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THE CONTRIBUTION OF MITOCHONDRIAL FUNCTION TO THE ENERGETICS OF CELL CYCLE PROGRESSION

By

SUSAN SWEET, B.Sc.

A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfilment of the Requirements

for the Degree

Doctor of Philosophy

McMaster University

MITOCHONDRIAL CONTRIBUTIONS TO CELL CYCLE PROGRESSION

This thesis is dedicated to Otto,
without whom there would be no thesis;
And to my parents,
without whom there would be no author.

Doctor of Philosophy (2000)
Medical Science

McMaster University
Hamilton, Ontario

TITLE: The Contribution of Mitochondrial Function to the Energetics of Cell Cycle Progression

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NUMBER OF PAGES: xiii, 151

ABSTRACT

Mitochondria play a critical role in the provision of energy to individual cells through the synthesis of ATP. The relationship between mitochondrial energy production and cell cycle progression has been explored in this work with the intention of defining the fuel requirements of the cell cycle engine. Because mitochondrial physiology and cell division proved to be intimately linked, we also examined the feasibility of disrupting mitochondrial properties in order to alter cell cycle dynamics.

We show that the rate of utilization of mitochondrially-derived ATP fluctuates throughout the cell cycle and that stages where ATP levels are lowest correspond to stages most sensitive to pharmacologic inhibition of mitochondrial function. These particular transition points comprise theoretical "energetic checkpoints" of cell cycle progression.

It is here that the biochemical events that are sensitive to changes in the cellular energy status will determine whether the cell continues through its cycle or pauses until a favourable energetic balance is restored. The increase in the number of cells in the G_1 component of the cell cycle that results from antagonizing mitochondrial function is accompanied by an augmented proportion of persistently active Retinoblastoma protein (Rbp). Failure to inactivate this tumour suppressor protein, whose role it is to brake cell cycle progression in response to suboptimal

conditions, does not result from an upregulation of the "classical" G_1 inhibitory proteins but rather does so secondary to a decrease in the availability of a critical G_1 cyclin. This sensitive regulatory protein, cyclin D, is essential for the activation of kinases responsible for the initial phosphorylation of Rbp, which renders it inactive and allows passage out of the G_1 component of the cell cycle.

This is the first work to report cell cycle-specific periods of increased ATP utilization which correlate with checkpoints through which a cell will not pass if its energetic balance is sufficiently disrupted. We also demonstrate that changes in mitochondrial function elicit changes in the core proteins of the cell cycle machinery suggesting some intracellular link between the energy balance within the cell and the proteins responsible for cell cycle progression. Finally this report confirms previous suggestions that mitochondria represent viable targets for altering the division characteristics of a cell population, particularly in the context of the altered mitochondrial phenotype of many tumour cells.

ACKNOWLEDGEMENTS

This document, my unsophisticated masterpiece, was a long time in the making and clearly involved participation of many noble souls other than my own. For their academic support and patience, I must thank my thesis supervisor and the members of my Supervisory Committee including Dr. Gurmit Singh, Dr. Bill Orr, Dr Larry Arsenault and Dr. Peter Whyte. It was my privilege to work alongside Dr. Steve Armstrong, Dr. Roger Moorehead and Dr. Ann Dorward, from whom I learned much about science and much more about friendship. The numerous graduate students who appeared and disappeared over the years from the labs on the fourth floor of the Cancer Centre were of the highest quality, both academically and personally and I am grateful to have shared their company. I am especially delighted to have met and frolicked with Anu Srivastava, Tanya Shaw, Lisa Porter, Tom Preston and Scott Su. Of course my foundation for this undertaking, as for all things, stems from the strength of my family and the endurance of my most precious Windsor Chicks, who have never been anything short of unconditionally supportive. Finally, and most directly important in the preparation of this thesis, I thank Rob Watering for being exactly who he is.

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LIST OF ABBREVIATIONS

ATP	adenosine triphosphate
ANT	adenine nucleotide translocase
Pi	inorganic phosphate
IMM	inner mitochondrial membrane
ETC	electron transport chain
CCCP	carbonyl cyanide phenylhydrazone
mV	millivolts
Rh123	Rhodamine123
NAO	10-N-nonyl acridine orange
deca	Dequalinium
CDKs	cyclin-dependent kinases
CAK	CDK-activating kinase
Rbp	Retinoblastoma protein
TPA	12-O-tetradecanoyl phorbol-13-acetate
CDKIs	cyclin dependent kinase inhibitors
CAK	CDK-activating kinase
DTT	dithiothreitol
FBS	fetal bovine serum
HRP	horse radish peroxide
λPPase	lambda protein phosphatase
SDS-PAGE	sodium dodedecyl sulphate-polyacrylamide gel electrophoresis

LIST OF PUBLICATIONS

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Sweets,S., Singh.G. Changes in Mitochondrial Mass, Membrane Potential and Whole Cell ATP Levels During the Cell Cycle of HL-60 Cells. J. Cell. Physiol. 180:91-96, 1999.

