NES (Peng and Hopper 2000). In that work and in identical experiments carried out here the time lag between the formation of the heterokaryon and image acquisition of the NLS<sub>SV40</sub>-Gal80-GFP was 1 to 2 hours. Consequently the results of the heterokaryon experiments can be explained by slow movement of NLS<sub>SV40</sub>-Gal80-GFP from the one nucleus to the cytoplasm, followed by rapid entry into the other nucleus, due to the strong SV40 NLS tag on Gal80. Therefore, we conclude that Gal80 does not exhibit a rate of nucleocytoplasmic exchange typical of shuttling proteins.

### 2.5.2 Gal3-GFP does not move rapidly into the nucleus:

If Gal3 enters the nucleus upon binding galactose we expected to see evidence of such redistribution by a decrease in cytoplasmic fluorescence and a corresponding increase in nuclear fluorescence in cells expressing Gal3-GFP. Our results show that the sub-cellular distribution observed for preexisting Gal3 (in non-induced cells) does not change rapidly in response to galactose. This conclusion is corroborated by the results of our FRAP experiments in which nuclear
Gal3-GFP was bleached, and only a small fractional recovery was detected 10 minutes after bleaching. Thus, it is very unlikely that galactose causes entry of Gal3 into the nucleus to account for the rapid the Gal4 mediated gene activation (induction).

2.5.3 Nuclear-localized Gal3 is required for rapid induction:

The fact that Gal3 is in the nucleus prior to galactose addition and does not show rapid movement from cytoplasm to the nucleus in response to galactose prompted us to test the significance of nuclear Gal3. We find that strongly reducing the nuclear level of Gal3 by fusing it to an exogenous NES impairs the kinetics of induction. Thus, some level of Gal3 is required in the nucleus for the normal rapid induction kinetics. This conclusion is at odds with the conclusion from previous work that Gal3 entry to the nucleus is not required for maximal levels of induction (Peng and Hopper 2002). That previous work showed that the levels of MEL1 gene encoded alpha-galactosidase enzyme activity produced by cells at 6 hours after galactose addition was similar for cells expressing wild type Gal3, myr- or mom-tagged Gal3 variants (Peng and Hopper 2002). To explain the contradictory conclusions, we reasoned that the myr and mom tags might not be as effective as the NES tag in excluding Gal3 from the nucleus, and that the magnitude of induction might not be as sensitive as the kinetics of induction to reduced nuclear levels of Gal3. The effectiveness of misplacing Gal3 with myr and mom tags was determined using conventional wide-field imaging in the previous work (Peng and Hopper 2002). As well, in this study when we used confocal imaging of non-array cells to compare the myr-, mom-, and NES tags we found that they similarly depleted
nuclear levels of Gal3-GFP. However, when we used a more sensitive photon-concentrating assay employing cells with the 32 Gal4 binding site (UAS\textsubscript{GAL}) array and expressing DBD-Gal80, we detected a difference in the effects of the tags. The myr- and mom-Gal3-GFP but not NES-Gal3-GFP formed spots at the array, indicating that the myr- and mom-tags are not as effective as the NES tag in reducing the nuclear concentration of Gal3. Thus, we surmise that in the previous work small pools of myr- or mom-tagged Gal3 in the nucleus were not detectable but were sufficient to support the maximal levels of induction observed at 6 hours, leading to the view at the time that Gal3 need not act in the nucleus.

That even a small fraction of myr- or mom-tagged Gal3 in the nucleus is likely sufficient to support high levels of \textit{GAL} gene expression is suggested also by our comparison of NES and mNES versions of Gal3-GFP. We found that mNES, like NES diminished nuclear Gal3 in non-array cells (figure 3B), but mNES-Gal3 supported induction kinetics very similar to wild-type Gal3 (figure 5). Expectedly, our sensitive array system showed mNES-Gal3-GFP spots at the Gal4 binding sites occupied by DBD-Gal80 (figure 4F). Taken together, these results indicate that some minimal nuclear level of Gal3 is required to support the rapid induction kinetics that is characteristic of the GAL gene switch.

2.5.4 A stable Gal3:Gal80:Gal4 complex is not detectable in galactose-induced cells: Our finding that a reduction in the nuclear pool of Gal3 slows the induction kinetics together with our evidence against rapid movements of Gal80 and Gal3 between nucleus and cytoplasm implies that the nuclear pool of galactose-activated
Gal3 might be critical for initiating rapid relief of Gal80 inhibition. Accordingly, nuclear Gal3 might act through binding to only free Gal80 or to both free Gal80 and Gal80 that is in complex with Gal4. Good evidence exists for Gal3 binding to free Gal80 in vivo, as Gal3-YFP:Gal80-CFP complexes have been detected in both cytoplasm and nucleus by 25 minutes after galactose addition (Wightman et al. 2008). And, Gal3 is able to bind to Gal80 in vitro in the absence of Gal4 (Yano and Fukasawa 1997). On the other hand, direct evidence for in vivo association of Gal3 with Gal4-associated Gal80 is lacking. In the course of these investigations we attempted to detect such a complex. We first confirmed that we could detect Gal3-GFP as a fluorescent spot at the array in galactose-exposed cells expressing DBD-Gal80 (Figure 4B). However, when such cells expressed Gal80 instead of DBD-Gal80, a Gal3-GFP spot did not appear at the array in response to galactose (Figure 4A). This result is inconsistent with a previously proposed model specifying non-dissociation of Gal80 from Gal4 in response to galactose (Leuther and Johnston 1992, Platt and Reece 1998), but is consistent with the observed galactose- and Gal3-dependent dissociation of Gal80 from Gal4 in live cells (Jiang et al. 2009). While our results are negative, they are not inconsistent with a mechanism wherein Gal3 interacts with Gal4-associated Gal80 to cause immediate dissociation of Gal80 (presumably as a Gal3:Gal80 complex) from Gal4.
2.5.5 Gal80 complexed with Gal4 on DNA does not exchange rapidly with free Gal80:

We are lacking any direct evidence that would distinguish between transient Gal3-binding to Gal4-associated Gal80 and Gal3 binding to only free Gal80. However, if the GAL gene switch works by Gal3 binding to only free Gal80, we expect to observe rapid exchange between Gal4-associated Gal80 and free Gal80. Otherwise, a kinetically stable Gal80:Gal4 complex would be rate-limiting for induction. To our knowledge the rate of exchange, or dynamics, between Gal4-associated Gal80 and free Gal80 has not been determined. To evaluate the kinetic stability of the Gal80:Gal4 complex on the DNA, we carried out FRAP experiments with diploid array cells. We monitored re-equilibration between bleached and unbleached Gal80-2GFP spots. Re-equilibration is possible only if either the Gal4:Gal80-2GFP complex dissociates from a UAS_GAL or the Gal80-2GFP molecules dissociate from the Gal4:UASgal complex at the array. Our results show that a bleached Gal80-2GFP spot did not re-equilibrate substantially with the unbleached spot over 10 minutes (Figure 8C). This lack of evident re-equilibration challenges a mechanism wherein Gal3 binds to only free Gal80.

On the other hand, Gal4 molecules exchanged between the two arrays within 5 minutes, when the cells were in galactose, but not in glycerol-lactic acid media. According to a previously proposed Gal4 cycle model, it was suggested that Gal4 molecules became more dynamically associated with the DNA binding sites after galactose induction (Muratani et al. 2005). However, more recent other work counters this idea by showing that Gal4 became more stable on the GAL promoters
of induced cells (Ferdous et al. 2007). Our results from the FRAP experiments using diploid array strains are consistent with Gal4 becoming less stable on the DNA upon induction.

2.5.6 Transient association of Gal3 with Gal4-associated Gal80: a plausible mechanistic feature of the GAL gene switch:

In light of the very rapid induction in this system the relatively slow exchange of Gal80 between its Gal4-bound and free states observed here and the importance of nuclear Gal3 suggests a new plausible model. Transient interaction of nuclear Gal3 with Gal4-associated Gal80 might be what initiates the rapid galactose-mediated dissociation of Gal80 from Gal4. This notion is seemingly at odds with the previously reported detection of a Gal3:Gal80:Gal4 ternary complex by DNA-electrophoretic mobility shift analyses (EMSA) in polyacrylamide gels (Platt and Reece 1998). However, the form of Gal3 used in those EMSA experiments was not the wild type Gal3 protein but rather a misense mutant Gal3 protein identified as not requiring galactose to bind to Gal80 (Blank et al. 1997). Moreover, it has been shown that exchange rates for macromolecular complexes can be 100 fold slower in gels than in free solution (Fried and Crothers 1981, Fried and Bromberg 1997, Vossen and Fried 1997). Thus, off-rates can be substantially underestimated by EMSA (Yang et al. 2002). Accordingly, we suggest the possibility that the association of Gal3 with Gal80:Gal4:DNA complexes observed previously might have been artificially stabilized by the gel environment and possibly also by the use of the Gal3 mutant protein.
The possibility of transient interaction of Gal3 with Gal4-associated Gal80 as a mechanistic feature of the GAL gene switch has two important testable implications. First, Gal3 interaction with Gal80 must occur on a surface separate from that to which Gal4 binds. Although the binding surface of Gal80 for Gal4 has been determined partially (Kumar et al. 2008), its surface for binding to Gal3 has not. Second, Gal3 transient binding to Gal80 must alter some property of Gal80 that is essential to its high affinity binding with Gal4. One property of Gal80 that has been implicated in its association with Gal4 is its capacity to dimerize (Pilauri et al. 2005, Kumar et al. 2008), but no direct evidence exists for whether Gal3 interaction with Gal80 affects Gal80 self-association. These issues highlight the fact that much remains to be learned concerning the mechanisms involved in this seemingly simple gene switch.
CHAPTER 3

GAL80 INTERACTS WITH PHOSPHOLIPIDS VIA A SURFACE EXPOSED ALPHA-HELIX.
3.1 ABSTRACT

Based on earlier work from our lab (Peng and Hopper 2000, Peng and Hopper 2002; Pilauri et al. 2005) it was hypothesized that (1) Gal3 is confined to cytoplasm, (2) galactose-triggered Gal3-Gal80 interaction sequestered Gal80 in the cytoplasm, and (3) Gal80p rapidly shuttles between the nucleus and cytoplasm. Here, we are presenting the results of the experiments that were carried out during our search of Gal80 nuclear export sequence (NES). Microscopy of cells expressing Gal80 peptide-GFP fusion construct revealed that GFP fused to a solvent-exposed alpha helix motif of Gal80 (aa 353-386; named the HALO fragment) did not diffuse into the nucleoplasm. This outcome would be expected from a NES-containing domain. However, Fluorescence Recovery After Photobleaching (FRAP) experiments indicated that Gal80 HALO-2GFP does not transport between nucleus and cytoplasm rapidly. These observations (and others, see chapter 2) suggested that Gal80 does not have a strong NES. Further investigations of the HALO fragment showed that this Gal80 peptide mediates binding of reporter molecules to specific phospholipids. Moreover, the full-length Gal80 molecules also interacted with the same phospholipids as the HALO fragment. Additionally, we here discovered that Gal4AD can interact stably with the membrane bound Gal80, and that Gal3p-Gal80p interaction caused dissociation of Gal80 from membranes. This suggested that the interplay between the three GAL regulatory proteins might be localized to specific nuclear membrane domains enriched with the Gal80-targeted phospholipids. These results, together with other observations and considerations led us to speculate that
perhaps Gal80-membrane interactions play a role to negatively regulate GAL genes at these specific nuclear domains.

