

# A model for restriction point control of the mammalian cell cycle<sup>☆</sup>

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## Abstract

Inhibition of protein synthesis by cycloheximide blocks subsequent division of a mammalian cell, but only if the cell is exposed to the drug before the “restriction point” (i.e. within the first several hours after birth). If exposed to cycloheximide after the restriction point, a cell proceeds with DNA synthesis, mitosis and cell division and halts in the next cell cycle. If cycloheximide is later removed from the culture medium, treated cells will return to the division cycle, showing a complex pattern of division times post-treatment, as first measured by Zetterberg and colleagues. We simulate these physiological responses of mammalian cells to transient inhibition of growth, using a set of nonlinear differential equations based on a realistic model of the molecular events underlying progression through the cell cycle. The model relies on our earlier work on the regulation of cyclin-dependent protein kinases during the cell division cycle of yeast. The yeast model is supplemented with equations describing the effects of retinoblastoma protein on cell growth and the synthesis of cyclins A and E, and with a primitive representation of the signaling pathway that controls synthesis of cyclin D.

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## 1. Introduction

The molecular controls of cell division are fundamentally similar in all eukaryotes (Nurse, 1990). Major events of the eukaryotic cell cycle are choreographed by periodic activation of several cyclin-dependent kinases (Cdks) and the enzymes and inhibitors that affect their activities. Unicellular organisms, like yeast, grow and divide as rapidly as nutritional conditions permit, but this strategy would be disastrous in multicellular organisms, for which cell growth and division must be highly constrained (Hanahan and Weinberg, 2000). These “social constraints” are enforced by a complex

network of inhibitions on Cdk activities (Sherr and Roberts, 1999).

The cells of multicellular organisms proliferate only when permitted by specific growth factors (GFs). If GFs are deprived, cells early in G1 phase leave the cycle and enter a resting state (G0); older cells finish the ongoing cycle and enter the resting state after mitosis. The point in G1, before which cells enter directly into the resting state, is called the *restriction point* (Pardee, 1989; Bartek et al., 1996; Planas-Silva and Weinberg, 1997). (A glossary of technical terms used in this paper is provided in Table 1.)

The goal of this paper is to understand the physiological properties of restriction point control in mammalian cells by computer simulations of a mathematical model of the underlying molecular mechanism. Zetterberg and Larsson (1995) have located the restriction point quite precisely 3–4 h after cell birth, whether cell proliferation is stopped by deprivation of GF or by partial inhibition of protein synthesis with cycloheximide. They also measured the kinetics of re-entry into the cell cycle, when GFs are added back or

<sup>☆</sup> Dedicated to the memory of Arthur T. Winfree, who played a major role in JJT's graduate education at the University of Chicago in the early 1970s. Though secondary to his focus on circadian rhythms and cardiac arrhythmias, Art was fascinated by the periodicity of cell cycle events, as described in chapter 22 of *The Geometry of Biological Time* (1980).

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Table 1  
Glossary

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*Cyclin-dependent kinase (Cdk1, Cdk2, etc.):* An enzyme that attaches phosphate groups ( $-\text{PO}_3^{2-}$ ) to serine or threonine side chains of specific target proteins. Cdks are enzymatically active only in combination with a cyclin partner. In budding yeast, Cdk1 is usually referred to by its original name, Cdc28 (where “Cdc” stands for “cell division cycle”)

*Cyclin (CycA, CycB, etc.):* A protein that associates non-covalently with Cdk subunits to form enzymatically active heterodimers (Cdk1/CycA, etc.). Budding yeast cells have nine different cell cycle-related cyclins, in two distinct families (Cln1-3 and Clb1-6)

*Cyclin-dependent kinase inhibitor (CKI):* A protein that binds to Cdk/cyclin dimers to form enzymatically inactive trimers (Cdk/cyclin/CKI). The primary CKI in budding yeast is called Sic1 (“substrate and inhibitor of Cdc28”). A functionally similar inhibitor in mammalian cells is p21<sup>Kip1</sup>

*Cycloheximide (CHX):* A drug that blocks peptide chain elongation on eukaryotic ribosomes

*Growth factors (GF):* Small proteins present in blood serum that are required for proliferation of normal mammalian cells in culture

*Restriction Point (RP):* A point-of-no-return in G1 phase of the mammalian cell cycle. If GFs are removed from a cell prior to the RP, the cell halts immediately in G1. If GFs are removed after the RP, the cell continues through G1, S, G2, M and halts in G1 phase of the next cell cycle

*Retinoblastoma protein (Rb):* A general inhibitor of RNA polymerases and a specific inhibitor of E2F transcription factors

*Transcription factor:* A protein that binds to DNA and regulates the expression of specific genes. For example, in budding yeast, Mbp1/Swi6 (also called MBF) is a heterodimeric transcription factor that activates the synthesis of S-phase cyclins and other proteins involved in DNA synthesis. E2F and DP proteins are transcription factors that play similar roles in mammalian cells

*Ubiquitin:* A small polypeptide used to label proteins for degradation by proteasomes

*Proteasome:* A multi-protein complex that disassembles polypeptide chains (i.e. opposite function of a ribosome)

*Anaphase Promoting Complex (APC):* A multi-protein complex that covalently links ubiquitin molecules to specific proteins. APC-dependent ubiquitination of B-type cyclins is directed by auxiliary proteins, called Cdc20 and Cdh1 in budding yeast

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cycloheximide is washed away. They found that cells treated early in the cell cycle (before the restriction point) suffer an immediate delay of 8 h plus the duration of the treatment; cells treated late in the cycle divide on schedule but are delayed significantly in the next division cycle; and cells treated shortly after the restriction point suffer no delay in either the first or second division cycles.

Our mathematical model explains these observations in quantitative detail by considering the interactions between cell growth and the dynamics of the Cdk-regulatory system. The model emphasizes the deep similarities of the Cdk-regulatory systems in yeast and mammalian cells, while also accounting for subtle interplays between “sizer” and “timer” functions characteristic of the mammalian cell cycle. In our opinion, the most promising way to understand the molecular basis of mammalian cell-cycle control is to build models of social constraints (like GF requirements) around a yeast-like core of cyclin–Cdk interactions.

## 2. Biochemical circuit theory

An understanding of the molecular network controlling DNA synthesis and mitosis in mammalian cells is

growing rapidly as research groups around the world explore the intricate details of Cdks and their associated activators and inhibitors (Bartek et al., 1996; Sherr, 1996; Sherr and Roberts, 1999; Cross and Roberts, 2001; Blagoskonny and Pardee, 2002). Kohn (1999) summarized this information in a comprehensive biochemical circuit diagram of such complexity that intuitive methods will never suffice to reveal all its properties with certainty. Just as electrical circuits are now designed and tested in silico by sophisticated simulation software, so also the characterization of gene–protein regulatory circuits will require computational tools for analysis and simulation. Many people have recognized this need and called for new theoretical approaches to molecular biology (Maddox, 1994; Hartwell et al., 1999; Lander and Weinberg, 2000; Nurse, 2000; Gilman and Arkin, 2002; Kumar and Feidler, 2003). Although everyone seems to agree that present theoretical techniques are not ready for a “frontal assault” on Kohn’s (1999) circuit diagram or a comprehensive explanation of the physiology of mammalian cell division, several groups of theoretical biologists have risen to the challenge of modeling parts of the mammalian cell cycle (Hatzimanikatis et al., 1995; Obeyesekere et al., 1997; Kohn, 1998; Aguda, 1999; Thron, 1999; Qu et al., 2003b). A recent model

published by Qu et al. (2003a) is quite similar in spirit to the model presented here.

We have approached the problem by modeling the simpler regulatory circuits in frog eggs and yeast cells (Novak and Tyson, 1993, 1997; Novak et al., 1998a, 1999). Our models have developed over the years from simple pictures of cyclin B/Cdk1 activation at the G2–M transition (Tyson, 1991) to comprehensive models of all the major cell cycle controls in budding yeast (Chen et al., 2000). With this experience in hand, we have begun to develop a mathematical model of the mammalian cell cycle patterned after the molecular controls operating in yeast cells. We take this approach, first of all, because the complex control mechanisms in multicellular organisms must have evolved from simpler controls in unicellular eukaryotes. Second, because the full mechanism is currently intractable, it is necessary to start with a simplified version of the control system and add important details stepwise, in the same way that experimental exploration of the problem started with an incomplete picture and patiently uncovered new pieces of the puzzle.

### 3. The model

We propose that, hidden within Kohn’s circuitry for replication and division of mammalian cells is a central cell-cycle engine shared with all eukaryotes. The underlying yeast-like controls can be seen with the help of Table 2, which identifies proteins that play functionally similar roles in yeast and mammalian cells. In many cases, these functionally related proteins share considerable sequence homology as well. Abstracting these proteins from Kohn’s figures and rearranging them to emphasize the analogy to our yeast model (Chen et al., 2000), we present our proposed mechanism (Fig. 1) for the central circuitry controlling mammalian cell proliferation. As stated, the model is an abstraction, a starting point for more realistic and comprehensive models of the future. Our purpose here is to propose a “skeletal” control system for mammalian cell division and to see how certain characteristic features of

restriction point control derive from the underlying skeleton.

We justify the skeletal model in four stages.

#### 3.1. Antagonism between CycB/Cdk1 and Cdh1/APC

Entry into mitosis in higher eukaryotes is triggered by the activity of Cdk1 in combination with B-type cyclins (Pines, 1995). The kinase subunit is present in excess, so dimer level is determined by cyclin availability. CycB is absent in G1 and accumulates in S/G2/M phases of the cycle. A similar pattern of B-type cyclin accumulation is observed in budding yeast, where the reason is clear (Nasmyth, 1996): in G1 phase, B-type cyclin genes are not transcribed and the proteins are rapidly degraded.