Sweet,S., Singh,G. Changes in the Regulation of the G_1 to S Phase Transition of the Cell Cycle in HL-60 Cells in Response to Inhibition of Mitochondrial ATP Production (manuscript in preparation)

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Singh,G., Moorehead,R., Dorward,A., **Sweet,S.** Novel mechanisms of resistance to cancer chemotherapy. The Cancer Journal 8(6):304-307, 1995.

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Chapter 1

INTRODUCTION

There is an extensive field of research concerned with describing the components and defining the pathways of the cell cycle engine. This engine drives a cell to increase in size and replicate its genetic material with the goal of subsequently dividing into functional daughter cells. Far less attention has been paid however, to understanding the fuel that drives that engine, an idea that not only has importance in basic cell biology, but also has practical implications in conditions where control over cell division becomes impaired.

In the realm of basic science, further defining the relationship between energy-generating mitochondria and the capacity for cell division would help solidify an existing foundation which states that cells with impaired mitochondria progress through their cell cycle more slowly, if at all (Van den Bogert et al., 1992; Giraud and Velours, 1997). It has also been shown that rapidly dividing cell types possess mitochondria with elevated membrane potentials when compared to cells at rest, further suggesting a link between the propensity of a cell to divide and the physiology of fuel provision (Chen, 1988). The experiments described herein will attempt to complement this story and broaden its relevance into practical application.

Cellular fuel in the form of adenosine triphosphate (ATP) is required for many cellular functions, with several taking priority over cell division. For example, the bulk of cellular ATP is dedicated to maintaining active transport across membranes,

particularly for establishing and maintaining the plasma membrane potential. ATP is also preferentially consumed in the synthesis and processing of informational macromolecules like DNA, RNA and proteins. As these processes are integral to cell survival, they are "protected"; i.e. only when their energy requirements have been satisfied can a cell consider expending excess ATP on cell division.

The demands on mitochondria to produce energy sufficient to facilitate cell division must be superimposed upon this organelle's own requirement to double its functional mass by the end of the cell cycle. Understanding this process will help to describe how mitochondria change in structure and function during the cell cycle and will also address issues such as "minimum thresholds" of mitochondrial function, below which a cell could not commit to another round of cell division. Specifically, it will define the energetic requirements of cell cycle progression. This will allow identification of cell cycle mechanisms that are most reliant upon, and therefore sensitive to, changes in mitochondrial fuel provision. Furthermore, it opens the door to discussions regarding the intracellular sensors of energy status that link mitochondria to the cell cycle machinery.

The practical implications of such investigations are apparent and applicable in situations where manipulating the rate of cell division is desirable as in the uncontrolled growth that is characteristic of neoplastic cells. Because rapidly dividing cells have the hardest working mitochondria, as evidenced by their elevated membrane potentials, a manipulation of mitochondrial function would manifest itself

preferentially and primarily as a decrease in the replication rate of these cells such that their uncontrolled proliferation was no longer a threat to the host.

Therefore, the relevance of delineating the interdependence of mitochondrial function and cell cycle progression lies not only in furthering our understanding of the influences that govern cell division but it also presents the mitochondrion as an alternative intracellular target for controlling otherwise uncontrolled cell growth.

Mitochondrial Structure and Function

Mitochondria are the predominant source of energy in most eukaryotic cells. They are organelles of approximately 1μm diameter and variable length, comprising up to 15% of total cellular protein, varying in accordance with the energetic demands of the tissue they supply (Attardi and Schatz, 1988; Schatz, 1995). They are enclosed by a highly permeable outer membrane, which contains transmembrane channels discriminating only against molecules with a molecular weight greater than 5 kDa. An intermembrane space separates the outer membrane from a relatively impermeable inner membrane that is responsible for inhibiting passage of polar molecules that lack specific transporters. The inner mitochondrial membrane (IMM) is folded into numerous cristae, increasing its surface area to accommodate thousands of copies of the enzyme complexes required for the generation of cellular energy in the form of adenosine triphosphate (ATP) Enclosed by the IMM is the mitochondrial matrix, containing mitochondrial DNA and ribosomes and the metabolic enzymes of the citric acid cycle and fatty acid oxidation (for details, consult Tzagoloff, 1982; Alberts et al., 1998). Of the hundreds of polypeptides found in mitochondria, only 13

are encoded on mitochondrial DNA and synthesized within the organelle, rendering it dependent on nuclear transcription and protein import for function and structure.

