

Quantitative Analysis of a Cell Cycle Checkpoint Response in *Xenopus laevis* Cell-Free Egg Extracts

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August 10th, 2005
Blacksburg, Virginia

Thesis submitted to the Faculty of the
Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements for the degree of

Masters of Science
in Biology

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Keywords: cell cycle, cyclin-dependent kinase (Cdk), nucleocytoplasmic ratio, *Xenopus laevis*.

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Abstract:

In somatic cells, checkpoint pathways trigger cell cycle arrest in response to unreplicated or damaged DNA by inhibiting the activity of cyclin-dependent kinases (Cdks). In the *Xenopus laevis* embryo, checkpoints are not operational until the midblastula transition (MBT). Studies in cell-free egg extracts indicate that a threshold concentration of nuclei, which approximates the MBT concentration, is required to elicit a checkpoint. The checkpoint response to unreplicated DNA in the extract prevents transition into mitosis by inhibiting Cdk1/cyclin B, causing an increase in the minimum amount of cyclin B necessary to enter mitosis, termed the cyclin threshold. Once the threshold of cyclin is maintained or exceeded, the system will proceed into mitosis after a lag time. We have investigated the relationship between nuclear concentration and cell cycle regulation in the extract. By precisely regulating the concentration of cyclin B and nuclear content in extract samples, we have found 1) the concentration of nuclei affects cyclin B thresholds and lag time of entry into mitosis, 2) elevated cyclin thresholds caused by DNA replication blocks are further increased by increasing the concentration of nuclei, and 3) double-stranded DNA breaks in the extract system do not affect cyclin thresholds or lag time of entry into mitosis within the range of nuclear concentrations that can be efficiently replicated. This data provides evidence of the importance of the nucleocytoplasmic ratio in normal cell cycle progression and its importance for checkpoint acquisition during early *Xenopus laevis* development.

Acknowledgements

The work presented here would not have been possible without the wealth of knowledge and support provided to me by my peers and mentors here at Virginia Tech. As I entered the lab as an undergraduate researcher, I was very lucky to have graduate students willing to put up with my incessant string of questions. Matt Petrus was a great mentor and I never tired of hearing about his trips abroad, which he often liked to recite. Wei Sha was never too busy to answer any questions after I took over her project as I entered graduate school, and she laid the groundwork for the work I present here. Wei was aided by Tony Lassaletta, a very bright and enthusiastic undergraduate who provided many of the protein reagents used in my project. Matt Tormenti was an experienced undergraduate who was always ready to lend a hand or a pint. Many thanks also go out to Brian Wroble. He has been a great asset as both a friend and lab mate, and I greatly appreciate all the help he has given me. Many of our current undergraduates have also proven to be invaluable in the laboratory. Ken Tian and Nick Fingland are both astounding young men, of whom I expect nothing less than the very best in their future endeavors. Amit Dravid was my collaborator in charge of modifying the mathematical model. I thank him for all the patience he showed me when working on the mathematical model. Iris Siegle interned in our lab last spring and her bright young mind was a pure joy to work with. Many people have aided me in my years here at Virginia Tech, and I have truly loved my time here. My committee members have been instrumental in the compilation of the work presented here. I would like to thank Jill Sible, John Tyson, and Rich Walker for all of the help they have provided me over the years. A few more people that I would also like to mention are: Ayesha Carter, Dayna Wilhelm, Bob Johnson, Jason Clement and all of the wonderful people I have worked with in the lab over the years.

I have encountered many hurdles during the development of my research, and there has always been an endless supply of advice and encouragement from my advisor, Jill Sible. Her compassion as a professor is only exceeded by her intelligence as a researcher. Under her tutelage I have learned more than I could ever thank her for, and I will always cherish my time in her laboratory.

Finally, I would like to thank my family: my parents David and Cathy for their unending patience and love as well as my sisters Lindsey and Alicia and brother Alan. I love you all.

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Chapter 1: Introduction

Biology

In order to maintain a healthy multi-cellular organism capable of reproduction, individual cells within the organism are constantly dividing. New cell division is necessary for growth of the overall organism as well as renewal of dead or dying cells. Human skin is a good example of this, as we replace some 30,000-40,000 cells per minute (Hyde, 2004). These cells rely on signals within their own micro-environment to promote or prevent cell growth and division. The factors and machinery necessary for cell growth and division are very complex, and the control mechanisms differ between species, but the overall question is the same: is it advantageous for the organism to allow a particular cell to divide? If the answer is yes, and the cell growth machinery cannot respond appropriately, functions such as wound healing and skin regeneration will not take place. If the answer is no, and the cell's machinery proceeds with growth and division, aberrations such as tumors can develop. It is important for us to understand how cells answer this question so that we may one day help steer them in the right direction when they answer incorrectly.

The Cell Cycle

The cell cycle is a term used to describe the progression of steps necessary for a cell to replicate itself. Progression through the cell cycle is typically visualized as shown in Figure 1.1, with the formation of a new daughter cell after each cell cycle. Every trip around the circle represents a single cell cycle and the formation of a daughter cell. The

cell cycle is broken down into smaller steps, known as phases, indicated by G₀, G₁, S, G₂, and M in Figure 1.1. The three G phases are also known as gap or growth phases. G₀ is used to describe a quiescent phase where limited cell growth takes place and the cell is not progressing through the cell cycle. G₁ represents a time where the cell is metabolically active in order to prepare for DNA synthesis. S phase is characterized by the synthesis of a complete copy of the cell's genome (Lodish *et al.*, 2004). Proper progression of DNA synthesis during S phase is vital to ensure that each new daughter cell receives all the genomic tools necessary for survival. During the G₂ phase, the cell

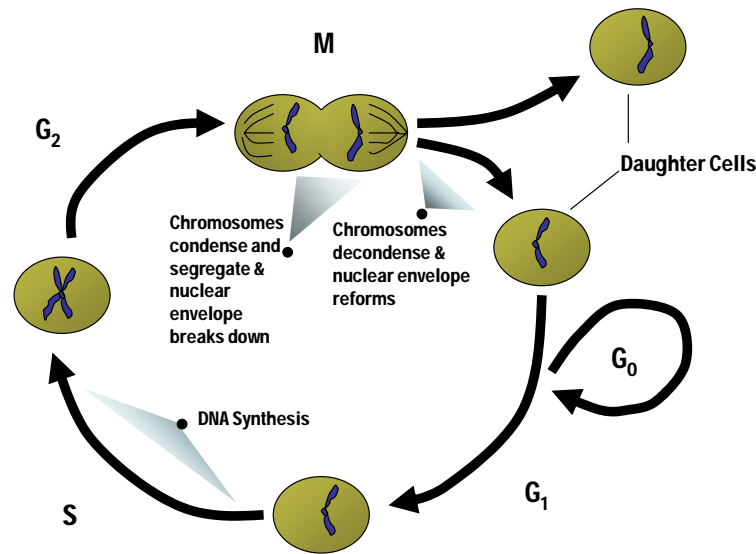


Figure 1.1 The somatic cell cycle. When a cell completes a trip around this circle, a new cell is formed. It is important for the cell to regulate the progression through each stage in order to ensure healthy daughter cells. Adapted from Lodish *et al.* (1999).

continues to grow and will manufacture the necessary components for mitosis. Once everything is in place in G₂, the cell rapidly enters mitosis or M phase. Once in M phase, the cell condenses its chromosomes and breaks down the nuclear envelope. Sister

chromatids then align on the mitotic spindle between the spindle poles on opposing sides of the cell and are pulled apart so that each new daughter cell will receive a full complement of genomic material. The cell completes the process by going through cytokinesis and reforming nuclear envelopes (Lodish *et al.*, 2004). Each of the two new cells may either continue the cell cycle or become quiescent in G₀.

When a problem arises during the cell cycle, it is normally sensed by the cell cycle checkpoint machinery, and the cell cycle is halted until the problem can be fixed. Cell cycle checkpoints are molecular signaling pathways that stop cell cycle progression. If the problem is irreparable due to large areas of damage to the DNA, the cell will die cleanly by a programmed cell death called apoptosis (Rich *et al.*, 1999). If a cell is unable to detect a problem such as DNA damage during the cell cycle and continues through multiple cell cycles, the amount of damage could compound with each spin around the cell cycle, losing and/or changing large amounts of genomic material. A primary mutation in the cell cycle checkpoint response machinery is usually the cause of a cell advancing through the cell cycle unregulated (Jacks and Weinberg, 1996; Neecke *et al.*, 1999). Mutations in a cell lacking a cell cycle checkpoint may be lethal if the mutation resides in a metabolic or other important signaling pathway, and the cell will eventually die by apoptosis or nonspecific death. In some instances, however, these cells, with an increased mutation rate, will acquire traits that are advantageous for cell growth and accelerate progression through the cell cycle (Hanahan and Weinberg, 2000). Cancer cells develop in this manner and have become a major contributor to human death as our lifespan increases. Because development of cancer depends on certain mutational events,

the longer humans live, the more likely we are to acquire the necessary mutations to form a tumor (Anderson, 2001).

The Cell Cycle during the Early Development of *Xenopus laevis*

Mature *Xenopus laevis* female frogs lay large volumes of eggs that are highly manipulatable and provide a good experimental system to study the cell cycle and how it is regulated. The size of newly laid eggs is approximately 1 mm in diameter, making them amenable for microinjection (Vize *et al.*, 1991). There are also well established protocols for making cytoplasmic extracts that contain all the necessary ingredients to complete multiple cell cycles (Murray, 1991).

During the first twelve cell cycles of the *Xenopus laevis* embryo, the cell cycle is rapid and synchronous as well as devoid of any transcription (Murray and Kirschner, 1989). In this way, the newly fertilized embryo relies completely on the RNA and protein inherited maternally to provide the tools necessary for early development. After fertilization, the cell cycle takes 90 minutes to produce two new daughter cells. The next 11 cell cycles each take 30 minutes, thus multiplying a two-cell embryo to 4096 cells in about six hours. These rapid cell cycles are devoid of the gap or growth G phases and oscillate between mitosis and S phase (interphase) (Newport and Kirschner, 1984; Frederick and Andrews, 1994). These rapid cell cycles also do not arrest at checkpoints in response to damaged or unreplicated DNA (Anderson *et al.*, 1997; Hensey and Gautier, 1997; Sible *et al.*, 1997; Carter and Sible, 2003).

Cell cycle checkpoints are active in healthy somatic cells and prevent cell cycle progression if conditions in the cell do not favor cell replication at a given time. These checkpoints are typically activated in response to damage found in the genomic material

of the cell (Furnari *et al.*, 1997; Nyberg *et al.*, 2002). After the twelfth division, the embryo enters the midblastula transition (MBT). During the MBT, the cell cycle is remodeled, becoming asynchronous with introduction of gap phases. At the MBT, transcription, cell motility, and the cell cycle checkpoint machinery also become functional (Newport and Dasso, 1989; Clute and Masui, 1997). Any damage incurred in the previous 12 divisions will activate an apoptotic program (Anderson *et al.*, 1997; Hensey and Gautier, 1997; Sible *et al.*, 1997; Stack and Newport, 1997; Carter and Sible, 2003). The switch from a rapidly dividing cell cycle with no operational cell cycle checkpoints to an extended cell cycle with checkpoints monitoring genomic integrity is somewhat the reverse of what occurs during tumorigenesis. The exact mechanism by which the cell senses it is time to start the MBT is not completely understood, but the nucleocytoplasmic ratio is thought to play a major role (Dasso and Newport, 1990; Howe and Newport, 1996), perhaps because DNA titrates a factor after 12 divisions (Newport and Kirschner, 1984). The early cell cycle in *Xenopus* embryos switch from rapid, synchronous, and unregulated cell cycles to that of asynchronous and regulated cell cycles at the MBT. These characteristics of the early development of *Xenopus laevis* and ease of manipulation render it a good model system for investigating cell cycle regulation.

Cyclins and Cyclin-Dependent Kinases

The advancement of the cell through each phase of the cell cycle is governed by specific cyclin-dependent kinases (Cdks) and their associated cyclins (Nigg, 2001). Cdks and cyclins form dimers that act as the molecular engines that drive the cell through the cell cycle (Guadagno and Newport, 1996; Arellano and Moreno, 1997; Krude *et al.*,

1997). The Cdk subunits remain at a constant level throughout the cell cycle, being primarily activated through association with their respective cyclins. The level of a cyclin at any specific time during the cell cycle is regulated stringently and usually oscillates through the cell cycle, hence the term “cyclin”. Cyclin proteins all have a destruction box motif on their amino terminus that targets them for ubiquitination and rapid degradation (Glotzer *et al.*, 1991). Once the Cdk/cyclin dimers form, they can also be regulated by a change in phosphorylation state as well as inhibited by Cdk inhibitors (CKIs) (Peter, 1997). The Cip family of CKIs (p21 and p27) inhibit Cdk/cyclin by binding the Cdk and preventing removal of inhibitory phosphate groups (Luo *et al.*, 1995; Su *et al.*, 1995; Shou and Dunphy, 1996). The INK family of CKIs (p15 and p16) also bind the Cdk subunit but cause degradation of the cyclin subunit (Sherr and Roberts, 1999). The regulation of cyclin levels and subsequent activity of Cdk/cyclin dimers is essential to prevent inappropriate progression through the cell cycle.

Checkpoints

There are requirements that need to be met in the cell before it is allowed to progress from one phase of the cell cycle to the next. In somatic cells, the cell must pass the restriction point and a DNA damage checkpoint in G1 to enter S phase. This requires exposure to specific growth factors in order to ensure regulated growth, an important control mechanism in multicellular organisms. Once in S phase, the cell must complete DNA synthesis before entering G2. The cell then enters G2, where it monitors the DNA for damage and completion of DNA replication. In G2, the cell also acquires the cell cycle machinery necessary to bypass the G2-M checkpoint and enter mitosis. The G2-M checkpoint that ensures genomic integrity is enforced through inhibition of a Cdk/cyclin

dimer called MPF or Mitosis Promoting Factor (Cdk1/ cyclin A/B). When conditions are permissive for M phase, and there are no genetic aberrations, MPF is rapidly activated through a positive feedback mechanism. When conditions are not permissive, MPF is inhibited from activation by several different mechanisms. Once in mitosis, the cell goes through two more checkpoints known as the metaphase and anaphase checkpoints. The metaphase checkpoint ensures proper alignment of the sister chromosomes along the metaphase plate. The anaphase checkpoint monitors for proper segregation of the chromosomes to either side of the (Gorbsky, 1997).

Somatic Cell Cycle Control

Normal plant and animal cells progressing through the cell cycle are controlled by similar molecular mechanisms. The proteins responsible for managing the cell cycle are highly conserved, especially in their active sites, across a variety of species (Lui, 2002). Some of the greatest advancements in biology over the last 50 years have been the discovery of the key proteins that drive the cell through the cell cycle (Masui and Markert, 1971).

Cdk/Cyclin Control in G1 Phase

During G phase, the somatic cell must increase cell mass, receive growth promoting factors, and have factors inhibiting growth removed. Also, during G1 phase, DNA is prepared for synthesis by the loading of licensing factors termed MCMs (Kubota *et al.*, 1997; Nishitani and Lygerou, 2002). MCMs may only load onto the DNA once per cell cycle (when Cdk activity is low at the beginning of G1) to ensure that the DNA is

only synthesized once. Towards the end of G1, the cell cycle encounters its first checkpoint, known as the restriction point in mammalian cells and START in yeast (Pardee, 2002).

If the aforementioned requirements are met, cyclin D will be synthesized and combine with Cdk4 and Cdk6 to promote progression through G1 phase. If all the necessary growth factors are present and anti-growth factors absent, cyclin E is also synthesized and forms a dimer with Cdk2 to push the cell cycle past the restriction point, through the remaining G1 phase and into S phase. After a cell passes the restriction point, growth factors may be removed and the cell will still complete the cell cycle because cyclin D will have already done its job (Golias *et al.*, 2004). Cdk2/cyclin E is regulated through phosphorylation on threonine-14 and tyrosine-15 of the Cdk subunit. After association with cyclin E, Cdk2 is phosphorylated on these sites by Wee1 and Myt1 (Hoffman *et al.*, 1994; Saha *et al.*, 1997). When the cell is ready to enter S phase, Cdc25A phosphatase will remove the inhibitory phosphate groups and block the CKI, p21 (Cip1), from binding Cdk2/cyclin E (Hoffman *et al.*, 1994; Saha *et al.*, 1997). Once Cdk2/Cyclin E is in its active form, it activates origin of replication complexes to start DNA synthesis by phosphorylation and loading of Cdc45 onto the MCM complexes (DNA helicases) (Weinreich *et al.*, 2001).

G1-S Phase Checkpoint

If DNA is damaged during the transition from G1 into S phase, branching molecular pathways responsible for cell cycle arrest become activated. The checkpoint is initiated through the DNA damage sensing proteins, ATR and ATM, which phosphorylate Chk1 and Chk2 in response to UV (ultra-violet) or IR (ionizing radiation), respectively (Costanzo *et al.*, 2000; Mailand *et al.*, 2000; Bartek and Lukas, 2001). Subsequently, Chk1 and Chk2 phosphorylate Cdc25A, targeting it for ubiquitination and proteolysis (Mailand *et al.*, 2000; Bartek and Lukas, 2001). In the absence of Cdc25A, inhibitory phosphorylations on Cdk2 will not be removed.

