

Chapter 1

Cell Cycle-Regulated Transcription: Effectively Using a Genomics Toolbox

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Abstract

The cell cycle comprises a series of temporally ordered events that occur sequentially, including DNA replication, centrosome duplication, mitosis, and cytokinesis. What are the regulatory mechanisms that ensure proper timing and coordination of events during the cell cycle? Biochemical and genetic screens have identified a number of cell-cycle regulators, and it was recognized early on that many of the genes encoding cell-cycle regulators, including cyclins, were transcribed only in distinct phases of the cell cycle. Thus, “just in time” expression is likely an important part of the mechanism that maintains the proper temporal order of cell cycle events. New high-throughput technologies for measuring transcript levels have revealed that a large percentage of the *Saccharomyces cerevisiae* transcriptome (~20 %) is cell cycle regulated. Similarly, a substantial fraction of the mammalian transcriptome is cell cycle-regulated. Over the past 25 years, many studies have been undertaken to determine how gene expression is regulated during the cell cycle. In this review, we discuss contemporary models for the control of cell cycle-regulated transcription, and how this transcription program is coordinated with other cell cycle events in *S. cerevisiae*. In addition, we address the genomic approaches and analytical methods that enabled contemporary models of cell cycle transcription. Finally, we address current and future technologies that will aid in further understanding the role of periodic transcription during cell cycle progression.

Key words *Saccharomyces cerevisiae*, Cell cycle, Periodic transcription, Transcription factor network, Cyclins, Cyclin-dependent kinases (CDKs)

1 Introduction

Successful cell division requires complete duplication of genetic material followed by equal segregation into two cell bodies, resulting in two identical daughter cells. Historically, the cell cycle has been divided into four phases—Gap 1 (G1), Synthesis (S), Gap 2 (G2), and Mitosis (M)—that describe the chronological order of different events observed in normally cycling cells. The bulk of duplication and segregation events occur in S and M phases. During S phase, both DNA and centrosomes are duplicated (Fig. 1). Duplicated centrosomes separate in order to form the poles of the mitotic spindle responsible for segregating sister

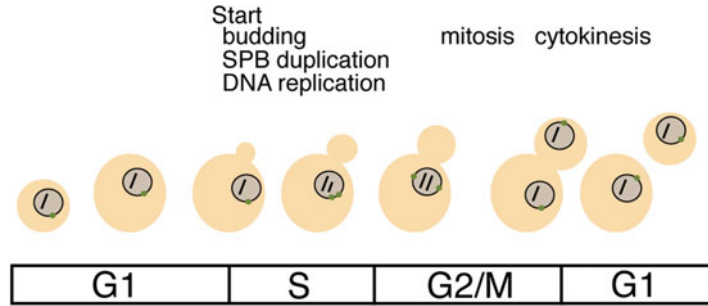


Fig. 1 Cell-cycle progression in *Saccharomyces cerevisiae*. Budding yeast serves as an excellent model system to study the cell cycle. Timing and regulation of events are conserved across species. More importantly, the phase of the cell cycle can be deduced by observing the state and size of the bud, the future daughter cell

chromatids (Fig. 1). Sister chromatid segregation is initiated during M phase, or mitosis (Fig. 1).

G1 and G2 were termed “gap” phases, as they separate the visibly observable events of S and M phase. Although no overt cellular changes or events are observed during G1 and G2, cells are interpreting signals from their extracellular and intracellular environments to ensure that conditions are appropriate for cellular division events. In early G1, cells interpret extracellular signals (e.g., nutrient abundance, mating pheromone) to decide whether to commit to a new cell cycle. Following this point of commitment (called *START* in *Saccharomyces cerevisiae*), cells prepare for entry into S phase by activating the expression of genes required for duplicating DNA and centrosomes (Fig. 1). In G2, cells interpret intracellular signals from checkpoint pathways that monitor whether duplication events have been completed with fidelity, and whether the mitotic spindle apparatus is functional.

Each cell cycle event—such as DNA replication, centrosome duplication, and chromosome segregation—is a complex process that requires the coordination of many different proteins acting together to complete the task at hand. In turn, each of these complex events must be coordinately controlled with the other events. What, then, are the mechanisms that orchestrate the complex set of events required for cellular division? Over the past three decades, an overwhelming number of studies have identified and characterized two proteins that act in a complex to trigger cell cycle events: cyclins and cyclin-dependent kinases (CDKs). Both biochemical and genetic approaches have shown that, throughout the cell cycle, CDKs are activated by different cyclins, whose role is to activate and inhibit different events at the proper time and in the proper order (reviewed in [1–3]). More recently, it has been shown that up to a fifth of the *S. cerevisiae* genome, including cyclins themselves,

is transcribed once per cell cycle [4–7]. This set of genes is often referred to as the cell cycle-regulated transcriptional program. The precise nature of this temporal transcriptional program may serve as another mechanism to ensure proper timing and ordering of cell cycle events.

Historical models of cell-cycle regulation have proposed that cyclin/CDK activity directs transcription factors to initiate gene expression at the proper time [8] (reviewed in [9–11]). However, recent studies have shown that cell cycle-regulated transcription has the capacity to occur largely in the absence of CDKs [5, 12]. These findings suggest that some other regulatory mechanism is responsible for controlling periodic transcription and coordinating cell cycle events. To better understand and characterize this control module, single gene studies have given way to genome-wide experimental approaches that measure global gene expression dynamics [4–7]. These studies require unbiased quantitative analyses tailored both to the experimental method and to the overarching biological question. Here, we will discuss both current and future experimental and analytical methods used to address the seemingly simple questions: What portion of the genome is cell cycle-regulated? And how is this transcription program coordinated with other cell cycle events?