Mitochondria contribute significantly to numerous important cellular processes including the anabolic process of regenerating the carbon skeleton required for synthesis of carbohydrate and lipids, and the recycling of NAD^+ for the catabolic process of glycolysis. They are essential for the production of heme and nucleotides, for the recovery of amino acids and for the maintenance of intracellular calcium homeostasis, acting as a reservoir for this metabolically active ion (reviewed in Tzagoloff, 1982; Lehninger et al., 1993). Their principle role, however, is in the generation of ATP, which functions as the energy currency of the cell. The hydrolysis of ATP is an energy yielding reaction constituting a ubiquitous source of chemical energy that is used to drive a wide variety of biochemical reactions, including active transport across biological membranes and powering of contractile elements within cells. It serves also as an activated precursor of DNA and RNA synthesis and in the production of other informational macromolecules, including peptides and proteins. The production of ATP is the result of a cascade of biochemical events beginning with the catabolism of proteins, carbohydrates and lipids and culminating in the reduction of oxygen and concurrent liberation of CO_2 and H_2O. This process, proposed by Otto Warburg in the mid 1920s is known as cellular respiration and the mechanism, described in the 1930s and 1940s by Kalckar, Kennedy and Lehninger, is oxidative phosphorylation.

A potential mechanism by which the cellular consumption of O_2 could drive the phosphorylation of ADP to ATP within the mitochondrial inner membrane was suggested by Peter Mitchell in 1961 and has become the doctrine of mitochondrial bioenergetics. Mitchell's Chemiosmotic Theory describes the mechanism by which the transfer of high-energy electrons along the components of the respiratory chain is accompanied by the removal of hydrogen ions from the mitochondrial matrix (Mitchell, 1961). This outward proton translocation results in a gradient of both charge and pH across the inner mitochondrial membrane, favouring the passive inward flow of H^+ through the IMM. It is this inward proton-motive force that is harnessed by the specialized rotary enzyme of the inner mitochondrial membrane, the ATP synthase, to generate ATP from ADP and inorganic phosphate (Pi) (Lehninger et al., 1993; Alberts et al., 1998).

Substrate Oxidation and the Mitochondrial Membrane Potential

Pyruvate, fatty acids and amino acids from carbohydrate, lipid and protein metabolism are converted to acetyl coA and funnelled into the citric acid cycle within the mitochondrial matrix. Oxidation of the acetyl groups liberates electrons that are carried on pyridine nucleotides (NAD/NADP) or flavin nucleotides (FMN/FAD) for transport to the respiratory chain of the IMM. The respiratory chain consists of a series of integral membrane proteins capable of accepting and donating electrons, followed in series by the ATP synthase. The electron transport chain (ETC) is composed specifically of I) the NADH dehydrogenase complex, II) succinate

dehydrogenase, III) the ubiquinone/ cytochrome bc_1 complex, and cytochrome c, and IV) the cytochrome oxidase complex.. Enzyme complexes I, III and IV pump protons outward as electrons are passed along in pairs, generating a gradient in both charge and pH across the IMM.

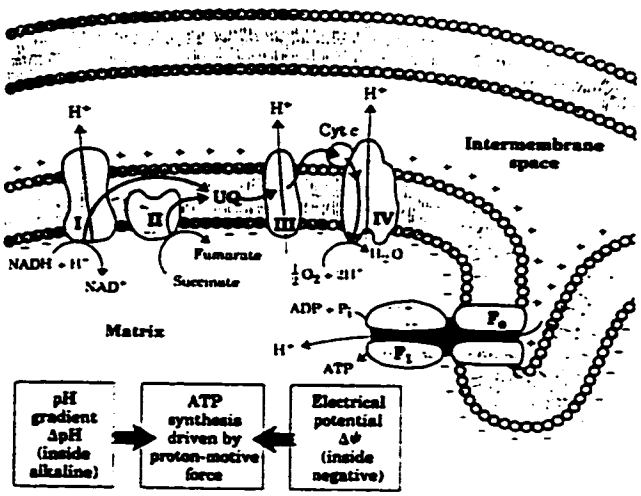


Figure 1. Enzymes of the Inner Mitochondrial Membrane (reprinted from Lehninger et al., 1993). This schematic representation of the inner mitochondrial membrane outlines the principles of the Chemiosmotic theory wherein electrons are passed along the enzymes of the electron transport chain with concurrent proton transfer from the matrix into the intermembrane space, generating both an electrical and a chemical gradient.

