Cell, Vol. 106, 697–708, September 21, 2001, Copyright ©2001 by Cell Press

Serial Regulation of Transcriptional Regulators in the Yeast Cell Cycle

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Summary

Genome-wide location analysis was used to determine how the yeast cell cycle gene expression program is regulated by each of the nine known cell cycle transcriptional activators. We found that cell cycle transcriptional activators that function during one stage of the cell cycle regulate transcriptional activators that function during the next stage. This serial regulation of transcriptional activators forms a connected regulatory network that is itself a cycle. Our results also reveal how the nine transcriptional regulators coordinately regulate global gene expression and diverse stage-specific functions to produce a continuous cycle of cellular events. This information forms the foundation for a complete map of the transcriptional regulatory network that controls the cell cycle.

Introduction

Regulation of the cell cycle clock is effected through a controlled program of gene expression and oscillations in the activity of the cyclin-dependent kinase (CDK) family of protein kinases. Much is known about the control of stage-specific functions by CDKs and their regulators during the cell cycle (Mendenhall and Hodge, 1998; Morgan, 1997; Nurse, 2000). A more complete understanding of cell cycle regulation is constrained, however, by our limited knowledge of the transcriptional regulatory network that controls the clock.

Transcription factors have been identified that have roles in regulating transcription of a small set of yeast genes whose expression is cell-cycle dependent; these include Mbp1, Swi4, Swi6, Mcm1, Fkh1, Fkh2, Ndd1, Swi5, and Ace2 (Koch and Nasmyth, 1994; Koranda et al., 2000; Kumar et al., 2000; McBride et al., 1999; McInerny et al., 1997; Pic et al., 2000; Zhu et al., 2000; reviewed in Mendenhall and Hodge, 1998 and Breeden, 2000). Based on these studies, the following model has emerged. MBF (a complex of Mbp1 and Swi6) and SBF (a complex of Swi4 and Swi6) control late G1 genes. Mcm1, together with Fkh1 or Fkh2, recruits the Ndd1 protein in late G2, and thus controls the transcription of G2/M genes. Mcm1 is also involved in the transcription of some M/G1 genes. Swi5 and Ace2 regulate genes at the end of M and early G1. It is not yet clear whether this model, developed using a small set of genes, will extrapolate to regulation of all cell cycle genes.

Microarray analysis has revealed that the expression levels of approximately 800 genes vary in a periodic fashion during the yeast cell cycle (Cho et al., 1998; Spellman et al., 1998), but little is known about the regulation of most of these genes. The set of genes controlled by MBF and SBF has recently been identified by using a genome-wide binding method, confirming that these factors are largely bound to genes expressed in late G1 and revealing how sets of functionally related genes are regulated during this time (Iyer et al., 2001). Identification of the genes regulated by all nine transcription factors in living cells should be considerably more informative, and is essential for further understanding how the cell cycle is regulated at the transcriptional level.

A fundamental question associated with any biological phenomenon is "how are the regulators regulated?" Most of the key cell cycle regulators, including transcription activators and CDK regulators, are themselves expressed in a cell cycle-dependent fashion, so it is important to understand how their expression is regulated. Previous studies have identified transcriptional activators for genes encoding three of the nine cell cycle transcription factors and many of the cyclins, but knowledge of how these regulators are regulated is most likely incomplete.

Coordinate expression of genes whose products contribute to stage-specific functions is a key feature of the cell cycle (Morgan, 1997; Nasmyth, 1996). The identification of genomic binding sites for SBF and MBF revealed how coordinate expression of genes involved in budding and in DNA replication is accomplished (Iyer et al., 2001). Knowledge of the genomic binding sites for all cell cycle transcription factors is needed to better understand how coordinate gene regulation is accomplished throughout the cell cycle.

We report here the genomic targets of all nine known cell cycle transcription activators in living yeast cells. The results reveal a fundamental feature of cell cycle regulation in living cells: cell cycle transcriptional activators that function during one stage of the cell cycle contribute to the regulation of transcriptional activators that function during the next stage, forming a fully connected regulatory circuit. The results also reveal how these cell cycle-specific transcriptional regulators control key temporal features of the cell cycle and how coordinate control of genes with shared stage-specific functions is accomplished. This information can be used to begin constructing a map of the transcriptional and posttranscriptional regulatory networks that control the complex and highly regulated processes that occur throughout the cell cycle.

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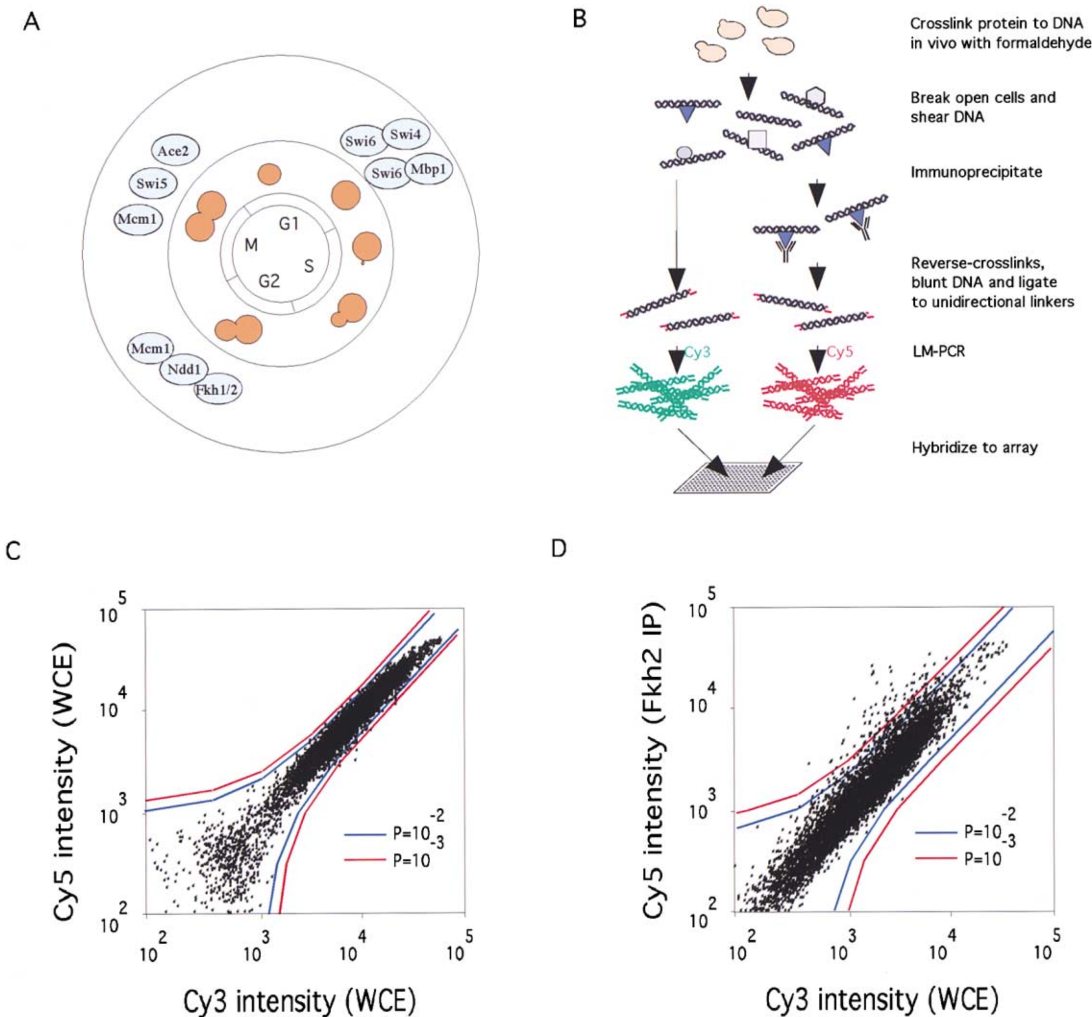