### 3.2 INTRODUCTION

Transcription initiation ensues only after multiple proteins and protein complexes act at the promoters to facilitate binding of RNA polymerase. The long established model pictures assembly of the transcription machinery wherever the promoters happen to be in the nucleus. A more recently emerging model based on high resolution fluorescence imaging of different chromosomal regions in vivo, however, turns this picture upside-down (Misteli 2007). These new data argue that the genes within the genome are not randomly arranged in the nucleus, and that the spatial organization of the genes correlates with their activity. This new model suggests the genes might be recruited to sites of active transcription or sites of strong repression rather than protein complexes being recruited to the genes (Tadei et al. 2004, Misteli 2007, Pascual-Garcia and Rodriguez-Navarro 2009, Tadei et al. 2010, Ahmed and Brickner 2010)

The nuclear periphery is directly implicated in correlation between the transcriptional activity and sub-nuclear compartmentalization of the genes. In mammalian cells, the heterochromatin is found at the nuclear periphery (Tadei et al. 2004). The regions of chromosomes with lesser gene abundance are also appears to be localized to the peripheral regions in the nucleus (Croft et al. 1999). In yeast,
similar observations for the correlation between transcription and nuclear periphery have been monitored. For example, the heterochromatin and the silenced telomeric regions are found at the periphery of the *S. cerevisiae* nuclei (Akhtar and Gasser 2007, Tadei et al. 2010). Altogether, all these observations attribute a transcriptional inhibitory role to the nuclear periphery and suggest active recruitment of the genes to this region as a mechanism of regulation.

On the other hand, studies on conditionally expressed genes in *S. cerevisiae* also indicate the nuclear periphery as a site of active transcription. So far, genes in carbon source metabolism, heat shock, and mating pheromone synthesis pathways have been reported to move to peripheral locations upon activation (Casolari et al. 2004, Brickner and Walter 2004, Tadei et al. 2006, Berger et al. 2008). For example, the GAL1-10-7 genes cluster on the chromosome II (Casolari et al. 2004, Brickner et al. 2007) and GAL2 gene on chromosome XII (Dieppois et al. 2006) move to the nuclear periphery from the nucleoplasm during a shift from glucose to galactose (Casolari et al. 2004, Brickner et al. 2007). Studies on mutants of the chromatin-remodeling SAGA complex, mRNA export machinery, and the nuclear pore complex (NPC) suggest that the GAL genes physically interact with and are recruited to the NPCs upon activation (Rodriguez-Navarro et al. 2004, Bhaumik et al. 2004, Cabal et al. 2006, Dieppois et al. 2006, Luthra et al. 2007). Moreover, artificial recruitment of reporter genes to the NPCs causes hyper-activation (Menon et al. 2005).

In addition to the studies showing activated GAL genes recruited to the NPCs at the periphery, a recent study shows that the uninduced GAL genes are also found at the periphery of cells grown in glycerol or raffinose (Green et al. 2012). Seemingly,
then, there might be two distinct domains at the nuclear envelope with respect to GAL gene activity. One region (most likely NPC occupied region) recruits active GAL genes, and another region (most likely lacking NPCs) recruits repressed GAL genes in absence of glucose. Hence, the latter implicates a negative regulatory mechanism at that specific domain of the nuclear periphery.

In the course of the experiments aimed at identifying the proposed Gal80 nuclear export sequence, we discovered a surface-exposed alpha helix, named the HALO fragment, which binds to the nuclear membrane. Here, we show that both the HALO fragment and full length Gal80 protein bind to specific phospholipids in vitro. Furthermore, we found that full-length Gal80 interacts with biological membranes derived from yeast extracts and remains membrane-associated when bound to Gal4AD. In contrast, our results showed that the amount of Gal80 that coprecipitates with the membrane fractions decreases in response to Gal3 and galactose. Based on these observations, we suggest that the interactions between phospholipids in the inner nuclear membrane, Gal80, Gal4 and GAL promoters might result in the previously reported recruitment of the uninduced GAL genes to the nuclear periphery (Green et al. 2012).
3.4 MATERIALS AND METHODS

3.4.1 Reporter molecule vectors.

The plasmids expressing the GFP-fused reporter molecules for the search of Gal80 functional domains were constructed in E. coli strain DH5α using standard methods. Previously published pLGin and pLGout plasmids (Butterfield-Gerson et al. 2006) were used to generate pOE131 (GFP) and pOE67 (NLS-2GFP). The Gal80 fragments to be tested were cloned into these two vectors using PCR and other molecular methods.

3.4.2 Microscopic imaging.

All microscopy experiments were carried out with a Nikon TE-2000U spinning disk confocal microscope that was equipped with a 100x /1.4 NA objective lens (Nikon, Melville, NY), 488-, 514-, and 568-nm argon ion lasers, and a charge-coupled device camera (ORCA-AG, Hamamatsu, Bridgewater, NJ). The wild-type Sc652 expressing the reporter molecule were placed on superfrost microscope slides, and were immobilized using 10 % gelatin solution.

3.4.3 Protein-lipid overlay assays.

The wild-type Sc652 yeast cells expressing HALO-GFP were grown at 30°C to an OD_{600} of approximately 0.8. Pelleted cells were disrupted by glass beads beating. Cell extracts were obtained by a 30 min centrifugation at 10 000 x at 4°C. The lipid
overlay assay was performed with commercially available reagent from Echelon Bioscience, according to manufacturers instructions. Briefly, the phospholipid spotted blots were blocked for 1 h in 2 ml of blocking buffer (3% fatty-acid-free BSA, 150 mM NaCl, 10 mM Tris pH 7.4). The blocked blots were then incubated for 1 h in the presence of cell extracts, washed and the GFP proteins bound to the phospholipids in each spot were immuno-detected with antiGFP antibodies (Invitrogen).

3.4.4 Yeast membrane extraction and membrane-protein binding assays.

The Sc745 cells (gal4Δ) were grown to mid-log phase at 30°C. The cells were pelleted, and whole cell extracts were obtained using standard cell lysis protocols with glass beads. The heavy cell debris was disposed after slow centrifugation at 3000 x g. The remaining supernatants containing small cellular membrane vesicle were centrifuged at 40 000 x g using polyallomer tubes suitable for ultracentrifugation at such high speeds. The pelleted membrane fractions were resuspended in sterile PBS solution, and were sonified briefly to break the pellet into small membranes. 50 µl of membrane containing buffer was mixed with 5 ng of proteins were mixed in binding reactions with 500 µl total volume. The molecules bound to the membranes were precipitated using an ultra-centrifuge at 40 000 x g. The pellets were resuspended in 50 ul SDS-PAGE loading buffer and the binding of GAL regulatory proteins were tested using standard western blot techniques.
3.3 RESULTS

3.3.1 The Gal80 fragment spanning amino acids 353-386 prevents diffusion of GFP into the nucleus.

To identify the hypothesized Gal80 NES (Peng and Hopper 2002), I screened twelve Gal80 fragments by expressing them in frame with GFP from GAL1 promoter on a plasmid (Figure 9A and 9B). The nuclear membrane is a semi-permeable barrier between the cytoplasm and nucleus. It restricts free diffusion of molecules bigger than approximately 50 kDa. A single 27 kDa GFP molecule (Prendergast and Mann 1978) is small enough to diffuse in and out of the nucleus freely (Figure 9C). As well, the Gal80 fragments fused to the GFP molecule were small enough that the fusion protein size would not exceed the permeability limit for the nuclear membrane. Only one out of twelve such fusions, the Gal80(353-386)–GFP was strictly cytoplasmic, consistent with it specifying a NES (Figure 9D).
Figure 9. *Gal80(353-386)-GFP does not localize to the nucleoplasm.* (A) The schematic representation of the microscopy assay for detection of NLS and/or NES containing fragments. The 27 kDa GFP molecule diffuses into the nucleus freely. The nuclear localization of GFP is not affected, when it is fused to a small fragment lacking a NLS or NES. If the fragment has a NLS function, the signal becomes exclusively nuclear. If the fragment has a NES function, the nucleus becomes void of GFP signal. (B) The schematic representation of the twelve fragments that were tested with the assay in (A). (C) Wild-type Sc652 cells bearing pOE131 and pOE79 were grown to mid-log phase, and GFP expression from pOE131 was induced with galactose for 2 hours. The cells were imaged with a confocal microscope. GFP molecules were evenly distributed between the nucleus, marked by Htb2-mCherry (pOE79), and cytoplasm. (D) The wild-type cells bearing pOE125 and pOE79 were grown to mid-log phase. *Gal80(353-386)-GFP* expression from pOE125 was induced with galactose for 2 hours, and the cells were imaged same as the cells in (B).
Figure 9:

A

B

Gal80

1  204  333  435

(continued)
Figure 9. continued...

C

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D

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3.3.2 The Gal80(353-386) fragment fused to an NLS exhibits dynamic localization to the nuclear periphery.

To test for NES function of the Gal80(353-386) fragment, we created a fusion of this Gal80 fragment to the strong NLS from SV40 large T antigen protein and 2 tandem copies of GFP, making a reporter of approximately 60 kDa. Interestingly, this molecule localized to the nuclear periphery, creating a halo-like effect. Hence, the name HALO fragment was assigned to this Gal80 fragment (Figure 10).

Typically, nucleoplasmic shuttling proteins are chaperoned across the NPC via their interactions with carrier proteins, called karyopherins. The reversible interactions between the NPC subunits and the karyopherins mediate the passage of the cargo through the channels within the NPC. In this case, it was possible that the HALO fragment was anchored to the membrane, rather than functioning as an NES. To find out whether the NLS-HALO-2GFP molecules were irreversibly bound to the NPC (i.e., “stuck” at that location), we carried out FRAP experiments were carried out to bleach a small segment of the NLS-HALO-GFP ring leaving a crescent shape after bleaching. The signal in the unbleached region re-distributed around the nuclear periphery under one second, indicating the molecules were rapidly moving and not “stuck” at the NPCs or at other parts of the nuclear envelope (Figure 10).

During these experiment with NLS-HALO-2GFP, some of the cells exhibited localization of NLS-HALO-2GFP unevenly around the nuclear periphery (Figure 11). Co-localization studies using Nup49-mCherry as a marker for the NPC localization indicated that these cells also had uneven distribution of NPCs at the nuclear envelope. Interestingly, the NPCs were clustered at regions that were not targeted
by NLS-HALO-2GFP, indicating there was a greater affinity of HALO fragment to the NPC-free regions of the nuclear periphery, or an active exclusion of it from NPC-rich regions.
Figure 10. The Gal80 HALO fragment facilitates dynamic association with the nuclear envelope. The wild-type Sc652 cells bearing pOE71 were grown to mid-log phase. Expression of NLS-HALO-2GFP molecules was induced with galactose for 2 hours. The GFP signal at the nuclear periphery was observed with a confocal microscope, and half of the nuclear membrane was bleached. Recovery of the GFP signal at the bleached region was observed within a second after bleaching.
Figure 10:
Figure 11. The HALO fragment tends to locate to NPC-free regions of the nuclear envelope. A sub-population of Sc652 expressing NLS-HAL-2GFP from pOE71 exhibited GFP localization only at one part of the nuclear periphery. Co-expression of Nup49-mCherry that in those cells revealed that the NPC was heavily localized to the region void of the GFP signal.
Figure 11:
3.3.3 The HALO fragment associates with specific phospholipids in vitro.