Synthesis of ATP

Complex V of the ETC is the enzyme ATP synthase, which exploits the potential energy stored in the proton gradient. It is a large, membrane-bound enzyme that, under normal physiological conditions, generates ATP from ADP and inorganic phosphate (Pi). In the absence of a pH gradient, this reversible ATPase functions as a proton pump, hydrolyzing ATP to move protons outward across the IMM (reviewed in Pedersen and Amzel, 1993; Pedersen, 1994). It is composed of 2 major sub-units: a) the transmembrane Fo portion forms a proton channel through the IMM and is

sensitive to inhibition by oligomycin, and b) the F1 portion of the enzyme that forms a stalk projecting into the matrix and is held to the IMM through its interaction with Fo (Figure 2). It is the F1 subunit that has catalytic activity with binding sites for adenine nucleotides and a highly efficient capacity for ATP production of approximately 100 molecules per second (reviewed in Wang and Oster, 1998, Yasuda et al., 1998). The required substrate for ATP production, adenosine diphosphate (ADP) is exchanged for ATP by the adenine nucleotide translocase (ANT) within the IMM. Each of three active sites within the F1 component of the ATP synthase cycles through stages of: i) ATP binding ("tight" state), ii) ADP and Pi binding ("loose" state) and iii) the empty or "open" state (Boyer, 1993) (Figure 3). The enzyme functions as a rotary motor with the three sites catalyzing synchronously, as the F1 subunit core undergoes an incremental 120° rotation, resulting in the release of one molecule of ATP (Yasuda et al., 1998). The proposal and elucidation of the unique binding exchange mechanism utilized by this enzyme earned its authors the Noble Prize in 1997 (Boyer, 1993; Abrahams et al., 1994).

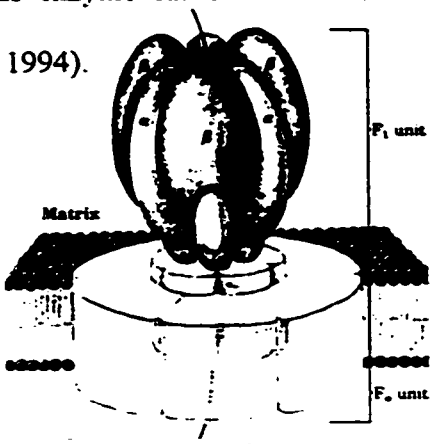


Figure 2. The ATP Synthase complex (reprinted from Lehninger et al., 1993). The proton-conducting Fo portion of the complex is embedded within the inner mitochondrial membrane while the catalytic F_1 subunit projects into the matrix. It is the F_1 subunit that functions as a rotating motor for the synthesis and release of ATP.

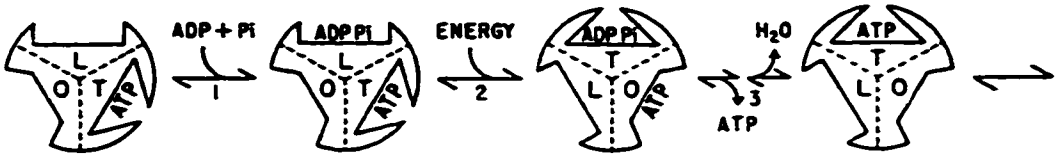


Figure 3. The binding change mechanism of the F_1 subunit for ATP synthesis (reprinted from Alberts et al., 1998). Rotation of the F_1 subunit forces the 3 catalytic sites to undergo conformational changes associated with substrate binding and product release. "Tight", "loose" and "open" configurations are represented by "T", "L" and "O" and refer to the affinity of the catalytic sites for ligand.

Regulation of oxidative phosphorylation

Oxidative phosphorylation is the final step in the extraction of energy from nutrient sources at the cellular level. It depends both on the supply and effective transfer of electrons along the ETC, and the efficient activity of the ATP synthase, which requires adequate substrate ADP and Pi. The function of the complexes of the ETC and ATP synthase is tightly coupled under normal physiologic conditions, such that inhibition of electron transfer with agents such as cyanide and rotenone will inhibit ATP synthesis. Conversely, depletion of substrate ADP (by inhibition of the ANT with atractyloside, for example) will reduce oxygen consumption (Senior, 1988). Additionally, oxygen utilization can be temporarily dissociated from ADP phosphorylation by uncoupling agents like dinitrophenol and carbonyl cyanide phenylhydrazone (CCCP). These compounds allow free passage of H^+ through the IMM, dissipating the membrane potential and thereby removing the force that drives the ATP synthase (Heytler and Prichard, 1962; Chen, 1988), while electron transfer and O_2 consumption transiently persist.