Figure 1. Cell Cycle Transcriptional Regulators: Study Design

(A) The stages of the cell cycle are depicted together with yeast cell morphology (brown) and transcriptional regulators (blue). The transcriptional regulators are positioned at the stage during which they have been reported to function (Breeden, 2000; Mendenhall and Hodge, 1998).

(B) Schematic summary of the experimental design. Detailed protocols can be obtained at the authors' website at http://web.wi.mit.edu/young/cellcycle and through the *Cell* website at http://www.cell.com/cgi/content/full/106/6/697/DC1.

(C) A scatter plot of Cy5 versus Cy3 intensities for a control experiment in which aliquots of whole cell extract (WCE) were independently labeled with Cy3 and Cy5 and hybridized to a DNA microarray containing all yeast intergenic regions. The red and blue lines border the regions with confidence levels of $p < 0.001$ and $p < 0.01$, respectively.

(D) A scatter plot of an experiment in which the Fkh2 IP-enriched DNA was labeled with Cy5 and the WCE was labeled with Cy3. The red and blue lines border the regions with confidence levels of $p < 0.001$ and $p < 0.01$, respectively. The spots whose values have confidence levels of $p < 0.001$ represent promoters most likely bound by the Fkh2 factor. Scatter plots for all the experiments can be found at the authors' web site.

Results

We used genome-wide location analysis (Ren et al., 2000) to identify the in vivo genome binding sites for each of the known cell cycle transcription factors (Figures 1A and 1B). Yeast strains, each containing a myc-tagged version of Mbp1, Swi4, Swi6, Mcm1, Fkh1, Fkh2, Ndd1, Swi5, or Ace2, were grown in asynchronous cultures to mid log phase and subjected to location analysis as described previously (Ren et al., 2000). Each experiment was carried out in triplicate, and a single-array error model was used to handle noise, to average repeated experiments with appropriate weights, and to rank binding sites by p value (Figures 1C and 1D). We used asynchronous cultures because previous studies showed that the results obtained for Swi4 in genome-wide location experiments are essentially identical in unsynchronized and arrested cultures (Iyer et al., 2001), and because it was not feasible to obtain high quality datasets in triplicate at multiple cell cycle time points for all nine factors.

The regulation of the cell cycle expression program by each of the nine factors is summarized in Figure 2. The binding of a transcriptional activator to the promoter region of a gene suggests that the activator has a regulatory effect on the gene, but it is also possible that the

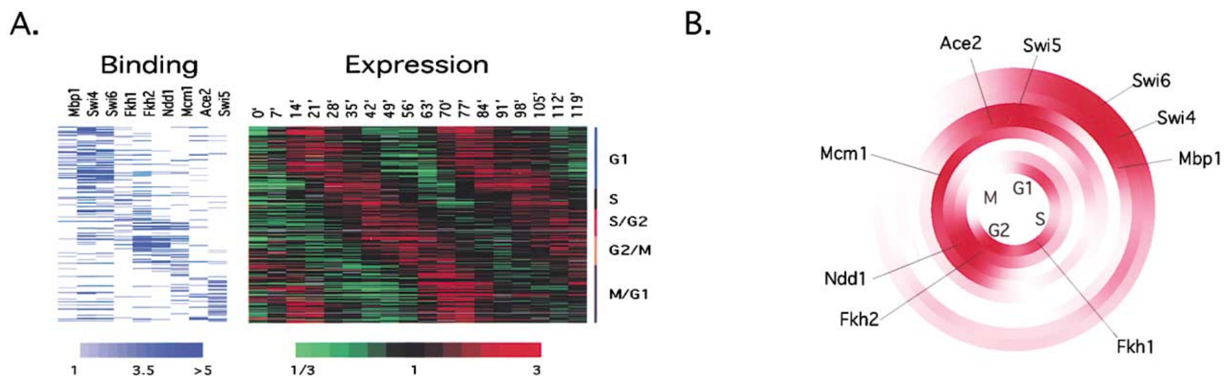

Figure 2. Genome-wide Location of the Nine Cell Cycle Transcription Factors

(A) 213 of the 800 cell cycle genes whose promoter regions were bound by a myc-tagged version of at least one of the nine cell cycle transcription factors ($p < 0.001$) are represented as horizontal lines. The weight-averaged binding ratios are displayed using a blue and white color scheme (genes with p value < 0.001 are displayed in blue). The expression ratios of an α factor synchronization time course from Spellman et al. (1998) are displayed using a red (induced) and green (repressed) color scheme.

(B) The circle represents a smoothed distribution of the transcription timing (phase) of the 800 cell cycle genes (Spellman et al., 1998). The intensity of the red color, normalized by the maximum intensity value for each factor, represents the fraction of genes expressed at that point that are bound by a specific activator. The similarity in the distribution of color for specific factors (with Swi4, Swi6, and Mbp1, for example) shows that these factors bind to genes that are expressed during the same time frame.

activator does not fully or even partially control the gene. For this reason, we have identified the set of genes where factor binding correlates with gene expression, an approach that produced highly accurate information on transcription factor function in previous studies with other factors (Ren et al., 2000). The set of genes bound by the nine cell cycle transcription factors was compared to the set of approximately 800 genes whose expression levels vary in a periodic fashion during the yeast cell cycle (Spellman et al., 1998). The proportion of the 800 genes whose promoters are bound by one or more of the nine transcription factors studied here varies with the stringency of the analysis criteria for binding data (27% at $p < 0.001$, 37% at $p < 0.01$; 50% at $p < 0.05$). We focus further discussion on results obtained with the highest stringency criteria ($p < 0.001$) because a previous investigation using this approach detected no false positives in followup studies (Ren et al., 2000) (see the authors' website at http://web.wi.mit.edu/young/cellcycle and through the *Cell* website at http://www.cell.com/cgi/content/full/106/6/697/DC1 for complete datasets and additional analysis).