ATM and ATR also initiate a checkpoint response to DNA damage, independent of Chk1 or Chk2, by initiating p21 (also termed Cip) synthesis through the p53 pathway (Bartek and Lukas, 2001). Following a DNA damaging event, ATM and or ATR kinases phosphorylate and stabilize p53 (Banin *et al.*, 1998; Canman *et al.*, 1998; Khanna *et al.*, 1998; Costanzo *et al.*, 2000; Mailand *et al.*, 2000). Once activated, p53 promotes synthesis of p21, a CKI that inhibits the Cdk2/cyclin E complex (Chen *et al.*, 1995; Luo *et al.*, 1995). In a checkpoint response at the end of G1, p21 inhibits the activity of the Cdk2/cyclin E complex as well as prevents any Cdc25A from binding Cdk2/cyclin E (Saha *et al.*, 1997). These events prevent cell cycle progression into S phase in the presence of genomic assault.

Cdk/Cyclin Control in S Phase

DNA synthesis is accomplished during S phase of somatic cell cycles. Late in G1 and during S phase, cyclin A is synthesized and forms a dimer with Cdk2. Active Cdk2/cyclin A and Cdk2/cyclin E initiate DNA synthesis (Heichman and Roberts, 1994; Jackson *et al.*, 1995; Strausfeld *et al.*, 1996; Arellano and Moreno, 1997) through activation of Cdc45, an origin of replication (ORC) initiator (Costanzo *et al.*, 2000). It is important that Cdk2/cyclin A remains active throughout DNA synthesis in order to progressively turn on early to late firing origins of replication (Diffley, 1998).

Intra-S Phase Checkpoint

An intra S-phase checkpoint can be initiated if stalled DNA replication or genomic damage is sensed. When activated, the cell must first stabilize replication forks that are in progress to prevent replication of damaged areas (Santocanale and Diffley, 1998; Shirahige *et al.*, 1998). The cell must then prevent further initiation of late firing origins that are in their pre-replication state (Lopes *et al.*, 2001; Tercero and Diffley, 2001). This checkpoint is initiated through phosphorylation of ATM in response to double-stranded breaks in the DNA (Luciani *et al.*, 2004) ATM subsequently phosphorylates and activates Chk2. Chk2 inhibits Cdk2/cyclin A by targeting Cdc25A for degradation, similar to the response at the checkpoint in the end of G1 (Bartek *et al.*, 2001; Falck *et al.*, 2001). Chk1 is also important to maintain proper progression through S phase. In the presence of Chk1 inhibitors during S phase, there is an increase in DNA damage and inappropriate firing of origins of replication (Syljuasen *et al.*, 2005).

Despite their obvious importance, Chk1 and Chk2 pathways are not the only pathways governing the intra-S phase checkpoint, as evidenced by initiation of a Chk1 or Chk2 independent cell-cycle checkpoint in the presence of Chk1 and Chk2-specific inhibitors (Luciani *et al.*, 2004). The exact mechanism of intra-S phase checkpoint engagement is still unclear, although many studies have shown high similarity to the checkpoints at the end of G1 and G2 in regards to how the Cdk/cyclin dimers are regulated. It also seems clear that while this checkpoint can slow the cell cycle, it does not completely stop progression through and eventual exit from S phase (Diffley, 2001). The intra-S phase checkpoint only stalls S phase progression without compromising the ability to replicate undamaged portions of the genome (Bartek and Lukas, 2001; Diffley, 2001).

G2 Control

Upon completion of DNA synthesis, the cell cycle progresses to the G2 phase. In somatic cells, the primary functions during G2 phase are to monitor genomic content for completeness of DNA replication and for DNA damage. As in earlier cell cycle phases, the progression through G2 is governed by cyclins and their associated Cdk(s). During G2 phase, Cdk1 associates with cyclins A and B, an important step for the eventual transition into mitosis (Golias *et al.*, 2004). The dimer of Cdk1 and cyclin B was found to be the major molecular factor driving the events in mitosis, and was termed mitosis or maturation promoting factor (MPF) (Gautier *et al.*, 1988). MPF was originally discovered by Masui and Markert (1971) in frog eggs. MPF accumulates during G2 and is maintained in an inactive, pre-MPF state through inhibitory phosphorylation of Cdk1 by Wee1 and Myt1 (Featherstone and Russell, 1991; Mueller *et al.*, 1995a). The length

of G2 depends upon the accumulation of enough pre-MPF molecules to activate the positive feedback loop responsible for driving the cell cycle into mitosis. The dynamics of this feedback loop are important for regulating the progression of the cell cycle and will be explained in more detail in the upcoming sections. Cyclin A also plays an important role in the transition from G2 into mitosis. Cdk1/cyclin A remains active in the early transition into mitosis but does not persist like MPF (Furuno *et al.*, 1999). Cdk1/cyclin A is thought to play an important role in inhibiting MCM loading during G2 and mitosis (Lei and Tye, 2001). MCM loading must only take place once per cell cycle to ensure DNA is synthesized only once. Cyclins A and B complexed with Cdk1 accumulate during G2 and are responsible for driving the cell cycle into mitosis.

MPF Regulation

The regulation of MPF is well documented in humans as well as many other eukaryotes, including *Xenopus laevis*. Before mitosis, inactive pre-MPF complexes accumulate in the cell. The pre-MPF complex refers to MPF with the Cdk1 subunit phosphorylated on the three regulatory sites (Figure 1.2, step 2). When the MPF heterodimer forms, it is almost immediately conferred into this inactive state where all three residues are phosphorylated. The inhibitory regulatory sites on Cdk1 are located on threonine-14 and tyrosine-15 (Draetta *et al.*, 1989; Dunphy and Newport, 1989) and the activating phosphorylation site is on threonine-161 (Solomon *et al.*, 1992). When the two inhibitory phosphate groups are removed and the activating site remains phosphorylated, MPF is catalytically active.

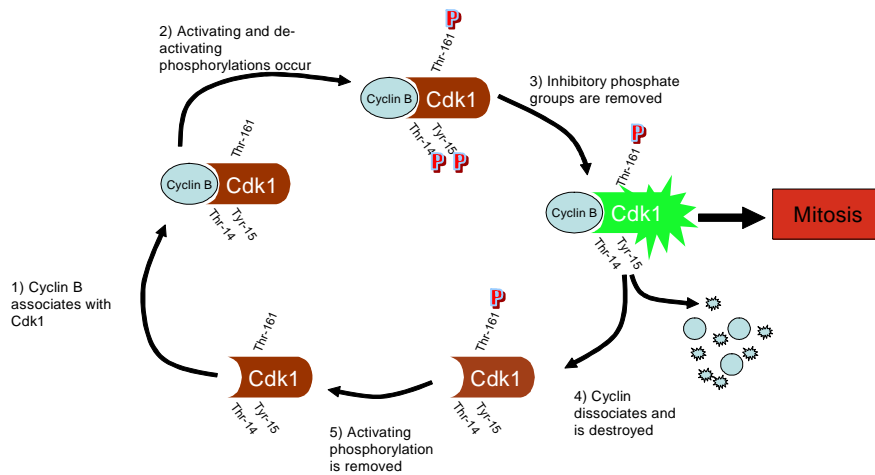


Figure 1.2 Diagram depicting the key regulatory phosphorylation sites on MPF and how they are modified as the cell cycle progresses. Adapted from Cooper (2000).

MPF and its activity are governed through a multitude of regulatory pathways. The limiting factor in formation of active MPF molecules depends upon the accumulation of the cyclin B subunit. Cyclin B accumulates during G2 phase and is highly susceptible to degradation. Upon dimerization of Cdk1 with cyclin B, CAK (Cdk Activating Kinase) phosphorylates Cdk1 on threonine-161 (Solomon *et al.*, 1992). This phosphorylation event is followed closely by phosphorylation on tyrosine-15 and threonine-14 by Wee1 and Myt1, rendering MPF catalytically inactive (Figure 1.2, step 2) (Featherstone and Russell, 1991; Parker and Piwnica-Worms, 1992; Mueller *et al.*, 1995a; Mueller *et al.*, 1995b; Watanabe *et al.*, 1995). In the absence of unreplacated or damaged DNA, MPF inhibits Wee1 through phosphorylation (Smythe and Newport, 1992; Lee *et al.*, 2001) and possibly targets Wee1 for proteolysis (Michael and Newport, 1998). Active MPF

also phosphorylates Cdc25C, increasing its phosphatase activity towards the inhibitory phosphate groups on MPF (Kumagai and Dunphy, 1992; Izumi and Maller, 1995).

The resulting activity of MPF on its activating and inhibitory regulators causes a positive feedback loop, which is engaged as threshold concentrations of cyclin are reached. The Cdc25C phosphatase promotes an increase in MPF activity, which creates a positive feedback loop whereby MPF activates Cdc25C (Kumagai and Dunphy, 1992; Izumi and Maller, 1995) and inactivates the inhibitory kinase Wee1 (Smythe and Newport, 1992). This positive feedback loop builds upon itself and creates a rapid increase in MPF kinase activity that triggers the abrupt transition into M phase.

Checkpoint Response

In a checkpoint response at the end of G₂, the cell cycle is halted through inhibition of MPF. This is accomplished by inhibiting the activators of MPF (Cdc25 phosphatases A and C) (Hoffman *et al.*, 1993; Mailand *et al.*, 2000) and activating the inhibitor kinase Wee1 (Mueller *et al.*, 1995a). Regulating Cdc25C and Wee1 in the above manner turns off the positive feedback loop with Cdc25C and turns on the inhibitors of MPF. This checkpoint response ensures cell cycle progression is halted until DNA synthesis and/or repair comes to completion.

In response to unreplicated or damaged DNA, the checkpoint proteins (Chk1 or Chk2) inhibit Cdc25C and remove Cdc25A by targeting it for degradation. When Cdc25C is phosphorylated by Chk1 or Chk2, it prevents MPF from adding an activating phosphate group (Bulavin *et al.*, 2003) and promotes binding of the Cdc25C to 14-3-3 cytoskeletal proteins. When Cdc25A is inhibited by either Chk1 or Chk2 by

phosphorylating serine-75 (Hasepass *et al.*, 2003), the protein is targeted for degradation through ubiquitination by the SCF complex (Mailand *et al.*, 2000; Xiao *et al.*, 2003).

Chk1 is normally activated through phosphorylation by ATM or ATR in response to genomic aberrations. When Chk1 is activated by a cell cycle checkpoint, it activates Wee1 by phosphorylating serine-549, causing binding of 14-3-3 cytoskeletal proteins (Lee *et al.*, 2001). When Wee1 is complexed with 14-3-3 proteins, it becomes a hyperactive kinase on the inhibitory phosphorylation site (tyrosine-15) of Cdk1.

The net result of the regulatory pathways activated during a checkpoint response is an inhibited MPF complex. The checkpoint proteins eliminate the positive feedback loop through Cdc25 proteins as well as increase the rate of inhibitory phosphorylation on Cdk1 by hyper-activating Wee1 (Kumagai *et al.*, 1998a; Kumagai *et al.*, 1998b; Lee *et al.*, 2001). The cell cycle checkpoints negatively influencing MPF are translating a problem with the genomic material through the Chk1 and Chk2 proteins. By inhibiting MPF, these checkpoint proteins ensure the cell cycle does not progress in the presence of damaged or unreplicated DNA.

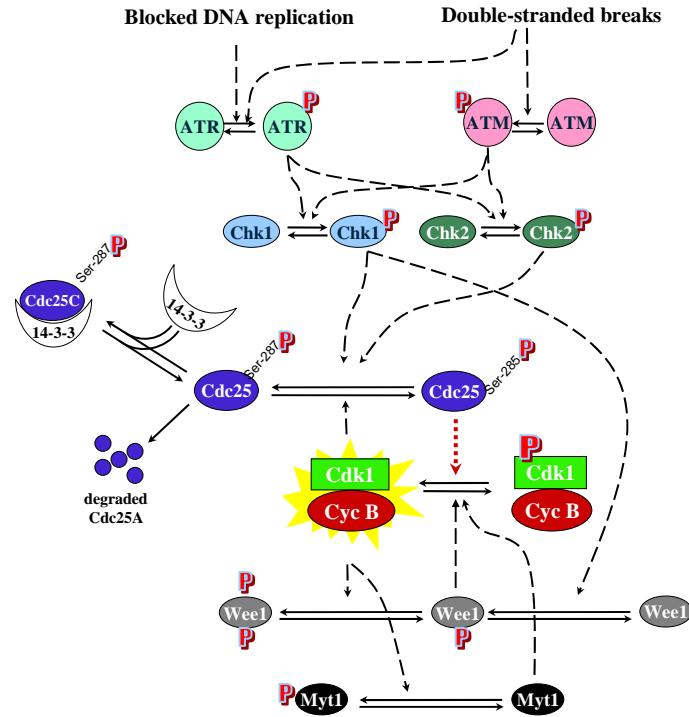


Figure 1.3 Wiring diagram depicting the regulation network surrounding Cdk1/cyclin B. The red letter P associated with the periphery of a molecule indicates phosphorylation. All black dashed lines indicate kinase activity. The red dashed arrow indicates phosphatase activity. When active, Cdk1/cyclin B will inhibit Wee1 and promote Cdc25 activity. When unreplicated or damaged DNA is present in the somatic cell, Cdc25 is inhibited or destroyed and Wee1 is hyper-activated through phosphorylation. Adapted from (Novak *et al.*, 2002).

Delayed Inhibition for Mitotic Exit

Once the cell cycle progresses into mitosis, the cell executes the required mechanical events initiated by active MPF and produces two new daughter cells. The events that take place during mitosis are completed in a relatively short time compared with the rest of the cell cycle (5% of a typical eukaryotic cell cycle (Cooper, 2000)). As soon as MPF becomes active, it initiates a signal that results in its eventual degradation. The degradation of MPF allows for a quick exit from mitosis (and entry into G1) once two new daughter cells are formed.

MPF engages a negative feedback loop when it activates the Anaphase-Promoting Complex (APC) through Fzy (King *et al.*, 1995; Sudakin *et al.*, 1995). Fzy is responsible for directing the ubiquitination of cyclin B by the APC. The activated APC attaches ubiquitin molecules to the cyclin B subunit of MPF. Ubiquitin-labeled cyclin B is then recognized by the proteasome and degraded. As levels of cyclin B decrease due to MPF-mediated degradation, Cdk1 becomes catalytically inactive, causing a decrease in MPF activity and exit from mitosis.

The net effect of these positive and negative feedback loops on MPF is an abrupt increase in kinase activity followed by degradation of cyclin B and subsequent inactivation of the Cdk1 subunit at the end of mitosis. This important dynamical control system of MPF allows a fast, regulated progression through mitosis. Fast progression is essential in order to limit the time the cell is unable to respond to environmental changes.

M Phase Control

Mitosis is the most dramatic of the cell phases and is where a new cell is formed via cytokinesis. The separation of sister chromatids to the opposing spindle poles in the dividing cell ensures that each new cell gets a complete copy of the genome (Rappaport, 1971; Oegema and Mitchison, 1997). Once each sister chromosome is pulled to its respective side of the cell, a cleavage furrow develops by the contraction of actin filaments and constricts until a new daughter cell is formed (Rappaport, 1971; Glotzer, 2005). These events are sequential and are governed by biochemical pathways to ensure proper division takes place.

Metaphase Regulation

Once MPF has been activated and the cell is past the G2 checkpoint, the cell must equally divide the genomic material for each new daughter cell. The DNA is first condensed into easily manipulable chromosomes facilitated by a protein complex known as condensin (Hirano, 2005). The nuclear envelope rapidly breaks down through the dissolution of the lamin network of protein (phosphorylated by MPF) that forms the scaffolding for the nuclear membrane (Nigg, 2001). The condensed sister chromosomes, now in the cytosol, are held together at the kinetochore by DNA binding proteins called cohesins (Heck, 1997). Microtubules originating from the centrosomes on opposing sides of the cell attach to each pair of chromosomes and shuttle them to the middle of the cell for alignment along the metaphase plate. This event involves microtubule filaments attaching to the kinetochore and arms of each chromatid and a mechanical pushing and pulling force exerted by the microtubules through motor proteins called dynein and kinesin (Banks and Heald, 2001). Once all of the chromosomes are attached to microtubules and aligned along the metaphase plate, the APC initiates a signal to separate the chromosomes (Yanagida, 2000). The APC is activated through MPF (King *et al.*, 1995; Sudakin *et al.*, 1995) and once all of the chromosomes are aligned and attached on the metaphase plate, the APC will destroy a chaperone protein known as securin (Darwiche *et al.*, 1999). Once securin is destroyed, it can no longer inhibit the protease separase. Active separase breaks down the cohesins responsible for holding the sister chromatids together.

If some of the chromosomes are out of alignment on the metaphase plate or unattached, the APC will be inhibited and cause a metaphase arrest. Mad2 and BubR1

are proteins that attach to the kinetochore and ensure chromosomes are correctly attached to mitotic microtubules along the metaphase plate. If even a single pair of sister chromosomes is misaligned, Mad2 and BubR1 inhibit Cdc20 and prevent it from associating with the APC. If the APC cannot associate with Cdc20, it cannot destroy securin and cyclin B, and so the cell cycle remains in metaphase (Tang *et al.*, 2001; Hagan and Sorger, 2005).

Anaphase Regulation

The cleavage of the cohesins that hold sister chromosomes together signals the beginning of anaphase in mitosis. During anaphase, all of the sister chromosomes are pulled (by microtubules) to opposite poles of the cell. The mechanical force driving the chromosomal movement is provided by the shortening of the polar microtubules and by microtubule-dependent motor proteins (Banks and Heald, 2001; Peters, 2002).

Progression through anaphase is dependent upon the APC associating with Cdh1. Cdh1 is phosphorylated by the Cdk1 prior to anaphase, rendering it inactive (Peters, 2002).