Generation of ATP via oxidative phosphorylation is limited by the availability of ADP. Normally, the ratio of [ATP] to [ADP] is high, with the bulk of the adenine nucleotide pool existing in the fully phosphorylated form. As ATP is utilized, ADP levels rise making available more substrate for ATP synthase, regenerating the ATP stock. In addition, ATP has a product-inhibition effect on several components of glycolysis and the citric acid cycle (Figure 4). Therefore, the relative concentrations of ATP and ADP control the rates of glycolysis, pyruvate oxidation, the citric acid cycle, electron transfer and oxidative phosphorylation. Thus, all the major catabolic pathways are regulated by the same signals of cellular energy status allowing their economical and coordinated functioning (Senior, 1988; Lehninger et al., 1993)

Mitochondrial Biogenesis

Mitochondrial biogenesis relies on the coordinated effort of both the nuclear and mitochondrial genomes. The major regulatory contribution to mitochondrial biogenesis comes from nuclear DNA, which controls the formation of new mitochondrial membrane (Grivell, 1995). A much smaller, but equally important role is played by mitochondrial DNA, which orchestrates the functional aspects of the newly synthesized membrane, outfitting mitochondria as the organelles of oxidative phosphorylation (Attardi and Schatz, 1988). Increases in cellular mitochondrial mass occur via the growth and fragmentation of existing mitochondria, and not as a result of *de novo* synthesis of new organelles. Thus, mitochondrial biogenesis relies on a complex system of protein import and sorting that coordinates cytoplasmic protein

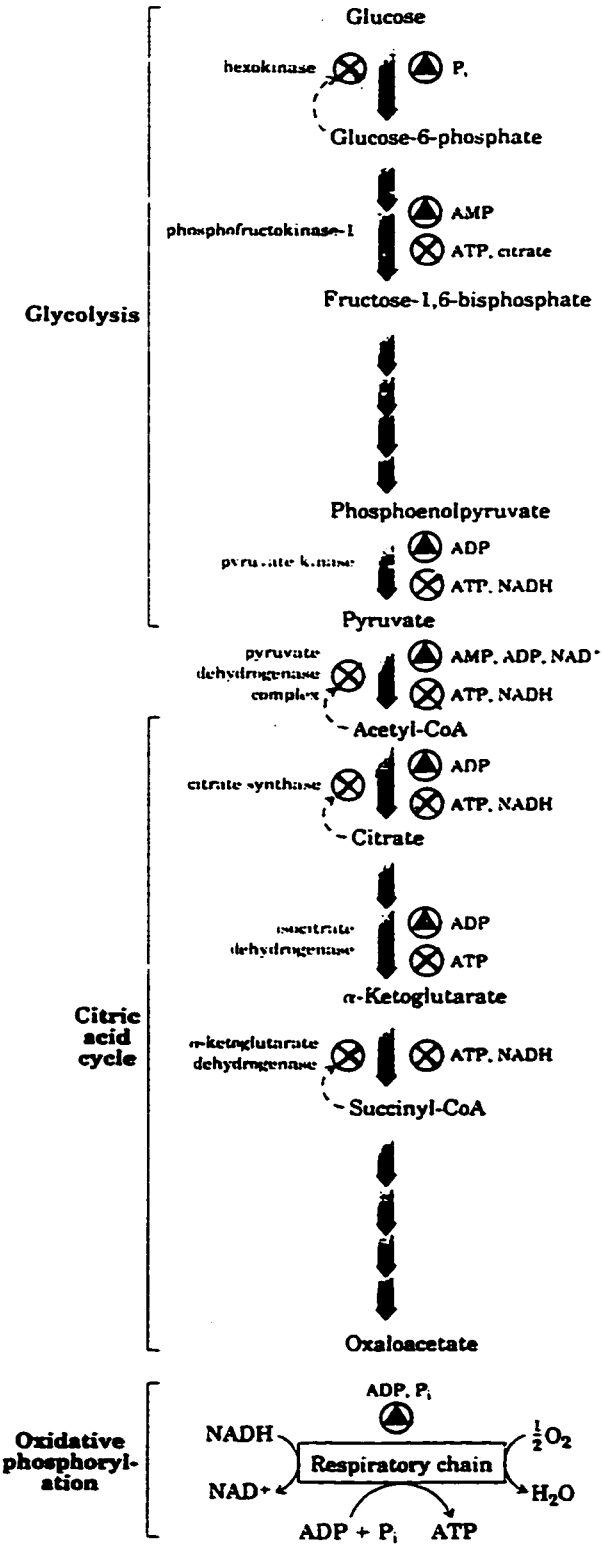


Figure 4. Regulation of cellular ATP production by substrate and product feedback (reprinted from Lehninger et al., 1993). The relative concentrations of ATP, ADP and AMP influence numerous steps in the generation of cellular energy. These pathways are accelerated when the ATP/ADP ratio is low, whereas, a high ATP/ADP ratio will reduce the rate of all of these processes.

production with synthesis of functional mitochondria (Grivell, 1995; Pfanner and Meijer, 1997). The growth of mitochondrial membranes and the division of these organelles occur throughout the cell cycle, although their precise patterns of expansion and activation remain unclear (Attardi and Schatz, 1988, Schatz, 1995, Cuezva et al., 1997). The striking differences in number, structure and activity of mitochondria in different cell types and their highly dynamic nature, including their variability in size and shape and their tendency to fuse and fragment, has made the definition of a pathway outlining mitochondrial biogenesis difficult to describe. These mutable characteristics of mitochondria were initially observed using electron microscopy techniques (Dewey and Fuhr, 1976; Posakony et al., 1977), which highlighted the limitations of this approach for direct mitochondrial quantification. What is required for the comprehensive analysis of mitochondrial changes during cell division is a reliable technique for assessing mitochondrial behaviour in living cells, at different stages of the cell cycle.

