Analysis of Transcriptional Regulatory Circuitry

by

Nicola J. Rinaldi
B.A., Chemistry
Johns Hopkins University, 1994

SUBMITTED TO THE DEPARTMENT OF BIOLOGY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY
at the
MASSACHUSETTS INSTITUTE OF TECHNOLOGY
September 2004

Signature of Author ______________________________
Department of Biology
July 26, 2004

Certified by ______________________________
Dr. Richard A. Young
Professor of Biology
Thesis Supervisor

Accepted by ______________________________
Steve Bell
Professor of Biology and
Co-Chairperson, Biology Graduate Committee

Dedication

In memory of my father, Anthony Sykes.

Acknowledgements

I owe thanks to many people for their help and support during my graduate career. Rick Young and David Gifford for providing an environment where I could pursue my interest in computational biology, and teaching me how to think about the problems and issues that arise with these large scale data. My thesis committee, consisting of David Page, Eric Lander, David Housman and Martha Bulyk for taking the time to offer suggestions on my work through the years, including on this thesis. In particular I'd like to thank Martha Bulyk for providing me with access to a manuscript pre-publication. All the people that have been in the Young lab with me over the years have been wonderful, providing both intellectual stimulation and help with scientific issues, as well as being really fun to hang out with! In particular I'd like to thank Tony Lee, Duncan Odom, Ann Schlesinger and Francois Robert for all their help and mentoring through my four years in the lab. Francois and Sarah Johnstone have also been super bench-mates! I owe many thanks to Elizabeth (Bizzy) Herbolsheimer without whose computational assistance I would never have been able to finish my thesis. I would also like to thank my other labmates who have made the Young lab so entertaining through the years – Craig Thompson, Jo Terragni, Nancy Hannett, Chris Harbison, Dmitry Pokholok, Jean-Bosco Tagne, Jane Yoo, Liz McReynolds, Ezra Jennings, Matt Guenther, David Reynolds, Heather Murray, Jerry Nau, Megan Cole, Richard Jenner, Julia Zeitlinger, Joerg Schreiber, Nora Zizlsperger, Lea Medeiros, Rhonda Harrison, John Barnett, Itamar Simon, Brett Chevalier, Laurie Boyer, Jen Love, Bruno Chazaro and Tom Volkert. Kathleen Blackett, Kercine Elie and Carolyn Carpenter were incredibly helpful with all kinds of administrative issues. I also would not have survived without advice and commiseration from fellow graduate students, particularly Julie Wallace and Natalia Comella. Finally, I would not have made it without my family. Particularly my father, who passed away in 1995, whom I had told I would be getting my PhD – nothing like that for motivation! Also, my husband Mark has been my biggest supporter through the years. My mom, sister, their husbands and my in-laws have also been such great sources of strength and encouragement. Thank you from the bottom of my heart.

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Submitted to the Department of Biology on July 26, 2004
in partial fulfillment of the requirements for the Degree of
Doctor of Philosophy in Biology

Abstract

The research in this thesis has focused on the analysis of data from two types of microarray technologies with the goal of improving understanding of transcriptional regulatory circuitry in yeast. These microarray technologies, expression analysis and location analysis (ChIP on CHIP), bring unique challenges for data analysis. I have adapted methods described previously and have developing new algorithms for addressing some of these challenges.

I generated yeast transcription factor location data as part of a team effort to determine how a large set of transcriptional regulators occupy the yeast genome and to facilitate discovery of regulatory network structures. I also contributed to another team effort to deduce a portion of the yeast transcriptional regulatory code, the set of genomic DNA sequences recognized by transcriptional regulators to effect gene expression programs. Finally, I used these experimental and analytical methods to explore changes in gene expression circuitry that occur after exposure to oxidative stress in yeast.

Thesis Supervisor:	Dr. Richard A. Young
Title:	Professor of Biology
Thesis Supervisor:	Dr. David K. Gifford
Title:	Professor of Computer Science and Engineering

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Chapter 1

Introduction: Regulation of Transcriptional Activation

Overview

"The discovery of regulator and operator genes, and of repressive regulation of the activity of structural genes, reveals that the genome contains not only a series of blue-prints, but a coordinated program of protein synthesis and the means for controlling its execution"

Jacob and Monod, 1961

The ability of a cell to respond to its environment is based on exquisite control of gene expression, protein synthesis, and enzymatic activity. The simple step of initiating transcription of genes from DNA into RNA is a carefully choreographed process, involving hundred of proteins. The RNA polymerase holoenzyme, associated general transcription factors, transcriptional activators and repressors, chromatin modifiers and remodelers, kinases, phosphatases, import and export proteins are all components of this coordinated program. Of particular interest is how all this machinery is controlled to regulate the expression of an individual gene. Transcriptional activators and repressors seem to provide the requisite specificity by binding to only a select set of DNA sequences. Combinatorial interactions of these gene-specific factors with other transcription factors, the chromatin modifying enzymes which control access of the basic transcriptional machinery to the DNA template, and various components of the initiation apparatus, determine which genes are transcribed in response to a particular stimulus. Therefore, this introduction will focus on the regulation of transcription at the level of these gene-specific transcription factors.

One of the keys to establishing this gene-specific regulation of transcription is that the transcription factors themselves must be responsive to varied environmental conditions. Without such changes that affect the DNA binding or protein-protein interaction abilities of transcription factors, all gene expression would necessarily be constitutive. Transcription factors have been shown to be regulated in many different ways including modification of the transcription factor protein, changes in the amount of protein present in the nucleus, and sequestration from the DNA.

Initial studies of transcription factors and their modifications focused on the function of a few regulators at the promoters of single genes or reporter constructs. With the sequencing of the yeast genome and advent of microarray technology, however, it is now possible to study the regulation of gene expression globally. Beyond gene expression, microarray technologies have been adapted to study the direct effects of transcription factors by finding the promoters to which the factors are bound *in vivo*. These data, in combination with additional information such as sequence conservation between species, are leading to a much deeper understanding of global mechanisms for controlling the cellular program, as suggested by Jacob and Monod in 1961.

This introduction will focus, in two sections, on the regulation of gene expression in *Saccharomyces cerevisiae* at the level of transcriptional activators. First, I will discuss the components involved in transcription and activation of expression, how those components are controlled by interactions with the gene-specific transcription factors, and how the activities of the transcription factors themselves are modulated. Following that, I will focus on new microarray technologies available for the study of transcriptional regulation and how use of these technologies has impacted our knowledge of the regulatory network of the cell. Later chapters will discuss methods for analyzing the data obtained from microarray experiments as well as my contributions to the data analysis arena, followed by examples of how these analyses have been used to further our understanding of transcriptional regulation in *Saccharomyces cerevisiae*.

Transcriptional machinery components

The current understanding of transcription regulation is that gene-specific transcription factors, activators and repressors, control the expression of individual genes or sets of genes. This regulation is based upon the specificity provided by two distinct domains in the transcription factor protein. The archetypal transcription factor consists of a DNA-binding domain, which contacts the specific DNA sequence, and an activation (or repression) domain that recruits specific pieces of the basal transcription machinery (Ptashne 1988). The crystal structures of a number of these domains have been solved. Activation domains tend to be highly unstructured (Donaldson and Capone 1992; Van Hoy *et al.* 1993; Schmitz *et al.* 1994; Cho *et al.* 1996), and make contacts with other proteins in a manner that depends on general properties of the activation domain rather than specific protein-protein contacts. For example, the strength of the interaction between Gal4 and the basal transcriptional machinery depends on the length of the acidic region of the activation domain as opposed to depending on specific amino acids (Wu *et al.* 1996). Crystal structures of many transcription factor DNA-binding domains complexed with DNA have also been solved, including those for the most well-studied activators Gcn4, Gal4 and Hsf1 (Ellenberger *et al.* 1992; Marmorstein *et al.* 1992; Harrison *et al.* 1994). In contrast to the activation domains, the DNA binding domains are highly structured and make conserved contacts with specific DNA sequences. These sequences vary from 4 to approximately 17 base pairs in length, with some degeneracy allowed (Maniatis *et al.* 1975; Pelham 1982; Stormo 2000; Bulyk *et al.* 2001). Because of the relatively short length of these recognition sequences, they are found throughout the genome. A four base pair site will arise about every 256 base pairs by random chance. The Gal4 binding site occurs 236 times in the *S. cerevisiae* genome, of which 186 instances are positioned within open reading frames. However, these sites appear to affect transcription only when located within promoters (Li and Johnston 2001; Topalidou and Thireos 2003). This is likely because of the additional elements such as the TATA-box that are contained in promoters, that help to stabilize the binding interaction between the transcription factor and DNA (Lee and Struhl 1995; Vashee and Kodadek 1995).

A typical promoter in yeast consists of three elements: the upstream activating sequences (UAS), at which these transcription factors bind, the TATA-box (consensus sequence TATAAA), which nucleates the assembly of the apparatus that actually performs the transcription, and the initiator element (Inr), where gene transcription begins. A promoter can also contain operator sequences, bound by transcriptional repressors, which act to turn off gene expression.

For the most part, these promoters are not readily accessible to the transcriptional machinery in the cell. Most cellular DNA is contained in chromatin, an ordered structure consisting of repeats of approximately 146 base pairs of DNA wrapped around a complex comprised of histone proteins, into a structure called a nucleosome. The nucleosomes serve to package DNA into a small volume, and also to repress transcription of genes. *In vitro*, the basal transcriptional machinery is able to bind to and transcribe DNA in the absence of transcription factors, but *in vivo* this machinery is unable to overcome the repression by chromatin. TATA-binding protein (TBP), for example, is unable to bind to a TATA-box wrapped in a nucleosome (Workman and Roeder 1987; Imbalzano *et al.* 1994). The transcriptional activators direct a series of additional enzymes to modify and remodel the chromatin to allow access to the DNA template by the transcriptional machinery.

The protein components of this transcriptional machinery (Struhl 1995; Ptashne and Gann 1997; Kornberg 1998; Martinez 2002; Roeder 2003; Hahn 2004) have been discovered and characterized through a series of biochemical studies. The machinery that transcribes DNA to RNA is the 12 subunit RNA polymerase II complex (Bushnell and Kornberg 2003). Tethering of polymerase subunits to DNA by attaching a DNA binding domain results in synthesis of the complementary messenger RNA molecule (Barberis *et al.* 1995; Farrell *et al.* 1996). However, without the physical attachment of the polymerase to DNA, other factors are required for transcription to take place. These are termed General Transcription Factors (GTFs), and provide many of the accessory functions necessary for the initiation of transcription. GTFs and polymerase alone are sufficient to transcribe DNA to RNA *in vitro* (Sayre *et al.* 1992) but *in vivo* gene-specific transcription factors are generally required to recruit both the enzymes to open the chromatin structure and the GTFs and polymerase. Additionally, a form of the polymerase called the holoenzyme is also required for activated transcription (Kelleher *et al.* 1990; Kim *et al.* 1994; Koleske and Young 1994). This consists of RNA polymerase II and another complex of approximately 20 proteins called Mediator (Kim *et al.* 1994).

The recruitment of these complexes by activators begins the assembly of a large group of proteins, called the pre-initiation complex (PIC). The assembly of the PIC is well understood *in vitro*, however, whether the same model is followed *in vivo* is not known. *In vitro* this assembly begins with a GTF called TATA-binding protein (TBP). TBP binds to the TATA box in the promoter with a defined orientation, establishing the direction in which transcription will occur (Struhl 1995). Other GTFs that are components of the PIC include TFIIB and TFIIF, which recruit the polymerase. These three complexes select the start site of transcription based on their contacts with the Initiator element in the promoter DNA (Kornberg 1998; Ziegler *et al.* 2003; Bushnell *et al.* 2004). Additional GTFs involved in the initiation super complex include TFIIE, which appears to be involved promoter melting, clearance, and recruitment of TFIIH (Goodrich and Tjian 1994; Lommel *et al.* 2000; Sakurai and Fukasawa 2000), and TFIIH itself.

TFIIH comprises two enzymatic functions: a DNA helicase, which opens the DNA at the promoter (Goodrich and Tjian 1994), and a kinase, for phosphorylating the C-terminal domain (CTD) of the large subunit of RNA polymerase (Feaver *et al.* 1991; Sakurai and Fukasawa 1998). After this complex is assembled, gene transcription begins. The transcriptional activator and some components of the PIC remain at the promoter to form a scaffold for reinitiation of transcription, while the polymerase traverses the DNA being transcribed (Zawel *et al.* 1995; Yudkovsky *et al.* 2000).

Model for transcriptional activation

As just mentioned, gene specific transcription factors are used by the cell to recruit most of the apparatus needed to activate gene transcription. In general, the first barrier to transcription that must be overcome is the packaging of DNA into chromatin. A few different models for how the changes in chromatin that allow gene transcription can occur have been described (Morse 2003). First, some promoters, which tend to be constitutively transcribed, do not complex with histones. This can be due either to constraints based on sequence, or binding of general regulatory factors such as Reb1 (Workman and Buchman 1993; Angermayr and Bandlow 2003; Morse 2003). Poly(dA:dT) sequences near a promoter are another mechanism by which nucleosome formation can be discouraged (Iyer and Struhl 1995).

In cases where the promoter is found in chromatin, activators can bind and destabilize the interaction between DNA and histones. For example, the activator Gal4 has been demonstrated to bind to DNA contained in nucleosomes. That binding alone seems to be sufficient to destabilize the DNA-histone interaction, as the activation domain is not necessary for the destabilization (Workman and Kingston 1992; Axelrod *et al.* 1993; Morse 2003). In fact, some activators have been demonstrated to have a higher affinity for DNA complexed with nucleosomes than for naked DNA, perhaps because the bending of the DNA around the histones exposes the binding sequence (Cirillo and Zaret 1999).

The chromatin structure is dynamic rather than static, with stretches of DNA being transiently exposed. This can allow for binding of sequence specific proteins to DNA packaged in nucleosomes. Restriction enzyme sites were engineered into the ends and middle of a 150bp DNA molecule that was known to form a positioned nucleosome. The equilibrium constants for binding of the restriction enzymes to each restriction site were calculated by comparing the rate of cleavage in the nucleosomal DNA versus free DNA. Contrary to previous theory, binding and cleavage was seen even at the DNA positioned in the middle of the nucleosome, indicating that the DNA does occasionally separate from the histone proteins for long enough to allow for binding of another protein (Polach and Widom 1995). This also can explain the cooperativity of binding of transcription factors to nucleosomal DNA even when the factors do not contact one another (Adams and Workman 1995). The binding of one activator to the nucleosome exposes the site to which the second factor binds, significantly reducing the free energy required for binding of the second factor (Polach and Widom 1996).

Another way in which activators can interfere with the nucleosomal structure is illustrated by the binding of the transcription factor Pho4 to the PHO5 promoter (Venter *et al.* 1994; Svaren and Horz 1997). Pho4 binds to a UAS that is located in the free DNA region between two nucleosomes. This binding event disrupts the adjacent nucleosome, allowing Pho4 binding to a second UAS which was previously complexed in the nucleosome and inaccessible. This model was confirmed in two ways. First, mutation of the free UAS so that Pho4 could no longer bind blocked the nucleosome remodeling (Svaren *et al.* 1994). Second, removal of the activation domain of Pho4 also interfered with the restructuring at the promoter (Svaren *et al.* 1994). Unlike the situation with Gal4 where the binding of the factor to the DNA is sufficient to remodel the chromatin, in this case the activation domain is required for the remodeling.

Finally, the binding of transcriptional activators to promoters can open chromatin structure through recruitment of additional proteins. Activators have been demonstrated to recruit various general transcription factors that can stabilize the interaction of the transcription factor with DNA by tilting the energy equilibrium. For example, Gal4 binding is enhanced in the presence of a TATA site, indicating that recruitment of TBP and its subsequent binding to DNA can stabilize the Gal4 - DNA interaction (Vashee and Kodadek 1995). Other proteins that can be recruited by the transcription factors are chromatin modifiers and remodelers. One group of these enzymes, Histone Acetyl-Tranferases (HATs) acetylates the N-terminal tails of the histone proteins (Allfrey *et al.* 1964), which is hypothesized to destabilize chromatin by disrupting nucleosome – nucleosome interactions (Bannister and Miska 2000; Narlikar *et al.* 2002).

The other class of complexes recruited by transcription factors consists of ATP dependent chromatin remodeling enzymes, which function to either slide histones along DNA to expose different stretches (Meersseman *et al.* 1992; Whitehouse *et al.* 1999; Narlikar *et al.* 2002), or to change the conformation of the histones to expose the DNA *in situ* (Lorch *et al.* 1998; Jaskelioff *et al.* 2000; Schnitzler *et al.* 2001). Interactions of the activation domain of transcription factors with at least three subunits of the yeast SWI/SNF complex, one of the complexes effecting chromatin remodeling, has been demonstrated (Neely *et al.* 1999; Neely *et al.* 2002).

Based on studies of how these various chromatin remodeling or modifying factors are recruited at two different promoters, PHO8 and HO, it appears that the order in which these events occur is promoter dependent (Cosma 2002; Neely and Workman 2002). Acetylation of the histones at the PHO8 promoter was measured in strains containing either a deletion or mutation of the catalytic subunit of the SWI/SNF chromatin remodeling complex, so that no remodeling could occur. Hyperacetylation of histones at the promoter region was observed in these strains, in contrast to the wild type strain, where acetylation at the promoter was somewhat lower than before activation of the gene. No hyperacetylation was found in a strain deleted for the HAT Gcn5, or in a strain with no Pho4. Therefore, the model for activation of transcription at this promoter is that Pho4 binds to its cognate sequence and recruits the Gcn5 containing

SAGA complex, which then acetylates the histones in the nucleosomes at the promoter. This acetylation leads to recruitment of SWI/SNF, which remodels the chromatin, with acetylation lost during the remodeling (Reinke *et al.* 2001). A subsequent study of the PHO5 promoter found a similar order of events, but with complete loss of the histones. This might explain the loss of acetylation due to SWI/SNF at the PHO8 promoter also (Reinke and Horz 2003).

The order of recruitment of these complexes was also studied at the HO promoter (Cosma *et al.* 1999). In this case, an epitope tag was added to the transcription factors or remodeling complex subunits thought to be involved. Chromatin immunoprecipitation was performed at various times throughout the cell cycle to detect the presence or absence of each tagged factor. The interdependence of the events was assessed by using deletion mutants of the various factors. At this promoter the model consists of the initial step of binding of the transcription factor Swi5, followed by Swi5 dependent recruitment of the SWI/SNF chromatin remodeling complex. Subsequent recruitment of SAGA to acetylate the histones is dependent upon the prior presence of SWI/SNF. Only after each of these events has occurred can the transcription factor complex SBF bind to the HO promoter and recruit the transcription apparatus. Finally, this entire chain of events is obviated when the transcription factor Ash1 is expressed – Ash1 binds to Swi5 and prevents interactions with the SWI/SNF complex.

Subsequent to the recruitment of activators and the release of repressive chromatin structures, the transcription initiation apparatus must be recruited. This is another step that takes place through interactions with the transcription factors. *In vitro* interactions between activators and many components of the basal machinery have been demonstrated: TFIIA (Kobayashi *et al.* 1995), TFIIB (Lin *et al.* 1991), TFIID, comprised of TBP (Stringer *et al.* 1990) and TAFs (Tjian and Maniatis 1994), TFIIF (Joliot *et al.* 1995), TFIIH (Xiao *et al.* 1994) and the Rpb5 subunit of RNA polymerase II (Lin *et al.* 1997). As mentioned earlier, artificial recruitment of many of these factors has also been shown to activate transcription *in vivo*, close to the levels seen with wild type transcription factor / basal machinery interactions. For example, a fusion of TBP to the LexA DNA binding domain activates transcription from a LexA promoter (Chatterjee and Struhl 1995; Ryan *et al.* 2000). Using fusions of a DNA binding domain and TBP with leucine zippers to promote dimerization and thus artificially recruit TBP to the promoter also causes activation of transcription (Klages and Strubin 1995). This artificial recruitment does not, however, circumvent the requirement for the prior opening of chromatin. A TBP-Gal4 fusion is unable to activate transcription from a promoter complexed with histones, in contrast to the normal activation from a less constrained promoter (Ryan *et al.* 2000). Artificial tethering of TAFs associated with TBP in TFIID, as well as artificial recruitment of TFIIB also activate transcription (Gonzalez-Couto *et al.* 1997).

In vivo, interactions between TFIIA and TBP are required for activation of genes regulated by Gcn4, Ace1 or Gal4 (Stargell and Struhl 1995). A mutant TBP unable to interact with TFIIA was also incapable of activating transcription from promoters regulated by these three activators. This implies that TFIIA is the GTF recruited by these activators, which then recruits the remainder of the transcription machinery. It is possible that this recruitment is mediated by other cofactors – in human cells a positive cofactor PC4 was required for an interaction between the activator VP16 and TFIIA to take place (Ge *et al.* 1994). Direct interactions of activation domains with TBP have also been demonstrated *in vivo.* Using a construct where the single cysteine in the activation domain of the human transcription factor E2F-1 was derivatized with maleimide-4-benzophenone, photocrosslinking demonstrated a specific interaction between this activator and TBP (Emili and Ingles 1995). Finally, TFIIB is also a bona fide target of transcriptional activators. A mutant TFIIB with a serine to proline substitution is unable to activate PHO5 transcription under conditions of phosphate starvation, although the basal level of transcription is unaffected. GST pull down experiments demonstrated an inability of the activator Pho4 to interact with this TFIIB S53P mutant. A similar defect in interaction, and thus in transcription, was seen with the transcription factor Adr1 (Wu and Hampsey 1999).

Regulation of transcription factor activity

In order that genes be expressed only when needed by the cell, the transcription factors themselves must be regulated. To effect all the transcriptional programs that are required by the cell upon various stimuli or environmental changes, a large number of mechanisms are used to control the transcription factors. These include modulation of the amount of a transcription factor present in the cell, covalent modification by phosphorylation, ubiquitination, or acetylation, direct repression by protein-protein interactions, indirect repression by competition for general transcription factors or chromatin modifiers, competition for binding sites within the DNA sequence, binding of a small molecule substrate, and alterations in subcellular protein localization (Struhl 1995; Reece and Platt 1997; Sharrocks 2000; Tansey 2001). These mechanisms have been studied in detail for a few canonical activators, as described below.

The most obvious way for a cell to regulate transcription factors is through controlling the amount of the factor present in the cell. This can be achieved through increasing or decreasing the amount of transcription of the gene, through the half-life of the mRNA, through the rate of protein synthesis, and finally through the control of protein degradation. Examples of transcription factors controlled in these varied ways include the the cell cycle factors Swi4, Swi5, and Ace2, as well as the amino acid biosynthesis master regulator, Gcn4 (Struhl 1995). The transcription of the cell cycle factors seems to be controlled by serial binding of transcriptional activators to the promoters of activators turned on later in the cycle (Simon *et al.* 2001). Swi4, for example, is activated by Swi6 at the M/G1 transition, and later shut down by Mcm1 in combination with Yox1 and Yhp1 (Breeden and Mikesell 1991; Foster *et al.* 1993; McInerny *et al.* 1997; Pramila *et al.* 2002).

The concentration of Gcn4 in the cell is controlled by numerous mechanisms, one of which is alteration in the rate of translation. The eukaryotic translation initiation factor 2 (eIF-2) is phosphorylated upon amino acid starvation, leading to an increase in the amount of Gcn4 protein synthesized. Mutation of the phosphorylated eIF-2 serine to an alanine ablates this increase in Gcn4. Conversely, a serine to aspartate mutation to mimic phosphorylation derepresses Gcn4 regardless of the amino acid content of the growth media (Dever *et al.* 1992). This regulation of Gcn4 occurs through small open reading frames occurring upstream of the start of the major exon, called uORFs (Hinnebusch 1984; Hinnebusch 1994). Ribosomes initiate translation at the first uORF. In the presence of unphosphorylated eIF-2 the ribosomes that continue scanning downstream of uORF1 are able to re-initiate translation of the downstream uORFs, particularly uORF4, which then blocks initiation at the Gcn4 coding region. The phosphorylated eIF-2 is unable to re-initiate as quickly. Therefore it cannot translate uORF4, but rather starts translation of Gcn4. Yap1 and Yap2 are also regulated through uORFs (Vilela *et al.* 1998). The Yap1 uORF is comparable to the Gcn4 uORF1, allowing leaky re-initiation of translation as the ribosomes scan through the mRNA. In contrast, the uORFs in the Yap2 leader sequence post a strong block to translation of the Yap2 ORF like the Gcn4 uORF4, and also mediate accelerated decay of the mRNA. How stress conditions increase synthesis of the Yap regulators when needed has not yet been described, but it seems likely that these uORFs will be involved, as they are for Gcn4.

Regulation of transcription factors by phosphorylation seems to be one of the most common mechanisms by which activity is controlled, based upon the number of factors that show alterations in phosphorylation state (Yeast Proteome

Database, (Costanzo *et al.* 2000; Csank *et al.* 2002)). This is because a change in phosphorylation state, as well as potentially modifying the DNA-binding properties of a transcription factor or activity of its activation domain, can also be the signal for regulation by another mechanism, such as nuclear exclusion or protein degradation.

The DNA-binding ability of the transcriptional repressor Rgt1, involved in regulation of glucose transporters, is modulated by the its phosphorylation state, which is dependent on the carbohydrate source in the growth medium (Kim *et al.* 2003; Mosley *et al.* 2003). In high glucose conditions, Rgt1 is hyperphosphorylated and unable to bind to DNA, and the hexose transport genes regulated by Rgt1 are expressed. On the other hand, in low glucose conditions the phosphates are removed and DNA binding and repression of the hexose transporters takes place. The observation was strengthened by assays showing that removal of the serine residues that undergo phosphorylation induces constitutive DNA binding and repression of the transporters. Other transcription factors that are regulated in this manner include Crt1, involved in DNA damage response (Huang *et al.* 1998), and Mac1, which regulates intracellular copper levels (Heredia *et al.* 2001).

Regulation of the level of activity of transcriptional activation domains by phosphorylation has been proposed to occur by altering the protein-protein contacts the activation domain is able to participate in. In one example, Pho4 is unable to bind to its partner Pho2 when a particular serine in the Pho4 activation domain is phosphorylated (Komeili and O'Shea 1999). Phosphorylation of Pho2 likewise inhibits the Pho4 – Pho2 interaction (Liu *et al.* 2000). The interaction between cell cycle regulators Fkh2 and Ndd1 is similarly affected by activation domain phosphorylation, altering the ability to Fkh2 activate gene expression (Darieva *et al.* 2003). Phosphorylation of activation domains has also been postulated to help with interactions of transcription factors with the general transcription machinery by increasing the negative charge / acidity of the activation domain (Struhl 1995), but no clear examples of this mechanism for alteration in activation function have been found.

Phosphorylation of transcription factors also affects interactions with nuclear import and export proteins. Pho4 contains five phosphorylation sites, one of which, as mentioned above, affects its interaction with Pho2. Phosphorylation at two of the remaining four sites is required for interaction with Msn5, the β-importin family member that exports Pho4 from the nucleus. Dephosphorylation at yet another site is required for interaction with Pse1, the β-importin that imports Pho4 (Komeili and O'Shea 1999). The subcellular localization of transcription factors is almost always signaled by a change in phosphorylation state – other examples include Msn2/4, Gat1, Gln3 (Beck and Hall 1999), Sko1 (Pascual-Ahuir *et al.* 2001) and cell cycle factors Ace2 and Swi5 (Nasmyth *et al.* 1990; Moll *et al.* 1991; O'Conallain *et al.* 1999), to name just a few.

Gal4 provides an example of a transcription factor regulated by direct repression through additional proteins. This transcription factor provides a switch highly sensitive to the carbohydrate source available to the cell. In media where glucose is present, GAL genes are transcriptionally inert, repressed by Mig1 (Nehlin *et al.* 1991). Under non-inducing, non-repressing conditions, such as growth in glycerol, Gal4 is expressed and binds in a complex with Gal80 to the promoters containing its cognate sequence (Leuther and Johnston 1992). Gal80 is thought to block access of the transcriptional machinery to the Gal4 activation domain. When the cell is grown in galactose, Gal3 binds to Gal80, likely inducing a conformational change that exposes the Gal4 activation domain, thus allowing the recruitment of TBP and TFIID, and activated transcription of the GAL genes (Suzuki-Fujimoto *et al.* 1996; Wu *et al.* 1996)

Regulation through the presence or absence of a small molecule was the first model described for regulation of a transcription factor in bacteria (Jacob and Monod 1961). Sensing of cellular conditions through binding of an intermediate in a biosynthetic pathway or binding of another small molecule to a transcription factor provides a sensitive mechanism for the cell to regulate gene transcription based on extracellular conditions. One example of the effects of small molecules is the regulation of DNA binding of Ace1 and Mac1 by copper ions. This allows the cell to tightly regulate the amount of copper and other metal ions available (Furst and Hamer 1989; Heredia *et al.* 2001). As the amount of heavy metal in the cell decreases, these factors can bind to DNA, activating transcription of heavy metal transporters. This increases the intracellular concentration of these ions, restoring the required balance. Another transcription factor regulated similarly is Leu3, which is regulated by the presence of α-Isopropylmalate, one of the intermediates in the biosynthesis of leucine (Sze *et al.* 1992). If the intermediate is present, Leu3 binds to the promoters of leucine synthesis genes but is transcriptionally inactive. As the levels of intermediate drop, the conformation of the activation domain changes such that components of the general transcription machinery are recruited (Sze *et al.* 1992). A final example of regulation through binding of a small molecule is illustrated by the interaction of the regulator Hap1 with heme. In the absence of heme Hap1 forms a high molecular weight complex and is sequestered from DNA through interactions with other proteins like Hsp90, showing yet another possible mechanism of regulation of regulators (Zhang and Guarente 1994; Zhang *et al.* 1998). The activation domain of Hap1 is also regulated by the presence of heme, through a heme responsive domain (Zhang and Guarente 1995). In this case, binding of heme seems to block the binding site for an additional repressive protein, perhaps Hsp90 (Zhang *et al.* 1998).

Changes that activate transcription factors to begin recruitment of the transcriptional machinery must be reversible in order that transcription be shut down when the stimuls is no longer present. An elegant model for how this is accomplished has been described (Chi *et al.* 2001; Tansey 2001; Ansari *et al.* 2002). Srb10, a component of the RNA polymerase II holoenzyme phosphorylates the transcription factor Gcn4 as the activator and holoenzyme make contacts at the promoter. This phosphorylation then leads to ubiquitination of the marked Gcn4 molecule, which is then degraded by the 26S proteasome. Similarly, Msn2 is phosphorylated by Srb10 during their interaction at the promoter, leading to the subsequent export of Msn2 from the nucleus. Using the time during which the transcriptional machinery is contacting the transcription factor to mark that factor for subsequent removal from the cell ensures that activation of a gene does not outlive the need for the gene product. Ste12 is also regulated in this manner (Nelson *et al.* 2003), and Gal4 and Sip4 are likewise phosphorylated by Srb10, although in these cases the phosphorylation is associated with activation of the transcription factors rather than degradation or removal (Sadowski *et al.* 1996; Hirst *et al.* 1999; Vincent *et al.* 2001).

Microarray technologies and the analysis of transcription

Many of the experiments used to elucidate the mechanisms and ideas described so far were performed on the level of a single gene. *In vitro* assays were used to determine the components necessary for transcription, and how different complexes were interacting with one another. *In vivo*, reporter constructs were used to test activation at a single promoter, or expression levels of individual genes were tested using Northern or Western blots. However, since the *S. cerevisiae* genome was fully sequenced (Goffeau *et al.* 1996), new technologies have been developed that enable us to ask similar questions, on a genome-wide scale.

The first technology to be developed was analysis of the mRNA expression level of essentially all *S. cerevisiae* genes using DNA microarrays (Schena *et al.* 1995; Shalon *et al.* 1996). These microarrays consist of cDNAs corresponding to each gene printed in spots on glass slides, or oligonucleotides synthesized *in situ* or printed on slides (Pease *et al.* 1994; Lipshutz *et al.* 1995).

Total RNA or purified mRNA is used as a template for reverse transcription with nucleotides conjugated to a fluorescent moiety, and the resulting labeled DNA is hybridized to the array. Genes of interest are found by scanning the array with a laser, using software to find spots and quantitate the level of fluorescence in each spot, and performing statistical manipulations and tests (Bowtell 1999; Holloway *et al.* 2002)

This technology has been used to gain many insights into the global transcriptional regulation in the cell. Initial experiments were focused on the changes in gene expression during a cellular process, or following an environmental perturbation. For example, some of the earliest studies used various methods to synchronize yeast cells in the cell cycle, then examined the transcriptional readout as the cells progressed through the cycle (Cho *et al.* 1998; Spellman *et al.* 1998). In another series of experiments, effects of various environmental stresses on the cell, including changes in pH, high salt concentration, oxidative and osmotic stress, and nutrient deprivation were examined (Gasch *et al.* 2000; Causton *et al.* 2001). These results highlighted the global changes in gene expression effected by transcription factors. In another landmark study, the effects on gene expression of over three hundred perturbations, including knockouts of selected genes such as transcription factors, were profiled (Hughes *et al.* 2000b). In these experiments, as well as others performed with transcription factor knockouts, a surprisingly small number of the genes with changes in expression contain a recognizable DNA binding site for the particular transcription factor being studied. This highlights the extent of secondary effects that occur based on the regulation of additional transcription factors, as well as other proteins involved in transcriptional regulation. Large surveys like these are complemented by hundreds of experiments in which microarrays have been used to elucidate components of individual pathways and understand the transcriptional regulation of those pathways (Ogawa *et al.* 2000; Natarajan *et al.* 2001; Agarwal *et al.* 2003; Schuller *et al.* 2004).

Aside from studying the downstream effects of transcriptional regulation, microarrays have also been used to analyze the cellular requirements for components of the general transcriptional apparatus. In a seminal study, the function of subunits of each of the main complexes involved in transcriptional initiation was abolished either through a knockout, a point mutation, or a temperature sensitive mutation (Holstege *et al.* 1998). The genome-wide requirement for each complex was then assessed. Many of the observations made about the function of these complexes at individual genes were confirmed and extended. For example, because of the equivalent requirement for RNA polymerase II and the Srb4 component of the Srb/Mediator complex at almost every gene, previous assertions about the *in vivo* requirement for the holoenzyme rather than just the polymerase were confirmed (Holstege *et al.* 1998). The kinase subunit of the GTF TFIIH was also required at almost every gene, giving added credence to the hypothesis that phosphorylation of RNA polymerase is required for transcriptional initiation to proceed to elongation. The genome-wide nature of these experiments also enables conclusions about transcriptional regulation that cannot be drawn from studies at a single gene.

Micoarray analysis of mutants of essential subunits of the chromatin remodeler Swi/Snf showed that nucleosome perturbations were localized to individual genes rather than chromosomal domains (Sudarsanam *et al.* 2000).

A technique that brings together the direct study of the targets of transcriptional components with DNA microarrays is variously termed "Chromatin-immunoprecipitation on a Chip", "ChIP-Chip", and "Genome-wide location analysis" (Ren *et al.* 2000; Iyer *et al.* 2001). When these experiments are performed in yeast, typically an epitope tag is introduced into the genomic locus of the protein of interest (Knop *et al.* 1999). The cells containing the epitope tagged factor are grown under a relevant condition, proteins are crosslinked to DNA using formaldehyde, and sonicated to shear chromatin. The cellular extract is then subjected to immunoprecipitation using an antibody to the epitope tag. Alternatively, an antibody raised directly against the protein can be used. This immunoprecipitation selectively purifies the factor of interest, along with any DNA to which it may be binding. The DNA is then amplified in the presence of fluorescently labeled nucleotides, genomic DNA from the same strain is labeled with a different fluorophore, and the two samples are hybridized to a microarray containing yeast intergenic regions. Statistical analyses (Hughes *et al.* 2000b; Lee *et al.* 2002) are used to assign a significance of enrichment to each spot, and spots with a high significance are likely to be bound by the protein of interest.

Genome-wide location analysis has led to a better understanding of the transcriptional regulation underlying the yeast cell cycle. The promoters bound by the complexes SBF, consisting of Swi4 and Swi6, and MBF, comprising Mbp1 and Swi6, were examined in one study (Iyer *et al.* 2001). Approximately 200 novel targets for these factors were found, and the genes downstream of these promoters were enriched for the functions that take place during the G1 phase of the cell cycle, when these factors are active. Sixteen of the promoters to which SBF and MBF were binding putatively regulate transcription factors. Genome-wide location analysis was performed on nine of these additional transcription factors, and these were found to bind to a number of genes performing additional functions necessary for the G1 phase (Horak *et al.* 2002). This idea of transcription factor cascades was delineated in another study, where the nine known cell cycle transcription factors were profiled (Simon *et al.* 2001). The transcriptional regulation of the entire cell cycle was shown to be cyclical, with transcription factors from each stage regulating the transcription of regulators from the next stage, as well as other signaling molecules that stimulate and shut down the activation function of these regulators. Additional transcriptional regulators regulating the cell cycle, as well as an increased number of genes regulated by the cell cycle transcription factors were found by using an algorithm to combine expression and location data (Lee *et al.* 2002; Bar-Joseph *et al.* 2003).

In an approach highly complementary to location analysis, binding of transcription factors and other sequence specific DNA binding proteins to microarrays has been used to analyze the specificity of that binding. Initial studies demonstrated sequence specific binding and cutting by restriction enzymes on DNA immobilized on an array (Bulyk *et al.* 1999). Subsequently, the sequence specificities of a number of zinc finger mutants were assessed (Bulyk *et al.* 2001). The second zinc finger of a three finger mouse transcription factor was mutagenized, and displayed on a phage. Binding of this protein to oligonucleotides containing all 64 variants of the second finger's binding sites was tested. Interestingly, different mutants showed varying levels of selectivity: some mutants were only able to bind to one or two of the sixty four oligonucleotides, while others were able to bind to almost all of the oligonucleotide variants. Finally, tagged transcription factors were bound directly to arrays containing most yeast intergenic regions to find all sequences to which DNA binding by the factors was possible (Mukherjee *et al.* 2004). A direct comparison with location data for the same factors (Lee *et al.* 2002) showed that while there was significant overlap in the intergenic regions bound for two out of three factors, there were intergenic regions that were bound only in one of the two assays (Mukherjee *et al.* 2004). Promoters at which these differences were seen could be used to study the different determinants of binding in the *in vitro* versus *in vivo* situations.