When sister chromosomes dissociate at the end of metaphase, the APC^{Cdc20} complex degrades cyclin B (inactivating Cdk1) and Cdh1 becomes active (Lukas *et al.*, 1999; Peters, 2002). Active Cdh1 combines with the APC complex (APC^{Cdh1}) and ubiquitinates the Cdc20 associated with the APC (APC^{Cdc20}), targeting it for degradation (Pfleger and Kirschner, 2000). However, during early embryonic development, cell cycle progression is regulated by the APC/Cdc20 complex as Cdh1 is not synthesized until later stages of development (Lorca *et al.*, 1998; Peters, 2002; D'Angiolella *et al.*, 2003). The cell then reforms nuclear envelopes around each group of chromosomes, the DNA decondenses, and the cell divides into two. The cell divides by forming a ring of actin filaments where

the metaphase plate was, and gradually tightens the ring. This pinches the cell in two, forming the new daughter cells.

Cell Cycle Control in Cell-Free Egg Extracts

Somatic cells are limited in their ability to provide an experimental system amenable to biochemical assays advantageous to the study of the cell cycle. A population of somatic cells will vary in the stage of the cell cycle they exist. In order to synchronize the cell cycle for a population of somatic cells, checkpoints must be initiated to halt all of the cells at the same stage. These methods range in severity from DNA damaging to cell-cell contact inhibition, but all impede the normal progression of the cell cycle for the population of cells to be studied (Davis *et al.*, 2001). Inducing a checkpoint into the cells affects the cell cycle, and may introduce artifacts into a cell cycle assay. To avoid complications associated with somatic cells, Andrew Murray developed a protocol for creating cell-free extracts from *Xenopus laevis* eggs (1991). The early stages of *Xenopus* development are relatively simple and minimize the complexity of the cell cycle, allowing for evaluation of some fundamental components of cell cycle regulation. The cell-free extract system of *Xenopus laevis* thus provides a highly tractable experimental system to study the cell cycle.

The procedure for obtaining a cell-free extract involves fractionating the cytoplasm of the eggs by centrifugation and maintaining that cytoplasmic fraction in a cytostatic factor (CSF)-arrested state. CSF maintains a high level of MPF activity prior to fertilization, locking the cell in meiosis II. Upon fertilization, Ca^{++} ions are released from the endoplasmic reticulum (ER) and trigger the destruction of cyclin B, thus destroying MPF. The Ca^{++} ions also signal the combination of maternal and paternal

nuclei. To release CSF-arrested extracts, CaCl_2 is added, mimicking fertilization and allowing the extract to exit meiosis II and enter its first interphase. These extracts will continue through interphase, and under permissible conditions, will enter mitosis. This procedure can provide large volumes of cytoplasm with all the necessary proteins required to drive the cell cycle (Lohka and Masui, 1983; Blow and Laskey, 1986; Hutchison *et al.*, 1987; Murray and Kirschner, 1989).

Xenopus laevis egg extracts mimic the cell cycles of the early developing embryo. Depending upon the concentration of nuclei used in an extract, one can create nucleocytoplasmic ratios found in a pre or post-MBT embryo. The cell cycles are very quick, oscillating between interphase (essentially S phase) and mitosis. During interphase, the cell rapidly synthesizes DNA and prepares for the next division by manufacturing more cyclin B. During early development in *Xenopus*, there is no cell growth or gap phases. The early embryo and extract alike complete the cell cycle every 30 minutes after the first cell cycle. Checkpoints in the extract can become activated (Costanzo and Gautier, 2004), and as we will discuss in Chapter 3, these checkpoints appear to require a certain nucleocytoplasmic ratio. The checkpoints operate under the same molecular controls as described for the somatic system, although in somatic cells, the nucleocytoplasmic ratio is high and remains fairly constant, whereas we can vary this ratio in the extract.

In the *Xenopus laevis* extract system and pre-MBT embryos, the rapid cell cycles are governed by the same basic cell cycle machinery discussed for somatic cells. The Cdk governing S phase is Cdk2/cyclin E, while Cdk1/cyclin A and Cdk1/cyclin B are responsible for M phase progression as in somatic cells. Since these cells divide so

quickly, however, the regulation of the Cdk/cyclin dimers is simplified. As opposed to somatic cells, cyclin E levels remain constant and Cdk2/cyclin E activity is probably controlled through its phosphorylation states (Kappas *et al.*, 2000; D'Angiolella *et al.*, 2001). Changes in the phosphorylation state of Cdk2/cyclin E were detected by D'Angiolella *et al.*, in 2001 and were described in a mathematical model constructed by Ciliberto *et al.*, (2003). As in somatic cells, progression from interphase (G2) into mitosis is governed by accumulation of the cyclin subunit for MPF (cyclin B). CKIs, which inhibit the cell cycle, are absent or at least at levels below detection in pre-MBT embryos and extracts (Su *et al.*, 1995; Shou and Dunphy, 1996). Therefore, the cell cycle is allowed to progress in a rapid fashion through multiple cell cycles, accumulating a large quantity of cells to start the development of *Xenopus laevis*.

Cell Cycle Checkpoints in the Extract System

As described earlier, cell cycle control in *Xenopus* extracts is governed in a similar fashion as in somatic cells. Consequently, cell cycle checkpoints inhibit the cell cycle by similar mechanisms found in the somatic cell (although not through CKIs (Su *et al.*, 1995; Shou and Dunphy, 1996)). In extracts, DNA damage caused by ultra-violet radiation (UV) initiates a checkpoint signal through phosphorylation of Chk1 (Kumagai *et al.*, 1998a). Double-stranded breaks induced by nuclei exposed to ionizing radiation (IR), also initiate a checkpoint response through Chk2 and Chk1 (Guo and Dunphy, 2000; Guo *et al.*, 2000; Kappas *et al.*, 2000). Unreplicated DNA also halts cell cycle progression through Chk1 phosphorylation (Dasso and Newport, 1990; Kumagai *et al.*, 1998a; Shimuta *et al.*, 2002). The checkpoint responses to either damaged or

unreplicated DNA impede progression into mitosis through either S phase or M phase Cdk/cyclin complexes as described for somatic cells (Blow and Laskey, 1986; Shimuta *et al.*, 2002; Luciani *et al.*, 2004). Therefore, *Xenopus* extracts provide a relevant system to study cell cycle checkpoint engagement.

The engagement of cell cycle checkpoints in extracts from cells that normally fail to elicit a checkpoint response is unique. The acquisition of checkpoints in the cell-free extract system is attributed to the effect the nuclei concentration has on MPF regulation. In normal *Xenopus* development, the embryo acquires checkpoints and gap phases at the MBT, when the nucleocytoplasmic ratio is approximately 4096 nuclei per μl of cytosol. In embryos, the MBT is where the cells normally turn on cell cycle checkpoints and gap phases are acquired. The embryo switches from a program of rapid, checkpoint-independent cell cycles to controlled, checkpoint-dependent cell cycles. We hypothesize it is likely the dramatic increase in the nucleocytoplasmic ratio could be responsible for the acquisition of checkpoints and gap phases at the MBT. Although difficult to compare nuclear concentrations between the early embryo and the cell-free extract system, the concentration of nuclei typically used in extracts (300-1500 nuclei per μl of extract for (Dasso and Newport, 1990)) could be comparable to the concentration of nuclei in the intact embryo at the MBT. Extracts with nuclei concentrations below 300 nuclei per μl of extract do not exhibit cell cycle checkpoints in response to DNA replication blocks (Dasso and Newport, 1990). The extract system allows for premature engagement of checkpoints in pre-MBT cytoplasm that are absent from the intact pre-MBT embryo system.

The way in which the developing embryo senses the nucleocytoplasmic ratio and uses it as a signal for cell cycle remodeling at the MBT remains unclear. Based on our current findings, it has become apparent that the nucleocytoplasmic ratio has a direct effect on the cell cycle engine in the cell-free extract system of *Xenopus laevis*. Nuclear concentration affects the amount of cyclin required to advance the cell cycle into mitosis in a dose-dependent fashion. These findings have aided our understanding of the importance of the nucleocytoplasmic ratio and its effects on the cell cycle.

Mathematics

Understanding how the eukaryotic cell cycle is regulated is an important goal of cell biology. The cell cycle is the underlying control mechanism for cell proliferation and proper regulation is important to allow for the proper development and growth of eukaryotes. In the past 40 years, our understanding of the cell cycle and the biochemical signals responsible for its control has increased dramatically. Due to the complexity of the control mechanisms discovered, it has become increasingly difficult to understand how a multitude of molecular signaling events regulate cell cycle progression. Therefore, our ability to predict how perturbations affect various aspects of cell cycle control is limited and near-sighted at best. Our ability to evaluate and cure cancer, however, relies on our understanding of the cell cycle control mechanisms and our ability to manipulate them to our advantage (Chen *et al.*, 1995; Basu and Haldar, 1998). Mathematical models of the cell cycle can help us achieve a comprehensive understanding of the cell cycle and its control mechanisms. By combining biochemical data from a multitude of laboratories, mathematical models can yield a systems-level view of the cell cycle and allows us to predict outcomes of perturbations of experimental systems using computer simulations. Mathematical models have already been successful in predicting that the cell cycle is hysteretic (Novak and Tyson, ; Novák and Tyson, 1993; Pomerening *et al.*, 2003; Sha *et al.*).

Novák-Tyson Mathematical Model

In 1993, John Tyson and Béla Novák published a mathematical model consisting of a set of differential equations relating the different proteins regulating MPF activity in *Xenopus laevis* eggs and egg extracts. At the time, the model was in agreement with current experimental findings of the control mechanisms that govern the cell cycle (Murray and Kirschner, 1989; Dasso and Newport, 1990; Felix *et al.*, 1990; Solomon *et al.*, 1990; Ferrell *et al.*, 1991). Using differential equations to describe the reaction network governing the cell cycle, Novák and Tyson performed model simulations of the cell cycle *in silico*. The core of this differential equation model was a quantitative depiction of the feedback loops affecting MPF activation. These feedback loops made the understanding of MPF and cell cycle control intuitively difficult. The mathematical model was able to show several facets of MPF control and behavior that were previously unappreciated. The mathematical model described a system that required a minimum amount of cyclin B (cyclin B threshold) to trigger entry into mitosis. As cyclin B levels reach this threshold level and overcome it, MPF is rapidly activated due to the self-sustaining positive feedback loop created between MPF and Cdc25 as well as MPF and Wee1.

Once this MPF “motor” is turned on, the amount of cyclin required to retain MPF in an active state to maintain mitosis is noticeably lower than that which is required to enter mitosis (Figure 1.4). This phenomenon is called hysteresis. A characteristic of such a hysteretic system is the correlation between cyclin levels (and thresholds) with lag times (Novák and Tyson, 1993; Sha *et al.*, 2003). The amount of time it takes a system (both experimental and simulated model) to enter mitosis once cyclin B is above the

minimum cyclin B threshold is termed lag time (Novák and Tyson, 1993). With the amount of cyclin B higher than the minimum threshold, the closer cyclin B is to the threshold, the larger the lag time (for example; .4 units of total MPF in Figure 1.4). As the difference between cyclin and the minimum cyclin threshold increases, the lag time until mitosis decreases (for example; .8 units of total MPF in Figure 1.4). The minimum lag time achievable in an extract system is reached once the cyclin B concentration equals the Cdk1 concentration. Any concentration of cyclin B above the concentration of Cdk1 will not have an effect on lag time. These lag times reflect the amount of time it takes to turn on the MPF positive feedback loop. The smaller the concentration of MPF molecules able to participate in these positive feedback loops, the longer it takes them to drive the cell into mitosis.

A characteristic of a hysteretic system is its bistability. The minimum concentration of cyclin required to enter mitosis is greater than that which is required for maintenance of a mitotic state after MPF is already turned on. This means there is some range of cyclin concentrations that could maintain either high (mitosis) or low (interphase) activity of MPF depending on where the system started. This can be visualized in Figure 1.4 by observing where the amount of active MPF crosses .3 total cyclin B. When total cyclin B equals .3, the system can have three different values of active MPF. Two of these values (active MPF=.0005 and .1) are stable steady state solutions of the model equations and represent interphase and mitosis in the extract system, respectively. The third state (MPF=.005) represents an unstable steady state (Figure 1.4, dashed line) solution produced by the mathematical model and is not directly observable in the extract system. Both stable and unstable steady states are solutions

from the mathematical model but differ in how the system responds when it is slightly perturbed from the solution. A marble placed at a sharp peak represents an unstable steady state. When the marble moves ever so slightly to either side, it will roll down to the bottom of one of the sides of the peak. Systems at a stable steady state behave like a marble in an inverted peak (or a trough). After a small perturbation, a stable steady state will return to the original answer like a marble returning to the bottom.

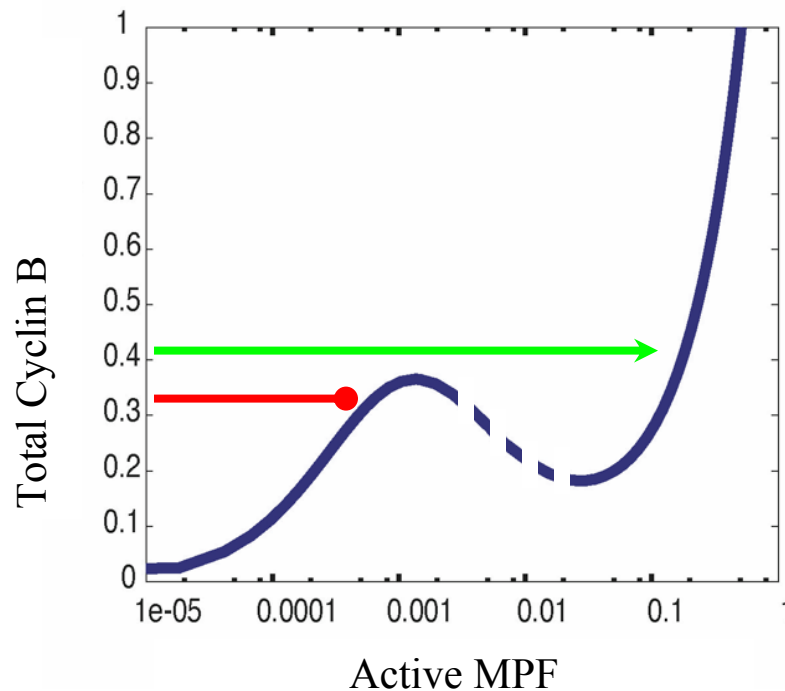


Figure 1.4 MPF activity is bistable and hysteretic. Following the graphed line, we can see that as total cyclin B increases, the amount of active MPF slowly increases as well. If the concentration of cyclin B is above the threshold level required, the concentration of active MPF molecules increases dramatically and the cell cycle will enter mitosis (green line). Anything below this threshold level will not enter mitosis and the concentration of active MPF molecules will be kept relatively low (red line). Adapted from Novák and Tyson (1993).

Another prediction of the Novák-Tyson model was that unreplicated DNA in the extract system would increase the cyclin B threshold that inhibits mitotic entry. Blocking DNA synthesis in the extract system initiates an unreplicated DNA checkpoint. This

unreplicated DNA checkpoint inhibits MPF, subsequently causing an increase in MPF molecules required to initiate the positive feedback loop. Sha et al. (2003) provided experimental evidence that the cyclin B threshold did increase in response to unreplicated DNA. The relative increase in cyclin B threshold due to unreplicated DNA is depicted in Figure 1.5.

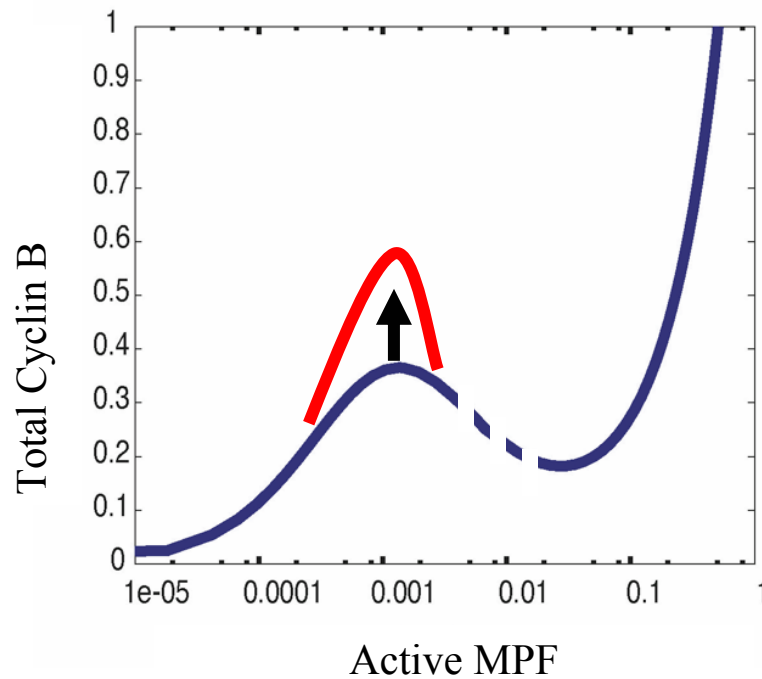


Figure 1.5 Change in cyclin B threshold due to unreplicated DNA. Graph represents the steady state level of active MPF as the total concentration of MPF (active and inactive) increases (as cyclin B accumulates). In the presence of unreplicated DNA, the minimum threshold level of cyclin B required for entry into mitosis increases. The increase is depicted as the red curve extending above the arrow and is greatly increased compared to the cyclin threshold in the absence of unreplicated DNA. Adapted from Novák and Tyson (1993).