Collaboration of Regulators in Periodic Gene Expression

A model for transcriptional control of cell cycle genes has been developed that is based on studies involving a relatively small number of genes. In this model, MBF and SBF control expression of late G1 genes (Koch and Nasmyth, 1994); a complex of Mcm1, Ndd1, and Fkh1/Fkh2 controls G2/M genes (Koranda et al., 2000; Kumar et al., 2000; Pic et al., 2000; Zhu et al., 2000); and Mcm1, Swi5, and Ace2 regulate genes expressed in M/G1 (McBride et al., 1999; McInerny et al., 1997). The genome-wide binding data for these activators support this model (Figure 2) and provide compelling evidence for collaboration among specific factors in genome-wide gene regulation. Mbp1, Swi4, and Swi6 bound predominantly to promoter regions of late G1 genes ($p < 10^{-14}$, $p < 10^{-18}$, and $p < 10^{-20}$ respectively), Swi5 and Ace2 to M/G1 genes ($p < 10^{-14}$ and $p < 10^{-3}$, respectively), and Mcm1, Fkh2, and Ndd1 to G2/M genes ($p < 10^{-14}$, $p < 10^{-15}$, and $p < 10^{-21}$, respectively). Thus, our data generally support the model for stage-specific regulation of gene expression by these activators and extend it to encompass promoters for several hundred cell cycle genes.

Our data also provide novel insights into stage-specific gene regulation by these factors. Previous studies suggested that Fkh1 and Fkh2 are homologs that function in concert with Mcm1 during G2/M (Zhu et al., 2000), but we find that Fkh1 and Fkh2 are also associated with genes expressed in G1 and S, where we could not detect Mcm1 binding (Figure 2). The combination of Mcm1, Fkh2, and Ndd1 bound predominantly to G2/M genes, as expected, but Mcm1 was also bound to genes expressed during M/G1 ($p < 10^{-6}$), where we could not detect binding by Fkh1, Fkh2, or Ndd1. These results suggest that differential regulation of Mcm1 and Forkhead target genes in different stages of the cell cycle may be governed by the association of these factors with different regulatory partners. Future identification of the genomic binding sites of all yeast transcriptional activators should reveal these partners.

Regulation of Transcriptional Regulators

We examined the extent to which the cell cycle transcriptional regulators regulate expression of other regulators. Previous studies established that genes encoding several of the cell cycle transcriptional regulators are themselves bound by other cell cycle regulators (Figure 3A). *SWI4* is regulated by Mcm1 and Swi6 (Foster et al., 1993; Mackay et al., 2001; McInerny et al., 1997), *SWI5* is regulated by the Mcm1/Fkh2/Ndd1 complex (Koranda et al., 2000; Kumar et al., 2000; Pic et al., 2000; Zhu et al., 2000), and expression of *ACE2* is affected by depletion of Mcm1 (Althoefer et al., 1995). The genome-wide location data confirmed these results. The location

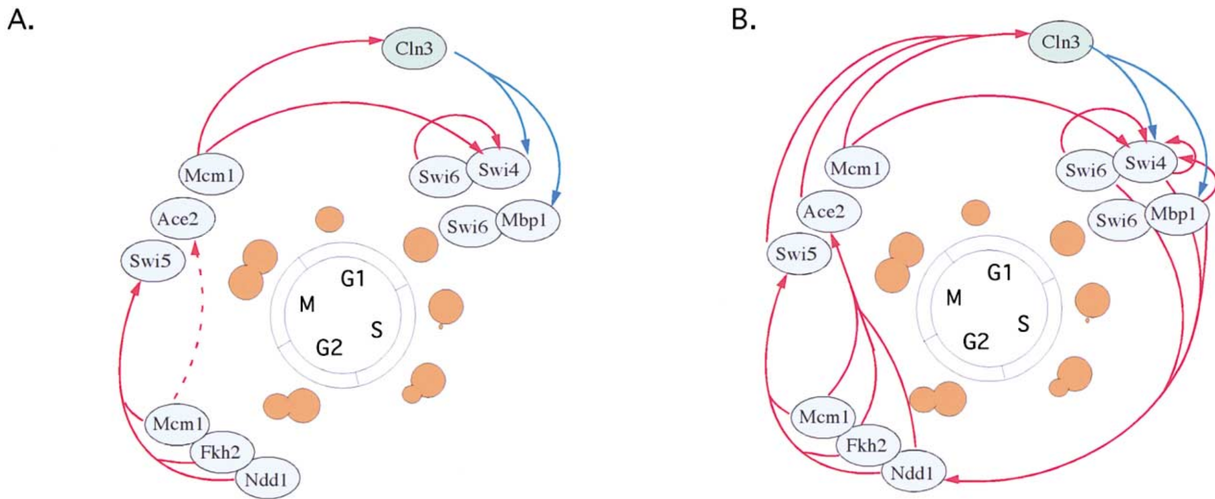

Figure 3. Transcriptional Regulation of Cell Cycle Transcription Factor Genes

(A) Summary of previous evidence for regulation of cell cycle transcription factor genes and *CLN3* by transcriptional regulators (Althoefer et al., 1995; Foster et al., 1993; Koranda et al., 2000; Kumar et al., 2000; Kuo and Grayhack, 1994; Loy et al., 1999; Lydall et al., 1991; Mackay et al., 2001; McInerny et al., 1997; Pic et al., 2000; Zhu et al., 2000). The relationships between the transcription factors and their target genes are indicated by red arrows; solid lines represent evidence for direct regulation by these factors, and dashed lines represent inferences from indirect evidence. The blue arrows represent posttranscriptional regulation by Cln3/Cdc28 (Dirick et al., 1995).

(B) Model for the closed regulatory circuit produced by cell cycle transcriptional regulators based on genome-wide binding data. The genome-wide location data indicate that each group of transcriptional activators regulates activators acting in the next cell cycle stage. The red arrows represent binding of a transcription factor to the promoter of another regulatory factor. The blue arrows represent posttranscriptional regulation.

data also revealed that the set of factors that regulates genes during each phase of the cell cycle also regulates expression of one or more activators involved in the next phase of the cell cycle, forming a fully connected regulatory network (Figure 3B).

The regulatory network we derive from the genomic binding data (Figure 3B) can be described as follows. SBF (Swi4/Swi6) and MBF (Mbp1/Swi6), which are active during late G1, both regulate *NDD1*. Ndd1 protein is a limiting component of the complex that activates G2/M genes; Mcm1 and Fkh2 are bound to promoters throughout the cell cycle, and activation of G2/M genes is dependent on recruitment of Ndd1 (Koranda et al., 2000). The Mcm1/Fkh2/Ndd1 complex regulates *SWI5* and *ACE2*. Swi5, Ace2, and Mcm1 activate M/G1 genes. Mcm1 binds to the *SWI4* promoter and contributes to its activation in M/G1, leading to accumulation of the Swi4 subunit of the SBF transcription factor in G1. All three M/G1 transcription factors regulate *CLN3*, whose protein product forms a complex with Cdc28, which in turn activates SBF and MBF during late G1 (Dirick et al., 1995). *Swi4* transcription is further regulated in late G1 by both SBF and MBF. Thus, the serial regulation of cell cycle regulators occurs throughout the cycle, forming a fully connected regulatory network that is itself a cycle.