2 Cell Cycle-Regulated Transcription

2.1 Identifying Periodic Transcripts

With the advent of modern molecular biology, measuring mRNA levels in cells became a regular test to address whether genes are regulated at the transcriptional level. For genes involved in the cell cycle, understanding gene regulation at the transcriptional level requires measuring mRNA abundance over time in synchronous populations of cells as they progress through the cell cycle. Histones were the first genes identified whose expression oscillates periodically during the cell cycle [13]. Classifying histone gene expression as cell cycle-regulated was done by correlating the timing of histone mRNA expression with the timing of DNA replication over the course of several cell cycles [13]. Over the following decade, ten more genes involved in cell cycle events were also identified as being expressed in a periodic manner—*HO* [14], *CDC21* [15], *CDC9* [16], *RAD6* [17], *SWI5* [18], *CDC8* [19], *POLI* [20], *DBF4* [21], *PRII* [22], and *DBF2* [23]. For each of these genes, the definition of periodic is anchored to the correlation of gene expression with an observable cell cycle event that is known to occur only once per cycle. The periodic expression of these genes was discovered while investigating the function of each gene during cell cycle events. Is periodic expression of cell cycle genes a global phenomenon or specific to just a small set of genes? In total, approximately 100 periodically expressed budding yeast genes were identified one

at a time using northern blots. However, a technology that is able to measure transcript dynamics at a genome-wide level was necessary not only to quantify the proportion of genes that is periodically transcribed but also to understand how cell cycle-regulated transcription is coordinated with cell cycle progression.

The microarray is one method developed to measure mRNA levels of many genes in an organism [24]. Several genome-wide studies utilizing microarrays have been reported that focus on identifying periodic genes with respect to the cell cycle in synchronized populations of budding yeast cells. Cho and colleagues identified 416 genes as being cell cycle-regulated at the transcriptional level by visual inspection of transcript dynamics [4]. Spellman and colleagues identified 800 genes that demonstrate oscillations in transcript levels during the cell cycle using quantitative methods including a Fourier transform and Pearson correlation [7]. Pramila and colleagues found 991 cell cycle-regulated transcripts using a permutation-based method developed by de Lichtenberg and colleagues [6, 25]. Orlando and colleagues identified 1,275 periodically expressed genes also using a permutation-based method [5, 25]. Overall, between the three studies using quantitative methods to identify periodic genes, 440 cell cycle-regulated genes are shared [5–7].

Although each study identifies slightly different sets of periodic genes, it is clear that many more genes are regulated at the transcriptional level during the cell cycle than previously thought. Differences between periodic gene lists from each study result from a combination of experimental design and quantitative analysis. Further discussion of these differences is addressed in a later section.

Is the phenomenon of cell cycle-regulated transcription specific only to budding yeast? Additional studies in fission yeast and human cells have measured gene expression dynamics in synchronized cells to determine the scope of periodic transcription in these organisms. In fission yeast, three genome-wide studies identified a limited number of periodic genes [26–28]. Unlike budding yeast, fewer genes were classified as cell cycle-regulated; less than 800 genes were identified by each study. Similar to budding yeast, the consensus between studies is very low, with only 171 genes shared between all analyses [26–28]. Two studies in human cell lines have classified fewer than 1,000 periodic genes [29, 30]. The low numbers of periodic genes may be due to the larger genome size, undetected alternative splicing of introns, or the difficulty involved in synchronizing fission yeast or human cell lines. While budding yeast has the largest number of identified periodic genes, cell cycle-regulated transcription is also clearly observed in fission yeast and human cell lines, suggesting that this phenomenon is conserved between organisms. Moreover, the transcriptional regulation of some orthologs in the evolutionarily diverged yeast species *S. cerevisiae* and *S. pombe* have been shown to be conserved [31]. With improved experimental approaches and mRNA measuring technology, the

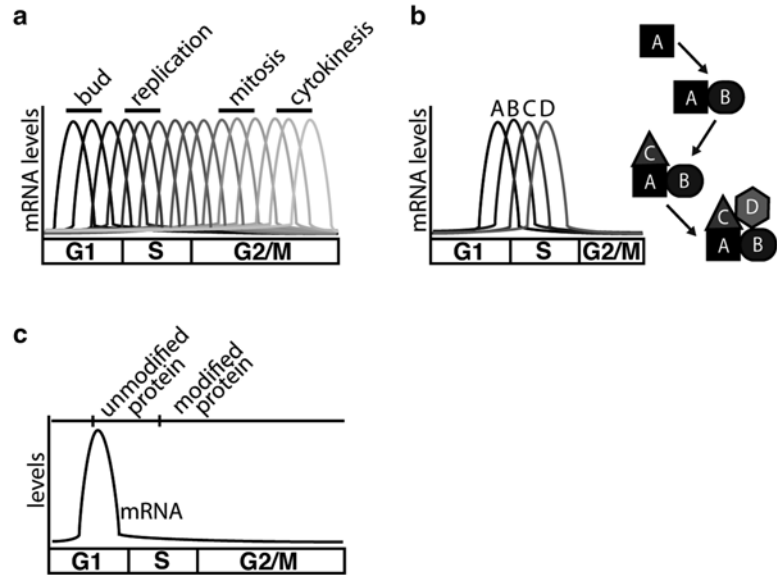


Fig. 2 Significance of the periodic transcription program. **(a)** Genes are expressed only during the cell-cycle phase needed. Genes required for DNA replication are expressed during S phase. **(b)** The temporal order of gene expression may aid in the construction of a protein complex only needed once per cycle. **(c)** While protein levels of cell-cycle regulators may remain constant, posttranslational modifications may alter the activity of the proteins

characterization of periodic gene expression will become more tractable in other model systems.

Two questions arise from the finding that the transcriptional program is conserved across eukaryotes: (1) what is the significance of cell cycle-regulated transcription and (2) what mechanisms coordinate this large transcriptional program with cell cycle progression?

2.2 Significance of Cell Cycle-Regulated Transcription

Many hypotheses have been posed to explain the importance of cell cycle-regulated transcription. The explanations can be generalized into three categories. While all are plausible reasons for regulating gene expression timing during the cell cycle, it is not currently possible to discriminate between the multiple hypotheses. Moreover, each potential hypothesis is not mutually exclusive and may be true for only a subset of cell cycle-regulated genes.