Mitochondrial Membrane Potential and Mass

The fact that mitochondria maintain an electrochemical gradient across their internal membrane not only makes them unique among organelles, but facilitates their analysis using positively-charged fluorescent compounds. Isolated mitochondria can maintain a maximal transmembrane gradient of 180-200 millivolts (mV) while, in living cells, the functional mitochondrial membrane potential is usually within the lower range of 120-170 mV, in contrast to the plasma membrane potential of

approximately 30-60 mV (Murphy, 1997). This electrochemical gradient across the IMM attracts lipophilic cations at a concentration [C] predicted by the Nerst equation

$$\text{membrane potential (mV)} = 61.5 \log \frac{[C] \text{ inside}}{[C] \text{ outside}}$$

wherein every 61.5 mV increase in membrane potential results in a 10-fold increase in cation accumulation. Therefore, mitochondria can theoretically amass more than 100 times as much of a given cation as compared to the cytoplasm and 1000-fold greater than the extracellular environment. Although polar cations require specific carriers to cross the IMM, lipophilic cations with deiocalized positive charges pass freely and are ideal tools for quantification of the mitochondrial membrane potential in living cells.

Studies in the early 1980s reported the use of Rhodamine123 (Rh123) for the detection of functional mitochondria in living cultured cells. The tendency of this lipophilic cation to accumulate within mitochondria in accordance with the Nerst equation, together with its stability, lack of toxicity, and its easy and sensitive detection, make it suitable for the quantification of the mitochondrial transmembrane potential (Johnson et al., 1980; Ronot et al., 1986; Chen, 1988). Ionophores, such as CCCP and DNP reduce the mitochondrial uptake of Rh123, as does simultaneous exposure to rotenone (an inhibitor of the electron transport chain) and oligomycin (an ATP synthase inhibitor)(Chen, 1988), suggesting that Rh123 is highly specific for and responsive to changes in the mitochondrial membrane potential. Its excitation wavelength (485 nm) and emission spectrum in the yellow/green band (525-545 nm) are well suited for detection via fluorescence microscopy (Johnson et al., 1980)

and/or flow cytometry (Darzynkiewicz et al., 1981; James and Bohman, 1981, Darzynkiewicz et al., 1982; Collins and Foster, 1983). Using these methods, it has been demonstrated that mitochondria within a single cell stain with equal intensity, such that a given membrane potential is maintained throughout the cell in response to a given set of intracellular conditions. The greatest degree of Rh123 accumulation has been observed in cardiac and skeletal muscle cells with the least uptake and retention reported in bladder epithelium (Chen, 1988). This ranking, taken together with the observation that exponentially growing cells accumulate up to 45% more Rh123 than quiescent cells (Chen, 1988), links the regulation of the membrane potential to the energetic requirements of the cell.

By its nature, however, Rh123 lacks the capacity to discriminate between increases in membrane potential within a finite number of mitochondria from increases in the number of functional mitochondria per cell. To this end, a derivative of acridine orange, 10-N-nonyl acridine orange (NAO), was described which binds specifically to negatively charged phospholipids, with an affinity for cardiolipin which is more than 30 times greater than that for phosphatidylserine or phosphatidlyinositol (Septinus et al., 1985; Maftah et al., 1989; LePrat et al., 1990; Petit et al., 1992). Because cardiolipin is the typical lipid of the inner mitochondrial membrane and is found almost exclusively at this site, it is considered to be a specific indicator of mitochondrial mass (Petit et al., 1992; Schlame and Haldar, 1993) Again, its lipophilicity, specificity, lack of toxicity and ability to stain mitochondria

independent of their energetic state make it an ideal tool for mitochondrial quantification (Maftah et al., 1989).

Tumour Cell Mitochondria

Since Warburg's original observation in 1926, it has been recognized that many tumour cells have a decreased capacity for respiration (Krebs, 1981) In fact, many cancer cell lines contain fewer mitochondria as compared to normal cells from the same tissue of origin. In addition, it is not uncommon for these tumour mitochondria to harbour alterations in their mitochondrial DNA (reviewed in Pederson, 1978; Baggetto, 1993). Most normal cells and slow growing tumours derive less than 10% of total ATP from glycolysis, whereas most solid tumours depend on glycolysis for 15-40% of their ATP. Despite this increased reliance on anaerobic metabolism, oxidative phosphorylation still accounts for at least half of the total ATP production in even "highly glycolytic" tumour cells (Pederson, 1978), suggesting that mitochondrial function remains an important process, and a viable target, in many aggressive cancers.