Cyclin/CDK Gene Regulation

The transition between stages of the cell cycle is associated with oscillations in the activity of Cdc28-cyclin complexes; cyclin synthesis is necessary for phase entry, and CDK-cyclin inhibition/degradation is necessary for phase exit (Morgan, 1997). The G1 and S cyclins Cln1, Cln2, Clb5, and Clb6 accumulate and associate with Cdc28 in late G1, and cyclins Clb1–Clb4 accumulate and associate with Cdc28 in G2 and M (Nasmyth, 1996). These cyclin-CDK complexes can be inhibited by specific cyclin-CDK inhibitors such as Sic1 and Far1 (Mendenhall and Hodge, 1998), or can be targeted for degradation by, for example, the anaphase promoting complex (APC) (King et al., 1996).

Previous studies identified the transcriptional regulators for most cyclin genes (Figure 4A). SBF and MBF control trancription of G1 and S cyclin genes (Iyer et al., 2001; Koch and Nasmyth, 1994). SBF also participates in the regulation of *CLB1* and *CLB2* (Iyer et al., 2001). The Mcm1/Fkh2/Ndd1 complex regulates the *CLB2* gene in G2/M (Koranda et al., 2000; Kumar et al., 2000; Pic et al., 2000; Zhu et al., 2000), and Mcm1 regulates transcription of *CLN3* in M/G1 (Mackay et al., 2001; McInerny et al., 1997). Our results confirm these observations and reveal that Fkh1 binds the *CLB4* promoter. The additional target genes we find to be bound by the cell cycle transcriptional regulators reveal that transcriptional regulation is more involved in cell cycle progression than previously reported. Transcription factors that regulate cyclin genes during each phase of the cell cycle also regulate genes encoding key components involved in transitioning to the next stage of the cycle (Figure 4B).

The location analysis indicates that SBF and MBF control transcription of G1/S cyclin genes, but also regulate expression of the G2/M cyclin Clb2, which inhibits further expression of the G1/S cyclins Cln1 and Cln2 (Amon et al., 1993) and promotes entry into mitosis (Surana et al., 1991). SBF and MBF also regulate the transcription of the transcription factor Ndd1, which also binds the *CLB2* promoter. Thus, SBF, MBF, and Ndd1 ultimately collaborate to regulate transcription of the

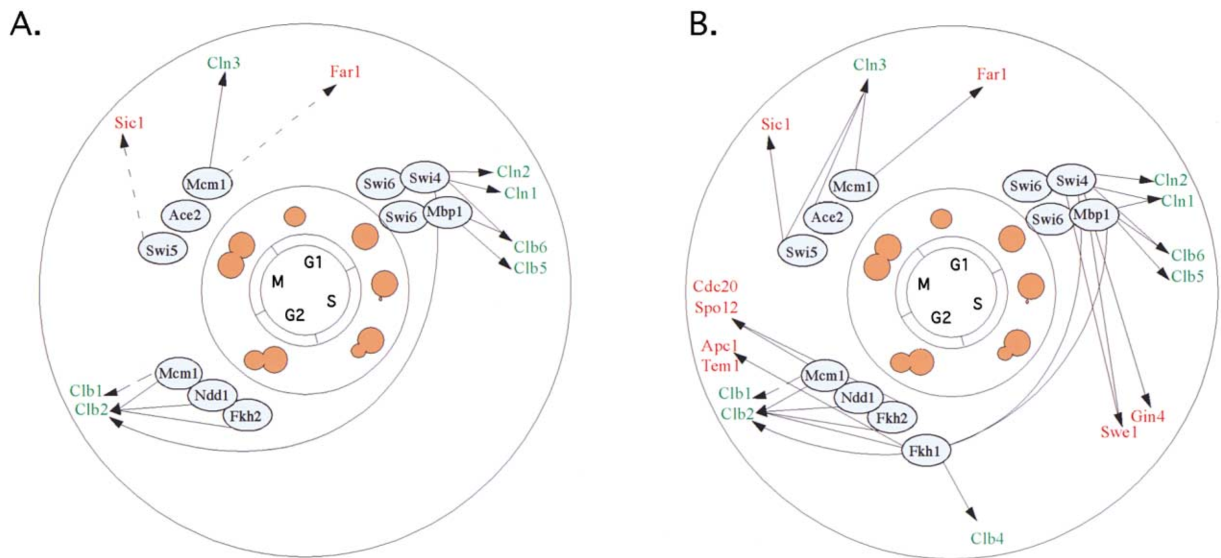

Figure 4. Transcriptional Regulation of Cyclin and Cyclin/CDK Regulator Genes

(A) Summary of previous evidence for transcriptional regulation of genes encoding the cyclins (green) and cyclin/CDK regulators (red) by the cell cycle transcription factors (Althoefer et al., 1995; Dirick et al., 1992; Hollenhorst et al., 2000; Iyer et al., 2001; Knapp et al., 1996; Koch et al., 1993, 1996; Koranda et al., 2000; Kumar et al., 2000; Kuo and Grayhack, 1994; Loy et al., 1999; Mackay et al., 2001; McBride et al., 1999; McInerny et al., 1997; Nasmyth and Dirick, 1991; Oehlen et al., 1996; Ogas et al., 1991; Partridge et al., 1997; Pic et al., 2000; Schwob and Nasmyth, 1993; Toyn et al., 1997; Zhu et al., 2000). The factors, as well as their targets, are positioned according to their approximate time of function. The relationships between the transcription factors and their target genes are indicated by arrows, solid lines represent evidence for direct regulation by these factors, and dashed lines represent inferences from indirect evidence.

(B) Model for transcriptional regulation of cyclin and cyclin/CDK regulators based on previous studies and on genome-wide binding data. Each group of transcription factors regulates key cell cycle regulators that are needed for progression through the cell cycle.

CLB2 gene. SBF and MBF therefore regulate genes necessary for the transition through G1/S, as well as genes whose products set the stage for further progression through the cell cycle.

The data also reveal that the G2/M activators (Mcm1/Fkh2/Ndd1) bind genes whose expression is necessary for both entry into and exit from mitosis. The G2/M activators bind and regulate transcription of *CLB2*, whose product is necessary to enter mitosis (Surana et al., 1991). They also set the stage for exit from mitosis by regulating the gene encoding Cdc20, an activator of the APC, which targets the APC to degrade Pds1 and thus initiate chromosome separation (Visintin et al., 1997). Cdc20-activated APC also degrades Clb5 (Shirayama et al., 1999) and thus enables Cdc14 to promote the transcription and activation of Sic1 (Shirayama et al., 1999) and to initiate the degradation of Clb2 (Jaspersen et al., 1998; Visintin et al., 1998). In addition, the G2/M activators Mcm1/Fkh2/Ndd1 regulate transcription of *SPO12*, which encodes a protein that also regulates mitotic exit (Grether and Herskowitz, 1999).