The first category postulates that cell cycle-regulated transcription is a mechanism to expend energy resources efficiently, as transcription and translation are energetically expensive. This concept is often referred to as “just in time” transcription, in which gene products that function at a specific cell cycle interval are expressed only when needed (Fig. 2a) (reviewed in [10, 32, 33]). A variation on this first explanation has been referred to as the “Sleeping Beauty” situation, which takes into account the full lifetime of a

cell or tissue, rather than the completion of a single cell cycle ([7], reviewed in [9]). Whether it is a single budding yeast or a population of cells that form tissues in an organism, active cellular division occurs during only a portion of a cell's overall life cycle. Microorganisms are subject to environmental constraints and will divide when conditions (nutrients, temperature, growth factor signaling, etc.) permit, but not when the local environment is not amenable to cell division. Therefore, much of the life of a single cell is spent outside the cell cycle, in a state of rest or quiescence. However, once a signal is received to initiate cellular division, the cells are poised to complete cell cycle events with the proper genes expressed at the correct time.

A second proposal for the importance of cell cycle-regulated transcription centers on building a required structure only once per cell cycle (Fig. 2b) (reviewed in [32, 33]). For example, proteins required for DNA replication are loaded onto DNA in different stages. The components of the replication complex are periodically transcribed themselves, lending to the temporal events that are required for DNA replication. A pre-initiation complex first binds to DNA replication origins and is only activated when elements are phosphorylated by S-phase cyclin/CDK. Other components required for replication are then synthesized, recruited to origins, and replicate DNA (reviewed in [34]). Further, mitotic cyclin/CDK activity inhibits the reformation of pre-initiation complexes until the following cell cycle [35]. This mechanism ensures that the complex required to trigger DNA replication is only built once and thus acts as a layer of control to prevent re-replication.

A third category of hypotheses centers on renewing pools of unmodified protein. Gene products that are posttranslationally modified may no longer be active or be responsive to additional signaling. Therefore, periodic transcription provides a pool of unmodified product that is able to carry out cell cycle events (Fig. 2c) (reviewed in [9]). For example, Swi6, a component of transcription factor complexes SBF and MBF, is phosphorylated in S phase after START to localize it to the cytoplasm [36]. Periodic transcription of *SWI6* may provide a new pool of the Swi6 protein to induce transcription at START.

Despite varying hypotheses on the physiological importance of the cell cycle-regulated transcriptional program, the underlying requirement for proper expression timing during the cell cycle has led to the development of a sophisticated program for cell cycle transcription control. Additionally, transcriptional regulation of these genes may represent only a single layer of control; post-translational modifications such as cyclin/CDK phosphorylation have also been shown to play a critical role in proper coordination of cell cycle events. Understanding how periodic transcription is

Table 1
Transcription factors that are known to play a role in activating or repressing periodic transcription during the cell cycle

TF	Phase	Function	Representative target	CDK target?	CDK regulation
SBF	G1/S	Activator	<i>CLN1</i>	Yes [52]	Inhibitory
MBF	G1/S	Activator	<i>POL1</i>	Yes [98]	Unknown
Yhp1	G1/S	Repressor	<i>CLN3</i>	No	N/A
Yox1	G1/S	Repressor	<i>SWI4</i>	No	N/A
Nrm1	G1/S	Co-repressor	N/A	Yes [99]	Unknown
Hcm1	S	Activator	<i>NDD1</i>	No	N/A
SFF	G2/M	Activator	<i>CLB2</i>	Yes [61, 63]	Activating
Ace2	M/G1	Activator	<i>NIS1</i>	Yes [64]	Inhibitory
Swi5	M/G1	Activator	<i>SIC1</i>	Yes [65]	Inhibitory

regulated and is coordinated with other cell cycle events may lead to insight into the importance of such a substantial periodic transcriptional program.

2.3 What Are the Regulators of Periodic Transcription?

How does the cell generate a large and continuous program of temporally ordered gene transcription throughout the cell cycle? In order to understand how this is done, we need to understand the transcriptional regulators. As more and more transcripts were identified as periodic during the cell cycle, focus turned to the regulators that activated or inhibited transcription—transcription factors (TFs). To identify the regulators that control activation or repression of periodic transcription in budding yeast, researchers utilized genetic tools, promoter sequence information, and physical localization studies [37–39]. Not surprisingly, a number of TFs were found to regulate distinct subsets of periodic genes throughout the cell cycle (reviewed in [9–11, 33]). A list of known TFs involved in cell cycle-regulated transcription and relevant information on their activation timing and regulation by cyclin/CDKs is shown in Table 1.

The TFs identified possess three striking qualities that suggest potential modes of regulation for the periodic transcription program. First, many of the TFs that play a role in controlling cell cycle-regulated transcription are themselves periodically transcribed (reviewed in [11]). For TFs that act in complexes, at least one TF is periodically expressed. This observation suggests that a portion of genes may be cell cycle-regulated due to the periodic expression of their regulators. Second, cyclin/CDK activity has been found to affect the activity of many of these transcription factors.

In these cases, cyclins that are expressed during any of these phases have the capacity to affect TF activity (Table 1). Additionally, this regulation can be either activating or inhibitory depending on the TF (Table 1). These findings, coupled with cyclin deletion experiments, imply that cell cycle-regulated gene expression is also modulated by cyclin/CDK activity [40–42]. Finally, genome-wide binding data have shown that these TFs also bind to the promoters of other TFs shown to regulate periodic gene expression [8, 43, 44]. In fact, TFs expressed late in the cell cycle have been shown to bind to the promoters of TFs responsible for early periodic gene expression [45]. This finding demonstrates that the TFs controlling the last wave of periodic transcription may also activate the first TFs in the following cycle. Taken together with global views of transcript dynamics from microarray experiments [7], it was proposed that a TF network could account for the periodic nature of the TFs themselves and the entire periodic transcription program ([8, 44], reviewed in [9–11]). Models for how the TF network is integrated with cyclin/CDK activity and cell cycle events will be discussed below.

Several versions of TF networks that control cell cycle-regulated transcription have been proposed [5, 6, 8, 12, 44]. Understanding which TFs are included in the network is an important outstanding question. For the purposes of this review, we will focus on how a transcriptional signal is transmitted through a version of the transcription network (Fig. 3).