Given that the FRAP experiments with full-length Gal80 fused to GFP determined that Gal80 did not have a fast-acting NES (chapter 2), we asked what the HALO fragment might be doing, if not a nuclear exporting function. One possibility was direct association with the nuclear periphery. The results above would be consistent with molecules binding to the inner nuclear membrane and moving fast laterally along the membrane. Cellular membranes are mosaic composite structures with a variety of moieties. One type of these membrane domains that proteins specifically target is the phospholipids. To test whether the HALO fragment binds to these molecules, and which ones if it does, we carried out a protein-phospholipid overlay experiment using Echelon Biosciences sheets containing spots of specific phospholipid species. Yeast extracts containing HALO-GFP molecules were incubated with these spots, and binding of HALO-GFP molecules at each spot monitored using an immuno-detection system with anti-GFP antibody. These experiments demonstrated that HALO-GFP molecules did indeed bind to various phospholipids in vitro (Figure 12B). The GFP molecules by themselves, on the other hand, failed to show any binding (Figure 12A), indicating the association of HALO-GFP with phosphoinosites and phosphatydic acid was mediated via the residues on the HALO fragment. Next, we tested whether full length Gal80 would interact with these phospholipids. Untagged Gal80 was expressed in E. coli cells from pXT49, and the above experiment was repeated with E. coli extracts from these cells. The results showed that full-length untagged Gal80 molecules were also able to bind the same phospholipids plus cardiolipin.
Figure 12. **HALO-GFP and full-length Gal80 molecules bind to phospholipids.** (A) GFP (pOE131) or (B) HALO-GFP (pOE125) molecules were expressed in Sc652 cells. (C) 6His-Gal80 was expressed in *E. coli* cells (pClaks82). The extracts obtained from the cells were incubated with the blots that were spotted with specific phospholipids. The binding was determined using immuno-detection after the blots were washed repeatedly. The antibodies used were against (A,B) GFP or (C) against Gal80. (D) The key shows the specific phospholipid species present in each spot.
Figure 12:

A

B

C

GFP

Halo-GFP

Gal80

D

Triglyceride
Diacylglycerol (DAG)
Phosphatidic Acid (PA)
Phosphatidylserine (PS)
Phosphatidylethanolamine (PE)
Phosphatidylcholine (PC)
Phosphatidyglycerol (PG)
Cardiolipin

○ ○ Phosphatidylinositol (PI)
○ ○ PtdIns(4)P
○ ○ PtdIns(4,5)P₂
○ ○ PtdIns(3,4,5)P₃
○ ○ Cholesterol
○ ○ Sphingomyelin
○ ○ 3-sulfogalactosylceramide (Sulfatide)
○ ○ Blue Blank
3.3.4 **Gal80 binds to isolated yeast cellular membranes.**

The results above suggested that Gal80 might be able to associate with the cellular membranes via an interaction between the HALO fragment and specific phospholipids. The protein-overlay experiments, however, involve the interaction of protein molecules with a flat surface containing one specific molecular species. Yeast cellular membranes, on the other hand, are curvature surfaces containing a mosaic of molecular species presenting motifs in certain spatial geometry. To test whether yeast cellular membranes would also bind to Gal80, we prepared membranes from yeast Sc745 cells bearing a gal4 deletion grown to mid-log phase. The membrane fraction from these extracts of these cells was isolated using standard centrifugation methods. I then carried out a binding experiment using purified 6His-Gal80 expressed in *E. coli* systems. The results showed binding of Gal80 associated the membranes (Figure 13A).

Next, we wanted to test whether or not the Gal80-membrane interactions were affected by Gal4AD and/or Gal3. I repeated the above Gal80-membrane binding experiments, including or leaving out Gal3 or Gal4 in the binding buffer during the incubation step. When Gal3 and galactose were incubated with Gal80 and membrane, the amount of Gal80 precipitated with the membranes was less than the control sample that did not have galactose in the binding mix (Figure 13B). A mutant Gal80 (Gal80S-2) defective in binding to Gal3 did not exhibit this effect due to presence of Gal3 and galactose (Figure 13B). Adding Gal4 to the binding reactions with Gal80 and membrane, on the other hand, did not dissociate Gal80 from the membrane fraction, but did show Gal80 facilitated Gal4 co-precipitation with the
membranes (Figure 13C). Gal4, by itself, also associated with the membranes at a lesser degree. This outcome would be expected as Gal4 has been implicated in interactions with components of the NPCs and other membrane bound elements (nuclear and other membranes would both be in my membrane preparations.

Taken together, these results suggested that Gal3-Gal80 interaction could cause dissociation of Gal80 from the cellular membranes, as suggested by the observation that HALO fragment is completely buried between Gal3 and Gal80 in the Gal3-Gal80 crystal structure (Lavy et al. 2012, Figure 14). On the other hand, Gal80-membrane interactions were compatible with the Gal4 binding to Gal80. Moreover, these results suggested that perhaps Gal80 facilitates recruitment of Gal4 to the membrane domains.
Figure 13. Interactions of GAL regulatory proteins and cellular membranes. (A) The Sc745 cells bearing a gal4 deletion mutation were grown to mid-log phase, and the membrane fractions were isolated with centrifugation techniques. 5 ng of purified 6His-Gal80 was incubated with or without the membranes. The samples were centrifuged at high speed, and all Gal80 was precipitated only when membranes were present. (B) The co-precipitation experiments were repeated in presence of Gal3. The Gal80 co-precipitated was diminished when galactose was added to the samples. This effect was not observed when a Gal80 variant defective in Gal3 binding (Gal80S-2) was used. (C) Gal4 was added to the binding reactions with Gal80 and membranes. Some Gal4 was precipitated with the membranes even in the absence of Gal80, but the amount of Gal4 precipitated was substantially enhanced when Gal80 was present in the binding reactions.
Figure 13:

**A**

<table>
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<th>Input</th>
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<tr>
<td><strong>Gal80</strong></td>
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**B**

Galactose
- +

WT

S-2

**C**

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</tr>
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<td>-</td>
<td>-</td>
<td>+</td>
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<tr>
<td>Membrane</td>
<td>-</td>
<td>+</td>
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Figure 14. The Gal80 HALO fragment is predicted to be buried in the Gal3:Gal80 complex. The crystal structure of Gal3 in complex with Gal80 (Lavy et al. 2012) was obtained from the protein databank depository (http://www.rcsb.org/). The complex was visualized and the orientations were arranged to display HALO. (A and B) Gal3 monomer (yellow) is in complex with Gal80 monomer (blue). The heterodimer is presented in cartoon format to show beta-sheets and alpha helices. The HALO fragment alpha helix (red) can be seen as protruding into the image along the interaction surface. (C and D) The structures in A and B are rendered in spacefill format to display the surface exposed residues of the Gal3-Gal80 complex. (E and F) The spacefill images in (C and D) were rotated 90 degrees along the vertical axis and Gal3 was hid to display the interaction surface containing the HALO fragment (F).
Figure 14:
3.4 DISCUSSION

Early investigations of the GAL gene loci were in support of activation-dependent peripheral recruitment hypothesis (Casolari et al. 2004, Cabal et al. 2006, Dieppois et al. 2006, Luthra et al. 2007, Brickner et al. 2007, Sarma et al. 2007). However, a more recent study showed that the non-induced genes were also nuclear-peripheral when the cells were grown in raffinose or glycerol (Green et al. 2012). That work drew attention to the fact that all previous reports on this issue compared galactose-induced cells to glucose-repressed cell (Green et al. 2012). Thus, those results suggested that the nucleoplasmic localization of GAL genes is an effect of glucose repression, rather than peripheral localization being a function of galactose.

If both non-induced and induced GAL genes are retained at the nuclear envelope, one has to ask whether the genes are at the same peripheral location or not. The nuclear envelope is clearly a mosaic structure of various distinct domains consisting of different elements. So, it may well be that the GAL genes are found in two completely different molecular environment depending on transcriptional activity. In fact, artificially tethering reporter genes to the NPCs has suggested that NPC abundance is a key determinant for transcriptional activation (Menon et al. 2005), and it appears that the sites of repression are lacking the NPCs (Akhtar and Gasser 2007).

Here, we report that the Gal80 fragment consisting of residues 353-386, the HALO fragment, specifically associates with the NPC-free regions of the nuclear envelope. The in vitro binding experiments suggest that Gal80 targets specific
phospholipid species, probably via its HALO fragment. Moreover, Gal4 precipitation with membrane fractions is enhanced in presence of Gal80, suggesting Gal4-Gal80 interactions can form while Gal80 is membrane bound. So, one possibility for the peripheral localization of non-induced GAL genes is that DNA-bound Gal4 binds to Gal80 at the nuclear membranes, recruiting the GAL genes to the NPC-free domains of the nuclear envelope. Such a mechanism would be also consistent with the observation that the GAL genes are not predominantly peripheral in glucose, as under those conditions Mig1-dependent transcriptional repression causes depletion of these regulatory proteins.

In contrast to Gal80 binding to Gal4AD, Gal80 binding to Gal3 appears not compatible with Gal80’s association with membranes, suggesting Gal3-Gal80 interaction causes dissociation of Gal80 from the nuclear membrane. In agreement with this notion, the recent crystal structure of Gal3 in complex with Gal80 (Lavy et al. 2012) shows that the HALO fragment is buried between the two molecules in the complex. This implies that the membrane binding HALO fragment of Gal80 would not be available to bind to membranes while in complex with Gal3.

What might be the functional relevance of Gal80-membrane interactions? For one, these interactions could localize the GAL genes close proximity the NPCs. This would enhance the induction rate, if localization to the NPCs indeed aids rapid galactose response. Another possibility is that the membrane binding might shield Gal80 from Gal3 under non-inducing conditions. Galactose-bound Gal3 attains conformations that are more apt to associate with Gal80. However, some lower level of Gal3-Gal80 interaction can be established even prior to induction. Under such
circumstances, Gal80-membrane binding might prevent the futile GAL induction due to the residual Gal3-Gal80 interaction in absence of galactose in the cell. Yet, another possibility consistent with the data to date is that the Gal3-Gal80 complex might require a competitor or facilitator to dissociate rapidly once galactose is depleted in the cell. Otherwise, a small transient induction of the GAL system would persist due to possibly stable Gal3-Gal80 complexes and strong Gal4 engagement with the transcription machinery. Specifically, membrane binding might compete with Gal3 and retain unbound Gal80 molecules as a means of rapidly repressing transiently activated GAL genes.
CHAPTER 4

GAL3-GAL80 INTERACT OCCURS AT THE EXPENSE OF GAL80 OLIGOMERS IN SACCHAROMYCES CEREVISIAE

Egriboz, Onur; Tao, Xiaorong; Schaffer, Christie; Hopper, James E.
4.1 ABSTRACT

The DNA-binding transcriptional activator, Gal4, and its controlling proteins, Gal80 and Gal3 of yeast constitute a transcriptional switch for GAL gene expression. In the absence of galactose, Gal80 binds to and inhibits the Gal4 dimer. Gal80 exists in monomer, dimer and multimer states, and the dimer is strongly implicated as the form that binds to the transcriptional activation domain (AD) of a DNA-associated Gal4 dimer. Galactose effects Gal3-Gal80 interaction that in turn relieves Gal80 inhibition of Gal4 to allow activation of the GAL genes. Just how the Gal3-Gal80 interaction alters Gal80 has been unknown. To address this we performed experiments employing co-immunoprecipitation, cross-linking followed by SDS PAGE denaturing gels, native PAGE and invivo fluorescence imaging of live cells. Our results collectively show that the Gal3-Gal80 interaction brings about a decrease in Gal80 self-association and/or the stability of Gal80 dimer and multimer forms. We suggest that this effect of Gal3 could explain how Gal3 relieves Gal80 inhibition of Gal4. We also report the results of a GSTGal4AD pull-down experiment in which we detect a small amount of Gal3 specifically retained by Gal4AD-associated Gal80. This and data of others is consistent with transient interaction of Gal3 with a Gal80-Gal4 complex.
4.2 INTRODUCTION

Regulation of transcription initiation by DNA-binding transcription activator proteins is a common strategy all cells use to modulate production of proteins adaptively in response to environmental changes. Such regulation often involves signaling molecules and a cascade of protein interactions and modifications that converge on the transcriptional activator to block or trigger transcriptional activator-mediated recruitment of RNA polymerase to targeted promoters (Lenburg et al. 1996, Calkhoven et al. 1996, Xu et al. 1999, Whitemarsh and Davis 2000, Desterro et al. 2000, Bannister and Miska. 2000). Several well-studied transcriptional activators have their activation domains masked by specific inhibitor proteins under one condition or another, in which case the signal meant to turn on the target gene acts to unmasked the activation domain (Carman and Henry 2007, Dyson 1998, Marine et al. 2007, Lue et al. 1987, Ma and Ptashne 1987, Jiang et al. 2009).