The M/G1 transcriptional regulators (Mcm1, Ace2, and Swi5) bind genes that are key to entering and progressing through G1. Swi5 binds to the *SIC1* promoter, and all three transcriptional regulators bind to the *CLN3* promoter. Sic1 inhibits Clb-Cdc28 during mitosis (Toyn et al., 1997), thus facilitating exit from mitosis. Cln3-Cdc28 activates SBF and MBF in late G1 (Dirick et al., 1995), thus setting the stage for another cell cycle circuit. In summary, knowledge of the global set of cyclin and CDK regulatory genes that are bound by each of the transcriptional activators provides a much enriched model to explain how transcriptional regulation contributes to cell cycle progression (Figure 4B).

Regulation of Stage-Specific Functions

The genomic location data revealed how specific factors regulate genes associated with stage-specific cell cycle functions (Figure 5). SBF regulates genes involved in the morphological changes associated with cell budding, and MBF controls genes involved in DNA replication and repair, confirming a previous study (Iyer et al., 2001). SBF is also bound to the promoters of several histone genes (*HTA1*, *HTA2*, *HTA3*, *HTB1*, *HTB2*, and *HHO1*), which makes it likely that SBF contributes to the increase in histone gene transcription observed at S phase. Fkh1 was found to bind various genes that encode proteins associated with chromatin structure and its regulation; these include histones (*HHF1* and *HHT1*), telomere length regulators (*TEL2* and *CTF18*), a shared component of the chromatin remodeling complexes Swi/Snf and RSC (*ARP7*), and a histone deacetylase (*HOS3*). The G2/M activators (Mcm1/Fkh2/Ndd1) bind genes that regulate the transition through mitosis (*SWI5*, *ACE2*, *CLB2*, *CDC20*, and *SPO12*). Ace2 and Swi5 regulate genes involved in cytokinesis (*CTS1* and *EGT2*), whereas Mcm1 (apparently in the absence of Fkh1, Fkh2, and Ndd1) regulates genes encoding proteins involved in prereplication complex formation (*MCM3*, *MCM5/CDC46*, *MCM6*, and *CDC6*) and in mating (*STE2*, *STE6*, *FAR1*, *MFA1*, *MFA2*, *AGA1*, and *AGA2*). A summary of binding data for each of the transcriptional regulators is presented in Table 1.

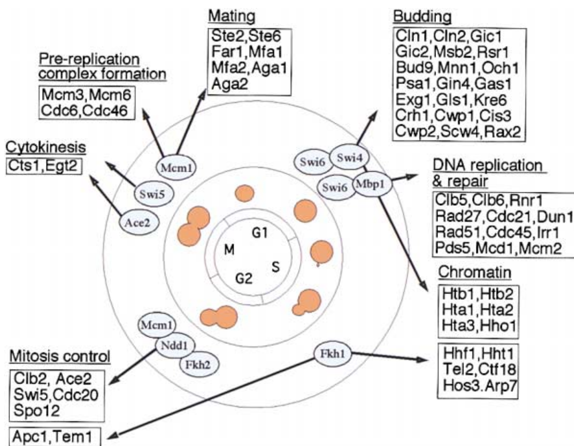

Figure 5. Regulation of Cell Cycle Functions by the Activators

Stage-specific cell cycle functions under the control of specific factors are shown. The budding category includes genes involved in budding and in cell wall biogenesis; the DNA replication category includes genes involved in replication, repair, and sister chromatid cohesion; the chromatin category includes genes encoding histones, chromatin modifiers, and telomere length regulators. The identity and function of genes in each category are listed in Table 1.

Functional Redundancy

The factor location data demonstrate that each of the nine cell cycle transcription factors binds to critical cell cycle genes, yet cells with a single deletion of *MBP1*, *SWI4*, *SWI6*, *FKH1*, *FKH2 ACE2*, or *SWI5* are viable; only *MCM1* and *NDD1* are essential for yeast cell survival (Breeden, 2000; Loy et al., 1999; Mendenhall and Hodge, 1998). The conventional explanation for this observation is that each nonessential gene product shares its function with another. Swi4 and Mbp1 share 50% identity in their DNA binding domains (Koch et al., 1993). Similarly, Fkh1 and Fkh2 are 72% identical (Kumar et al., 2000), and Swi5 and Ace2 are 83% identical in their respective DNA binding domains (McBride et al., 1999). Each of these pairs of proteins recognizes similar DNA motifs, so it is possible that functional redundancy rescues cells with mutations in individual factors. However, it was not clear whether each of the pairs of factors had truly redundant functions in normal cells, or whether they exhibit redundant function only in mutant cells that lack the other factor.

Our data demonstrate that each of the cell cycle factor pairs discussed above does bind overlapping sets of genes in wild-type cells, revealing that the two members of each of the pairs are partially redundant in normal cell populations (Figure 6). Mbp1 and Swi4 share 34% of their target genes, Fkh1 and Fkh2 share 22%, and Ace2 and Swi5 share 25%. It is also clear, however, that this redundancy does not apply to all genes regulated by a pair of related activators in wild-type cells. The partial overlap in genes under the control of pairs of regulators explains why one gene of a pair can rescue defects in the other, yet each member of the pair can be responsible for distinct functions in wild-type cells.

Discussion

Identification of the transcriptional regulatory network that controls the cell cycle clock is essential to fully understand how cell cycle control is effected. We have now identified the genomic targets of each of the nine known yeast cell cycle regulators by using a combination of genome-wide location and expression analysis. The investigation revealed that a connected, circular transcriptional regulatory network has evolved to control the cell cycle, and showed how each of the transcriptional regulators contributes to diverse stage-specific functions.

Cell Cycle Transcriptional Regulatory Network

A key concept that emerged from this study is that cell cycle transcriptional control is effected by a connected regulatory network of transcriptional activators. The cell cycle transcriptional regulators that function during one stage of the cell cycle regulate the transcriptional regulators that function during the next stage, and this serial regulation of transcriptional regulators forms a complete regulatory circuit. Thus, the transcriptional regulatory network that controls the cell cycle is itself a cycle of regulators regulating regulators. The discovery of this connected transcriptional regulatory network is important for several reasons. It provides additional understanding of the regulatory mechanisms by which cells ensure transitions from one stage into the appropriate next stage. It supplies the foundation for future work on the mechanisms that coordinate gene expression and other aspects of cell cycle regulation. Furthermore, it suggests that a connected, circular transcriptional regulatory network may also be a fundamental feature of cell cycle regulation in other, more complex, organisms.

It is interesting to consider why cells have pairs of cell cycle transcriptional regulators with partially redundant functions. This configuration may help ensure that the cell cycle is completed efficiently, which is critical since the inability to complete the cycle leads to death. At the same time, devoting each of the pair to distinct functional groups of genes enables coordinate regulation of those functions. It may also be that partial redundancy helps the cell to make a smoother temporal transition from one mode of operation to another during the cell cycle.