Concurrent with passage through START and the commitment to the cell cycle, the heterodimeric TFs SBF and MBF activate a large program of periodic genes involved in budding, centrosome duplication, and DNA replication. SBF and MBF share a trans-activating subunit, Swi6 [46], and each have a distinct DNA-binding subunit, Swi4 and Mbp1, respectively [47, 48]. Activation of SBF and MBF centers on feedback loops that include G1 cyclin/CDKs and the transcriptional co-repressor Whi5 [40, 41, 49, 50] (Fig. 3). Activation begins when Cln3/Cdk1 phosphorylates Whi5, triggering its dissociation from SBF complexes and the activation of transcription of SBF targets. Two of these targets are the genes encoding the G1-cyclins; Cln1 and Cln2. Cln1/Cdk1 and Cln2/Cdk1 kinase complexes also phosphorylate Whi5, triggering further dissociation from SBF complexes and export from the nucleus. Following activation, a series of transcriptional repressors and B-type cyclins inactivate SBF and MBF in a series of negative feedback loops. SBF activates the *YOX1* and *YHP1* genes, and in turn, their gene products repress the transcription of the gene encoding the SBF component, Swi4 [51]. Moreover, SBF transcriptional activity is repressed by Clb2 [52], after a cascade of transcriptional activation that triggered the expression of the *CLB2* gene (Fig. 3). The transcriptional activity of MBF is modulated by its direct target and co-repressor *NRMI* [53] (Fig. 3). Thus, positive feedback loops contribute to the full activation of SBF and MBF, while negative feedback loops serve as the “OFF switch” for their activity.

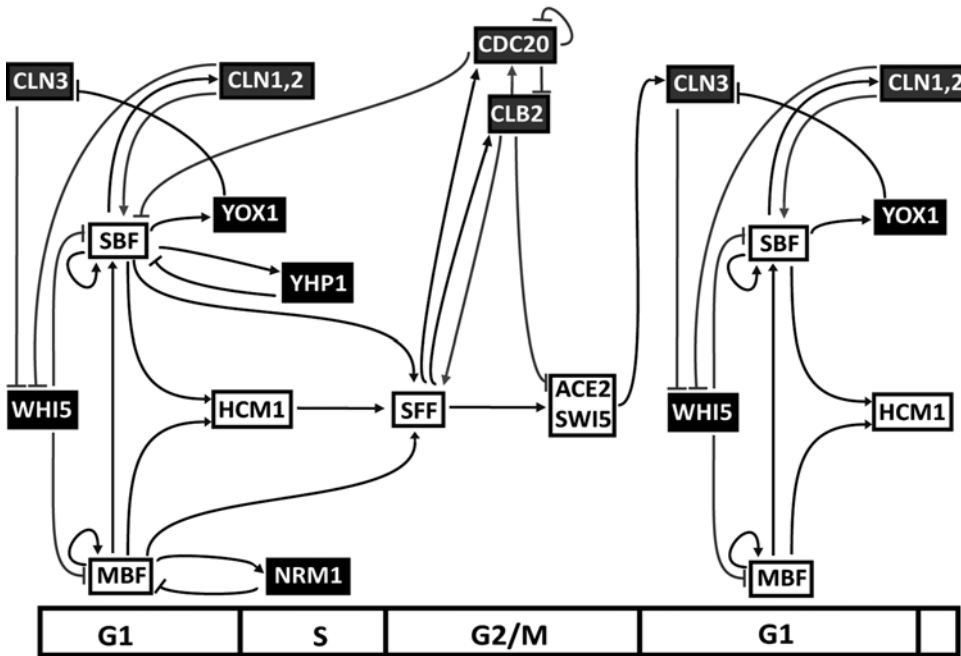


Fig. 3 Cell-cycle transcription network. An interconnected network of transcription factors that demonstrate how a transcriptional signal could be passed through the cell cycle. Note that this is just one representation of a TF network. Based on significance cutoffs and TFs included, different networks may be constructed. *Boxes* are nodes. *Green*, transcriptional activators; *red*, transcriptional repressors; *blue*, posttranslational modifications. *Arrows* signify either an upstream promoter binding to the promoter of the downstream target (*black arrows*) or a posttranslational modification that affects the activity of the TF (*blue arrows*). Nodes are placed on a cell-cycle timeline based on time of peak expression in wild-type cells (Color figure online)

SBF and MBF transmit a transcriptional signal to activator Hcm1 [54] that is responsible for expression of genes required for chromosome segregation, centrosome dynamics, and budding during late S phase [6] (Fig. 3). *HCM1* is periodically transcribed, and its protein levels are also periodic, closely mirroring the behavior of *HCM1* mRNA [6]. Moreover, Hcm1 activates the synthesis of the SBF co-repressor *WHI5* and Swi-five factor (SFF) subunit *NDD1* [6] (Fig. 3). SFF, a TF complex composed of Fkh1, Fkh2, Ndd1, and Mcm1 [55–59], activates a set of periodic genes referred to as the “*CLB2* cluster” during G2/M phase ([7, 60], reviewed in [9–11]). SFF activity is modulated through a positive feedback loop with the B-type cyclin Clb2 [52]. SFF activates *CLB2* gene transcription [60], which in turn binds CDK and further stimulates components of SFF to increase its transcriptional activity [61–63] (Fig. 3). SFF transmits the periodic transcriptional signal by activating transcription of genes encoding TFs *ACE2* and *SWI5* [60] (Fig. 3). Ace2 and Swi5 share a number of targets (Ace2 also activates a number of unique targets only in daughter cells) and activate periodic transcripts involved in the transition between late M phase and the beginning of early G1 of the subsequent cell cycle.

While Ace2 and Swi5 are transcribed during G2/M, their activity is inhibited by Clb2/CDK-dependent cytoplasmic sequestration [64, 65]. Mitosis triggers the destruction of Clb2, and thus, Swi5 and Ace2 can return to the nucleus and bind to the promoters of their target genes. Swi5 and Ace2 bind to the promoter of *CLN3* to activate *CLN3* expression and thus begin a new cycle of transcription [45] (Fig. 3). Currently, these TFs, in addition to other TFs that potentially regulate periodic transcription, are being further studied to better understand the connections with each other and with cyclin/CDKs.

2.4 Roles of Cyclin/CDKs and a Transcription Factor Network in Controlling the Periodic Transcriptional Program

Although the periodic transcriptional program during the cell cycle could be explained by a network of sequentially activated transcription factors, cyclin/CDK regulation of network TF activities could be critical for the proper execution of the program.