Recently, the hysteresis loop proposed by the Novák-Tyson mathematical model was experimentally verified by Sha et al. (2003). Sha's experiments confirmed three predictions made by the Novák-Tyson model: 1) the threshold concentration of cyclin B required to activate MPF is measurably higher than the threshold concentration required to inactivate MPF, 2) for cyclin levels marginally above the activation threshold

concentration of cyclin B, there is a dramatic “slowing-down” of MPF activation, and 3) a DNA replication checkpoint increases the activation threshold concentration of cyclin B by enlarging the hysteresis loop.

The Novák-Tyson model is based on experimental findings in *Xenopus* egg extracts. Novák and Tyson (1993) assembled a diagram that represented the regulation of MPF based on known signaling pathways at that time. They translated this model into sets of differential equations, which were then analyzed numerically. Differential equations are used because they can describe how the level of a protein or other entity changes as a function of time. Parameters were estimated based on experimental literature and adjusted through trial and error. The mathematical model produced included an equation to describe the changes in the level of each different form of MPF as well as the different forms of the enzymes involved in MPF regulation (Figure 1.3). Through computational simulation of the model, the experimenter can view how each species or enzyme (described by the model using differential equations) changes with time. Through computer-based numerical analysis, this model of MPF regulation in *Xenopus* eggs and egg extracts was designed to follow a bistable and hysteretic control pattern.

As described earlier, the cell cycle is regulated in a complex manner. Our ability as scientists to comprehend how vast molecular signaling networks behave is reaching its limit. The ability of mathematical modelers to tie together information from a multitude of research laboratories allows for systems-level understanding of cell cycle regulation. As shown previously in our lab, there are many facets of the cell cycle (hysteretic and bistable) that might have gone undiscovered if it were not for the close interaction

between experimental and computational biologists. Mathematical modeling is broadening our understanding of how the cell cycle is regulated and will hopefully yield therapeutic solutions for diseases involving aberrant cell cycle regulation such as cancer.

We have investigated the unreplicated DNA checkpoint in the extract system and how it affects the cell cycle motor, MPF. We have expanded upon the Novák-Tyson model of frog extract cell cycle control to reflect more current data (Dravid *et al.*, 2004). The mathematical model can now predict the outcome of the cell-free experimental system with various components affecting MPF removed or added to the system. This updated model has provided a clearer picture of how a multitude of regulatory components seem to act in a concerted manner to regulate cell cycle progression in the presence of unreplicated DNA. Working on this model has also shown us there are still several components of MPF regulation that will require future clarification in order to become well outlined in the mathematical model. We hope to further clarify the regulation of MPF in response to unreplicated DNA in the cell-free extract system by pairing rigorous quantitative experimentation with computational modeling. Using cell-free extract system as a biochemical proving ground, we are providing quantitative data regarding the changes in cyclin thresholds in response to a variety of treatments.

Chapter 2: Materials and Methods

Inducing Ovulation of *Xenopus laevis*

Frogs were injected with 75 U/ frog of pregnant mare serum gonadotropin (PMSG) three to eight days before desired induction of ovulation. The frogs were kept in chlorine-free water at 16° C. Sixteen to eighteen hours before eggs were required, ovulation was induced by injecting 500 U/ frog of human chorionic gonadotropin (HCG). After injection with HCG, frogs were kept at 20-22° C in chlorine-free water with 100 mM NaCl.

CSF Extract

Extract preparation was performed as described by Murray et al., 1989: Laid eggs were collected in 100 mM NaCl, de-jellied with 2% (w/v) cysteine in XB salt (0.1 M KCl, 1 mM CaCl₂, 1 mM MgCl₂, pH 7.6-7.8) for 6-8 minutes and subsequently washed four times in XB buffer (0.1 M KCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, 50 mM sucrose, pH 7.7), and then twice in CSF-XB buffer (XB buffer plus 5 mM EGTA; pH 7.7, 1 mM MgCl₂ (for final concentration of 2 mM MgCl₂), and 10 µg/mL each of leupeptin, chymostatin, and pepstatin). The eggs were then transferred to a pre-chilled centrifuge tube at 4° C containing 5 mL of CSF-XB plus 100 µg/mL cytochalasin B. One mL of Versilube F-50 oil (General Electric, MIL-S-81087) was added to the top of the centrifuge tube to displace excess buffer. The tube was then centrifuged in a tabletop swinging bucket centrifuge at 1000 RPM (168 g) for one minute, and then 2000 RPM (671 g) for 30 seconds. Excess buffer and any eggs appearing lytic at top of centrifuge tube were then removed and replaced with a minimal amount of fresh Versilube oil. The

packed eggs were then centrifuged for 15 minutes at 15,680 g at 4° C (10,000 RPM, JS 13.2 swinging bucket rotor, Beckman Avanti J-20 XP centrifuge). The cytoplasmic fraction was collected and 1/20 volume of CSF energy mix (150 mM creatine phosphate, 20 mM ATP; pH 7.4, 20 mM MgCl₂, 200 µg/mL each of leupeptin, chymostatin, and pepstatin, and 200 µg/mL cytochalasin B) was added. The extract was then given a clarifying centrifugation for 15 minutes, at 15,680 g, at 4° C (10,000 RPM, JS 13.2 swinging bucket rotor, Beckman Avanti J-20 XP centrifuge). Sperm nuclei were diluted into the extract at appropriate concentrations from a stock concentration of 60,000 nuclei/µl.

To arrest extracts in interphase, 100 µg/mL of cycloheximide was added to CSF extracts at the zero time point to block synthesis of endogenous proteins. At the zero time point, 0.4 mM CaCl₂ was added to activate the extract, and extracts were then incubated at room temperature. After 30-40 minutes post-activation, a viable extract entered interphase. To induce DNA replication blocks, aphidicolin (10 mg/mL in DMSO) was diluted into the extract to a final concentration of 100 µg/mL.

Expression of Recombinant Nondegradable Human Δ87 Cyclin B Protein and Purification from *Spodoptera frugiperda* Gut Endothelial Cells.

The Δ87 cyclin B is a mutated version of cyclin B that is truncated via deletion of the first 87 N-terminal amino acids. This deletion removes the cyclin destruction box that is targeted by the APC for polyubiquitination. The Δ87 cyclin B protein is His-tagged and can be prepared as described by Kumagai and Dunphy, 1991. Human Δ87

cyclin B baculovirus (originally from Dr. William Dunphy's lab) was provided by Dr. Jonathan Moore, ICRF.

Spodoptera frugiperda gut endothelial (Sf9) cells (Invitrogen) were cultured in SF-900 II SFM™ (Invitrogen) media to a cell density of 2×10^6 before infecting with the baculovirus encoding $\Delta 87$ cyclin B. Seventy-two hours post-infection, the 100 mL cultures were harvested by centrifugation at 500 g at 4° C for 5 minutes, and cell pellets were stored at -80° C. Cell pellets from six cultures were resuspended in 30 mL of HBS (10 mM HEPES; pH 7.5, 150 mM NaCl, 0.5 mM EGTA, 0.5% (v/v) Triton-X, and 10 μ g/mL each of leupeptin, chymostatin, and pepstatin). The resuspension was then lysed by sonication using a microtip at the highest allowable setting (6 x 10 second pulses with 10 second gaps between pulses). The lysate was centrifuged for 20 minutes at 15,680 g at 4° C (10,000 RPM, JS 13.2 swinging bucket rotor, Beckman Avanti J-20 XP centrifuge). The supernatant was then applied to a pre-equilibrated (with HBS) column of Ni-NTA agarose beads (Gibco) kept at 4° C throughout procedure. The column was then washed with 20 mL HBS wash buffer (HBS buffer plus 20 mM imidazole and 0.5% (v/v) NP-40) and then washed with 25 mL HBS. The $\Delta 87$ cyclin B was eluted from the Ni-NTA column using HBS with 200 mM imidazole and collected as elution fractions and were stored at 4° C in the dark. The eluted fractions were then evaluated for purity and concentration using Coomassie staining on SDS-PAGE gels and using BSA as concentration standards.

Preparation of Sperm Nuclei

Method based on Gurdon (1976):

Four male frogs were injected with 25 U/ frog of PMSG three days before sperm collection, and then injected with 125 U/ frog of HCG the day before the collection. The frogs were then anesthetized by immersion in ice water for at least 20 minutes or until the animal was unresponsive. Testes were then surgically removed and rinsed three times in cold MMR (0.1 M NaCl, 2 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 0.1 mM Na₂EGTA, and 5 mM HEPES; pH 7.8), then washed twice in NPB (250 mM sucrose, 15 mM HEPES; pH 7.4, 1 mM EDTA; pH 8.0, 0.5 mM spermidine trihydrochloride (Sigma), 0.2 mM spermidine tetrahydrochloride (Sigma), 1 mM dithiothreitol (Sigma), 10 ug/mL leupeptin, 0.3 mM PMSF (Sigma)). Testes were then macerated in a small Petri dish and 2 mL NPB was added. Testes were then filtered and rinsed with 8 mL NPB and centrifuged at 3000 RPM for 10 minutes. The sperm pellet was then resuspended in 1 mL NPB with 10 mg/mL lysolecithin and incubated for five minutes at room temperature. Ten mL cold NPB containing 3% (w/v) BSA was then added. The sperm were then centrifuged at 3000 RPM for 10 minutes, resuspended in 5 mL NPB containing 3% (w/v) BSA and centrifuged at 3000 RPM for 10 minutes. The sperm were resuspended again in one mL NPB without PMSF and containing 0.3% (w/v) BSA and 30% (v/v) glycerol. The sperm concentration was determined using a hemocytometer and adjusted to a final concentration of 10⁷ sperm nuclei/mL. After appropriate dilution, sperm were snap frozen in 25 µL aliquots in liquid nitrogen and stored at -80° C.

Irradiation of Sperm Nuclei

Sperm nuclei were exposed to ionizing radiation (IR) using a Mini-Shot cabinet X-ray machine (TFI Corporation). The machine has an IR output of five Gy/minute when samples are placed in the center of the top shelf of the cabinet. Nuclei were irradiated in 0.7 mL capacity microcentrifuge tubes for varying lengths of time, depending upon the degree of damage desired. Throughout this treatment, the nuclei were suspended in NPB without PMSF at a density of 10^7 sperm nuclei/ μ L.

Monitoring MPF Activity by Sperm Nuclear Morphology

The progression of the cell cycle from interphase into mitosis in CSF extracts was monitored by examining sperm nuclear morphology. Two μ l of the extract sample was added to 2 μ l of 4',6-Diamidino-2-phenylindole (DAPI (Sigma)) fix (11.1% (v/v) formaldehyde, 48% (v/v) glycerol, 1 X MMR, 1 μ g/mL DAPI) and was mixed on a glass microscope slide. A cover slip was placed on the combined drops and extract was viewed using fluorescence and phase contrast microscopy (Olympus Provis AX70 microscope with a Olympus Colorview 12 digital camera). The nuclear membranes were resolved under phase contrast microscopy, while DNA morphology was viewed using fluorescent microscopy. The nuclear morphology for an extract in mitosis is condensed DNA with no nuclear membrane. Dispersed or decondensed DNA surrounded by a nuclear membrane is indicative of interphase.

Assay for Caspase Activity

Extract samples were assayed for caspase activity by the cleavage of recombinant poly ADP-ribose polymerase (PARP) substrate, as modified from the protocol of Hensey and Gautier (1997) and Carter and Sible (2003). 5 μ l of extract sample was collected at the times indicated, snap-frozen on dry ice, and stored at -80° C. Samples were then diluted 1:1 into caspase elution buffer (CEB) (80 mM β -glycerophosphate, 15 mM $MgCl_2$, 20 mM EGTA, 10 mM DTT). Lysates were then incubated with 2–5 ng/ml recombinant human PARP (Alexis Biochemicals) at 30° C for 15–30 min, then resolved on 7.5% Anderson SDS-polyacrylamide gels (Kappas et al., 2000), and transferred to nitrocellulose membranes. Western analysis of PARP was performed by hybridizing membranes with anti-PARP antibody (Alexis Biochemicals) diluted 1:5000 in 10% nonfat dry milk in PBS. Immunoreactivity of proteins was visualized using horseradish peroxidase-conjugated secondary antibody (Jackson), and chemiluminescence from the secondary antibody was detected with ECL plus western blotting detection system (Amersham Biosciences).

**Chapter 3: Quantitative Analysis of the Effect
of Nuclear Concentration in *Xenopus laevis*
Cell-Free Egg Extracts**

Abstract:

At the midblastula transition or MBT, gap phases and cell cycle checkpoints are acquired. The mechanism responsible for the initiation of these important features of the cell cycle is still not fully understood. In this study, we investigated effects of nuclear concentration upon the underlying kinetics of the cell cycle engine in cell-free egg extracts. We found that increases in the nuclear concentration affect cell cycle progression in a dose-dependent fashion. We quantified these effects by evaluating Δ cyclin B thresholds. By treatment of extracts with varying concentrations of sperm nuclei, we examined the change in requirement for cyclin B necessary for entry into mitosis. Using *Xenopus laevis* cell-free extracts we found that 1) the concentration of nuclei affects lag time of entry into mitosis, 2) the lag time of entry into mitosis and Δ cyclin B thresholds required to enter mitosis increases with increasing concentrations of nuclei, and 3) elevated cyclin thresholds caused by DNA replication blocks are further increased by increasing the concentration of nuclei. In the developing embryo, the nuclei concentration doubles every 30 minutes and could be responsible for the introduction of gap phases and checkpoints at the MBT. This study examines the effect of nuclear concentration in the cell-free *Xenopus* extract system with and without unreplicated DNA. This may help identify the mechanism by which the developing embryo turns on checkpoints and gap phases at the necessary time during development based upon our findings in the cell-free extract system and accompanying predictions made by the Novák-Tyson mathematical model (Novák and Tyson, 1993).

Introduction:

During early development of *Xenopus laevis*, the cell cycles are rapid and synchronous. These rapid divisions increase the cell number from one at fertilization, to 4096 at the midblastula transition (MBT) or Stage 8 of development (Nieuwkoop and Faber, 1975; Newport and Kirschner, 1984). In approximately six hours, the embryo is able to accomplish this by starting out with a large volume of egg material and with all the necessary RNA and many proteins to drive the cell cycle through the first twelve cell divisions. This allows for the rapid divisions because the cells do not require any time to grow or rearrange themselves in the developing embryo. After the first 90 minute cycle that follows fertilization, the cell cycle is accelerated and oscillates between S and M phase every 30 minutes. Gap phases are not present nor are any checkpoints that halt the cell cycle in the presence of damaged or unreplicated DNA (Anderson *et al.*, 1997; Hensey and Gautier, 1997; Kappas *et al.*, 2000). The egg is essentially prepared with all the necessary cell cycle tools it needs to progress through the first twelve divisions. The embryo also contains enough fuel to allow for development of the complete tadpole, the first chance the organism has to acquire external nutrients.

Working in *Xenopus* egg extracts, Dasso and Newport found that the DNA replication checkpoint could be engaged by concentrations of sperm nuclei above 250/ μ l in the presence of aphidicolin (Dasso and Newport, 1990). Aphidicolin induces blocks to DNA replication by interfering with DNA polymerase α (Dasso and Newport, 1990). This study showed that the checkpoint machinery responsible for recognizing unreplicated DNA and halting the cell cycle in response to an explicit block to DNA

replication is functional in pre-MBT egg extracts. Dasso and Newport's findings also implied that a minimum concentration of nuclei is required to activate the unreplicated DNA checkpoint (Dasso and Newport, 1990). This checkpoint could be overcome by addition of Cdc25C or a constitutively active form of MPF into extract samples treated with aphidicolin (Dasso and Newport, 1990; Kumagai and Dunphy, 1991). These experiments implied that the unreplicated DNA checkpoint was operating through phosphorylation of the tyrosine-15 and or threonine-14 on the Cdk1 subunit of MPF. Addition of a non-degradable (Δ) form of the cyclin B subunit of MPF can also overcome the checkpoint response to unreplicated DNA (Sha *et al.*, 2003). In 2003, Sha *et al.* identified the minimal threshold amount of Δ cyclin B to overcome the DNA replication checkpoint, as hypothesized by Novák and Tyson in 1993. Novák and Tyson produced this hypothesis through the construction and simulation of a mathematical model (see Chapter 1 Math section).

In Sha *et al.*'s study, the investigators used *Xenopus laevis* cell-free extracts to evaluate cyclin threshold levels. In the experiments they performed, cycloheximide was used to block protein synthesis. Upon activation, extracts degrade cyclin B as they exit meiosis II and will stay in interphase until enough cyclin B is introduced to the system to push it into mitosis (for review, see Chapter 1). Sha *et al.* (2003) used a non-degradable form of cyclin B (Δ cyclin B) to supplement the extracts. This non-degradable form of cyclin B was created by removing the destruction box containing the binding sites for ubiquitin (Kumagai and Dunphy, 1995). They found that Δ cyclin B concentrations of 40 nM or above were enough to push an extract (with 1200 nuclei per μ l) into mitosis. Any concentration of Δ cyclin B less than 40 nM would keep the extract sample in interphase

for the duration of the experiment. When an extract sample was treated with aphidicolin and 1200 nuclei per μl , the amount of Δ cyclin B needed to allow the extract to enter mitosis was 100 nM. These experiments proved the validity of the Novák-Tyson model regarding effect of unreplicated DNA on the cell cycle engine as well as opened the door for the study of how changes in the extract system can affect the Δ cyclin B thresholds.