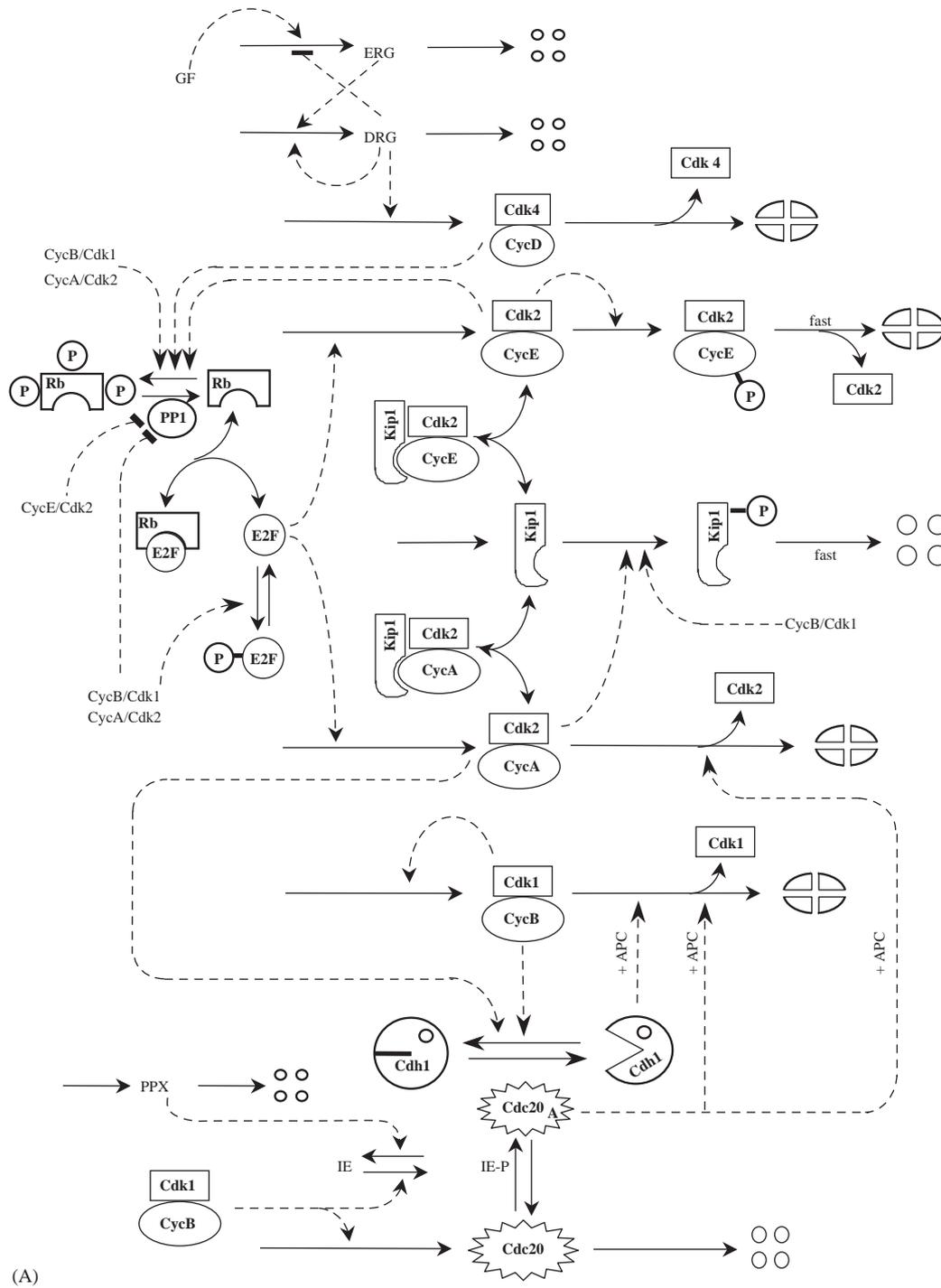
Regarding synthesis, in budding yeast mitotic cyclins (Clb1 and Clb2) activate their own transcription factors (Amon et al., 1993); hence, they are not synthesized in G1, when their related kinase activities are low, and they accumulate autocatalytically in S phase, as their activities rise. Although there is no experimental evidence to support the idea of autocatalytic CycB production in mammalian cells, we adopt the hypothesis temporarily as the simplest way to model the fact of negligible CycB synthesis in G1 phase (Brandeis and Hunt, 1996).

Cyclin B degradation is initiated by the anaphase-promoting complex (APC), which ubiquitinates its substrates, thus targeting them for proteolysis by proteasomes (Morgan, 1999). At the heart of our model is an antagonistic relation between CycB and the APC (Novak et al., 1998b). APC-dependent degradation of mitotic cyclins is mediated by “adaptors,” Cdc20 and Cdh1, which apparently recognize CycB and present it to the APC core for ubiquitination (Morgan, 1999; Zachariae and Nasmyth, 1999). The adaptors are regulated differently during the cell cycle. Cdc20 is activated (indirectly) by CycB/Cdk1 and functions chiefly at anaphase. Cdh1 is inhibited by CycB/Cdk1 (and other cyclin/Cdk holoenzymes) and functions chiefly during G1, when Cdk activity is low (Kotani et al., 1999; Kramer et al., 2000). Hence, between CycB/Cdk1 and Cdh1/APC there is a fundamental antagonism, which creates two stable steady states: a G1 state with active Cdh1 and low CycB activity, and an S/G2/M state with high CycB level and Cdh1 turned off (Novak et al., 1998b).

Progress through the cell cycle is, in essence, periodic switching between these two stable states (Nasmyth, 1996; Chen et al., 2000; Tyson et al., 2001). The transition from G1 to S (i.e. commitment to a new round of DNA synthesis and division) is traditionally called START; the reverse transition (i.e. the completion of the chromosome cycle, which occurs at anaphase, when any remaining cyclins are finally destroyed by the APC) is reasonably called FINISH.

Table 2  
Functional relations among cell-cycle regulatory proteins

Budding yeast	Mammalian cell	Role
Cdc28	Cdk1,...	Cyclin-dependent kinase
Cln3/Cdc28	CycD/Cdk4	Growth-factor sensor
Cln2/Cdc28	CycE/Cdk2	Starter kinase
Clb5/Cdc28	CycA/Cdk2	Initiate DNA synthesis
Sic1	Kip1	Cdk inhibitor in G1
SBF, MBF	Rb, E2F	Regulate transcription at G1/S
Clb2/Cdc28	CycB/Cdk1	Mitosis promoting factor
Cdh1	Cdh1	Degradation of B-type cyclins
Cdc20	p55cdc	Proteolysis at anaphase



(A)

Fig. 1. Molecular network regulating the progression of mammalian cells through the cell cycle. (A) In the center of the diagram we propose a yeast-like cell-cycle “engine” composed of Cdk2/CycE, Cdk2/CycA, Cdk1/CycB, and some ancillary proteins (Kip1, Cdh1 and Cdc20). This part of the diagram should be compared to [Chen et al. \(2000\)](#). To the engine we attach three components characteristic of mammalian cell-cycle controls: (i) the retinoblastoma protein, Rb, which binds to and inhibits E2F, a transcription factor for production of CycA and CycE, (ii) the cyclin-dependent kinase, Cdk4/CycD, which phosphorylates and inactivates Rb, and (iii) the signal-transduction pathway, GF–ERG–DRG, which controls CycD synthesis in response to GF stimulation. Although not indicated on the wiring diagram, the model includes the fact that Kip1 binds to CycD/Cdk4 but does not inhibit its activity. (B) A more detailed representation of the binding and phosphorylation reactions that govern Rb–E2F interactions. In deriving the model equations ([Table 3](#)), we assume that binding and release reactions are fast compared to phosphorylation and dephosphorylation reactions.

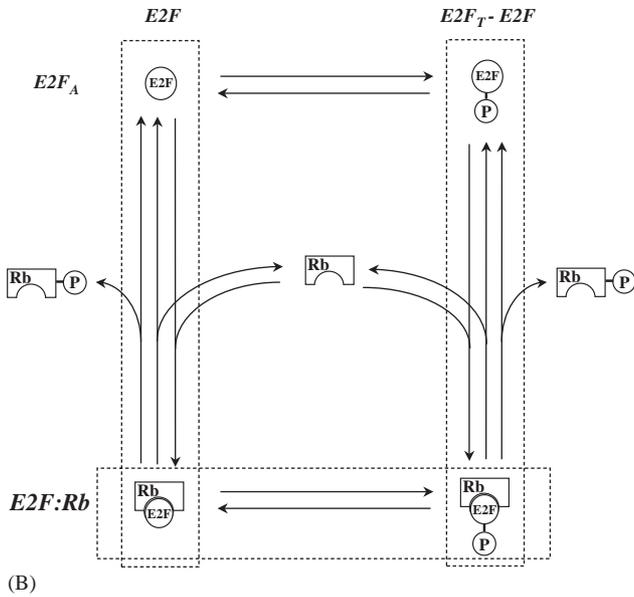


Fig. 1 (continued).

At START, Cdh1 must be inactivated so that mitotic cyclins may reappear. In budding yeast, this is the job of Clb5/Cdc28 (Shirayama et al., 1999), and in mammalian cells, of CycA/Cdk2 (Lukas et al., 1999). More on this shortly. At FINISH, Cdh1 must be reactivated. In budding yeast, this process depends on Cdc20 (Fang et al., 1998, 1999; Ohtoshi et al., 2000), whose mammalian homolog is p55cdc (Weinstein, 1997). In addition to degrading cyclins A and B at anaphase, Cdc20 indirectly activates Cdh1 (Bardin and Amon, 2001). To model the fact that Cdc20 (p55cdc, as well) accumulates in S/G2/M and disappears in G1 (Weinstein, 1997), we assume that it is synthesized in a CycB-dependent manner. Newly synthesized Cdc20 is inactive. It becomes active at anaphase, in a process that depends indirectly on CycB/Cdk1 (Step 13 in Fig. 1) and can be delayed (Step 14) if chromosomes are not properly aligned on the mitotic spindle. The mechanistic details of the model for exit from mitosis have been inherited from our earlier models of frog eggs and yeast.