A series of studies have been carried out to determine the relative contributions of cyclin/CDKs and the transcription factor network on periodic transcription during the cell cycle. The first study to address this question measured the effect of S phase and mitotic cyclin/CDKs on periodic transcription by deleting all six of these cyclins (*clb1,2,3,4,5,6*) in budding yeast. These cells are kept alive by the inducible overexpression of Clb1; in the absence of Clb1, cells arrest due to the absence of all S-phase and mitotic cyclin/CDK activity, resulting in their inability to initiate DNA replication, centrosome duplication, or mitosis. However, the arrested cells continue to carry out G1 events, including budding and G1-specific transcription [66]. What happens to the rest of the periodic transcriptional program in these cells? In a synchronous population of early G1 cells lacking all S-phase and mitotic cyclins (synchronized by centrifugal elutriation), global gene expression dynamics were measured by microarray. Strikingly, compared to the expression dynamics of genes normally periodic in wild-type cells, 70 % of genes remain periodic in the absence of both S-phase and mitotic cyclins and in the absence of cell cycle progression, with a period very similar to normally cycling cells [5]. These findings suggest that S-phase and mitotic CDKs are not required for the execution of the majority of the cell cycle-transcriptional program, and that this program can continue to oscillate even in arrested cells.

How then is the periodic transcriptional program maintained in arrested cells? Included in the 70 % of genes that remain periodic in these cells are many of the TFs involved in modulating periodic transcription throughout the cell cycle [5, 8, 44]. Using these periodic TFs and binding information, Orlando and colleagues were able to construct a mathematical model of the TF network. Model simulations indicated that the TF network itself could sustain oscillation independent of S-phase and mitotic cyclin/CDKs and cell cycle progression [5]. This finding led to the proposal that a TF

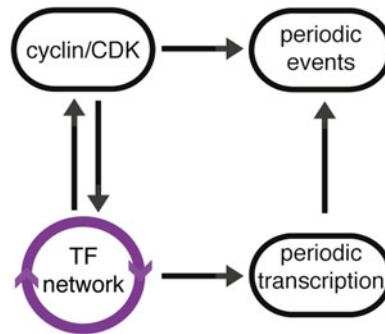


Fig. 4 Model of cell-cycle regulation. A transcription factor network is responsible for regulating the timing of the periodic transcriptional program, including cyclins. Cyclins, in complex with CDKs, then act as effectors to trigger events at the proper time after periodic synthesis

network may function as an underlying cell cycle oscillator that controls the periodic transcriptional program ([5], reviewed in [33]).

In yeast and somatic cells, several experiments have shown that cyclin/CDKs have the capacity to alter TF activity. What is the effect that cyclin/CDK feedback acting on TF activity has on transcriptional oscillations? Comparing transcriptional dynamics reveals that the overall amplitude of periodic gene expression dynamics decreases as cyclin/CDK activity is removed [12]. Additionally, the period of transcriptional oscillations also increase with decreasing cyclin/CDK activity [12]. These observations suggest that cyclin/CDK activity plays an important role in regulating the amplitude and period of transcriptional oscillations. In addition to cyclin/CDK feedback on the TF network, cyclins themselves are periodically transcribed (reviewed in [9–11, 33]). Yet in the absence of CDK activity, cell cycle progression is halted. A study showed that in the absence of all S-phase and mitotic cyclins, except for a single S-phase cyclin, periodic cycles of DNA replication occur together with transcriptional activation of the S-phase cyclin [12]. This observation implies that cyclin/CDK activity also acts as an effector of the TF network oscillator.

Taken together, these results led to the proposal of a new model of cell-cycle regulation (Fig. 4) [12]. A TF network acts as an oscillator that drives the timing of periodic transcription, including transcription of cyclin genes. Cyclins (in complex with CDKs) then feedback onto the TF network via phosphorylation to contribute robust transcriptional oscillations. Phosphorylation of a TF is capable of enhancing or reducing transactivation of the TF's target genes, thus “fine-tuning” the TF network output during the cell cycle. Additionally, cyclin/CDKs also act as effectors of the TF network to trigger cell cycle events in the proper order (Fig. 4). This model is different from previous cell cycle models in that a TF

network, rather than cyclin/CDK activity, acts as the oscillator that keeps the timing and ordering of cell cycle progression, and functions to control the temporal program of transcription. Although the mechanism by which the timing of cell cycle oscillations is different, the process by which cell cycle events are triggered by cyclin/CDKs is not different. The activation of different events in the proper order is dependent both on transcriptional oscillations and cyclin/CDK activity. However, it remains to be determined how a TF network oscillator is coupled to other cell cycle events and cyclin/CDK activity.

An important factor in designing experiments to address still-open questions is the use of technologies and analytical tools available to measure periodic transcription. Here we will discuss many of the technologies that have been used in the past as well as the computational tools that exist to identify periodic behaviors. Each technology and analytical tool has its own advantages and drawbacks, and it is critical to choose the right combination of the two in order to most completely distinguish between the models described above.

3 Assaying Cell Cycle-Regulated Transcription

Over the past three decades, we have learned that periodic gene transcription is not simply a phenotype of a small subset of cell cycle-regulated genes. In fact, a large portion of the budding yeast genome is periodically transcribed [4–7]. Models of cell cycle-regulated transcription have evolved over the years and this evolution was enabled by new experimental approaches and analytical methods. First, single gene approaches demonstrated that a handful of genes were transcribed periodically. Then, with the advent of genome-wide approaches, a global view of transcript dynamics demonstrated that many more genes have the capacity to oscillate. The analytical methods used to define periodicity also changed with each of these experimental approaches. Here, we will discuss the strengths and limitations of each.

3.1 *Single Gene Approaches*

The first sets of periodic genes were identified in budding yeast by northern blotting [67] (reviewed in [68, 69]). Although this method was able to classify a number of genes as periodically transcribed, the major limitation of northern blotting is scalability. Northern blotting is limited to testing only a handful of genes at a time. So while it was useful for asking whether any specific gene might be cell cycle-regulated, it was not a particularly useful tool for discovering new periodic genes. Additionally, because only a small number of genes could be assayed on each blot, the temporal and quantitative relationships between all periodic genes could not be determined. Thus, a global view of the program and the regulatory

mechanisms that governed them could not be easily discerned by this approach. Often, a gene was defined as periodically transcribed if dynamics matched the period of observable events. For example, histones were shown to be transcribed in concert with DNA replication during every cell cycle [13].