Based on Dasso and Newport's work in the *Xenopus* extract system (1990), it seemed there might be a correlation between nuclear concentration in the extracts and the unreplicated DNA checkpoint. A correlation between nuclear concentration and checkpoint engagement seems likely in the embryo, as the unreplicated DNA checkpoint is not engaged until after a seemingly minimum concentration of nuclei is obtained at the MBT. It is easy to manipulate the concentration of nuclei in an extract system (Murray, 1991), and we know the approximate concentration of nuclei in the intact embryo at the MBT and subsequent stages. The cytoplasmic volume of an embryo is thought to be around 1 μl /embryo (Murray, 1991). Based on the size of the eggs, the maximum amount of cytoplasm would be 1.4 μl ($4/3\pi r^3$, $r = .7\text{mm}$). Due to all of the energy and material requirements of the developing cell, much of the intercellular space is reserved for storage of yolk platelets and other insoluble material. This leaves approximately 1 μl of the eggs total volume dedicated to comprise the cytoplasm. It is plausible that utilization of nuclei concentrations equivalent to post-MBT concentrations is what permitted the extract to engage the DNA replication block checkpoint.

Based on the understanding of the biochemical signaling network governing MPF control in 1993, Novák and Tyson constructed a mathematical model of how MPF is controlled at the G2/M transition. Using this model, they made predictions about the

kinetics of the MPF control mechanism, hypothesizing that it was bistable and hysteretic (see Chapter 1, math section). Sha et al., confirmed that 1) MPF is regulated by hysteresis, 2) the time it takes the extract to enter mitosis increases with decreasing cyclin B concentrations, approaching the minimum threshold amount required to activate MPF, and 3) the hysteresis loop is enlarged when the DNA replication block checkpoint is engaged. These experimental findings were the first published validation of the 1993 Novák-Tyson model. Pomerening et al. (2003) independently confirmed the findings of Sha et al. later in 2003, further validating the ability of the Novák-Tyson mathematical model to predict MPF control in *Xenopus laevis* extracts.

Since the initial Novák-Tyson model was constructed in 1993, several discoveries have broadened our understanding of MPF control at the G2-M transition. Many of these discoveries involve how MPF is regulated in response to DNA replication blocks. Chk1 becomes phosphorylated by the ATR kinase (Guo *et al.*, 2000) in the presence of unreplicated DNA (Kumagai *et al.*, 1998a). ATR binds with DNA (Hekmat-Nejad *et al.*, 2000), and Chk1 acts as an intermediary protein in a checkpoint response (Kumagai *et al.*, 1998a; Kumagai *et al.*, 1998b). Once activated by ATR, Chk1 phosphorylates Cdc25C, thereby preventing removal of the inhibitory phosphate groups (threonine-14 and tyrosine-15) on the Cdk1 subunit of MPF (Kumagai *et al.*, 1998a; Kumagai *et al.*, 1998b). These discoveries link physical events affecting DNA structure with MPF, the molecular motor driving cell cycle progression.

During the cell cycle in *Xenopus* embryos, there are three different forms of Cdc25 present at any given time. All of the Cdc25 proteins share the ability to act as phosphatases on Cdk1. Cdc25A and C have been characterized as important regulators

of MPF in *Xenopus laevis*. Although Cdc25B has a well understood role in mammalian systems (Lammer *et al.*, 1998), its role in *Xenopus laevis* is still unclear (Chen *et al.*, 2000; Kanemori *et al.*, 2005), so we will focus on the A and C forms of Cdc25. Cdc25A can be phosphorylated on multiple phosphorylation sites by Chk1 (Shimuta *et al.*, 2002), signaling it for degradation. Cdc25C is also phosphorylated by Chk1 on serine-287, signaling it for binding to 14-3-3 structural proteins (Kumagai *et al.*, 1998a; Lee *et al.*, 2001). When Cdc25C is bound to 14-3-3 proteins, it is found localized in the cytoplasm. This removal of Cdc25C from the nucleus is believed to isolate it from MPF, which is mainly located in the nucleus.

In pre-MBT embryos, the proteins necessary for the DNA replication checkpoint signaling cascade are present (Costanzo *et al.*, 2000; Guo *et al.*, 2000; Kappas *et al.*, 2000). Similar to Sha *et al.*'s finding of a threshold amount of Δ cyclin B needed to initiate mitosis, a minimum concentration of nuclei must be reached to activate the DNA replication block checkpoint (Dasso and Newport, 1990). The influence nuclear concentrations have upon the kinetics governing MPF, in the absence of an explicit block to DNA replication, has not been investigated. Our findings clarify the effect nuclear concentration with and without explicit blocks to DNA replication has on the cell cycle engine in the *Xenopus laevis* extract system.

Results

The Concentration of Nuclei in the *Xenopus laevis* Extract System Affects Lag Time of Entry into Mitosis.

A wide range of nuclear concentrations have been used in the *Xenopus laevis* cell-free extract system. Higher concentrations (200-10,000 nuclei per μl) have been utilized to elicit cell-cycle checkpoints (Dasso and Newport, 1990; Kumagai *et al.*, 1998a; Kumagai *et al.*, 1998b), while lower concentrations (≤ 1000 nuclei per μl) have been used to determine cyclin thresholds or monitor cell-cycle stage (Sha *et al.*, 2003). Dasso and Newport (1990) showed that the unreplicated DNA checkpoint in extracts was dependent upon having at least 250 nuclei per μl of extract. *In vivo*, nuclei concentration was predicted to play an important role in the timing of the introduction of gap phases and checkpoints at the MBT in *Xenopus laevis* embryos (Dasso and Newport, 1990; Novák and Tyson, 1993). The effect nuclear concentration has on cell cycle progression is not well studied outside of a checkpoint response pathway. We wanted to know how nuclei concentration could affect cell cycle progression in the *Xenopus laevis* cell-free extract system.

For our study, we utilized a CSF-released cell-free extract system. This allowed us to activate all of the extract samples at the same time after purification of the cytoplasmic fraction. We split each extract into multiple samples and treated them with a range of concentrations of nuclei. Samples were periodically collected and monitored for mitotic entry. A given sample was mixed with DAPI stain/fix solution and analyzed under fluorescent and phase-contrast microscopy. Samples with less than 90% of nuclei

in mitosis were scored as an interphase extract. If the sample had greater than 90% of the nuclei in mitosis, clearly exhibiting nuclear envelope breakdown and DNA condensation, it was scored as mitotic. The transition from interphase into mitosis is very abrupt and normally corresponds to an all or none response in terms of nuclear morphology (Figure 3.1).

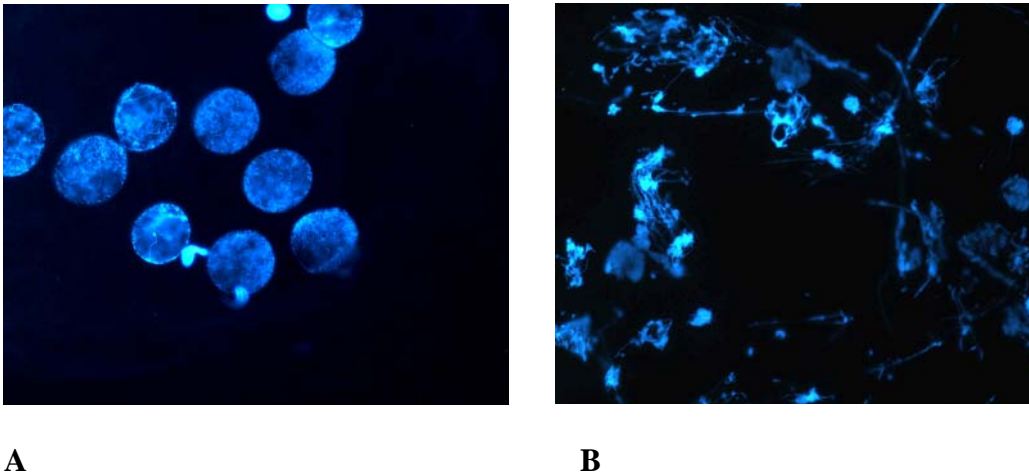


Figure 3.1 Cell-free extract samples evaluated for cell cycle phase using DAPI fixative stain and resolved using fluorescent microscopy **A:** A representative *Xenopus laevis* cell-free extract in interphase. A nuclear envelope clearly contains the dispersed chromatin. **B:** Upon entry into mitosis, the chromatin is condensed and DNA is no longer contained because the nuclear envelope has broken down.

In Figure 3.2, a graphical representation of the length of interphase for each sample is presented. Samples were evaluated approximately every 30 minutes for mitotic entry, as previously described. Nuclear concentrations spanned from 550 to 4400 nuclei/ μ l. The experiment was carried out until 210 minutes. We start to observe aberrant nuclear morphology in extract samples still in interphase after approximately

200 minutes post-activation. This is most likely due to the proteolysis of key cell cycle components or autolysis of the extract.

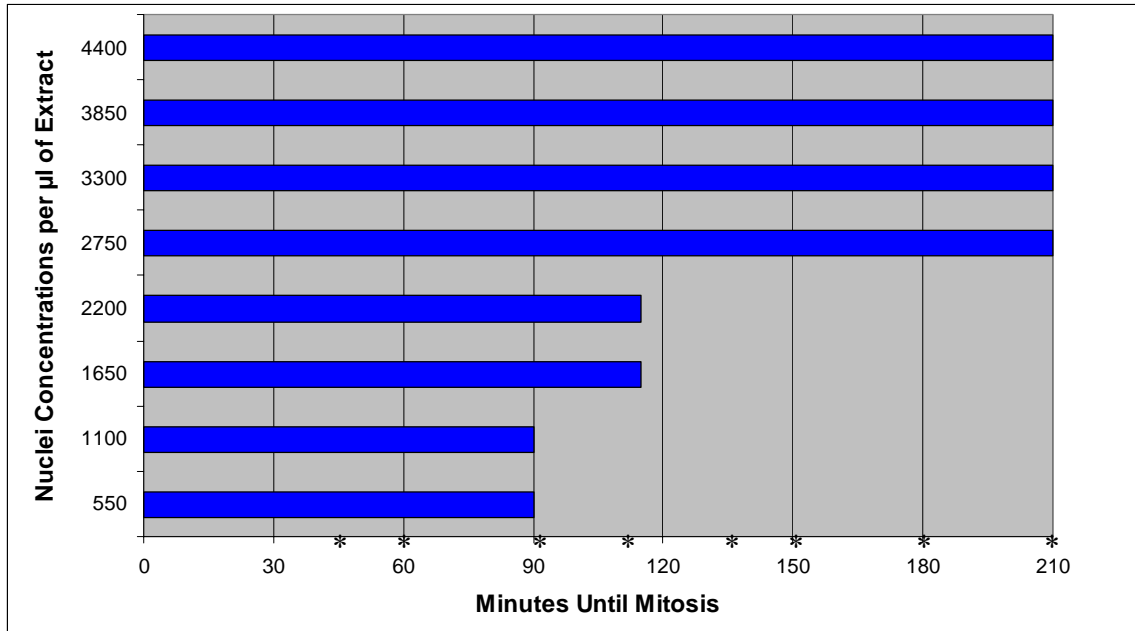


Figure 3.2 The change in time until mitosis due to increasing nuclear concentrations. The samples were treated with a range of nuclei concentrations and monitored for entry into mitosis at time points indicated by an asterisk (*). Two μl of each sample was mixed with two μl of DAPI fix/stain and analyzed under fluorescent and phase-contrast microscopy. Extract samples still in interphase exhibited dispersed chromatin and well defined nuclear envelopes. Mitotic samples exhibited condensed chromatin as well as an absence of a nuclear envelope. Samples were examined approximately every 15 minutes, and representative pictures of nuclei from each sample were taken. The experiment was completed by 210 minutes due to our observation of aberrant nuclear morphology in samples that remain in interphase at approximately 200 minutes after extract activation. As exhibited in the figure, samples with a nuclei concentration of 550, 1100, 1650, and 2200 per μl all went into mitosis during the experiment. However, 2750, 3300, 3850, and 4400 nuclei per μl did not enter mitosis by the end of the experiment. There is also an increase in the lag time of entry into mitosis between the samples treated with 550/1100 and 1650/2200 nuclei per μl . Increases in lag times are typically associated with an increase in cyclin threshold levels required to enter mitosis (Sha *et al.*, 2003). Experiment adapted from Dasso and Newport (1990).

As visualized in Figure 3.2, we can see that not all of the samples entered mitosis during the experiment. It appears that any concentration of DNA above 2200 nuclei/ μl did not enter mitosis. As we will discuss in later sections, this is an important finding we repeated multiple times. Nuclei concentrations were verified using a hemocytometer and the same DAPI fix/stain used for nuclei imaging in the extract samples.

These experiments gave us a good range of nuclei an extract could handle without dramatically affecting mitotic timing. This is important for the question we try to answer in later experiments. This experiment gives us direct evidence of a mitotic delay in the extract system induced by increasing nuclei concentrations. It is important to note that no DNA replication blocks or changes to the endogenous system were performed other than addition of nuclei.

Nuclei Concentration Affects Cyclin Thresholds and Lag Times of Entry into Mitosis

In our current study, nuclei concentration exhibits a direct effect upon the kinetics governing MPF control. These changes in MPF control are observed through alterations of cyclin thresholds and the lag time of entry until mitosis. In the cell-free extract system, the cyclin threshold is defined by the minimum amount of cyclin B required to drive the extract into mitosis. Below the minimum cyclin threshold, the number of pre-MPF molecules formed is insufficient to progress the cell cycle into mitosis. The amount of cyclin required to progress the cell cycle changes in response to regulatory signals targeting MPF for inhibition or activation (respectively raising and lowering the cyclin threshold).

The lag time of entry into mitosis is tightly correlated with the cyclin threshold. The more cyclin above and beyond the minimum threshold, the faster the extract system will enter mitosis, and shorter the subsequent lag time. As cyclin levels decrease and approach the minimum threshold level, entry into mitosis will be delayed and lag times until mitosis are increased. The lag time of entry into mitosis is the time required to activate the positive feedback loops responsible for progressing the cell cycle into mitosis. The less cyclin available to form pre-MPF molecules, the fewer pre-MPF molecules capable of participating in the initiation of the positive feedback loops activating MPF.

Using our evaluation of the effect nuclei concentration has upon the cell cycle progression in an extract as a starting point, we expanded our studies to evaluate the

effect of nuclear concentrations on cyclin threshold levels. Changes in the cyclin thresholds at later stages of development in *Xenopus laevis* could explain how the cell cycle is remodeled at the MBT. The nucleocytoplasmic ratio has long been thought to play an important role in the engagement of MBT checkpoints (Masui and Markert, 1971; Newport and Kirschner, 1982). An abrupt shift from rapid cell cycle divisions to a controlled, checkpoint-responsive cell cycle takes place at the MBT. Perhaps an accumulation of DNA during the first twelve divisions creates a concentration of nuclei that increases the cyclin threshold until it reaches a level high enough to make cyclin and its regulation a limiting factor. This could be an additive effect whereby each new nucleus formed contributes a small percentage of inhibitory signal until the additive effect of 4096 nuclei at the MBT creates enough substrate for the cell cycle checkpoint proteins to slow the cell cycle and activate cell cycle checkpoints if necessary.

Previous studies showed us that 1) a minimum amount of nuclei in the extract system was necessary to activate a checkpoint in response to unreplicated DNA (Dasso and Newport, 1990), and 2) once activated, the unreplicated DNA checkpoint increases the cyclin B threshold by a factor of 2.5 times in the presence of 1200 nuclei/ μl (Sha *et al.*, 2003). Since a minimum concentration of nuclei is necessary to activate a checkpoint, it follows that nuclei concentration may have an effect upon the cell cycle even without explicit blocks to DNA replication. In order to test this, we evaluated cyclin B thresholds for different nuclei concentrations (Figure 3.3).

In the cell-free extract system of *Xenopus laevis*, nuclei concentration has an effect on cyclin thresholds in a dose-dependent fashion. Figure 3.3 displays the change in time until mitosis due to increasing cyclin thresholds with increasing nuclei

concentrations. By using cycloheximide, we blocked all protein synthesis in order to prevent any endogenous cyclin B from accumulating. We then separated the extract into two groups, containing either high (2200 nuclei/ μ l) or low (1100 nuclei/ μ l) concentrations of nuclei. These two groups were then treated with an increasing concentration of Δ cyclin B and evaluated for mitotic entry. Samples were fixed with DAPI fix/stain and observed by fluorescent and phase contrast microscopy approximately every 30 minutes after activation with CaCl_2 . The samples were scored for the percent of nuclei in interphase or mitosis, and representative nuclei were photographed (not shown). The amount of time each sample spent in interphase is represented in the bar graph (Figure 3.3), with the last samples observed at 210 minutes.

As shown in Figure 3.3, the amount of cyclin required to initiate mitosis is greater in extract samples containing 2200 nuclei/ μ l than for samples treated with 1100 nuclei/ μ l. Evaluation of the samples treated with 17.5 and 35 nM Δ cyclin B for 1100 nuclei/ μ l, shows one sample entering mitosis while the other stays in interphase for the length of the experiment. Somewhere between 17.5 and 35 nM is the exact threshold needed for entry into mitosis with 1100 nuclei/ μ l. When using 2200 nuclei/ μ l, the minimum cyclin threshold was 42 nM Δ cyclin B. This experiment was repeated four more times with similar results and provides direct evidence for an increase in Δ cyclin B required to enter mitosis with increasing concentrations of nuclei in the *Xenopus laevis* extract system.