Our results identify how the cyclin genes are regulated by the nine transcriptional activators. In addition, the results reveal that transcription factors that regulate the cyclin genes during each phase of the cell cycle also regulate genes that are involved in transitioning to the next stage of the cycle (Figure 4). For example, the G1/S activators SBF and MBF control transcription of G1/S cyclin genes, but also regulate expression of the G2/M cyclin Clb2, which subsequently inhibits further expression of the G1/S cyclins Cln1 and Cln2 and promotes entry into mitosis. Thus, the cell cycle transcriptional regulatory network has evolved so that some transcriptional regulators contribute to the control of both stage entry and exit.

The identification of sets of genes that are bound by each of these regulators reveals how coordinate regulation of a wide variety of stage-specific cell cycle functions is regulated (Figure 5). For example, the G1/S activators regulate genes involved in cell budding, DNA replication and repair, and chromosome maintenance. The G2/M activators bind genes that regulate the transi-

Table 1. Selected Targets of the Cell Cycle Activators

	Gene	SBF	MBF	Fkh1	Fkh2	Mcm1/ Fkh2/ Ndd1	Mcm1	Ace2	Swi5	Short description
Cell cycle control	PCL9								+	Cyclin that associates with Pho85p
	CDC6	+	+				+			Protein that regulates initiation of DNA replication
	SIC1								+	P40 inhibitor of Cdc28p-Clb protein kinase complex
	SWI4	+	+				+			Transcription factor that participates in the SBF complex
	PCL2	+						+	+	Cyclin, found partly in association with Pho85p
	CLB6	+	+		+					B-type cyclin appearing late in G1
	CLB5		+							B-type cyclin appearing late in G1
	SWE1	+	+							Serine/tyrosine dual-specificity protein kinase
	PCL1	+	+		+		+			G1/S-specific cyclin
	CLN2	+								G1/S-specific cyclin
	CLN1	+	+	+	+					G1/S-specific cyclin
	OPY2		+							Protein that may be involved in cell-cycle regulation
	NDD1	+								Protein required for nuclear division
	CLB4			+						G2/M-phase-specific cyclin
	SIM1	+			+		+			Protein involved in the aging process and in cell cycle regulation
	PCL7								+	Cyclin, associates with Pho85p
	HSL7				+					Negative regulatory protein of the Swe1p protein kinase
	APC1			+/−						Component of the anaphase-promoting complex (APC)
	ACE2			+	+	+				Metallothionein expression activator with similarity to Swi5p
	CLB2	+		+	+	+				G2/M-phase-specific cyclin
	SWI5				+	+				Transcription factor that controls cell cycle-specific transcription of HO
	HDR1			+						Protein involved in meiotic segregation
	TEM1			+	+					GTP-binding protein of the ras superfamily involved in termination of M-phase
	CDC20				+	+				Protein required for microtubule function at mitosis
	SPO12		+		+	+				Sporulation protein required for chromosome division in meiosis I
	CLN3						+	+	+/−	G1/S-specific cyclin
	DBF2						+			Serine/threonine protein kinase related to Dbf20p
	FAR1						+			Inhibitor of Cdc28p-Cln1p and Cdc28p-Cln2p kinase complexes
Cell wall biogenesis, budding, and cytokinesis	CHS1								+	Chitin synthase I
	TEC1								+	Transcriptional activator
	EGT2							+	+	Cell-cycle regulation protein, may be involved in cytokinesis
	GIC2	+	+							Putative effector of Cdc42p, important for bud emergence
	SCW11							+	+	Putative cell wall protein
	GIN4	+					+			Serine/threonine-protein kinase
	BUD9	+		+			+	+	+	Protein required for bipolar budding
	OCH1	+								Alpha-1,6-mannosyltransferase
	CTS1				+			+	+	Endochitinase
	RSR1	+								GTP-binding protein of the ras superfamily involved in bud site selection
	CRH1	+	+						+	Protein for which overproduction suppresses bud emergence defects
	MSB2	+								Cell wall protein
	MNN1	+								Exo-beta-1,3-glucanase (I/II)
	EXG1	+	+	+	+			+	+	Alpha-1,3-mannosyltransferase
	GLS1	+								Component of beta-1,3-glucan synthase

(*continued*)

Table 1. Continued

	Gene	SBF	MBF	Fkh1	Fkh2	Mcm1/ Fkh2/ Ndd1	Mcm1	Ace2	Swi5	Short description
Cell wall biogenesis, budding, and cytokinesis	GAS1	+								Glycophospholipid-anchored surface glycoprotein
	PSA1	+						+		Mannose-1-phosphate guanyltransferase
	KRE6	+								Glucan synthase subunit required for synthesis of beta-1,6-glucan
	GIC1	+			+					Putative effector of Cdc42p, important for bud emergence
	CWP1	+			+					Mannoprotein of the cell wall; member of the PAU1 family
	CIS3	+			+					Cell wall protein
	CWP2	+	+	+	+			+		Protein that controls interaction of bud-neck cytoskeleton with G2 nucleus
	BUD4			+	+	+				Protein required for axial budding but not for bipolar budding
	WSC4							+		Protein required for maintenence of cell wall integrity
	BUD8			+						Protein required for bipolar budding
	SCW4	+								Cell wall protein; similar to gulcanases
	RAX2	+			+	+				Protein involved in bipolar budding
	SKN1						+			Glucan synthase subunit
DNA replication	RNR1	+	+		+					Ribonucleotide reductase large subunit
	RAD27		+							Single-stranded DNA endonuclease and 5′-3′ exonuclease
	CDC21		+							Thymidylate synthase, converts dUMP to dTMP
	IRR1		+							Component of cohesin complex
	MCD1		+							Cohesin, protein required for mitotic chromatid cohesion
	PDS5		+	+	+					Protein required for sister chromatid cohesion
	RAD51		+		+					Protein that stimulates pairing and strand-exchange between homologous
	DUN1		+							Protein kinase required for induction of DNA repair genes after DNA damage
	ALK1						+			DNA damage-responsive protein
Chromatin	CTF18			+						Protein required for maintenance of normal telomere length
	HHF1			+/−						Histone H4, identical to Hhf2p
	HHT1			+/−						Histone H3, identical to Hht2p
	HTB2	+								Histone H2B, nearly identical to Htb1p
	HTB1	+								Histone H2B
	HTA1	+								Histone H2A, identical to Hta2p
	HTA2	+								Histone H2A, identical to Hta1p
	HHO1	+								Histone H1
	TEL2			+						Protein involved in controlling telomere length and telomere position effect
	ARP7			+						Component of SWI-SNF and RSC chromatin remodeling complex
	HTA3	+								Histone-related protein that can suppress histone H4 point mutation
	HOS3			+						Protein with similarity to Hda1p, Rpd3p, Hos2p, and Hos1p
Prereplication complex	MCM3						+			Protein that acts at ARS elements to initiate replication
	CDC6	+	+				+			Protein that regulates initiation of DNA replication
	CDC46						+			Protein that acts at ARS elements to initiate replication
	CDC45		+							Protein required for initiation of chromosomal DNA replication
	MCM2		+							Protein that acts at ARS elements to initiate replication