Quantitative real time PCR (qRT-PCR) is a more recent technology developed to measure mRNA levels at a single gene level. While not many studies have utilized qRT-PCR to measure mRNA dynamics during the cell cycle, this approach is also able to provide transcript dynamics.

3.2 Transcription Microarrays

Transcription microarrays have proven to be very useful in revealing genome-wide transcriptional behaviors in a variety of different systems. Microarray approaches facilitated the discovery of new cell cycle-regulated genes. In fact, several studies demonstrated that a substantial portion of the budding yeast genome is periodically transcribed ([4–7], reviewed in [32]). This approach also revealed that transcripts rise and fall in a continuum throughout the budding yeast cell cycle, and that clusters of genes with similar behaviors were likely to be co-regulated [7].

However, the substantial differences in periodic gene lists generated by these studies demonstrate that even global studies must be analyzed critically to understand methods used to define cell cycle-regulated transcription. Several factors, including experimental methods and definition of periodicity, play a role in the different periodic gene lists generated by each report.

A series of differences in experimental approach may contribute to low agreement between the results of these studies. First, the methods to synchronize populations of cells varied between each study, resulting in starting populations that were released from a variety of cell cycle phases. Depending on the starting population, periodic mRNAs expressed during cell cycle phases immediately following release may be more synchronous than those mRNAs in later cell-cycle phases as populations become more asynchronous. This observation may be due to the following factors: (1) normally cycling cells complete cellular division at different rates and (2) different synchronizations result in starting populations that vary in level of synchrony [31]. Second, each research group used different microarrays with different sets of oligos to represent the budding yeast genome. These differences may play a role in discrepancies between mRNA measurements. Third, the method of labeling and hybridizing mRNAs to the chips varied between the groups, which may result in differences in the quantification of mRNA levels. Fourth, Spellman and colleagues and Pramila and colleagues hybridized mRNA from an asynchronous population of cells labeled with a different fluorescent probe to each chip as a control in addition to the synchronized pools of labeled mRNA [6, 7]. This was meant as a way to control for

differences across different microarray chips. Alternatively, Cho and colleagues and Orlando and colleagues only hybridized the synchronized pools of mRNA to each chip [4, 5]. This approach generates a direct measure of mRNA levels relative to other time points from the synchronized time course. Finally, each group used different methods to normalize the microarrays with each other for consistent measures of gene expression both within and across microarrays, which can affect the resulting dynamic range of expression measurements. An important factor to consider is the differences in experimental approaches that measure and quantify gene expression dynamics when comparing the various studies centered on identifying cell cycle-regulated transcription.

In addition to differences in experimental approaches, each study utilized different methods to classify genes as periodically transcribed. Similar yet distinct definitions of periodic are embedded within each of the different methods applied to identify cell cycle-regulated transcripts. As described in Subheading 2.1, these four groups exploited different methods to classify a set of periodic genes. Two features play prominent roles in describing periodicity; each method integrates these features differently. The first feature addresses the oscillatory nature of gene expression dynamics. Whether by visual inspection or by some quantitative approach, a necessary component of any of these analyses requires identifying genes that are expressed once per cell cycle across multiple cycles. The second feature addresses the dynamic range of the queried genes. This attribute is much more subjective in nature, as it is unclear what minimum dynamic range is above stochastic noise within a synchronous population of cells. Additional quantitative methods exist to measure the periodicity of gene expression dynamics and define periodic behavior in a variety of ways [70]. Therefore, it is important to have an understanding of how you want to define periodic genes and select a computational algorithm that best matches your assumptions.

3.3 RNA Sequencing

A more recent experimental approach to measuring gene expression in cells is RNA-Sequencing (RNA-seq). This method provides a new way to measure transcript levels in cells and can benefit many biological systems when gene expression microarrays are not efficient [71]. First, RNA-seq requires much less starting RNA. This is advantageous when studying a system in which cells are in short supply. Second, RNA-seq allows for measuring the abundance of multiple transcript isoforms. This benefit is especially helpful in organisms that possess introns because microarrays usually do not distinguish between different splice variants of the same transcribed gene. Finally, RNA-seq has a much larger dynamic range compared to gene expression microarrays [71]. A major drawback of current RNA-seq methodologies is the lack of standardized normalization approaches, especially in time-series experiments.

Table 2
A summary of approaches used to determine cell-cycle transcript abundance

Approach	Detection	Population/ single cell	Target measurement	Target amplification
Northern blot	RNA	Population	Preselected probes	No
Microarray	RNA	Population	Preselected ORFs (all annotated genes)	Yes
RNA-sequencing	RNA	Population	Unbiased	Yes/no
RNA-FISH	RNA	Single cells	Preselected probes	No
Reverse transcription PCR	RNA	Population	Preselected primers	Yes

This drawback is critical when comparing time points across the cell cycle. While few groups have used RNA-seq to study cell cycle-regulated transcription, with continued development of experimental and analytical techniques, this technology may provide a way to gain a better insight into the scope of periodic gene expression.

3.4 Mapping Relationships Between Transcription Factors and Their Targets by Physical Interaction Approaches

The past 15 years have seen massive strides in the characterization of cell cycle gene expression. Many techniques have allowed us to elucidate the dynamics of cell cycle transcription (Table 2). However, much still needs to be done to understand the molecular mechanisms governing transcription dynamics during the cell cycle.

One important method for developing regulatory models involves detecting the physical interaction between a TF and a target gene promoter. Using Chromatin Immunoprecipitation approaches (ChIP) coupled with microarray or RNA-sequencing (ChIP-chip or ChIP-seq) provides information on where a particular TF binds genome-wide. Such localization studies enable the construction of global transcription factor networks that describe the regulatory interactions of the cell cycle transcription program [8, 39, 43, 44, 54, 72, 73].