If we look at the green bars in Figure 3.3 representing 1100 and 2200 nuclei/ μ l, we can see a difference in Δ cyclin B threshold levels. As described earlier, the cyclin threshold represents the minimum amount of cyclin B necessary for advancement of the cell cycle into mitosis. This experiment represents a case in which an increase in nuclei

concentration alone increases the cyclin B threshold. The discovery of this increase in cyclin threshold implies a change in the kinetics of MPF control has taken place. The change in MPF could be contributed to an increase in the activity of negative regulators of MPF (Wee1 and Myt1), a decrease in the positive regulators (Cdc25A and Cdc25C), or change in both. Based on our current understanding of the signaling network connecting DNA and MPF, the negative and positive regulators of MPF are most likely being signaled through the Chk1 pathway (Bartek and Lukas, 2003). The relationship between checkpoint activation and nuclear concentrations could be due to a checkpoint inhibitor(s) being titrated out or an increase in basal checkpoint strength due to increasing nuclear concentrations.

Figure 3.3 shows the effect on lag time that various concentrations of Δ cyclin B has on the entry into mitosis. The concentrations used were surrounding the lower threshold of Δ cyclin B needed to enter mitosis. Under these extract conditions (1100 nuclei/ μ l, no DNA replication block, cycloheximide-treated), the minimum concentration of Δ cyclin B required to enter mitosis fell between 17.5 nM to 35 nM. Δ cyclin B concentrations of 35 nM or higher entered mitosis with varying lag times. The samples entered mitosis in a predictable fashion with the higher concentrations entering the earliest, soon followed by the next highest concentration.

In Figure 3.3, if we compare the two nuclei concentrations treated with 42 nM Δ cyclin B, we see a difference in the time each sample spent in interphase. As shown by Sha et al. (2003), an increase in lag time of entry into mitosis implies an increase in cyclin thresholds. For these nuclei concentrations, 42 nM Δ cyclin B was above the threshold of cyclin required to push both samples into mitosis. As described earlier, the higher the

concentration of cyclin is above the threshold, the faster the system will enter mitosis. As the difference between the amount of cyclin used and the threshold decrease, the lag time of entry into mitosis increases. As shown earlier, the cyclin threshold for 2200 nuclei/ μ l is between 35 and 42 nM Δ cyclin B, while the threshold for 1100 nuclei/ μ l is between 17.5 and 35 nM Δ cyclin B. Therefore, when both nuclei concentrations are exposed to 42 nM Δ cyclin B, we can expect both to enter mitosis, but at different times, based on the difference in their respective cyclin thresholds. Using 1100 nuclei/ μ l, we were well above the cyclin threshold with 42 nM Δ cyclin B, and therefore, the extract entered mitosis at a relatively early time (130 minutes after extract activation, 100 minutes after Δ cyclin B addition). The extract sample containing 2200 nuclei/ μ l had a delay in entry into mitosis when compared to 1100 nuclei/ μ l because 42 nM of Δ cyclin B is only slightly above the threshold for 2200 nuclei/ μ l. This experiment has repeatedly yielded similar results, confirming our theory that nuclei concentration does have a direct and quantifiable effect on the cell cycle engine.

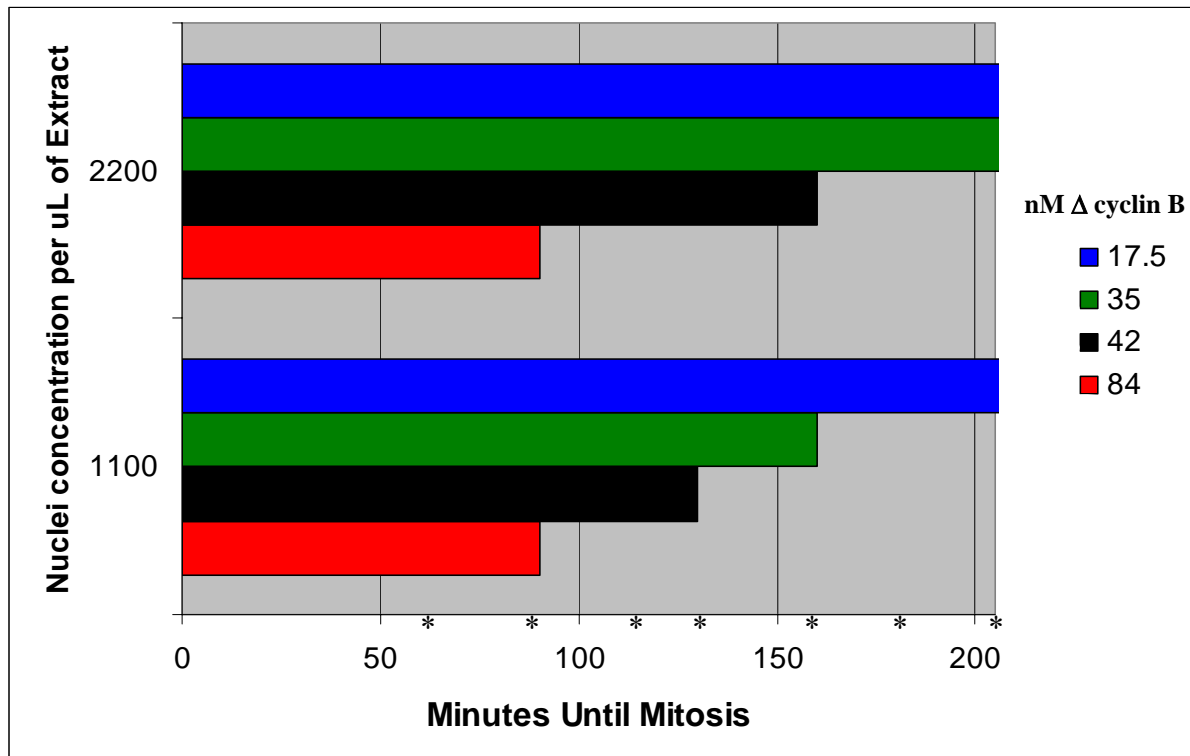


Figure 3.3 Change in cyclin thresholds due to change in nuclear concentration. This graph shows time each particular extract sample spent in interphase. Time until mitosis is measured from the time of activation of each sample with CaCl_2 . Any bars that extend to the edge of the graph were still in interphase when the experiment ended, 210 minutes after activation. Samples were evaluated at time points indicated by an asterisk (*). All samples were treated with cycloheximide to prevent any endogenous synthesis of cyclin B and insure cyclin B concentration is controlled by the experimenter. Two sets of samples with either 2200 or 1100 nuclei/ μL of extract were treated with a range of Δ cyclin B. Treatment of both nuclei concentrations with 17.5 nM Δ cyclin B resulted in interphase arrest, as neither sample was able to overcome the cyclin threshold necessary to enter mitosis. Treatment with 35 nM Δ cyclin B allowed 1100 nuclei/ μL to enter mitosis, but 2200 nuclei/ μL was unable to enter mitosis. This showed that there was a difference in cyclin thresholds in extracts treated with different concentrations of nuclei. 42 nM Δ cyclin B pushed extracts with both nuclei concentrations into mitosis. This means that the threshold for 2200 nuclei/ μL was between 35 and 42 nM while the threshold for 1100 nuclei/ μL was between 17.5 and 35 nM. This experiment also shows a difference in lag time or amount of time it takes each sample to enter mitosis: 130 minutes for 1100 nuclei/ μL and 160 minutes for 2200 nuclei/ μL . As discussed earlier, using concentrations slightly above the minimum threshold of Δ cyclin B required to enter mitosis increases lag times as the difference between the actual threshold and amount used in the sample decreases. Treatment of both nuclei concentrations with a level of Δ cyclin B that is far above both of their respective Δ cyclin B thresholds yields an entry into mitosis that is quick, and any slight difference in thresholds or lag times is unnoticeable. This experiment proves, through changes in lag times and thresholds, that nuclei concentration has an impact upon the kinetics of the cell cycle engine, MPF.

Elevated Cyclin Thresholds Caused by DNA Replication Blocks are Further Increased by Increasing the Concentration of Nuclei.

As suggested by the Novák-Tyson model in 1993, the cyclin thresholds are elevated by blocks to DNA replication. This was experimentally verified by Sha et al. (2003) in the *Xenopus laevis* cell-free extract system. When DNA replication is blocked in the extract system, the DNA replication checkpoint is engaged and prevents entry into mitosis.

Sha et al. (2003) showed experimental proof for the Novák-Tyson (1993) prediction that DNA replication blocks inhibited the cell cycle by increasing the cyclin B threshold required for mitosis. Based on our finding earlier in this chapter of increasing cyclin concentrations with increasing concentrations of nuclei, we wanted to explore the effect nuclei concentration had upon the DNA replication checkpoint thresholds. Would more DNA with replication blocks increase the requirement for cyclin B to enter mitosis?

In cell-free extracts, we evaluated extract samples (treated with a high (2000 nuclei per μl) or low (1000 nuclei per μl) concentration of nuclei supplemented with a range of non-degradable (Δ) cyclin B) for mitotic entry. These samples were then monitored for mitotic entry as described earlier. A representative graph of our results is shown in Figure 3.4. Length of interphase is graphed as a function of time after activation of the extract. An observable change in cyclin thresholds between the two samples is evidenced by the change in entry into mitosis within the 70-140 nM range of Δ cyclin B concentrations. For 1000 nuclei per μl , the cyclin threshold is between 70 and 105 nM Δ cyclin B whereas the 2000 nuclei per μl sample required between 105 and 140

nM Δ cyclin B to enter mitosis. These data provide evidence for an increase in cyclin threshold due to an increase in nuclear concentrations with DNA replication blocks.

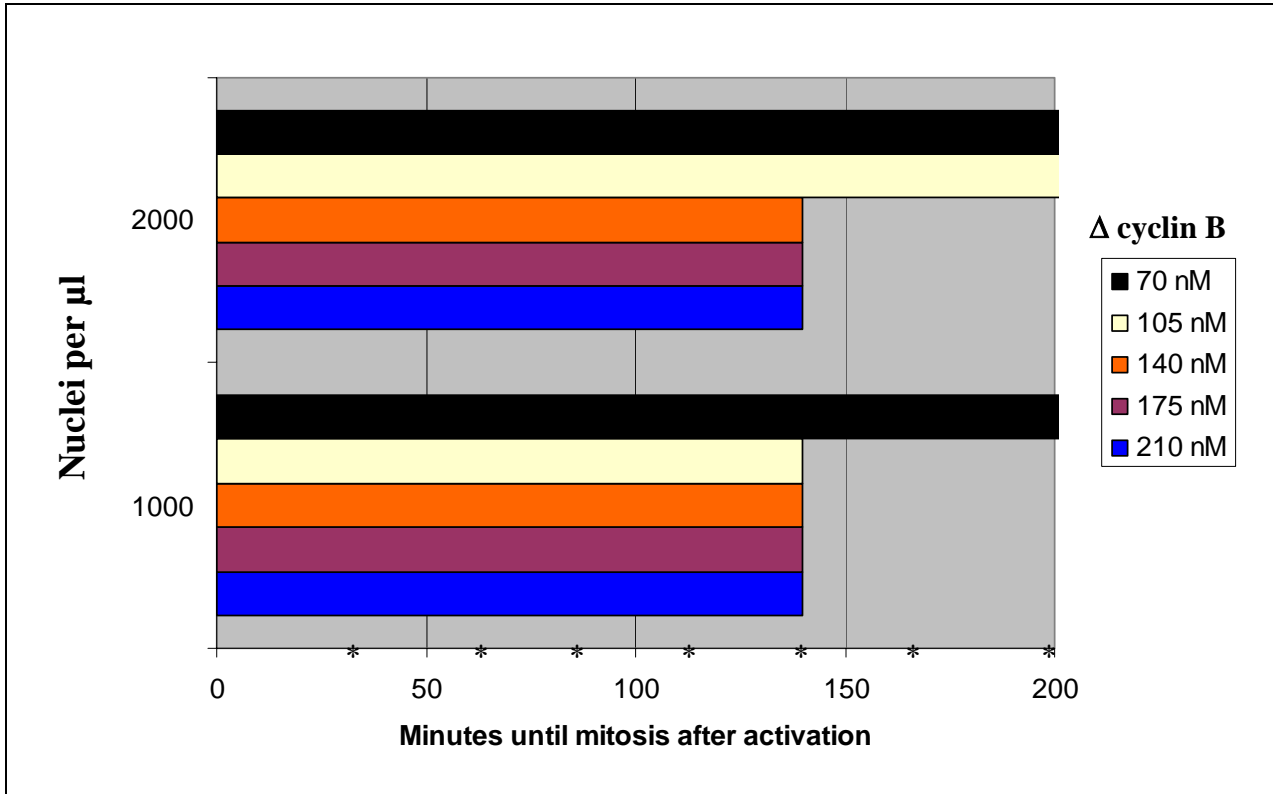


Figure 3.4 Increasing nuclear concentrations with DNA replication blocks further increase cyclin thresholds. The graph displays the time until entry into mitosis for two different nuclear concentrations treated with a range of Δ cyclin B. All samples were treated with 100 $\mu\text{g/ml}$ aphidicolin to block DNA replication and cycloheximide to prevent endogenous protein synthesis, specifically translation of cyclin B. The asterisks represent when the extract samples were evaluated for cell cycle stage using fluorescent and phase microscopy as described earlier. As indicated in the graph, for 1000 nuclei per μl , the cyclin threshold is between 70 and 105 nM Δ cyclin B whereas the 2000 nuclei per μl sample required between 105 and 140 nM Δ cyclin B to enter mitosis. These data provide evidence for an increase in cyclin thresholds due to increasing concentrations of unreplicated DNA.

In order to get a better appreciation for the change in cyclin thresholds due to nuclei concentrations, we also evaluated the same extract with and without DNA replication blocks. These experiments were carried out as described earlier and tested for cyclin thresholds by treatment with cycloheximide and a range of Δ cyclin B

concentrations. As shown in Figure 3.5, cyclin thresholds increased with an increase in nuclear concentration. Figure 3.5 also shows that this increase in cyclin thresholds can be further amplified when DNA replication blocks were induced with aphidicolin.

The effect nuclei concentration has upon cyclin thresholds and lag times was shown earlier in Figure 3.3. Figure 3.5 verifies our earlier finding if we evaluate the difference between samples treated with both nuclei concentrations and 42 nM Δ cyclin B (no aphidicolin). The sample with 1100 nuclei per μ l has an increased lag time of entry into mitosis when compared to the sample treated with 550 nuclei per μ l. Similar results are shown in Figure 3.3 and indicate that an increase in nuclei concentration can increase the cyclin B thresholds required for mitosis and associated lag time of entry.

As originally shown by Sha et al. (2003), and proposed by Novák and Tyson (1993), the cyclin threshold for entry into mitosis is increased in response to unreplicated DNA. Sha et al. showed that under normal conditions, the minimum amount of cyclin B required to push an extract system into mitosis was between 32 and 40 nM Δ cyclin B. In the presence of DNA replication blocks, the amount of Δ cyclin B required for mitosis was between 80 and 100 nM, approximately 2.5 times greater than the amount of Δ cyclin B required in the absence of DNA replication blocks. Our findings in Figure 3.5 confirm Sha et al.'s (2003) observations. Our concentration of 1100 nuclei per μ l displayed a similar change in the amount of Δ cyclin B required to enter mitosis with or without DNA replication blocks. 42 nM Δ cyclin B was adequate for entry into mitosis with 1100 nuclei per μ l, while 21 nM Δ cyclin B was below the cyclin threshold required and the extract sample remained in interphase in the absence of DNA replication blocks.

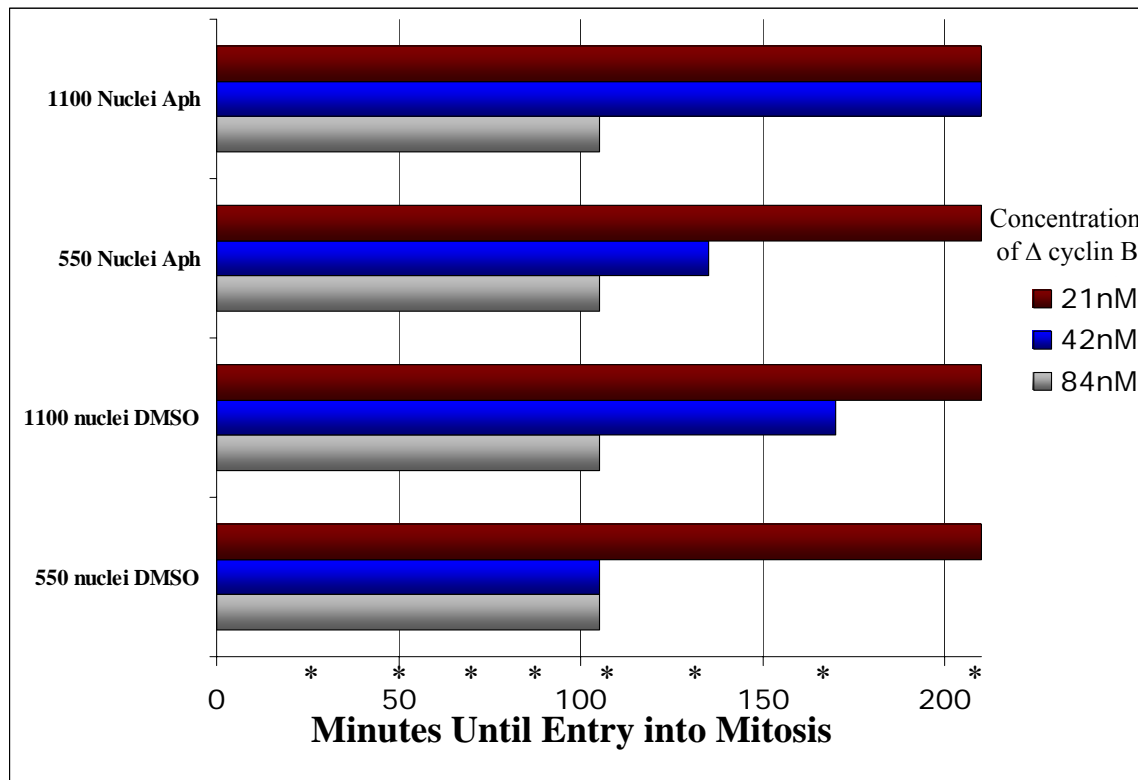


Figure 3.5 The difference between cyclin thresholds and lag times with changing nuclear concentrations. The graph displays the time until entry into mitosis for various samples with and without treatment with 100 $\mu\text{g/ml}$ aphidicolin. The asterisks represent when the extract samples were evaluated for cell cycle stage using fluorescent and phase microscopy as described earlier. Two nuclei concentrations were used (1100 and 550 nuclei per μl of extract). All samples were treated with cycloheximide to prevent endogenous protein synthesis, specifically translation of cyclin B. Samples were treated with 21, 42 or 84 nM Δ cyclin B to induce mitosis. All samples were evaluated approximately every 15 minutes for entry into mitosis by fluorescent and phase-contrast microscopy, and representative nuclei from each sample were photographed (not shown). Samples with $>90\%$ nuclei with complete nuclear envelope breakdown were considered mitotic.