Each of these sources of microarray data has been used computationally to attempt to determine the actual sequence specificity of the gene-specific transcription factors. Prior to the advent of the microarray technologies, the sequence motifs to which a few factors bound had been painstakingly identified. The first motifs were defined in the bacterium E. coli by searching for patterns by eye: the sequence to which the lambda repressor binds (Maniatis *et al.* 1975), and the –10 promoter element (Pribnow 1975). In *S. cerevisiae*, the consensus motif for the transcription factor Hsf1 was one of the first to be identified (Pelham 1982). The DNA sequence necessary for upregulation of the heat shock protein Hsp70 by Hsf1 was narrowed down to a 20 base pair stretch by deletion analysis, then a palindromic sequence within this region was also found upstream of several other genes regulated by Hsf1. It was quickly realized that unlike restriction enzymes, the sequence specificity of transcription factors was much less stringent (Pribnow 1975). This led to the use of Position Weight Matrices (PWMs) to account for the variable affinity of transcription factor proteins for variations in their binding sites (Stormo 2000).

Microarray data were initially used to find consensus sequences or PWMs by clustering genes with similar expression patterns then searching for sequence motifs, based on the assumption that the similar expression patterns were effected by the same regulators (DeRisi *et al.* 1997; Brazma *et al.* 1998; Roth *et al.* 1998; Spellman *et al.* 1998; van Helden *et al.* 1998). Since then, many other methods have been developed or used to take advantage of these data, using varied statistical sampling to determine sensitivity and specificity. These include expectation-maximization (EM) (Lawrence and Reilly 1990; Spellman *et al.* 1998), and Gibbs sampling (Hughes *et al.* 2000a; Liu *et al.* 2001). The inclusion of additional data sources, such as location data, has been used to further narrow the set of input sequences to raise the signal to noise ratio of the site specific sequence within the background random DNA (Ren *et al.* 2000; Iyer *et al.* 2001; Simon *et al.* 2001; Lee *et al.* 2002; Liu *et al.* 2002; Zeitlinger *et al.* 2003;

Harbison *et al.* 2004). The sequencing of multiple similar species has added an additional constraint, making motif finding easier with the assumption that the nucleotides important for transcription factor binding will be more conserved between species than nucleotides without constraint on mutation (Cliften *et al.* 2003; Kellis *et al.* 2003; Harbison *et al.* 2004).

The knowledge of the sequence specificity of transcription factors allows prediction of genes regulated by the factor, and provides an important component of any network model of the cell. While large leaps in the ability to do this have been achieved over the past decade as described above, we are still far from a complete understanding of the phenomenon. For example, transcriptional activators seem to bind to and regulate genes where no sequence motif for the factor can be found (Lieb *et al.* 2001; Lee *et al.* 2002). Conversely, there are hundreds of copies of factor specific motifs throughout the genome, many of which are not bound by the site's cognate transcription factor (Lieb *et al.* 2001; Harbison *et al.* 2004). And for a large percentage of additional genes, no site specific regulator can be found (Harbison *et al.* 2004).

The holy grail in the study of transcriptional regulation is to determine the complete network of interactions that drives gene expression in the living cell. Each of the microarray technologies described provides information towards attaining this goal. Expression analysis provides a functional readout of the results of the transcriptional regulation, but with expression data one cannot deconvolute direct effects of transcription factor binding from indirect effects. Location analysis complements the expression data by providing the information about which genes are likely to be the direct targets of a transcription factor. This method has also been used to examine the regulation of transcription at other levels. Histone density (D. Pokholok, unpublished data), chromatin modifications (Kurdistani *et al.* 2002; Ng *et al.* 2002; Robyr *et al.* 2002; Wang *et al.* 2002; Ng *et al.* 2003), and the promoters at which other components of the complete transcription apparatus are acting ((Odom *et al.* 2004), D. Pokholok, unpublished data) have all been assayed using location analysis. Binding of transcription factors directly to arrays, particularly arrays containing intergenic regions, allows determination of every possible genomic sequence to which a particular factor can bind. The derived sequence specificity of these factors based on these data will also play a part in the construction of a complete network model, along with additional high throughput data sources. The *in vivo* subcellular localization of most yeast proteins (Huh *et al.* 2003), systematic analysis of synthetic lethality between genes (Tong *et al.* 2004), and high-throughput analysis of protein complexes using mass spectrometry (Krogan *et al.* 2004) all provide a wealth of additional data towards the goal.

My contributions to this work

When I started my graduate studies, I was interested in doing both experimental and computational work. At that time, microarray analysis of gene expression was in full swing, opening exciting avenues to explore many aspects of biology. The number of experiments using expression microarrays was exploding, and the technique of genome-wide location analysis was being developed for publication in Ren *et al.* 2000. Both of these experimental approaches generate vast amounts of data, but in 1999 computational support and analytical methods were in their infancy and still required development and refinement. I joined the Young lab because their studies with microarrays afforded me the opportunity to work both at the bench and on the computer.

My initial contribution to the lab came in the computational arena, when I set up a public website for Itamar Simon's paper on the cell-cycle. This website was among the first to make data from genome-wide studies publicly available and searchable. Through this open approach, I set up a forum to not only share our data, but also to address the need to standardize microarray data and the analytical approaches in this burgeoning field.

My first summer, I started performing location analysis experiments with the aim of elucidating networks of interactions between transcription factors. I was interested in several fundamental metabolic systems; my first experiments were performed with factors involved in glucose metabolism, Rgt1, Mth1 and Mig1 and I followed up by investigating factors from the nitrogen and phosphate metabolic pathways. These experiments yielded a wealth of data which led me to believe that the approach which would most benefit the yeast community consisted of profiling as many factors as possible. I decided to work as a team with several other investigators in the Young lab, synergizing our efforts to perform hundreds of chips worth of location-analysis experiments.

I also focused on improving the analysis of the data we were acquiring. This analysis occurred on two levels: basic analysis for each experiment to determine which intergenic regions were bound by the profiled transcription factor, and then meta-analyses to gain a deeper understanding of the overall biology. The framework of the analysis for each experiment had been laid out in Ren *et al.* (2001), based on an error model described for gene expression data in Hughes TR *et al.* (2000). I expanded significantly on this model. I put in place a series of templates for use by researchers for analyzing individual experiments, making them as versatile and user-friendly as possible. In addition, I wrote scripts to perform batch analyses for making comparisons between data sets, a key step to integrate data from different experiments and expand comparative analyses. I also took advantage of the large number of data sets that we had generated to help with noise reduction (see discussion in chapter 2). Another of my computational contributions consisted of a number of meta-analyses, which ranged from determining various statistics about the data in general to collaborating with Tony Lee to determine the definitions of the network motifs. Based on these analyses, I developed computational tools such as a series of scripts for finding network motifs. The results from the genome-wide location analysis, interpreted using the tools I developed, was published (Lee, Rinaldi, Robert *et al.* 2002), and is included in chapter 3.

I next extended our results, by addressing one of the weaknesses of our approach. Looking at transcription under a single condition, in most cases the rich medium Yeast extract – Peptone – Dextrose, affords a snapshot of the cell's function. It does not, however, help to understand the most important role of the cellular machinery in the life cycle of that cell: adapting to changes. Cells constantly need to adjust the amount of metabolic enzymes based on the available nutrients, to respond to cellular damage caused by external forces, and to remove toxic elements from the cell. The work we published in Lee *et al.* did not investigate cellular adaptation. We were able to find transcription factors interacting with the promoters of only approximately 30% of the genome. Additionally, although we did use expression data from conditions in which the external environment was manipulated, without the matching binding data it was difficult to assess which promoter-transcription factor interactions were leading to productive regulation. We described regulatory network motifs based on the binding data in rich media, but could not determine which of the motifs were essential to transcription of the genes involved, as opposed to those that might be used in another condition. To remedy this, location analysis under 12 additional conditions on a number of factors was performed. I focused mainly on the experiments in hydrogen peroxide, and performed the data analysis for the >400 experiments we had accumulated. These data and analyses were published (Harbison *et al.* 2004) and are included in chapter 4.

In order to further improve the analysis of microarray data, I collaborated with Ron Dror and Jon Murnick on a project that originated in the "Computational Functional Genomics" course taught by Rick Young, David Gifford and Tommi Jaakkola. We were concerned with the practice that was common at the time of "flooring" Affymetrix intensity results to an arbitrary small number, and of averaging repeated measurements without regard for their reliability. Instead, we devised a noise model to incorporate both additive and multiplicative noise introduced throughout the experiments, and used a Bayesian estimation method to provide a principled way of dealing with negative results, combining repeated measurements, and determining differentially expressed genes. This work was published as Dror *et al.* 2003. I also took part in the development of the Genetic RegulAtory Modules algorithm, performing the large scale analysis of location data that was required, collecting and curating over 500 expression data sets from publicly available sources, and participating in a series of discussions during the development. This work was published (Bar-Joseph *et al.* 2003), and can be found in Appendix A.

Through my research and reading I have come to understand many of the nuances involved in the various steps necessary for thorough analysis of genome-wide data. My experience with both bench work and computational analysis has allowed me a well-rounded view of the field of genomics. I have included, in Chapter 2 of this thesis, many of my findings and observations in this arena.

References

Adams CC and Workman JL (1995). "Binding of disparate transcriptional activators to nucleosomal DNA is inherently cooperative." Mol Cell Biol **15**(3): 1405-21.

Agarwal AK, Rogers PD, Baerson SR, Jacob MR, Barker KS, Cleary JD, Walker LA, Nagle DG and Clark AM (2003). "Genome-wide expression profiling of the response to polyene, pyrimidine, azole, and echinocandin antifungal agents in Saccharomyces cerevisiae." J Biol Chem **278**(37): 34998-5015.

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

Angermayr M and Bandlow W (2003). "Permanent nucleosome exclusion from the Gal4p-inducible yeast GCY1 promoter." J Biol Chem **278**(13): 11026-31.

Ansari AZ, Koh SS, Zaman Z, Bongards C, Lehming N, Young RA and Ptashne M (2002). "Transcriptional activating regions target a cyclin-dependent kinase." Proc Natl Acad Sci U S A **99**(23): 14706-9.

Axelrod JD, Reagan MS and Majors J (1993). "GAL4 disrupts a repressing nucleosome during activation of GAL1 transcription in vivo." Genes Dev **7**(5): 857-69.

Bannister AJ and Miska EA (2000). "Regulation of gene expression by transcription factor acetylation." Cell Mol Life Sci **57**(8-9): 1184-92.

Barberis A, Pearlberg J, Simkovich N, Farrell S, Reinagel P, Bamdad C, Sigal G and Ptashne M (1995). "Contact with a component of the polymerase II holoenzyme suffices for gene activation." Cell **81**(3): 359-68.

Bar-Joseph Z, Gerber GK, Lee TI, Rinaldi NJ, *et al.* (2003). "Computational discovery of gene modules and regulatory networks." Nat Biotechnol **21**(11): 1337-42.

Beck T and Hall MN (1999). "The TOR signalling pathway controls nuclear localization of nutrient-regulated transcription factors." Nature **402**(6762): 689-92.

Bowtell DD (1999). "Options available--from start to finish--for obtaining expression data by microarray." Nat Genet **21**(1 Suppl): 25-32.

Brazma A, Jonassen I, Vilo J and Ukkonen E (1998). "Predicting gene regulatory elements in silico on a genomic scale." Genome Res **8**(11): 1202-15.

Breeden L and Mikesell GE (1991). "Cell cycle-specific expression of the SWI4 transcription factor is required for the cell cycle regulation of HO transcription." Genes Dev **5**(7): 1183-90.

Bulyk ML, Gentalen E, Lockhart DJ and Church GM (1999). "Quantifying DNA-protein interactions by double-stranded DNA arrays." Nat Biotechnol **17**(6): 573-7.

Bulyk ML, Huang X, Choo Y and Church GM (2001). "Exploring the DNA-binding specificities of zinc fingers with DNA microarrays." Proc Natl Acad Sci U S A **98**(13): 7158-63.

Bushnell DA and Kornberg RD (2003). "Complete, 12-subunit RNA polymerase II at 4.1-A resolution: implications for the initiation of transcription." Proc Natl Acad Sci U S A **100**(12): 6969-73.

Bushnell DA, Westover KD, Davis RE and Kornberg RD (2004). "Structural basis of transcription: an RNA polymerase II-TFIIB cocrystal at 4.5 Angstroms." Science **303**(5660): 983-8.

Causton HC, Ren B, Koh SS, Harbison CT, *et al.* (2001). "Remodeling of yeast genome expression in response to environmental changes." Mol Biol Cell **12**(2): 323-37.

Chatterjee S and Struhl K (1995). "Connecting a promoter-bound protein to TBP bypasses the need for a transcriptional activation domain." Nature **374**(6525): 820-2.

Chi Y, Huddleston MJ, Zhang X, Young RA, Annan RS, Carr SA and Deshaies RJ (2001). "Negative regulation of Gcn4 and Msn2 transcription factors by Srb10 cyclin-dependent kinase." Genes Dev **15**(9): 1078-92.

Cho HS, Liu CW, Damberger FF, Pelton JG, Nelson HC and Wemmer DE (1996). "Yeast heat shock transcription factor N-terminal activation domains are unstructured as probed by heteronuclear NMR spectroscopy." Protein Sci **5**(2): 262-9.

Cho RJ, Campbell MJ, Winzeler EA, Steinmetz L, *et al.* (1998). "A genome-wide transcriptional analysis of the mitotic cell cycle." Mol Cell **2**(1): 65-73.

Cirillo LA and Zaret KS (1999). "An early developmental transcription factor complex that is more stable on nucleosome core particles than on free DNA." Mol Cell **4**(6): 961-9.

Cliften P, Sudarsanam P, Desikan A, Fulton L, Fulton B, Majors J, Waterston R, Cohen BA and Johnston M (2003). "Finding functional features in Saccharomyces genomes by phylogenetic footprinting." Science **301**(5629): 71-6.

Cosma MP (2002). "Ordered recruitment: gene-specific mechanism of transcription activation." Mol Cell **10**(2): 227-36.

Cosma MP, Tanaka T and Nasmyth K (1999). "Ordered recruitment of transcription and chromatin remodeling factors to a cell cycle- and developmentally regulated promoter." Cell **97**(3): 299-311.

Costanzo MC, Hogan JD, Cusick ME, Davis BP, *et al.* (2000). "The yeast proteome database (YPD) and Caenorhabditis elegans proteome database (WormPD): comprehensive resources for the organization and comparison of model organism protein information." Nucleic Acids Res **28**(1): 73-6.

Csank C, Costanzo MC, Hirschman J, Hodges P, *et al.* (2002). "Three yeast proteome databases: YPD, PombePD, and CalPD (MycoPathPD)." Methods Enzymol **350**: 347-73.

Darieva Z, Pic-Taylor A, Boros J, Spanos A, Geymonat M, Reece RJ, Sedgwick SG, Sharrocks AD and Morgan BA (2003). "Cell cycle-regulated transcription through the FHA domain of Fkh2p and the coactivator Ndd1p." Curr Biol **13**(19): 1740-5.

DeRisi JL, Iyer VR and Brown PO (1997). "Exploring the metabolic and genetic control of gene expression on a genomic scale." Science **278**(5338): 680-6.

Dever TE, Feng L, Wek RC, Cigan AM, Donahue TF and Hinnebusch AG (1992). "Phosphorylation of initiation factor 2 alpha by protein kinase GCN2 mediates gene-specific translational control of GCN4 in yeast." Cell **68**(3): 585-96.

Donaldson L and Capone JP (1992). "Purification and characterization of the carboxyl-terminal transactivation domain of Vmw65 from herpes simplex virus type 1." J Biol Chem **267**(3): 1411-4.

Ellenberger TE, Brandl CJ, Struhl K and Harrison SC (1992). "The GCN4 basic region leucine zipper binds DNA as a dimer of uninterrupted alpha helices: crystal structure of the protein-DNA complex." Cell **71**(7): 1223-37.

Emili A and Ingles CJ (1995). "Promoter-dependent photocross-linking of the acidic transcriptional activator E2F-1 to the TATA-binding protein." J Biol Chem **270**(23): 13674-80.

Farrell S, Simkovich N, Wu Y, Barberis A and Ptashne M (1996). "Gene activation by recruitment of the RNA polymerase II holoenzyme." Genes Dev **10**(18): 2359-67.

Feaver WJ, Gileadi O, Li Y and Kornberg RD (1991). "CTD kinase associated with yeast RNA polymerase II initiation factor b." Cell **67**(6): 1223-30.

Foster R, Mikesell GE and Breeden L (1993). "Multiple SWI6-dependent cis-acting elements control SWI4 transcription through the cell cycle." Mol Cell Biol **13**(6): 3792-801.

Furst P and Hamer D (1989). "Cooperative activation of a eukaryotic transcription factor: interaction between Cu(I) and yeast ACE1 protein." Proc Natl Acad Sci U S A **86**(14): 5267-71.

Gasch AP, Spellman PT, Kao CM, Carmel-Harel O, Eisen MB, Storz G, Botstein D and Brown PO (2000). "Genomic expression programs in the response of yeast cells to environmental changes." Mol Biol Cell **11**(12): 4241-57.

Ge H, Zhao Y, Chait BT and Roeder RG (1994). "Phosphorylation negatively regulates the function of coactivator PC4." Proc Natl Acad Sci U S A **91**(26): 12691-5.

Goffeau A, Barrell BG, Bussey H, Davis RW, *et al.* (1996). "Life with 6000 genes." Science **274**(5287): 546, 563-7.

Gonzalez-Couto E, Klages N and Strubin M (1997). "Synergistic and promoter-selective activation of transcription by recruitment of transcription factors TFIID and TFIIB." Proc Natl Acad Sci U S A **94**(15): 8036-41.

Goodrich JA and Tjian R (1994). "Transcription factors IIE and IIH and ATP hydrolysis direct promoter clearance by RNA polymerase II." Cell **77**(1): 145-56.

Hahn S (2004). "Structure and mechanism of the RNA polymerase II transcription machinery." Nat Struct Mol Biol **11**(5): 394-403.

Harbison CT, Gordon DB, Lee TI, Rinaldi NJ, *et al.* (2004). "Transcriptional regulatory code of a eukaryotic genome." Nature **431**(7004): 99-104.

Harrison CJ, Bohm AA and Nelson HC (1994). "Crystal structure of the DNA binding domain of the heat shock transcription factor." Science **263**(5144): 224-7.

Heredia J, Crooks M and Zhu Z (2001). "Phosphorylation and Cu+ coordination-dependent DNA binding of the transcription factor Mac1p in the regulation of copper transport." J Biol Chem **276**(12): 8793-7.

Hinnebusch AG (1984). "Evidence for translational regulation of the activator of general amino acid control in yeast." Proc Natl Acad Sci U S A **81**(20): 6442-6.

Hinnebusch AG (1994). "Translational control of GCN4: an in vivo barometer of initiation-factor activity." Trends Biochem Sci **19**(10): 409-14.

Hirst M, Kobor MS, Kuriakose N, Greenblatt J and Sadowski I (1999). "GAL4 is regulated by the RNA polymerase II holoenzyme-associated cyclin-dependent protein kinase SRB10/CDK8." Mol Cell **3**(5): 673-8.

Holloway AJ, van Laar RK, Tothill RW and Bowtell DD (2002). "Options available--from start to finish--for obtaining data from DNA microarrays II." Nat Genet **32 Suppl**: 481-9.

Holstege FC, Jennings EG, Wyrick JJ, Lee TI, Hengartner CJ, Green MR, Golub TR, Lander ES and Young RA (1998). "Dissecting the regulatory circuitry of a eukaryotic genome." Cell **95**(5): 717-28.

Horak CE, Luscombe NM, Qian J, Bertone P, Piccirrillo S, Gerstein M and Snyder M (2002). "Complex transcriptional circuitry at the G1/S transition in Saccharomyces cerevisiae." Genes Dev **16**(23): 3017-33.

Huang M, Zhou Z and Elledge SJ (1998). "The DNA replication and damage checkpoint pathways induce transcription by inhibition of the Crt1 repressor." Cell **94**(5): 595-605.

Hughes JD, Estep PW, Tavazoie S and Church GM (2000a). "Computational identification of cis-regulatory elements associated with groups of functionally related genes in Saccharomyces cerevisiae." J Mol Biol **296**(5): 1205-14.

Hughes TR, Marton MJ, Jones AR, Roberts CJ, *et al.* (2000b). "Functional discovery via a compendium of expression profiles." Cell **102**(1): 109-26.

Huh WK, Falvo JV, Gerke LC, Carroll AS, Howson RW, Weissman JS and O'Shea EK (2003). "Global analysis of protein localization in budding yeast." Nature **425**(6959): 686-91.

Imbalzano AN, Kwon H, Green MR and Kingston RE (1994). "Facilitated binding of TATA-binding protein to nucleosomal DNA." Nature **370**(6489): 481-5.

Iyer V and Struhl K (1995). "Poly(dA:dT), a ubiquitous promoter element that stimulates transcription via its intrinsic DNA structure." Embo J **14**(11): 2570-9.

Iyer VR, Horak CE, Scafe CS, Botstein D, Snyder M and Brown PO (2001). "Genomic binding sites of the yeast cell-cycle transcription factors SBF and MBF." Nature **409**(6819): 533-8.

Jacob F and Monod J (1961). "Genetic regulatory mechanisms in the synthesis of proteins." J Mol Biol **3**: 318-56.

Jaskelioff M, Gavin IM, Peterson CL and Logie C (2000). "SWI-SNF-mediated nucleosome remodeling: role of histone octamer mobility in the persistence of the remodeled state." Mol Cell Biol **20**(9): 3058-68.

Joliot V, Demma M and Prywes R (1995). "Interaction with RAP74 subunit of TFIIF is required for transcriptional activation by serum response factor." Nature **373**(6515): 632-5.

Kelleher RJ, 3rd, Flanagan PM and Kornberg RD (1990). "A novel mediator between activator proteins and the RNA polymerase II transcription apparatus." Cell **61**(7): 1209-15.

Kellis M, Patterson N, Endrizzi M, Birren B and Lander ES (2003). "Sequencing and comparison of yeast species to identify genes and regulatory elements." Nature **423**(6937): 241-54.

Kim JH, Polish J and Johnston M (2003). "Specificity and regulation of DNA binding by the yeast glucose transporter gene repressor Rgt1." Mol Cell Biol **23**(15): 5208-16.

Kim YJ, Bjorklund S, Li Y, Sayre MH and Kornberg RD (1994). "A multiprotein mediator of transcriptional activation and its interaction with the C-terminal repeat domain of RNA polymerase II." Cell **77**(4): 599-608.

Klages N and Strubin M (1995). "Stimulation of RNA polymerase II transcription initiation by recruitment of TBP in vivo." Nature **374**(6525): 822-3.

Knop M, Siegers K, Pereira G, Zachariae W, Winsor B, Nasmyth K and Schiebel E (1999). "Epitope tagging of yeast genes using a PCR-based strategy: more tags and improved practical routines." Yeast **15**(10B): 963-72.

Kobayashi N, Boyer TG and Berk AJ (1995). "A class of activation domains interacts directly with TFIIA and stimulates TFIIA-TFIID-promoter complex assembly." Mol Cell Biol **15**(11): 6465-73.

Koleske AJ and Young RA (1994). "An RNA polymerase II holoenzyme responsive to activators." Nature **368**(6470): 466-9.

Komeili A and O'Shea EK (1999). "Roles of phosphorylation sites in regulating activity of the transcription factor Pho4." Science **284**(5416): 977-80.

Kornberg RD (1998). "Mechanism and regulation of yeast RNA polymerase II transcription." Cold Spring Harb Symp Quant Biol **63**: 229-32.

Krogan NJ, Peng WT, Cagney G, Robinson MD, *et al.* (2004). "High-definition macromolecular composition of yeast RNA-processing complexes." Mol Cell **13**(2): 225-39.

Kurdistani SK, Robyr D, Tavazoie S and Grunstein M (2002). "Genome-wide binding map of the histone deacetylase Rpd3 in yeast." Nat Genet **31**(3): 248-54.

Lawrence CE and Reilly AA (1990). "An expectation maximization (EM) algorithm for the identification and characterization of common sites in unaligned biopolymer sequences." Proteins **7**(1): 41-51.

Lee M and Struhl K (1995). "Mutations on the DNA-binding surface of TATA-binding protein can specifically impair the response to acidic activators in vivo." Mol Cell Biol **15**(10): 5461-9.

Lee TI, Rinaldi NJ, Robert F, Odom DT, *et al.* (2002). "Transcriptional regulatory networks in Saccharomyces cerevisiae." Science **298**(5594): 799-804.

Leuther KK and Johnston SA (1992). "Nondissociation of GAL4 and GAL80 in vivo after galactose induction." Science **256**(5061): 1333-5.

Li Q and Johnston SA (2001). "Are all DNA binding and transcription regulation by an activator physiologically relevant?" Mol Cell Biol **21**(7): 2467-74.

Lieb JD, Liu X, Botstein D and Brown PO (2001). "Promoter-specific binding of Rap1 revealed by genome-wide maps of protein-DNA association." Nat Genet **28**(4): 327-34.

Lin Y, Nomura T, Cheong J, Dorjsuren D, Iida K and Murakami S (1997). "Hepatitis B virus X protein is a transcriptional modulator that communicates with transcription factor IIB and the RNA polymerase II subunit 5." J Biol Chem **272**(11): 7132-9.

Lin YS, Ha I, Maldonado E, Reinberg D and Green MR (1991). "Binding of general transcription factor TFIIB to an acidic activating region." Nature **353**(6344): 569-71.

Lipshutz RJ, Morris D, Chee M, Hubbell E, Kozal MJ, Shah N, Shen N, Yang R and Fodor SP (1995). "Using oligonucleotide probe arrays to access genetic diversity." Biotechniques **19**(3): 442-7.

Liu C, Yang Z, Yang J, Xia Z and Ao S (2000). "Regulation of the yeast transcriptional factor PHO2 activity by phosphorylation." J Biol Chem **275**(41): 31972-8.

Liu X, Brutlag DL and Liu JS (2001). "BioProspector: discovering conserved DNA motifs in upstream regulatory regions of co-expressed genes." Pac Symp Biocomput: 127-38.

Liu XS, Brutlag DL and Liu JS (2002). "An algorithm for finding protein-DNA binding sites with applications to chromatin-immunoprecipitation microarray experiments." Nat Biotechnol **20**(8): 835-9.

Lommel L, Gregory SM, Becker KI and Sweder KS (2000). "Transcription-coupled DNA repair in yeast transcription factor IIE (TFIIE) mutants." Nucleic Acids Res **28**(3): 835-42.

Lorch Y, Cairns BR, Zhang M and Kornberg RD (1998). "Activated RSC-nucleosome complex and persistently altered form of the nucleosome." Cell **94**(1): 29-34.

Maniatis T, Ptashne M, Backman K, Kield D, Flashman S, Jeffrey A and Maurer R (1975). "Recognition sequences of repressor and polymerase in the operators of bacteriophage lambda." Cell **5**(2): 109-13.

Marmorstein R, Carey M, Ptashne M and Harrison SC (1992). "DNA recognition by GAL4: structure of a protein-DNA complex." Nature **356**(6368): 408-14.

Martinez E (2002). "Multi-protein complexes in eukaryotic gene transcription." Plant Mol Biol **50**(6): 925-47.

McInerny CJ, Partridge JF, Mikesell GE, Creemer DP and Breeden LL (1997). "A novel Mcm1-dependent element in the SWI4, CLN3, CDC6, and CDC47 promoters activates M/G1-specific transcription." Genes Dev **11**(10): 1277-88.

Meersseman G, Pennings S and Bradbury EM (1992). "Mobile nucleosomes--a general behavior." Embo J **11**(8): 2951-9.

Moll T, Tebb G, Surana U, Robitsch H and Nasmyth K (1991). "The role of phosphorylation and the CDC28 protein kinase in cell cycle-regulated nuclear import of the S. cerevisiae transcription factor SWI5." Cell **66**(4): 743-58.

Morse RH (2003). "Getting into chromatin: how do transcription factors get past the histones?" Biochem Cell Biol **81**(3): 101-12.

Mosley AL, Lakshmanan J, Aryal BK and Ozcan S (2003). "Glucose-mediated phosphorylation converts the transcription factor Rgt1 from a repressor to an activator." J Biol Chem **278**(12): 10322-7.

Mukherjee S, Berger MF, Jona G, Wang XS, Muzzey D, Snyder M, Young RA and Bulyk ML (2004). "Rapid analysis of the DNA binding specificities of transcription factors with DNA microarrays." Nat Genet.

Narlikar GJ, Fan HY and Kingston RE (2002). "Cooperation between complexes that regulate chromatin structure and transcription." Cell **108**(4): 475-87.

Nasmyth K, Adolf G, Lydall D and Seddon A (1990). "The identification of a second cell cycle control on the HO promoter in yeast: cell cycle regulation of SW15 nuclear entry." Cell **62**(4): 631-47.

Natarajan K, Meyer MR, Jackson BM, Slade D, Roberts C, Hinnebusch AG and Marton MJ (2001). "Transcriptional profiling shows that Gcn4p is a master regulator of gene expression during amino acid starvation in yeast." Mol Cell Biol **21**(13): 4347-68.

Neely KE, Hassan AH, Brown CE, Howe L and Workman JL (2002). "Transcription activator interactions with multiple SWI/SNF subunits." Mol Cell Biol **22**(6): 1615-25.

Neely KE, Hassan AH, Wallberg AE, Steger DJ, Cairns BR, Wright AP and Workman JL (1999). "Activation domain-mediated targeting of the SWI/SNF complex to promoters stimulates transcription from nucleosome arrays." Mol Cell **4**(4): 649-55.

Neely KE and Workman JL (2002). "Histone acetylation and chromatin remodeling: which comes first?" Mol Genet Metab **76**(1): 1-5.

Nehlin JO, Carlberg M and Ronne H (1991). "Control of yeast GAL genes by MIG1 repressor: a transcriptional cascade in the glucose response." Embo J **10**(11): 3373-7.

Nelson C, Goto S, Lund K, Hung W and Sadowski I (2003). "Srb10/Cdk8 regulates yeast filamentous growth by phosphorylating the transcription factor Ste12." Nature **421**(6919): 187-90.

Ng HH, Robert F, Young RA and Struhl K (2002). "Genome-wide location and regulated recruitment of the RSC nucleosome-remodeling complex." Genes Dev **16**(7): 806-19.

Ng HH, Robert F, Young RA and Struhl K (2003). "Targeted recruitment of Set1 histone methylase by elongating Pol II provides a localized mark and memory of recent transcriptional activity." Mol Cell **11**(3): 709-19.

O'Conallain C, Doolin MT, Taggart C, Thornton F and Butler G (1999). "Regulated nuclear localisation of the yeast transcription factor Ace2p controls expression of chitinase (CTS1) in Saccharomyces cerevisiae." Mol Gen Genet **262**(2): 275-82.

Odom DT, Zizlsperger N, Gordon DB, Bell GW, *et al.* (2004). "Control of pancreas and liver gene expression by HNF transcription factors." Science **303**(5662): 1378-81.

Ogawa N, DeRisi J and Brown PO (2000). "New components of a system for phosphate accumulation and polyphosphate metabolism in Saccharomyces cerevisiae revealed by genomic expression analysis." Mol Biol Cell **11**(12): 4309-21.

Pascual-Ahuir A, Posas F, Serrano R and Proft M (2001). "Multiple levels of control regulate the yeast cAMP-response element-binding protein repressor Sko1p in response to stress." J Biol Chem **276**(40): 37373-8.

Pease AC, Solas D, Sullivan EJ, Cronin MT, Holmes CP and Fodor SP (1994). "Light-generated oligonucleotide arrays for rapid DNA sequence analysis." Proc Natl Acad Sci U S A **91**(11): 5022-6.

Pelham HR (1982). "A regulatory upstream promoter element in the Drosophila hsp 70 heat-shock gene." Cell **30**(2): 517-28.

Polach KJ and Widom J (1995). "Mechanism of protein access to specific DNA sequences in chromatin: a dynamic equilibrium model for gene regulation." J Mol Biol **254**(2): 130-49.

Polach KJ and Widom J (1996). "A model for the cooperative binding of eukaryotic regulatory proteins to nucleosomal target sites." J Mol Biol **258**(5): 800-12.

Pramila T, Miles S, GuhaThakurta D, Jemiolo D and Breeden LL (2002). "Conserved homeodomain proteins interact with MADS box protein Mcm1 to restrict ECB-dependent transcription to the M/G1 phase of the cell cycle." Genes Dev **16**(23): 3034-45.

Pribnow D (1975). "Bacteriophage T7 early promoters: nucleotide sequences of two RNA polymerase binding sites." J Mol Biol **99**(3): 419-43.

Ptashne M (1988). "How eukaryotic transcriptional activators work." Nature **335**(6192): 683-9.

Ptashne M and Gann A (1997). "Transcriptional activation by recruitment." Nature **386**(6625): 569-77.

Reece RJ and Platt A (1997). "Signaling activation and repression of RNA polymerase II transcription in yeast." Bioessays **19**(11): 1001-10.

Reinke H, Gregory PD and Horz W (2001). "A transient histone hyperacetylation signal marks nucleosomes for remodeling at the PHO8 promoter in vivo." Mol Cell **7**(3): 529-38.

Reinke H and Horz W (2003). "Histones are first hyperacetylated and then lose contact with the activated PHO5 promoter." Mol Cell **11**(6): 1599-607.

Ren B, Robert F, Wyrick JJ, Aparicio O, *et al.* (2000). "Genome-wide location and function of DNA binding proteins." Science **290**(5500): 2306-9.

Robyr D, Suka Y, Xenarios I, Kurdistani SK, Wang A, Suka N and Grunstein M (2002). "Microarray deacetylation maps determine genome-wide functions for yeast histone deacetylases." Cell **109**(4): 437-46.

Roeder RG (2003). "Lasker Basic Medical Research Award. The eukaryotic transcriptional machinery: complexities and mechanisms unforeseen." Nat Med **9**(10): 1239-44.

Roth FP, Hughes JD, Estep PW and Church GM (1998). "Finding DNA regulatory motifs within unaligned noncoding sequences clustered by whole-genome mRNA quantitation." Nat Biotechnol **16**(10): 939-45.

Ryan MP, Stafford GA, Yu L and Morse RH (2000). "Artificially recruited TATA-binding protein fails to remodel chromatin and does not activate three promoters that require chromatin remodeling." Mol Cell Biol **20**(16): 5847-57.

Sadowski I, Costa C and Dhanawansa R (1996). "Phosphorylation of Ga14p at a single C-terminal residue is necessary for galactose-inducible transcription." Mol Cell Biol **16**(9): 4879-87.

Sakurai H and Fukasawa T (1998). "Functional correlation among Gal11, transcription factor (TF) IIE, and TFIIH in Saccharomyces cerevisiae. Gal11 and TFIIE cooperatively enhance TFIIH-mediated phosphorylation of RNA polymerase II carboxyl-terminal domain sequences." J Biol Chem **273**(16): 9534-8.

Sakurai H and Fukasawa T (2000). "Functional connections between mediator components and general transcription factors of Saccharomyces cerevisiae." J Biol Chem **275**(47): 37251-6.

Sayre MH, Tschochner H and Kornberg RD (1992). "Reconstitution of transcription with five purified initiation factors and RNA polymerase II from Saccharomyces cerevisiae." J Biol Chem **267**(32): 23376-82.

Schena M, Shalon D, Davis RW and Brown PO (1995). "Quantitative monitoring of gene expression patterns with a complementary DNA microarray." Science **270**(5235): 467-70.

Schmitz ML, dos Santos Silva MA, Altmann H, Czisch M, Holak TA and Baeuerle PA (1994). "Structural and functional analysis of the NF-kappa B p65 C terminus. An acidic and modular transactivation domain with the potential to adopt an alpha-helical conformation." J Biol Chem **269**(41): 25613-20.

Schnitzler GR, Cheung CL, Hafner JH, Saurin AJ, Kingston RE and Lieber CM (2001). "Direct imaging of human SWI/SNF-remodeled mono- and polynucleosomes by atomic force microscopy employing carbon nanotube tips." Mol Cell Biol **21**(24): 8504-11.

Schuller C, Mamnun YM, Mollapour M, Krapf G, Schuster M, Bauer BE, Piper PW and Kuchler K (2004). "Global phenotypic analysis and transcriptional profiling defines the weak acid stress response regulon in Saccharomyces cerevisiae." Mol Biol Cell **15**(2): 706-20.

Shalon D, Smith SJ and Brown PO (1996). "A DNA microarray system for analyzing complex DNA samples using two-color fluorescent probe hybridization." Genome Res **6**(7): 639-45.

Sharrocks AD (2000). "Introduction: the regulation of eukaryotic transcription factor function." Cell Mol Life Sci **57**(8-9): 1147-8.

Simon I, Barnett J, Hannett N, Harbison CT, *et al.* (2001). "Serial regulation of transcriptional regulators in the yeast cell cycle." Cell **106**(6): 697-708.

Spellman PT, Sherlock G, Zhang MQ, Iyer VR, Anders K, Eisen MB, Brown PO, Botstein D and Futcher B (1998). "Comprehensive identification of cell cycle-regulated genes of the yeast Saccharomyces cerevisiae by microarray hybridization." Mol Biol Cell **9**(12): 3273-97.

Stargell LA and Struhl K (1995). "The TBP-TFIIA interaction in the response to acidic activators in vivo." Science **269**(5220): 75-8.

Stormo GD (2000). "DNA binding sites: representation and discovery." Bioinformatics **16**(1): 16-23.

Stringer KF, Ingles CJ and Greenblatt J (1990). "Direct and selective binding of an acidic transcriptional activation domain to the TATA-box factor TFIID." Nature **345**(6278): 783-6.

Struhl K (1995). "Yeast transcriptional regulatory mechanisms." Annu Rev Genet **29**: 651-74.

Sudarsanam P, Iyer VR, Brown PO and Winston F (2000). "Whole-genome expression analysis of snf/swi mutants of Saccharomyces cerevisiae." Proc Natl Acad Sci U S A **97**(7): 3364-9.