Most publicly available large-scale ChIP-chip datasets were derived from asynchronous populations of cells. Thus, these experiments lack any temporal information about dynamic TF binding. Moreover, TFs that bind very strongly to a target gene promoter for only a short amount of time yield a “low” signal that resembles a regulator that only binds weakly to a promoter throughout the cell cycle. Thus, it is likely that short-lived TF/promoter interactions are under-represented in these data sets. Both of these issues could be rectified by performing ChIP approaches on synchronized cells. However, performing a single replicate of a ChIP-chip or ChIP-seq experiment over 10 time points for all *S. cerevisiae* TFs

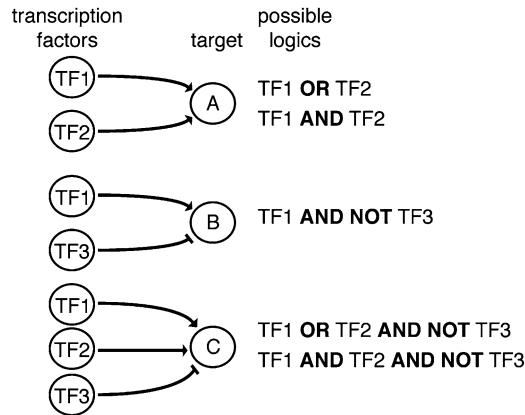


Fig. 5 Logics of multiple transcription factors regulating a single target affect its synthesis. Depending on the combinations of transcription factors that bind to the promoter of a single target, they may work together (AND logic) or may work separately (OR logic). Repressors most likely override any activators that may be bound at the same time (AND NOT logic). Depending on the number and combination of potential regulators, other logics may be possible

would require ~2,000 ChIP experiments followed by the same number of microarray hybridizations or sequencing runs. Presently, this is an effort that is currently beyond the capacity of most labs.

In budding yeast, ChIP-chip and associated computational studies have shown that many different TFs can bind within the promoter region of a single gene [8, 39, 43, 44, 54, 72, 73]. While ChIP studies have been informative in determining the physical association of a DNA-binding protein with a given promoter, these studies do not reveal the degree to which multiple TFs bind in a coordinated or exclusive fashion. Furthermore, binding studies do not reveal the functional relationships between multiple TFs bound at the same promoter. Since transcriptional regulators can promote or repress transcription (some TFs can perform both tasks), there are several different logical combinations that can define the transcription of a given gene (Fig. 5).

A primary goal of the field is to understand the functional targets of cell cycle transcription factors. However, physical interaction data can only predict binding, and binding does not predict function. Therefore, to determine the downstream targets of TFs in vivo, one can use genetic approaches to observe changes in target gene behavior directly.

3.5 Genetic Approaches to Transcriptional Target Discovery

Gene knockout experiments are straightforward methods to begin characterizing TF-target relationships in budding yeast. Nonessential TFs may be deleted and, in theory, their targets should have altered expression. This method had been used successfully to identify gene targets with altered expression upon deletion of cell cycle TFs [74]. The method can even be combined with microarray or

other whole-genome analysis to determine expression changes among large numbers of genes [75]. However, this method does not necessarily demonstrate direct TF-target relationships between the deleted TF and gene expression. In some instances, the deletion of TF1 could alter the expression of another TF2, resulting in expression changes for the targets of TF2. Therefore, these methods are often tied to physical interaction data to strengthen the argument for a direct relationship [74, 75].

An alternative method to TF deletion is conditional TF expression. Using an inducible TF, one can determine the state of transcription prior to and post TF induction, while controlling and monitoring the timescale of gene activation/repression. Budding yeast researchers have long relied on inducible promoter constructs to turn on gene expression and monitor the outcome. The replacement of an endogenous promoter with the GAL promoter has allowed for induction of transcription via a shift to galactose-containing medium [76]. The use of the GAL-inducible promoter, among others, allows a researcher to turn on gene expression of a TF and then monitor the effect on target genes. However, after the shift to galactose medium, the TF must be expressed, translated, and then activate/repress gene expression of its targets. Thus, there is a desire to shorten the time from induction to the activation/repression of target genes.

Instead of inducing TF expression, one can induce TF localization to the nucleus using a hormone-induced localization. To construct an estradiol-induction system, a Gal4-DNA binding domain was fused to the human estrogen receptor [77, 78]. The addition of estradiol hormone results in the localization of the fusion protein to the nucleus and binding to Gal4 transcriptional targets. This massive shift in localization leads to the rapid binding of Gal4 targets without concern for protein level thresholds for transactivation. McIsaac and colleagues employed microarrays to monitor temporal changes in global transcription in asynchronous populations and found that the targets of Gal4 transactivation were quickly upregulated, as soon as 5 min after estradiol treatment [79]. By sampling mRNA levels at several time points after induction, one can determine direct transcriptional targets and observe the activation/repression dynamics. Using a similar system, one can identify the targets of many of the yeast cell-cycle TFs employing an induction system in asynchronous populations of cells and monitoring the first genes to respond, thus identifying the direct targets of the TF. The forced expression of these targets should be readily detectable over the background expression in an asynchronous population (low amplitude changes may require cell cycle synchronization to be observed).

As mentioned for physical interactions in Subheading 3.4, temporal dynamics are important for determining first-order targets of a TF. A systematic analysis of cell cycle TFs would be possible, using 4–5 time points after induction, thus reducing the time and cost of

such an experiment compared to ChIP analysis of TFs throughout the cell cycle. Combining the identification of TF targets with existing data on expression dynamics of the TFs themselves will help to build a temporal network of transcription regulation during the cell cycle.

Ideally, data from physical interaction studies can be combined with data from genetic experiments in order to develop high confidence molecular models. Additional information can be gained by approaches that identify global changes in chromatin structure, such as nucleosome dynamics. Nucleosome positioning experiments identify chromatin that is accessible to TFs. However, recent work on temporal dynamics of nucleosome binding at promoters suggests that TFs can actually induce nucleosome position changes once they bind a site, while some sites which are “accessible” to TFs remain transcriptionally inactive [80]. These experiments help to build a model describing the transactions at a promoter. By correlation, this information can be associated with expression timing and provide additional information to describe mechanisms of gene activation and repression during the cell cycle. As these experiments do not directly probe the activation/repression of genes, expression analysis upon induction of TF localization to the nucleus remains a more direct observation of transcriptional activity at TF targets.