A prominent example of regulation via masking and unmasking of a transcriptional activation domain is the GAL gene switch regulatory system that allows switching off or on expression of the galactose pathway genes expression in the budding yeast, Saccharomyces cerevisiae dependent on absence or presence of galactose. Primarily, three proteins constitute the GAL gene switch: Gal4, the DNA-binding transcriptional activator, Gal80, the Gal4 inhibitor, and Gal3, the Gal80 inhibitor (Carlson 1987, Johnston 1987, Johnston and Carlson 1992, Lohr et al. 1995, Rubio-Texeira 2005).
Alternative interactions among the three GAL gene switch proteins determine whether the Gal4AD is masked (-galactose) or not masked (+galactose) by Gal80 (Reece and Platt 1997, Travern et al. 2006). In the absence of galactose the Gal80 protein binds to a small peptide (aa 855-870) within the Gal4 transcription activation domain (Gal4AD; aa 768-881) to mask the Gal4AD and prevent binding reactions that are required to recruit RNA polymerase to the GAL gene promoters (Lue et al. 1987, Ma and Ptashne 1987, Wu et al. 1996). Galactose converts Gal3 to a form that readily binds to Gal80, and it is Gal3-Gal80 complex formation that somehow leads to relief of Gal80 inhibition of Gal4AD and Gal4-mediated activation of the GAL genes (Bhat and Hopper 1992, Blank et al. 1997, Yano and Fukasawa 1997, Vollenbroich, V. et al 1999). These events occur rapidly, resulting in readily detectable Gal4-mediated transcriptional activation of the galactose pathway genes (GAL genes) in yeast cells within 3 to 4 minutes of exposure to galactose (John and Davis 1981, Yarger, J. et al 1984, Bryant and Ptashne 2003).

Although much is known concerning the Gal4, Gal80 and Gal3 proteins and their overall roles in the operation of the GAL gene switch, the central question of how the Gal3-Gal80 interaction relieves Gal80 inhibition of Gal4 remains controversial. This question can be rephrased as two highly interrelated questions. First: Does Gal80 dissociate from Gal4 or remain associated with Gal4 in response to the Gal3-Gal80 interaction? On this issue there is solid evidence in support of dissociation from our lab (Sil et al 1999, Peng and Hopper 2002, Jiang et al 2009) as well as evidence for non-dissociation (Leuther and Johnston 1992, Platt and Reece 1998, Abramczyk et al. 2012). Second: how does the binding of Gal3 to Gal80 bring
about dissociation from Gal4, or a conformational change in the Gal80-Gal4 complex for unmasking Gal4AD? It is this second question that concerns us here.

Just how the binding of Gal3 to Gal80 works to relieve Gal80 inhibition of Gal4 might come down to simple competition between Gal4 and Gal3 for Gal80 binding, as has been proposed for the somewhat similar GAL gene switch of the distantly related yeast, Kluvermyces lactis (K. lactis or Kl) (Salmeron and Johnston 1986, Webster and Dickson 1988, Meyer et al. 1991). In K. lactis the binding of KlGal1, a Gal3 homologue, to KlGal80 overcomes its inhibition of KlGal4 activity (Zenke et al 1996). In that system there is experimental evidence indicating that KlGal1 and KlGal4 binding to KlGal80 is mutually exclusive and a heterotetrameric KlGal80\textsubscript{2}-KlGal1\textsubscript{2} complex forms in response to galactose. Moreover, mathematical modeling of that system points to a mechanism wherein two KlGal1 monomers somehow compete with KlGal80 dimer-KlGal4 dimer interaction (Anders et al. 2006).

Possibly, in the S. cerevisiae GAL gene switch as well, it could be competition between Gal80-Gal4 and Gal80-Gal3 binding events that couples Gal3-Gal80 binding to activation of Gal4, as over-expression of either Gal4AD or Gal3 relieves Gal80 inhibition of Gal4 in the absence of galactose (Johnston and Hopper 1982, Hashimoto et al. 1983, Johnston et al. 1986, Ma and Ptashne 1987, Bhat and Hopper 1992) and increasing Gal80 concentration reverses this effect (Nogi, Y. et al. 1984).

However, a consideration of early discoveries concerning Gal80 associations led us to suspect that there might be more to the coupling between Gal3-Gal80 interaction and activation of Gal4 than simple competition between Gal3 and Gal4
for binding to Gal80. First, the predominant stable Gal3:Gal80 complex is a 1:1 complex (REF; Timson and Reece 2002). Second, Gal80 self associates to form a dynamic dimer with very high affinity (REF; Melcher and Xu 2001). And, third, the complex of Gal80 with Gal4 conforms to a 2:2 heterotetramer: (Melcher and Xu 2001, Kumar et al. 2008). Additionally, a genetic selection for Gal80 mutants impaired in binding to Gal3 revealed that such mutants were also defective in self-association and binding to Gal4 (Pilauri et al. 2005). The behavior of those Gal80 variants and the fact that the predominant stable form of the Gal80:Gal3 complex consists of a monomer of each protein (Timson and Reece 2002) led us to propose that Gal3 binding to Gal80 somehow competes with Gal80-Gal80 self-association (Pilauri et al. 2005). More recently, structural data has corroborated the notion that a Gal80 dimer is the species of Gal80 that interacts with, and inhibits the Gal4 dimer. (Kumar et al 2008).

In this work we present results from in vitro molecular studies and spinning disk confocal fluorescence imaging of live S.cerevisiae yeast cells that show that Gal3 interaction with Gal80 reduces Gal80 self-association. We propose that this represents the mechanism by which Gal3-Gal80 binding couples to the relief inhibition of Gal4. This notion is consistent with the data of others that highlights the dimer of Gal80 as the likely form of Gal80 that binds to and inhibits Gal4. We also provide data suggesting that Gal3 is able to interact with a Gal80 molecule that is either directly associated with the Gal4AD or with another molecule of Gal80 that is. We discuss the implications of our results and interpretations thereof for explaining the rapid response of the GAL gene switch. We also discuss how the
mechanism elucidated in this work distinguishes between the GAL regulatory systems of Saccharomyces cerevisiae and Kluyveromyces lactis.

4.3 MATERIALS AND METHODS

4.3.1 Yeast strains and plasmids:

The assays to test protein-protein interactions on an array of Gal4 binding sites were carried out in Sc952 cells (MATa ade1 ile leu2-3,112 ura3-52 trp1-HIII his3-Δ1 LacOx64::LEU2 (P\text{GAL1}\text{-GST})x8::Kan r gal4Δ::NAT), which was derived from Sc902 (Jiang et al. 2009) by deleting GAL4 with deletion cassette PCR amplified from pFJ68N (Egriboz et al. 2011) with primers OE174 (ATC ATT TTA AGA GAG GAC AGA GAA GCA AGC CTC CTG AAA GGT AAT ATA GAT CTG TTT AGC) and OE175 (GAA GTG AAC TTG CGG GGT TTT TCA GTA TCT ACG ATT CAT TGG CGG CGT TAG TAT CGA ATC). Protein extracts for the in vitro binding assay were obtained from Sc745 cells (MATa ade1 ile leu2-3,112 ura3-52 trp1-HIII his3-Δ1 MEL1 LYS2::P\text{GAL1}\text{-HIS3} gal4Δ::LEU2).

The plasmids used in this study are generated using standard molecular cloning and PCR techniques. A more detailed description for construction of each plasmid can be provided upon request. A list of all the vectors can be found in the table below.
Table 3. The plasmids used in chapter 4

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Genotype</th>
<th>Source</th>
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<tbody>
<tr>
<td>pOE145</td>
<td>CEN ARS1 TRP1 ( P_{ADH2} )-GAL80-2mYFP</td>
<td>This study</td>
</tr>
<tr>
<td>pGP23A</td>
<td>CEN ARS1 URA3 ( P_{ADH2} )-HA-GAL80</td>
<td>Peng and Hopper 2002</td>
</tr>
<tr>
<td>pMPW66</td>
<td>CEN ARS1 TRP1 ( P_{ADH2} )-GAL3</td>
<td>Pilauri et al. 2005</td>
</tr>
<tr>
<td>pRS414</td>
<td>CEN ARS1 TRP1</td>
<td>NE biolabs</td>
</tr>
<tr>
<td>pOE165</td>
<td>CEN ARS1 URA3 ( P_{ADH2} )-DBD-GAL80</td>
<td>This study</td>
</tr>
<tr>
<td>pOE167</td>
<td>CEN ARS1 URA3 ( P_{ADH2} )-DBD-GAL3</td>
<td>This study</td>
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<tr>
<td>pOE169</td>
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<td>This study</td>
</tr>
<tr>
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<tr>
<td>pME9</td>
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<td>Egriboz et al. 2011</td>
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<tr>
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<tr>
<td>pPXGSTG4AD</td>
<td>GST-GAL4AD Amp(^R)</td>
<td>This study</td>
</tr>
</tbody>
</table>
4.3.2 Microscopy:

All microscopy experiments were carried out with a Nikon TE-2000U spinning disk confocal microscope that was equipped with a 100x/1.4 NA objective lens (Nikon, Melville, NY), 488-, 514-, and 568-nm argon ion lasers, and a charge-coupled device camera (ORCA-AG, Hamamatsu, Bridgewater, NJ). To observe the effect of galactose on protein interactions in a single cell, the cells were grown to mid-log phase and were immobilized in the Callasik Y04C plates. The temperature of the media in the plates was kept at 28°C with a Biotech lens heater. The images were taken before and after adding galactose into the media circulated around the cells.

4.3.3 In-vitro pulldown assays

Sc745 cells expressing HA-Gal80 (pGP23A) and Gal80-2YFP (pOE145) were grown to mid-log phase in the appropriate synthetic drop out media. The cells were pelleted and re-suspended in lysis buffer (20 mM HEPES, pH 7.4, 0.5% Triton X-100, 200 mM NaCl, 0.5 mM EDTA, 2 mM DTT, 5 mM MgCl₂) with protease inhibitors (1 µg/ml leupeptin, 1 µg/ml pepstatin, 1 µg/ml chymostatin, 1 mM PMSF, 0.5 µg/ml benzamidine, and 1 µg/ml aprotinin). 0.5 µm acid washed beads were added to cells in 2 ml tubes, and the cells were mechanically lysed by vortexing with glass beads as previously described (Mylin et al. 1989, Blank et al. 1997, Pilauri et al. 2005). 1 µg of
total protein from the whole cell extracts were mixed with 20 µl of *E. coli* extract containing Gal3 (pXT52) in binding buffer that was previously shown suitable for Gal80 self-association interactions (Melcher and Xu 2001, 25 mM Tris-HCl, pH 7.5, 50 mM KCl, 5 mM MgCl₂, 0.125 mM EDTA, 10% glycerol, 1 mM DTT). Agarose beads conjugated to anti-HA antibody were added, and the samples were rotated in 4°C for 2 hours. The targeted proteins were precipitated at 3000 x g with the beads, and the beads were washed 3 times with lysis buffer with or without galactose accordingly. The final pellets were subjected to SDS-PAGE, and were assessed on a western blot probed with anti-Gal80 (VVA883, 1:5000 dilution), and anti-Gal3 (#138, 1:1000 dilution).

The GST pulldown experiments were carried out with GST-Gal4AD (pPXGSTG4AD), 6his-Gal80 (pCLAKS82), 6his-Gal80S-2 (pAK118), and Gal3 (pXT52) expressed in *E. coli* cells. 100 ml of cells were grown overnight with IPTG at 18°C. The collected cells were divided into tubes with 250 ul of cell pellet in each. The pellets were resuspended in 1.5 ml of PBS buffer. The cells were incubated with lysozyme (5 µg/ml) for 1 h on ice, and subsequently lysed by sonification. The debris disposed after centrifugation, and the cell extracts were collected. 250 ul of 6his-Gal80 or 6his-Gal80S-2 containing extract was incubated with 150 ul of GST-Gal4AD containing extract at 4°C for 1 h. Then, the samples were incubated with 50 ul of 50% slurry of glutathione-S-agarose beads for another 1 h. 350 ul of Gal3 extracts was added to the samples with or without ATP and galactose. The samples were incubated for an additional 2 h, and the pellets were collected by centrifugation at
3000 x g. The samples were, then washed 3 times, and the beads were resuspended in 50 ul of SDS-PAGE loading buffer. The proteins precipitated with the beads were analyzed on a 7.5 % denaturing SDS-polyacyrlamide gel that was stained with coomassie blue.

The pulldown experiments using NTA beads to target 6his tagged Gal80, were carried out with E. coli extracts containing 6his-Gal80 (pCLACKS82) or Gal3 (pXT52). 100 ul of Gal3 containing extract was mixed with 0, 10, 25, 50, 100, 200, 300 and 400 µl of 6his-Gal80 containing extracts in a series of samples. Total volume of the binding reaction was topped to 500 µl in each sample. The samples were incubated at 4°C for 1 h. The NTA beads were, then, added to the samples. After an additional 1 h incubation at 4°C, the beads were centrifuged at 3000 x g. The samples were washed with wash buffer containing ATP and galactose 3 times. The precipitated proteins were analyzed on a SDS-polyacrylamide gel that was stained with coomassie blue.