Suzuki-Fujimoto T, Fukuma M, Yano KI, Sakurai H, Vonika A, Johnston SA and Fukasawa T (1996). "Analysis of the galactose signal transduction pathway in Saccharomyces cerevisiae: interaction between Gal3p and Gal80p." Mol Cell Biol **16**(5): 2504-8.

Svaren J and Horz W (1997). "Transcription factors vs nucleosomes: regulation of the PHO5 promoter in yeast." Trends Biochem Sci **22**(3): 93-7.

Svaren J, Schmitz J and Horz W (1994). "The transactivation domain of Pho4 is required for nucleosome disruption at the PHO5 promoter." Embo J **13**(20): 4856-62.

Sze JY, Woontner M, Jaehning JA and Kohlhaw GB (1992). "In vitro transcriptional activation by a metabolic intermediate: activation by Leu3 depends on alpha-isopropylmalate." Science **258**(5085): 1143-5.

Tansey WP (2001). "Transcriptional activation: risky business." Genes Dev **15**(9): 1045-50.

Tjian R and Maniatis T (1994). "Transcriptional activation: a complex puzzle with few easy pieces." Cell **77**(1): 5-8.

Tong AH, Lesage G, Bader GD, Ding H, *et al.* (2004). "Global mapping of the yeast genetic interaction network." Science **303**(5659): 808-13.

Topalidou I and Thireos G (2003). "Gcn4 occupancy of open reading frame regions results in the recruitment of chromatin-modifying complexes but not the mediator complex." EMBO Rep **4**(9): 872-6.

van Helden J, Andre B and Collado-Vides J (1998). "Extracting regulatory sites from the upstream region of yeast genes by computational analysis of oligonucleotide frequencies." J Mol Biol **281**(5): 827-42.

Van Hoy M, Leuther KK, Kodadek T and Johnston SA (1993). "The acidic activation domains of the GCN4 and GAL4 proteins are not alpha helical but form beta sheets." Cell **72**(4): 587-94.

Vashee S and Kodadek T (1995). "The activation domain of GAL4 protein mediates cooperative promoter binding with general transcription factors in vivo." Proc Natl Acad Sci U S A **92**(23): 10683-7.

Venter U, Svaren J, Schmitz J, Schmid A and Horz W (1994). "A nucleosome precludes binding of the transcription factor Pho4 in vivo to a critical target site in the PHO5 promoter." Embo J **13**(20): 4848-55.

Vilela C, Linz B, Rodrigues-Pousada C and McCarthy JE (1998). "The yeast transcription factor genes YAP1 and YAP2 are subject to differential control at the levels of both translation and mRNA stability." Nucleic Acids Res **26**(5): 1150-9.

Vincent O, Kuchin S, Hong SP, Townley R, Vyas VK and Carlson M (2001). "Interaction of the Srb10 kinase with Sip4, a transcriptional activator of gluconeogenic genes in Saccharomyces cerevisiae." Mol Cell Biol **21**(17): 5790-6.

Wang A, Kurdistani SK and Grunstein M (2002). "Requirement of Hos2 histone deacetylase for gene activity in yeast." Science **298**(5597): 1412-4.

Whitehouse I, Flaus A, Cairns BR, White MF, Workman JL and Owen-Hughes T (1999). "Nucleosome mobilization catalysed by the yeast SWI/SNF complex." Nature **400**(6746): 784-7.

Workman JL and Buchman AR (1993). "Multiple functions of nucleosomes and regulatory factors in transcription." Trends Biochem Sci **18**(3): 90-5.

Workman JL and Kingston RE (1992). "Nucleosome core displacement in vitro via a metastable transcription factor-nucleosome complex." Science **258**(5089): 1780-4.

Workman JL and Roeder RG (1987). "Binding of transcription factor TFIID to the major late promoter during in vitro nucleosome assembly potentiates subsequent initiation by RNA polymerase II." Cell **51**(4): 613-22.

Wu WH and Hampsey M (1999). "An activation-specific role for transcription factor TFIIB in vivo." Proc Natl Acad Sci U S A **96**(6): 2764-9.

Wu Y, Reece RJ and Ptashne M (1996). "Quantitation of putative activator-target affinities predicts transcriptional activating potentials." Embo J **15**(15): 3951-63.

Xiao H, Pearson A, Coulombe B, Truant R, *et al.* (1994). "Binding of basal transcription factor TFIIH to the acidic activation domains of VP16 and p53." Mol Cell Biol **14**(10): 7013-24.

Yudkovsky N, Ranish JA and Hahn S (2000). "A transcription reinitiation intermediate that is stabilized by activator." Nature **408**(6809): 225-9.

Zawel L, Kumar KP and Reinberg D (1995). "Recycling of the general transcription factors during RNA polymerase II transcription." Genes Dev **9**(12): 1479-90.

Zeitlinger J, Simon I, Harbison CT, Hannett NM, Volkert TL, Fink GR and Young RA (2003). "Program-specific distribution of a transcription factor dependent on partner transcription factor and MAPK signaling." Cell **113**(3): 395-404.

Zhang L and Guarente L (1994). "HAP1 is nuclear but is bound to a cellular factor in the absence of heme." J Biol Chem **269**(20): 14643-7.

Zhang L and Guarente L (1995). "Heme binds to a short sequence that serves a regulatory function in diverse proteins." Embo J **14**(2): 313-20.

Zhang L, Hach A and Wang C (1998). "Molecular mechanism governing heme signaling in yeast: a higher-order complex mediates heme regulation of the transcriptional activator HAP1." Mol Cell Biol **18**(7): 3819-28.

Ziegler LM, Khaperskyy DA, Ammerman ML and Ponticelli AS (2003). "Yeast RNA polymerase II lacking the Rpb9 subunit is impaired for interaction with transcription factor IIF." J Biol Chem **278**(49): 48950-6.

Chapter 2

Microarray Data Analysis for Biological Insight

Overview

The link between the projects I have undertaken while a member of the Young Lab is analysis of the enormous amount of microarray data we have generated. Both expression and location experiments are complicated by a multitude of factors, not the least of which are the scale of the data and inconsistencies in experimental performance. The analysis process needs to account for all of these issues. There are two levels at which analysis occurs: technically, in manipulating the data to remove bias based on those experimental inconsistencies and determining which genes are expressed or enriched, and biologically, in assessing the implications of these results.

In the technical phase of the analysis, normalization is used to mathematically remove many of the sources of noise present in the data. I will discuss the methods available for normalization and the optimal choices for removing bias. The second technical issue is assessing the quality of the data. I will discuss some current suggestions for metrics for spot quality, as well as offering an initial statistic for determining the overall array quality. One other key technical question is which method to use to determine differentially expressed or enriched genes. I will describe many of the algorithms available for this purpose, as well as adjustments that I made to the Rosetta error model to account for some chromatin immunoprecipitation specific issues.

The second level of analysis is in interpreting the data for biological understanding. One of the common approaches to this is to assess the significance of the overlap between one expression or location dataset and annotations, categories, or other datasets. I will describe some of the techniques for performing these comparisons, as well as a novel method I developed. Another area in which I have made contributions is in determining which regulators and genes are involved in minimal regulatory motifs. Finally, I will cover various approaches to visualizing these microarray data and analyses to improve our ability to make biological discoveries.

Introduction

Just as the 1980's heralded the coming of age of molecular biology, with cloning, sequencing and mutagenizing genes becoming commonplace, the 1990's saw the beginning of the genomic revolution. Coming on the heels of the first complete sequences of bacterial (Fleischmann *et al.* 1995) and eukaryotic genomes (Goffeau *et al.* 1996), microarray technology enabled researchers for the first time to examine the expression of every gene in a population of cells. This meant that for the first time scientists were able to see the full spectrum of changes occurring during normal cell growth and division (Cho *et al.* 1998; Chu *et al.* 1998; Spellman *et al.* 1998), brought on by alteration of a cell's environment (DeRisi *et al.* 1997; Gasch *et al.* 2000; Causton *et al.* 2001), or caused by perturbation of the normal genetic complement (Hughes *et al.* 2000). These microarray data were used both to assign tentative functions to previously unannotated genes, as well as defining cohorts of similarly expressed genes defining each of the cellular responses.

In a typical expression experiment, cDNA is reverse transcribed from messenger RNA in the cell type of interest, labeled with a fluorophore and hybridized to an appropriate microarray. Data are collected, analyzed, and the differentially expressed genes discovered and investigated for biological relevance. Another technology being widely used is location analysis. In these experiments, proteins and DNA are crosslinked and an immunoprecipitation is performed using an antibody to the transcription factor of interest. Immunoprecipitated DNA is labeled with a fluorophore, then hybridized. Data collection and analysis are performed similarly to an expression experiment. Despite the seeming simplicity of these processes, each stage of the experiment requires that a series of non-trivial choices be made that can drastically affect the outcome. These options involve everything from the type of microarray to use to which labeling method to employ. Once the experiment has been physically completed and the raw data are in hand, a new series of decisions about each step of the analysis process begins.

Removal of noise and systematic bias from the data through normalization is the first component of the analysis. This should be followed by assessment of the quality of the data, both for individual spots and for whole arrays. At this point, a statistical model can be used to determine which spots in the test sample(s) are different from the control. Then, the biological analysis begins. Comparisons are made with other expression data, with annotations, and with additional genome-wide data such as protein-protein interactions or subcellular localization data. Various visualization tools are used for reducing the data to a space where intuition becomes more feasible. Finally, biological conclusions can be drawn.

Normalization

In order to use data from microarrays effectively, it is important to be able to compare between samples at many levels. First, in two-color arrays, one must be able to make reliable comparisons between the control and the test channel. Unequal amounts of RNA as starting material could cause biases, as could the preferential incorporation of one fluorophore over the other, as well as differential response of the fluorophores to the laser. At a higher level, comparison across arrays is almost more important, allowing meta-analysis such as how the expression of a gene changes throughout a timecourse (Cho *et al.* 1998; Spellman *et al.* 1998; Cho *et al.* 2001), or construction of a regulatory network (Ideker *et al.* 2001; Tegner *et al.* 2003). Finally, the ability to compare results across different studies could allow for discovery of inter-relations between pathways and modules that are not assessed in single experimental series.

While it was recognized in the early microarray experiments that some degree of consistency was necessary between channels or arrays in order to make valid comparisons, the approaches used were highly subjective, if they were even discussed. In the first reports of genome-wide expression analysis experiments, normalization was performed at the scanning stage, by adjusting the scan power to obtain a ratio as close to 1.0 as possible in spots containing total genomic DNA (DeRisi *et al.* 1997). In another study, normalization was mentioned as using spiked-in controls, but no specifics were given (Holstege *et al.* 1998). Others did not report any normalization step in the data processing at all (Cho *et al.* 1998; Spellman *et al.* 1998).

Subsequently, biologists have begun to understand the importance of both using and reporting statistical methods for analysis. The most straightforward method for normalization involves computing a single constant by which to multiply one channel on an array in order to account for gross differences. Various approaches to this achieve the same ends: normalizing so that total, mean or median intensity in the two channels or across chips are the same, or using the same operation on a subset of spots, such as control spots. While these methods have been widely used (Richmond *et al.* 1999; Ren *et al.* 2000; Natarajan *et al.* 2001; Newton *et al.* 2001; Dasgupta *et al.* 2002; Lee *et al.* 2002b; Moqtaderi and Struhl 2004) and are effective in eliminating gross differences between channels or arrays, they definitely do not remove all bias introduced to this point (Schuchhardt *et al.* 2000; Yang *et al.* 2002a; Yang *et al.* 2002b). In particular, there is an intensity dependent bias, and can be spatial array effects, that are not removed by global normalization. Figure 1 shows how median normalization does not correct for localized or regional spatial anomalies, or for intensity dependent bias.

Based on these intensity dependent effects, a normalization procedure based on a locally weighted linear regression (lowess) method (Cleveland 1979) has been adapted for use with microarrays and is rapidly becoming the standard

Figure 1: Methods for Normalization

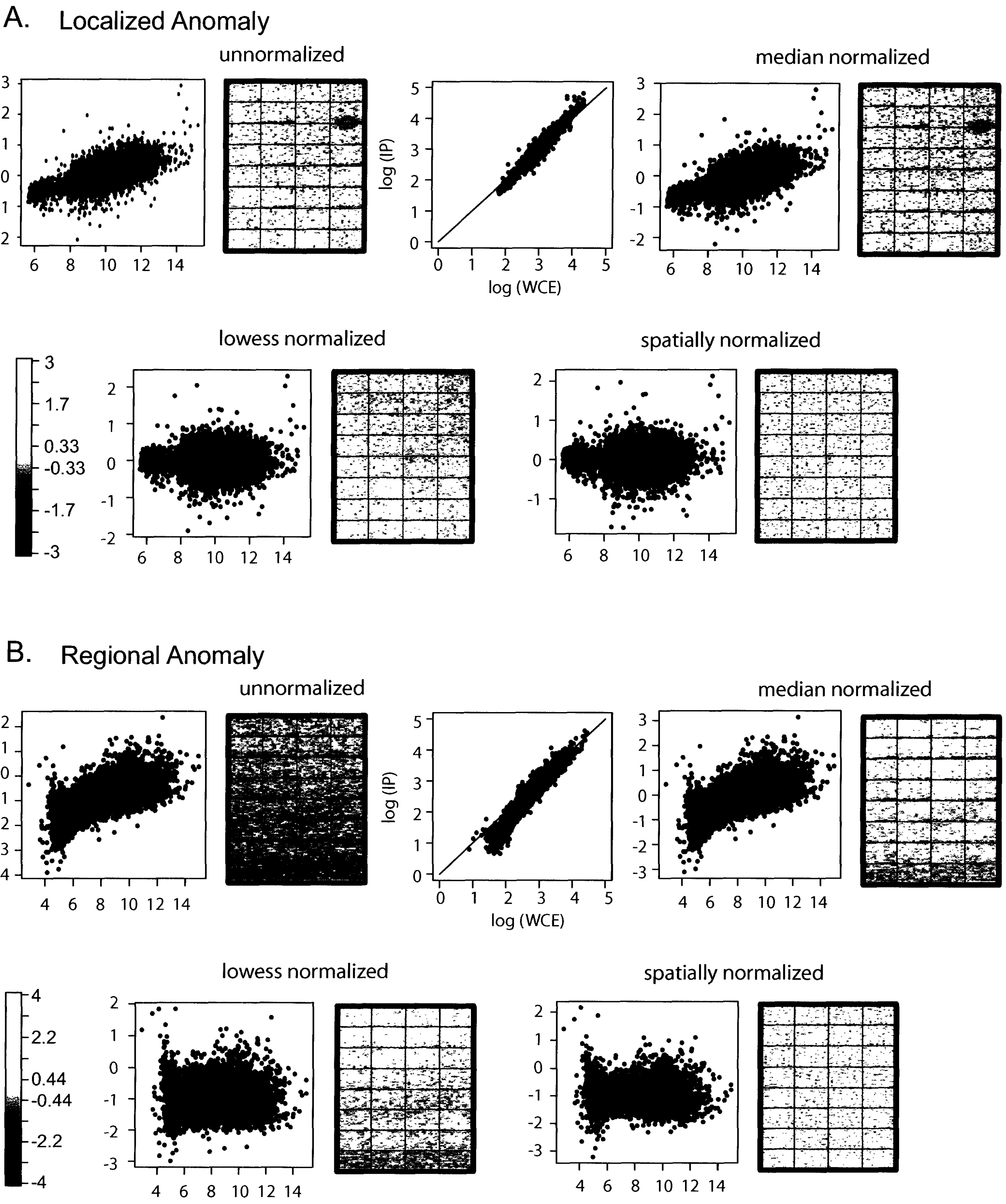


Figure 1: Methods for Normalization

In each panel, four methods of normalization are compared. Unnormalized data are shown, with an M-A scatter plot with the log of the sum of intensities on the x axis plotted against the log of the ratio of intensities on the y axis. The red line indicates the intensity dependent bias. A spatial representation of the ratios is shown on a virtual chip. Finally, the unnormalized data are plotted on an R-G scatter plot, with the log of the control intensity on the x axis versus the log of the immunoprecipitated DNA on the y axis. The M-A and spatial representations are show for each of the additional three normalization methods, median, lowess and spatial lowess. A) Normalization of a chip with a small spatial anomaly. B) Normalization of a chip with a regional anomaly, an intensity gradient down the chip.

for normalization (Finkelstein *et al.* 2002; Quackenbush 2002; Leung and Cavalieri 2003). The lowess procedure determines the dependency of the log ratio on the log of the intensity, and subtracts this function from each observed log ratio (Yang *et al.* 2002a; Yang *et al.* 2002b; Smyth and Speed 2003; Wilson *et al.* 2003). This procedure can also be modified to normalize the group of spots printed by each individual print tip separately. This can correct for defects during the printing process that might be pin specific (Yang *et al.* 2002b). The removal of intensity dependent bias is illustrated in Figure 1. This figure shows, however, that the lowess normalization still cannot correct for regional anomalies that might be due to uneven hybridization. A spatial normalization filter following lowess normalization has been suggested to correct for these biases (Wilson *et al.* 2003). This could prove to be an important addition to any normalization procedure, as ANOVA tests on arrays produced for the Arabidopsis Functional Genomics Consortium indicated that approximately 20% of arrays have significant spatial effects, explaining more than 10% of the variance in log ratios (Finkelstein *et al.* 2002). This spatial smoothing is accomplished by computing the median log ratio, for each spot, of the surrounding spots in some grid size: from 3x3 to 7x7. The data are then rescaled by dividing by the median absolute deviation (Wilson *et al.* 2003). This method removes both the intensity dependent bias and spatial anomalies, as shown in Figure 1.

One issue with all of these normalization methods is that each makes the assumption that only a small proportion of spots are differentially affected between the two channels or samples. This is usually true in the case of perturbations of single genes, or in immunoprecipitated DNA from a single gene-specific transcription factor. However, serious environmental perturbations or immunoprecipitations from general transcription factors, or 'boutique' arrays designed to capture a specific gene set, can significantly deviate from this in practice. In these cases it is imperative to use normalization based upon a selected set of probes rather than the entire array. Housekeeping genes have been suggested as controls under certain circumstances, although these are not as consistently expressed as had been imagined (Savonet *et al.* 1997; Lee *et al.* 2002a). For experiments like location analysis, the use of DNA sequences found within long ORFs, or DNA from regions of the genome with low gene density, "desert regions", has been suggested. However, as with the housekeeping genes, there is no guarantee that a particular factor is not binding to any one of these regions. Exogenous controls added during one or more steps of the experiment are likely to be the most reliable set of data to which to normalize in such cases.

Just because arrays are normalized within channels, or one array to another, does not mean that an entire set of arrays is comparable. There are still likely to be differences between arrays, based mostly upon different amounts of RNA used as starting material, but also because of differential labeling of samples or differential scanning. Two methods have been proposed to correct for these between slide errors. First, a Singular Value Decomposition (SVD), also called Principle Components Analysis (PCA) approach has been suggested (Alter *et al.* 2000). In this algorithm, the data undergo a linear transformation so that the expression ratios for each gene are based upon a sum of biologically relevant expression patterns. In re-analyzing data from the yeast cell cycle (Spellman *et al.* 1998), the most predominant pattern found was determined to be noise based. Other patterns included sinusoid waves with peaks at different times, as would be expected from the cell cycle with genes peaking at various cell cycle stages (Alter *et al.* 2000). The noise pattern was subtracted from each array to normalize the expression values. Problems with this method include its requirement for no missing data, as well as unclear assignment of the biological function of each of the determined patterns. The second method of normalization between arrays normalizes for different levels of variation on different slides by scaling each array to have the same median absolute deviation (Yang *et al.* 2002b). This assumes that variation should be the same across slides, which is not necessarily the case, for example, in a series of expression measurements after a perturbation over time. So, while these methods may be an improvement over no between slide normalization, it is clear that more work is required in this area.

Quality Control

There are many steps in a microarray experiment during which things can go awry. Poor purification of the initial starting material, contamination of reagents or samples, loss of pellets, leaky or uneven hybridization are just some of the problems that can arise. However, even if one of these issues has occurred, it is often without knowledge of the researcher, and does not prevent obtaining labeled material to hybridize to an array or the subsequent data analysis. Datasets affected by one of these difficulties should be discarded as they are likely to be irreproducible and to give spurious results. Especially as the use of microarrays becomes more high-throughput, this means that there must be some qualitative, or preferably quantitative, measure of array data quality.

As with every other aspect of microarray technology, quality has to be assessed on different levels. The first check occurs at the level of individual spots. During initial analysis of the scanned image, spots that are missing, have an aberrant shape or contain an artifact, can be flagged as 'bad' and discarded from further analysis. This is performed automatically by spot-finding programs to some extent, but frequently requires manual curation. Another quality standard that has been proposed is to use a secondary stain to assess the amount of DNA physically present in a spot, and discard those spots where no signal is present. Some stains that have been used include Sybr Green I for dsDNA arrays (Mukherjee *et al.* 2004), Sybr Green II for ssDNA arrays (Battaglia *et al.* 2000), DAPI (Finkelstein *et al.* 2002), or spiking a fluorophore labeled dNTP into the printing reaction (Shearstone *et al.* 2002).

A composite spot quality score based on a quantitative assessment of five common problems with microarray spots has recently been proposed (Wang *et al.* 2001; Wang *et al.* 2003). This score incorporates the size of the spots, their signal to noise ratio, variability in the local background of the spot, the absolute intensity of the background, and whether the spot is saturated or not. On arrays containing replicated spots, there is a high correlation between a good quality score and low variability between replicates. The same is seen when duplicate chips are tested, and the high quality spots are significantly more consistent between different image processing programs than are the low quality spots. Possible uses for this spot quality score are to remove spots with a score below some threshold from further analysis (Wang *et al.* 2001; Wang *et al.* 2003), or to use the score to weight each spot in the analysis, such as normalization (Smyth and Speed 2003).

Some attention has been paid recently to quality control for microarrays at the level of the entire array. Various researchers have advocated examinations of visual representations of data to determine chip quality. M-A scatter plots graph the log ratio of each spot versus the log of the sum of intensities in the two channels (Dudoit *et al.* 2000; Tseng *et al.* 2001; Yang *et al.* 2002a; Yang *et al.* 2002b; Petri *et al.* 2004). This is similar to the more traditional scatter plots where the log intensity in one channel is plotted against the log intensity in the other channel, but the M-A plots can make significant effects more obvious. Spatial representation of the intensities on a virtual array can also be used as a quality check, as it can reveal hybridization or other spatial artifacts (Petri *et al.* 2004). Examples of each of these displays are shown in Figure 1. Each of these methods requires the researcher to make a determination about whether an array is acceptable based on individual inspection of these displays. In order to remove the variability introduced by this subjective measure, a quantitative metric is essential. One such metric that has been proposed offhand is to examine the distribution of coefficients of variation for multiple spots corresponding to the same gene in a given slide, or for single spots across replicated arrays (Tseng *et al.* 2001). Again, however, there is no empirical measure for determining whether a slide should be used or not, just the subjectivity of the researcher.

I have developed a quality control statistic for genome-wide location analysis experiments, similar to the spot quality score proposed by Wang *et al.* (2001). It does incorporate some elements that are specific to chromatin immunoprecipitation versus traditional gene expression microarrays, but could easily be adapted to the latter. The quality statistic is based upon a number of metrics that have been chosen to incorporate various aspects of both raw and normalized microarray data. Examples of chips that would be discarded due to each of these metrics are shown in Figure 2. The first metric incorporates a simple count of the number of spots with low intensity or missing data in each channel, and the difference between the two channels, as well as the median and maximum intensity in each channel (Figure 2A). Additional metrics include the distribution of the intensities in the control channel (Figure 2B), the standard deviation of the log ratios (Figure 2C), and the number of spots with a significant p-value (<0.001) versus the number with a non-significant p-value (>0.999) (Figure 2D).

The first two metrics are appropriate for all microarray experiments. A large number of spots with low signal intensity generally indicates a poor labeling reaction. It is also important that there not be a much greater number of spots with low intensity in one channel than the other. Both of these mean that a high percentage of the spots will not yield meaningful data, confounding interpretation of the results. The median and maximum intensity in each channel indicate whether a large proportion of measurements are towards the low end of the scale, regardless of how many spots fall below the intensity threshold, although the two are related. In general results obtained from low intensity spots are less reliable (Wang *et al.* 2001), so the lower the median and maximum intensity, the less reliable the results. The second metric compares the distribution of intensities in the control channel with the average distribution across all chips. A distribution for an individual chip that is significantly different from the average is indicative of problems during the experiment (Figure 2B).

The other metrics are more specific for chromatin immunoprecipitation experiments, and have only been examined for gene-specific transcription factors with a small percentage of enriched spots. The expectation in a location analysis experiment performed in a cell containing no antibody target is that the distribution of DNA species in the immunoprecipitated sample will be the same as the distribution in the genomic control. This is due to non-specific binding of the DNA to the beads and antibody. Therefore, the histogram of log-ratios for each spot should have a low standard deviation. In practice, we find this to be true for a majority of experiments. However, when the experiment fails for any number of reasons, including a low amount of starting material, poor DNA cleanup, or low quality linker incorporated prior to ligation mediated PCR (LM-PCR), the result is a much higher standard deviation than expected. Finally, we expect enrichment ($p < 0.001$) to occur only in the immunoprecipitated channel.

Figure 2: Components of the Quality Metric

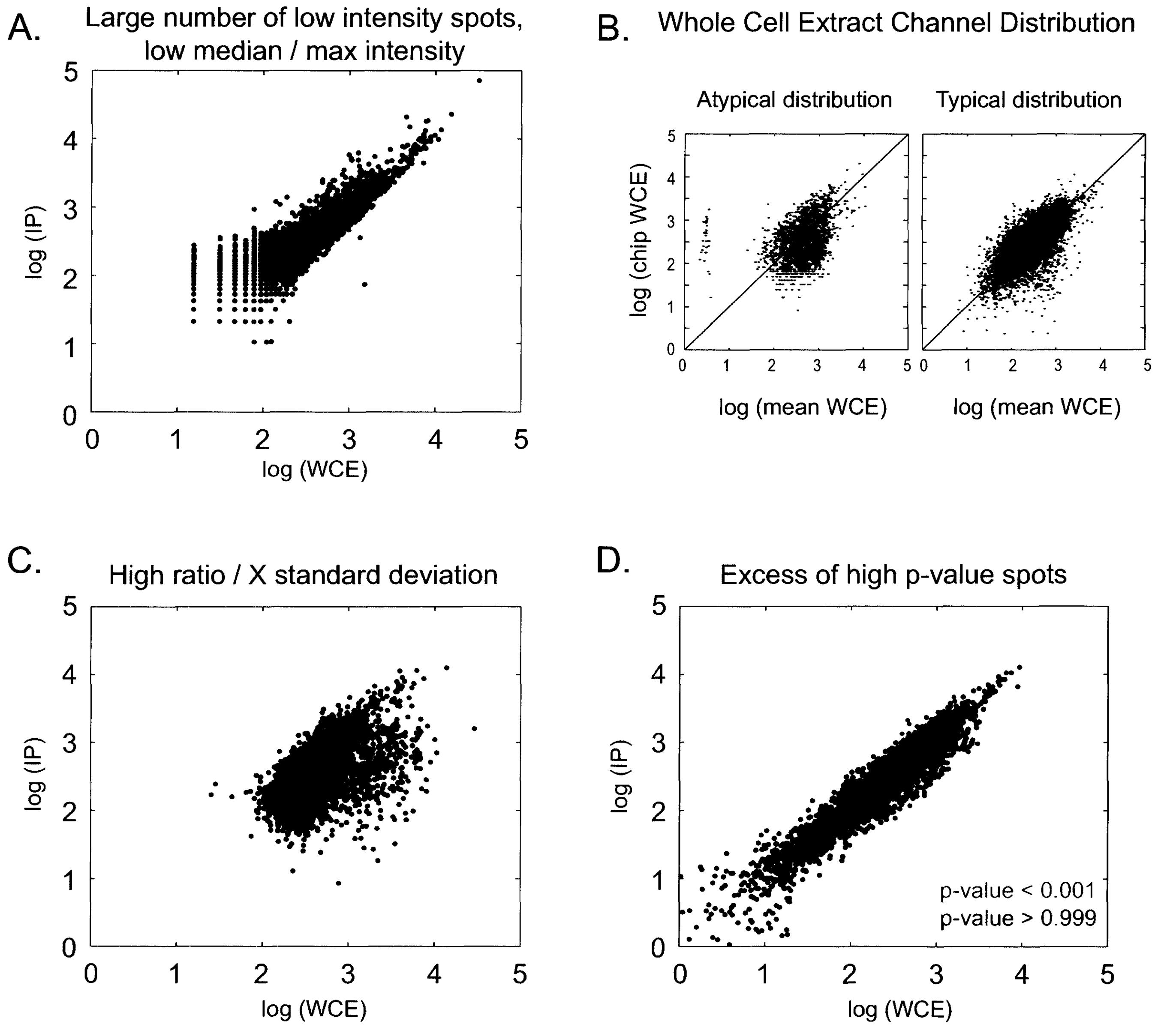


Figure 2: Components of the Quality Metric

R-G scatter plots with the log of the median normalized control intensity on the x axis versus the log of the median normalized immunoprecipitated DNA on the y axis are shown to illustrate the components of the quality metric.

A) Large number of low intensity spots, low overall intensity.

B) In these plots, the average log control intensity of each spot over >1000 chips is plotted on the x axis. The log control intensity for each spot on individual chips is plotted on the y axis. The chip on the left has an atypical distribution, as seen by the cluster of spots on the left side of the plot, and the low density and banding of the remaining group of spots, indicating a large number of missing spots. The chip on the right has a typical distribution that clusters more tightly around the 1:1 line.

C) A chip with ratios with a high standard deviation.

D) A chip with significantly more high p-value ($p > 0.999$, in red) spots than low p-value ($p < 0.001$, in green) spots.

If the enrichment seen is due to experimental error, however, there are also correspondingly more spots on the other tail of the distribution (p > 0.999), so the difference between the two is used as the final metric (Figure 2D).

Each of these metrics is computed for each individual array, and averaged across all arrays. The quality score for each array is computed as the weighted sum of the differences from this average, if the difference indicates poor array quality. That is, no value is added to the array score if it is better than average, only if it is worse. The weights are determined so that components of the score that are at different orders of magnitude (for example the average number of low intensity spots is in the hundreds, whereas the standard deviation of the log ratios is less than one) have equal weight in computation of the array score.

The final stage at which quality control is necessary is to ensure that the data from arrays that are intended to be replicates are in fact concordant. One approach to this is to determine the coefficient of variation (CV, standard deviation divided by mean) for each spot across multiple replicated slides. A high variance in a plot of this CV versus intensity can indicate a poor slide, or poor replication between slides (Tseng *et al.* 2001). Another method is to examine the correlation between pairs of replicated slides (Yang *et al.* 2002a; Yu and Wolfinger 2004).

Determining differential expression

Once the data have been normalized and determined to be of acceptable quality, the next step is to determine which genes are expressed differently between samples. The naïve method is to select a fold change ratio cutoff, then consider any gene with a larger increase or decrease in expression as differentially expressed. This heuristic is still in common use, but has some serious drawbacks. First, it has been well documented that expression changes at low intensity are much less reliable than those at high intensity (Tusher *et al.* 2001; Wang *et al.* 2001; Yang *et al.* 2002a), but this method does not account for that. Second, some genes are inherently more variable in expression than others, which can lead to a high rate of false positives (Hughes *et al.* 2000; Tusher *et al.* 2001). For other genes that are expressed at a much more constant level, a small amount of change in an experiment could be extremely significant, but missed by using a simple fold-change criterion.

A burgeoning literature in this field has proposed many statistics for quantifying differential expression and significance thereof. There are three major problems with this literature: most of it is incomprehensible to the typical biologist who could benefit from using these analyses, tools for performing the analysis are not provided in a user-friendly form, and there is generally no comparison of the results with those of other methods. Standards have been developed for the reporting of microarray experimental data, and are now largely in place (Ball *et al.* 2002). Similarly, the community interested in the analysis of these data must develop principles for assessing each new method, and should perform these tests on a common data set(s). The leukemia dataset from Golub *et al.* (1999) seems to be becoming one such data set. A standardization of the reported results would also be useful. Two authors have recently performed comparative studies of selected analysis methods using the leukemia data (Pan 2002; Broberg 2003), but as they report different statistics, it is difficult to compare between the two.

Despite the difficulties in comparing individual methods to decide which is optimum for a given analysis, some consensus has emerged as to the properties of different techniques. First of all, statisticians uniformly agree that the use of a straightforward fold-change criterion for selecting differentially expressed genes is a poor metric. Because this method does not take into account the variability of expression for each gene, nor the intensity of the measurements the fold change is based on, the fold-change cutoff leads to an unacceptable rate of both false positives and false negatives (Ideker *et al.* 2000; Baldi and Long 2001; Tusher *et al.* 2001; Wang and Ethier 2004). A conventional t-test, also widely used, is a poor metric as well, because it assumes many more replicates than are typically performed in a microarray experiment, as well as assuming that the data are normally distributed, which is not the case (Baldi and Long 2001; Pan 2002; Broberg 2003; Wang and Ethier 2004).

Beyond that consensus, a de facto standard for analysis of microarray data does seem to have emerged, Significance Analysis of Microarrays (SAM) (Tusher *et al.* 2001). In this method, each gene is assigned a score based on its change in expression relative to its standard deviation. A significance threshold is then set, based on permutations of the data, to allow the researcher to choose an acceptable level of false positives within the list of genes passing the threshold. This technique makes three major advances over the more simplistic measures discussed above. First, the use of an empirical calculation of the false discovery rate rather than making assumptions about the distribution of the data for determining significance. Second, the incorporation of a gene-specific metric to account for differences in variance between mRNA species. Finally, a regularizing parameter is used to remove the dependence of variance on intensity, thus making inferences at lower intensities more sound. More recently, a proposal has been made to improve this analysis by modifying the test statistic, but it remains to be seen whether the improvement is significant enough to warrant general use (Broberg 2003).

There is one drawback with SAM – because it relies on replicated data both to calculate the metric for differential expression and to assess the significance of this difference, it cannot be used on a single chip level. This weakness is not present in the other most commonly used method for assessing differential expression, an error model developed originally for use with ink-jet synthesized expression arrays (Hughes *et al.* 2000). Like SAM, this algorithm improves over the basic analyses in that the larger variability at low intensities is incorporated into the model. The test statistic incorporates a measure of the background variance, as well as normalizing the statistic by intensity so that it becomes intensity independent. However, it does assume normality of the data in order to calculate a probability. This is the error model we adopted for use with genome-wide location analysis.

It is important, with whatever statistical method is chosen for analysis of differential expression, to keep the underlying biology in mind. For example, after performing location analysis on approximately fifty different transcription factors in rich media conditions, we began to notice that there were some intergenic regions to which our model was assigning a high probability of being bound in every experiment. That these intergenic regions were truly bound by this many proteins seemed highly unlikely given the physical characteristics of the DNA and proteins – there simply isn't enough physical space to allow for the binding of all of these factors.

It seemed likely that this binding was an experimental artifact. In order to test this hypothesis, we performed a chromatin immunoprecipitation in wild type cells, containing no epitope tag. These immunoprecipitations were performed as per the standard operating procedure in one case, in another, a different antibody and type of beads was used. Not surprisingly, in the immunoprecipitation using the standard antibody and beads, the same set of intergenic regions found in most experiments was assigned a high probability of being enriched. On the other hand, using the different antibody and beads this set of intergenic regions showed no enrichment, implying that the large amount of enrichment we were seeing was an artifact.

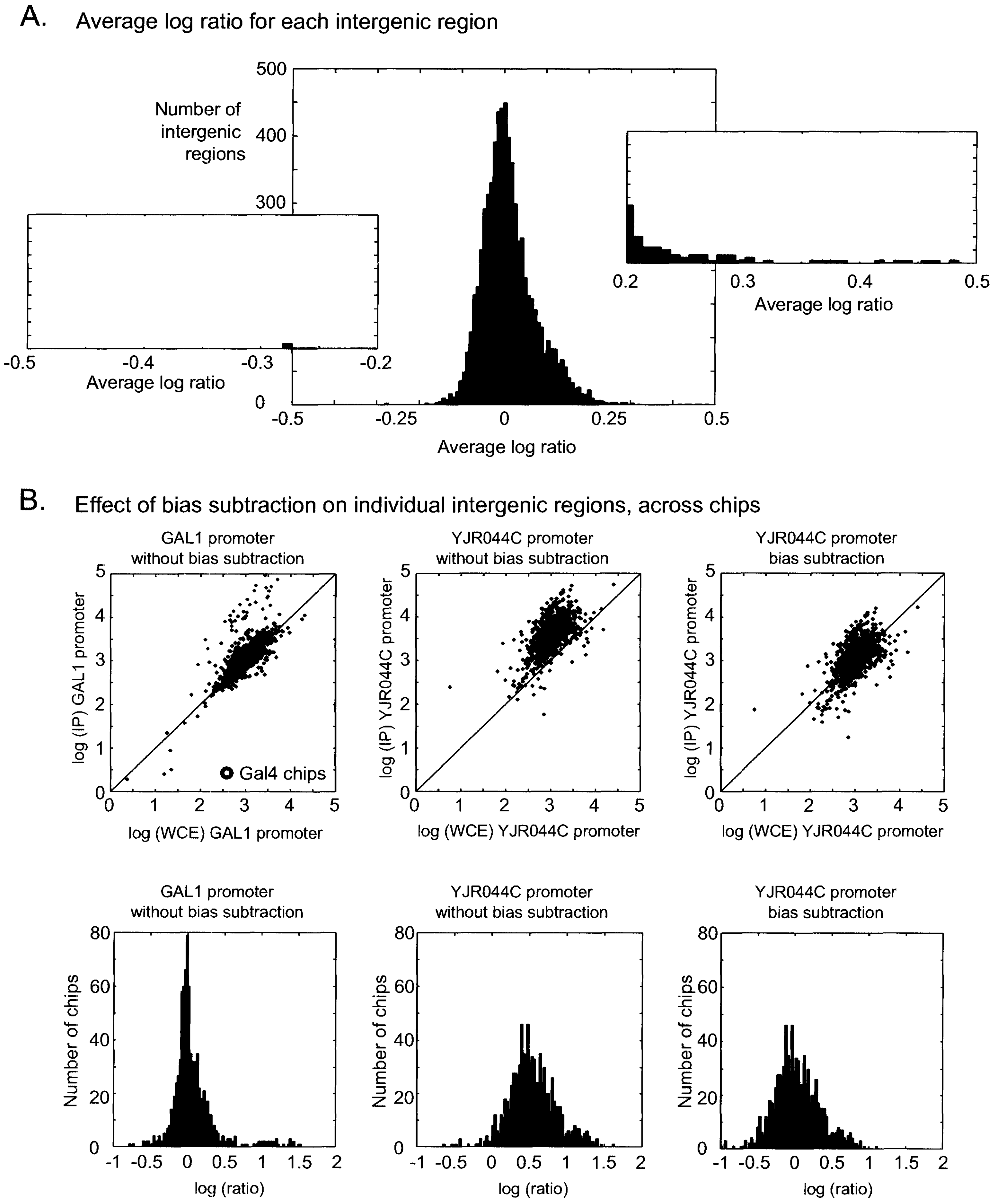
This led me to the add a bias correction step in the data analysis algorithm for location analysis. For each new version of analysis results, the average log ratio for each intergenic region is computed across all chips. A histogram of these results is shown in Figure 3A. There is a much larger proportion of spots with an enriched ratio than would be expected. To remove this bias from the results, the log ratio for each intergenic region on each chip is adjusted to bring the average log ratio for that intergenic region across all chips to zero. This is achieved by adjusting the intensity values in the immunoprecipitated channel, as that is the channel in which the bias is introduced. Figure 3B illustrates the effects of this bias removal. For the Gal1 promoter the bias is minimal. The average log ratio is approximately zero (average ratio of 1), and the majority of enrichment occurs in experiments where Gal4 is the tagged factor, as expected. This is in contrast to the YJR044C promoter, where enrichment is seen in almost every experiment prior to bias removal. Note that unlike the Gal1 promoter, there is no separation of "enriched" spots from non-enriched. The bias removal brings the average log ratio to zero, and removes much of the "enrichment".