Much stands to be gained from leveraging both physical and genetic approaches. The data eventually yielded from these approaches will ultimately allow us to develop quantitative methods to integrate and interpret present and future “big data.” Designing future experiments in the context of cell cycle dynamics and with the understanding that several regulators may contribute to target gene regulation will provide the information necessary for the challenging analyses to come. The use of genetic experiments may direct the strategic implementation of physical interaction studies that may prove too costly on a larger scale (or vice versa). Thus, collection of physical and genetic data will only strengthen our ability to understand cell-cycle TF regulation.

3.6 Gaps in Knowledge and the Future

The ability to quantify the levels of RNA in a cell at any given moment, in an unbiased manner, is the Holy Grail for developing a precise understanding of cell cycle-regulated transcription. The field has come a long way from original expression studies, and new technologies are continuously being developed to address issues with sensitivity, noisy data, and population effects. However, in overcoming some of the current obstacles, one must understand potential pitfalls and biases introduced by the various methods used to measure transcript levels, which are especially important while implementing methods that measure expression over time. Improving current approaches and developing novel assays will allow the field to continue to move forward.

One of the key improvements needed in cell cycle gene expression analysis is the removal of amplification bias. Microarray analysis and RNA sequencing protocols typically use a reverse transcription step to produce a complementary DNA or RNA, which is then amplified to add a fluorophore to the nucleic acid. The introduction of these amplification steps during any RNA detection assay increases the risk that some RNA species are preferentially amplified over others. To prepare libraries representing an mRNA population, the use of poly-thymidine primers to amplify cDNA from purified RNA leads to a preference for mRNAs with longer polyadenine tails, altering the representative population in the amplified pool.

One possible solution is the use of single-molecule sequencing technologies to eliminate the amplification steps of RNA-seq and microarray methods [71]. So-called third-generation single-molecule sequencing removes the requirement for RNA amplification from the assay [81, 82]. These sequencing technologies have longer read lengths than current deep-sequencing technologies, making sequencing of an entire mRNA possible. Critically, a formidable problem for these technologies is the improvement of the sequencing accuracy which currently sits at ~85 % for PacBio sequencing and ~75–90 % for Nanopore sequencing [83, 84]. With these accuracy rates, the possibility that a read does not identify the correct gene is substantial. Recently, it has been shown that aligning single molecule sequencing with reads from amplification based sequencing (such as Illumina) greatly enhances the accuracy of single molecule sequencing while still allowing for quantitation to be performed in a no-amplification manner, increasing the viability of the technology, but increasing the cost and effort involved [85, 86]. With the introduction of these technologies to RNA-Seq, the biases of amplification-based methods should be greatly reduced.

While great advances have been made in removing artifacts and biases from microarray and sequencing methods, they do not address a main concern that many researchers harbor: the fact that these methods detect average transcript levels in a population. To eliminate this problem, many have made use of RNA fluorescence in situ hybridization, or RNA-FISH [87]. RNA-FISH uses a fluorophore-conjugated nucleotide probe to detect target RNAs in fixed cells. The foci generated by the probe are visualized by microscopy, and each focus represents an mRNA contained within a cell at that moment. Barcoding FISH probes and superresolution microscopy has led to the potential for identifying and quantifying more transcripts than available fluorophore colors [88, 89]. Using RNA-FISH, one can determine the actual number of transcripts at a time point in a single cell. Observing multiple cells, one can determine the range of transcript abundance at a given time in a collection of single cells, which helps understand the distribution

of RNA abundance in a population in a way that cannot be determined using methods that rely on purified RNA from a population of cells (microarray, RNA-seq, etc.).

The drawbacks to this method are also inherent to working with single cells. For each time point, many cells must be labeled and analyzed to obtain statistically significant results. Furthermore, the method requires cells to be fixed. Hence, transcript dynamics cannot be measured in the same cells over a period of time. Therefore, a time course using an RNA-FISH approach still samples a population, albeit in a manner that allows for individual mRNA molecules to be quantified. The ability to determine the number of mRNA transcripts in cells at a time point serves as a complementary approach to the whole transcriptome approaches described above.

Methods for tracking RNA in live cells have been developed to expand temporal observations and localization behavior [90]. These methods rely on inserting hairpin-forming sequences to the noncoding regions of the mRNA. These hairpin constructs are recognized by virus-derived hairpin-recognition proteins. The best characterized of these systems is the MS2 reporter [91, 92]. The hairpin-binding protein domain can be detected as they are typically fused to a fluorescent reporter. MS2 stem-loop repeats are integrated into the mRNA of interest and co-expressed in a cell with the bacteriophage derived MS2-binding protein fused to a fluorescent reporter. When the mRNA is expressed, the stem-loop structures fold and are recognized by the MS2-binding domain, generating fluorescent foci that can be tracked within a living cell.

The system was originally described in yeast, where it was used to follow *ASH1* mRNA as it was trafficked from the nucleus to the bud [93]. Since the introduction of the system, it has been effectively used in a variety of eukaryotes and expanded to a two-color system [94, 95]. Still, live-cell imaging using reporters has typically shied away from quantitation and has been used more extensively for localization experiments. The RNA hairpin-binding approach is, so far, relatively unproven in systems where mRNA levels are dynamically regulated over time (though some efforts have been made to quantify mRNA by foci in prokaryotes [96, 97]). For example, the *ASH1* experiment described above observed mRNA localization dynamics, not abundance. A major limitation of probing mRNAs by FISH or by hairpin-binding proteins is the introduction of bias to the analysis while limiting scale. Using these methods, an experimenter can only determine the transcript levels of the specific mRNAs that they have targeted for analysis. Therefore, examining correlation or coherence with other transcripts is not yet possible with this approach. In time, the ability to multiplex probes may expand the experimental arsenal of probe targets. Certainly, with advances in fluorescent probe development and live cell imaging, the potential remains for RNA binding

probes to yield fruitful results in the field of cell cycle transcription. Currently, the field still benefits from data collected at the population level as well as in single cells.

Presently, there does not appear to be a “cure-all” experiment that generally addresses the concerns of those studying cell-cycle transcription. The most benefit appears to come from integrating the data the field has already collected. Using a growing body of correlative data can increase the confidence in understanding the coordination of periodic transcription with other cell cycle events and the coordination of two major regulators: a TF network and cyclin/CDKs. Quantitative methods that integrate existing data with an understanding of all possible limitations will greatly increase our current knowledge and help direct specific experiments to address new hypotheses in cell-cycle transcription.

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