Inhibitory tyrosine-phosphorylation of Cdk1 subunits plays an important role in the transition from G2 to M-phase (Nurse, 1990), and we have well-developed models of these events in frog eggs (Novak and Tyson, 1993) and fission yeast (Novak et al., 1998a). However, because the current model focuses on events in G1 phase rather than S/G2/M, we have chosen to neglect tyrosine phosphorylation of Cdk1 for the time being.

### 3.2. Early and delayed-response genes

GFs bind to specific receptors in the plasma membrane, stimulating an intracellular signal-transduction pathway (Ras–Raf–MAP kinase) that activates so-called *early response genes*, which in turn activate a

second group, the *delayed-response genes* (Alberts et al., 1994; Sherr, 1995). Among the delayed-response genes are those for D-type cyclins, which can be considered as GF sensors (Sherr, 1995; Bartek et al., 1996). D-type cyclins, combined with Cdk4 and Cdk6, set in motion the cell-cycle engine that drives rounds of DNA replication, mitosis and cell division.

The kinetics of MAP-kinase signaling has been studied in great detail by Ferrell (Bagowski et al., 2003), who has shown convincingly that this signal-transduction pathway functions as a bistable switch in frog eggs. According to Ferrell’s evidence, the switch is created by a positive-feedback loop superimposed on a highly nonlinear (sigmoidal) signal–response curve of the kinase cascade (Ferrell and Machleder, 1998; Ferrell, 2002). We assume that a similar positive-feedback mechanism creates a bistable response to GF signaling in cultured mammalian cells. In the absence of a carefully worked out mechanism for the regulation of CycD transcription, we propose a highly stylized account (Fig. 1). GF stimulates the synthesis of two classes of transcription factors: ERG and DRG (early and delayed-response gene products). ERG stimulates DRG synthesis and DRG inhibits ERG synthesis. In addition, DRG feeds back and activates the kinase in its own signaling pathway. Finally, DRG stimulates CycD synthesis. We write this system as a pair of phenomenological Eqs. (1) and (2) in Table 3.

### 3.3. The retinoblastoma protein

CycD/Cdk4 stimulates cell growth and division by phosphorylating the retinoblastoma protein, Rb (Weinberg, 1995; Planas-Silva and Weinberg, 1997). Rb is a general inhibitor of RNA polymerases (White, 1997) and a specific inhibitor of E2F, a transcription factor for the CycA and CycE genes (Bartek et al., 1996; DeGregori, 2002). Phosphorylated Rb releases its hold on E2F, which stimulates synthesis of CycA and CycE. Because these cyclins, in combination with Cdk2, can phosphorylate Rb (Planas-Silva and Weinberg, 1997), CycA and CycE activate their own synthesis. Indeed, we assume that all four cyclin/Cdk complexes (A, B, D, and E) phosphorylate Rb, although their efficiencies may differ (see Eq. (20)).

In this version of the model, we assume that the total levels of E2F and Rb do not fluctuate ( $E2F_T$  and  $Rb_T$  are constants). Rb is distributed between two forms: (1) an active, hypophosphorylated form ( $Rb_{hypo}$ ), which binds to and inhibits E2F; and (2) an inactive, phosphorylated form ( $Rb_T - Rb_{hypo}$ ), which does not bind E2F.

Dephosphorylation of Rb is catalysed by a type-1 protein phosphatase (PP1), whose activity is inhibited by cyclin/Cdk complexes (Dohadwala et al., 1994; Kwon et al., 1997). PP1 phosphorylation could be started by

Table 3  
Mathematical model of mammalian cell-cycle controls

$$\frac{d[\text{ER}G]}{dt} = \varepsilon \frac{k_{15}}{1 + ([\text{DR}G]/J_{15})^2} - k_{16}[\text{ER}G] \quad (1)$$

$$\frac{d[\text{DR}G]}{dt} = \varepsilon \left( k'_{17}[\text{ER}G] + \frac{k_{17}([\text{DR}G]/J_{17})^2}{1 + ([\text{DR}G]/J_{17})^2} \right) - k_{18}[\text{DR}G] \quad (2)$$

$$\frac{d[\text{cycD}]}{dt} = \varepsilon k_9[\text{DR}G] + V_6[\text{CycD} : \text{Kip1}] + k_{24r}[\text{CycD} : \text{Kip1}] - k_{24}[\text{CycD}][\text{Kip1}] - k_{10}[\text{CycD}] \quad (3)$$

$$\frac{d[\text{cycD} : \text{Kip1}]}{dt} = k_{24}[\text{CycD}][\text{Kip1}] - k_{24r}[\text{CycD} : \text{Kip1}] - V_6[\text{CycD} : \text{Kip1}] - k_{10}[\text{CycD} : \text{Kip1}] \quad (4)$$

$$\frac{d[\text{cycE}]}{dt} = \varepsilon(k'_7 + k_7[\text{E2F}_A]) - V_8[\text{cycE}] - k_{25}[\text{CycE}][\text{Kip1}] + k_{25r}[\text{CycE} : \text{Kip1}] + V_6[\text{CycE} : \text{Kip1}] \quad (5)$$

$$\frac{d[\text{cycE} : \text{Kip1}]}{dt} = k_{25}[\text{CycE}][\text{Kip1}] - k_{25r}[\text{CycE} : \text{Kip1}] - V_6[\text{CycE} : \text{Kip1}] - V_8[\text{CycE} : \text{Kip1}] \quad (6)$$

$$\frac{d[\text{cycA}]}{dt} = \varepsilon k_{29}[\text{E2FA}][\text{mass}] - k_{30}[\text{Cdc20}][\text{cycA}] - k_{25}[\text{CycA}][\text{Kip1}] + k_{25r}[\text{CycA} : \text{Kip1}] + V_6[\text{CycA} : \text{Kip1}] \quad (7)$$

$$\frac{d[\text{cycA} : \text{Kip1}]}{dt} = k_{25}[\text{CycA}][\text{Kip1}] - k_{25r}[\text{CycA} : \text{Kip1}] - V_6[\text{CycA} : \text{Kip1}] - k_{30}[\text{Cdc20}][\text{CycA} : \text{Kip1}] \quad (8)$$

$$\begin{aligned} \frac{d[\text{Kip1}]}{dt} = & \varepsilon k_5 - V_6[\text{Kip1}] - k_{24}[\text{CycD}][\text{Kip1}] + k_{24r}[\text{CycD} : \text{Kip1}] + k_{10}[\text{CycD} : \text{Kip1}] - k_{25}[\text{Kip1}]( [\text{CycE}] + [\text{CycA}] ) \\ & + k_{25r}([\text{CycE} : \text{Kip1}] + [\text{CycA} : \text{Kip1}]) + V_8[\text{CycE} : \text{Kip1}] + k_{30}[\text{Cdc20}][\text{CycA} : \text{Kip1}] \end{aligned} \quad (9)$$

$$\frac{d[\text{E2F}]}{dt} = k_{22}([\text{E2FT}] - [\text{E2F}]) - (k'_{23} + k_{23}([\text{CycA}] + [\text{CycB}])([\text{E2F}]) \quad (10)$$

$$\frac{d[\text{cycB}]}{dt} = \varepsilon \left( k'_1 + \frac{k_1([\text{CycB}]/J_1)^2}{1 + ([\text{CycB}]/J_1)^2} \right) - V_2[\text{CycB}] \quad (11)$$

$$\frac{d[\text{Cdh1}]}{dt} = (k'_3 + k_3[\text{Cdh20}]) \frac{1 - [\text{Cdh1}]}{J_3 + 1 - [\text{Cdh1}]} - V_4 \frac{[\text{Cdh1}]}{J_4 + [\text{Cdh1}]} \quad (12)$$

$$\frac{d[\text{Cdc20}_T]}{dt} = \varepsilon(k'_{11} + k_{11}[\text{CycB}]) - k_{12}[\text{Cdc20}_T] \quad (13)$$

$$\frac{d[\text{Cdc20}]}{dt} = k_{13}[\text{IEP}] \frac{[\text{Cdc20}_T] - [\text{Cdc20}]}{J_{13} + [\text{Cdc20}_T] - [\text{Cdc20}]} - k_{14} \frac{[\text{Cdc20}]}{J_4 + [\text{Cdc20}]} - k_{12}[\text{Cdc20}] \quad (14)$$

$$\frac{d[\text{PPX}]}{dt} = \varepsilon k_{33} - k_{34}[\text{PPX}] \quad (15)$$

$$\frac{d[\text{IEP}]}{dt} = k_{31}[\text{CycB}] \frac{1 - [\text{IEP}]}{J_{31} + 1 - [\text{IEP}]} - k_{32}[\text{PPX}] \frac{[\text{IEP}]}{J_{32} + [\text{IEP}]} \quad (16)$$

$$\frac{d[\text{GM}]}{dt} = k_{27}[\text{mass}]H\left(\frac{[\text{Rb}_{hyp}]}{[\text{Rb}_T]}\right) - k_{28}[\text{GM}] \quad (17)$$

$$\frac{d[\text{mass}]}{dt} = \varepsilon \mu [\text{GM}] \quad (18)$$

Steady-state relations

$$[\text{PP1}_A] = \frac{[\text{PP1}_T]}{1 + K_{21}(\phi_E([\text{CycE}] + [\text{CycA}]) + \phi_B[\text{CycB}])} \quad (19)$$

$$[\text{Rb}_{hyp}] = \frac{[\text{Rb}_T]}{1 + \frac{k_{20}(\lambda_D[\text{CycD}_T] + \lambda_E[\text{CycE}] + \lambda_A[\text{CycA}] + \lambda_B[\text{CycB}])}{k'_{19}([\text{PP1}_T] - [\text{PP1}_A]) + k_{19}[\text{PP1}_A]}} \quad (20)$$

$$[\text{E2F}_A] = \frac{([\text{E2F}_T] - [\text{E2F} : \text{Rb}])([\text{E2F}]}{[\text{E2F}_T]} \quad (21)$$