Determining biological meaning – comparison with other data sets

The ultimate goal of performing microarray experiments is to gain a deeper biological understanding of the phenomenon being studied. This can occur in some cases by using microarrays as a screen to find genes implicated in a particular process. For example, microarrays were used to compare the efficiency of transformation of cells containing deletions of all non-essential genes with linearized versus circular plasmids, to find genes involved in the non-homologous end joining pathway (Ooi *et al.* 2001). However, in most cases the true power of microarray data is exploited by comparison of one dataset with another, or with other genome-wide information.

One technique that has been used to leverage the information in microarray data is clustering, or grouping together, of gene expression profiles

Figure 3: Bias reduction



A. Average log ratio for each intergenic region

B. Effect of bias subtraction on individual intergenic regions, across chips

Figure 3: Bias Reduction

A) A histogram of the average log base 10 ratio for each spot across > 1000 chips. The inset shows a blowup of the histogram for log ratios > 0.25. Average log ratios greater than zero indicate enrichment. Note that the tail on the enriched side is heavier than the tail on the non-enriched (left) side.

B) R-G scatter plots and ratio histograms for individual promoters across > 1000 chips. In each R-G plot the control intensity for the selected spot (x-axis) is plotted against the immunoprecipitated DNA intensity for that spot on each chip (y-axis). The GAL1 promoter is an unbiased spot, the average log ratio is close to zero, and enriched chips are separated from non-enriched chips. GAL1 is known to be regulated by Gal4, so spots corresponding to chips where Gal4 was the tagged factor are marked in red. Other highly enriched spots correspond to tagged factors Mig1 and Mth1, also known to regulate galactose synthesis. YJR044C is a highly biased promoter, with a log ratio greater than zero in almost every experiment, and no separation between spot populations, indicating a low likelihood of enrichment. The bias reduction reduces the average log ratio to 0.

generated through perturbations. This is based on the hypothesis that genes involved in the same function will have similar effects on gene expression when deleted. Likewise, extracellular perturbations such as application of a drug or other small molecule that affect a particular pathway will cause analogous changes. Clustering of such expression profiles has been used to annotate previously uncharacterized genes (Hughes *et al.* 2000; Wu *et al.* 2002), to find targets of drugs (Hughes *et al.* 2000; Parsons *et al.* 2004), and to find novel genes involved in peroxisome biogenesis (Smith *et al.* 2002).

Comparison amongst various types of genome-wide data has been particularly productive for determining functional significance of microarray results. Genes in common between expression data and the binding of gene-specific transcription factors (Ren *et al.* 2000; Bar-Joseph *et al.* 2003) indicate likely transcriptional regulation. The overlap between genes whose upstream region contains a particular sequence motif and expression data can correlate that motif with gene functions (Kellis *et al.* 2003). Overlaps with location data can indicate which transcription factor is binding to the selected sequence motif. Comparison of genes or sequences culled from any of these types of experiments with functional annotation based on literature curation has been widely used to determine pathways or functions affected (Robinson *et al.* 2002; Doniger *et al.* 2003) These comparisons are performed by selecting genes from one dataset that meet a particular threshold, then counting the number of genes in common with the comparison gene set. A probability is calculated using the hypergeometric distribution or its binomial approximation. The categories with the most significant enrichment are taken as likely to inform on the biological function of the set of genes being tested, and in many cases have been validated experimentally as such.

The biggest drawback to this approach is the use of a fixed cutoff in selection of one or both gene sets being examined. A novel algorithm called "Gene Set Enrichment Analysis" (GSEA) which avoids this shortcoming has been proposed (Mootha *et al.* 2003). The expression profiles of muscle tissue from both diabetic patients and normal controls were obtained. After correcting for multiple testing, there were no genes which met the threshold for significance. Instead, each gene was ranked based on the average difference in expression between the patients and controls, and an enrichment score for overrepresentation of genes in a particular category or biological pathway was computed. Significance of the enrichment was assessed by comparison with permuted data. In this study, the functional class with the highest enrichment score was genes involved in oxidative phosphorylation. Examination of the genes so classified showed a highly significant decrease in expression of approximately 20% across this set of genes (Mootha *et al.* 2003). Thus by using a rank ordering of genes for comparison with functional annotations, the problem of selection of an appropriate significance threshold for a single gene was avoided.

Despite the improvement that GSEA makes over the previously used heuristics, it does face the same problem in that the gene sets must be static. The groups of genes culled from literature annotation, containing particular sequence motifs, or clusters of expression data meet this criterion, but genes selected as highly differentially expressed in other microarray studies do not. Another case where the use of a significance cutoff can prove problematic is in comparison of location data for different factors. Determining the overlap of binding between factors can be used to decide if the factors are functioning at the same promoters (Zeitlinger *et al.* 2003). Particularly if one or both factors are more general, the use of a significance cutoff for binding can mask detection of a true overlap, or the extent of an overlap.

I have developed a simple algorithm and statistic for overcoming these obstacles. Rather than comparing a fixed number of genes or intergenic regions from each dataset, a sliding comparison is performed. Similar to GSEA, genes in each dataset are first ranked by significance. Then, for the top N genes the number of genes in common between the two datasets is plotted, with N varying from 1 to the size of the dataset. The area under the curve is used as the test statistic. Significance is computed based on the number of higher test statistics seen when testing the overlap with other data. This test statistic could be improved – an enrichment score computed as in GSEA would probably be more sensitive (Mootha *et al.* 2003).

The differences between computing overlaps between static, thresholded sets of genes and the sliding overlap method are highlighted in Figure 4. The first comparison, Figure 4A, is between the binding of each of four factors in rich media versus peroxide. Using the static method, overlaps between the two conditions are significant for three of the four factors, Hsf1, Reb1, and Yap4. The

Figure 4: Overlaps Between Datasets

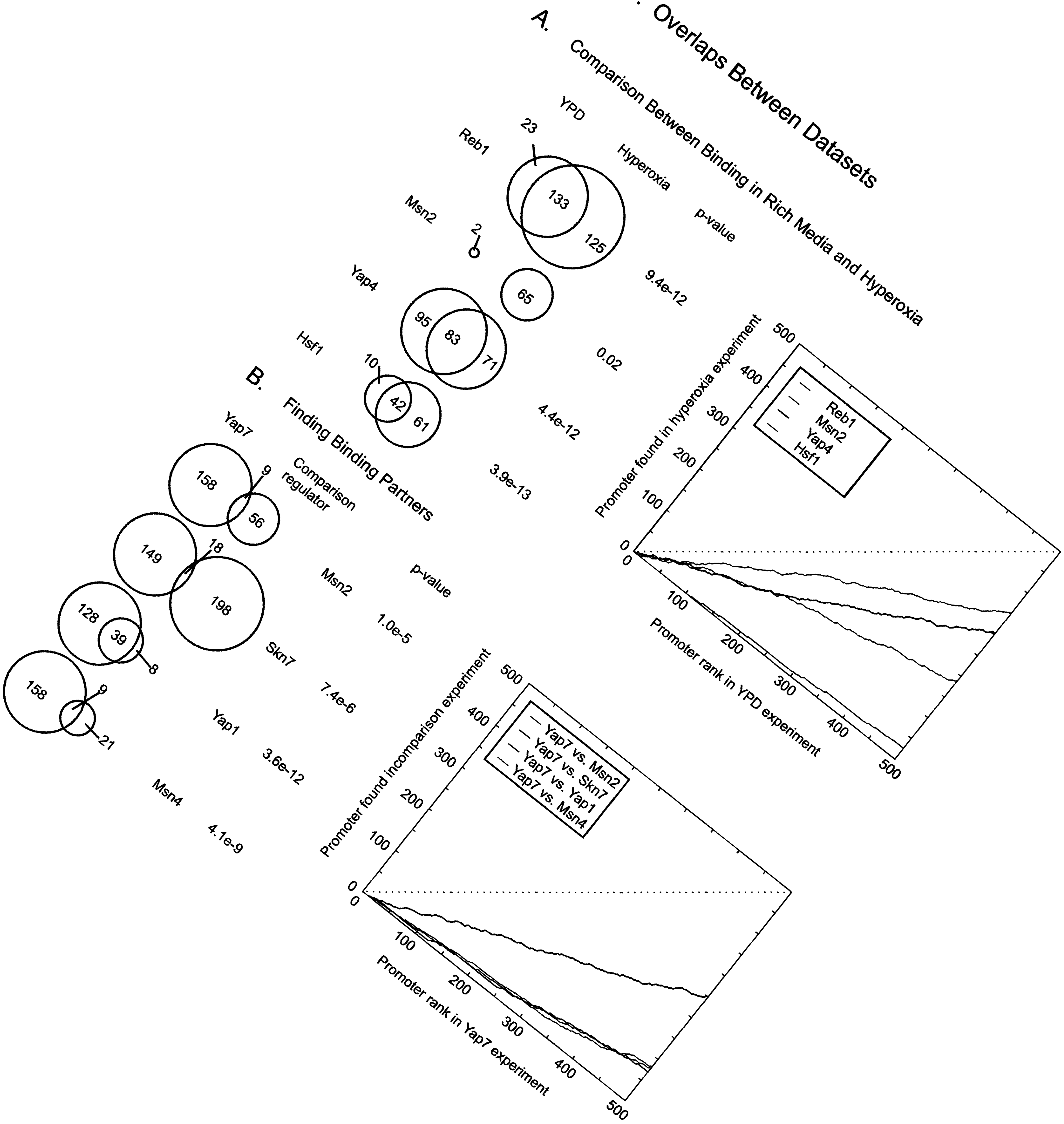


Figure 4: Overlaps Between Datasets

Two methods of assessing overlaps between datasets. The static method, where genes are selected for comparison if they pass a threshold, in this case a p-value <0.001. The overlaps between selected genes are counted, and a probability computed using the hypergeometric test. These overlaps are shown using Venn diagrams, where the size of the circle or overlap is proportional to the number of genes. In the sliding overlap method, genes in one dataset are ranked in order of enrichment (or expression). For each N, the number of the top N ranked genes in that dataset found in the top N ranked genes of the comparison dataset is counted, and this overlap is plotted from 1 to N. An overlap probability is computed by comparing with randomized data.

A) Comparison between binding of factors in rich media with peroxide. The factors chosen for comparison were Reb1, Msn2, Yap4 and Hsf1. A separate Venn diagram with associated p-value is shown for each factor for the static overlap method. Using this tool, significant overlaps are seen for Yap4, Hsf1 and Reb1. No overlap is found for Msn2 in rich media versus peroxide. The Yap4 and Hsf1 overlaps are equally significant according to the hypergeometric test, with a slightly less significant overlap for Reb1. For the sliding overlap technique, a line showing the extent of the overlap is graphed for each factor, Reb1 (green), Msn2 (red), Yap4 (blue) and Hsf1 (magenta). The black dotted line indicates the maximum possible overlap. In contrast to the results seen with the static overlap method, Reb1 has the highest overlap between the YPD and peroxide conditions, followed by Yap4 and Hsf1. In agreement with the static method, minimal overlap is seen with Msn2.

B) Comparison between binding of Yap7 and four hyperoxia regulators in peroxide – Msn2 (green), Skn7 (red), Yap1 (blue) and Msn4 (magenta). Significant overlaps are computed for each factor compared with Yap7 with the static method. Yap1 has the highest overlap, followed by Msn4. Again, this is contrasted with the results from the sliding overlap technique, where the only significant overlap is between Yap7 and Yap1, and the overlaps with the other three factors are equivalent.

overlap is most significant for Hsf1, followed by Reb1 and Yap4. On the other hand, the sliding overlap method makes it clear that the binding of Reb1 is actually the most similar between the two conditions, followed by Yap4 then Hsf1. In Figure 4B, comparisons are made between binding of regulators in the same condition, hyperoxia, to determine whether any of the regulators might be functioning at the same promoters. The binding of Yap7 is compared with the binding of the four master regulators of the hyperoxic stress response. With the static method, despite only small overlaps between Yap7 and Msn2, Msn4 and Skn7, all overlaps are assigned a reasonably significant p-value. The overlap between Yap7 and Yap1 is most significant, followed by the overlap of Yap7 with Msn4. However, the sliding overlap method shows that only the overlap between Yap7 and Yap1 is significant, and that the overlaps with the other three factors are almost identical. This illustrates how use of a fixed cutoff can introduce artifacts into the analysis.

Determining biological meaning – network motifs

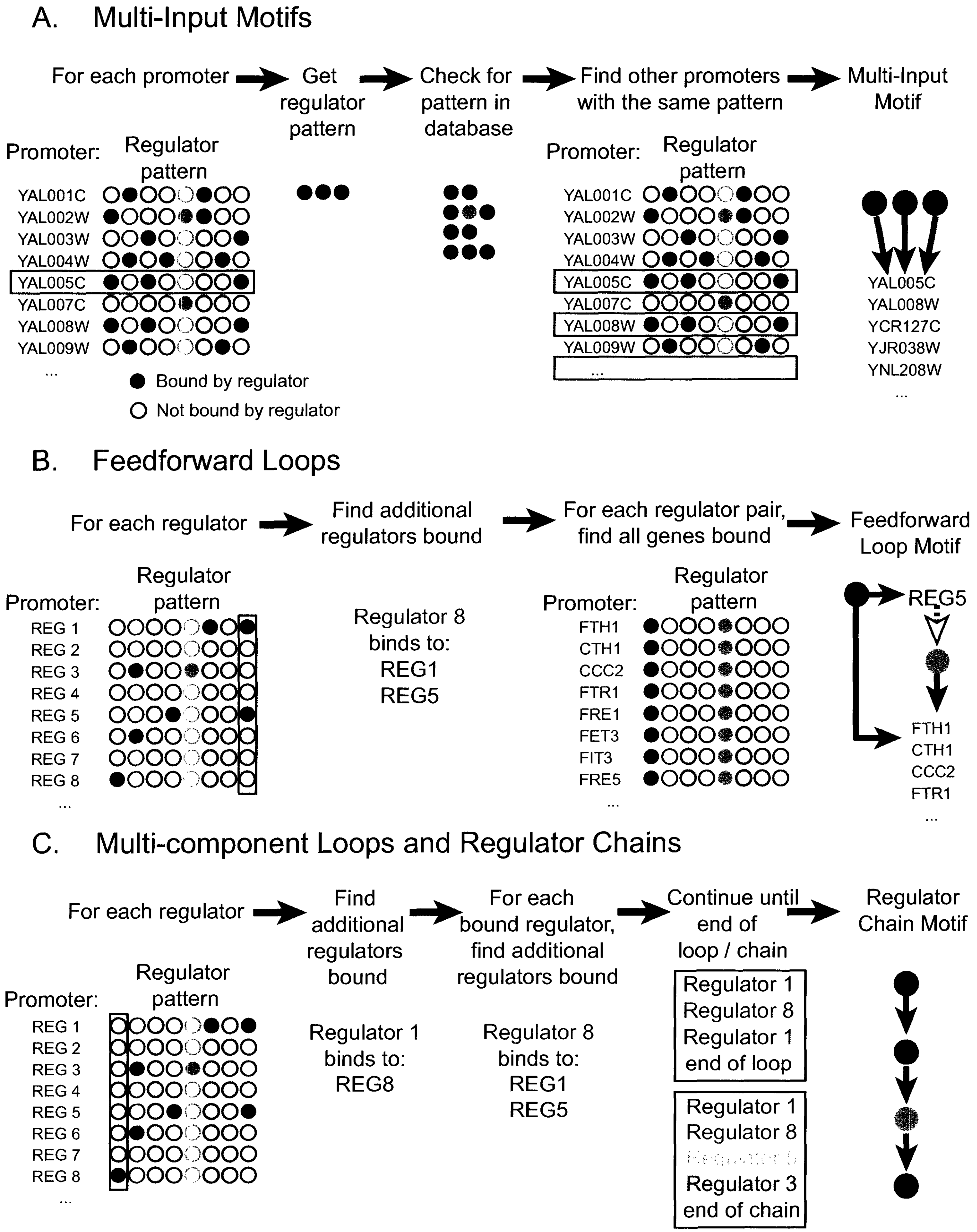
One way that the overlaps between the sets of genes bound by transcription factors have been used is to find factors that might be binding as a complex, or cooperatively regulating genes in some other way. Groups of regulators jointly regulating expression of groups of genes has been termed a “multi-input motif” (Lee *et al.* 2002b), or “dense overlapping regulon” (Shen-Orr *et al.* 2002). This is one example of a network motif, patterns of regulation that occur more frequently than expected by chance (Milo *et al.* 2002; Shen-Orr *et al.*

2002). Other network motifs include autoregulation, feedforward loops, single-input motifs, multi-component loops, and regulator chains (Lee *et al.* 2002b). Individual network motifs for a small subset of regulators have been defined on a gene by gene basis, but until genome-wide location analysis was used to profile most regulators, enumeration of the possible network motifs used by the cell globally was not feasible.

I developed a set of algorithms for finding all of the network motifs present in the genome-wide binding data. These motifs are not definitive, but instead designed to provide hypotheses for further testing. As with other analyses we performed with these location data, we used a fixed p value threshold of 0.001 to minimize the number of false positives, but this does mean that we are probably not capturing all network motifs. The methods for discovering the motifs ranged from the trivial determination of the autoregulatory motifs, the set of regulators found to be binding to their own promoters, to the complexities of finding the multi-component loops and regulator chains.

Single-input motifs consisted of simply listing the genes bound by each regulator. Multi-input motifs were found by testing each gene (see schematic in Figure 5A), first to determine if it was bound by multiple regulators, then to find out if the pattern of bound regulators had already been cataloged. If the regulatory pattern was novel, the remaining genes were searched to find any additional instances of the pattern. This reduced computational time significantly over testing each possible regulatory pattern. To obtain the list of feedforward loops, any additional regulators bound by a given factor were found, followed by

Figure 5: Algorithms to Find Network Motifs



A. Multi-Input Motifs

B. Feedforward Loops

C. Multi-component Loops and Regulator Chains

Figure 5: Algorithms to Find Network Motifs

For these schematics, the promoter for a given gene is labeled with capital letters. The pattern of regulators binding to a promoter is indicated by circles, which are filled if the regulator is binding, or empty if the regulator is not binding. The promoter or regulator being examined is boxed in red. In the motif schematics, regulator proteins are denoted by circles, the promoters to which they are binding are denoted by rectangles. A binding interaction is shown by an arrow point from the regulator to the promoter to which it binds. This figure shows schematics for how network motifs were found as follows:

A) Multi-Input Motifs. The list of genes is scanned through, one by one. The pattern of regulators binding to the current gene is found. If that pattern has not already been used, all other genes that are bound by the same pattern of regulators are added to the motif.

B) Feedforward Loops. Each regulator is examined to find promoters to which it binds that could regulate additional regulators. All genes that are bound by both regulators are added to the motif.

C) Multi-component Loops and Regulator Chains. This is a recursive algorithm, where the list of regulators bound by the factor being examined is obtained. The list of regulator bound by each of those downstream regulators is then found, and so on. The motif terminates when a downstream regulator does not bind to any additional regulators, leading to a Regulator Chain, or the downstream regulator binds to a regulator earlier in the series, leading to a Multi-Component Loop.

the group of genes bound by these two regulators (Figure 5B). Since multi-component loops and regulator chains are similar in that they consist of multiple sequential regulator-regulator promoter interactions, they were found using a single recursive algorithm (Figure 5C). In each loop of the recursion, one more regulator was added onto a loop or chain, until a regulator earlier in the sequence was repeated (loop), or no further regulators were bound by the terminal factor (chain). After the final list of loops and chains was determined, any motif that was a subset of another motif was removed. Some improvements that could be made to these methods include incorporating additional sources of data such as sequence motifs, conservation and expression information in order to relax the p-value threshold used.

While the biological effects of the motifs we discovered have yet to be demonstrated, network motifs discovered using traditional biological experiments have been shown to effect particular patterns of transcriptional regulation. Positive autoregulatory events increase the amount of a transcription factor available to regulate expression of additional genes upon a specific change in environment. Transcription of Pdr3, a regulator involved in response to various drugs, is initially stimulated by binding of Pdr1 followed by autoregulation (Delahodde *et al.* 1995). These two regulators are also involved in a feedforward loop: Pdr1 binds to and activates Pdr3, and the two activators together regulate expression of a number of transporters, including Pdr5, Pdr10, Pdr15, Snq2 and Yor1 (Decottignies *et al.* 1994; Decottignies *et al.* 1995; Wolfger *et al.* 1997; Decottignies *et al.* 1998). Examples of multi-input motifs abound; in the regulation of response to various stresses, Hsf1 and Msn2/4 coordinate the response of some genes (Treger *et al.* 1998; Amoros and Estruch 2001), while Skn7 and Hsf1 regulate others (Raitt *et al.* 2000).

Determining biological meaning – data visualization

Comparing microarray data with annotations or other data sets can yield biological discovery in terms of which pathways might be regulated by a particular perturbation, or which regulators might be causing those changes, for example. However, it can be very easy to miss key discoveries because of the size of the data sets and our inherent inability to cope with that much information. Methods for visualizing the data have proved invaluable in spotting global trends.

The first type of visualization used was developed for viewing the results of clustering analyses. In this depiction, now commonly called a 'heat map', ratios that indicate an increase in expression are colored in red, those indicating a decrease are colored green, and the intensity of the color is proportional to the magnitude of the change (Eisen *et al.* 1998; Wen *et al.* 1998). The data are clustered using one of a number of metrics, then displayed. Patterns of changes that might be missed just by examination of the numerical data are now clearly obvious. In analyzing the response of yeast cells to varying stress conditions (Gasch *et al.* 2000; Causton *et al.* 2001), for example, the fact that there is a common response to all the stresses becomes obvious when the data are clustered and visualized in this manner. Besides being effective with expression data, this method of visualizing data can aid discoveries with other technologies as well. A heat map of clustered location data for the cell cycle regulators shows the waves of regulation over time (Simon *et al.* 2001). Heat maps of location data for the binding of 106 yeast transcription factors to promoters shows pairs and groups of regulators that are functioning together, as well as the sets of genes being regulated (Lee *et al.* 2002b).

Another useful way of displaying both expression and location data is by chromosomal location. Genes that are changing in expression, or that are bound by a transcription factor are colored on a depiction of each chromosome. Visualizing changes in expression upon depletion of histone H4 in this manner led to the insight that histones do not seems to be generally repressive to gene expression except at the 20kb most proximal to telomeres (Wyrick *et al.* 1999). This contrasted strikingly with the pattern seen upon deletion of the silencing proteins, Sir2, 3 and 4, where the repressive effect did not spread nearly as far. In another instance, the chromosomal coloring was used to illustrate the gene-specificity of Swi/Snf dependent remodeling (Sudarsanam *et al.* 2000). An open question had been whether this chromatin remodeling complex was altering the nucleosome structure of entire chromatin domains, or whether the changes were localized to individual promoters. Visualization of the expression changes occurring in Swi/Snf mutants showed no clustering of changes by chromosomal location, indicating that the remodeling action of Swi/Snf occurs on a very limited basis. Microarrays for genome-wide location analysis are constantly being improved; the latest generations include tiled promoter regions. Similar representations of these tiled regions could assist in discovering the global positions at which general regulatory factors or chromatin modifiers are acting.

Other displays of genome-scale data are often used, but more to illustrate discoveries than to make them. Venn diagrams of overlaps between data sets or between data sets and annotations are frequently used to give a visual sense of the scale of the overlap. Scatter plots of intensities or ratios for a single experiment can show the effectiveness of the normalization and analysis methods; scatter plots comparing different experiments illustrate the extent of differences between the two data sets. Graphs of clustered expression profiles, for example, illustrated the changes seen in the profile over time or through experiments.

Because we are much better able to process the amount of information generated in a microarray experiment visually rather than by examining numbers, it behooves the community to develop even more methods by which biological discoveries can be made. With so many novel genome-wide data sets of which to take advantage, methods that can distill these into clear representations will greatly assist in helping to use these data to make global discoveries rather than serving in the same way as gene by gene approaches have for the past century.

References:

Alter O, Brown PO and Botstein D (2000). "Singular value decomposition for genome-wide expression data processing and modeling." Proc Natl Acad Sci U S A **97**(18): 10101-6.

Amoros M and Estruch F (2001). "Hsf1p and Msn2/4p cooperate in the expression of Saccharomyces cerevisiae genes HSP26 and HSP104 in a gene- and stress type-dependent manner." Mol Microbiol **39**(6): 1523-32.

Baldi P and Long AD (2001). "A Bayesian framework for the analysis of microarray expression data: regularized t -test and statistical inferences of gene changes." Bioinformatics **17**(6): 509-19.

Ball CA, Sherlock G, Parkinson H, Rocca-Sera P, *et al.* (2002). "Standards for microarray data." Science **298**(5593): 539.

Bar-Joseph Z, Gerber GK, Lee TI, Rinaldi NJ, *et al.* (2003). "Computational discovery of gene modules and regulatory networks." Nat Biotechnol **21**(11): 1337-42.

Battaglia C, Salani G, Consolandi C, Bernardi LR and De Bellis G (2000). "Analysis of DNA microarrays by non-destructive fluorescent staining using SYBR green II." Biotechniques **29**(1): 78-81.

Broberg P (2003). "Statistical methods for ranking differentially expressed genes." Genome Biol **4**(6): R41.

Causton HC, Ren B, Koh SS, Harbison CT, *et al.* (2001). "Remodeling of yeast genome expression in response to environmental changes." Mol Biol Cell **12**(2): 323-37.

Cho RJ, Campbell MJ, Winzeler EA, Steinmetz L, *et al.* (1998). "A genome-wide transcriptional analysis of the mitotic cell cycle." Mol Cell **2**(1): 65-73.

Cho RJ, Huang M, Campbell MJ, Dong H, *et al.* (2001). "Transcriptional regulation and function during the human cell cycle." Nat Genet **27**(1): 48-54.

Chu S, DeRisi J, Eisen M, Mulholland J, Botstein D, Brown PO and Herskowitz I (1998). "The transcriptional program of sporulation in budding yeast." Science **282**(5389): 699-705.

Cleveland WS (1979). "Robust locally weighted regression and smoothing scatterplots." J Amer Stat Assoc **74**: 829-836.

Dasgupta A, Darst RP, Martin KJ, Afshari CA and Auble DT (2002). "Mot1 activates and represses transcription by direct, ATPase-dependent mechanisms." Proc Natl Acad Sci U S A **99**(5): 2666-71.

Decottignies A, Grant AM, Nichols JW, de Wet H, McIntosh DB and Goffeau A (1998). "ATPase and multidrug transport activities of the overexpressed yeast ABC protein Yor1p." J Biol Chem **273**(20): 12612-22.

Decottignies A, Kolaczkowski M, Balzi E and Goffeau A (1994). "Solubilization and characterization of the overexpressed PDR5 multidrug resistance nucleotide triphosphatase of yeast." J Biol Chem **269**(17): 12797-803.

Decottignies A, Lambert L, Catty P, Degand H, Epping EA, Moye-Rowley WS, Balzi E and Goffeau A (1995). "Identification and characterization of SNQ2, a new multidrug ATP binding cassette transporter of the yeast plasma membrane." J Biol Chem **270**(30): 18150-7.

Delahodde A, Delaveau T and Jacq C (1995). "Positive autoregulation of the yeast transcription factor Pdr3p, which is involved in control of drug resistance." Mol Cell Biol **15**(8): 4043-51.

DeRisi JL, Iyer VR and Brown PO (1997). "Exploring the metabolic and genetic control of gene expression on a genomic scale." Science **278**(5338): 680-6.

Doniger SW, Salomonis N, Dahlquist KD, Vranizan K, Lawlor SC and Conklin BR (2003). "MAPPFinder: using Gene Ontology and GenMAPP to create a global gene-expression profile from microarray data." Genome Biol **4**(1): R7.

Dudoit Y, Yang YH, Callow MJ and Speed T (2000). Statistical methods for identifying differentially expressed genes in replicated cDNA microarray experiments. Technical Report 578, Department of Statistics. UC Berkeley, CA.

Eisen MB, Spellman PT, Brown PO and Botstein D (1998). "Cluster analysis and display of genome-wide expression patterns." Proc Natl Acad Sci U S A **95**(25): 14863-8.

Finkelstein D, Ewing R, Gollub J, Sterky F, Cherry JM and Somerville S (2002). "Microarray data quality analysis: lessons from the AFGC project. Arabidopsis Functional Genomics Consortium." Plant Mol Biol **48**(1-2): 119-31.

Fleischmann RD, Adams MD, White O, Clayton RA, *et al.* (1995). "Whole-genome random sequencing and assembly of Haemophilus influenzae Rd." Science **269**(5223): 496-512.

Gasch AP, Spellman PT, Kao CM, Carmel-Harel O, Eisen MB, Storz G, Botstein D and Brown PO (2000). "Genomic expression programs in the response of yeast cells to environmental changes." Mol Biol Cell **11**(12): 4241-57.

Goffeau A, Barrell BG, Bussey H, Davis RW, *et al.* (1996). "Life with 6000 genes." Science **274**(5287): 546, 563-7.

Holstege FC, Jennings EG, Wyrick JJ, Lee TI, Hengartner CJ, Green MR, Golub TR, Lander ES and Young RA (1998). "Dissecting the regulatory circuitry of a eukaryotic genome." Cell **95**(5): 717-28.

Hughes TR, Marton MJ, Jones AR, Roberts CJ, *et al.* (2000). "Functional discovery via a compendium of expression profiles." Cell **102**(1): 109-26.

Ideker T, Thorsson V, Ranish JA, Christmas R, *et al.* (2001). "Integrated genomic and proteomic analyses of a systematically perturbed metabolic network." Science **292**(5518): 929-34.

Ideker T, Thorsson V, Siegel AF and Hood LE (2000). "Testing for differentially-expressed genes by maximum-likelihood analysis of microarray data." J Comput Biol **7**(6): 805-17.

Kellis M, Patterson N, Endrizzi M, Birren B and Lander ES (2003). "Sequencing and comparison of yeast species to identify genes and regulatory elements." Nature **423**(6937): 241-54.

Lee PD, Sladek R, Greenwood CM and Hudson TJ (2002a). "Control genes and variability: absence of ubiquitous reference transcripts in diverse mammalian expression studies." Genome Res **12**(2): 292-7.

Lee TI, Rinaldi NJ, Robert F, Odom DT, *et al.* (2002b). "Transcriptional regulatory networks in Saccharomyces cerevisiae." Science **298**(5594): 799-804.

Leung YF and Cavalieri D (2003). "Fundamentals of cDNA microarray data analysis." Trends Genet **19**(11): 649-59.

Milo R, Shen-Orr S, Itzkovitz S, Kashtan N, Chklovskii D and Alon U (2002). "Network motifs: simple building blocks of complex networks." Science **298**(5594): 824-7.

Mootha VK, Lindgren CM, Eriksson KF, Subramanian A, *et al.* (2003). "PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes." Nat Genet **34**(3): 267-73.

Moqtaderi Z and Struhl K (2004). "Genome-wide occupancy profile of the RNA polymerase III machinery in Saccharomyces cerevisiae reveals loci with incomplete transcription complexes." Mol Cell Biol **24**(10): 4118-27.

Mukherjee S, Berger MF, Jona G, Wang XS, Muzzey D, Snyder M, Young RA and Bulyk ML (2004). "Rapid analysis of the DNA binding specificities of transcription factors with DNA microarrays." Nat Genet.

Natarajan K, Meyer MR, Jackson BM, Slade D, Roberts C, Hinnebusch AG and Marton MJ (2001). "Transcriptional profiling shows that Gcn4p is a master regulator of gene expression during amino acid starvation in yeast." Mol Cell Biol **21**(13): 4347-68.

Newton MA, Kendziorski CM, Richmond CS, Blattner FR and Tsui KW (2001). "On differential variability of expression ratios: improving statistical inference about gene expression changes from microarray data." J Comput Biol **8**(1): 37-52.

Ooi SL, Shoemaker DD and Boeke JD (2001). "A DNA microarray-based genetic screen for nonhomologous end-joining mutants in Saccharomyces cerevisiae." Science **294**(5551): 2552-6.

Pan W (2002). "A comparative review of statistical methods for discovering differentially expressed genes in replicated microarray experiments." Bioinformatics **18**(4): 546-54.

Parsons AB, Brost RL, Ding H, Li Z, *et al.* (2004). "Integration of chemical-genetic and genetic interaction data links bioactive compounds to cellular target pathways." Nat Biotechnol **22**(1): 62-9.

Petri A, Fleckner J and Matthiessen MW (2004). "Array-A-Lizer: a serial DNA microarray quality analyzer." BMC Bioinformatics **5**(1): 12.

Quackenbush J (2002). "Microarray data normalization and transformation." Nat Genet **32 Suppl**: 496-501.

Raitt DC, Johnson AL, Erkine AM, Makino K, Morgan B, Gross DS and Johnston LH (2000). "The Skn7 response regulator of Saccharomyces cerevisiae interacts with Hsf1 in vivo and is required for the induction of heat shock genes by oxidative stress." Mol Biol Cell **11**(7): 2335-47.

Ren B, Robert F, Wyrick JJ, Aparicio O, *et al.* (2000). "Genome-wide location and function of DNA binding proteins." Science **290**(5500): 2306-9.

Richmond CS, Glasner JD, Mau R, Jin H and Blattner FR (1999). "Genome-wide expression profiling in Escherichia coli K-12." Nucleic Acids Res **27**(19): 3821-35.

Robinson MD, Grigull J, Mohammad N and Hughes TR (2002). "FunSpec: a web-based cluster interpreter for yeast." BMC Bioinformatics **3**(1): 35.

Savonet V, Maenhaut C, Miot F and Pirson I (1997). "Pitfalls in the use of several "housekeeping" genes as standards for quantitation of mRNA: the example of thyroid cells." Anal Biochem **247**(1): 165-7.

Schuchhardt J, Beule D, Malik A, Wolski E, Eickhoff H, Lehrach H and Herzel H (2000). "Normalization strategies for cDNA microarrays." Nucleic Acids Res **28**(10): E47.

Shearstone JR, Allaire NE, Getman ME and Perrin S (2002). "Nondestructive quality control for microarray production." Biotechniques **32**(5): 1051-2, 1054, 1056-7.

Shen-Orr SS, Milo R, Mangan S and Alon U (2002). "Network motifs in the transcriptional regulation network of Escherichia coli." Nat Genet **31**(1): 64-8.

Simon I, Barnett J, Hannett N, Harbison CT, *et al.* (2001). "Serial regulation of transcriptional regulators in the yeast cell cycle." Cell **106**(6): 697-708.

Smith JJ, Marelli M, Christmas RH, Vizeacoumar FJ, *et al.* (2002). "Transcriptome profiling to identify genes involved in peroxisome assembly and function." J Cell Biol **158**(2): 259-71.

Smyth GK and Speed T (2003). "Normalization of cDNA microarray data." Methods **31**(4): 265-73.

Spellman PT, Sherlock G, Zhang MQ, Iyer VR, Anders K, Eisen MB, Brown PO, Botstein D and Futcher B (1998). "Comprehensive identification of cell cycle-regulated genes of the yeast Saccharomyces cerevisiae by microarray hybridization." Mol Biol Cell **9**(12): 3273-97.

Sudarsanam P, Iyer VR, Brown PO and Winston F (2000). "Whole-genome expression analysis of snf/swi mutants of Saccharomyces cerevisiae." Proc Natl Acad Sci U S A **97**(7): 3364-9.

Tegner J, Yeung MK, Hasty J and Collins JJ (2003). "Reverse engineering gene networks: integrating genetic perturbations with dynamical modeling." Proc Natl Acad Sci U S A **100**(10): 5944-9.

Treger JM, Schmitt AP, Simon JR and McEntee K (1998). "Transcriptional factor mutations reveal regulatory complexities of heat shock and newly identified stress genes in Saccharomyces cerevisiae." J Biol Chem **273**(41): 26875-9.

Tseng GC, Oh MK, Rohlin L, Liao JC and Wong WH (2001). "Issues in cDNA microarray analysis: quality filtering, channel normalization, models of variations and assessment of gene effects." Nucleic Acids Res **29**(12): 2549-57.

Tusher VG, Tibshirani R and Chu G (2001). "Significance analysis of microarrays applied to the ionizing radiation response." Proc Natl Acad Sci U S A **98**(9): 5116-21.