Chen *et. al.* conducted a comprehensive study of mitochondrial membrane potential in over 200 cell lines derived from a panel of tumours from all common tissues of origin (Chen, 1988). It was reported that mitochondria of the transformed cells consistently maintained elevated membrane potentials with respect to their non-transformed counterparts. The increase in magnitude invariably exceeded 60 mV in the tumour cells, which, according to the Nerst equation, dictates that these cancer

cells would take up more than ten times the amount of a monovalent cation than normal cells with a lower membrane potential. Therefore, this characteristic of tumour cells, which promotes positively-charged drug accumulation in excess of a full order of magnitude as compared to normal cells, is currently being exploited for improving selectivity of chemotherapy (Singh and Moorehead, 1992, Koya et al., 1996).

Agents that affect Mitochondria

Oligomycin is a specific inhibitor of the Fo portion of the ATP synthase located in the inner mitochondrial membrane. It is known to reduce mitochondrially-derived ATP by causing a conformational change in the Fo portion of the complex, blocking the conductance of H^+ ions through the proton channel thereby removing the proton-motive force that drives the synthesis of ATP from this enzyme. The effect of oligomycin is also transmitted through to the F1 portion, reversibly decreasing the binding of substrate in the catalytic sites (Penefsky, 1985). Oligomycin is useful in the study of the effects of inhibiting mitochondrial ATP production on cell function; however, it shows no preference for tumour cell mitochondria.

Dequalinium (deca), Rhodamine123 and MKT-077 are lipophilic cations used to investigate the effects of selective alteration of tumour cell mitochondrial function (Lampidis et al., 1983; Weiss et al., 1987; Modica-Napolitano et al., 1996). The elevated mitochondrial membrane potential maintained by many tumour cells causes such positively charged agents to reach significantly greater concentrations within these

organelles. A high intramitochondrial concentration of lipophilic cations decreases the electrical gradient across the IMM and increases its proton permeability, disrupting the function of IMM-related enzymes, and inhibiting efficient respiration (Murphy, 1997). These agents have all been shown to be selectively toxic to tumour cell mitochondria *in vitro* (Lampidis et al., 1983; Weiss et al., 1987; Chen, 1988; Modica-Napolitano et al., 1996), while MKT-077 is currently in phase I clinical trials (Britten et al., 2000)

Relationship between Mitochondria and Cell Division

The dependence of normal cell division on functional mitochondria has been reported in various cell types. Numerous studies employing agents that selectively disrupt mitochondrial function have demonstrated that cell division is sensitive to alterations in the cellular energy pool (Van den Bogert et al., 1986; Hapala, 1989; Van den Bogert et al., 1992; Heerdt et al., 1997). For example, inhibition of oxidative phosphorylation by inactivation of the gene encoding a single subunit of the ATP synthase or blockage of the adenine nucleotide exchanger by bongkrekic acid in *Saccharomyces cerevisiae* leads to termination of cell division (Hapala, 1989; Giraud and Velours, 1997). In human leukemic MOLT-4 cells, inhibition of mitochondrial protein synthesis by doxycycline compromises the ability of the ETC to function as a proton pump and subsequently results in a cytostatic population (Van den Bogert et al., 1988). However, the precise bioenergetic requirements of the mechanical processes that drive cell division remain to be specifically described. Such

associations will link the known protein components of the cell cycle engine to the physiology of fuel provision.

Cell Cycle

Unravelling the mechanisms of cell cycle control has allowed a more thorough understanding of the manner in which a cell successfully transfers its genetic information to its progeny, and how this process can go awry in cancer cells. The necessity that each daughter cell receives an exact copy of the full complement of parental DNA while maintaining undiminished genomic integrity highlights the importance of rigorous control over this process.

The cell division cycle can be broadly divided into 2 distinct phases: i) interphase, which involves cell growth and replication of genetic material and ii) mitosis, which involves segregation of chromosomes and physical cell division. Mitosis is further subdivided into phases based upon the status of the replicated chromosomes. It begins with prophase, a period during which the duplicated genetic material becomes condensed and appears as sister chromatids, and the nuclear envelope begins to disintegrate. During metaphase, the paired chromatids are aligned along the equatorial plane of the cell, from whence they are pulled toward opposite poles during anaphase. Telophase involves the synthesis of a new nuclear envelope surrounding the DNA at each pole and cytokinesis divides the cytoplasm into two daughter cells (Alberts et al., 1998) (Figure 5).