Table 3 (Continued)

$$[\text{E2F} : \text{Rb}] = \frac{2[\text{E2F}_T][\text{Rb}_{\text{hypo}}]}{[\text{E2F}_T] + [\text{Rb}_{\text{hypo}}] + L + \sqrt{([\text{E2F}_T] + [\text{Rb}_{\text{hypo}}] + L)^2 - 4[\text{E2F}_T][\text{Rb}_{\text{hypo}}]}} \quad (22)$$

*Definitions*

$$V_2 = k_2'(1 - [\text{Cdh1}]) + k_2[\text{Cdh1}] + k_2''[\text{Cdc20}] \quad (23)$$

$$V_4 = k_4(\gamma_A[\text{CycA}] + \gamma_B[\text{CycB}]) \quad (24)$$

$$V_6 = k_6' + k_6(\eta_E[\text{CycE}] + \eta_A[\text{CycA}] + \eta_B[\text{CycB}]) \quad (25)$$

$$V_8 = k_8' \frac{k_8(\psi_E([\text{CycE}] + [\text{CycA}]) + \psi_B[\text{CycB}])}{J_8 + [\text{cycE}_T]} \quad (26)$$

$$L = \frac{k_{26r}}{k_{26}} + \frac{k_{20}}{k_{26}}(\lambda_D[\text{CycD}] + \lambda_E[\text{CycE}] + \lambda_A[\text{CycA}] + \lambda_B[\text{CycB}]) \quad (27)$$

*Rate constants ( $h^{-1}$ )*

$k_1' = 0.1$ ,  $k_1 = 0.6$ ,  $k_2' = 0.05$ ,  $k_2 = 20$ ,  $k_2'' = 1$ ,  $k_3' = 7.5$ ,  $k_3 = 140$ ,  $k_4 = 40$ ,  $k_5 = 20$ ,  $k_6' = 10$ ,  $k_6 = 100$ ,  $k_7' = 0$ ,  $k_7 = 0.6$ ,  $k_8' = 0.1$ ,  $k_8 = 2$ ,  $k_9 = 2.5$ ,  $k_{10} = 5$ ,  $k_{11}' = 0$ ,  $k_{11} = 1.5$ ,  $k_{12} = 1.5$ ,  $k_{13} = 5$ ,  $k_{14} = 2.5$ ,  $k_{15} = 0.25$ ,  $k_{16} = 0.25$ ,  $k_{17}' = 0.35$ ,  $k_{17} = 10$ ,  $k_{18} = 10$ ,  $k_{19}' = 0$ ,  $k_{19} = 20$ ,  $k_{20} = 10$ ,  $k_{22} = 1$ ,  $k_{23}' = 0.005$ ,  $k_{23} = 1$ ,  $k_{24} = 1000$ ,  $k_{24r} = 10$ ,  $k_{25} = 1000$ ,  $k_{25r} = 10$ ,  $k_{26} = 10.000$ ,  $k_{26r} = 200$ ,  $k_{27} = 0.2$ ,  $k_{28} = 0.2$ ,  $k_{29} = 0.05$ ,  $k_{30} = 20$ ,  $k_{31} = 0.7$ ,  $k_{32} = 1.8$ ,  $k_{33} = 0.05$ ,  $k_{34} = 0.05$ ,  $\mu = 0.061$

*Dimensionless constants*

$J_1 = 0.1$ ,  $J_3 = J_4 = 0.01$ ,  $J_8 = 0.1$ ,  $J_{13} = 0.005$ ,  $J_{14} = 0.005$ ,  $J_{15} = 0.1$ ,  $J_{17} = 0.3$ ,  $J_{31} = J_{32} = 0.01$ ,  $K_{21} = 1$ ,  $[\text{E2F}_T] = 5$ ,  $[\text{PP1}_T] = 1$ ,  $[\text{Rb}_T] = 10$ ,  $\phi_E = 25$ ,  $\phi_B = 2$ ,  $\gamma_A = 0.3$ ,  $\gamma_B = 1$ ,  $\eta_E = 0.5$ ,  $\eta_A = 0.5$ ,  $\eta_B = 1$ ,  $\lambda_D = 3.3$ ,  $\lambda_E = 5$ ,  $\lambda_A = 3$ ,  $\lambda_B = 5$ ,  $\psi_E = 1$ ,  $\psi_B = 0.05$ ,  $\varepsilon = 1$

*Notes on equations*

We write DEs for proteins only and neglect mRNAs. That is, we assume rapid message turnover, so that mRNAs are always in steady state. As a consequence, although the rate of synthesis of each protein is proportional to the level of its message,  $[\text{mRNA}]$  never shows up in the equations. All the rate-of-synthesis terms for proteins have a factor  $\varepsilon$ , which represents the translation efficiency of the ribosomes.  $\varepsilon$  is a number between 0 and 1; its value is influenced by growth factors and by translation inhibitors like cycloheximide

(3), (5), (7), (11). We assume that all the Cdks are in excess over their cyclin partners, so their concentrations are not rate-limiting in the formation of cyclin/Cdk complexes. For this reason, the concentrations of Cdks do not show up in the equations, and each cyclin/Cdk complex is named for its cyclin subunit

(9) We do not write an extra DE for the phosphorylated form of Kip1, because we assume that it is rapidly ubiquitinated and degraded

(10)  $[\text{E2F}]$  is the total concentration of unphosphorylated E2F (free E2F, and E2F complexed with Rb)

(15) IE is an “intermediary enzyme” that creates a time delay between CycB accumulation and Cdc20 activation. IEP is dephosphorylated by a phosphatase whose basal activity  $= k_{32}$ . We assume that the activity of this phosphatase (in the nucleus, where it opposes the action of CycB/Cdk1) is proportional to translation efficiency,  $\varepsilon$ . Hence, when  $\varepsilon$  drops to 0.5, both kinase and phosphatase acting on IE are halved

(16)  $H([\text{Rb}_{\text{hypo}}]/[\text{Rb}_T])$  is a Heaviside function, which equals 0, if  $[\text{Rb}_{\text{hypo}}]/[\text{Rb}_T] > 0.8$ , or 1, if  $[\text{Rb}_{\text{hypo}}]/[\text{Rb}_T] \leq 0.8$

(17) We assume that a cell divides,  $[\text{mass}] \rightarrow [\text{mass}]/2$ , when  $[\text{Cdh1}]$  crosses 0.2 from below

(18)  $\text{PP1}_A$  is the active (dephosphorylated) form of PP1.  $[\text{CycE}] = [\text{CycE}_T] - [\text{CycE}:\text{Kip1}]$

(19)  $[\text{Rb}_{\text{hypo}}]$  is the total concentration of dephosphorylated forms of Rb, including complexes with E2F and E2FP.  $[\text{CycD}_T] = [\text{CycD}] + [\text{CycD}:\text{Kip1}]$ , because binding of Kip1 does not inhibit CycD/Cdk4 complexes

(20)  $\text{E2F}_A$  is the active form of E2F, i.e. unphosphorylated and not complexed with Rb

(21)  $[\text{E2F}:\text{Rb}]$  represents E2F (either phosphorylated or unphosphorylated) complexed with Rb

CycE/Cdk2, with CycA/Cdk2 and CycB/Cdk1 keeping PP1 phosphorylated until the end of mitosis (Mittnacht, 1998). Down-regulation of PP1 activity could be an essential event at the restriction point, since constitutively active PP1 arrests proliferation of  $\text{Rb}^+$  cells (Berndt et al., 1997). In this model, the total concentration of PP1 ( $\text{PP1}_T$ ) is constant throughout the cycle and distributed between more active ( $\text{PP1}_A$ ) and less active forms ( $\text{PP1}_T - \text{PP1}_A$ ) in a Cdk-dependent manner (see Eq. (19)). Furthermore, the phosphorylation and dephosphorylation of Rb is assumed to be fast enough that the

hypo- and hyper-phosphorylated forms are always in equilibrium (Eq. (20)).

Another role of Rb is to repress the transcription of housekeeping genes by inhibiting RNA polymerases I and III (White, 1997). By this mechanism, Rb interferes with general cell growth as well as the synthesis of E2F-specific gene products. To model this effect, Eq. (17) describes the production of general machinery (GM) for protein synthesis in an Rb-dependent manner, which machinery is then used (Eq. (18)) to increase the overall mass of the cell.

### 3.4. Antagonism between Kip1 and cyclins A and E

p27<sup>Kip1</sup> binds to CycA/Cdk2 and CycE/Cdk2 dimers to form inactive trimers (Sherr and Roberts, 1999). In the model, Kip1 is synthesized at a constant rate, consistent with the observed constant level of Kip1 mRNA (Hengst and Reed, 1996). Degradation of Kip1 depends on ubiquitination by a protein complex called the SCF (Amati and Vlach, 1999). Cdk-catalysed phosphorylation of threonine-187 of Kip1 promotes its ubiquitination (Sheaff et al., 1997; Vlach et al., 1997). CycA-, CycE- and CycB-complexes are allowed to phosphorylate Kip1 (although with different efficiencies—see Eq. (25)). Clearly, Kip1 and the CycA- and CycE-complexes are mutual antagonists (Sheaff et al., 1997), much like Cdh1/APC and CycB/Cdk1. Either Kip1 is abundant and the cyclins are inactive, or Kip1 is absent and at least one of the cyclins is active.

During G1 phase, several events occur in succession. First, CycD/Cdk4 helps to rid the cell of the enemies of CycA and CycE (Sherr and Roberts, 1999), by phosphorylating Rb and by binding to and titrating away Kip1. (Notice that CycD/Cdk4, though it binds Kip1, is not inhibited by Kip1 (Blain et al., 1997).) A little help from CycD is enough to allow rapid, autocatalytic rise of cyclins A and E, as they phosphorylate Kip1 and Rb, thus destroying their inhibitor and turning on their own transcription factor, E2F. At this stage, the cell is past the restriction point, as we shall see. As CycA rises, it initiates DNA synthesis and turns off Cdh1, allowing CycB to accumulate, so that the cell will eventually be able to enter mitosis.