Wang S and Ethier S (2004). "A generalized likelihood ratio test to identify differentially expressed genes from microarray data." Bioinformatics **20**(1): 100-4.

Wang X, Ghosh S and Guo SW (2001). "Quantitative quality control in microarray image processing and data acquisition." Nucleic Acids Res **29**(15): E75-5.

Wang X, Hessner MJ, Wu Y, Pati N and Ghosh S (2003). "Quantitative quality control in microarray experiments and the application in data filtering, normalization and false positive rate prediction." Bioinformatics **19**(11): 1341-7.

Wen X, Fuhrman S, Michaels GS, Carr DB, Smith S, Barker JL and Somogyi R (1998). "Large-scale temporal gene expression mapping of central nervous system development." Proc Natl Acad Sci U S A **95**(1): 334-9.

Wilson DL, Buckley MJ, Helliwell CA and Wilson IW (2003). "New normalization methods for cDNA microarray data." Bioinformatics **19**(11): 1325-32.

Wolfger H, Mahe Y, Parle-McDermott A, Delahodde A and Kuchler K (1997). "The yeast ATP binding cassette (ABC) protein genes PDR10 and PDR15 are novel targets for the Pdr1 and Pdr3 transcriptional regulators." FEBS Lett **418**(3): 269-74.

Wu LF, Hughes TR, Davierwala AP, Robinson MD, Stoughton R and Altschuler SJ (2002). "Large-scale prediction of Saccharomyces cerevisiae gene function using overlapping transcriptional clusters." Nat Genet **31**(3): 255-65.

Wyrick JJ, Holstege FC, Jennings EG, Causton HC, Shore D, Grunstein M, Lander ES and Young RA (1999). "Chromosomal landscape of nucleosome-dependent gene expression and silencing in yeast." Nature **402**(6760): 418-21.

Yang IV, Chen E, Hasseman JP, Liang W, *et al.* (2002a). "Within the fold: assessing differential expression measures and reproducibility in microarray assays." Genome Biol **3**(11): research0062.

Yang YH, Dudoit S, Luu P, Lin DM, Peng V, Ngai J and Speed TP (2002b). "Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation." Nucleic Acids Res **30**(4): e15.

Yu X and Wolfinger R (2004). A mixed model approach to identify yeast transcription regulatory motifs via microarray experiments, personal communication.

Zeitlinger J, Simon I, Harbison CT, Hannett NM, Volkert TL, Fink GR and Young RA (2003). "Program-specific distribution of a transcription factor dependent on partner transcription factor and MAPK signaling." Cell **113**(3): 395-404.

Chapter 3

Transcriptional Regulatory Networks in Saccharomyces cerevisiae

Published as: Lee TI, Rinaldi NJ, Robert F, Odom DT, Bar-Joseph Z, Gerber GK, Hannett NM, Harbison CT, Thompson CM, Simon I, Zeitlinger J, Jennings EG, Murray HL, Gordon DB, Ren B, Wyrick JJ, Tagne JB, Volkert TL, Fraenkel E, Gifford DK and Young RA (2002). "Transcriptional regulatory networks in Saccharomyces cerevisiae." Science **298**(5594): 799-804.

Summary

We have determined how most of the transcriptional regulators encoded in the eukaryote Saccharomyces cerevisiae associate with genes across the genome in living cells. Just as maps of metabolic networks describe the potential pathways that may be used by a cell to accomplish metabolic processes, this network of regulator-gene interactions describes potential pathways yeast cells can use to regulate global gene expression programs. We use this information to identify network motifs, the simplest units of network architecture, and demonstrate that an automated process can use motifs to assemble a transcriptional regulatory network structure. Our results reveal that eukaryotic cellular functions are highly connected through networks of transcriptional regulators that regulate other transcriptional regulators.

Introduction

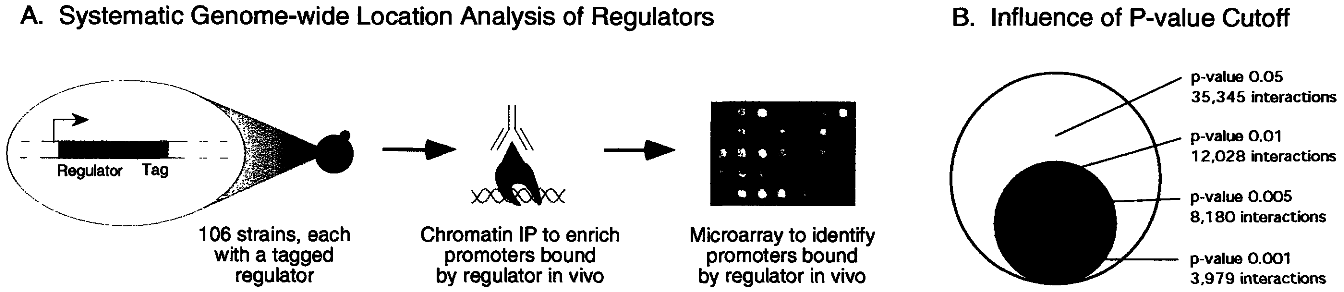
Genome sequences specify the gene expression programs that produce living cells, but how the cell controls global gene expression programs is far from understood. Each cell is the product of specific gene expression programs involving regulated transcription of thousands of genes. These transcriptional programs are modified as cells progress through the cell cycle, in response to changes in environment, and during organismal development (DeRisi *et al.* 1997; Cho *et al.* 1998; Spellman *et al.* 1998; Gasch *et al.* 2000; Causton *et al.* 2001).

Gene expression programs depend on recognition of specific promoter sequences by transcriptional regulatory proteins (Lee and Young 2000; Garvie and Wolberger 2001; Orphanides and Reinberg 2002; Ptashne and Gann 2002). Because these regulatory proteins recruit and regulate chromatin modifying complexes and components of the transcription apparatus, knowledge of the sites bound by all the transcriptional regulators encoded in a genome can provide the information necessary to nucleate models for transcriptional regulatory networks. With the availability of complete genome sequences and development of a method for genome-wide binding analysis (also known as genome-wide location analysis), investigators can identify the set of target genes bound in vivo by each of the transcriptional regulators that are encoded in a cell's genome. This approach has been used to identify the genomic sites bound by nearly a dozen regulators of transcription (Ren *et al.* 2000; Iyer *et al.* 2001; Lieb *et al.* 2001; Simon *et al.* 2001) and several regulators of DNA synthesis (Wyrick *et al.* 2001) in yeast.

Experimental Design

We have used genome-wide location analysis to investigate how yeast transcriptional regulators bind to promoter sequences across the genome (Fig. 1A). All 141 transcription factors listed in the Yeast Proteome Database (Costanzo *et al.* 2000) and reported to have DNA-binding and transcriptional activity were selected for study. Yeast strains were constructed so that each of the transcription factors contained a myc epitope tag. To increase the likelihood that tagged factors were expressed at physiologic levels, we introduced epitope tag coding sequences into the genomic sequences encoding the COOH terminus

Figure 1



A. Systematic Genome-wide Location Analysis of Regulators

B. Influence of P-value Cutoff

Regulator
Tag
106 strains, each with a tagged regulator
Chromatin IP to enrich promoters bound by regulator in vivo
Microarray to identify promoters bound by regulator in vivo
p-value 0.05
35,345 interactions
p-value 0.01
12,028 interactions
p-value 0.005
8,180 interactions
p-value 0.001
3,979 interactions

Figure 1. Systematic genome-wide location analysis for yeast transcription regulators.

(A) Methodology. Yeast transcriptional regulators were tagged by introducing the coding sequence for a c-myc epitope tag into the normal genomic locus for each regulator. Of the yeast strains constructed in this fashion, 106 contained a single epitope-tagged regulator whose expression could be detected in rich growth conditions. Chromatin immunoprecipitation (ChIP) was performed on each of these 106 strains. Promoter regions enriched through the ChIP procedure were identified by hybridization to microarrays containing a genome-wide set of yeast promoter regions.

(B) Effect of p-value threshold. The sum of all regulator-promoter region interactions is displayed as a function of varying p-value thresholds applied to the entire location dataset for the 106 regulators. More stringent p-values reduce the number of interactions reported, but decrease the likelihood of false positive results.

of each regulator as described (Knop *et al.* 1999). We confirmed appropriate insertion of the tag and expression of the tagged protein by polymerase chain reaction and immunoblot analysis. Introduction of an epitope tag might be expected to affect the function of some transcriptional regulators; for 17 of the 141 factors, we were not able to obtain viable tagged cells, despite three attempts to tag each regulator. Not all the transcriptional regulators were expected to be expressed at detectable levels when yeast cells were grown in rich medium, but immunoblot analysis showed that 106 of the 124 tagged regulator proteins could be detected under these conditions.

We performed a genome-wide location analysis experiment (Ren *et al.* 2000) for each of the 106 yeast strains that expressed epitope-tagged regulators.[1] Each tagged strain was grown in three independent cultures in rich medium (yeast extract, peptone, dextrose). Genome-wide location data were subjected to quality control filters and normalized, and the ratio of immunoprecipitated to control DNA was determined for each array spot. We calculated a confidence value (P value) for each spot from each array by using an error model (Hughes *et al.* 2000). The data for each of the three samples in an experiment were combined by a weighted average method (Hughes *et al.* 2000); each ratio was weighted by P value and then averaged. Final P values for these combined ratios were then calculated.[2]

Given the properties of the biological system studied here (cell populations, DNA-binding factors capable of binding to both specific and non-

[1] Additional information is available at the authors' Web site: http://web.wi.mit.edu/young/regulator_network. See supporting data on *Science* online.
[2] *Ibid.*

specific sequences) and the expectation of noise in microarray-based data, it was important to use error models to obtain a probabilistic assessment of regulator location data. The total number of protein-DNA interactions in the location analysis dataset, using a range of P value thresholds, is shown in Fig. 1B. We selected specific P value thresholds to facilitate discussion of a subset of the data at a high confidence level, but note that this artificially imposes a "bound or not bound" binary decision for each protein-DNA interaction.

We will generally describe results obtained at a P value threshold of 0.001 because our analysis indicates that this threshold maximizes inclusion of legitimate regulator-DNA interactions and minimizes false positives. Various experimental and analytical methods indicate that the frequency of false positives in the genome-wide location data at the 0.001 threshold is 6% to 10%.[3] For example, conventional, gene-specific chromatin immunoprecipitation experiments have confirmed 93 of 99 binding interactions (involving 29 different regulators) that were identified by location analysis data at a threshold P value of 0.001. Use of a high-confidence threshold should underestimate the regulator-DNA interactions that actually occur in these cells. We estimate that about one-third of the actual regulator-DNA interactions in cells are not reported at the 0.001 threshold.[4]

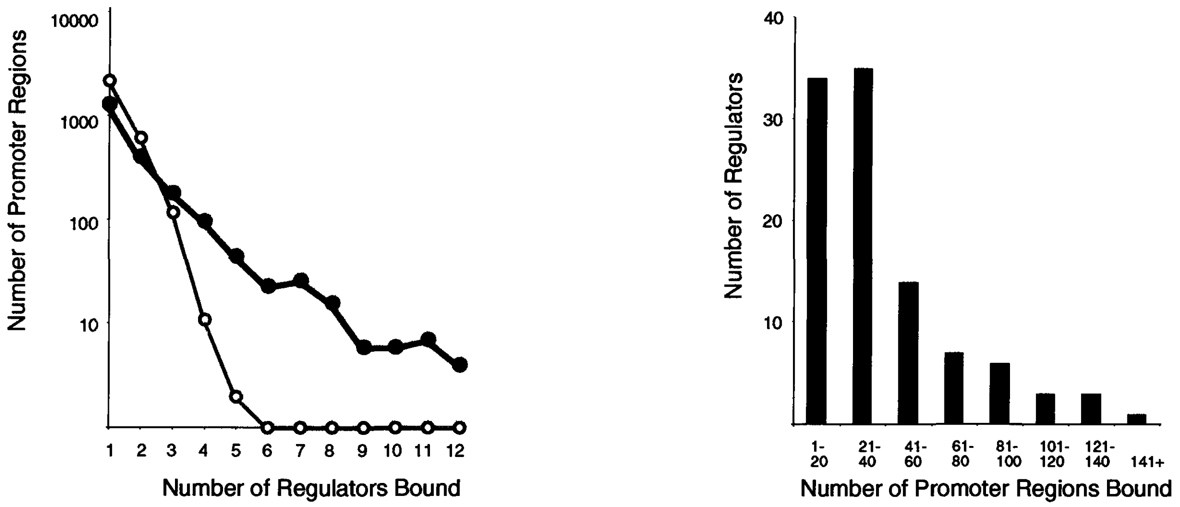
[3] *Ibid.*
[4] *Ibid.*

Regulator Density

We observed nearly 4000 interactions between regulators and promoter regions at a P value threshold of 0.001. The promoter regions of 2343 of 6270 yeast genes (37%) were bound by one or more of the 106 transcriptional regulators in yeast cells grown in rich medium. Many yeast promoters were bound by multiple transcriptional regulators (Fig. 2A), a feature previously associated with gene regulation in higher eukaryotes (Lemon and Tjian 2000; Merika and Thanos 2001), suggesting that yeast genes are also frequently regulated through combinations of regulators. More than one-third of the promoter regions that are bound by regulators were bound by two or more regulators (P value threshold = 0.001), and, relative to the expected distribution from randomized data, a disproportionately high number of promoter regions were bound by four or more regulators. Because of the stringency of the P value threshold, we expect that this represents an underestimate of regulator density.

The number of different promoter regions bound by each regulator in cells grown in rich medium ranged from 0 to 181 (P value threshold = 0.001), with an average of 38 promoter regions per regulator (Fig. 2B). The regulator Abf1 bound the largest number (181) of promoter regions. Regulators that should be active under growth conditions other than yeast extract, peptone and dextrose were typically found, as expected, to bind the smallest number of promoter regions. For example, Thi2, which activates transcription of thiamine biosynthesis genes under conditions of thiamine starvation (Kawasaki *et al.* 1990; Nishimura *et al.* 1992), was among the regulators that bound the smallest number (3) of

Figure 2



A. Number of Regulators Bound Per Promoter Region

B. Number of Promoter Regions Bound Per Regulator

Figure 2. Genome-wide distribution of transcriptional regulators.

(A) A plot of the number of regulators bound per promoter region. The distribution for the actual location data (red circles) is shown alongside the distribution expected from the same set of p-values randomly assigned among regulators and intergenic regions (white circles). At a p-value threshold of 0.001, significantly more intergenic regions bind 4 or more regulators than expected by chance.

(B) Distribution of the number of promoter regions bound per regulator.

promoters. Identification of a set of promoter regions that are bound by specific regulators allowed us to predict sequence motifs that are bound by these regulators.[5]

Network Motifs

The simplest units of commonly used transcriptional regulatory network architecture, or network motifs, provide specific regulatory capacities such as positive and negative feedback loops. We used the genome-wide location data to identify six regulatory network motifs: autoregulation, multi-component loops, feedforward loops, single input, multi-input and regulator chains (Fig. 3). These motifs suggest models for regulatory mechanisms that can be tested. Descriptions of the algorithms used to identify motifs and a complete compilation of motifs can be obtained at http://web.wi.mit.edu/young/regulator_network.

An autoregulation motif consists of a regulator that binds to the promoter region of its own gene. We identified 10 autoregulation motifs with genome-wide location data for the 106 regulators (P value threshold = 0.001), which suggests that about 10% of yeast genes encoding regulators are autoregulated. This percentage does not change substantially at less stringent P value thresholds. In contrast, studies of *Escherichia coli* genetic regulatory networks indicate that most (52% to 74%) prokaryotic genes encoding transcriptional regulators are autoregulated (Thieffry *et al.* 1998; Shen-Orr *et al.* 2002).

Autoregulation is thought to provide several selective growth advantages, including reduced response time to environmental stimuli, decreased biosynthetic

[5] *Ibid.*

Figure 3

Examples of Network Motifs in the Yeast Regulatory Network

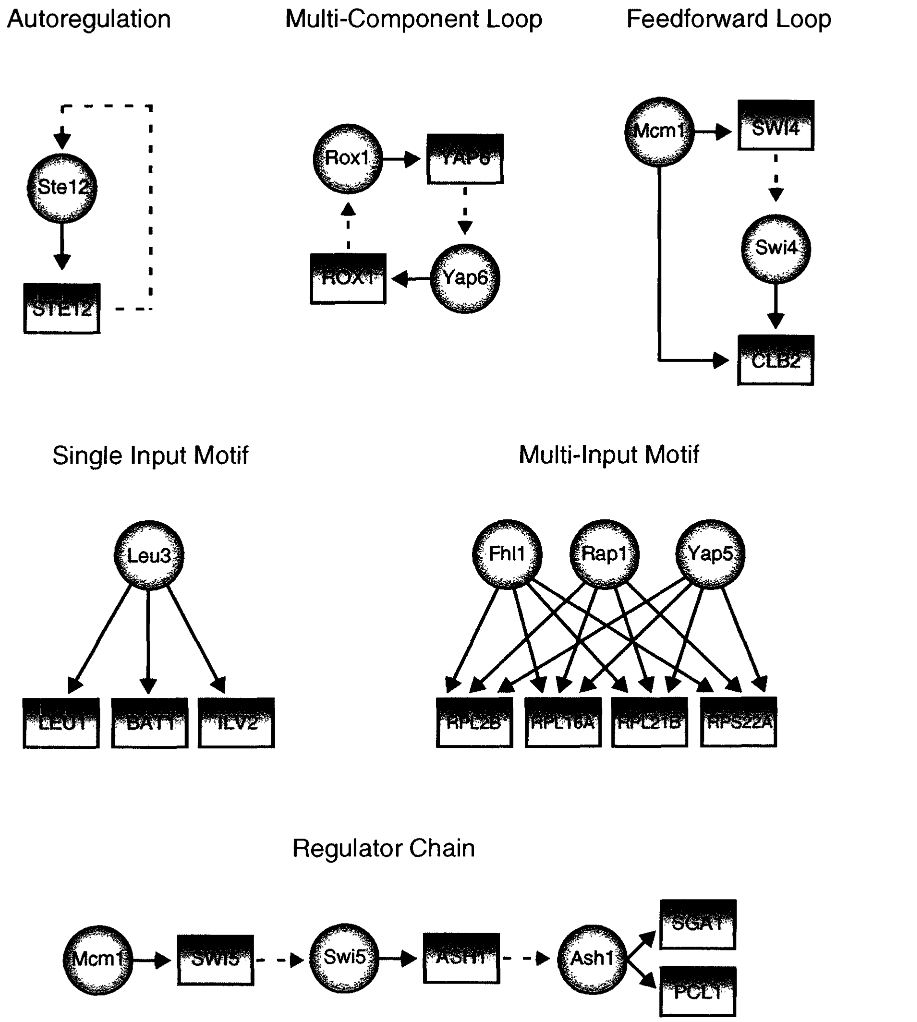


Figure 3. Examples of network motifs in the yeast regulatory network. Regulators are represented by blue circles, and gene promoters are represented by red rectangles. Binding of a regulator to a promoter is indicated by a solid arrow. Genes encoding regulators are linked to their respective regulators by dashed arrows. For example, in the autoregulation motif, the Ste12 protein binds to the STE12 gene, which is transcribed and translated into Ste12 protein. These network motifs were uncovered by searching binding data with various algorithms. For details on the algorithms used, and a full list of motifs found, see http://web.wi.mit.edu/young/regulator_network.

cost of regulation, and increased stability of gene expression (McAdams and Arkin 1997; Thieffry *et al.* 1998; Becskei and Serrano 2000; Guelzim *et al.* 2002; Shen-Orr *et al.* 2002). For example, upon exposure to mating pheromone, the concentrations of the pheromone-responsive Ste12 transcriptional regulator rapidly increase because Ste12 binds to and up-regulates its own gene (Dolan and Fields 1990; Ren *et al.* 2000) (Fig. 3). The consequent increase in Ste12 protein leads to the binding of other genes required for the mating process (Ren *et al.* 2000).

A multi-component loop motif consists of a regulatory circuit whose closure involves two or more factors (Fig. 3). We observed three multi-component loop motifs in the location data for 106 regulators (P value threshold = 0.001). The closed-loop structure provides the capacity for feedback control and offers the potential to produce bistable systems that can switch between two alternative states (Ferrell 2002). The multi-component loop motif has yet to be identified in bacterial genetic networks (Thieffry *et al.* 1998; Shen-Orr *et al.* 2002).

Feedforward loop motifs contain a regulator that controls a second regulator, and have the additional feature that both regulators bind a common target gene (Fig. 3). The regulator location data reveal that feedforward loop architecture has been highly favored during the evolution of transcriptional regulatory networks in yeast. We found that 39 regulators are involved in 49 feedforward loops potentially controlling 240 genes in the yeast network (about 10% of genes that are bound in the genome-wide location dataset).

In principle, a feedforward loop can provide several features to a regulatory circuit. The feedforward loop may act as a switch that is designed to be sensitive to sustained rather than transient inputs (Shen-Orr *et al.* 2002). Feedforward loops have the potential to provide temporal control of a process because expression of the ultimate target gene may depend on the accumulation of adequate levels of the master and secondary regulators. Feedforward loops may provide a form of multistep ultrasensitivity (Goldbeter and Koshland 1984) as small changes in the level or activity of the master regulator at the top of the loop might be amplified at the ultimate target gene because of the combined action of the master regulator and a second regulator that is under the control of the master regulator.

Single-input motifs contain a single regulator that binds a set of genes under a specific condition. Single-input motifs are potentially useful for coordinating a discrete unit of biological function such as a set of genes that code for the subunits of a biosynthetic apparatus or enzymes of a metabolic pathway. For example, several genes of the leucine biosynthetic pathway are controlled by the Leu3 transcriptional regulator (Fig. 3).

Multi-input motifs consist of a set of regulators that bind together to a set of genes. We found 295 combinations of two or more regulators that could bind to a common set of promoter regions. This motif offers the potential for coordination of gene expression across a wide variety of growth conditions. For example, each of the regulators bound to a set of genes can be responsible for regulating those genes in response to a unique input. In this manner, two different regulators responding to two different inputs would allow coordinate expression of the set of genes under these two different conditions.

Regulator chain motifs consist of chains of three or more regulators in which one regulator binds the promoter for a second regulator, the second binds the promoter for a third regulator, and so forth (Fig. 3). This network motif is observed frequently in the location data for yeast regulators; we found 188 regulator chain motifs, which varied in size from 3 to 10 regulators. The chain represents the simplest circuit logic for ordering transcriptional events in a temporal sequence. The most straightforward form of this appears in the regulatory circuit of the cell cycle where regulators functioning at one stage of the cell cycle regulate the expression of factors required for entry into the next stage of the cell cycle (Simon *et al.* 2001).

The regulatory motifs described above suggest models for gene regulatory mechanisms whose predictions can be tested with experimental data. One regulatory motif that caught our attention involved ribosomal protein genes; ribosomes are important protein biosynthetic machines, but transcriptional regulation of ribosomal protein genes is not well understood. Fhl1, a protein whose function was not previously known, forms a single-input regulatory motif consisting of essentially all ribosomal protein genes, but little else. No other regulator studied here exhibited this behavior. This predicts that loss of Fhl1 function should have a profound effect on ribosome biosynthesis if no other regulators are capable of taking its place. Indeed, a mutation in Fhl1 causes severe defects in ribosome biosynthesis (Hermann-Le Denmat *et al.* 1994), an observation that was difficult to interpret previously in the absence of the genome-wide location data. Many ribosomal protein genes are also components of a multi-input motif involving Fhl1 and additional regulators (Fig. 3), which suggests that expression of these genes may be coordinated by multiple regulators under various growth conditions. This model and others suggested by regulatory motifs can be addressed with future experiments.

Assembling Motifs into Network Structures

We assume that regulatory network motifs form building blocks that can be combined into larger network structures. An algorithm was developed that explores all the genome-wide location data together with the expression data from over 500 expression experiments to identify groups of genes that are both coordinately bound and coordinately expressed. In brief, the algorithm begins by defining a set of genes, G, that are bound by a set of regulators, S, with a P value threshold of 0.001. We find a large subset of genes in G that are similarly expressed over the entire set of expression data, and we use those genes to establish a core expression profile. Genes are then dropped from G if their expression profile is significantly different from this core profile. The remainder of the genome is scanned for genes with expression profiles that are similar to the core profile. Genes with a significant match in expression profiles are then examined to see if the set of regulators S are bound. At this step, the probability of a gene being bound by the set of regulators is used instead of the individual probabilities of that gene being bound by each of the individual regulators.

Because we are assaying the combined probability of the set of regulators being bound, and are relying on similarity of expression patterns, we can relax the P value for individual binding events and thus recapture information that is lost because of the use of an arbitrary P value threshold. The process is repeated until all combinations of genes bound by regulators have been considered. Additional details of the algorithm are available upon request. The resulting sets of regulators and genes are essentially multi-input motifs refined for common expression (MIM-CE). We expect these to be robust examples of coordinate binding and expression and therefore useful for nucleating network models.

We used the refined motifs to construct a network structure for the yeast cell cycle by an automatic process that requires no prior knowledge of the regulators that control transcription during the cell cycle. We selected the cell cycle regulatory network because of the importance of this biological process, the availability of extensive genome-wide expression data for the cell cycle (Cho *et al.* 1998; Spellman *et al.* 1998) and the extensive literature that can be used to explore features of a network model. Our goal was to determine whether the computational approach would construct the regulatory logic of cell cycle from the location and expression data without previous knowledge of the regulators involved. We reasoned that MIM-CEs that are significantly enriched in genes whose expression oscillates through the cell cycle (Spellman *et al.* 1998) would identify the regulators that control these genes. We identified 11 regulators with this approach. To construct the cell cycle network, we generated a new set of

MIM-CEs by using only the 11 regulators and the cell cycle expression data (Spellman *et al.* 1998).

To produce a cell cycle transcriptional regulatory network model, we aligned the MIM-CEs around the cell cycle on the basis of peak expression of the genes in the group by means of an algorithm described previously (Bar-Joseph *et al.* 2002) (Fig. 4). Three features of the resulting network model are notable. First, the computational approach correctly assigned all the regulators to stages of the cell cycle where they were shown to function in previous studies (Simon *et al.* 2001). Second, two regulators that have been implicated in cell cycle control but whose functions were ill-defined (Morgan *et al.* 1995; Bouquin *et al.* 1999; Ho *et al.* 1999), could be assigned within the network on the basis of direct binding data. Third, and most important, reconstruction of the regulatory architecture was automatic and required no prior knowledge of the regulators that control transcription during the cell cycle. This approach should represent a general method for constructing other regulatory networks.

Coordination of Cellular Processes

Transcriptional regulators were often bound to genes encoding other transcriptional regulators (Fig. 5). For example, there were many instances in which transcriptional regulators within a functional category (for example, cell cycle) bound to genes encoding regulators within the same category. We have noted that cell cycle regulators bound to other cell cycle regulators (Simon *et al.* 2001), and this phenomenon was also apparent among transcriptional regulators

Figure 4

A Model for the Yeast Cell Cycle Transcriptional Regulatory Network

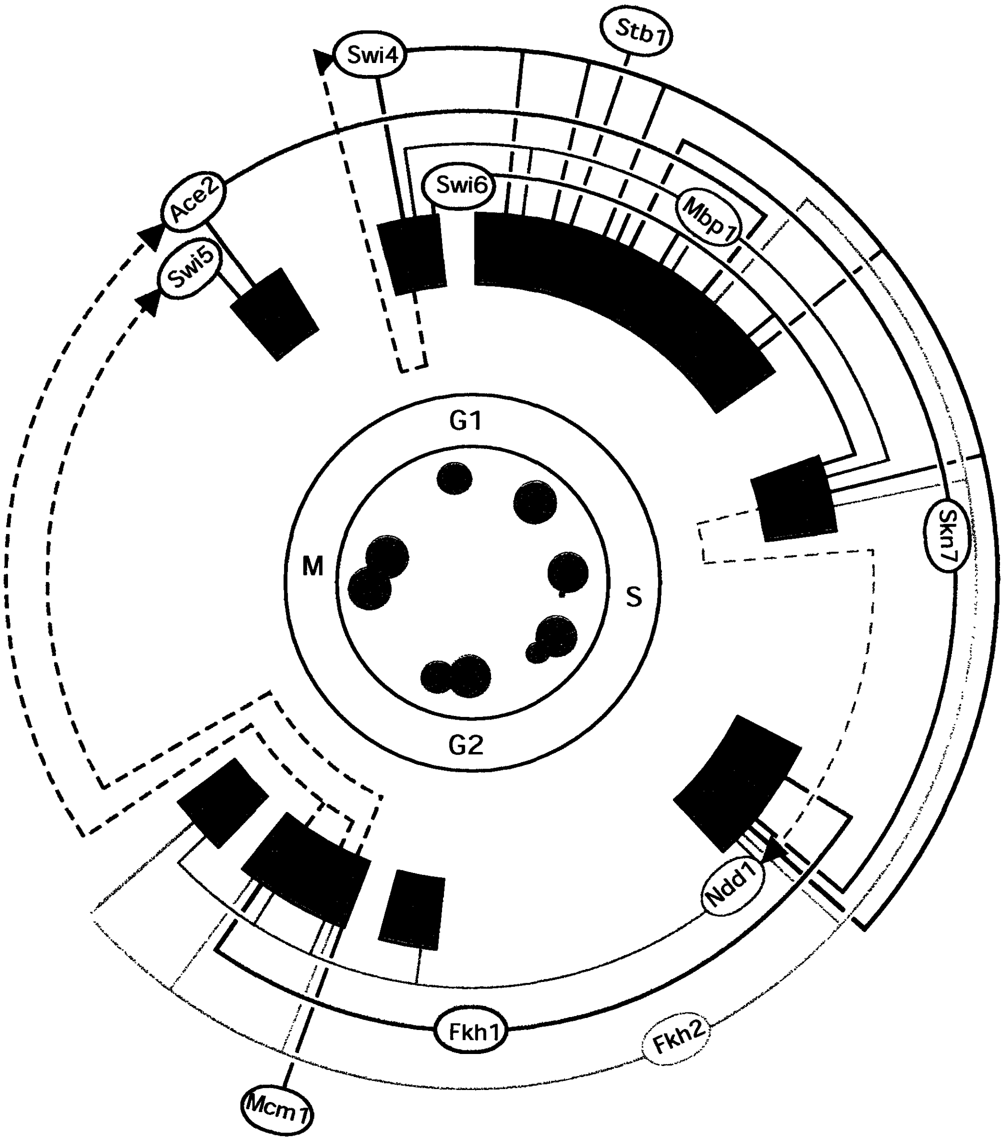
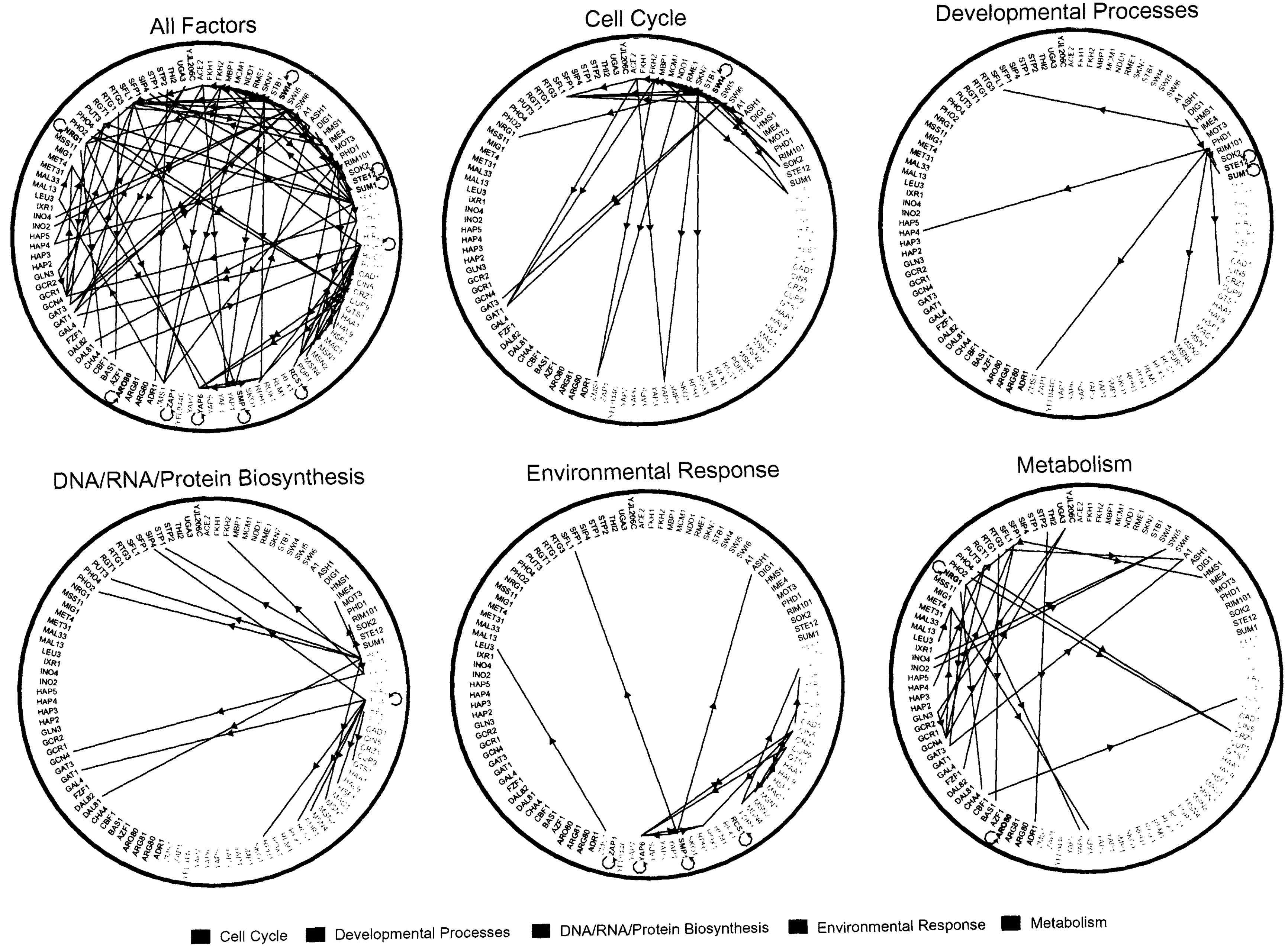


Figure 4. Model for the yeast cell cycle transcriptional regulatory network. A transcriptional regulatory network for the yeast cell cycle was derived from a combination of binding and expression data as described in the text. Yeast cell morphologies are depicted during the various stages of the cell cycle. Each blue box represents a set of genes that are bound by a common set of regulators and co-expressed throughout the cell cycle. The text inside each blue box identifies the common set of regulators that bind to the set of genes represented by the box. Each box is positioned in the cell cycle according to the time of peak expression levels for the genes represented by the box. Regulators, represented by ovals, are connected to the sets of genes they regulate by solid lines. The arc associated with each regulator effectively defines the period of activity for the regulator. Dashed lines indicate that a gene in the box encodes a regulator found in the outer rings.

Figure 5

Diverse Cellular Functions are Connected Through Transcriptional Networks



All Factors

Cell Cycle

Developmental Processes

DNA/RNA/Protein Biosynthesis

Environmental Response

Metabolism

Figure 5. Network of transcriptional regulators binding to genes encoding other transcriptional regulators.

All 106 transcriptional regulators that were subjected to location analysis in rich media are displayed in a circle, segregated into functional categories based on the primary functions of their target genes (Cell Cycle in red, Development in black, DNA/RNA/Protein Biosynthesis in tan, Environmental Response in green, and Metabolism in blue). Lines with arrows depict binding of a regulator (0.001 p-value threshold) to the gene encoding another regulator. Circles with arrows depict binding of a regulator to the promoter region of its own gene.

that fall into the metabolism and environmental response categories. For example, the metabolic regulator Gcn4 bound to promoters for PUT3 and UGA3, genes that encode transcriptional regulators for amino acid and other metabolic functions. The stress response activator Yap6 bound to the gene encoding the Rox1 repressor, and vice versa, suggesting positive and negative feedback loops.

We also found that multiple transcriptional regulators within each category were able to bind to genes encoding regulators that are responsible for control of other cellular processes. For example, the cell cycle activators bind to genes for transcriptional regulators that play key roles in metabolism (GAT1, GAT3, NRG1, SFL1); environmental responses (ROX1, YAP1, ZMS1); development (ASH1, SOK2, MOT3); and DNA, RNA and protein biosynthesis (ABF1). These observations are likely to explain, in part, how cells coordinate transcriptional regulation of the cell cycle with other cellular processes. These connections are generally consistent with previous experimental information about the relationships between cellular processes. For example, the developmental regulator Phd1 has been shown to regulate genes involved in pseudohyphal growth during certain nutrient stress conditions; we found that Phd1 also binds to genes that are key to regulation of general stress responses (MSN4, CUP9 and ZMS1) and metabolism (HAP4).

These observations have several important implications. The control of most, if not all, cellular processes is characterized by networks of transcriptional regulators that regulate other regulators. It is also evident that the effects of transcriptional regulator mutations on global gene expression as measured by expression profiling (DeRisi *et al.* 1997; Chu *et al.* 1998; Jelinsky and Samson 1999; Madhani *et al.* 1999; Gasch *et al.* 2000; Hughes *et al.* 2000; Lopez and Baker 2000; Lyons *et al.* 2000; Roberts *et al.* 2000; Shamji *et al.* 2000; Travers *et al.* 2000; Causton *et al.* 2001; Epstein *et al.* 2001; Natarajan *et al.* 2001; Devaux *et al.* 2002) are as likely to reflect the effects of the network of regulators as they are to identify the direct targets of a single regulator.

Significance of regulatory network information

This study identified network motifs that provide specific regulatory capacities for yeast, revealing the regulatory strategies that were selected during evolution for this eukaryote. These motifs can be used as building blocks to construct large network structures through an automated approach that combines genome-wide location and expression data in the absence of prior knowledge of regulator functions. The network of transcriptional regulators that control other transcriptional regulators is highly connected, suggesting that the network substructures for cellular functions such as cell cycle and development are themselves coordinated at a transcriptional level.