4.3.4 Protein expression and purification

We used affinity purification to purify untagged Gal80 and Gal3 with 6xhis-Gal3 and 6xhis-Gal80, respectively. Gal80 from pXT49, Gal3 from pXT52, 6xhis-Gal80 from pClaks82 and 6xhis-Gal3 from pAKS123 were expressed in the Rossetta (DE3) strain. A single colony was inoculated in 5 ml Lb medium with the selection antibiotics for 8 hours at 37°C. The resulting culture was transferred into 1 L terrific broth (Research product international Corp.) with glycerol (0.4%), kanamycin (50
µg/ml) and chloramphenicol (35 µg/ml). The cells were grown in shaker flasks at 37°C to a culture density corresponding to OD<sub>600</sub> 0.6-0.8, and then they were shifted to room temperature. The cells were, then, induced with 0.1 mM IPTG overnight. The next day, the cultures were centrifuged to obtain cell pellets. The pellets were stored at -80°C or used immediately. The cells were resuspended in 45 ml lysis buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl and 10 mM imidazole, pH 8.0) with one tablet of protease inhibitor cocktail (Roche) and were lysed in a French press with pressure of 1200 psi. The cell lysates were centrifuged at 15 000 rpm for 1 hour and the supernatants were transferred into a 50 ml falcon tube. 6xhis-Gal3 extract from cells bearing pAKS123 mixed with Gal80 extract from cells bearing pXT49. 6xhis-Gal80 extract from cells bearing pClaks82 was mixed with Gal3 extract from cells bearing pXT52. Galactose and ATP were added to the mixtures to the final concentrations of 25 mM and 2 mM, respectively. 2 ml of Ni-NTA resin (Qiagen) was added into the extracts and the samples were rocked/rotated for 2 hours at 4°C in 15 ml falcon tubes. Samples were centrifuged, and the precipitate resin was washed with wash buffer (20 mM imidazole, 2 mM ATP, 25 mM Galactose, 50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, pH 8.0) once. The resin was then transferred to a plastic gravity chromatography column (BioRad) and washed twice with the wash buffer. The untagged proteins were then eluted with elution buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, pH 8.0) lacking ATP and galactose.
4.3.5 Cross-linking of Gal80 and Gal3

The cross-linking reaction was carried out as described elsewhere (Adams A.C et al., 2010. J. Mol. Biol. (2009) 389, 248–263). 2 μM purified Gal80 was incubated with an increasing amount of purified Gal3 in the presence of 500 μM ATP and 25 mM Galactose in 30μL total reaction volume at 4°C for 1 hr. 5 mM formaldehyde was added to the samples, which were incubated at 4°C for an additional 2hr to crosslink the proteins. 10μL 6xSDS electrophoresis loading buffer was added to each sample. The samples were then incubated at room temperature for additional 10 min. The samples were analyzed on a 7.5% SDS-polyacrylamide gel followed by coomassie blue staining.

4.3.6 Discontinuous blue native protein gel Electrophoresis

The native gel electrophoresis was carried out as described by Niepmann and Zheng (Electrophoresis 2006, 27, 3949–3951), which allows the separation of proteins according to their size, oligomeric state, and shape. 2.5 μM Gal80 was incubated with varied amounts of Gal3 in the presence or absence of 2mM ATP and 25mM Galactose at 4°C for 2 hr. The samples were then mixed with loading buffer (100 mM Tris-Cl pH 8.0, 40% glycerol, 0.5% Serva Blue G) and incubated for 10 min at room temperature. The protein species in the samples were analyzed on a non-denaturing 4-16% polyacrylamide gradient gel. 100 mM histidine adjusted to pH 8.0 using Tris–base (without chloride) and 0.002% Serva Blue G were added to the
cathode buffer. The gels were destained with several changes of 7.5% acetic acid and 5% ethanol. 20 µg catalase (230 kDa), 9 µg GAPDH (143 kDa) and 7.5 µg BSA (69 kDa) were used as molecular markers.

4.4 RESULTS

4.4.1 Conditions favoring Gal3-Gal80 interaction reduce the level of HA-Gal80-Gal80-2mYFP complexes in yeast extracts:

To determine whether Gal3-Gal80 association reduces Gal80 self-association we performed co-immunoprecipitation assays. Yeast extracts containing co-expressed HA-Gal80 and Gal80-2mYFP were incubated with E.coli-extracts containing wild type Gal3 (WT), or no Gal3 in presence or absence of galactose and ATP. Repeated experiments consistently showed that markedly less Gal80-2mYFP was co-precipitated with HA Ab-coupled beads in the presence compared to the absence of Gal3 and galactose (Figure 15). As well, we detected Gal3 in the co-precipitates only in the presence of galactose. Moreover, these results were not observed in similar experiments where in place of wt Gal3 a mutant of Gal3 incapable of binding to Gal80 was used (data not shown), suggesting a mechanism whereby Gal3 binding to Gal80 interferes with Gal80 self-association and/or the stability of pre-existing Gal80 oligomers.
Figure 15. *Gal3 binding interferes with Gal80 self-association*. HA-Gal80 (pGP23a) and Gal80-2mYFP (pOE145) are expressed in Sc745. The binding of the two Gal80 species is tested by co-precipitation with anti-HA antibody-conjugated agarose beads in presence or absence of galactose and/or Gal3 that was expressed in *E. coli* (pXT52).
Figure 15:

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<thead>
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<td>+</td>
</tr>
<tr>
<td><em>Gal3</em></td>
<td>-</td>
<td>+</td>
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</table>

**anti-Gal80**

**anti-Gal3**
4.4.2 Increasing [Gal3] vs a fixed [Gal80] leads to 1:1 and higher order Gal3:Gal80 complexes at the expense of Gal80 oligomers.

In the above experiments our assay for Gal80 self-association detected complexes containing two forms of Gal80, an N-ter HA-tagged form and a C-ter GFP-tagged form. Because the tags could possibly influence the formation or stability of such Gal80 oligomers, we next turned to an assay involving only untagged molecules for determining the effect of Gal3 on Gal80 self-association. We incubated increasing amounts of Gal3 (~ 58kDa) with a constant 2 \( \mu \text{M} \) of Gal80 (~48kDa), added formaldehyde to capture cross-linked species, and then subjected samples to SDS-PAGE (Figure 16). When Gal80 alone was treated with formaldehyde we observed species with mobilities consistent with monomer, 80, dimer, 80\(_2\), trimer 80\(_3\) and tetramer, 80\(_4\) (lane 3). Strikingly, the abundance of all three of the oligomeric Gal80 species decreased in response to increasing amounts of Gal3 (lanes 4-9). This result was correlated with the appearance of two distinct new species having mobilities consistent with Gal3-Gal80 heterodimer and heterotrimer species.

To determine the composition of the putative Gal3-Gal80 complexes indicated above we used native gels followed by denaturing gels to display complexes and their constituent proteins. Two separate binding reaction series were carried out in which [Gal3] was increased against a constant 2.5 \( \mu \text{M} \) (Figure 17A) or 4 \( \mu \text{M} \) of Gal80 (Figure 18A). Complexes formed were detected by electrophoresis on 4-16% gradient non-denaturing polyacrylamide gels containing
ATP and galactose. We detected Gal80 oligomers with mobilities consistent with dimers and trimers (Figure 17A) and, in the case of higher Gal80 concentration, tetramers (Figure 17A). In both the lower and higher concentration titration series the levels of the oligomeric species of Gal80 decreased in response to increased levels of Gal3. (Figure 17A and Figure 18A, respectively). Again, as above, titration of Gal3 against fixed Gal80 levels resulted in the appearance of two new bands (1 and 2, Figure 18A). The mobility of the faster migrating species is consistent with a 1:1 complex, and is expected on the basis of a 1:1 complex reported previously (Timson and Reece 2002). The mobility of the slower migrating band is consistent with a 1:2 or 2:1 complex. In parallel experiments, we used a Gal80S-2 variant incapable of binding to Gal3 instead of wt Gal80. There was no observed decrease in the levels of the Gal80 oligomers and no detectable appearance of candidate Gal3:Gal80 complexes (Figure 17C).

The new mobility species bands appearing with increasing Gal3 in Figures 17A and 18A were excised and subjected to SDS PAGE (7.5%) analyses (Figure 17B and Figure 18B, respectively). All three bands excised from the native gels yielded two distinct proteins with mobilities consistent with Gal3 and Gal80 (Figure 17B and Figure 18B, respectively). The faster migrating band (1) in Figure 18A yielded approximately equal levels of Gal3 and Gal80, indicative of a 1:1 complex (Figure 18B), whereas the slower migrating band (2) in Figure 18A yielded somewhat less Gal3 than Gal80, indicative of a 1:2 complex (Figure 18B). Altogether, these results show that ATP- and galactose-dependent binding of Gal3 to Gal80 markedly reduces levels of Gal80 oligomers. Moreover, the Gal3-Gal80 interaction gives rise to two
prominent electrophoretically resolved Gal3-Gal80 complexes that we suggest represent stoichiometries of 1:1 and 1:2.
Figure 16. *Increasing levels of Gal3 binding reduced cross-linked Gal80 molecules.* Untagged Gal3 (3 µM) alone that was incubated with 500 µM ATP and 25 mM galactose in 5 mM formaldehyde did not show any Gal3 oligomers (lane 1). Without the cross-linking reagent, untagged Gal80 molecules (2 µM) were found in a single band, indicating the mobility of the monomer (lane 2). 2 µM Gal80 was incubated without Gal3 (lane 3) or with a series of increasing Gal3 levels (0.3 µM, 0.6 µM, 1.2 µM, 3 µM, 6 µM, 12 µM) in the presence of 500 µM ATP, 25 mM galactose and 5 mM formaldehyde at 4°C (lanes 4-9). The samples were resolved on a 7.5% SDS-polyacrylamide gel, and were subsequently stained with coomassie blue.
Figure 16:

<table>
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<td>0 μM</td>
<td>0.3 μM</td>
<td>0.6 μM</td>
<td>1.2 μM</td>
<td>3 μM</td>
<td>6 μM</td>
<td>12 μM</td>
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- 80_{(2)}
- 80_{(3)}
- 80_{(4)}
- 80_{(2)}-3
- 80-3
Figure 17. Gal3:Gal80 complex competes with Gal80 oligomerization. (A) 2.5 μM untagged Gal80 was incubated in series with increasing amounts of untagged Gal3 (0.4 μM, 1 μM, 2 μM, 4 μM, 10 μM) in the presence of 2 mM ATP and 25 mM galactose at 4°C for 2 hr. The samples were then incubated with loading buffer (100 mM Tris-Cl pH 8.0, 40% glycerol, 0.5% Serva Blue G) for 10 minutes, and were subsequently resolved at 4°C on a 4–16% polyacrylamide gradient gel containing 2 mM ATP and 25 mM galactose. (B) The new band that appeared with increasing amounts of Gal3 in the native gel was cut out, and resolved on a 7.5% SDS-polyacrylamide gel to yield two distinct peptides with molecular weights corresponding to Gal3 and Gal80. (C) The native gel in (A) is repeated with Gal80S-2 mutant that is defective in Gal3-binding.
Figure 17:

A

B

C

120
Figure 18. Higher order Gal3-Gal80 hetero-oligomers appear on native gels at relatively higher protein concentrations. (A) The native gel experiment in figure 17A was repeated with 4µM of untagged Gal80 and series of Gal3 concentration (0.6 µM, 3 µM, 6 µM, 12 µM), and ATP and galactose were added into the gel at concentrations 1 mM and 25 mM, respectively. (B) The indicated bands in (A) were cut out from the native gel and resolved on a 7.5% SDS-polyacrylamide gel.
Figure 18:
4.4.3 Gal80-2mYFP dissociates from DBD-Gal80 in a Gal3 and galactose-dependent manner in live cells:

Taken all together the analyses presented above constitute strong evidence that under in vitro conditions Gal3 association with Gal80 reduces Gal80 self-association. To test for such Gal3 action in live yeast cells we set-up a spinning disk confocal microscopy-based experiment that takes advantage of a previously developed UAS\textsubscript{GAL} array system (Jiang et al. 2009). The UAS\textsubscript{GAL} array in yeast S. cerevisiae provides a stretch of genomic locus containing 8 copies of GST genes under GAL1 promoters, which contains 32 Gal4 binding sites in total. This array of UAS\textsubscript{GAL} is located close to an array of 64 LacO elements. We expressed Gal80 fused to Gal4 DNA binding domain (DBD-Gal80), Gal80-2mYFP, and Gal3-mCherry (Figure 19A) or LacI-mCherry (Figure 19C) from yeast ADH2 promoters in the array cells bearing a gal4-deletion (Sc952). In cells harboring the DBD-Gal80, Gal80-2mYFP and Gal3-mCherry constructs we observed DBD-Gal80:Gal80-2mYFP complexes at the arrays in the absence of galactose (Figure 19A). To monitor the effect of galactose and Gal3 on the DBD-Gal80-Gal80-2mYFP interaction in a single cell, we utilized a microfluidics system (Cellasic inc.) In response to galactose addition, the fluorescence intensity due to Gal80-2mYFP at the array diminished while fluorescence intensity due to Gal3-mCherry increased (Figure 19A). These changes in fluorescence intensity were quantified, as illustrated in Figure 19B. Galactose did not affect the intensity of the DBD-Gal80:Gal80-2mYFP spots in cells expressing no Gal3 protein (Figure 19C). These results provide strong evidence that in live cells the galactose triggered Gal3 association with Gal80 reduces Gal80 self-association.
Figure 19. Real-time destabilization of Gal80 oligomers in response to Gal3-Gal80 association in live cells. (A) DBD-Gal80 (pOE165), Gal80-2mYFP (pOE145), and Gal3-mCherry (pOE208) were expressed in array cells lacking Gal4 (Sc952). The cells were grown to the mid-log phase in glycerol-lactic acid media to, and loaded into Cellasic microfluidics plate chambers. After acquiring the initial images, galactose was added to the media in the plates, and images were acquired 2 hours later. (B) The intensity of fluorescence of Gal80-Gal80 and Gal80-gal3 complexes was obtained as follows. A line was drawn centering the fluoro-spots in the cell indicated with the arrow in figure 19A. The intensity of the pixels on the line was quantified using ImageJ software. The graphs demonstrate the change in the intensity of each pixel before and after galactose. (C) LacI-mCherry (pME9) was expressed in the cells instead of Gal3-mCherry.
Figure 19:

A

Gly-Lac → Galactose

Gal80-2mYFP

Gal3-mCherry

Composite

B

Gal80-2mYFP spot

Gal3-mCherry spot

(Continued)
Figure 19. continued...

C

![Image of fluorescence microscopy results showing changes from Gly-Lac to Galactose transition for Gal80-2mYFP, LacI-mCherry, and Composite.

Gal80-2mYFP

LacI-mCherry

Composite
4.4.4 Galactose-dependent association of Gal3 with Gal4AD-bound Gal80:

How might a Gal3-mediated decrease in Gal80 self-association relates to the induction mechanism? A number of considerations that we discuss below led us previously (Egriboz et al 2011) to propose that Gal3 binding to free and Gal4-associated Gal80 dimers would elicit faster induction than would occur if Gal3 interacts with only Gal80 monomers. Such putative 1:2 Gal3-Gal80 hetero-complexes would need to be transient in accordance with the predominant 1:1 Gal3-Gal80 complexes previously observed by gel filtration chromatography (Timson and Reece 2002). We reasoned that the slower mobility Gal3-Gal80 complex we detect (Figure 18A) in this work might represent such a transient complex of Gal3 with a dimer of Gal80. We further reasoned that we might be able to capture Gal3 in transient complex with Gal4-associated Gal80 dimer by using a scaled-up pull-down assay using GST-Gal4AD. Indeed, we observed that Gal3 is co-precipitated with GST-Gal4AD when galactose and wild type (wt) Gal80 were present but not when either galactose was absent or the Gal80S-2 (Gal3 non-binder) was used in place of wt Gal80. (Figure 20, lanes 1-3) Significantly, the appearance of Gal3 in the GSTGal4AD pull-down in the presence of galactose and wt Gal80 correlates with a substantial decrease in the yield of Gal80 (Figure 20, lane 2). We discuss the implications of these results for reconciliation of contradictory models of the GAL gene switch mechanism.
Figure 20. **Gal3 binds to Gal4AD-associated Gal80.** E.coli cell extract containing GST-Gal4AD (pPXGSTG4AD) incubated with extract containing 6hisGal80 (pCLAKS82) for 1 hour. Then, extract containing Gal3 (pXT52) is added to the mix with or without ATP and galactose to incubate for additional 2hr. The protein complexes in the samples were precipitated with glutathione-sepharose beads, and were analyzed on denaturing SDS polyacrylimide gel.
Figure 20:

<table>
<thead>
<tr>
<th></th>
<th>Gal80 Wt</th>
<th>Gal80-S-2</th>
<th>Gal80-Wt</th>
</tr>
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<tbody>
<tr>
<td>ATP/Gal</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

- Gal3
- Gal80
- GST4AD

No Gal3
4.4.5 Nuclei of live cells display clusters of Gal80-2mYFP that dissipate in response to Gal3 and galactose:

The evidence for Gal80 dimer-dimer interactions, and the existence of tetrameric forms of Gal80 evident from gels presented above (Figures 17 and 18C), together with octomeric forms observed from higher concentration preps on gels (data not shown) led us to use spinning disk confocal microscopy to look for Gal80-2mYFP clusters in live yeast cells that do not have an array. We consistently observe a large fraction of cells that show two or more densely packed foci of Gal80-2mYFP fluorescence in the nuclei of wild-type cells grown in glycerol-lactic acid media (Figure 21A). Tracking of these foci over time indicated that they were highly dynamic within the nucleus (data not shown). Using photon counting calculations based on Cse4-mYFP as a standard (Coffman et al. 2011), we find that Gal80-2mYFP foci contain an average of 56 molecules, but the sizes of individual clusters varied considerably. Addition of galactose to such cells results in marked diffusion of the Gal80-2mYFP fluorescence from the defined foci within 30 minutes (figure 21B). The same result is observed with cells maintained in the presence of cycloheximide at levels that prevent protein synthesis (data not shown). When the galactose is removed and the prior-induced cells are re-incubated in non-inducing glycerol-lactic acid media, the Gal80-2mYFP fluorescence foci begin to noticeably reappeared (figure 21B) within 30 minutes. Moreover, the dissipation of the dense Gal80-2mYFP foci did not occur in an isogenic gal3Δ strain (not shown). These results reveal that there is a coalescence of Gal80-mYFP within subnuclear regions in the
absence of galactose and that these clusters are dispersed in response to a galactose and Gal3-dependent mechanism.
Figure 21. Observed clusters of Gal80-2mYFP molecules within the nucleus dissipate in response to galactose. Genomic Gal80 was tagged with 2 copies of monomeric YFP (mYFP) in otherwise wild-type cells (Sc856). (A) Sec63 was also tagged with mCherry in the cells to depict the nuclear and cellular peripheries (Sc1029). (B) The Sc856 cells were grown to mid-log phase in glycerol-lactic acid media and were loaded into Cellasic microfluidics plate chambers. Localization of Gal80-2mYFP in the uninduced cells was imaged. Galactose was, then, added to the media in the plates together with cycloheximide (to prevent galactose-induced synthesis of new Gal80 molecules) and incubated for 30 minutes. Then, the cells were incubated with media lacking galactose for 30 minutes. The Gal80-2mYFP clusters dissipated in response to galactose, but reappeared when galactose was removed.
Figure 21:

A

Gal80-2mYFP | Sec63-mCherry | Composite | Bright field

B

30 minutes

Brightfield | Gly/Lac | Galactose | Gly/Lac
4.4.6 Intra-nuclear clusters of Gal80-2mYFP co-localize with the GAL1 gene:

Next we were curious whether Gal80-2mYFP molecules clustered away from Gal4 or Gal4 interacted with Gal80 within the clusters. Low levels of Gal4 in the cell did not allow us to carry out co-localization studies. However, we were able to compare the nuclear position of GAL1 gene with the clusters. If the Gal4 molecules that are bound to the GAL1 promoter interacted with Gal80-2mYFP, GAL1 gene would be found in close proximity. Accordingly, we inserted an array of 64 LacO elements downstream of GAL1 gene in cells that express Gal80-2mYFP (Sc1020). In these cells, we then expressed LacI-mCherry, which was tightly bound to LacO array and indicated the GAL1 locus. Subsequently, we determined that GAL1 was in one of the Gal80 clusters (Figure 22), indicating at least one of the Gal80 packed sub-nuclear regions pointed to the GAL1 gene under non-inducing conditions.
Figure 22. The GAL1 gene is recruited into the Gal80p clusters. The GAL1 genomic locus in Sc856 cells was tagged with Lac0x64 array (pOE177). The cells (Sc1020) expressing Lacl-mCherry (pME9) were grown to mid-log phase in glycerol-lactic acid media. The GAL1 gene marked by Lacl-mCherry was observed localized within one of the Gal80-2mYFP clusters.
Figure 22:  

<table>
<thead>
<tr>
<th></th>
<th>Gal80-2mYFP</th>
<th>LacI-mCherry</th>
<th>Composite</th>
</tr>
</thead>
<tbody>
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<td><img src="image2.jpg" alt="Image" /></td>
<td><img src="image3.jpg" alt="Image" /></td>
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4.5 DISCUSSION

The in vitro and in vivo studies presented here reveal for the first time that the binding of Gal3 to Gal80 reduces the levels of dimer and higher-order multimers of Gal80. Our in vitro data was obtained using three approaches. One involved co-immunoprecipitation wherein the amount of one tagged version of Gal80 precipitated with antibody directed against the tag of the other version of Gal80 was a measure of Gal80 self-association assemblies. The other two approaches involved detection of Gal80 monomer, dimer and oligomer species by gel electrophoresis with or without chemical cross-linking. All approaches allowed detection of Gal3-Gal80 complexes and demonstrated substantial reductions of Gal80 dimer and oligomer species only under conditions favoring Gal80-Gal80 complexes formation. Our in vivo data came from fluorescence microscopy of live yeast cells that contained a UAS$_\text{GAL}$ array and expressed two interacting species of Gal80, Gal80-2YFP and DBDGal80. The Gal80-2YFP is known to retain all normal Gal80 regulatory functions (Jiang et al. 2009), and the DBDGal80 retains ability to bind to Gal4AD and Gal3 (Pilauri et al. 2005). Using such cells we observed galactose- and Gal3-dependent dissociation of Gal80-2YFP from UAS$_\text{GAL}$-associated DBD-Gal80. And, expectedly, the dissociation of Gal80-2YFP was coincident with association of Gal3mCherry with DBDGal80 at the array. In addition, we present data from GST-Gal4AD pull-down experiments that provide evidence for interaction of wild type Gal3 protein with Gal4AD-associated Gal80. These findings considered in light of other data for Gal3, Gal80 and Gal4 lead us to propose that galactose-activated Gal3
binding to Gal80 relieves Gal80 inhibition of Gal4 through destabilization of the Gal80 dimer, the form of Gal80 found in complex with Gal4.

Both native and formaldehyde- treated samples of Gal80 showed Gal80 forms having electrophoretic mobilities consistent with dimers, trimers and, at higher [Gal80], tetramers. All such forms of Gal80 were diminished in response to increases in the concentration of Gal3 (+galactose). And, this response correlated with the appearance of Gal3-Gal80 complexes. The relative yields of the cross-linked Gal80 dimer and multimer forms as well as the two differently migrating Gal3-Gal80 heteromers are likely not reflective of the relative abundance the native species, as the cross-linking efficiency is low and a higher number of cross-links are required to capture trimers and tetramers relative to dimers. Nevertheless, these results highlight the occurrence of three multimeric forms of Gal80 in the absence of galactose as well as the galactose-induced appearance of two differently migrating Gal3-Gal80 complexes. The fact that the latter complexes appear at the expense of dimer and multimer forms of Gal80 provides compelling evidence that Gal3-Gal80 association competes with Gal80 self-association.