Ubiquitin-mediated degradation of CycE is also a phosphorylation-dependent process (Clurman et al., 1996; Won and Reed, 1996), mediated presumably by CycE/Cdk2 itself and perhaps other Cdk complexes (Eq. (26)). In addition, cyclins A and B have a negative effect on E2F-dependent transcription, by phosphorylating DP1 (the partner of E2F) and thereby down-regulating synthesis of cyclins A and E (Krek et al., 1994). Rather than introduce another variable for DP1, the model equations regulate E2F by phosphorylation; Eq. (10) in Table 3. Rb is assumed to bind to both unphosphorylated and phosphorylated forms of E2F, and only the free, unphosphorylated form of E2F is transcriptionally active, Eq. (21). (In these equations, the concentration of all forms of E2F is  $E2F_T$ , of all non-phosphorylated forms is  $E2F$ , of all phosphorylated forms is  $E2F_T - E2F$ , and of the only active form is  $E2F_A$ .) Notice that, when E2F turns off, CycE level will drop precipitously because it is heavily phosphorylated, but CycA- and CycB-associated kinases will take over CycE's role in phosphorylating Rb and Kip1.

## 4. Results

### 4.1. Simulation of the normal cell cycle

Fig. 2 shows numerically simulated cell cycles of normal mammalian cells, growing exponentially in the presence of GFs, based on the differential equations and parameter values in Table 3. The parameter values used here are inherited, for the most part, from our earlier models of yeast and frog cell cycles. In those cases, the kinetic parameters were carefully estimated by comparison of model simulations to numerous experimental observations, including some quantitative time-course measurements. For details, see especially Marlovits et al. (1998), Chen et al. (2000), and Zwolak et al. (2004). For those parts of the mechanism novel to mammalian cells (CycD, Rb, E2F, ERG, DRG), the kinetic constants were adjusted to bring our simulations into quantitative agreement with data from Zetterberg's group, as described below. We propose these parameter values as a first guess of the effective rate constants for this skeleton model of the mammalian cell cycle. Of course, as the model matures, through comparison with other characteristic features of mammalian cell proliferation, we can expect that the parameter values will evolve along with the wiring diagram.

Referring to Fig. 2, we see that, for a newborn cell ( $t = 0$ ) in early G1, CycD/Cdk4 represents the only kinase phosphorylating Rb, and cyclins A, B and E all lose to their antagonists, Kip1, Rb and Cdh1. However, CycE synthesis is increasing because E2F is slowly recovering from its phosphorylated state caused by CycA- and CycB-kinase in the previous cycle ( $t < 0$ ). As a consequence, at approximately 3 h after cell division, Rb phosphorylation by CycD-kinase (fixed level) and CycE-kinase (increasing level) reaches a threshold, and the positive-feedback loop in CycE transcription turns on. The explosive rise in CycE-dependent kinase activity is reinforced by the simultaneous elimination of its inhibitor Kip1 (a second positive-feedback loop). Because E2F is active at this time, CycA rises and turns off Cdh1 at about half-way through the cycle. Together, CycA- and E-dependent kinases stimulate DNA synthesis. For lack of a better event marker, we presume that the G1/S transition is roughly coincident with the inactivation of Cdh1. Somewhat later, high CycB-kinase activity drives the cell into mitosis. After a suitable time delay, introduced by the intermediary enzyme (IE), Cdc20 is activated, enabling cells to exit from mitosis. The cycle then repeats itself.

An essential feature of this model is balanced growth and division, i.e. interdivision time = mass doubling time. In the model, the rate of mass increase is determined by the level of "general machinery;" hence, by the dynamics of Rb and in turn by the presence or absence of GF. No matter how fast or slow cells are

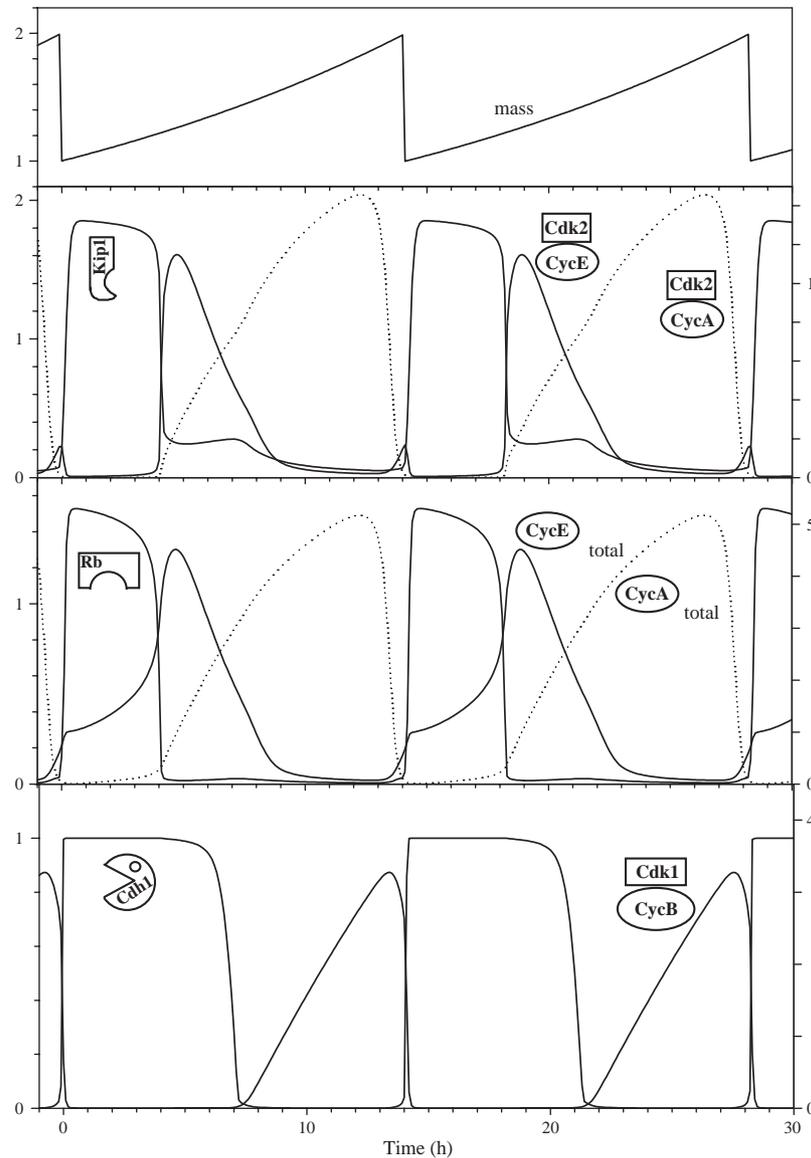


Fig. 2. Numerical simulation of the mammalian cell cycle. Two full cell cycles are shown. Cells are accumulating cytoplasmic mass exponentially (panel A) and dividing when Cdc20 and Cdh1 are activated at the end of the cycle. The curves in the lower panels represent the cellular concentration of different cell-cycle regulators. Panel B: total Kip1 level (free and in complex with cyclins A, D and E), and the active forms of CycA- and CycE-associated kinase (not in complex with Kip1). Panel C: total CycA and CycE levels (dimers with Cdk2 and trimers with Cdk2 and Kip1), and the hypophosphorylated form of Rb (free and in complex with E2F). Panel D: active form of Cdh1, and CycB/Cdk1 level (only one form in this model).

growing, cell division is always driven by a doubling of mass. Growth and division are connected by a built-in requirement that cells reach a critical size before starting DNA synthesis. If mass at birth is larger than average (one arbitrary unit), then cells have a shorter cycle time than average (14 h). If smaller, then the cycle time is longer. Consistent with experimental data (Killander and Zetterberg, 1965), size-control operates at the G1/S boundary, when Cdh1/APC is inactivated by rising CycA-kinase (Fig. 3A).

Cell size enters the model through the “mass” factor in the synthesis of CycA (Eq. (7) in Table 3). This factor causes the nuclear concentration of CycA to increase as

the cell grows, thereby introducing size control at the G1/S boundary. This feature of size control is necessary in our model to explain the results of Zetterberg and Larsson, to be described in the next section. We believe that some coupling between cell growth and progression through the chromosome cycle (DNA synthesis and mitosis) is crucial for an understanding of the proliferation of normal and cancerous cells. However, we recognize that size control of the mammalian cell division cycle has been a contentious issue for years, and therefore we refer the interested reader to other points of view (Brooks, 1981; Baserga, 1984; Conlon and Raff, 2003).

#### 4.2. Zetterberg–Larsson experiments

When proliferating cells are treated with cycloheximide (an inhibitor of protein synthesis), they stop dividing. Cells treated early in G1 stop immediately, whereas cells treated more than a few hours after division complete the current cycle and stop in G1 of the next cycle. The “point of no return” was called the restriction point by Pardee (1989). In our model, the restriction point is about 3 h after division (Fig. 3B). Withdrawing GFs from the culture medium also shows a point-of-no-return, which seems to be identical to the restriction point for cycloheximide treatment. If GFs are added back (or cycloheximide removed), cells re-enter the division cycle after a considerable delay.

To accurately measure the timing of events around the restriction point, Zetterberg and Larsson (1995) cultivated mouse fibroblast cells under a photomicroscope, measuring the cycle times of individual cells in response to transient deprivation of GF at different stages in the cycle. In continuous presence of GF, the cells divided (on average) every 14 h, spending 7 h in G1 phase and 7 h in S/G2/M. Cells that were deprived transiently of GF in the first 3 h after cell division experienced a long delay of the next cell division (delay = duration of GF deprivation + 8 h), whereas cells deprived after 4 h experienced no delay of the next division. These observations led Zetterberg and Larsson to split G1 into two subphases, G1pm and G1ps (“post-mitosis” and “pre-S”), with the dividing line being the restriction point at 3–4 h into G1.