It is possible to envision mapping the regulatory networks that control gene expression programs in considerable depth in yeast and in other living cells. More complete understanding of transcriptional regulatory networks in yeast will require knowledge of regulator binding sites under various growth conditions[6] and experimental testing of models that emerge from computational analysis of regulator binding, gene expression and other information. The approach described here can also be used to discover transcriptional regulatory networks in higher eukaryotes. Knowledge of these networks will be important for understanding human health and designing new strategies to combat disease.

[6] *Ibid.*

Acknowledgements

We thank J. Terragni, D. Pokholok and E. Kanin for experimental support. We thank J. Liang for assistance with the supporting website. We thank R. DeShaeis, T. Ideker and T. Jaakkola for helpful discussions. Supported by fellowships from National Cancer Institute of Canada (F.R.), Human Frontier Sceince Program (J.Z.), Sloan/DOE Program for Computational Molecular Biology (D.O.), Program in Mathematics and Molecular Biology at Florida State University and Burroughs Wellcome Fund Interfaces Program (Z.B.J.), Helen Hay Whitney foundation (B.R.). Supported by grants from the National Institutes of Health and Corning, Inc.

References

Bar-Joseph Z, Gerber GK, Gifford DK, Jaakkola TS and Simon I (2002). A new approach to analyzing gene expression time series data. Sixth Annual International Conference on Research in Computational Molecular Biology.

Becskei A and Serrano L (2000). "Engineering stability in gene networks by autoregulation." Nature **405**(6786): 590-3.

Bouquin N, Johnson AL, Morgan BA and Johnston LH (1999). "Association of the cell cycle transcription factor Mbp1 with the Skn7 response regulator in budding yeast." Mol Biol Cell **10**(10): 3389-400.

Causton HC, Ren B, Koh SS, Harbison CT, *et al.* (2001). "Remodeling of yeast genome expression in response to environmental changes." Mol Biol Cell **12**(2): 323-37.

Cho RJ, Campbell MJ, Winzeler EA, Steinmetz L, *et al.* (1998). "A genome-wide transcriptional analysis of the mitotic cell cycle." Mol Cell **2**(1): 65-73.

Chu S, DeRisi J, Eisen M, Mulholland J, Botstein D, Brown PO and Herskowitz I (1998). "The transcriptional program of sporulation in budding yeast." Science **282**(5389): 699-705.

Costanzo MC, Hogan JD, Cusick ME, Davis BP, *et al.* (2000). "The yeast proteome database (YPD) and Caenorhabditis elegans proteome database (WormPD): comprehensive resources for the organization and comparison of model organism protein information." Nucleic Acids Res **28**(1): 73-6.

DeRisi JL, Iyer VR and Brown PO (1997). "Exploring the metabolic and genetic control of gene expression on a genomic scale." Science **278**(5338): 680-6.

Devaux F, Carvajal E, Moye-Rowley S and Jacq C (2002). "Genome-wide studies on the nuclear PDR3-controlled response to mitochondrial dysfunction in yeast." FEBS Lett **515**(1-3): 25-8.

Dolan JW and Fields S (1990). "Overproduction of the yeast STE12 protein leads to constitutive transcriptional induction." Genes Dev **4**(4): 492-502.

Epstein CB, Waddle JA, Hale Wt, Dave V, Thornton J, Macatee TL, Garner HR and Butow RA (2001). "Genome-wide responses to mitochondrial dysfunction." Mol Biol Cell **12**(2): 297-308.

Ferrell JE, Jr. (2002). "Self-perpetuating states in signal transduction: positive feedback, double-negative feedback and bistability." Curr Opin Cell Biol **14**(2): 140-8.

Garvie CW and Wolberger C (2001). "Recognition of specific DNA sequences." Mol Cell **8**(5): 937-46.

Gasch AP, Spellman PT, Kao CM, Carmel-Harel O, Eisen MB, Storz G, Botstein D and Brown PO (2000). "Genomic expression programs in the response of yeast cells to environmental changes." Mol Biol Cell **11**(12): 4241-57.

Goldbeter A and Koshland DE, Jr. (1984). "Ultrasensitivity in biochemical systems controlled by covalent modification. Interplay between zero-order and multistep effects." J Biol Chem **259**(23): 14441-7.

Guelzim N, Bottani S, Bourgine P and Kepes F (2002). "Topological and causal structure of the yeast transcriptional regulatory network." Nat Genet **31**(1): 60-3.

Hermann-Le Denmat S, Werner M, Sentenac A and Thuriaux P (1994). "Suppression of yeast RNA polymerase III mutations by FHL1, a gene coding for a fork head protein involved in rRNA processing." Mol Cell Biol **14**(5): 2905-13.

Ho Y, Costanzo M, Moore L, Kobayashi R and Andrews BJ (1999). "Regulation of transcription at the Saccharomyces cerevisiae start transition by Stb1, a Swi6-binding protein." Mol Cell Biol **19**(8): 5267-78.

Hughes TR, Marton MJ, Jones AR, Roberts CJ, *et al.* (2000). "Functional discovery via a compendium of expression profiles." Cell **102**(1): 109-26.

Iyer VR, Horak CE, Scafe CS, Botstein D, Snyder M and Brown PO (2001). "Genomic binding sites of the yeast cell-cycle transcription factors SBF and MBF." Nature **409**(6819): 533-8.

Jelinsky SA and Samson LD (1999). "Global response of Saccharomyces cerevisiae to an alkylating agent." Proc Natl Acad Sci U S A **96**(4): 1486-91.

Kawasaki Y, Nosaka K, Kaneko Y, Nishimura H and Iwashima A (1990). "Regulation of thiamine biosynthesis in Saccharomyces cerevisiae." J Bacteriol **172**(10): 6145-7.

Knop M, Siegers K, Pereira G, Zachariae W, Winsor B, Nasmyth K and Schiebel E (1999). "Epitope tagging of yeast genes using a PCR-based strategy: more tags and improved practical routines." Yeast **15**(10B): 963-72.

Lee TI and Young RA (2000). "Transcription of eukaryotic protein-coding genes." Annu Rev Genet **34**: 77-137.

Lemon B and Tjian R (2000). "Orchestrated response: a symphony of transcription factors for gene control." Genes Dev **14**(20): 2551-69.

Lieb JD, Liu X, Botstein D and Brown PO (2001). "Promoter-specific binding of Rap1 revealed by genome-wide maps of protein-DNA association." Nat Genet **28**(4): 327-34.

Lopez MC and Baker HV (2000). "Understanding the growth phenotype of the yeast gcr1 mutant in terms of global genomic expression patterns." J Bacteriol **182**(17): 4970-8.

Lyons TJ, Gasch AP, Gaither LA, Botstein D, Brown PO and Eide DJ (2000). "Genome-wide characterization of the Zap1p zinc-responsive regulon in yeast." Proc Natl Acad Sci U S A **97**(14): 7957-62.

Madhani HD, Galitski T, Lander ES and Fink GR (1999). "Effectors of a developmental mitogen-activated protein kinase cascade revealed by expression signatures of signaling mutants." Proc Natl Acad Sci U S A **96**(22): 12530-5.

McAdams HH and Arkin A (1997). "Stochastic mechanisms in gene expression." Proc Natl Acad Sci U S A **94**(3): 814-9.

Merika M and Thanos D (2001). "Enhanceosomes." Curr Opin Genet Dev **11**(2): 205-8.

Morgan BA, Bouquin N, Merrill GF and Johnston LH (1995). "A yeast transcription factor bypassing the requirement for SBF and DSC1/MBF in budding yeast has homology to bacterial signal transduction proteins." Embo J **14**(22): 5679-89.

Natarajan K, Meyer MR, Jackson BM, Slade D, Roberts C, Hinnebusch AG and Marton MJ (2001). "Transcriptional profiling shows that Gcn4p is a master regulator of gene expression during amino acid starvation in yeast." Mol Cell Biol **21**(13): 4347-68.

Nishimura H, Kawasaki Y, Kaneko Y, Nosaka K and Iwashima A (1992). "Cloning and characteristics of a positive regulatory gene, THI2 (PHO6), of thiamin biosynthesis in Saccharomyces cerevisiae." FEBS Lett **297**(1-2): 155-8.

Orphanides G and Reinberg D (2002). "A unified theory of gene expression." Cell **108**(4): 439-51.

Ptashne M and Gann A (2002). Genes and Signals. Cold Spring Harbor, Cold Spring Harbor Laboratories Press.

Ren B, Robert F, Wyrick JJ, Aparicio O, *et al.* (2000). "Genome-wide location and function of DNA binding proteins." Science **290**(5500): 2306-9.

Roberts CJ, Nelson B, Marton MJ, Stoughton R, *et al.* (2000). "Signaling and circuitry of multiple MAPK pathways revealed by a matrix of global gene expression profiles." Science **287**(5454): 873-80.

Shamji AF, Kuruvilla FG and Schreiber SL (2000). "Partitioning the transcriptional program induced by rapamycin among the effectors of the Tor proteins." Curr Biol **10**(24): 1574-81.

Shen-Orr SS, Milo R, Mangan S and Alon U (2002). "Network motifs in the transcriptional regulation network of Escherichia coli." Nat Genet **31**(1): 64-8.

Simon I, Barnett J, Hannett N, Harbison CT, *et al.* (2001). "Serial regulation of transcriptional regulators in the yeast cell cycle." Cell **106**(6): 697-708.

Spellman PT, Sherlock G, Zhang MQ, Iyer VR, Anders K, Eisen MB, Brown PO, Botstein D and Futcher B (1998). "Comprehensive identification of cell cycle-regulated genes of the yeast Saccharomyces cerevisiae by microarray hybridization." Mol Biol Cell **9**(12): 3273-97.

Thieffry D, Huerta AM, Perez-Rueda E and Collado-Vides J (1998). "From specific gene regulation to genomic networks: a global analysis of transcriptional regulation in Escherichia coli." Bioessays **20**(5): 433-40.

Travers KJ, Patil CK, Wodicka L, Lockhart DJ, Weissman JS and Walter P (2000). "Functional and genomic analyses reveal an essential coordination between the unfolded protein response and ER-associated degradation." Cell **101**(3): 249-58.

Wyrick JJ, Aparicio JG, Chen T, Barnett JD, Jennings EG, Young RA, Bell SP and Aparicio OM (2001). "Genome-wide distribution of ORC and MCM proteins in S. cerevisiae: high-resolution mapping of replication origins." Science **294**(5550): 2357-60.

Chapter 4

Transcriptional Regulatory Code of a Eukaryotic Genome

Published as: Harbison CT, Gordon DB, Lee TI, Rinaldi NJ, Macisaac KD, Danford TW, Hannett N, Tagne JB, Reynolds DB, Yoo JY, Jennings EG, Zeitlinger J, Pokholok DK, Kellis M, Rolfe PA, Takusagawa KT, Lander ES, Gifford DK and Young RA (2004). "Transcriptional regulatory code of a eukaryotic genome." Nature. **431**(7004): 99-104.

Summary

DNA-binding transcriptional regulators interpret the genome's regulatory code by binding to specific sequences to induce or repress gene expression (Jacob and Monod 1961). Comparative genomics has recently been used to identify potential cis-regulatory sequences within the yeast genome on the basis of phylogenetic conservation (Blanchette and Tompa 2003; Cliften *et al.* 2003; Kellis *et al.* 2003; Wang and Stormo 2003; Pritsker *et al.* 2004), but this information alone does not reveal if or when transcriptional regulators occupy these binding sites. We have constructed an initial version of yeast's transcriptional regulatory code by mapping the sequence elements that are bound by regulators under various conditions and that are conserved among Saccharomyces species. The organization of regulatory elements in promoters and the environment-dependent use of these elements by regulators are discussed. We find that environment-specific use of regulatory elements predicts mechanistic models for the function of a large population of yeast's transcriptional regulators.

We used genome-wide location analysis (Ren *et al.* 2000; Iyer *et al.* 2001; Lieb *et al.* 2001; Lee *et al.* 2002) to determine the genomic occupancy of 203 DNA-binding transcriptional regulators in rich media conditions and, for 84 of these regulators, in at least one of twelve other environmental conditions (Supplementary Table 1, Supplementary Figure 1, http://web.wi.mit.edu/young/regulatory_code). These 203 proteins are likely to include nearly all of the DNA-binding transcriptional regulators encoded in the yeast genome. Regulators were selected for profiling in an additional environment if they were essential for growth in that environment or if there was other evidence implicating them in regulation of gene expression in that environment. The genome-wide location data identified 11,000 unique interactions between regulators and promoter regions at high confidence ($P \leq 0.001$).

To identify the cis-regulatory sequences that likely serve as recognition sites for transcriptional regulators, we merged information from genome-wide location data, phylogenetically conserved sequences, and prior knowledge (Figure 1A). We used six motif discovery methods (Bailey and Elkan 1995; Roth *et al.* 1998; Liu *et al.* 2002) to discover 68,279 DNA sequence motifs for the 147 regulators that bound more than ten probes (Supplementary Methods; Supplementary Figure 2). From these motifs we derived the most likely specificity for each regulator through clustering and stringent statistical tests. This motif discovery process identified highly significant ($P \leq 0.001$) motifs for each of 116 regulators. We determined a single high-confidence motif for 65 of these

Figure 1

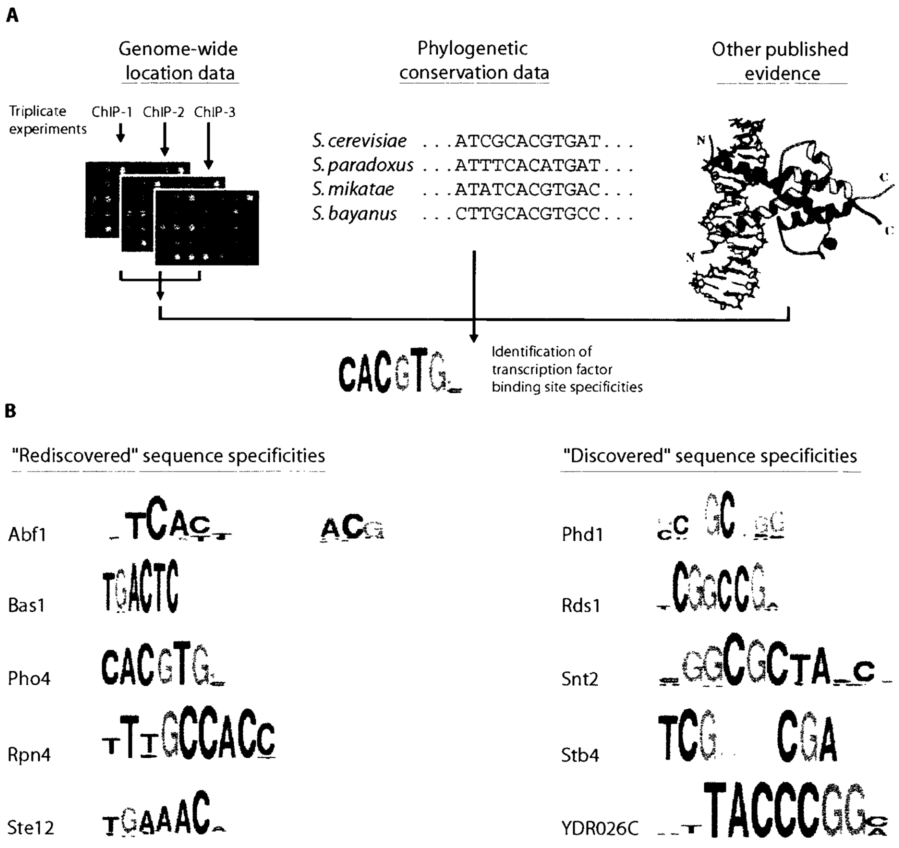

A
Genome-wide location data
Phylogenetic conservation data
Other published evidence
Triplicate experiments
ChIP-1
ChIP-2
ChIP-3
S. cerevisiae . . . ATCGCACGTGAT . . .
S. paradoxus . . . ATTTCACATGAT . . .
S. mikatae . . . ATATCACGTGAC . . .
S. bayanus . . . CTTGCACGTGCC . . .
N
C
N
C
CACGTG
Identification of transcription factor binding site specificities
B
"Rediscovered" sequence specificities
"Discovered" sequence specificities
Abf1
Bas1
Pho4
Rpn4
Ste12
Phd1
Rds1
Snt2
Stb4
YDR026C

Figure 1. Discovering binding site specificities for yeast transcriptional regulators.

A) Cis-regulatory sequences that likely serve as recognition sites for transcriptional regulators were identified by combining information from genome-wide location data, phylogenetically conserved sequences, and previously published evidence, as described in Supplementary Methods. The compendium of regulatory sequence motifs can be found in Supplementary Table 3.

B) Selected sequence specificities that were “rediscovered” and were newly discovered are displayed. The total height of the column is proportional to the information content of the position, and the individual letters have height proportional to the product of their frequency and the information content (Schneider and Stephens 1990).

Figure 2

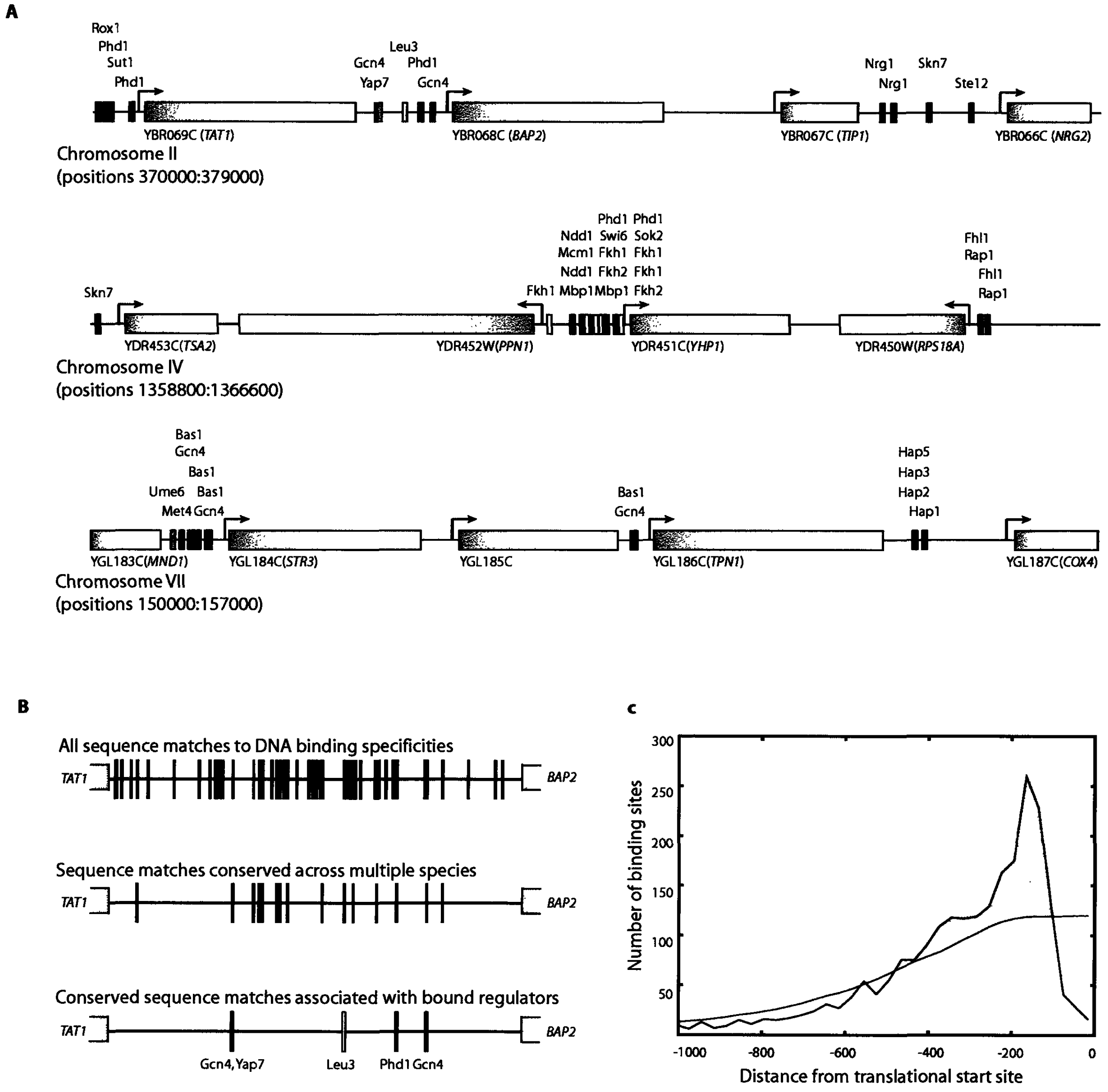

A
Rox1
Phd1
Sut1
Phd1
Leu3
Gcn4
Phd1
Yap7
Gcn4
Nrg1
Skn7
Nrg1
Ste12
YBR069C (TAT1)
YBR068C (BAP2)
YBR067C (TIP1)
YBR066C (NRG2)
Chromosome II
(positions 370000:379000)
Phd1 Phd1
Ndd1 Swi6 Sok2
Mcm1 Fkh1 Fkh1
Ndd1 Fkh2 Fkh1
Fkh1 Mbp1 Mbp1 Fkh2
Skn7
Fhl1
Rap1
Fhl1
Rap1
YDR453C(TSA2)
YDR452W(PPN1)
YDR451C(YHP1)
YDR450W(RPS18A)
Chromosome IV
(positions 1358800:1366600)
Bas1
Gcn4
Bas1
Ume6 Bas1
Met4 Gcn4
Bas1
Gcn4
Hap5
Hap3
Hap2
Hap1
YGL183C(MND1)
YGL184C(STR3)
YGL185C
YGL186C(TPN1)
YGL187C(COX4)
Chromosome VII
(positions 150000:157000)
B
All sequence matches to DNA binding specificities
TAT1
BAP2
Sequence matches conserved across multiple species
TAT1
BAP2
Conserved sequence matches associated with bound regulators
TAT1
BAP2
Gcn4,Yap7
Leu3
Phd1 Gcn4
C
300
250
200
150
100
50
Number of binding sites
-1000
-800
-600
-400
-200
0
Distance from translational start site

Figure 2. Drafting the yeast transcriptional regulatory map.

A) Portions of chromosomes illustrating locations of genes (grey rectangles) and conserved DNA sequences (coloured boxes) bound *in vivo* by transcriptional regulators.

B) Combining binding data and sequence conservation data. The diagram depicts all sequences matching a motif from our compendium (top), all such conserved sequences (middle) and all such conserved sequences bound by a regulator (bottom).

C) Regulator binding site distribution. The red line shows the distribution of distances from the start codon of open reading frames to binding sites in the adjacent upstream region. The green line represents a randomized distribution.

used to construct the map includes binding data from multiple growth environments, the map describes transcriptional regulatory potential within the genome. During growth in any one environment, only subsets of the binding sites identified in the map are occupied by transcriptional regulators, as we describe in more detail below.

Where the functions of specific transcriptional regulators were established previously, the functions of the genes they bind in the regulatory map are highly consistent with this prior information. For example, the amino acid biosynthetic regulators Gcn4 and Leu3 bind to sites in the promoter of BAP2 (chromosome II), which encodes an amino acid transporter (Figure 2A). Six well-studied cell cycle transcriptional regulators bind to the promoter for YHP1 (chromosome IV), which has been implicated in regulation of the G1 phase of the cell cycle. The regulator of respiration Hap5, binds upstream of COX4 (chromosome VII), which encodes a component of the respiratory electron transport chain. Where regulators with established functions bind to genes of unknown function, these target genes are newly implicated in such functional processes.

The utility of combining regulator binding data and sequence conservation data is illustrated in Figure 2B. All sequences matching the regulator DNA binding specificities described in this study (Supplementary Table 2) that occur within the 884 base-pair intergenic region upstream of the gene BAP2 are shown in the upper panel. The subset of these sequences that have been conserved in multiple yeast species, and are thus likely candidates for regulator interactions, are shown in the middle panel. The presence of these conserved regulatory sites indicates the potential for regulation via this sequence, but does not indicate whether the site is actually bound by a regulator under some growth condition. The incorporation of binding information (bottom panel) identifies those conserved sequences that are utilized by regulators in cells grown under the conditions examined.

The distribution of binding sites for transcriptional regulators reveals there are constraints on the organization of these sites in yeast promoters (Figure 2C). Binding sites are not uniformly distributed over the promoter regions, but rather show a sharply peaked distribution. Very few sites are located in the region 100 base pairs (bp) upstream of protein coding sequences. This region typically includes the transcription start site and is bound by the transcription initiation apparatus. The vast majority (74%) of the transcriptional regulator binding sites lie between 100 and 500 bp upstream of the protein coding sequence, far more than would be expected at random (53%). Regions further than 500 bp contain fewer binding sites than would be expected at random. It appears that yeast transcriptional regulators function at short distances along the linear DNA, a property that reduces the potential for inappropriate activation of nearby genes. We note that specific arrangements of DNA binding site sequences occur within promoters, and suggest that these promoter architectures provide clues to regulatory mechanisms (Figure 3). For example, the presence of a DNA binding site for a single regulator is the simplest promoter architecture and, as might be expected, we found that sets of genes with this feature are often involved in a common biological function (Supplementary Table 4). A second type of promoter

Figure 3

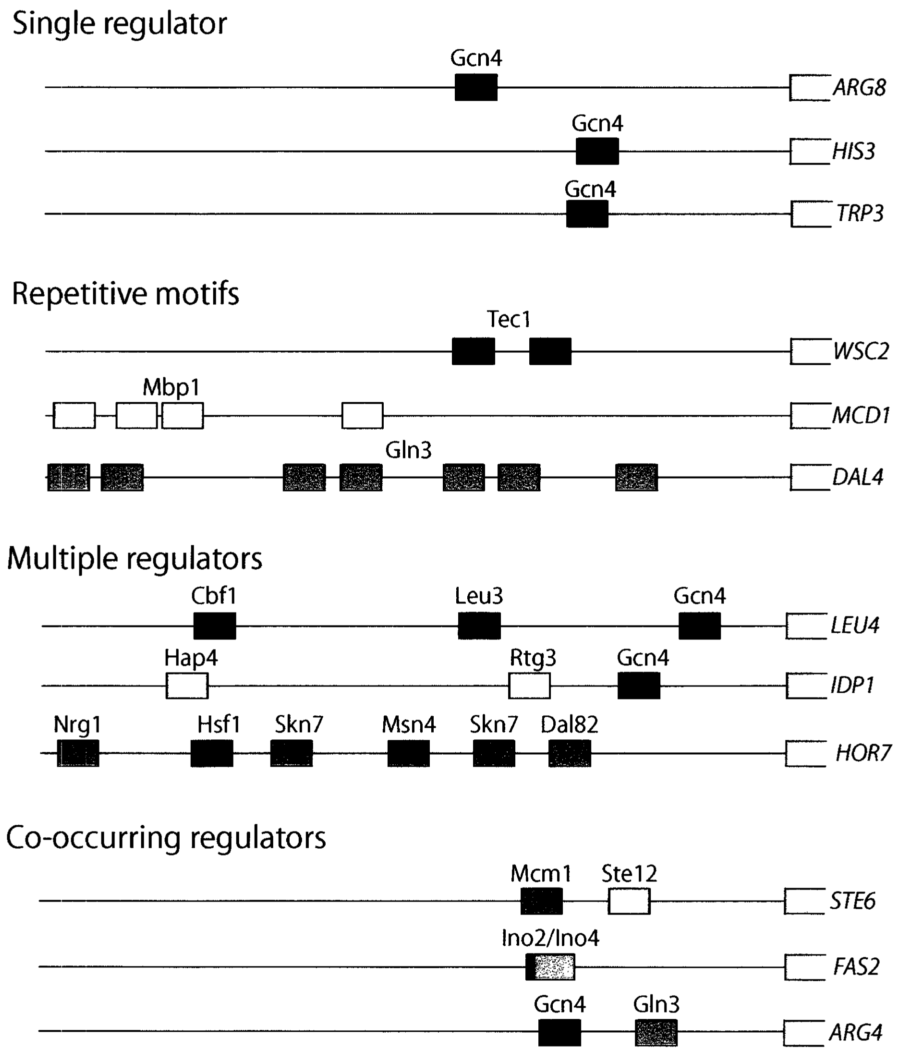

Single regulator
Gcn4
ARG8
Gcn4
HIS3
Gcn4
TRP3
Repetitive motifs
Tec1
WSC2
Mbp1
MCD1
Gln3
DAL4
Multiple regulators
Cbf1
Leu3
Gcn4
LEU4
Hap4
Rtg3
Gcn4
IDP1
Nrg1
Hsf1
Skn7
Msn4
Skn7
Dal82
HOR7
Co-occurring regulators
Mcm1
Ste12
STE6
Ino2/Ino4
FAS2
Gcn4
Gln3
ARG4

Figure 3. Yeast promoter architectures. Single regulator architecture: promoter regions that contain one or more copies of the binding site sequence for a single regulator. Repetitive motif architecture: promoter regions that contain multiple copies of a binding site sequence of a regulator. Multiple regulator architecture: promoter regions that contain one or more copies of the binding site sequences for more than one regulator. Co-occurring regulator architecture: promoters that contain binding site sequences for recurrent pairs of regulators. For the purposes of illustration, not all sites are shown and scale is approximate. Additional information can be found in Supplementary Tables 4-6.

architecture consists of repeats of a particular binding site sequence. Repeated binding sites have been shown to be necessary for stable binding by the regulator Dal80 (Cunningham and Cooper 1993). This repetitive promoter architecture can also allow for a graded transcriptional response, as has been observed for the HIS4 gene (Donahue *et al.* 1983). A number of regulators, including Dig1, Mbp1, and Swi6 show a statistically significant preference for repetitive motifs (Supplementary Table 5). A third class of promoter contains binding sites for multiple different regulators. This promoter arrangement implies that the gene may be subject to combinatorial regulation, and we expect that in many cases the various regulators can be used to execute differential responses to varied growth conditions. Indeed, we note that many of the genes in this category encode products that are required for multiple metabolic pathways and are regulated in an environment-specific fashion. In the fourth type of promoter architecture we discuss here, binding sites for specific pairs of regulators occur more frequently within the same promoter regions than would be expected by chance (Supplementary Table 6). This "co-occurring" motif architecture implies that the two regulators physically interact or have shared functions at multiple genes.

By conducting genome-wide binding experiments for some regulators under multiple cell growth conditions, we learned that regulator binding to a subset of the regulatory sequences is highly dependent on the environmental conditions of the cell (Supplementary Figure 4). We observed four common patterns of regulator binding behaviour (Figure 4, Supplementary Table 7). Prior

Figure 4

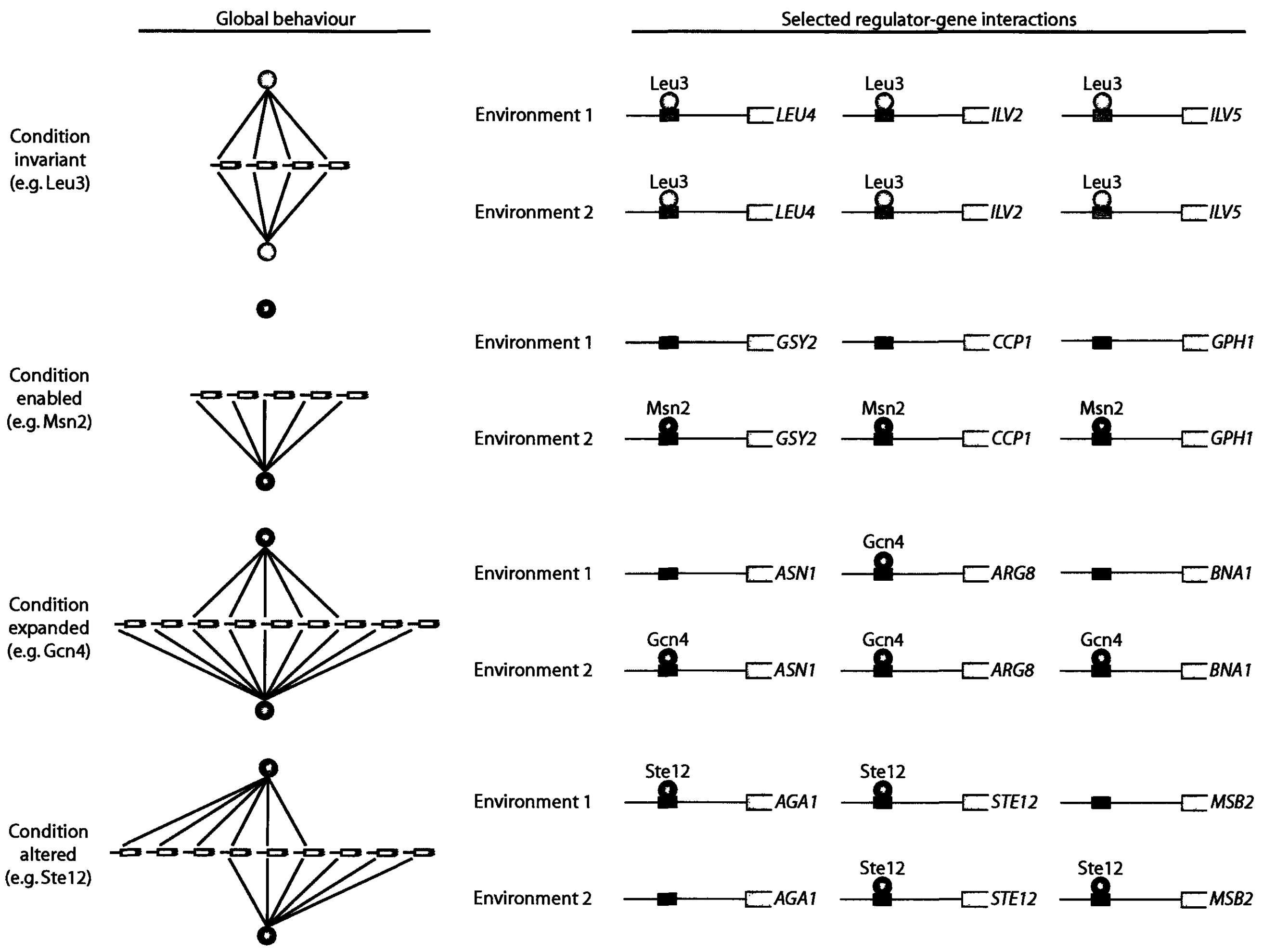

Global behaviour
Selected regulator-gene interactions
Condition invariant (e.g. Leu3)
Environment 1
Leu3
LEU4
Leu3
ILV2
Leu3
ILV5
Environment 2
Leu3
LEU4
Leu3
ILV2
Leu3
ILV5
Condition enabled (e.g. Msn2)
Environment 1
GSY2
CCP1
GPH1
Environment 2
Msn2
GSY2
Msn2
CCP1
Msn2
GPH1
Condition expanded (e.g. Gcn4)
Environment 1
ASN1
Gcn4
ARG8
BNA1
Environment 2
Gcn4
ASN1
Gcn4
ARG8
Gcn4
BNA1
Condition altered (e.g. Ste12)
Environment 1
Ste12
AGA1
Ste12
STE12
MSB2
Environment 2
AGA1
Ste12
STE12
Ste12
MSB2

Figure 4. Environment-specific utilization of the transcriptional regulatory code. Four patterns of genome-wide binding behaviour are depicted in a graphic representation on the left, where transcriptional regulators are represented by coloured circles and are placed above and below a set of target genes/promoters. The lines between the regulators and the target genes/promoters represent binding events. Specific examples of the environment-dependent behaviours are depicted on the right. Coloured circles represent regulators and coloured boxes represent their DNA binding sequences within specific promoter regions. We note that regulators may exhibit different behaviours when different pairs of conditions are compared.

information about the regulatory mechanisms employed by well-studied regulators in each of the four groups suggests hypotheses to account for the environment-dependent binding behaviour of the other regulators.

"Condition invariant" regulators bind essentially the same set of promoters (within the limitations of noise) in two different growth environments (Figure 4). Leu3, which is known to regulate genes involved in amino acid biosynthesis, is among the best studied of the regulators in this group. Binding of Leu3 *in vivo* has been shown to be necessary, but not sufficient for activation of Leu3-regulated genes (Kirkpatrick and Schimmel 1995). Rather, regulatory control of these genes requires association of a leucine metabolic precursor with Leu3 to convert it from a negative to positive regulator. We note that other zinc cluster type regulators that show "condition invariant" behaviour are known to be regulated in a similar manner (Ma and Ptashne 1987; Axelrod *et al.* 1991). Thus, it is reasonable to propose that the activation or repression functions of some of the other regulators in this class will be independent of DNA binding.

"Condition enabled" regulators do not bind the genome detectably under one condition, but bind a substantial number of promoters with a change in environment. Msn2 is among the best-studied regulators in this class, and the mechanisms involved in Msn2-dependent transcription provide clues to how the other regulators in that class may operate. Msn2 is known to be excluded from the nucleus when cells grow in the absence of stresses, but accumulates rapidly in the nucleus when cells are subjected to stress (Beck and Hall 1999; Chi *et al.* 2001). This condition-enabled behaviour was also observed for the thiamine biosynthetic regulator Thi2, the nitrogen regulator Gat1, and the developmental regulator Rim101. We suggest that many of these transcriptional regulators are regulated by nuclear exclusion or by another mechanism that would cause this extreme version of condition-specific binding.

"Condition expanded" regulators bind to a core set of target promoters under one condition, but bind an expanded set of promoters under another condition. Gcn4 is the best-studied of the regulators that fall into this "expanded" class. The levels of Gcn4 are reported to increase 6-fold when yeast are introduced into media with limiting nutrients (Albrecht *et al.* 1998), due largely to increased nuclear protein stability (Kornitzer *et al.* 1994; Chi *et al.* 2001), and under this condition we find Gcn4 binds to an expanded set of genes. Interestingly, the probes bound when Gcn4 levels are low contain better matches to the known Gcn4 binding site than probes that are bound exclusively at higher protein concentrations, consistent with a simple model for specificity based on intrinsic protein affinity and protein concentration (Supplementary Figure 5). The expansion of binding sites by many of the regulators in this class may reflect increased levels of the regulator available for DNA binding.