We wanted to evaluate how varying concentrations of nuclei with DNA replication blocks affected cyclin B thresholds. In Figure 3.5, we can observe that the elevated cyclin threshold for entry into mitosis induced by DNA replication blocks is further increased with increasing concentrations of nuclei. When 550 nuclei per μl was used in a sample treated with 42 nM Δ cyclin B, it entered mitosis at approximately 135

minutes after activation. The extract sample treated with 42 nM Δ cyclin B but with 1100 nuclei per μ l was still in interphase at the end of the experiment (210 minutes post extract activation). This data suggests elevated cyclin thresholds caused by DNA replication blocks are further increased by increasing the concentrations of nuclei. This data further implies that nuclei content can have an additive effect that inhibits MPF and helps the argument for nucleocytoplasmic ratio as an important mechanism for gap phase acquisition and checkpoint engagement at the MBT.

Discussion:

Using the *Xenopus laevis* extract system, our lab has quantified the link between DNA concentration and the cell cycle engine. Using the highly manipulable *Xenopus laevis* extract system, we were able to tightly regulate the amount of cyclin B present in the system. By regulating the amount of cyclin B, we essentially limited the number of MPF molecules able to form and were able to evaluate the effect on the aforementioned thresholds and lag times. We have shown that 1) the concentration of Δ cyclin B required to trigger entry into mitosis increases with increasing concentrations of nuclei, 2) the lag time of entry into mitosis increases with increasing concentrations of nuclei, and 3) elevated cyclin thresholds caused by DNA replication blocks are further increased by increasing the concentrations of blocked nuclei.

In many previous studies, the molecular signaling network coupling DNA replication blocks and the cell cycle machinery has been intensively studied (Dasso *et al.*, 1992; Kumagai *et al.*, 1998a; Kumagai *et al.*, 1998b; Hekmat-Nejad *et al.*, 2000). In this study, we were able to carefully regulate the DNA concentration and cyclin B levels and evaluate the effect of on the cell cycle engine. We have built upon our previous work evaluating cyclin thresholds in the *Xenopus laevis* extract system. Our collaboration with the Tyson lab also gives us an advantage due to their experience in predictive mathematical modeling (Novák and Tyson, 1993).

Based upon a change in the Δ cyclin B threshold as shown in Figure 3.3, we can predict that a change is occurring in the molecular signaling pathway governing MPF control. The exact way in which nuclear concentration is altering the molecular pathway

governing MPF is not known. Based on what is known about the MPF control, it seems possible that an increase in nuclear concentrations is increasing the activity of the Chk1/Chk2 kinases through ATR and/or ATM (Guo *et al.*, 2000 2001; Piwnicka-Worms and Zhao, 2001 2001). This could be due to the fact that we are saturating the DNA replication machinery and it is taking longer to finish DNA replication. If a DNA replication checkpoint were fully engaged, however, we would expect a much larger increase in Δ cyclin B thresholds based on finding from Sha *et al.* (2003). If the increase in nuclear concentration causes an increase in the amount of time it takes for complete DNA replication, then the Δ cyclin B activation thresholds would not change. As shown in Figure 3.3, we can clearly see that there is a change in the Δ cyclin B threshold levels. This leads us to believe that the cell cycle machinery is sensing the increase in nuclear concentration based on slight increases in the basal activity of the checkpoint machinery (Chk1). If the basal levels of Chk1/Chk2 activity reflected the concentration of nuclei, this would result in a slight increase in the activation threshold as nuclei concentrations increased. Chk1/Chk2 activity could be measured in these extract samples by performing kinase assays or Western blot detection of Chk1/Chk2 phosphorylation sites. Validation of this assumption would fit nicely with the mathematical model of the cell cycle recently updated by Dravid *et al.* (unpublished).

With each division in the pre-MBT embryo, the nuclei concentration doubles. At the MBT, the concentration of nuclei could be reaching a high enough concentration to strong enough to affect MPF and halt the cell cycle. This theory is amenable to our findings in the extract and was hypothesized by Novák and Tyson in 1993 based on Dasso and Newport's findings (1990).

Chapter 4: Evaluation of a DNA Damage Response (Induced by Ionizing Radiation (IR)) in the *Xenopus laevis* Extract System

Abstract:

A DNA damage checkpoint is found in somatic cells of all eukaryotic organisms that have been studied. DNA damage differs from DNA replication blocks in that it causes a physical break or mutation in the DNA strand. DNA is easily damaged by ionizing radiation (IR) as well as reactive compounds found within the cell. DNA exposed to IR acquires double-stranded breaks in a dose-dependent fashion (Bakkenist and Kastan, 2003). A DNA damage checkpoint responds to these double-stranded breaks by transducing a molecular signal cascade that halts the cell cycle. The DNA damage checkpoint in the extract system is not as well understood as the response to unreplicated DNA. We have evaluated the DNA damage checkpoint response induced by IR in *Xenopus laevis* extract system within the range of nuclei concentrations that can be replicated in the absence of DNA damaging agents. As shown previously (Figure 3.2, page 49) the extract system can only efficiently replicate DNA within a range of nuclear concentrations. Our results show that exposure of nuclei to IR is insufficient to create a DNA damage checkpoint or appreciable change in mitotic entry.

Introduction:

In the early development of *Xenopus laevis*, a DNA damage checkpoint is not active (Anderson *et al.*, 1997; Hensey and Gautier, 1997). At the MBT, however, the *Xenopus laevis* embryo acquires gap phases, and checkpoints are able to respond to damaged DNA (Anderson *et al.*, 1997; Hensey and Gautier, 1997). In the extract system,

the ability of the DNA replication checkpoint is well known and described in Chapter 3. DNA damage induced by IR has been shown to activate key regulatory proteins responsible for cell-cycle control in the extract (Kumagai *et al.*, 1998a; Kumagai *et al.*, 1998b), but how DNA damage affects mitotic timing is not well characterized.

Many of the checkpoint proteins involved with the DNA replication checkpoint also function in the damage checkpoint. Chk1 and Chk2 proteins fall into this category and help translate a damage or replication checkpoint from the DNA sensing machinery to the cell cycle engine. We know this signaling pathway is functional in the extract and responds in a predictable fashion to unrepligated DNA (Chapter 3). Studies performed in the extract system showed that when DNA was exposed to IR, the checkpoint pathway was activated through phosphorylation of Chk2 (Guo and Dunphy, 2000; Sagata, 2002) but not Chk1. In the intact embryo however, embryos exposed to IR acquired hyper-phosphorylated, activated forms of Chk1 (Kappas *et al.*, 2000). Based on studies done in the extract, it seems that Chk2 responds to DNA damage only when using a concentration of nuclei of around 3000-10,000 nuclei per μL of extract (Kumagai *et al.*, 1998a; Kumagai *et al.*, 1998b). This concentration is much higher than the concentration required for a DNA replication checkpoint (~ 250 nuclei per μl).

Previous studies performed in the extract evaluating the effect of IR damage monitored mobility shifts of Chk1 on SDS-PAGE gels (Kumagai *et al.*, 1998a). These gels separate proteins based on their size and shape, and when a protein is phosphorylated, it generally becomes retarded in its electrophoretic mobility. When inducing DNA damage in the *Xenopus laevis* extract system, the researchers were looking for a shift in mobility of Chk2 which would correspond to its activation in a

checkpoint response. Based on the change in phosphorylation state of Chk2, the researchers inferred that the DNA damage checkpoint was being activated (Guo and Dunphy, 2000). Kappas et al. (2000) found that IR elicited Chk1 phosphorylation as well, pointing to a possible overlap between Chk1 and Chk2 function in response to double-stranded breaks in DNA. However, Guo and Dunphy (2000) did not observe any change in the phosphorylation state of Chk1 in response to double-stranded breaks. It is also possible that Kappas et al. (2000) elicited stalled replication forks as a secondary effect of DNA damage. Therefore, there remains some discrepancy between the studies in extracts versus the intact embryo.

Our lab wanted to further investigate the effect a DNA damage checkpoint had on the cell cycle engine, MPF. We outlined a series of experiments that would test the DNA damage response in a similar way as we tested the unreplicated DNA response. Previous studies showed that 3000 nuclei/ μl was sufficient to initiate a DNA damage response (Kumagai *et al.*, 1998a). Since checkpoint proteins were being phosphorylated in response to IR exposed nuclei, it follows that there is also a corresponding increase in cyclin thresholds required for entry into mitosis. We wanted to analyze the Δ cyclin B thresholds for various concentrations of damaged nuclei in the extract system.

Ionizing radiation (IR) is a common mechanism of inducing DNA damage (Anderson *et al.*, 1997). IR causes double-stranded breaks in the DNA strand in a dose-dependent fashion. In somatic cells, these breaks will halt the cell cycle through activation of ATM (Sancar *et al.*, 2004). ATM recognizes double-stranded breaks and phosphorylates the Chk2 protein. Once activated, Chk2 inhibits MPF through Cdc25 (Guo and Dunphy, 2000) and possibly Wee1. Since there should be a direct correlation

with the exposure of DNA to IR and the number of double-stranded breaks formed, we could tightly regulate the amount of damage the DNA is incurring from IR exposure.

In the *Xenopus laevis* embryo, it is apparent that a minimum number of nuclei is required before gap phases and checkpoints become present in the cell cycle. When the embryo is exposed to IR in a pre-MBT developmental stage, it will continue to progress in development up to the MBT and then activate the apoptosis pathway (Anderson *et al.*, 1997; Kappas *et al.*, 2000). Exposure to IR post-MBT will activate a checkpoint at the stage the IR dose was given and allow for DNA repair or apoptosis, depending on the severity of genomic damage (Kappas *et al.*, 2000; Finkielstein *et al.*, 2001; Wroble and Sible, 2005). It seems possible that the DNA damage signal could be occurring in a dose-dependent fashion with regards to the concentration of nuclei present. To evaluate the effect of increasing the total nuclear content in the embryo system, double-stranded oligomers were injected into a single blastomere of a 2-cell embryo were found to slow the cell cycle (Conn *et al.*, 2004). In Conn *et al.*'s (2004) experiments, they found that the cell cycle was slowing down due to the double-stranded oligomers initiating a checkpoint response through Chk1. This checkpoint was typical of Chk1 and displayed inhibition of Cdk1 through the degradation and inhibition of Cdc25A and Cdc25C, respectively. Conn *et al.* (2004) also showed that the total amount of DNA was important in initiating the checkpoint, not just the crude number of double-stranded breaks. Precocious engagement of a checkpoint response required a minimum concentration of DNA, not all of which is required to contain double-stranded breaks (Conn *et al.*, 2004). In this manner, pre-MBT embryos exposed to IR do not have enough total genomic

content to elicit a DNA damage response, whereas post-MBT embryos have acquired enough total DNA to successfully activate the DNA damage response.

Conn et al., (2004) gives us some reasoning behind the lack of a DNA damage response in pre-MBT embryos. Using IR to elicit double-stranded breaks in DNA, we can control exactly how many breaks we introduce into the nuclei as well as total nuclei concentration in the extract. By using varying doses of IR on varying concentrations of nuclei, we tested for a similar response in the extract system as were found by Conn et al. (2004). In order to examine the cell-free extracts ability to evaluate DNA damage, we wanted to test what was more important: total nuclear content or, total number of double-stranded breaks. For example, would 600 nuclei/ μl with double stranded breaks every 500 base pairs elicit the same checkpoint response as 300 nuclei/ μl with breaks every 250 base pairs? We wanted to evaluate the aforementioned question in the cell-free extract system and look for differences in cyclin thresholds and lag time of entry into mitosis.

Our lab was interested in the possibility of measuring any changes in the cell cycle engine due to IR exposure in the *Xenopus laevis* cell-free extract. This could be done through Δ cyclin B threshold experiments. Evaluation of any changes in the Δ cyclin B threshold after exposure to IR would give us some insight as to how strong a checkpoint signal was elicited. Our hypothesis was that increasing the IR exposure (and consequently number of double-stranded breaks) would yield an increase in Δ cyclin B thresholds required to push the extract system into mitosis due to a stronger checkpoint response. We feel that evaluating the Δ cyclin B thresholds allows us to get a better understanding of the effects checkpoints can induce upon the cell-cycle.

Results:

Analysis of a DNA Damage Checkpoint and its Effect on MPF.

The *Xenopus laevis* cell-free CSF extract system was utilized to evaluate DNA damage checkpoint response. Extracts were treated with CaCl_2 to trigger exit from meiosis II and entry into the first interphase. Upon activation, the cell-free extracts were supplemented with different concentrations of nuclei pre-treated with ionizing radiation (IR) as well as cycloheximide to prevent any protein synthesis. After a healthy interphase was achieved (~25 minutes after activation) as determined by nuclear morphology, the extract samples were also treated with Δ cyclin B and evaluated for mitotic entry. In the early stages of testing, we used nuclei concentrations of 1100 and 3300 of damaged nuclei per μL of extract. After several attempts were made, we failed to elicit any amount of change in Δ cyclin B threshold levels needed for mitotic entry. We were unable to measure any difference in mitotic timing between the samples using a variety of templates as sources of double-stranded breaks (Figure 4.1).

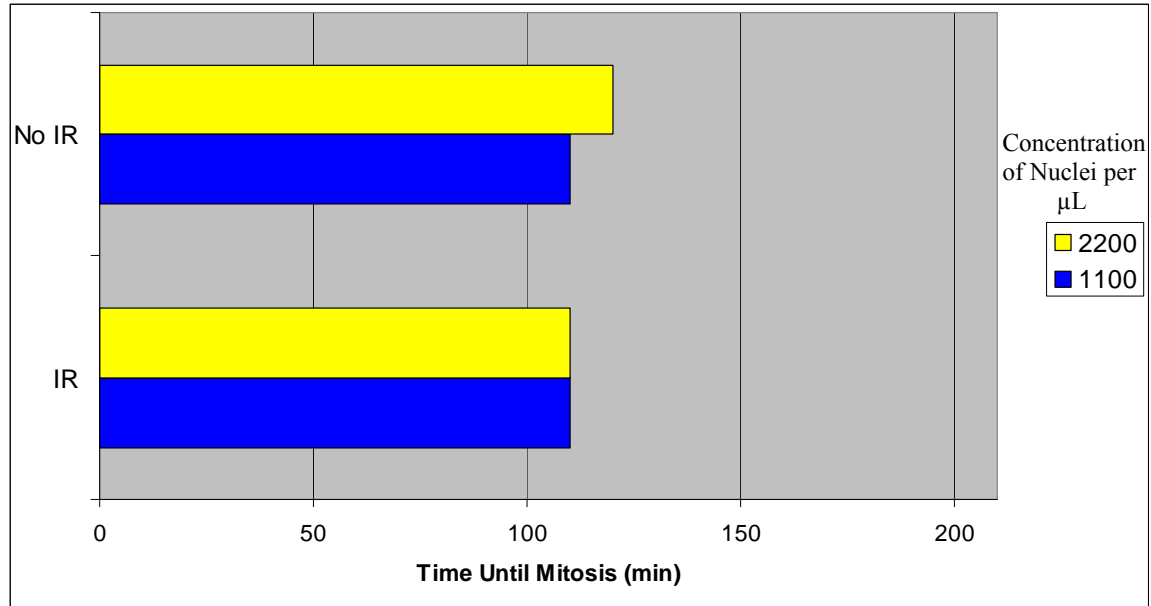


Figure 4.1- Time until mitosis in extracts with irradiated vs. unirradiated nuclei. The time until mitosis in this experiment was considered to be time after addition of CaCl_2 . Nuclei exposed to 40 Gy IR were added to the IR-labeled extract samples to a total concentration of either 1100 or 2200 per μl (as indicated by key). No significant difference in mitotic entry was observed. Samples were observed every 15 minutes initially, then every 5 minutes after first sample entered mitosis. Samples were deemed mitotic once greater than 90% of nuclei observed had nuclear envelope breakdown.