GF deprivation causes a general two-fold depression in the rate of protein synthesis, and this depression seems to be responsible for the characteristic cell-cycle response, because the same response is induced by a sublethal dose of cycloheximide (CHX) that causes a 50% decrease in overall rate of protein synthesis (Zetterberg and Larsson, 1985). For this reason, the Zetterberg–Larsson experiments are modeled by reducing the rate of translational efficiency on ribosomes to 50% ( $\varepsilon = 0.5$ ). Because GFs undoubtedly have more specific roles than general support of protein synthesis, our simulations cannot be expected to reproduce all the subtle responses of cells to GF withdrawal.

When translational efficiency ( $\varepsilon$ ) drops below 0.6, the stable steady state of high DRG activity is lost and DRG is rapidly destroyed (half-life = 4 min). With DRG gone, CycD synthesis turns off, and then, because CycD is also unstable (half-life = 8 min), CycD-kinase activity disappears quickly. For cells in G1pm, when CycD/Cdk4 is the only kinase present, the loss of CycD has profound consequences. Rb cannot be inactivated and Kip1 cannot be degraded, so cells in G1pm cannot enter G1ps.

Figs. 4A and B show numerical simulations of 1 h treatments (GF deprivation or CHX exposure), in the same format as Fig. 2 of (Larsson et al., 1985). Cells treated early in the cycle (age 3 h or less at the onset of treatment), experience an 8.5–9.5 h delay of their first post-treatment mitosis, but their second cycle is normal. By contrast, cells treated later in the cycle (age > 3 h) are not delayed in their first post-treatment division, but

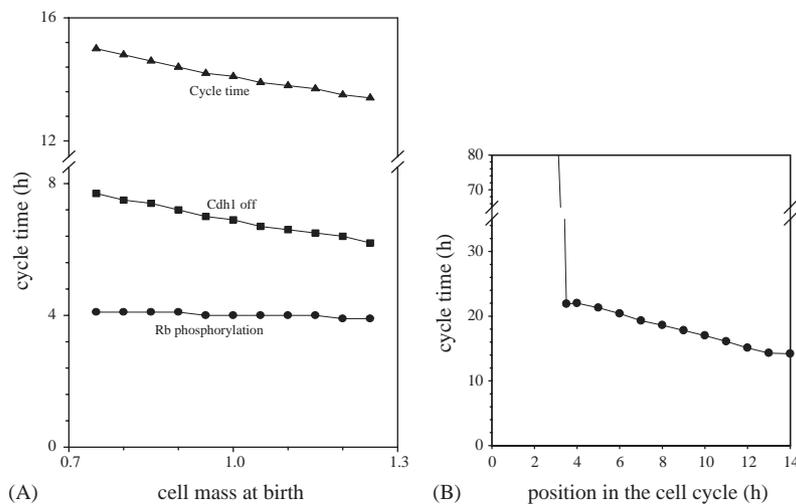


Fig. 3. (A) Timing of cell-cycle events as a function of birth size. Rb is phosphorylated 4 h after birth, regardless of birth size. By contrast, Cdh1 phosphorylation (which we associate with the onset of DNA synthesis) occurs earlier in cells that are larger at birth. Interdivision time is also shorter in cells larger at birth; hence, cell size at birth regresses to the mean (size control). The black symbols denote the time after birth ( $t = 0$ ) at which a specific event occurs in a model simulation: triangle, cell division; square, Cdh1 phosphorylated 50%; circle, Rb phosphorylated 50%. (B) Effects of continuous cycloheximide treatments. At increasing times after birth, cells are exposed to a sublethal dose of cycloheximide (translation rate reduced by 50%). If the treatment is started less than 3.5 h after birth, the cell is prevented from dividing. After 3.5 h, the treatment cannot block the next cell division. Compare to (Pardee, 1989). The black circles denote the results of model simulations; they are connected by straight lines to indicate the trend in the simulations.

some of them experience significant lengthening of their second mitotic cycle. By comparing Figs. 2 and 3, we see that passage through the restriction point coincides with phosphorylation of Rb (Bartek et al., 1996; Planas-Silva and Weinberg, 1997).

The fixed time to reach the restriction point (3 h) is determined by the kinetic constants governing the interactions among CycD, CycE, Rb, E2F, and Kip1. During that interval, as E2F is dephosphorylated and CycD helps to phosphorylate Rb, CycE begins to form. Prior to 3 h, if CycD is lost, there is not yet enough active CycE to carry on the job of phosphorylating Rb, so E2F remains bound to Rb and inactive, and CycE synthesis shuts off. After 3 h, there is enough CycE to keep Rb phosphorylated and to destroy Kip1, even if CycD disappears.

Longer treatments leave the position of the restriction point unchanged, but the delay experienced by G1pm cells is always 7–8 h longer than the duration of treatment (calculations not shown), as observed (Zetterberg and Larsson, 1985).

Fig. 5 shows simulations for 1 h treatments applied in early G1 and G2 phases. In both cases, the level of CycD drops quickly to zero and returns to its normal steady-state value about 8 h after the treatment ends. However, loss of CycD has very different effects in different phases of the cycle. In G1pm cells, CycD/Cdk4 is the only kinase able to phosphorylate Rb. When CycD level drops due to treatment, Rb is immediately dephosphorylated and consequently inactivates its targeted transcription factors. Cell growth slows, and cells enter the G0 state. Since it takes about 8 h after the end of treatment for CycD to come back and phosphorylate

Rb again, the first cycle is delayed by 8 h. The second cycle will be normal.

In G2 cells, on the other hand, other cyclin/Cdk complexes are present, and they dominate over Rb, Kip1 and APC even in the absence of CycD. For this reason, G2-treated cells continue their progress to cell division and keep growing until they finish their cycle. At the end of mitosis, when CycA and CycB get degraded and CycD is still absent (<8 h since GF readdition), Rb becomes hypophosphorylated and cell growth is repressed until CycD returns. This period of reduced growth induces a delay in the second mitotic cycle. The later are cells treated in their first cycle, the longer will be the delay in their second cycle.

### 4.3. Cell cycle mutants

#### 4.3.1. $Rb^-$

Cell lacking Rb ( $Rb_T=0$ ) are about half the size of normal cells (see Fig. 6,  $0 < t < 15$  h); Herrera et al. (1996) reported that  $Rb^-$  cells are 40% smaller than  $Rb^+$  cells. Unlike wild-type cells,  $Rb^-$  cells are not arrested by sublethal doses of CHX (Herrera et al., 1996). Reducing  $\varepsilon$  by 50% when  $Rb_T=0$  does not inhibit cell cycling (Fig. 6,  $t > 15$  h), because, in the absence of Rb, CycD is unnecessary (Bartek et al., 1996). As CycB disappears after cell division, E2F turns on immediately because there is no Rb to inhibit it; hence, CycE appears soon after birth and phosphorylates Kip1. Twelve hours later, the cell has accumulated enough CycA to inactivate Cdh1 and commit to a new round of DNA synthesis and division. The only effect is a lengthening of all phases of the cell cycle, because

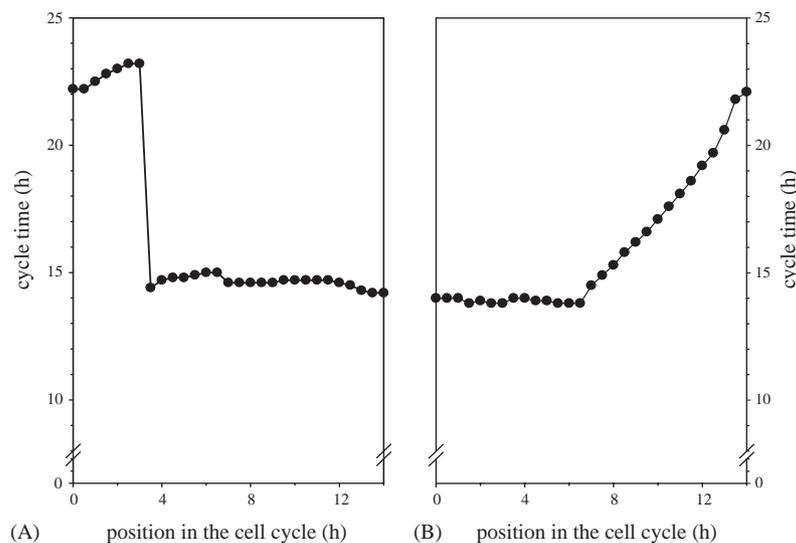


Fig. 4. The effect on cell-cycle progression of transient deprivation of GF or transient exposure to cycloheximide. At different time points during the cell cycle, the value of  $\varepsilon$  was temporarily decreased from 1 to 0.5 for 1 h. The lengths of the first (A) and the second cycles (B) were determined and plotted as functions of cell age at onset of the treatment, each black circle representing one simulation. These simulations should be compared to the experimental observations in Zetterberg and Larsson (1985), Fig. 5, and in Larsson et al. (1985), Fig. 2.