"Condition altered" regulators exhibit altered preference for the set of promoters bound in two different conditions. Ste12 is the best studied of the regulators whose binding behaviour falls into this "altered" class. Depending on the interactions with other regulators, the specificity of Ste12 can change and alter its cellular function (Zeitlinger *et al.* 2003). For example, under filamentous growth conditions, Ste12 interacts with Tec1, which has its own DNA-binding specificity (Baur *et al.* 1997). This condition-altered behaviour was also observed for the transcriptional regulators Aft2, Skn7, and Ume6. We propose that the binding specificity of many of the transcriptional regulators may be altered through interactions with other regulators or through modifications (e.g., chemical) that are environment-dependent.

Substantial portions of eukaryotic genome sequence are believed to be regulatory (Waterston *et al.* 2002; Cliften *et al.* 2003; Kellis *et al.* 2003), but the DNA sequences that actually contribute to regulation of genome expression have been ill-defined. By mapping the DNA sequences bound by specific regulators in various environments, we identify the regulatory potential embedded in the genome and provide a framework for modeling the mechanisms that contribute to global gene expression. We anticipate that the approaches used here to map regulatory sequences in yeast can also be used to map the sequences that control genome expression in higher eukaryotes.

Methods

Strain Information

For each of the 203 regulators, strains were generated in which a repeated Myc epitope coding sequence was integrated into the endogenous gene encoding the regulator. Polymerase chain reaction (PCR) constructs containing the Myc epitope coding sequence and a selectable marker flanked by regions of homology to either the 5' or 3' end of the targeted gene were transformed into the W303 yeast strain Z1256 (Ren *et al.* 2000; Lee *et al.* 2002). Genomic integration and expression of the epitope-tagged protein were confirmed by PCR and Western blotting, respectively.

Genome wide location analysis

Genome-wide location analysis was performed as previously described (Ren *et al.* 2000; Lee *et al.* 2002). Bound proteins were formaldehyde-crosslinked to DNA *in vivo*, followed by cell lysis and sonication to shear DNA. Crosslinked material was immunoprecipitated with an anti-myc antibody, followed by reversal of the crosslinks to separate DNA from protein. Immunoprecipitated DNA and DNA from an unenriched sample were amplified and differentially fluorescently labeled by ligation-mediated PCR. These samples were hybridized to a microarrray consisting of spotted PCR products representing the intergenic regions of the *S. cerevisiae* genome. Relative intensities of spots were used as the basis for an error model that assigns a probability score (P value) to binding interactions. All microarray data are available from ArrayExpress (accession number: E-WMIT-10) as well as from the authors' web site.

Growth environments

We profiled all 203 regulators in rich medium. In addition, we profiled 84 regulators in at least one other environmental condition. The list of regulators is given in Supplementary Table 1.

Regulator Binding Specificity

The putative specificities of regulators were identified by applying a suite of motif discovery programs to the intergenic sequences identified by the binding data. The resulting specificity predictions were filtered for significance using uniform metrics and then clustered to yield representative motifs (Supplementary Figure 2).

We used six methods to identify the specific sequences bound by regulators: AlignACE (Roth *et al.* 1998), MEME (Bailey and Elkan 1995), Mdscan (Liu *et al.* 2002), the method of Kellis *et al.* (Kellis *et al.* 2003) and two additional new methods that incorporate conservation data: MEME_c and CONVERGE. MEME_c uses the existing MEME program without change, but applies it to a modified set of sequences in which bases that are not conserved in the *sensu stricto Saccharomyces* species were replaced with the letter "N". CONVERGE is a novel expectation-maximization (EM)-based algorithm for discovering specificities using sequence information from multiple genomes. Rather than searching for sites that are identical across the *sensu stricto* species, as is the case for MEME_c, CONVERGE searches for loci where all aligned sequences are consistent with the same specificity model. See Supplementary Methods for runtime parameters and additional details for all of these methods.

Each of the programs we used attempts to measure the significance of its results with one or more statistical scores. However, we observed that these programs report results with high scores even when applied to random selections of intergenic regions. To distinguish the true motifs, we chose a set of statistical measures that are described in the Supplementary Methods, and we converted these scores into the empirical probability that a motif with a similar score could be found by the same program in randomly selected sequences. To estimate these P values, we ran each program 50 times on randomly selected sets of sequences of various sizes. We accepted only those motifs that were judged to be significant by these scores ($P \leq 0.001$).

Significant motifs from all programs were pooled together and clustered using a k-medoids algorithm. Aligned motifs within each cluster were averaged together to produce consensus motifs and filtered according to their conservation. This procedure typically produced several distinct consensus motifs for each regulator. To choose a single specificity for each regulator, we compared the results with information in the TRANSFAC (Matys *et al.* 2003), YPD (Hodges *et al.* 1999), and SCPD (Zhu and Zhang 1999) databases. When no prior information was available, we chose the specificity with the most significant statistical score.

Regulatory Code

Potential binding sites were included in the map of the regulatory code if they satisfied two criteria. First, a locus had to match the specificity model for a regulator in the *Saccharomyces cerevisiae* genome and at least two other *sensu stricto cerevisiae* genomes with a score ≥ 60% of the maximum possible. Second, the locus had to lie in an intergenic region that also contained a probe bound by the corresponding regulator in any condition ($P \leq 0.001$). All analyses of promoter architecture and environment-specific binding were based on this map, and can be found in Supplementary Information.

Supplementary Methods

More detailed information concerning all the methods used in this paper can be found in at http://web.wi.mit.edu/young/regulatory_code and in Supplementary Information.

Acknowledgements

We thank T. Ideker and S. McCuine for help in selecting regulators to study in environmental conditions. We thank E. Herbolsheimer, G. Bell, R. Latek and F. Lewitter for computational assistance, and E. McReynolds for technical assistance. E. F. is a Whitehead Fellow and was funded in part by Pfizer. D.B.G. was supported by NIH/NIGMS NRSA Award GM068278. This work was supported by NIH grant HG002668.

References:

Albrecht G, Mosch HU, Hoffmann B, Reusser U and Braus GH (1998). "Monitoring the Gcn4 protein-mediated response in the yeast Saccharomyces cerevisiae." J Biol Chem **273**(21): 12696-702.

Axelrod JD, Majors J and Brandriss MC (1991). "Proline-independent binding of PUT3 transcriptional activator protein detected by footprinting in vivo." Mol Cell Biol **11**(1): 564-7.

Bailey TL and Elkan C (1995). "The value of prior knowledge in discovering motifs with MEME." Proc Int Conf Intell Syst Mol Biol **3**: 21-9.

Baur M, Esch RK and Errede B (1997). "Cooperative binding interactions required for function of the Ty1 sterile responsive element." Mol Cell Biol **17**(8): 4330-7.

Beck T and Hall MN (1999). "The TOR signalling pathway controls nuclear localization of nutrient-regulated transcription factors." Nature **402**(6762): 689-92.

Blanchette M and Tompa M (2003). "FootPrinter: A program designed for phylogenetic footprinting." Nucleic Acids Res **31**(13): 3840-2.

Chi Y, Huddleston MJ, Zhang X, Young RA, Annan RS, Carr SA and Deshaies RJ (2001). "Negative regulation of Gcn4 and Msn2 transcription factors by Srb10 cyclin-dependent kinase." Genes Dev **15**(9): 1078-92.

Cliften P, Sudarsanam P, Desikan A, Fulton L, Fulton B, Majors J, Waterston R, Cohen BA and Johnston M (2003). "Finding functional features in Saccharomyces genomes by phylogenetic footprinting." Science **301**(5629): 71-6.

Cunningham TS and Cooper TG (1993). "The Saccharomyces cerevisiae DAL80 repressor protein binds to multiple copies of GATAA-containing sequences (URSGATA)." J Bacteriol **175**(18): 5851-61.

Donahue TF, Daves RS, Lucchini G and Fink GR (1983). "A short nucleotide sequence required for regulation of HIS4 by the general control system of yeast." Cell **32**(1): 89-98.

Hodges PE, McKee AH, Davis BP, Payne WE and Garrels JI (1999). "The Yeast Proteome Database (YPD): a model for the organization and presentation of genome-wide functional data." Nucleic Acids Res **27**(1): 69-73.

Iyer VR, Horak CE, Scafe CS, Botstein D, Snyder M and Brown PO (2001). "Genomic binding sites of the yeast cell-cycle transcription factors SBF and MBF." Nature **409**(6819): 533-8.

Jacob F and Monod J (1961). "Genetic regulatory mechanisms in the synthesis of proteins." J Mol Biol **3**: 318-56.

Kellis M, Patterson N, Endrizzi M, Birren B and Lander ES (2003). "Sequencing and comparison of yeast species to identify genes and regulatory elements." Nature **423**(6937): 241-54.

Kirkpatrick CR and Schimmel P (1995). "Detection of leucine-independent DNA site occupancy of the yeast Leu3p transcriptional activator in vivo." Mol Cell Biol **15**(8): 4021-30.

Knuppel R, Dietze P, Lehnberg W, Frech K and Wingender E (1994). "TRANSFAC retrieval program: a network model database of eukaryotic transcription regulating sequences and proteins." J Comput Biol **1**(3): 191-8.

Kornitzer D, Raboy B, Kulka RG and Fink GR (1994). "Regulated degradation of the transcription factor Gcn4." Embo J **13**(24): 6021-30.

Lee TI, Rinaldi NJ, Robert F, Odom DT, *et al.* (2002). "Transcriptional regulatory networks in Saccharomyces cerevisiae." Science **298**(5594): 799-804.

Lieb JD, Liu X, Botstein D and Brown PO (2001). "Promoter-specific binding of Rap1 revealed by genome-wide maps of protein-DNA association." Nat Genet **28**(4): 327-34.

Liu XS, Brutlag DL and Liu JS (2002). "An algorithm for finding protein-DNA binding sites with applications to chromatin-immunoprecipitation microarray experiments." Nat Biotechnol **20**(8): 835-9.

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

Matys V, Fricke E, Geffers R, Gossling E, *et al.* (2003). "TRANSFAC: transcriptional regulation, from patterns to profiles." Nucleic Acids Res **31**(1): 374-8.

Pritsker M, Liu YC, Beer MA and Tavazoie S (2004). "Whole-genome discovery of transcription factor binding sites by network-level conservation." Genome Res **14**(1): 99-108.

Ren B, Robert F, Wyrick JJ, Aparicio O, *et al.* (2000). "Genome-wide location and function of DNA binding proteins." Science **290**(5500): 2306-9.

Roth FP, Hughes JD, Estep PW and Church GM (1998). "Finding DNA regulatory motifs within unaligned noncoding sequences clustered by whole-genome mRNA quantitation." Nat Biotechnol **16**(10): 939-45.

Schneider TD and Stephens RM (1990). "Sequence logos: a new way to display consensus sequences." Nucleic Acids Res **18**(20): 6097-100.

Wang T and Stormo GD (2003). "Combining phylogenetic data with co-regulated genes to identify regulatory motifs." Bioinformatics **19**(18): 2369-80.

Waterston RH, Lindblad-Toh K, Birney E, Rogers J, *et al.* (2002). "Initial sequencing and comparative analysis of the mouse genome." Nature **420**(6915): 520-62.

Zeitlinger J, Simon I, Harbison CT, Hannett NM, Volkert TL, Fink GR and Young RA (2003). "Program-specific distribution of a transcription factor dependent on partner transcription factor and MAPK signaling." Cell **113**(3): 395-404.

Zhu J and Zhang MQ (1999). "SCPD: a promoter database of the yeast Saccharomyces cerevisiae." Bioinformatics **15**(7-8): 607-11.

Chapter 5

Conclusions: The Future of Systems Biology

The ultimate goal of systems biology is to have a complete understanding of all the elements that comprise a cell and how those elements interact to effect the functions of the cell (Aggarwal and Lee 2003). We should be able to construct a working computational model of a cell that responds to stimuli exactly as a real cell does. This will allow us to make predictions about cellular responses and to understand every component of that response. In the medical field, such models will enable thorough understanding of the mechanisms behind diseases as well as suggesting the best pathways and steps at which to interfere to cure or alleviate illness.

The elements of a cell that we need to catalog in order to enable a complete model include, but are not limited to, genomic elements, proteomic elements, and the interactions between the two. We need to know what elements are encoded by the DNA in the genome: their location, function, and presence under varied environmental conditions. We need to catalog the components of the proteome, their functions, modifications and different complexes that are formed. In addition, the interactions between the genome and proteome that control the content of the proteome must be understood and cataloged.

Of each of these levels of knowledge required to model a cell, we currently have the most thorough understanding of the genome and its elements. Complete sequences of the genomes of many organisms (excluding the hard to sequence highly repetitive regions) have been published. In addition to this knowledge of the DNA sequence, the list of elements encoded is approaching completion, particularly for smaller organisms such as the yeast *Saccharomyces cerevisiae* (Cliften *et al.* 2003; Kellis *et al.* 2003). One set of these elements is comprised of the sequence of protein coding genes. Many complementary methods have been used for finding these elements, including computational prediction based on searching for sequence patterns that match those associated with known genes (Burge and Karlin 1998; Katoh 2002; Yada *et al.* 2003). A second technique is sequencing the messenger RNA population that is intermediate between the genome and the proteome, via Expressed Sequence Tags (ESTs) (Williamson *et al.* 1995; Eckman *et al.* 1998). Comparison of sequences between related organisms is also used to find protein coding genes, based on the assumption that these genes are more likely to be conserved across species than random DNA sequence (Morgenstern *et al.* 2002; Cliften *et al.* 2003; Kellis *et al.* 2003; Taher *et al.* 2004). These techniques enumerate all the elements that are present in the genome; genome-wide expression analysis (Schena *et al.* 1995; Shalon *et al.* 1996) provides an assessment of which elements are being utilized by the cell under particular conditions by measuring the mRNA population, or other genomic components.

Similar methods have been used to find elements in the genome that code for RNA molecules that are not translated into proteins (Eddy 2001). These include tRNAs that assist in the translation of messenger RNA into proteins by linking the RNA sequence code with the appropriate amino acids. MicroRNAs are small RNA molecules that bind to and cause degradation of specific messenger RNAs, thus participating in the regulation of the level of protein coded for by the mRNA (Lagos-Quintana *et al.* 2001; Lau *et al.* 2001; Lee and Ambros 2001). These and other types of RNA molecules, such as ribozymes, are also found using computational predictions based on pattern recognition and cross species comparison (Grad *et al.* 2003; Lai *et al.* 2003; Ohler *et al.* 2004).

In addition to these coding sequences, the genome also contains regulatory elements. These are short sequences that serve as the points of interaction between the genome and proteome. They include sequences that nucleate the assembly of the general transcriptional machinery, as well as the more specific sequences that are bound by gene-specific transcription factors. Transcription factors can control the assembly or disassembly of the transcriptional machinery as well as the rate of transcription, by varied affinities for the DNA sequences as well as the recruited general transcription apparatus. While various methods for finding these regulatory elements have been described (Lawrence and Reilly 1990; Roth *et al.* 1998; Spellman *et al.* 1998; Hughes *et al.* 2000; Liu *et al.* 2001), these elements have proved more elusive than the protein and RNA coding genes. The main reason for this is that a wide range of sequences can be bound by each transcription factor, in contrast to the single defined sequences that codes for a protein or RNA message.

The proteome is the second level studied in systems biology. Mass spectrometry provides an assessment of the protein complement of cells under various conditions as well as how that complement changes as the cellular environment is altered. Mass spec in addition to the yeast-two-hybrid system has provided information about which proteins are present in complex with one another (Dziembowski and Seraphin 2004). Information about the function of proteins has been obtained on a genome-wide scale by deletion or knockdown of genes followed by phenotypic assessment (Winzeler *et al.* 1999). Deletion or knockdown of multiple proteins in concert provides information about which genes are acting in the same pathways (Krogan *et al.* 2004; Tong *et al.* 2004). Covalent linkage of small molecules to proteins can have a marked effect on protein functionality. These modifications can also be measured by mass spectrometry (Wilkins *et al.* 1999; Sickmann *et al.* 2001; Sickmann *et al.* 2002), but this has not been accomplished on a genome-wide level to date.

Another level of knowledge necessary to model a cell is of the interactions between the genome and the proteome. As mentioned, transcriptional regulators bind to specific DNA sequences close to genes and recruit other proteins to either increase or decrease transcription of the associated gene. In addition, DNA is complexed with histone proteins into chromatin, which packages the DNA into a small volume inside the nucleus (Allfrey *et al.* 1964). Various modifications of the histone proteins, as well as modifications of the transcription factors, have been shown to have an effect on regulation of gene synthesis (Allfrey *et al.* 1964; Struhl 1995; Reece and Platt 1997; Sharrocks 2000). Chromatin immunoprecipitation has long been used to study interactions between both regulatory transcription factors or histones and the genome. Using microarrays to examine the immunoprecipitates has turned this technique into another that can assess interactions on a genome-wide scale (Ren *et al.* 2000; Iyer *et al.* 2001). Computational analysis of the sequences that are reported to be bound by these various factors is one way in which the sequence elements involved are discovered (Harbison *et al.* 2004).

At the moment, we have gathered knowledge about the pieces of a cell that we need in order to build these cellular models, and in fact, have managed to build models of small systems within cells (Hartemink *et al.* 2001; Ideker *et al.* 2001; Davidson *et al.* 2002; Ideker 2004; Oliveri and Davidson 2004). The goal of modeling a complete cell is still somewhat farfetched, however. The regulatory sequences in the genome present one major challenge. As mentioned, these sequences are hard to find because a single protein has affinity for a range of sequences as opposed to only a single sequence. This means that for many genes known to be bound or regulated by a given transcription factor, we are unable to discover the particular sequence to which the protein binds, using the high throughput analyses and experiments that are essential to the systems biology approach. Improvements in analysis of groups of sequences to detect motifs with higher variability than is currently possible will assist in overcoming this limitation, as will more sensitive binding assays. The other side of this problem is that these regulatory sequences are comprised of only a few nucleotides and thus are found randomly throughout the genome. We do not understand yet why these sequences are bound only in a subset of their locations across the genome, and why even fewer of these sequences cause changes in gene expression.

There are also aspects of the system that we do not currently measure on a genome-wide level that could enhance our current understanding. One such aspect is complementary to genome-wide location analysis – a high throughput method of measuring, at each individual promoter, which proteins are present. For an individual promoter this could be accomplished using an oligonucleotide column to capture proteins bound to the sequence of interest, followed by mass spectrometry to identify those proteins. This assumes that the interactions between proteins and DNA are sufficiently strong to survive the column washing. Otherwise some form of crosslinking might be required. To do this in a high throughput fashion, one could possibly create oligonucleotides containing a barcode and linked to beads. After capturing the cognate DNA sequences from a lysed cell, mass spectrometry on individual beads could identify the bound proteins, and the barcode could identify the captured DNA sequence. Another technology that would assist is a method for directly reading the transcriptional output of the cell. Current technologies measure the RNA complement of a cell at a given time, but one cannot deconvolute effects of differential rates of synthesis and degradation to assess the direct effect of perturbations on transcription. Additional technological improvements to enable measuring changes occurring in cells in real time will enable clearer understanding of effects of perturbations. Finally, being able to make measurements on a single cell to avoid population effects will allow us to directly link cause and effect.

In the decade since the first eukaryotic genome was sequenced, we have made great strides towards the goal of a computational model of a complete cell. With some of the new technologies suggested here, as well as improvements in existing methods and analysis, the virtual cell should become a reality in the not too distant future.

References:

Aggarwal K and Lee KH (2003). "Functional genomics and proteomics as a foundation for systems biology." Brief Funct Genomic Proteomic **2**(3): 175-84.

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

Burge CB and Karlin S (1998). "Finding the genes in genomic DNA." Curr Opin Struct Biol **8**(3): 346-54.

Cliften P, Sudarsanam P, Desikan A, Fulton L, Fulton B, Majors J, Waterston R, Cohen BA and Johnston M (2003). "Finding functional features in Saccharomyces genomes by phylogenetic footprinting." Science **301**(5629): 71-6.

Davidson EH, Rast JP, Oliveri P, Ransick A, *et al.* (2002). "A provisional regulatory gene network for specification of endomesoderm in the sea urchin embryo." Dev Biol **246**(1): 162-90.

Dziembowski A and Seraphin B (2004). "Recent developments in the analysis of protein complexes." FEBS Lett **556**(1-3): 1-6.

Eckman BA, Aaronson JS, Borkowski JA, Bailey WJ, Elliston KO, Williamson AR and Blevins RA (1998). "The Merck Gene Index browser: an extensible data integration system for gene finding, gene characterization and EST data mining." Bioinformatics **14**(1): 2-13.

Eddy SR (2001). "Non-coding RNA genes and the modern RNA world." Nat Rev Genet **2**(12): 919-29.

Grad Y, Aach J, Hayes GD, Reinhart BJ, Church GM, Ruvkun G and Kim J (2003). "Computational and experimental identification of C. elegans microRNAs." Mol Cell **11**(5): 1253-63.

Harbison CT, Gordon DB, Lee TI, Rinaldi NJ, *et al.* (2004). "Transcriptional regulatory code of a eukaryotic genome." Nature **431**(7004): 99-104.

Hartemink AJ, Gifford DK, Jaakkola TS and Young RA (2001). "Using graphical models and genomic expression data to statistically validate models of genetic regulatory networks." Pac Symp Biocomput: 422-33.

Hughes JD, Estep PW, Tavazoie S and Church GM (2000). "Computational identification of cis-regulatory elements associated with groups of functionally related genes in Saccharomyces cerevisiae." J Mol Biol **296**(5): 1205-14.

Ideker T (2004). "A systems approach to discovering signaling and regulatory pathways--or, how to digest large interaction networks into relevant pieces." Adv Exp Med Biol **547**: 21-30.

Ideker T, Thorsson V, Ranish JA, Christmas R, *et al.* (2001). "Integrated genomic and proteomic analyses of a systematically perturbed metabolic network." Science **292**(5518): 929-34.

Iyer VR, Horak CE, Scafe CS, Botstein D, Snyder M and Brown PO (2001). "Genomic binding sites of the yeast cell-cycle transcription factors SBF and MBF." Nature **409**(6819): 533-8.

Katoh M (2002). "Paradigm shift in gene-finding method: From bench-top approach to desk-top approach (review)." Int J Mol Med **10**(6): 677-82.

Kellis M, Patterson N, Endrizzi M, Birren B and Lander ES (2003). "Sequencing and comparison of yeast species to identify genes and regulatory elements." Nature **423**(6937): 241-54.

Krogan NJ, Peng WT, Cagney G, Robinson MD, *et al.* (2004). "High-definition macromolecular composition of yeast RNA-processing complexes." Mol Cell **13**(2): 225-39.

Lagos-Quintana M, Rauhut R, Lendeckel W and Tuschl T (2001). "Identification of novel genes coding for small expressed RNAs." Science **294**(5543): 853-8.

Lai EC, Tomancak P, Williams RW and Rubin GM (2003). "Computational identification of Drosophila microRNA genes." Genome Biol **4**(7): R42.

Lau NC, Lim LP, Weinstein EG and Bartel DP (2001). "An abundant class of tiny RNAs with probable regulatory roles in Caenorhabditis elegans." Science **294**(5543): 858-62.

Lawrence CE and Reilly AA (1990). "An expectation maximization (EM) algorithm for the identification and characterization of common sites in unaligned biopolymer sequences." Proteins **7**(1): 41-51.

Lee RC and Ambros V (2001). "An extensive class of small RNAs in Caenorhabditis elegans." Science **294**(5543): 862-4.

Liu X, Brutlag DL and Liu JS (2001). "BioProspector: discovering conserved DNA motifs in upstream regulatory regions of co-expressed genes." Pac Symp Biocomput: 127-38.

Morgenstern B, Rinner O, Abdeddaim S, Haase D, Mayer KF, Dress AW and Mewes HW (2002). "Exon discovery by genomic sequence alignment." Bioinformatics **18**(6): 777-87.

Ohler U, Yekta S, Lim LP, Bartel DP and Burge CB (2004). "Patterns of flanking sequence conservation and a characteristic upstream motif for microRNA gene identification." Rna **10**(9): 1309-1322.

Oliveri P and Davidson EH (2004). "Gene regulatory network controlling embryonic specification in the sea urchin." Curr Opin Genet Dev **14**(4): 351-60.

Reece RJ and Platt A (1997). "Signaling activation and repression of RNA polymerase II transcription in yeast." Bioessays **19**(11): 1001-10.

Ren B, Robert F, Wyrick JJ, Aparicio O, *et al.* (2000). "Genome-wide location and function of DNA binding proteins." Science **290**(5500): 2306-9.

Roth FP, Hughes JD, Estep PW and Church GM (1998). "Finding DNA regulatory motifs within unaligned noncoding sequences clustered by whole-genome mRNA quantitation." Nat Biotechnol **16**(10): 939-45.

Schena M, Shalon D, Davis RW and Brown PO (1995). "Quantitative monitoring of gene expression patterns with a complementary DNA microarray." Science **270**(5235): 467-70.

Shalon D, Smith SJ and Brown PO (1996). "A DNA microarray system for analyzing complex DNA samples using two-color fluorescent probe hybridization." Genome Res **6**(7): 639-45.

Sharrocks AD (2000). "Introduction: the regulation of eukaryotic transcription factor function." Cell Mol Life Sci **57**(8-9): 1147-8.

Sickmann A, Marcus K, Schafer H, Butt-Dorje E, Lehr S, Herkner A, Suer S, Bahr I and Meyer HE (2001). "Identification of post-translationally modified proteins in proteome studies." Electrophoresis **22**(9): 1669-76.

Sickmann A, Mreyen M and Meyer HE (2002). "Identification of modified proteins by mass spectrometry." IUBMB Life **54**(2): 51-7.

Spellman PT, Sherlock G, Zhang MQ, Iyer VR, Anders K, Eisen MB, Brown PO, Botstein D and Futcher B (1998). "Comprehensive identification of cell cycle-regulated genes of the yeast Saccharomyces cerevisiae by microarray hybridization." Mol Biol Cell **9**(12): 3273-97.

Struhl K (1995). "Yeast transcriptional regulatory mechanisms." Annu Rev Genet **29**: 651-74.

Taher L, Rinner O, Garg S, Sczyrba A and Morgenstern B (2004). "AGenDA: gene prediction by cross-species sequence comparison." Nucleic Acids Res **32**(Web Server issue): W305-8.

Tong AH, Lesage G, Bader GD, Ding H, *et al.* (2004). "Global mapping of the yeast genetic interaction network." Science **303**(5659): 808-13.

Wilkins MR, Gasteiger E, Gooley AA, Herbert BR, *et al.* (1999). "High-throughput mass spectrometric discovery of protein post-translational modifications." J Mol Biol **289**(3): 645-57.

Williamson AR, Elliston KO and Sturchio JL (1995). "The Merck Gene Index, a public resource for genomics research." J NIH Res **7**: 61-63.

Winzeler EA, Shoemaker DD, Astromoff A, Liang H, *et al.* (1999). "Functional characterization of the S. cerevisiae genome by gene deletion and parallel analysis." Science **285**(5429): 901-6.

Yada T, Takagi T, Totoki Y, Sakaki Y and Takaeda Y (2003). "DIGIT: a novel gene finding program by combining gene-finders." Pac Symp Biocomput: 375-87.

Appendix B

Computational Discovery of Gene Modules and Regulatory Networks

Published as: Bar-Joseph Z, Gerber GK, Lee TI, Rinaldi NJ, Yoo JY, Robert F, Gordon DB, Fraenkel E, Jaakkola TS, Young RA and Gifford DK (2003). "Computational discovery of gene modules and regulatory networks." Nat Biotechnol **21**(11): 1337-42.

Summary

We present a new algorithm, GRAM (Genetic RegulAtory Modules), which fuses information from genome-wide location and expression data sets to discover regulatory networks of gene modules. A gene module is defined as a set of genes that are both co-expressed and bound by the same set of transcription factors. Unlike previous approaches (Eisen *et al.* 1998; Pilpel *et al.* 2001; Berman *et al.* 2002; Ihmels *et al.* 2002; Segal *et al.* 2003) that have relied primarily on functional information from expression data, the GRAM algorithm explicitly links genes to the factors that regulate them by using DNA binding data to incorporate direct physical evidence of regulatory interactions. We use the GRAM algorithm to discover a genome-wide regulatory network using binding information for 106 transcription factors in *Saccharomyces cerevisiae* in rich media conditions and over 500 expression experiments. Additionally, we present a new genome-wide location analysis data set for regulators in yeast cells treated with rapamycin, and use the GRAM algorithm to provide biological insights in this regulatory network.

High-throughput biological data sources hold the promise of revolutionizing molecular biology by providing large-scale views of genetic regulatory networks. Many genome-wide expression data sets are now readily available, and typical computational analyses have applied clustering algorithms to expression data to find sets of co-expressed and potentially co-regulated genes (Eisen *et al.* 1998). Recent approaches have used more sophisticated algorithms, such as the work of Segal *et al.*, in which they construct a probabilistic model that uses expression data to link regulators to regulated genes (Segal *et al.* 2003). Their method relies on the assumption that expression levels of regulated genes will depend on expression levels of regulators, which is a limitation in cases in which the expression level of the regulator does not change appropriately (e.g., cases of post-transcriptional modification). Other approaches have combined expression data with additional information, such as shared DNA binding motifs or MIPS categories (Pilpel *et al.* 2001; Berman *et al.* 2002; Ihmels *et al.* 2002), but the use of these data sources provides essentially only functional or *indirect* evidence of genetic regulatory interactions. These methods cannot distinguish among genes that have similar expression patterns but are under the control of different regulatory networks (see Supplementary Notes online for further details).

Large scale, genome-wide location analysis for DNA-binding regulators offers a second means for identifying regulatory relationships (Lee *et al.* 2002). Location analysis identifies physical interactions between regulators and DNA regions providing strong *direct* evidence for genetic regulation. While useful, binding information is also limited, as the presence of the regulator at a promoter region indicates binding but not function: the regulator may act positively, negatively or not at all. In addition, as with all microarray based data sources, location analysis data contains substantial experimental noise. Since expression and location analysis data provide complementary information, our goal was to develop an efficient computational method for integrating these data sources. We expected that such an algorithm could provide assignments of groups of genes to regulators that would be both more accurate and more biologically relevant than assignment based solely on either data source alone.

The GRAM algorithm begins by performing an efficient, exhaustive search over all possible combinations of transcriptional regulators indicated by the DNA-binding data with a stringent criterion for determining binding. Once a set of genes bound by a common set of transcriptional regulators is found, the algorithm identifies a subset of these genes with highly correlated expression, which serves as a "seed" for a gene module. The algorithm then revisits the binding data, and seeks to add additional genes to the module that are similarly expressed and bound by the same set of transcriptional regulators using a relaxed binding criteria. Our algorithm allows genes to belong to more than one module. See the Methods section for a complete description of the GRAM algorithm.

The GRAM algorithm was applied to genome-wide location data for 106 transcription factors and over 500 expression experiments (details on the data used are available in Supplementary Table 1 online). One-hundred six gene modules were identified, containing 655 distinct genes and regulated by 68 of the transcription factors. Figure 1 presents a visualization of these results as a graph with edges between gene modules and regulators.

The gene modules abstraction allowed us to label regulator-module edges in the graph to indicate whether there is significant evidence that regulators may be functioning as activators. Since a gene module provides a link between a set of regulators and the common expression pattern of a set of bound genes, we can use the relationship between a regulator's expression pattern and the common expression pattern of genes in a module to infer whether a regulator acts as an activator. In contrast, the use of genomic location data alone only allows us to infer the presence of regulators at promoters, but can give no information about the type of interaction. We searched for activator relationships by examining regulators with expression profiles that are positively correlated with the expression profiles of genes in the corresponding modules. Positive correlation indicates that higher levels of regulator expression correlate with higher levels of expression of genes in the module and suggests that the transcription factor positively regulates the expression of genes in the module. We determined the statistical significance of the activator relationships by computing correlation coefficients between all transcriptional regulators studied and all gene modules and taking the 5% positive tail of the distribution of correlation coefficients. Supplementary Table 2 online presents the eleven activators determined using the method described above. Ten of the eleven activators were previously identified in the literature, suggesting that this analysis

Figure 1

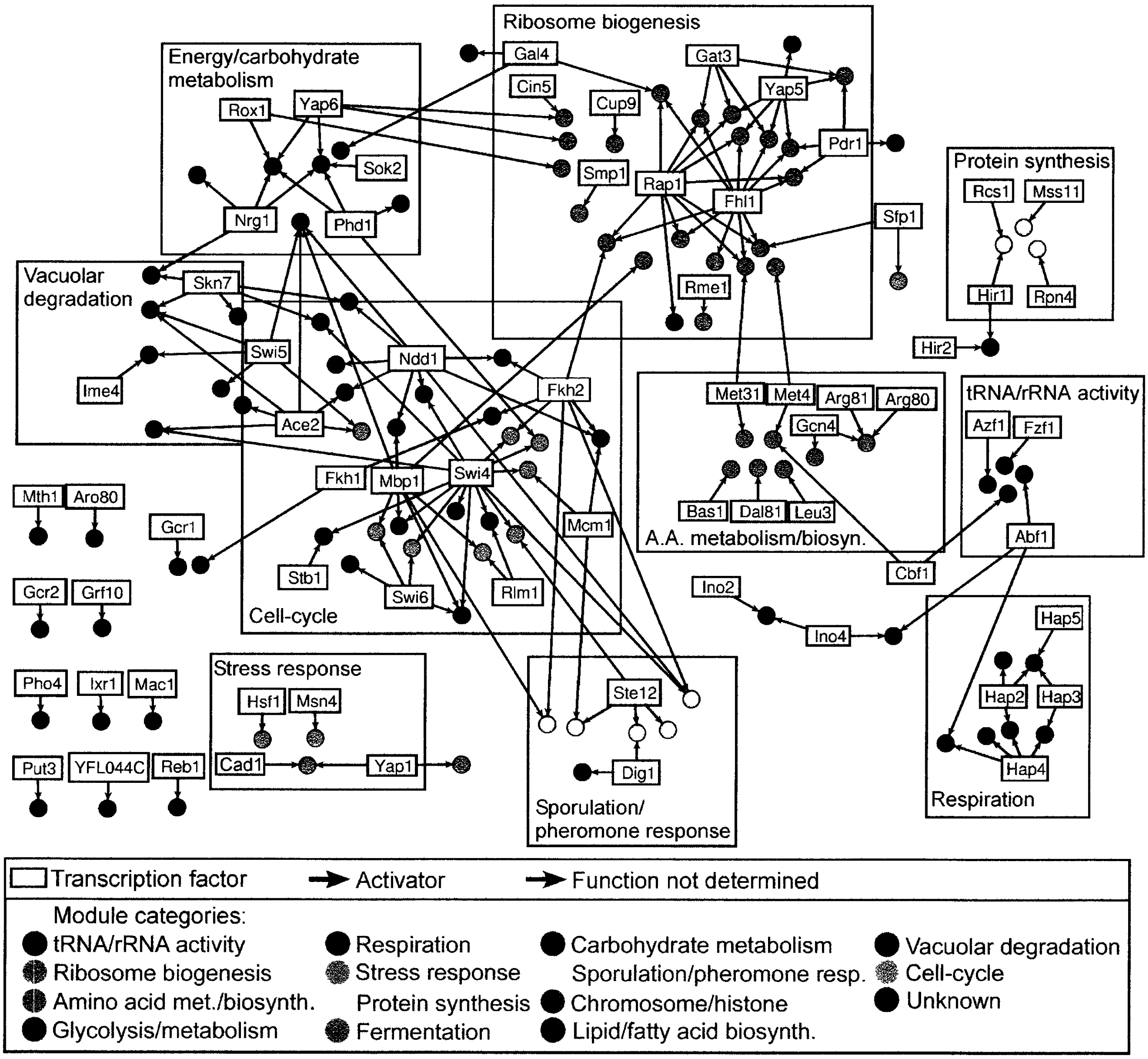

Energy/carbohydrate metabolism
Rox1
Yap6
Sok2
Nrg1
Phd1
Ribosome biogenesis
Gal4
Cin5
Cup9
Smp1
Gat3
Yap5
Pdr1
Rap1
Fhl1
Rme1
Sfp1
Protein synthesis
Rcs1
Mss11
Hir1
Rpn4
Hir2
Vacuolar degradation
Skn7
Swi5
Ime4
Ace2
Ndd1
Fkh2
Fkh1
Mbp1
Swi4
Mcm1
Stb1
Swi6
Rlm1
Cell-cycle
Met31
Met4
Arg81
Arg80
Gcn4
Bas1
Dal81
Leu3
A.A. metabolism/biosyn.
Cbf1
tRNA/rRNA activity
Azf1
Fzf1
Abf1
Mth1
Aro80
Gcr1
Gcr2
Grf10
Pho4
Ixr1
Mac1
Put3
YFL044C
Reb1
Stress response
Hsf1
Msn4
Cad1
Yap1
Ste12
Dig1
Sporulation/ pheromone response
Ino2
Ino4
Hap5
Hap2
Hap3
Hap4
Respiration
Transcription factor
Activator
Function not determined
Module categories:
tRNA/rRNA activity
Ribosome biogenesis
Amino acid met./biosynth.
Glycolysis/metabolism
Respiration
Stress response
Protein synthesis
Fermentation
Carbohydrate metabolism
Sporulation/pheromone resp.
Chromosome/histone
Lipid/fatty acid biosynth.
Vacuolar degradation
Cell-cycle
Unknown

Figure 1: Rich media gene modules network

Visualization of the transcriptional regulatory network discovered by the GRAM algorithm as a graph with edges between gene modules and regulators shows that there are many groups of connected gene modules/regulators involved in similar biological processes. The network consists of 106 modules containing 655 distinct genes regulated by 68 transcription factors. In most cases in which a gene module is controlled by one or more regulators, there was previous evidence suggesting that these regulators physically or functionally interact (see Supplementary Table 3 online for details). The directed arrows point from transcription factors to the gene modules that they regulate. Blue arrows indicate discovered activator regulatory relationships (see Supplementary Table 2 online and the text for details). Gene modules are colored according to the MIPS category to which a significant number of genes belong (significance test using the hypergeometric distribution $p < 0.005$). Modules containing many genes with unknown function or an insignificant number belonging to the same MIPS category are uncolored. When the gene modules discovered by the GRAM algorithm were compared to results generated using location data alone, the GRAM algorithm yielded an almost three-fold increase in modules significantly enriched for genes in the same MIPS category.

produces biologically meaningful results.