Taking a step back from evaluation of the Δ cyclin B thresholds, we treated extracts with varying concentrations of nuclei, with or without IR-induced damage (90 Gy IR). These extracts were not treated with cycloheximide because we were just testing the ability to illicit a DNA damage checkpoint. Using concentrations of nuclei between 300-10,000 nuclei/ μL , we were unable to elicit any change in mitotic entry between irradiated and unirradiated samples. These trials were done multiple times with similar results occurring each time. Any concentration of 3000 irradiated nuclei per μL or less would enter mitosis at the same time as unirradiated samples (with the same nuclei concentration), while higher concentration remained in an interphase-like state.

During these experiments, we observed that the samples with the higher concentrations of nuclei that did not enter mitosis with the controls would develop aberrant morphology. A typical sample with a nuclei concentration above 3000 per μL

would begin to exhibit a decreased nuclei size 150 minutes past activation (Figure 4.2A). These small nuclei would either disappear through some undefined method of degradation or become extremely condensed within the nuclear envelope (Figure 4.2B). As shown in Chapter 3, concentrations of unirradiated nuclei above approximately 3300 nuclei per μL of extract stalls advancement of the cell cycle. Any concentration below this amount will enter mitosis but with an increase in lag time of entry into mitosis as the concentration of nuclei approaches 3300 nuclei/ μl . Observations of irradiated nuclei with concentrations below 3300 nuclei/ μl entered mitosis with lag times similar to those observed in experiments performed in Chapter 3 (Figure 3.2).

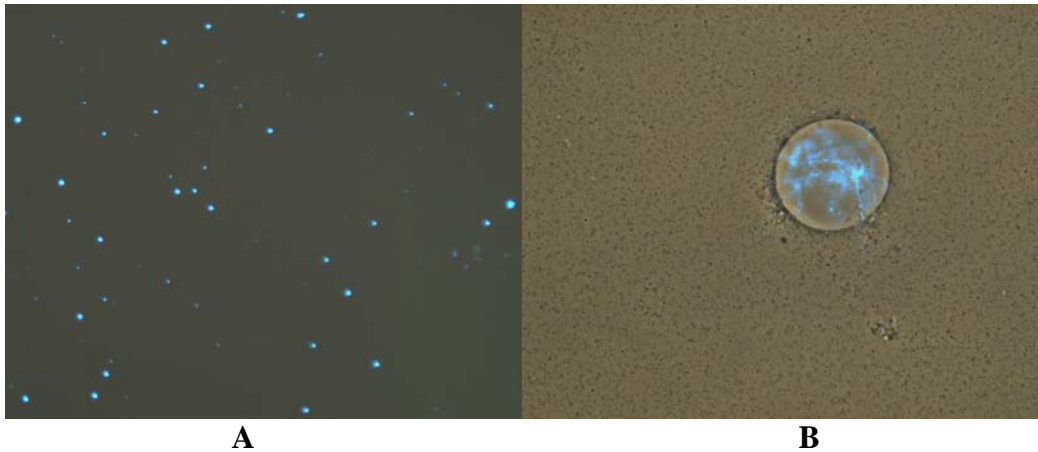


Figure 4.2 **A)** Aberrant nuclear morphology exhibited by extracts exposed to nuclear concentrations above 2500 nuclei/ μl . Cell free extract with 4400 nuclei per μL collected at 150 minutes after activation. This extract was collected and mixed with an equal volume of DAPI fixative stain. This image was taken using fluorescent microscopy. The small dots are what is left of the nuclei 180 minutes after the sample was activated. The magnification is equivalent to that found in figure 3.1. Under phase contrast microscopy no nuclear envelopes were apparent. **B)** This is an extract sample (4400 nuclei per μL treated with IR) photographed 140 minutes after activation. The image is an overlap of the fluorescent and phase-contrast images so that we can see the blue fluorescing DNA contained in the nuclear envelope. Both samples were photographed under the same magnification (400X).

Figure 4.2 shows typical results obtained when evaluating an extract exposed to IR-damaged nuclei. The nuclei would be exposed to IR right before dilution into the extract samples using a Minishot X-ray cabinet device (TFI). Extracts were not treated

with any protein translation or DNA replication blocking agent. Samples were evaluated by taking 2 μL of sample and mixing with an equal volume of DAPI fixative stain. Samples were then be visualized under fluorescent and phase-contrast microscopy approximately every 15-30 minutes after activation. Samples were deemed in either interphase or mitosis as shown and described in Figure 3.1. Figure 4.1 shows the results of the observed time until mitosis for each experiment. These results were repeated multiple times, and showed there was no significant difference between samples treated with or without IR.

Nuclear morphology observed in extracts containing high concentrations of nuclei suggested that the extract may be undergoing apoptosis. Apoptosis is a program of cell death initiated as a response to conditions in the cell that render it useless or detrimental to the surrounding tissue or organism(s) (Columbano, 1995). To test for apoptosis in extracts treated with high concentrations of nuclei, we utilized an assay for caspase activity. The PARP substrate is cleaved in response to an activated apoptosis signal through caspase 3 (Carter and Sible, 2003). The results of the assay showed that there was no caspase activity detectable in the extract samples (Figure 4.3). Cleaved PARP runs faster on the SDS-PAGE gel and is apparent in lane one. Uncleaved PARP runs slower on the gel and is seen in lanes two through nine. In the first two lanes, IR treated and untreated embryos were used as positive and negative controls, respectively. The next seven lanes represent samples taken from an extract treated with either 1000 or 4000 nuclei per μl of extract. This leads us to believe the extracts are degenerating by some other mechanism or that caspase activity is below our limit of detection. The DNA

content may be just too great for the replication machinery, and the cell cycle is unable to progress.

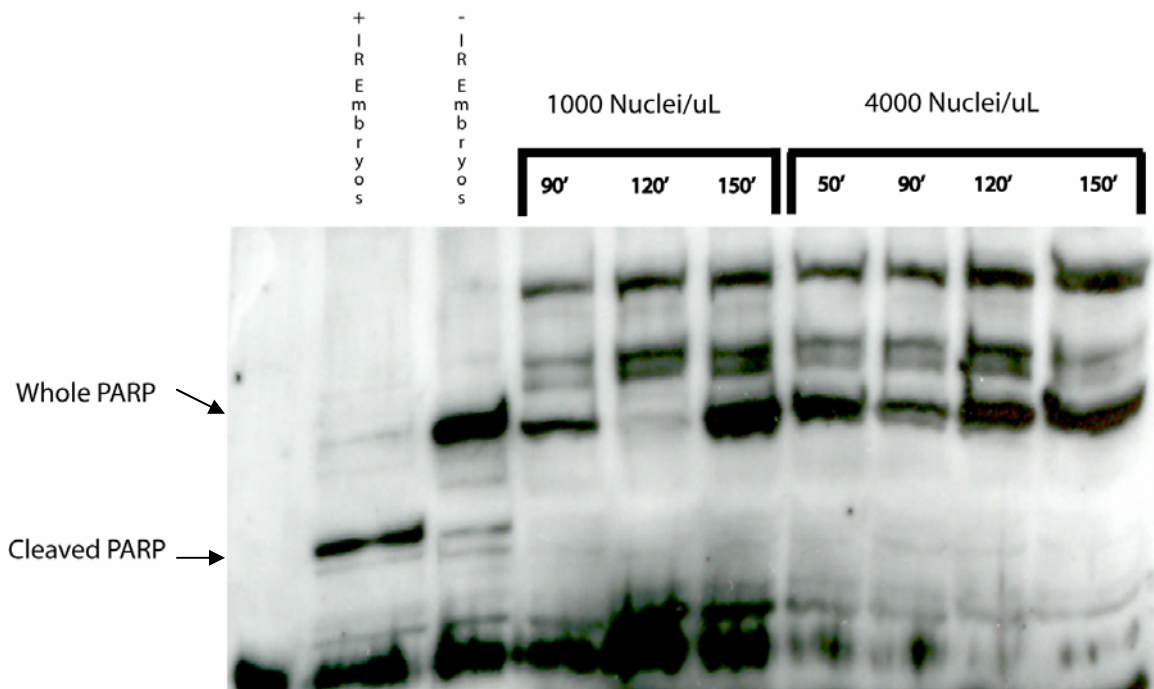


Figure 4.3 Extract samples were treated with 1000 and 4000 nuclei per μl and analyzed for cleavage of exogenous PARP. After incubation with the extract samples taken at various times during the experiment, samples were run on a 12.5% SDS-PAGE gel. A Western blot was then performed using the PARP antibody to evaluate samples for PARP cleavage. IR-treated (pre-MBT irradiated) and untreated embryos were collected at stage 14 (neurula) and used for positive and negative controls, respectively.

Discussion:

The inability of the *Xenopus laevis* cell-free system to illicit a DNA damage checkpoint was disappointing but nonetheless provides us with a valuable perspective on the appropriateness of cell-free extracts as a model system for studying DNA damage checkpoints. Based on our work in the extract system, nuclei concentrations above approximately 2500 nuclei per μL can elicit artifactual results. While using these high nuclei concentrations in an extract, it may be possible to activate checkpoint signals as determined by the presence of activating phosphorylations of Chk2 (Guo and Dunphy, 2000) and ATM (Costanzo *et al.*, 2000) that can be visualized using Western blots to determine phosphorylation events. However, these studies may be confounded by the fact that nuclei concentration overwhelms the extract system, and even control extracts lacking explicitly damaged DNA, cannot enter mitosis.

Our ability to determine cyclin thresholds is a very powerful tool when building mathematical models. The DNA damage response upon MPF is a crucial piece of information necessary to broaden the scope of current models. Unfortunately, our results were unable to define any correlation between DNA damage and MPF control. However, we have established that nuclei concentration plays an important role in regulating the cell cycle.

Xenopus laevis cell-free extracts have been extremely successful in outlining the roles for many cell cycle regulators (Dasso and Newport, 1990 1995, Kappas *et al.*, 1999, Sha *et al.*, 2003; Kumagai and Dunphy, 1995 1995, Kappas *et al.*, 1999, Sha *et al.*, 2003; Kappas *et al.*, 2000 1995, Kappas *et al.*, 1999, Sha *et al.*, 2003; Sha *et al.*, 2003 1995,

Kappas et al., 1999, Sha et al., 2003). There are many factors that may have contributed to the inability of the extract to respond to DNA damage. After the cell cycle is remodeled at the MBT, zygotic transcription begins and an important factor in the recognition of DNA damage may be introduced. During preparation of the extract, an insoluble DNA damage response element could be lost as well, yielding in unresponsive DNA damage checkpoint that normally becomes active once there is enough nuclear content in the embryo. There could also be a direct inhibitor of the DNA damage response signal that is not degraded until the MBT. Another possibility is that an important element of the DNA damage signal is not present until the MBT when zygotic transcription is activated.

In order to incorporate a DNA damage response into the mathematical model, it is important we work within the nuclei concentrations defined by Figure 3.1. Other sources of DNA damage may need to be incorporated into the DNA in order to elicit a functional DNA damage response. At this time however, we are limited by the capabilities of the extract to respond to DNA damage. Like any experimental system, the *Xenopus laevis* extract system is not without its flaws. The embryo may be better suited to evaluate the DNA damage response. However, even the embryo has its limitations and is not as easily manipulated as cell-free extracts. It is important we recognize these characteristics of the system and incorporate them into our interpretations from data gathered. This is especially important when generalizing data obtained in the extract system to that of the *in vivo* system.

Our observations in the extract system show that 1) a DNA damage checkpoint using concentrations of nuclei between 300 and 3300 treated with IR is unable to halt cell

cycle progression 2) a DNA damage checkpoint may be present based on observations in other labs evaluating phosphorylations of DNA damage checkpoint proteins but at nuclei concentrations high enough to halt the cell cycle in the absence of DNA damage, and 3) IR damaged nuclei had no influence upon cyclin B thresholds and lag times of entry into mitosis within the range of nuclei concentrations that can be efficiently replicated. Our findings elucidate several facets of the cell-free extract system that are important for interpretations of data collected therein. When constructing a mathematical model, our observation will be valuable when interpreting the literature from various labs using the cell-free extract system.

Chapter 5: Conclusion

Understanding the control mechanisms governing the cell cycle is extremely important for developing therapies against cancer. Utilizing the *Xenopus laevis* cell-free extract system, we have found a direct link between nuclei concentration and cell cycle regulation. This helps explain the phenomenon found in the early development of *Xenopus*, whereby the cell cycle switches from an unregulated, rapidly progressing cell cycle, to a tightly regulated, checkpoint-dependent cell cycle. We have found a correlation between nuclei concentration in the extract system and the Δ cyclin B concentration required for entry into mitosis (Figure 3.3). It is unclear how the nuclei concentration signals the cell cycle engine but is thought to act through Chk1 and/or Chk2. An increase in available nuclei content could be causing a slight increase in the basal Chk1 response in a dose-dependent fashion, reflected in the change in cyclin B threshold requirements. We know it is not activating a true checkpoint strength response due to the degree of increase in cyclin B thresholds. As found by Sha et al. (2003), when a checkpoint response is engaged in the extract system, an increase to cyclin B thresholds was found to be 2.5 times greater than controls.

Correlating with our data on increasing cyclin B thresholds required for entry into mitosis with increasing nuclei, we also found an increase in lag time of entry into mitosis with increasing nuclei concentrations (Figure 3.2, Figure 3.3). We showed that two different extract samples treated with two different concentrations of nuclei exhibited a difference in lag time of entry into mitosis despite equal Δ cyclin B concentrations (Figure 3.3, 1100 and 2200 nuclei/ μ l, 42 nM Δ cyclin B) due to a difference in the cyclin

thresholds between samples. This data validates our findings that nuclei concentrations in the cell-free extract system have a direct impact upon the cell cycle.

As found by Sha et al. (2003), the hysteretic loop determining cyclin B thresholds is further increased in response to unreplicated DNA found in the extract system. Using aphidicolin, we further tested the effect of various concentrations of unreplicated DNA upon cyclin thresholds. We found that elevated cyclin thresholds caused by DNA replication blocks are further increased with increasing concentrations of nuclei (Figure 3.4 and Figure 3.5). This further increases our appreciation for the impact of nuclei concentration in the extract. The checkpoint response to unreplicated DNA can vary in strength and is not reaching its potential maximum using nuclei concentrations below 2200 per μl . In the early development of *Xenopus* embryos, unreplicated DNA does not elicit a checkpoint until around the MBT (stage 12) (Anderson *et al.*, 1997; Hensey and Gautier, 1997). Based on our results, this is likely due to the fact that a threshold of DNA is not present and any checkpoint response is insufficient to elicit cell cycle arrest.

When we tried to evaluate the effect of DNA damage in the extract system, we encountered some difficulty in eliciting any checkpoint engagement. When evaluating a damage response induced by IR treated nuclei, within the range of concentrations (300-2200) outlined in Chapter 3 (Figure 3.2), a checkpoint was not observed. The amount of radiation used was far above that required to elicit a checkpoint in the post-MBT embryo (Kappas *et al.*, 2000; Wroble and Sible, 2005). Our original goal of these experiments was to evaluate the effect of damaged DNA on cyclin thresholds and see if it was dose-dependent as the case for DNA replication blocks. Previous studies using IR identified a checkpoint response using 3000 nuclei/ μl of extract (Kumagai *et al.*, 1998b). This data

conflicts with our findings in Chapter 3 (Figure 3.2), where we evaluated the effect of nuclei concentrations on cell cycle progression.

There are several reasons for the discrepancy between our lab and that of the Dunphy laboratory. In their paper, Guo and Dunphy (2000) evaluated extract samples for checkpoint engagement by looking at Chk2 phosphorylation after treating the extract with nuclei exposed to IR using γ -rays instead of X-rays. They used Western analysis to look at Chk2 phosphorylation but did not evaluate the extract samples for entry into mitosis via fluorescent and/or phase microscopy. By using concentrations of nuclei above what we found to be the maximum able to enter mitosis, the researchers could have been artificially inducing the checkpoint in system already halted in interphase. Our findings elucidate features of the cell-free system that are important in interpreting DNA damage checkpoint responses evaluated in the *Xenopus laevis* extract system. Future work will involve the evaluation of a checkpoint response due to nuclei concentrations alone. We believe that excessive concentrations of nuclei (≥ 2200 nuclei/ μ l) could be initiating a checkpoint response in the cell-free extract. This could be due to the fact that we are saturating the system with nuclei and overwhelming the DNA synthesis/maintenance mechanisms, initiating a cell cycle checkpoint even in the absence of explicit DNA damage or replication blocks.

Evaluation of cyclin thresholds is a reliable tool for broadening the mathematical model. In future studies, we hope to look at the effects several cell cycle regulatory proteins have upon MPF kinetics based on changes in cyclin thresholds. Preliminary data collected using Cdc25A phosphatase in extract samples has shown a decrease in cyclin thresholds as well as lag time of entry into mitosis. Using Cdc25A in extracts has shown

us the importance of its regulation of MPF in the extract system and the need to include the effect it has on MPF regulation in the mathematical model. The cell-free extract system provides an easily manipulatable, cell cycle competent, experimental proving ground where we can test a multitude of cell cycle regulators in a quantitative manner. Our experience in protein purification and the availability of gene constructs for many of the cell cycle regulators will allow for rapid and large scale expansion of how we understand cell cycle regulation.

Data we have described in the preceding chapters will be useful in the construction of a mathematical model of cell cycle control. The multitude of molecular signaling pathways governing cell cycle progression is too expansive to understand by intuitive methods alone. Using differential equations to describe the reactions of the known molecular signaling pathways gives us a systems-level understanding of how cell cycle progression is regulated. Pairing mathematical modeling techniques with an easily manipulatable experimental system, we can expand the scope and accuracy of the mathematical model allowing for more precise predicative power and a better understanding of the underlying kinetics governing cell cycle progression.

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