Another surprising result in this body of work was the observation that Gal80-2mYFP molecules did not localize to the nucleus in an evenly distributed manner. Spinning-disc confocal images of cells that were wild-type except for the 2mYFP tag at the 3’end of genomic Gal80 open reading frame showed that Gal80 molecules were abundant in clustered nuclear formation. Tracking of these clusters over time indicated that they were very dynamic structures. We believe this characteristic of Gal80 nuclear localization had not been observed to date, as the
conventional microscopy lacks the ability to resolve fast moving objects in a confined space. Spinning-disc feature of our microscope, however, was a kind of apparatus that allow us to elucidate the novel nuclear localization of Gal80. Photon counting experiments taking 120 Cse4-mYFP molecules at the centromeres as a standard showed about 40-60 molecules of Gal80 in each nuclear cluster. Intriguingly, the GAL1 genomic locus was recruited into the Gal80 clusters under non-inducing conditions, suggesting these are sites of GAL gene inhibition. It is tempting to postulate that these accumulations of Gal80 is representative of Gal80 oligomerization in live cells; however, Gal80 binding to other molecules may also be playing a role in stabilization of such big oligomers. Interesting, galactose induction caused dissipation of the clusters in a Gal3-dependent manner. Moreover, this observation was directly linked to Gal3-Gal80 interaction, as Gal80S-2 mutants did not dissipate long after galactose addition to the system. Assuming the nuclear clusters were directly linked to Gal80 self-association interaction, these observations, together, was in agreement of the notion that Gal80 oligomerizes in vivo and Gal3 binding to Gal80 causes destabilization of these oligomeric structures.

What might be the biological relevance of Gal3’s effect on Gal80-Gal80 self-association? The work of Melcher and Xu (Melcher and Xu 2001) bears on this question. They provided evidence that Gal80 self-association may well be critical to its activity as an inhibitor of Gal4. They showed that Gal80 assembles into a 2:2 complex with a Gal4 dimer residing at its target UASGAL DNA site. They determined that Gal80 dimerizes with high affinity ($K_D \sim 0.2 \text{ nM}$), and that Gal80 dimers interact with a $K_D$ of $\sim 50 \text{ nM}$. Moreover, their in vivo experiments provided evidence for
interaction between a Gal80 dimer associated with one UAS\textsubscript{GAL}-bound Gal4 dimer and a second Gal80 dimer associated with an adjacent UAS\textsubscript{GAL}-bound Gal4 dimer. Such evidence provided a plausible explanation for why the MEL1 gene that has a single UAS\textsubscript{GAL} site has a higher basal level of expression (i.e., weaker Gal80 inhibition) than Gal4-regulated genes that have multiple UASgal sites. (Melcher and Xu 2001). Both dimer and tetramer forms of Gal80 as well as a 2:2 heterotetrameric Gal80:Gal4 complex have been determined by x-ray crystallography (Kumar et al. 2008). Thus, the emerging picture is one which highlights the Gal80 dimer and Gal80 dimer-dimer interaction as potentially relevant to Gal80’s capacity to function as an antagonist of Gal4-mediated gene expression. In this light, the capacity of Gal3’s capacity to reduce Gal80 self-association may be a central feature of the GAL gene switch mechanism.

Although we show here that Gal3- Gal80 interaction is required for the observed galactose-mediated reduction in the levels of Gal80 self-assemblies we do not know what form of Gal80 initially interacts with Gal3. Clearly, different complexes of Gal3-Gal80 can be detected in the presence of galactose, depending on the method of assay. Using gel electrophoresis in this work we detected one complex consistent with a 1:1 stoichiometry and second complex of slower mobility consistent with a 1:2, 2:1 or 2:2 stoichiometry. The likelihood of the latter being of 2:1 stoichiometry is low in view of our cross-linking data and the observations by others that point to the Gal3 monomer as the prevalent form in solution. Previously it was reported that a Gal3-Gal80 complex of 1:1 stoichiometry was the only form of Gal3-Gal80 complexes resolved by Superdex S75 gel filtration chromatography (Timson et al. 2000).
2002). However, just as this manuscript was in preparation, it was reported that
Gal3-Gal80 complexes of 2:2 stoichiometry were detected Superdex 200 (10/300)
gel filtration chromatography, but no data was provided (Lavy et al. 2012). In that
most recent work a Gal3-Gal80-a-D-galactose-Mg$^{2+}$-ATP complex was crystallized as
a 2:2 heterotetramer to 2.1 angstrom resolution. Thus, a variety of forms of
Gal3:Gal80 are detectable under different conditions. And, although the cumulative
evidence seems to favor 1:1 and 2:2 forms, which one(s) of the various Gal3-Gal80
complexes are biologically relevant remains to be determined.

An important related question is whether or not the wild type Gal3 protein
interacts with a Gal80 molecule in complex with UAS$_{GAL}$-associated Gal4 in yeast
cells. Direct evidence for interaction of Gal3 with Gal4-associated Gal80 comes
from three types of experiments. First, EMSA experiments showed that addition of a
5 fold excess of a mutant form of Gal3 (Gal3C) over Gal80 could supershift the
mobility of a Gal80-Gal4-UAS$_{GAL}$-DNA complex (REF: Platt & Reece EMBO J. 1998
17:4086). Second, over-expression of an inner-nuclear membrane-tethered form of
Gal4 localizes Gal80 to the inner nuclear membrane, and this complex shows
Eukaryotic Cell 11:334). Third, in this present work we detect some Gal3 retained
by Gal4AD-associated wild type Gal80 in the presence of galactose. Importantly, the
amount of Gal3 retained is small relative to the very large amount of Gal80 that
dissociates from GSTGal4AD-Gal80 in response to galactose. Taken together, the
data from these diverse experiments suggest that Gal3 interacts transiently with
Gal4-associated Gal80.
How might the effect of Gal3 on Gal80 self-assembly release Gal4 from the Gal80 inhibitory activity? Consideration of this depends on whether Gal80 does or does not dissociate from Gal4 in response to the Gal3-Gal80 interaction. At this time there is conflicting evidence from widely contrasting experiments on the issue of dissociation vs. non-dissociation. On one hand, the work of Abramczyk and coworkers is consistent with the view that Gal80 does not dissociate from Gal4. Their results further suggest that during the initial response to galactose Gal3 interacts with Gal4-associated Gal80, but is replaced several minutes later by Gal1 (a Gal3 paralog). In striking contrast, work from this laboratory using a UAS\textsubscript{GAL} array strain expressing wild type levels of Gal4, Gal80 and Gal3 provided results consistent with dissociation of Gal80 from UAS\textsubscript{GAL}-associated Gal4 in response to galactose effected Gal3-Gal80 interaction (Jiang et al. 2009). Clearly, additional work is required to reconcile the two sets of data and dissonant interpretations.

We postulate that under either a non-dissociation or dissociation mechanism or, as we describe below, a hybrid mechanism, the Gal80-Gal4AD complex is destabilized through Gal3’s capacity (+galactose) to reduce formation and stability of Gal80 self-assemblies. The consequences would differ in the context of the different models. A reduction in the fractional occupancy of any and all regions of Gal4 by Gal80 would ensue under a dissociation model. Under a non-dissociation model Gal80 would dissociate from its binding site within the Gal4AD and re-associate with another part of Gal4, as implied or suggested previously (Leuther and Johnston 1992, Platt and Reece 1998, Sil et al. 1999) Under yet a third possible mechanism, referred to as a hybrid non-dissociation-dissociation (ND-D) model, we
envision that Gal80 multimers could mask Gal4AD by spanning (bridging) between
the Gal4AD binding site and another region of Gal4. The Gal4 middle region may
well be such a region, as a yeast two-hybrid interaction between Gal80 and the
middle region of Gal4 was previously reported (Sil et al. 1999). A transient
interaction of Gal3 with the Gal4-associated Gal80 multimers would then destabilize
Gal80-Gal80 bonds and, in turn, Gal80-Gal4AD complexes causing dissociation of
Gal80 molecules from Gal4AD. This ND-D model accommodates galactose-induced
transient interaction of Gal3 with Gal4-associated Gal80, Gal3-mediated reduction in
Gal80 self-assemblies, dissociation of Gal80 from Gal4AD, and retention of Gal80 on
other regions of Gal4. Regardless of which model is correct, dissociation, non-
dissociation or ND-D, our work here points out that Gal3-Gal80 interaction modifies
the quaternary structure of Gal80. Based on this outcome, we propose that this
effect of Gal3 on Gal80 triggers the GAL gene switch.
CHAPTER 5

CONCLUSIONS AND FUTURE WORK

A central characteristic of the GAL gene switch is the rapid response to galactose (Yarger, 1984, Bryant and Ptashne), and any model for mechanisms must explain the rapid response. Prior to my studies, the working model from our lab had been based on several hypotheses describing how the Gal3-Gal80 interaction led to unmasking of Gal4AD. It had been proposed that the Gal80 molecules were rapidly exchanged between the nucleus and cytoplasm. The Gal3 molecules, which were only detected in the cytoplasm at the time, would then sequester Gal80 in the cytoplasm upon galactose induction (Peng and Hopper 2000 and 2002). This became referred to as the Gal80 shuttling model (Pilauri et al Genetics, 2005). Accordingly, the dissociation of Gal80 from the Gal4AD was viewed as a central mechanism required for transcription initiation.

During the course of this thesis work, Dr. Fenglei Jiang, a postdoc in the lab, published her work strongly supporting the dissociation of Gal80 from Gal4. In same work, she also showed that Gal3 was uniformly distributed between nucleus
and cytoplasm (Jiang et al. 2009). The latter observation was the basis of one critical argument against cytoplasmic sequestering of Gal80. Presence of Gal3 in the nucleus implied that Gal80 shuttling between the two compartments was not required to explain rapid induction. In addition, my repeated attempts to identify Gal80 fragments containing nuclear localization signals and/or nuclear export sequences did not yield these proposed Gal80 functional domains despite the fact that my assay was very specific and robust.

The previous studies supporting the nucleo-cytoplasmic shuttling of Gal80 were based on two experiments. Existence of Gal80 NLS was proposed due to the observation that two distinct Gal80 fragments could facilitate nuclear localization of beta-galactosidase proteins (Nogi and Fukasawa 1989). Gal80 NES was hypothesized, because the heterokaryon studies indicated NLS-Gal80-GFP molecules could move out of one nucleus and move into the other (Peng and Hopper 2000). In those previous experiments, reporter molecule localization was assayed at a steady state. Kinetics of transport across the nuclear envelope was not tested. Therefore, FRAP was a better technique to test for rapid-acting NLS and NES functions, as it allows monitoring of molecular movement in real-time. Using this approach, I showed that Gal80 did not rapidly exchange between the nucleus and cytoplasm. Since fast shuttling of Gal80 was critical for rapid response according to the GAL switch model invoking this feature, the results of the FRAP experiments directly refuted cytoplasmic sequestering of Gal80. Any Gal80 NLS or NES, if they exist, would function too slowly to be consistent with the shuttling model and rapid emergence of GAL mRNA. Thus, we later proposed the nucleus for the critical
cellular compartment, where the initial Gal3-Gal80 interaction occurred. Indeed, my experiments with NES-Gal3 showed that diminishing nuclear Gal3 caused slower \textit{GAL} gene expression.