(*continued*)

Table 1. Continued

	Gene	SBF	MBF	Fkh1	Fkh2	Mcm1/ Fkh2/ Ndd1	Mcm1	Ace2	Swi5	Short description
Prereplication complex	MCM6						+			Protein involved in DNA replication; member of the MCM/P1 family of proteins
Mating	ASH1								+	GATA-type transcription factor, negative regulator of HO expression
	AGA2						+			a-Agglutinin binding subunit
	AGA1	+	+				+			a-Agglutinin anchor subunit
	HO	+								Homothallic switching endonuclease
	MFA1						+			Mating pheromone a-factor; exported from cell by Ste6p
	MFA2						+		+	Mating pheromone a-factor; exported from cell by Ste6p
	STE6						+			Membrane transporter responsible for export of “a” factor mating pheromone
	STE2						+			Pheromone alpha-factor receptor; has seven transmembrane segments
	FAR1						+			Inhibitor of Cdc28p-Cln1p and Cdc28p-Cln2p kinase complexes

A partial list of cell cycle genes whose promoter regions were bound by the indicated cell cycle regulators. + indicates binding with $P < 0.001$, +/− indicates binding with $P < 0.0015$. A full list of target genes is available at the author’s web site (http://web.wi.mit.edu/young/cellcycle). The DNA replication category includes genes that function in DNA synthesis, in DNA repair and in sister chromatid cohesion.

tion through mitosis. The late M factors regulate genes involved in cytokinesis and prereplication complex formation.

A more comprehensive picture of cell cycle regulation emerges when existing knowledge of cell cycle regulatory mechanisms is combined with the new information on the transcriptional regulatory network. Several key features of this integrated view have important implications for cell cycle regulation. Cells commit to a new cell cycle at START, but only after cell growth is sufficient to ensure completion of the cycle, since the inability to complete the cell cycle can be lethal (Mendenhall and Hodge, 1998). The emphasis on regulation at the G1/S boundary is evident from the regulatory events involving Swi4 in the model shown in Figure 3B. The Swi4 regulator becomes functionally active at START, via a mechanism that is dependent on Cln3-Cdc28, when the cell reaches a critical size (Dirick et al., 1995). The *SWI4* promoter is bound by Swi4 itself, suggesting that a positive feedback loop exists to ensure that adequate levels of Swi4, and thus SBF, are present prior to commitment. The observation that the G1/S regulators SBF and MBF both regulate *NDD1* suggests how adequate levels of Ndd1 are produced to initiate the G2/M transcriptional program. Ndd1 protein is a limiting component of the complex that activates G2/M genes; Mcm1 and Fkh2 are bound to promoters throughout the cell cycle, and activation of G2/M genes is dependent on recruitment of Ndd1 (Koranda et al., 2000). The Mcm1/Fkh2/Ndd1 complex regulates *SWI5* and *ACE2*, whose products become functional only in late anaphase after relocalization to the nucleus in a mechanism that is dependent on low Clb-Cdc28 activity (Nasmyth et al., 1990; Shirayama et al., 1999). Later in the cell cycle, the Swi5, Ace2, and Mcm1 factors all bind to the *CLN3* promoter, thus assuring adequate levels of the Cln3 cyclin at START.

The cell cycle trancriptional regulatory network model may account for several observations relevant to cell

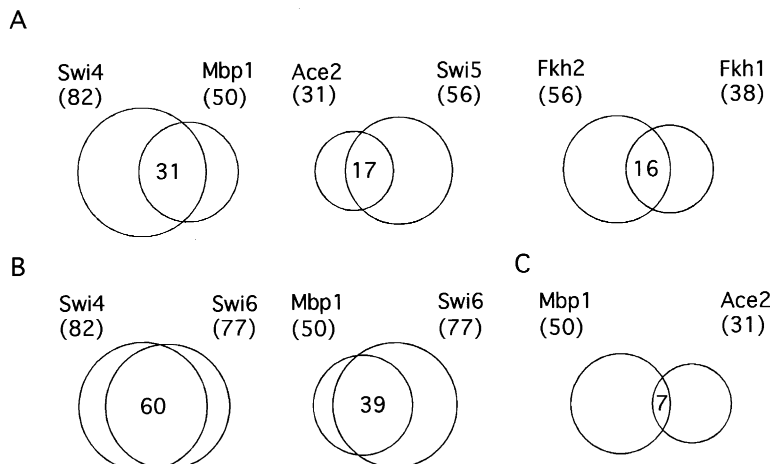

Figure 6. Partial Redundancy between Homologous Activators

(A) Venn diagrams depicting the overlap between the targets of pairs of homologous cell cycle transcriptional regulatory proteins. The numbers in parentheses under each activator represent the sum of cell cycle genes whose promoters were bound by the protein. The number in the intersection between two circles reflects the number of genes whose promoters were bound by both proteins.

(B) Venn diagrams representing the overlap in target sites between pairs of regulatory proteins that reside within the same complex.

(C) A Venn diagram representing the overlap in target sites between two transcriptional regulators that are not known to be related.

cycle regulation. The use of multiple transcription factors to regulate key transcription and cyclin regulators explains why mutations in single transcription factors generally have only limited effects on progression through the cell cycle, whereas mutations in activator pairs can have substantial effects (Breeden, 2000; Koch et al., 1993; Mendenhall and Hodge, 1998). Nutrient limitation causes yeast cells to arrest cell cycle progression, but rather than occurring at the time of nutrient limitation, the arrest is delayed until the cells reach G1 (Mendenhall and Hodge, 1998). Cells that have entered the cell cycle at START may progress through an entire cycle because of the design of the connected transcriptional regulatory network (Figure 3B), and perhaps then arrest in G1 because of the requirement for adequate levels of Cln3/Cdc28. Several cell cycle checkpoint controls are mediated by regulation of Cdc28 activity (Mendenhall and Hodge, 1998), but how Cdc28 activity affects the transcription program is not well understood. Since the activity of several of the cell cycle transcriptional regulators is dependent on Cdc28 activity, some checkpoint controls may effect arrest by perturbing the connected transcriptional regulatory circuit.

Importance of Direct Binding Information

An impetus for the development of methods that identify the genomic binding sites of factors in vivo was the realization that regulatory networks cannot be accurately deduced from global expression profiles because it is not possible to discriminate between direct and indirect effects due to genetic or other perturbations in living cells (Ren et al., 2000; Iyer et al., 2001). A further challenge for understanding global gene regulation is that comparisons of wild-type and mutant expression profiles produce valuable information on dependencies when the mutant gene is essential, but it is more difficult to interpret such information when the mutant gene can be rescued by functionally redundant gene products. We found that the direct binding data obtained in the present study was remarkably confirming of previous evidence for gene regulation by specific transcription factors when that evidence was direct. In contrast, we did not obtain evidence in support of many studies in which the involvement of a factor in the regulation of a gene was deduced from indirect evidence (Althoefer et al., 1995; Gordon and Campbell, 1991; Igual et al., 1996; Koch et al., 1993; Lowndes et al., 1991; Piatti et al., 1995; Pizzagalli et al., 1988; Toone et al., 1995; Verma et al., 1991).