Several results obtained by analysis of the discovered gene modules suggest that the algorithm identifies biologically relevant groupings of genes. First, we found that gene modules generally identify groups of genes that function in a similar biological pathway as defined by the MIPS functional categorization (Mewes *et al.* 2000) (see Fig. 1 and Supplementary Table 3 online for details). Second, we found the gene modules to be generally accurate in assigning regulators to sets of genes whose functions are consistent with the regulators' known roles. As an example, Gcr1 is a well-characterized regulator of glucose metabolism (Baker 1986; Holland *et al.* 1987); 6 of the 7 genes identified in the Gcr1 module are enzymes involved in glycolysis and gluconeogenesis. Additionally, we found that in most cases in which a gene module is controlled by one or more regulators, there was previous evidence suggesting that these regulators physically or functionally interact (see Supplementary Table 4 online). For example, gene modules identify Hap2/3/4/5, Hap4/Abf1, Ino2/Ino4, Hir1/Hir2, Mbp1/Swi6, and Swi4/Swi6 interactions. Taken together, these results provide evidence that the GRAM algorithm identifies not only biologically related sets of genes, but also relevant factors that are interacting to control the genes.

While genome-wide location data alone is potentially useful for deriving transcriptional regulatory networks, a key feature of the GRAM algorithm is its ability to compensate for technical limitations in the location data through the integration of expression data. To determine binding events in location data, Lee *et al.* (Lee *et al.* 2002) used a statistical model and chose a relatively stringent p-value threshold (.001) with the intention of reducing false positives at the expense of false negatives. The GRAM algorithm presents a powerful alternative to using a single p-value threshold to predict binding events, since our method allows the p-value cutoff to be relaxed if there is sufficient supporting evidence from expression data. As an example, consider Hap4, a well-characterized regulator of genes involved in oxidative phosphorylation and respiration (Forsburg and Guarente 1989). The Hap4 modules contain twenty-eight genes that are involved in respiration and show a high degree of co-regulation over the collected expression data sets (Fig. 2). Six of these genes (PET9, ATP16, KGD2, QCR6, SDH1, and NDI1) would not have been identified as Hap4 targets using the stringent .001 p-value threshold (p-values range from .0011 to .0036). Overall, 627 out of 1560 unique regulator-gene interactions (40%) in the rich media network discovered by the GRAM algorithm would not have been detected using only location data and the stringent p-value cutoff.

To further verify the ability of the GRAM algorithm to improve the rate of false negatives without contributing significantly to the rate of false positives, we performed gene-specific chromatin-IP experiments for the factor Stb1 and 36 genes. The profiled genes were picked randomly from the full set of yeast genes, with representatives selected from four p-values ranges. In these experiments, three additional genes were determined to be bound by Stb1 that had p-values between .01 and .001 in the genomic location experiments and had thus been excluded with the stringent cutoff. The GRAM algorithm identified *all* three genes as bound by Stb1 without adding any additional genes that were not

Figure 2

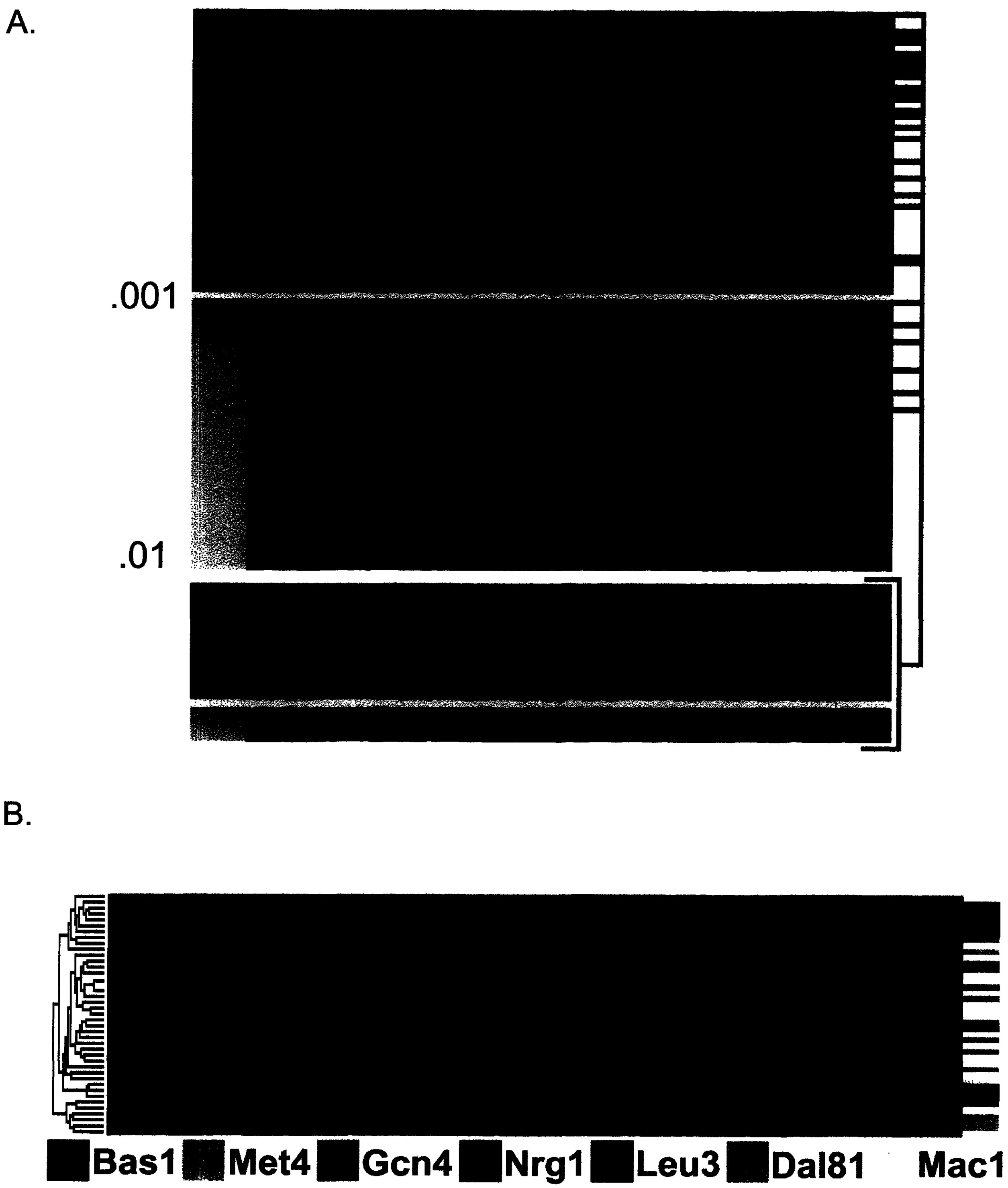


Figure 2: The GRAM algorithm integrates genome-wide binding and expression data and improves on either data source alone. A) Binding data: the GRAM algorithm can improve the quality of DNA-binding information, since it uses expression data to avoid a strict statistical significance threshold. Shown is DNA-binding and expression information for the 99 genes bound by the regulator Hap4 with a p-value < .01 using the statistical model in Lee et al6. The blue-white column on the left indicates binding p-values, and the horizontal yellow line denotes the strict significance threshold of .001. As can be seen, the p-values form a continuum and a strict threshold is unlikely to produce good results. The blue horizontal lines on the right indicate the 28 genes that were selected for modules by the GRAM algorithm. As can be seen, 22 (79%) have a p-value < .001, but 6 (21%) have p-values above this threshold. The lower portion of the figure shows together the 28 genes selected by the GRAM algorithm, and it can be seen that they exhibit coherent expression. Further, all the selected genes are involved in respiration. Six of these genes (PET9, ATP16, KGD2, QCR6, SDH1, and NDI1) would not have been identified as Hap4 targets using the stringent .001 p-value threshold (p-values range from .0011 to .0036).

B) Expression data: the GRAM algorithm can assign different regulators to genes with similar expression patterns that cannot be distinguished using expression clustering methods alone. Hierarchical clustering of expression data was used to obtain the sub-tree on the left. On the right, the regulators assigned to genes by the GRAM algorithm are color coded. As can be seen, many genes with very similar expression patterns are regulated by different transcription factors.

detected in the gene-specific chromatin-IP experiments (see Supplementary Table 5 and Methods online for full details).

We also expected that the gene modules derived by the GRAM algorithm would improve on the biological relevance of gene groupings that could be inferred from location data only. Since genes that participate in the same biological pathway often have similar expression patterns, and genes in a module share not only a common set of transcription factors but also similar expression patterns, we expected that genes in modules would more likely be functionally related than sets of genes identified by location data alone. Indeed, we found that gene modules derived using the GRAM algorithm were almost three times more likely to show enrichment for genes in the same MIPS functional category than were sets of genes derived solely from location data.

We similarly expected that genes in modules derived by the GRAM algorithm would be more likely to show independent evidence of co-regulation by the regulators assigned to the module when compared to sets of genes obtained using location data alone. One line of evidence for such an improvement would be enrichment for specific DNA sequence motifs. We identified 34 transcriptional regulators that bind to genes in at least one module and have well-characterized DNA binding motifs in the TRANSFAC database (Matys *et al.* 2003). For each of these 34 transcriptional regulators, we generated a list of genes in modules bound by the regulator and a second list of genes bound by the regulator using location data alone (stringent p-value cutoff of .001). We then computed the percentage of genes from each list that contained the appropriate known motif in the upstream region of DNA. We found that in most cases, the percentage of genes containing the correct motif was higher when modules generated using the GRAM algorithm were used as compared to sets of genes generated from location data alone (see Fig. 3 and Supplementary Table 6).

For the gene modules discussed above, we used a very large set of genome-wide location and expression data, which allowed us to validate the results of the GRAM algorithm comprehensively with searches of the prior literature, independent chromatin-IP experiments, and analysis for enrichment for genes in the same MIPS category and for known DNA binding motifs. The results of this large-scale validation gave us confidence that the GRAM algorithm would be useful in analyzing new data sources. Since biological insights are often gained by examining responses to specialized treatments or environmental conditions, we were interested in exploring the performance of the GRAM algorithm on a smaller, more biologically targeted data set than the rich media data. So, we chose to examine a transcriptional regulatory sub-network involved in the response to Tor kinase signaling.

The Tor proteins are highly conserved and function as critical regulators in the response to nutrient stress (Hardwick *et al.* 1999; Raught *et al.* 2001; Crespo and Hall 2002; Jacinto and Hall 2003). Tor kinase signaling can be inhibited by the addition of the small macrolide rapamycin, which mimics nutrient starvation and results in a wide range of physiological responses including cytoskeleton reorganization, decreased translation initiation, decreased ribosome biogenesis,

Figure 3

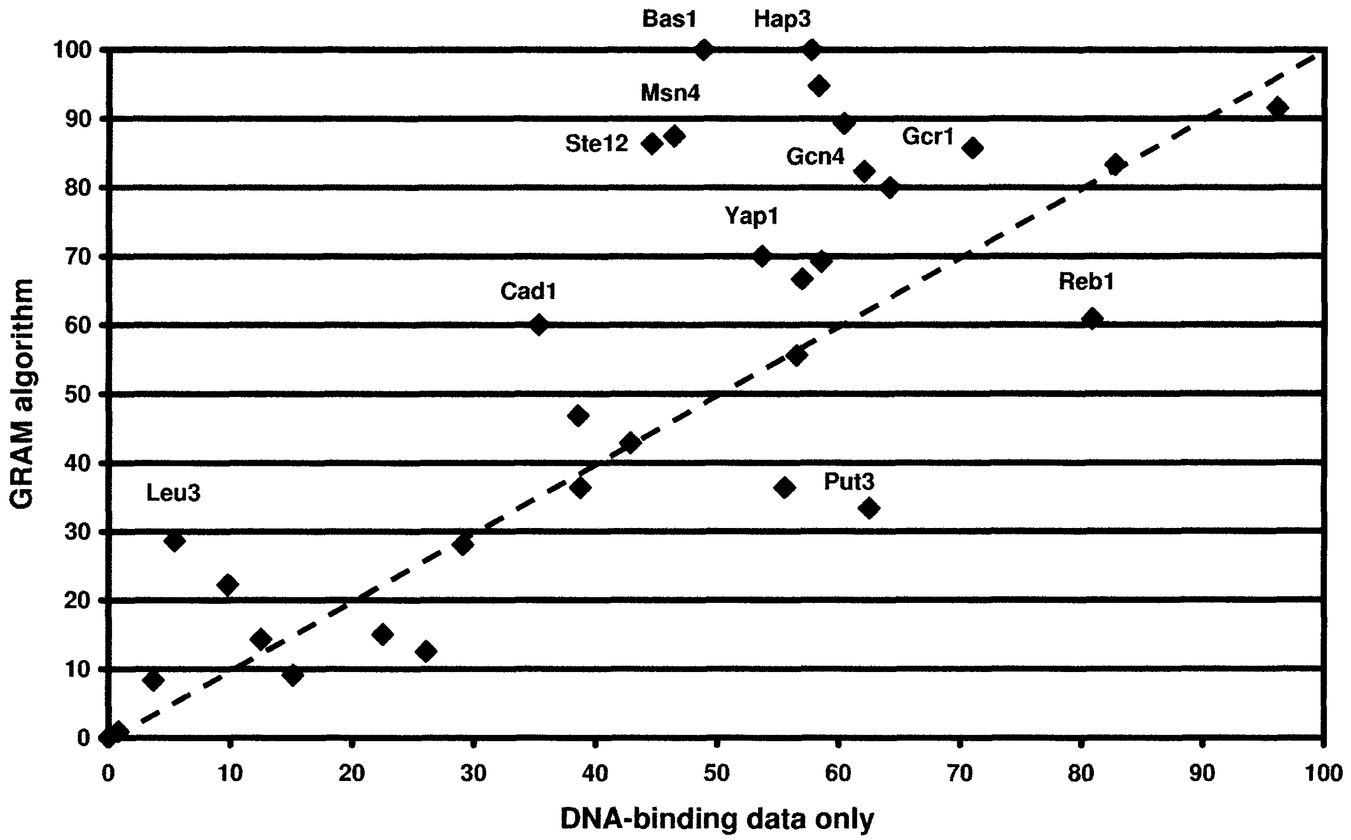

Bas1
Hap3
Msn4
Ste12
Gcr1
Gcn4
Yap1
Cad1
Reb1
Leu3
Put3
100
90
80
70
60
50
40
30
20
10
0
GRAM algorithm
0
10
20
30
40
50
60
70
80
90
100
DNA-binding data only

Figure 3: Motif enrichment

Genes in modules discovered by the GRAM algorithm are more likely to show independent evidence of co-regulation by the regulators assigned to the module when compared to sets of genes obtained using genomic location analysis data alone, as demonstrated by an enrichment for the presence of known DNA-binding motifs. We identified 34 transcriptional regulators that bind to genes in at least one module and have well-characterized DNA binding motifs in the TRANSFAC database (Matys *et al.* 2003). For each of these 34 transcriptional regulators, we generated a list of genes in modules bound by the regulator and a second list of genes bound by the regulator using location analysis data alone (stringent p-value cutoff of .001). We then computed the percentage of genes from each list that contained the appropriate known motif in the upstream region of DNA. In most cases, the percentage of genes containing the correct motif was higher when modules generated using the GRAM algorithm were used versus sets of genes generated from location analysis data alone. See Supplementary Table 6 online for a complete list of transcription factors.

amino acid permease regulation, and autophagy (Cardenas *et al.* 1999; Rep *et al.* 2000; Shamji *et al.* 2000; Hasan *et al.* 2002). Expression analysis indicates that Tor signaling also controls transcriptional regulation of metabolic pathways involving nitrogen metabolism, glycolysis and the TCA cycle (Cardenas *et al.* 1999; Hardwick *et al.* 1999; Shamji *et al.* 2000).

The rapamycin response presented an ideal opportunity for applying the GRAM algorithm to analyzing a novel transcriptional regulatory sub-network. Previous studies suggest a specific set of regulators that are likely to function in the transcriptional response to rapamycin (Hardwick *et al.* 1999; Shamji *et al.* 2000). Also, several publicly available genome-wide expression datasets measuring response after rapamycin treatment are available (Hardwick *et al.* 1999; Shamji *et al.* 2000). More importantly, the fact that there is little information about the transcriptional regulatory network involved and how this transcriptional network may contribute to the overall response to rapamycin treatment presented an opportunity for new biological insights.

We selected 14 transcriptional regulators that seemed likely to function in the rapamycin response in *S. cerevisiae* based on evidence from the literature, and performed genome-wide location analysis experiments (see the Methods section and Supplementary Table 7 online for full details). We ran the GRAM algorithm using the location data for the 14 transcription factors in rapamycin and 22 previously published expression experiments relevant to rapamycin conditions. Thirty-nine gene modules containing 317 unique genes and regulated by 13 transcription factors were discovered (see Fig. 4 and

Figure 4

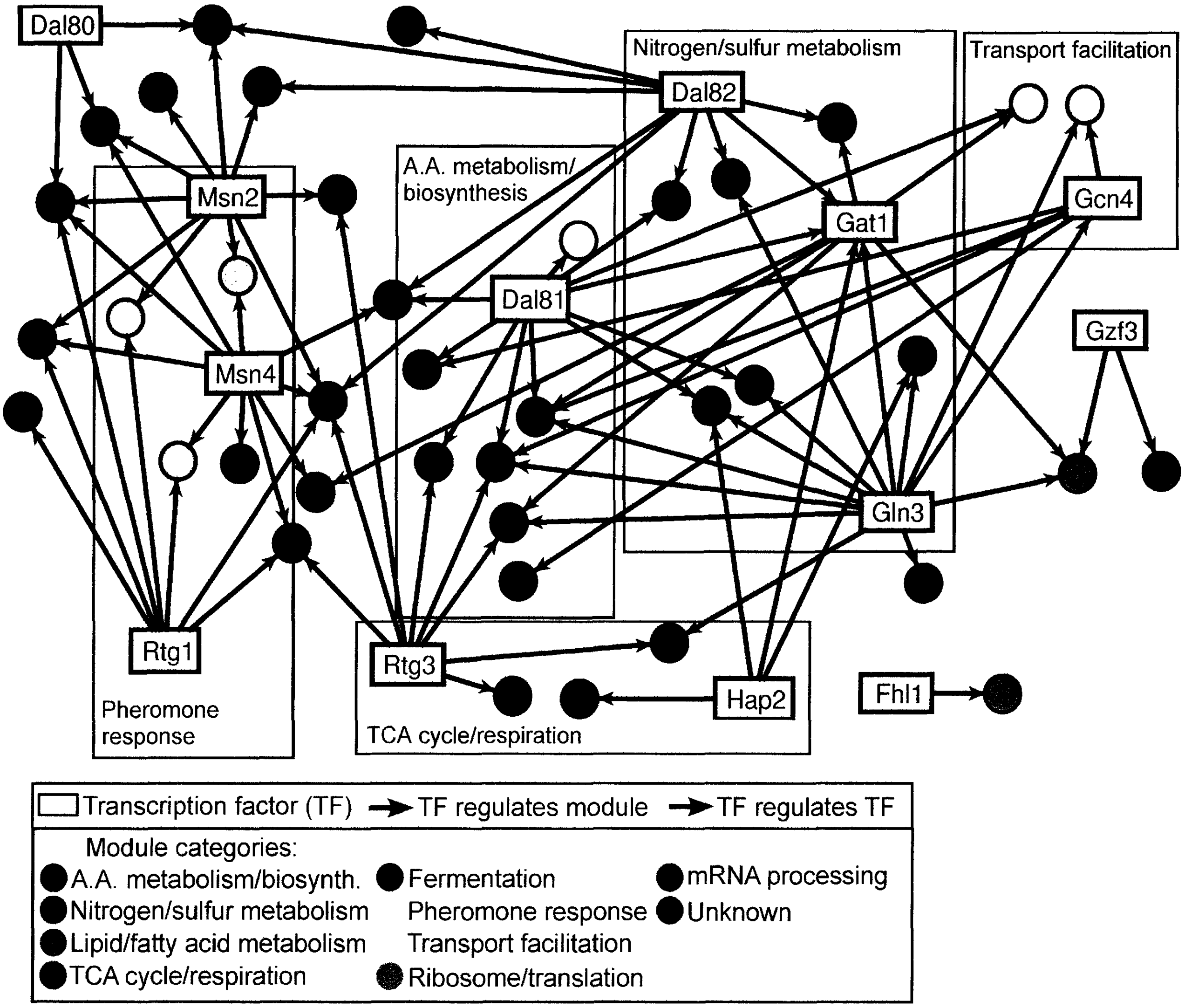

Dal80
Dal82
Nitrogen/sulfur metabolism
Transport facilitation
Msn2
A.A. metabolism/
biosynthesis
Gat1
Gcn4
Dal81
Gzf3
Msn4
Gln3
Rtg1
Rtg3
Hap2
Fhl1
Pheromone
response
TCA cycle/respiration
Transcription factor (TF)
TF regulates module
TF regulates TF
Module categories:
A.A. metabolism/biosynth.
Nitrogen/sulfur metabolism
Lipid/fatty acid metabolism
TCA cycle/respiration
Fermentation
Pheromone response
Transport facilitation
Ribosome/translation
mRNA processing
Unknown

Figure 4: Rapamycin gene modules network

Analysis of the rapamycin transcriptional regulatory sub-network revealed a number of novel biological insights, including evidence that some transcriptional regulators may control genes involved in biological pathways different from those generally associated with these regulators. Further, analysis of the network suggested more complex regulatory interactions in which there is communication among modules. Such complicated network topologies may be important for facilitating rapid and flexible responses to changing environmental conditions. See the text for further details. Thirty-nine modules containing 317 unique genes and regulated by 13 transcription factors were discovered. Red arrows between transcriptional regulators indicate that the source transcription factor binds at least one module containing the target transcription factor. Modules are colored according to the MIPS category to which a significant number of genes belong (significance test using the hypergeometric distribution $p < .05$).

Supplementary Table 8 online). The GRAM algorithm added 192 gene-regulator interactions that would not have been identified with a strict p-value (.001) in the location analysis experiments. Since genome-wide binding experiments for the rapamycin regulatory network have not previously been performed, it was not possible to verify these interactions comprehensively using literature searches.

As in the case for the rich media gene modules network, many features of the rapamycin regulatory network discovered by the GRAM algorithm were consistent with expectations from the literature. Twenty-three of the gene modules were found to contain a significant number of genes (p-value < 0.05) belonging to a single MIPS category. There were a total of 9 categories, all corresponding to biological responses associated with rapamycin treatment (Raught *et al.* 2001; Crespo and Hall 2002; Jacinto and Hall 2003). We also found that in general, regulators were assigned to genes that reflect functions described in previously published results.

In addition to identifying established regulatory interactions, analysis of the rapamycin gene modules suggested several unexpected interactions in which regulators typically assigned to a particular biological response also appear to bind genes acting in different biological pathways. Below we give several examples of such regulatory interactions. These findings suggest models of transcriptional regulation of the rapamycin response that can be validated in further more directed studies. A first example of an unexpected regulatory interaction involves the factors Msn2 and Msn4, which are generally regarded as stress response factors and have been well-studied as activators of such stress-related responses (Martinez-Pastor *et al.* 1996; Boy-Marcotte *et al.* 1998; Rep *et al.* 2000; Hasan *et al.* 2002). Unexpectedly, there were five gene modules in which Msn2 and Msn4 were bound to a significant number of genes involved in the mating pheromone response pathway. A second example involves the factors Rtg1 and Rtg3, which are generally thought to regulate directly genes of the TCA cycle and indirectly contribute to nitrogen metabolism (Liao and Butow 1993; Komeili *et al.* 2000; Crespo *et al.* 2002; Schuller 2003) (products of the TCA cycle are shunted to nitrogen metabolism pathways in low or poor nitrogen conditions). The gene modules network suggests that Rtg regulators may directly regulate genes involved in nitrogen metabolism.

A third example of an unexpected regulatory interaction involves Hap2, a part of the Hap2/3/4/5 complex which has been well-characterized as a regulator of genes involved in respiration (Pinkham and Guarente 1985; Schuller 2003). Indeed, in the rich media gene modules network, members of the Hap complex are unique among the 106 regulators profiled as the only regulators controlling modules that are significantly enriched for genes involved in respiration. As expected, Hap2 regulates a module of respiration genes under rapamycin conditions. Unexpectedly, Hap2 was also found to regulate two modules containing genes involved in nitrogen metabolism. There is some genetic evidence for such cross-pathway regulation, as Hap2 was previously implicated as a regulator of two nitrogen metabolism genes (Dang *et al.* 1996a; Dang *et al.* 1996b). Our results indicate that Hap2 participates in cross-pathway regulation more extensively than previously reported.

In addition to suggesting that some transcriptional regulators may control genes involved in biological pathways different from those generally associated with these regulators, analysis of the gene modules network suggests more complex regulatory interactions in which there is communication among gene modules. Such complicated network topologies may be important for facilitating rapid and flexible responses to changing environmental conditions. As an example, we found that several transcriptional regulators may be involved in a feed-forward regulatory loop in which the gene encoding a regulator is bound by another regulator and both regulators bind to a set of common genes (Lee *et al.* 2002; Shen-Orr *et al.* 2002). The regulator Gat1 has been previously identified as a general activator of nitrogen responsive genes (Coffman *et al.* 1996). We found that Gat1 is itself contained in several modules along with genes involved in nitrogen metabolism. These gene modules are bound by the transcriptional regulators Dal81, Dal82, Gln3 and Hap2. Interestingly, Gat1 also binds several gene modules along with Dal81, Dal82, and Gln3 (see Fig. 4). Feed-forward mechanisms may be important in regulatory responses (such as the response to rapamycin) by modulating regulatory sensitivity to sustained rather than transient inputs, providing temporal control, or amplifying the transcriptional response (Shen-Orr *et al.* 2002). These findings can be validated in further directed experimental studies.

The above analyses indicate that the GRAM algorithm can be useful for studying transcriptional regulatory networks using genome-wide location and expression data sources. We have made a Java implementation of the algorithm publicly available (see Supplementary Methods online), and believe that as new genome-wide location data becomes increasingly available other researchers will find the algorithm helpful. As demonstrated, the algorithm can integrate sources of genome-wide location and expression data to help compensate for technical limitations in the data. Further, the inferred gene modules networks can give a clearer view of regulation than can either location or expression data sources alone. We have found the algorithm is particularly useful for uncovering how certain regulators may act in multiple biological pathways. Overall, the GRAM algorithm facilitates a genome-wide approach to analysis of transcriptional regulatory networks that can suggest specific novel regulatory models, which can then be validated in more directed experimental studies.

Methods

The GRAM (Genetic RegulAtory Modules) algorithm

Below we describe the operation of the algorithm. Some details are omitted due to space constraints; see the Supplementary Methods online for complete information as well as a Java implementation of the algorithm.

Let e_i denote an expression vector and b_i a vector of binding p-values for gene i, where there are n_g genes. Let $B(i,t)$ denote the set of all transcription factors that bind to gene i with p-value less than t, i.e., the list of indices j such that $b_{ij} < t$. Let $F \subseteq B(i,t)$ denote a subset of the transcription factors that are bound to i. Let $G(F,t)$ be the set of all genes i such that for any gene $i \in G(F,t)$, $F \subseteq B(i,t)$, i.e., genes to which all the factors in F bind with a given significance threshold. The algorithm begins by going over all genes, and assigning each gene i to all possible sets $G(F,t_1)$, where t_1 is a high stringency binding threshold and F ranges over all subsets of $B(i,t)$.

For every set of transcription factors F, the genes in $G(F,t_1)$ serve as candidates for a module regulated by F. For each such set $G(F,t_1)$ with a sufficient number n of genes (e.g., $n \geq 5$), the algorithm attempts to find a "core" expression profile. That is, we are seeking a point c in expression space such that for an expression similarity threshold s_n, the ball centered at c of radius s_n contains as many genes in $G(F,t_1)$ as possible. Denote by $C(F,t_1,c)$ the "core" set of genes such that $C(F,t_1,c) \subseteq G(F,t_1)$ and for each gene $i \in C(F,t_1,c)$, $d(e_i,c) < s_n$, where d is the Euclidian distance between two points. The threshold s_n is determined by using all genes, and randomly sampling subsets of size n to determine the distribution of expression distances from a subset to all genes. The problem of finding a point *c* for a set of expression vectors is non-trivial, and cannot be optimally solved in reasonable time given the dimensionality of the expression space (>500). Thus, we use a theoretically motivated approximation algorithm which looks for the central point in all triplets of genes in $G(F,t_1)$. See the Supplementary Methods online for more details.

The genes in $C(F,t_1,c)$ are used to initialize a module $M(F)$. Conceptually, we would like to expand this module by relaxing our criteria for binding if a gene's expression profile is sufficiently similar to those in the "core." In order to do so, the algorithm calculates a combined p-value p_i for each gene *i* that belongs to the expanded set $C(F,t_2,c)$ and does not belong to $C(F,t_1,c)$, where $t_2 > t_1$. The p-value p_i is arrived at by computing independent p-values for gene *i* and each transcription factor in *F* and then combining the p-values using the Fisher method. A gene *i* from $C(F,t_2,c)$ is then included in $M(F)$ if $p_i < t_1$. This module initialization and expansion is completed for each feasible *F*, starting with the sets containing the largest number of factors and proceeding to the smallest. If a gene is included in a module $M(F)$, it is masked out (not considered) when forming modules with factor subsets, $M(F')$ where $F' \subseteq F$. That is, the algorithm will seek to explain a gene's expression using the most specific regulatory patterns. The thresholds t_1=.001 and t_2=.01 were chosen based on experiments reported in Lee *et al.* (Lee *et al.* 2002) that suggested very low false positive rates for a significance threshold of .001. Further, the rate of false negatives was found to be relatively high for p-values between .01 and .001, but decreased dramatically (to less than 3%) thereafter.

Strains

Epitope-tagged strains were generated as described previously (Lee *et al.* 2002). Briefly, regulators were tagged at the C-terminus by using homologous recombination to insert multiple copies of the Myc epitope coding sequence into the normal chromosomal loci of these genes. Insertion of the epitope coding sequence was confirmed by PCR and expression of the epitope-tagged protein was confirmed by Western blotting analysis.

Growth conditions

Strains containing epitope-tagged regulators were grown in 50 ml YPD (yeast extract – peptone – dextrose) at 30 degrees C. Cells were grown to an OD600 of 0.7–0.8 and rapamycin was then added to a final concentration of 100 nM. Cells were grown for 20 minutes at 30 degrees C in the presence of rapamycin.

Genome-wide Location Analysis

Genome-wide location analysis was performed as previously described (Lee *et al.* 2002). Briefly, cells containing an epitope-tagged regulator were fixed with formaldehyde (1% final concentration) and then harvested by centrifugation. Cells were lysed and then sonicated to shear DNA. DNA fragments representing chromosomal regions crosslinked to a protein of interest were enriched by immunoprecipitation with an anti-epitope antibody. After reversal of crosslinking, enriched DNA was purified. The ends of DNA fragments were then blunted using T4 DNA polymerase and ligated to previously prepared linkers. The enriched DNA was then amplified and labeled with a fluorescent dye by ligation-mediated PCR (LM-PCR). A sample of control DNA was similarly processed and labeled with a different fluorophore. Both IP-enriched and control DNA were then hybridized to a single DNA microarray. For each factor, three independently grown cell cultures were processed and scanned to generate binding information as previously described.

References:

Baker HV (1986). "Glycolytic gene expression in Saccharomyces cerevisiae: nucleotide sequence of GCR1, null mutants, and evidence for expression." Mol Cell Biol **6**(11): 3774-84.

Berman BP, Nibu Y, Pfeiffer BD, Tomancak P, Celniker SE, Levine M, Rubin GM and Eisen MB (2002). "Exploiting transcription factor binding site clustering to identify cis-regulatory modules involved in pattern formation in the Drosophila genome." Proc Natl Acad Sci U S A **99**(2): 757-62.

Boy-Marcotte E, Perrot M, Bussereau F, Boucherie H and Jacquet M (1998). "Msn2p and Msn4p control a large number of genes induced at the diauxic transition which are repressed by cyclic AMP in Saccharomyces cerevisiae." J Bacteriol **180**(5): 1044-52.

Cardenas ME, Cutler NS, Lorenz MC, Di Como CJ and Heitman J (1999). "The TOR signaling cascade regulates gene expression in response to nutrients." Genes Dev **13**(24): 3271-9.

Coffman JA, Rai R, Cunningham T, Svetlov V and Cooper TG (1996). "Gat1p, a GATA family protein whose production is sensitive to nitrogen catabolite repression, participates in transcriptional activation of nitrogen-catabolic genes in Saccharomyces cerevisiae." Mol Cell Biol **16**(3): 847-58.

Crespo JL and Hall MN (2002). "Elucidating TOR signaling and rapamycin action: lessons from Saccharomyces cerevisiae." Microbiol Mol Biol Rev **66**(4): 579-91, table of contents.

Crespo JL, Powers T, Fowler B and Hall MN (2002). "The TOR-controlled transcription activators GLN3, RTG1, and RTG3 are regulated in response to intracellular levels of glutamine." Proc Natl Acad Sci U S A **99**(10): 6784-9.

Dang VD, Bohn C, Bolotin-Fukuhara M and Daignan-Fornier B (1996a). "The CCAAT box-binding factor stimulates ammonium assimilation in Saccharomyces cerevisiae, defining a new cross-pathway regulation between nitrogen and carbon metabolisms." J Bacteriol **178**(7): 1842-9.

Dang VD, Valens M, Bolotin-Fukuhara M and Daignan-Fornier B (1996b). "Cloning of the ASN1 and ASN2 genes encoding asparagine synthetases in Saccharomyces cerevisiae: differential regulation by the CCAAT-box-binding factor." Mol Microbiol **22**(4): 681-92.

Eisen MB, Spellman PT, Brown PO and Botstein D (1998). "Cluster analysis and display of genome-wide expression patterns." Proc Natl Acad Sci U S A **95**(25): 14863-8.

Forsburg SL and Guarente L (1989). "Identification and characterization of HAP4: a third component of the CCAAT-bound HAP2/HAP3 heteromer." Genes Dev **3**(8): 1166-78.

Hardwick JS, Kuruvilla FG, Tong JK, Shamji AF and Schreiber SL (1999). "Rapamycin-modulated transcription defines the subset of nutrient-sensitive signaling pathways directly controlled by the Tor proteins." Proc Natl Acad Sci U S A **96**(26): 14866-70.

Hasan R, Leroy C, Isnard AD, Labarre J, Boy-Marcotte E and Toledano MB (2002). "The control of the yeast H2O2 response by the Msn2/4 transcription factors." Mol Microbiol **45**(1): 233-41.

Holland MJ, Yokoi T, Holland JP, Myambo K and Innis MA (1987). "The GCR1 gene encodes a positive transcriptional regulator of the enolase and glyceraldehyde-3-phosphate dehydrogenase gene families in Saccharomyces cerevisiae." Mol Cell Biol **7**(2): 813-20.

Ihmels J, Friedlander G, Bergmann S, Sarig O, Ziv Y and Barkai N (2002). "Revealing modular organization in the yeast transcriptional network." Nat Genet **31**(4): 370-7.

Jacinto E and Hall MN (2003). "Tor signalling in bugs, brain and brawn." Nat Rev Mol Cell Biol **4**(2): 117-26.

Komeili A, Wedaman KP, O'Shea EK and Powers T (2000). "Mechanism of metabolic control. Target of rapamycin signaling links nitrogen quality to the activity of the Rtg1 and Rtg3 transcription factors." J Cell Biol **151**(4): 863-78.

Lee TI, Rinaldi NJ, Robert F, Odom DT, *et al.* (2002). "Transcriptional regulatory networks in Saccharomyces cerevisiae." Science **298**(5594): 799-804.

Liao X and Butow RA (1993). "RTG1 and RTG2: two yeast genes required for a novel path of communication from mitochondria to the nucleus." Cell **72**(1): 61-71.

Martinez-Pastor MT, Marchler G, Schuller C, Marchler-Bauer A, Ruis H and Estruch F (1996). "The Saccharomyces cerevisiae zinc finger proteins Msn2p and Msn4p are required for transcriptional induction through the stress response element (STRE)." Embo J **15**(9): 2227-35.

Matys V, Fricke E, Geffers R, Gossling E, *et al.* (2003). "TRANSFAC: transcriptional regulation, from patterns to profiles." Nucleic Acids Res **31**(1): 374-8.

Mewes HW, Frishman D, Gruber C, Geier B, *et al.* (2000). "MIPS: a database for genomes and protein sequences." Nucleic Acids Res **28**(1): 37-40.

Pilpel Y, Sudarsanam P and Church GM (2001). "Identifying regulatory networks by combinatorial analysis of promoter elements." Nat Genet **29**(2): 153-9.

Pinkham JL and Guarente L (1985). "Cloning and molecular analysis of the HAP2 locus: a global regulator of respiratory genes in Saccharomyces cerevisiae." Mol Cell Biol **5**(12): 3410-6.

Raught B, Gingras AC and Sonenberg N (2001). "The target of rapamycin (TOR) proteins." Proc Natl Acad Sci U S A **98**(13): 7037-44.

Rep M, Krantz M, Thevelein JM and Hohmann S (2000). "The transcriptional response of Saccharomyces cerevisiae to osmotic shock. Hot1p and Msn2p/Msn4p are required for the induction of subsets of high osmolarity glycerol pathway-dependent genes." J Biol Chem **275**(12): 8290-300.

Schuller HJ (2003). "Transcriptional control of nonfermentative metabolism in the yeast Saccharomyces cerevisiae." Curr Genet **43**(3): 139-60.

Segal E, Shapira M, Regev A, Pe'er D, Botstein D, Koller D and Friedman N (2003). "Module networks: identifying regulatory modules and their condition-specific regulators from gene expression data." Nat Genet **34**(2): 166-76.

Shamji AF, Kuruvilla FG and Schreiber SL (2000). "Partitioning the transcriptional program induced by rapamycin among the effectors of the Tor proteins." Curr Biol **10**(24): 1574-81.

Shen-Orr SS, Milo R, Mangan S and Alon U (2002). "Network motifs in the transcriptional regulation network of Escherichia coli." Nat Genet **31**(1): 64-8.