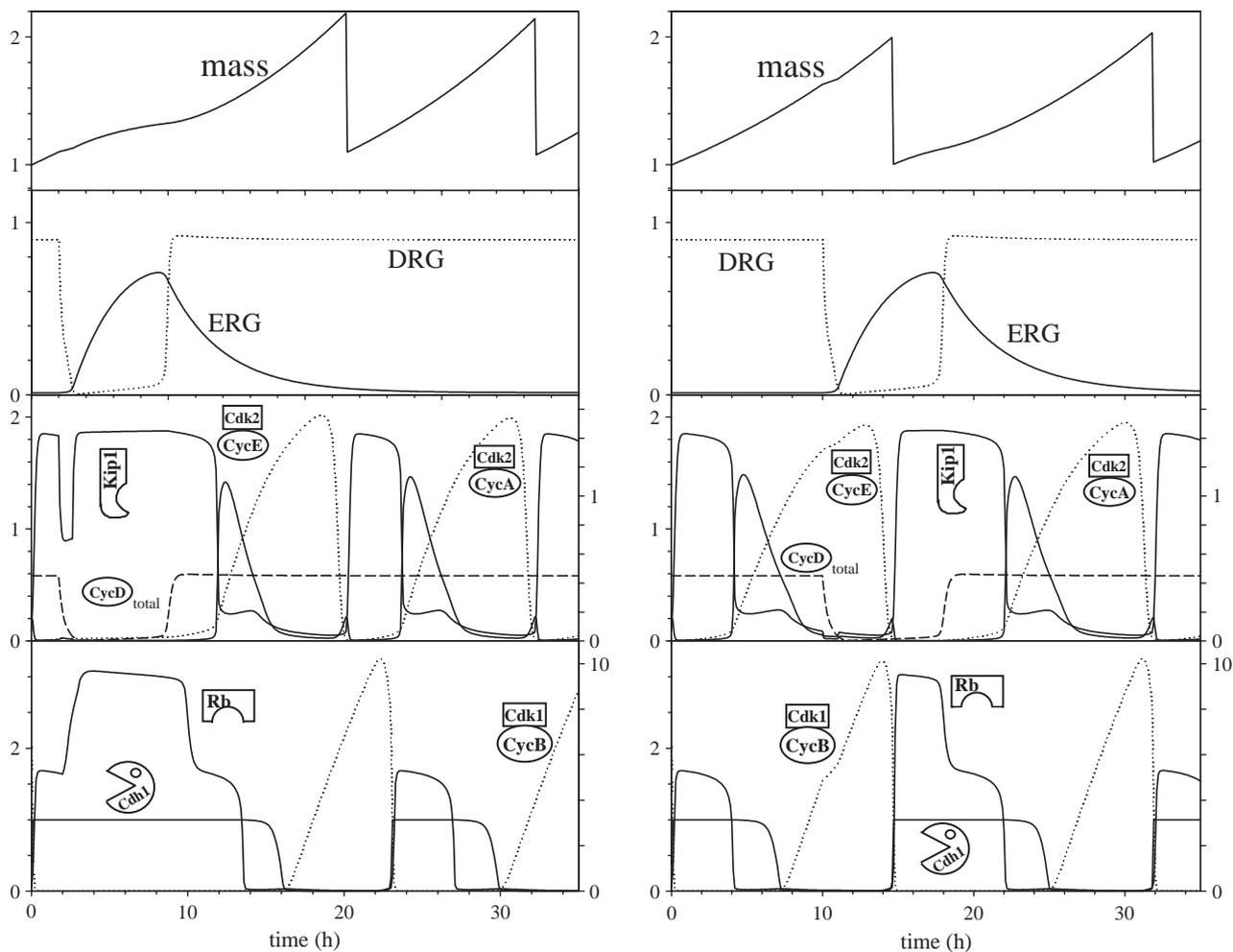


Fig. 5. The effect of 1-h exposure to cycloheximide on early G1 cells and G2 cells.  $\epsilon$  is reduced from 1 to 0.5 between 2 and 3 h after birth (left panel), or 10–11 h after birth (right panel).

overall cell growth is slower in constant low dose of CHX.

Although  $Rb^-$  cells lack any restriction point for protein synthesis inhibition, their proliferation can be stopped by GF deprivation (Herrera et al., 1996), suggesting that GFs have other effects on the cell-cycle engine, mediated presumably by Rb-related proteins, p130 and p107. If all three “pocket proteins” (Rb, p130 and p107) are deleted, then cells undergo significant proliferation in the absence of GFs (Sage et al., 2000).

#### 4.3.2. $Kip1^-$

In the simulated mutant ( $k_5 = 0$ ), without Kip1 to inhibit CycE in early G1, Rb is rapidly inactivated, allowing CycE to be synthesized even faster. As a consequence,  $Kip1^-$  cells lack the G1pm phase. In simulations (not shown), cells deprived of GF or treated with CHX keep on proliferating, which agrees with the observation that  $Kip1^-$  cells are less serum-dependent than  $Kip1^+$  cells (Coats et al., 1996; Rivard et al., 1996).

#### 4.3.3. $CycE^{op}$

Overexpression of CycE decreases cell size, shortens the duration of G1, and diminishes the serum requirement for the G1/S transition (Ohtsubo and Roberts, 1993; Ohtsubo et al., 1995). In the model (simulations not shown), excess production of CycE ( $k_7 = 0.15$ ) quickly titrates away Kip1 and then inactivates Rb, without needing help from CycD (i.e. GF-independent). Furthermore, higher concentration of CycE means that APC is inactivated at a smaller size. If the G2/M transition were also size dependent, as it should be, then G2 phase would be extended and G1 shortened.

Although  $CycE^{op}$  cells and  $Kip1^-$  cells are less serum-dependent than normal cells, they still require low levels of GFs (Ohtsubo and Roberts, 1993; Coats et al., 1996). Our model does not capture this dependence, presumably because it does not yet incorporate all the redundant proteins (multiple cyclins, Rb-like proteins, and stoichiometric inhibitors) in the regulatory network (Kohn, 1999).

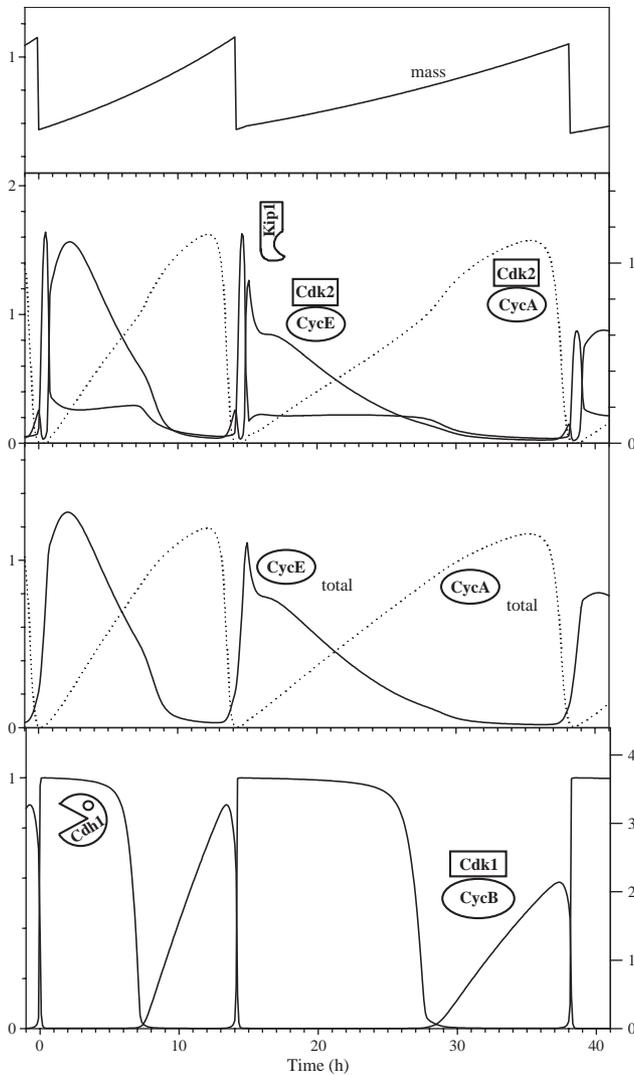


Fig. 6. Cells lacking Rb are not blocked by cycloheximide. In this simulation,  $Rb_T=0$ . During the first cycle ( $0 < t < 15$  h), growth is normal ( $\varepsilon = 1$ ). At  $t = 15$  h,  $\varepsilon$  is reduced to 0.5; the cell continues to divide, although its cycle time is twice as long because its growth is half as fast.

## 5. Discussion

A simple, realistic molecular mechanism for restriction point control of the mammalian cell cycle, when translated into a computational model, can account for the response of cells to transient exposure to cycloheximide and for the loss of restriction point control in certain mutants. The model is based on the idea that G1 events are driven by the “switching” properties of positive-feedback loops and antagonistic interactions (Tyson et al., 1995; Kohn, 1998; Novak et al., 1998b; Aguda, 1999; Thron, 1999).

### 5.1. The restriction point

Our account of the Zetterberg–Larsson experiments differs considerably from Cooper’s (1998). Cooper

argues that the timing of the first and second cycles after GF deprivation (or CHX treatment) can be explained by a simple model of exponential growth plus a cell-size requirement at the restriction point (RP), analogous to models of bacterial cell cycles (Donachie, 1968). Cells treated before the RP are not yet committed to S/G2/M, so, if their growth is interrupted, they experience a long delay before cell growth is resumed and they can complete the first cycle. Their second cycle is unaffected because they are now properly sized and normally growing. Cells treated after the RP are already committed to S/G2/M, so they divide on schedule (no delay). But, because their growth was interrupted during the treatment, they divide at an abnormally small size and produce small daughter cells, which have an extended interdivision time (second cycle), because it takes them longer than normal to grow to the critical size for RP. In Cooper’s scenario, all cells are delayed by an equal amount (whether treatment occurs before or after the RP); the only difference is whether the delay shows up in the first or second division after treatment.