The identification of the set of promoters bound in vivo by each of the cell cycle regulators allowed us to identify consensus binding sequence motifs (for a table containing these motifs, see the authors' website at http://web.wi.mit.edu/young/cellcycle). Two general insights emerged from this analysis. First, the binding motifs identified for some factors are found in most, but not all, of the promoters that they bind, suggesting that variations of the consensus sequence exist that are not easily recognized by search algorithms or that the transcription factor is modified or associated with binding partners that generate a new binding preference at some genes. In this context, it is interesting that the Mcm1 binding motif is somewhat different in the promoters of its G2/M targets than in its M/G1 targets, probably reflecting the influence of its binding partners. Second, the presence of the DNA binding motif in genomic DNA is not by itself a predictor of protein binding in vivo, as the predicted motifs are found at many sites in the genome other than those bound in vivo. There is, therefore, a need for empirical binding data such as that described here in order to accurately identify genuine binding sites.

Discovering Genetic Regulatory Networks

Understanding how biological processes are regulated on a genomic scale is a fundamental problem for the coming decades. Maps of metabolic pathways have been key to studying basic biology, uncovering disease mechanisms, and discovering new drugs over the last century. Maps of genome regulatory networks will almost certainly play an equally important role in future biological discovery.

The location data that we have presented in this paper are well adapted to new computational approaches to discovering genetic regulatory networks. The binding of a transcriptional activator to the promoter region of a gene suggests that the activator has a regulatory effect on the gene. However, it is also possible that the activator does not fully or even partially control the gene. Thus, location information must be fused with other data, such as expression data, to fully elaborate the complete mechanism of transcriptional regulation and the form of regulatory networks. We anticipate that new computational approaches will synergistically combine location data with other data types to form a well-focused picture of cellular function. For example, one way to combine location and expression data is to use the location data to first suggest tentative factor-target pairs with associated p-values. These factor-target pairs represent constraints on the possible genetic regulatory network models, and they can be used to guide the search of network models based on expression data. This process can discover alternative models of regulatory networks, with a principled measure of likelihood assigned to each hypothesis. The likelihood measure appropriately reflects how consistent the hypothesis is with both location and expression data. This likelihood-based approach can accommodate location data, expression data, and other forms of data (Ross-Macdonald et al., 1999; Uetz et al., 2000) that can be usefully employed to assign probabilities to potential interactions.

We have described how genome-wide location and expression analysis can be used to derive information useful for genetic network mapping, and have depicted some of the yeast cell cycle transcriptional regulatory network within figures displayed here. The discovery of additional transcriptional regulatory networks should lead to a network map that connects the cell cycle and other cellular functions.

Experimental Procedures

Tagging and Yeast Strains

The cell cycle activators Swi4, Mbp1, Swi6, Fkh1, Fkh2, Ndd1, Mcm1, and Ace2 were tagged with a multicopy myc epitope by inserting the epitope coding sequence into the normal chromosomal loci of these genes. Vectors developed by Cosma et al. (1999) were used for amplifying a fragment that contains the repeated myc tag

coding sequence flanked by 50 bp from both sides of the stop codon of the gene. The PCR products were transformed into the W303 strain Z1256 (MATa, *ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3*) to generate the tagged strains (Z1335, Z1372, Z1373, Z1448, Z1370, Z1369, Z1321, and Z1371, respectively). Clones were selected for growth on TRP plates, the insertion of the tagged sequence was confirmed by PCR, and expression of the epitope-tagged protein was confirmed by Western blotting using an anti-Myc antibody (9E11). A strain containing a myc-tagged version of Swi5 (Z1407) was obtained from K. Nasmyth.

Genome-wide Location Analysis

We used genome-wide location analysis as described (Ren et al., 2000) to identify genome binding sites for the transcription factors. Briefly, yeast strains containing a myc-tagged version of the protein of interest were grown to mid log phase (OD 0.6–1.0), fixed with 1% formaldehyde for 30 min, harvested, and disrupted by sonication. The DNA fragments crosslinked to the protein were enriched by immunoprecipitation with anti-myc specific monoclonal antibody (9E11), thus obtaining an enrichment of the in vivo binding sites. After reversal of the crosslinks, the enriched DNA was amplified and labeled with a fluorescent dye (Cy5) with the use of a ligation-mediated polymerase chain reaction (LM-PCR). A sample of DNA that was not enriched by immunoprecipitation was subjected to LM-PCR in the presence of a different fluorophore (Cy3), and both immunoprecipitation (IP)-enriched and -unenriched pools of labeled DNA were hybridized to a single DNA microarray containing all yeast intergenic sequences. Microarray design and production was as described in Ren et al. (2000).

Images of Cy3 and Cy5 fluorescence intensities were generated by scanning the arrays using a GSI Lumonics Scanner. The Cy3 and Cy5 images were analyzed using ArrayVision software, which defined the grid of spots and quantified the average intensity of each spot and the surrounding background intensity. The background intensity was subtracted from the spot intensity to give the final calculated spot intensity. The intensity of the two channels was normalized according to the median. For each spot, the ratio of corrected Cy5/Cy3 intensity was computed. Each experiment was carried out in triplicate, and a single-array error model was used to handle noise, to average repeated experiments with appropriate weights, and to rank binding sites by p value as described (Ren et al., 2000). Detailed protocols can be obtained at the authors' website at http://web.wi.mit.edu/young/cellcycle.

The intergenic regions present on the array were assigned to the gene or genes found transcriptionally downstream. Where a single intergenic region contains promoters for two divergently transcribed genes, the intergenic region was assigned to the gene or genes expressed during the cell cycle according to the Spellman et al. (1998) analysis. The Spellman et al. (1998) analysis was chosen because it incorporates all available yeast cell cycle expression data. Promoter regions detected with a p value < 0.001 were included for further analysis.

Statistics

In order to explore the statistical significance of the overlap between the set of targets of a factor and the genes expressed in a particular cell cycle stage, we used the hypergeometric distribution as described (Tavazoie et al., 1999).

Acknowledgments

We are grateful to Pronob Bardhan, Thomas Hinman, and Uwe Muller for encouraging and supporting this study. We thank Steve Bell, Angelika Amon, and Gerry Fink for advice and comments on the manuscript, Kim Nasmyth for reagents, and Ezra Jennings, Joerg Schreiber, and Elenita Kanin for technical assistance. Supported by funds from Corning, Inc., NIH, European Molecular Biology Organization (I.S.), NSF (J.J.W.), and the Human Frontier Science Program (J.Z.).

Received June 13, 2001; Revised August 28, 2001.

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