Subsequently, my FRAP results above prompted me to ask another fundamental question. Does Gal3 interact with only Gal80 that is not bound to Gal4 or does it target Gal80-Gal4 complex as well as binding to unbound Gal80? The Gal80 shuttling model, implying cytoplasm is the only location for Gal3-Gal80 interaction, certainly required that Gal3 bind to only free Gal80. This cytoplasmic sequestering feature necessitated a very dynamic Gal80-Gal4 complex in addition to rapid nucleo-cytoplasmic Gal80 shuttling, as discussed above, to be consistent with rapid induction rate of the \textit{GAL} genes. A very stable Gal80-Gal4 complex would be rate limiting to \textit{GAL} gene induction. However, my tests of the off-rate of Gal80 from Gal4 using FRAP and two different Gal4 binding arrays indicated a very slow dissociation of Gal80 from Gal4. These results argued for a direct physical interaction of Gal3 with Gal4-bound Gal80, enabling rapid Gal80 dissociation in response to galactose. But, my repeated attempts to localize Gal3 at an array of GAL genes were seemingly against the notion of a physical interaction between Gal3 and Gal80-Gal4 complex at the promoters. I showed that Gal3 was localized to the array of \textit{GAL} genes only when I covalently linked Gal4DBD to Gal80 (DBDGal80). These results were inconsistent with formation of a stable Gal3-Gal80-Gal4 complex. Thus, I proposed a model, wherein Gal3 would transiently bind to Gal80 on Gal4 to cause a structural change and destabilize the Gal80-Gal4 complex, resulting in dissociation of Gal80 with Gal3.
The idea of transient interaction of Gal3 with Gal4-associated Gal80 could also explain previous observations of others. Platt and Reece had showed binding of a mutant Gal3 molecule (Gal3C-322) with the Gal80-Gal4-DNA complex in an electrophoretic mobility shift assay (Platt and Reece 1998). The authors of that study had proposed a non-dissociation model that predicted Gal3 binding to Gal4-bound Gal80 at the UAS\textsubscript{GAL} to produce a stable Gal3-Gal80-Gal4 ternary complex in which Gal80 no longer masked the Gal4AD. The results of Dr. Fenglei Jiang's microscopy experiments strongly refuted the notion of a stable ternary complex by showing Gal80 molecules leaving the promoter after induction in real time (Jiang et al. 2009). So, how might one explain the results of Platt and Reece? Perhaps their assay yielded a ternary complex due to the use of Gal3 mutant and/or due to a possible stabilization of an otherwise transient complex in the gel matrix. In fact, the latter case has been shown to occur in studies of other protein complexes (Fried and Crothers 1981, Fried and Bromberg 1997, Vossen and Fried 1997).

Transient interaction of Gal3 with Gal4-bound Gal80 would invoke a structural change in Gal80 to cause dissociation from Gal4. So, next we asked, what is this structural change? Previous observations showed that Gal80 formed a heterodimer with Gal3 (Timson et al. 2002), but was a heterotetramer with Gal4 (Melcher and Xu 2001, Kumar et al. 2008). It was plausible that the structural change required for Gal3-mediated Gal80 dissociation involved an alteration in the quaternary form of Gal80. This notion was also proposed previously on the basis of a study of Gal80 mutants (Pilauri et al. 2005). In that work, Gal80 mutants selected for impaired binding to Gal3 were also impaired for both self-association and
binding to Gal4AD. Accordingly, it was postulated that self-association of Gal80 was required for binding to Gal4, and that Gal3 might act to relieve Gal80 inhibition of Gal4 by interfering with Gal80 self-association.

Here, in collaboration with Dr. Xiaorong Tao, we directly tested the idea that Gal3 might impair Gal80 self-association both in vivo and in vitro. Dr. Tao showed in native gels that the purified Gal80 protein showed several oligomers, which diminished in response to Gal3 and galactose. Using a co-immunoprecipitation approach, I also found decreased Gal80-Gal80 interactions in response to Gal3 and galactose. I also performed in vivo experiments showing that Gal3-mCherry molecules replaced Gal80-2mYFP on DNA-bound DBDGal80 in vivo soon after the cells encountered galactose. These results and others (chapter 4) were in support of the notion that Gal3 binding to Gal80 competes with Gal80 self-association interactions and/or disrupts Gal80 dimers and multimers. This model could also explain how Gal80 would dissociate from Gal4, even though Gal3 and Gal4 do not appear to share a common binding surface on Gal80 according to recent crystal structures (Kumar et al. 2008, Lavy et al. 2012).

In agreement with the notion of Gal80 oligomerization in vivo, I observed Gal80 localizing within two or more highly concentrated clusters in the nucleus (most cells having two), rather than the previously envisioned uniform distribution. Using photon-counting calculations established in the Wu lab (Coffman et al. 2011) I determined that approximately 40-60 molecules of Gal80 resided in each nuclear cluster. The native gels, on the other hand, did not detect oligomers bigger than tetramers. Our inability to see higher order Gal80 structures in vitro might be due to
the in vitro techniques not being suitable to visualize such huge complexes, or the
Gal4 binding in live cells might nucleate and stabilize high order Gal80 structures.
Moreover, we do not know whether Gal80 molecules require binding to other
moieties (such as phospholipids- see below) that are not present in in-vitro assays
for stability of higher order complexes. Clearly, the nature of these clusters and how
they assemble will be an interesting direction for future work.

Assuming that the Gal80 clusters were dependent on Gal80 self-association
interactions, I tested the effect of galactose induction on Gal80 localization.
Strikingly, the clusters dissipated soon after I added galactose to the media. I also
observed that the clusters of Gal80\textsuperscript{S-2} mutant, which does not bind to Gal3, do not
dissipate in galactose. Thus, the dissipation is a direct result of Gal3-Gal80
interaction. All together these results were consistent with the above model
invoking a competition between Gal3-Gal80 and Gal80 self-association interactions.

If the clusters were functional (i.e. they were marking a site of repression
within the nucleus) one would expect that the GAL genes should be co-localized with
the clusters under non-inducing conditions. Indeed, I found that the GAL1-10-7
genomic locus is almost always within one of the clusters when the cells are grown
in absence of galactose. The fact that there was more than one cluster in each cell
led me to ask which other GAL genes might be located within the other clusters. In
the \textit{S. cerevisiae} genome, there is no other locus (other than GAL1-GAL10-GAL7) that
is densely packed with Gal4 binding sites to accumulate such high number of Gal80
molecules (40-60). One possibility was that the UAS\textsubscript{GAL} from several genomically
dispersed GAL genes coalesced at a specific site to generate these Gal80 assemblies.
To test this idea, I monitored separately the localization of GAL2 and GAL80 genes. Surprisingly, only a fraction of the cells exhibited localization of these genes within the Gal80 protein clusters. So, the formation of clusters does not require either GAL2 or GAL80 loci. Additionally, these results showed that the GAL80 and GAL2 genes, in contrast to GAL1-10-7 region, only occasionally co-localized with the Gal80 clusters under non-inducing conditions. The difference between the transcriptional activities of these genes under non-inducing conditions might explain these observations. The GAL1-10-7 genes are tightly repressed. In contrast, GAL2 and GAL80 genes have considerable basal expression. Thus, I hypothesized that the $GAL$ gene repression is a result of gene recruitment to the sites marked by Gal80 molecules, and basal level expression occurs when the gene escapes from those sites. Accordingly, the GAL1-10-7 genes might be tightly repressed due to strong recruitment, while genes such as GAL80 and GAL2 that have noticeable basal expressions are not strongly recruited.

If the $GAL$ repression is consequence of gene recruitment into specific nuclear domains marked by Gal80 clusters, where might be these sites of repression? According to Green et al. 2012, the GAL1-10-7 genes were localized to the nuclear periphery in non-inducing carbon sources such as raffinose or glycerol. I have shown that this locus was almost always co-localized with a Gal80 cluster. So, it can be deduced that at least one of the Gal80 clusters should be at the periphery. However, I have not directly tested the relationship between the Gal80 clusters and nuclear periphery in this work. Assuming Gal80 clusters and the GAL1-10-7 region locate to the nuclear periphery under non-inducing conditions, what contributes to
such localization? My discovery that Gal80 protein interacts with the phospholipids found in cellular membranes might be relevant here.

While searching for a NES, I found that Gal80(353-386) peptide (the HALO fragment) localized GFP to the nuclear periphery. Further investigations suggested that this fragment might facilitate Gal80 binding to the nuclear envelope at domains enriched with the specific phospholipids, and not the NPCs where the induced GAL1-10-7 genes are apparently recruited (Cabal et al. 2006, Brickner et al. 2007). Therefore, the Gal80-membrane interactions might be recruiting the GAL genes to transcriptionally inactive sites at the periphery. Consistent with this notion, I showed that Gal4 binding to membranes extracted from yeast cells was enhanced in co-precipitation experiments, when Gal80 was present. Those results suggested that the Gal80-Gal4 interaction could facilitate association of the DNA regions target by Gal4DBD with the nuclear membrane domains containing phospholipids targeted by Gal80.

The observation of Gal80 clusters in vivo together with Gal80-Gal4 interactions on the membranes in vitro implied that Gal80 clusters (or oligomers) were sandwiched between DNA-bound Gal4 and the phospholipids at the nuclear periphery. If this were how the recruitment of GAL genes to repression sites occurs, one would expect Gal3 to cause dissociation of Gal80 from the membranes to counteract GAL inhibition. Indeed, the results of my binding assays showed that Gal3 binding diminished the amount of Gal80 precipitated with the membrane faction. The crystal structure of Gal3 complexed with Gal80 is consistent with this notion (Lavy et al. 2012). It shows that the HALO fragment (the proposed
phospholipid binding domain of Gal80) is completely masked by Gal3. All of these results converge on a model wherein the Gal3-Gal80 interactions would break the Gal80-membrane interactions in addition to Gal80 self-association, effectively causing dissipation of Gal80 clusters. Alternatively, perhaps only Gal80 dimers and multimers interact with membranes, and thus, Gal3 binding to Gal80 causes dissociation of Gal80 from membranes due to its action on Gal80 self-association. Either way, Gal3-Gal80 interaction would trigger the release of Gal4-bound \textit{GAL} genes from the sites of repression to be recruited to transcriptionally activated NPCs.

The possible physiological function of the Gal80-membrane interactions remains to be determined. But clearly, Gal80 repression might involve more than just masking of Gal4 activation domain, as the long-standing model suggests. It might also involve gene recruitment to sites of active repression, where other molecular events might be involved in inhibition of Gal4 activity. This might occur by a mechanism similar to silencing of genes, such as HM, located within the telomeric regions of chromosomes in yeast. These loci are co-localized with the Sir4 clusters at nuclear periphery in yeast, and this recruitment plays a role in transcriptional inhibition of the genes (Taddei 2010). Interestingly, recent findings show that the telomeric \textit{HM} gene is not always co-localized with the Sir4 clusters, indicating the silencing and recruitment are two different events (Taddei et al. 2010). The recruitment to the Sir4 clusters only facilitates the repression, which can be maintained even when the \textit{HM} locus briefly dissociates from the site. Similarly, the Gal80 inhibition of the \textit{GAL} gene switch might involve recruitment of
the genes to a repression site, where other molecular events operate. More work on Gal80-membrane interactions in the context of GAL gene activity will be crucial to understand whether such a repression mechanism, as occurs via Sir4, operates in the GAL system.
REFERENCES


CLER, E., G. PAPAI, P. SCHULTZ and I. DAVIDSON, 2009 Recent advances in understanding the structure and function of general transcription factor TFIID. Cellular and Molecular Life Sciences 66: 2123-2134.


DIEPPOIS, G., N. IGLESIAS and F. STUTZ, 2006 Cotranscriptional recruitment to the mRNA export receptor Mex67p contributes to nuclear pore anchoring of activated genes. Molecular and Cellular Biology 26: 7858-7870.

DOUGLAS, H. C., and HAWTHORN.DC, 1966 Regulation of Genes Controlling Synthesis of Galactose Pathway Enzymes in Yeast. Genetics 54: 911-&.


JACOB, F., and J. MONOD, 1965 Genetic Mapping of Elements of Lactose Region in Escherichia Coli. Biochemical and Biophysical Research Communications 18: 693-&.


LUTHRA, R., S. C. KERR, M. T. HARREMAN, L. H. APPONI, M. B. FASKEN et al., 2007 Actively transcribed GAL genes can be physically linked to the nuclear pore by the SAGA chromatin modifying complex. Journal of Biological Chemistry 282: 3042-3049.


NOGI, Y., H. SHIMADA, Y. MATSUZAKI, H. HASHIMOTO and T. FUKASAWA, 1984
Regulation of Expression of the Galactose Gene-Cluster in Saccharomyces-Cerevisiae