We share with Cooper only the conviction that the interaction between cell growth and commitment to cell division must be understood in order to make sense of the Zetterberg–Larsson experiments. We do not agree with his proposal that treatment interferes with cell growth equally at all stages in the cell cycle. Contrary to Cooper’s depiction (Fig. 2 of Cooper, 1998), the experimental results of Larsson et al. (1985), Fig. 2 show clearly that (i) the second-cycle delay experienced by post-RP cells increases as treatment is given later in the cycle, and (ii) cells treated briefly just after RP experience no delay in either the first or second cycles. Our simulations (Fig. 4) are consistent with both observations. In our model, it is not the growth-response that is the same at all stages in the cell cycle, but the CycD-response. Treatment at any cell age causes an abrupt disappearance of CycD, which does not reappear until about 8 h after reversal of treatment (see Fig. 5). How cell growth responds to the absence of CycD depends on cell cycle stage and the phosphorylation state of Rb. Between the RP and cell division, there is no excess inhibition of cell growth in our model because CycA, CycE and CycB keep Rb phosphorylated. Only between cell division and RP (when CycA, CycE and CycB are naturally missing), if CycD is also missing because of treatment, does most Rb become hypophosphorylated and the cell experiences prolonged depression of growth. Cells treated briefly just after RP never enter the non-growing state ( $[Rb_{hypo}]/[Rb_T] > 0.8$ ) and so experience little or no delay of either the first or second division. Cells treated ever later in the cycle carry ever more time into the next G1pm when CycD is missing,  $[Rb_{hypo}]$  is large, and growth is depressed, so they experience ever more delay of the second cell division.

We disagree with Cooper's contention that there are no distinctive molecular events in G1 phase. Our account of the Zetterberg–Larsson experiments relies on biochemical differences between G1pm and G1ps. Furthermore, we accept that G0 is a distinctly different state than G1. In our model, Cdks keep Rb phosphorylated in G1, so that the cell may grow. In G1pm, only CycD/Cdk4 is effective, so, if some treatment destroys CycD, then Rb becomes hypophosphorylated and cell growth is repressed (the G0 state, in our model). The cell can enter G0 only from G1pm, because, later in the cycle, cyclins E, A and B keep Rb phosphorylated.

Most cancer cells lack G0 (Zetterberg and Larsson, 1995). In response to GF withdrawal, they experience a perturbation in growth and division rates, but they do not leave the cell cycle. In our model, G0 corresponds to a deficit of cyclins and an excess of hypophosphorylated Rb. Therefore, cells can stay out of G0 if they overexpress cyclins or are missing Rb: two characteristic mutations associated with tumorigenesis (Sherr, 1996).

## 5.2. Size control

Fig. 1 bears superficial similarity to other published models of mammalian cell-cycle controls (Hatzimanikatis et al., 1995; Obeyesekere et al., 1997; Kohn, 1998; Aguda, 1999; Qu et al., 2003b), because everyone agrees on the principal molecular components and their interactions in G1 phase. Nonetheless, several features set our model apart. First of all, it is consciously designed to resemble the control system in yeast. Undoubtedly, the control of cell division in higher eukaryotes evolved from the simpler control systems in single-celled eukaryotes like yeast, by GF requirements grafting onto an underlying, yeast-like cell-cycle engine, in order to enforce the social constraints on cell proliferation necessary to multicellularity. Therefore, the way to make sense of the extreme complexity of components and interactions in mammalian cell division is to look for the underlying framework of yeast-like controls, and then to see how features unique to multicellularity are attached to this framework.

This model of the mammalian cell cycle inherits the G1 checkpoint controls designed into earlier yeast models. Progress through the cell cycle is intimately tied to cell growth by a size requirement for Cdh1 inactivation, about midway through the cycle. Unlike other mathematical descriptions (Hatzimanikatis et al., 1995; Obeyesekere et al., 1997), our model is not based on spontaneous limit-cycle oscillations. In the absence of GF, cell size is constant and the cell cycle is halted at a stable steady state (G0), with low cyclin levels, including cyclin D. In the presence of GF, various Cdks keep Rb phosphorylated, cell size increases exponentially, and interdivision time is identical to mass doubling time. The time from birth to division does

not depend on kinetic parameters of the Cdk control system, as in most models, but solely on the rate of mass increase. In this model, during the latter part of G1 phase, the cell must wait until it accumulates enough cyclin A to initiate S phase and to inactivate Cdh1. After Cdh1 is turned off, cyclin B accumulates and eventually drives the cell into mitosis. The model links cell size to the accumulation of cyclin A in the nucleus, and hence links mass doubling to progress through the cell cycle.

The size-controlled transition in our model occurs about the time of the G1/S transition, consistent with evidence from Zetterberg's group (Killander and Zetterberg, 1965; Zetterberg and Larsson, 1995). The RP is something entirely different: it occurs several hours earlier and is not size-controlled, occurring at a fixed time after birth, regardless of birth size (Zetterberg and Larsson, 1995). In our model, interactions that govern the RP are dissociated from the events that drive the G1/S transition. The RP is determined by activation of a cyclin-transcription factor (E2F) and degradation of a Cdk inhibitor (Kip1), events that occur a fixed time after cell birth, independent of cell size. The G1/S transition, on the other hand, is driven by accumulation of CycA, a process that is dependent on cell growth in our model. These differences are apparent in Fig. 3A, where the temporal location of the restriction point (when Rb is phosphorylated in the model) is seen to be independent of cell size at birth, whereas the initiation of DNA synthesis (when Cdh1/APC turns off in the model) occurs sooner in cells that are larger at birth.

## 6. Conclusion

The cell-cycle model presented here is grossly simplified from what is currently known about the molecules controlling DNA synthesis, mitosis and division in mammalian cells (Kohn, 1999). However, the full regulatory circuit is much too complex to be modeled computationally at present. Just as experimental characterization of the control system proceeded from simple, incomplete diagrams to increasingly complex and realistic circuitry, so a computational representation of the system must start with a simple "skeleton," capturing the basic topology of the network, on which later can be attached the complicated details that will make realistic models of specific cell types and physiological circumstances.

Our proposal for the skeleton of the mammalian cell-cycle control system (Fig. 1) is closely analogous to a model that has proved successful in accounting for most of the complexity of cell proliferation in budding yeast (Chen et al., 2000). In a whimsical sense, we are "getting in touch with our inner yeast." That is, from the complex machinery regulating mammalian cell proliferation, we are pulling out the underlying yeast-like

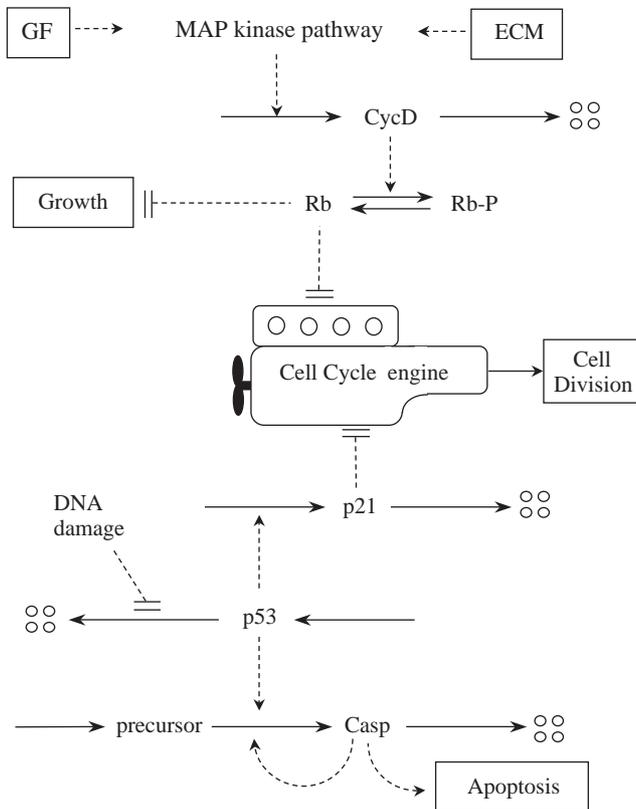


Fig. 7. Schematic diagram of some of the signal transduction pathways that control mammalian cell proliferation. The “cell-cycle engine” represents the interactions involving cyclins E, A and B in Fig. 1(A). Progress through the cell cycle ( $G1 \rightarrow S \rightarrow G2 \rightarrow M$ ) is repressed by two major negative regulators: Rb (which inhibits the transcription of cyclin genes) and p21 (a stoichiometric inhibitor of cyclin/Cdk complexes). The “default state” is Rb “on” and p21 “off”, i.e., no proliferation. In response to permissive signals from GF stimulation and extracellular matrix (ECM) attachments, mediated through MAP kinase pathways, the cell up regulates CycD-dependent kinase activity, which phosphorylates and inactivates Rb. Hence, these signals remove the brake on cell growth and division. Successful completion of the cell cycle depends now on “checkpoint” pathways that monitor DNA integrity (damage, incomplete replication, faulty chromosome alignment at metaphase). For example, DNA damage stabilizes a transcription factor, p53, whose accumulation drives the synthesis of p21. If the damage can be repaired, then p53 disappears, followed by disappearance of p21. If the damage cannot be repaired in a timely fashion, then a sustained high level of p53 seems to drive an irreversible activation of caspases (proteolytic enzymes that execute the cell death program).

controls, which presumably were inherited from the earliest ancestors of the eukarya. Then we are asking the question: what properties of mammalian cell division can be understood in terms of the basic cell-cycle controls that are common to most eukaryotic cells? One cannot expect a model at this beginning level to include everyone’s favorite protein, to explain everyone’s latest experiment, or even to predict some crucial experimental test of the theory. Rather, its function is to bring together in computer-readable form a reasonable picture of the basic molecular networks underlying cell division

in higher vertebrates. If the skeleton is sound, it should serve as a solid framework for building more realistic, comprehensive, predictive, computational models of the future.

In this paper, we have tested our vision of the basic molecular network (Fig. 1) against a classical set of experiments, carried out by Zetterberg and colleagues to characterize the restriction point in the mammalian cell cycle. Our yeast-like control system, supplemented by a crude representation of the growth-factor signaling pathway can account for all the unusual features of Zetterberg’s experiments. In future work, we intend to pursue our strategy for understanding the growth and division of mammalian cells, by adding more details to the underlying cell-cycle machinery and attaching additional signal-transduction pathways to regulate proliferation in response to internal and external cues (Fig. 7).

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