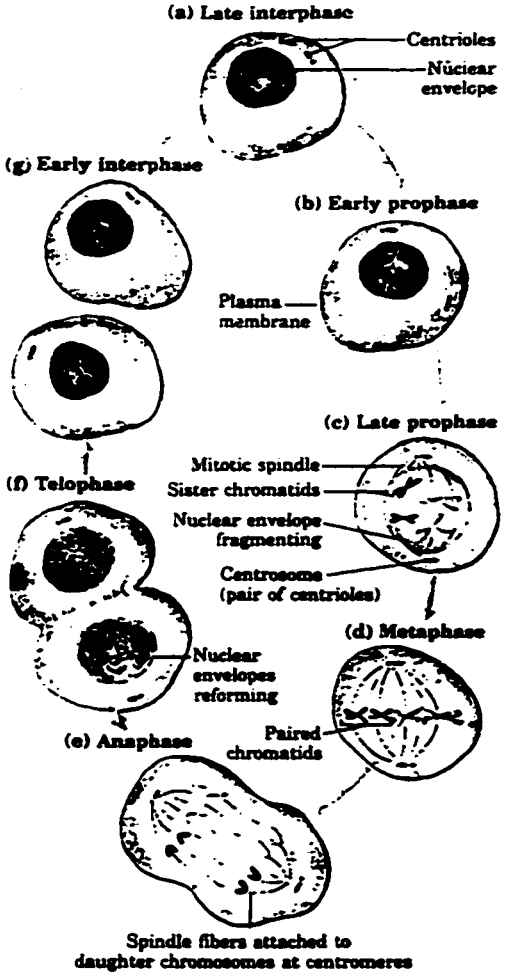


Figure 5. Mitosis and cytokinesis in animal cells (reprinted from Lehninger et al., 1993).

Interphase is likewise divided into discreet phases (G_0, G_1, S and G_2) that either prepare the cell for mitosis or allow the cell to exit the cycle. If extracellular conditions are not favourable for division, for instance, in the absence of growth factors or inappropriate temperatures, the cell cycle control machinery will direct the cell into G_0, a state of quiescence (Pardee, 1989; Sherr, 1994). When the environment is deemed to be conducive to cell division, extracellular signals initiate a period known as G_1, during which time the cell increases in volume and synthesises the precursor materials it will

require for completion of the cell cycle. Toward the end of G_1, when an adequate cell size is achieved and the cell has stockpiled the necessary macromolecules, it reaches the first "checkpoint" in the cell cycle. Known as the Restriction point, it represents a critical stage at which the cell must commit to a full round of cell division or pause until it is capable of doing so (Hartwell, 1992; Zetterberg et al., 1995, Elledge, 1996). Passage through the Restriction point renders the cell refractory to extracellular growth signals and initiates replication of the entire genome as the cell enters the Synthesis ("S") phase. Following DNA replication there is another short gap phase, G_2, during which the integrity of the copied genetic material is assessed, and corrected if required. In mammalian somatic cells, this entire process requires approximately 24 hours, although it varies depending on the cell type (MacLachlan et al., 1995).

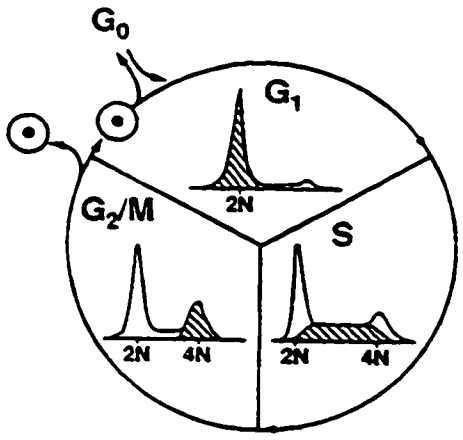


Figure 6. Stages of the cell cycle with representative DNA profiles. Cells entering the G_1 phase of the cell cycle from the quiescent G_0 phase will have a 2N complement of DNA. During the synthesis "S" phase, DNA is replicated so that prior to cell division, cells will posses a 4N complement of DNA as represented in the G_2M population.

Cell Cycle Proteins

The core cell cycle machinery involves complexes of protein kinases and their regulatory cyclin partners. The first cell cycle kinase was described in 2 different strains of yeast concurrently, and was designated CDC28 in *S. cerevisiae* (Hartwell et al., 1974) and CDC2 is *S. pombe* (Nurse et al., 1976) (for cell division cycle) Its human homologue, characterized more than a decade later, was termed cdc2 (Lee and Nurse, 1987). Many cdc2-related kinases have since been described with distinct roles in cell cycle progression. Due to their requirement for association with a cyclically expressed partner protein, members of the cdc2-related group of enzymes were renamed cyclin-dependent kinases (CDKs), a family which currently includes 9 related serine/threonine kinases (Morgan, 1995; Fisher, 1997; Hengstschlager et al., 1999; Pavletich, 1999; Gray et al., 1999).

The activity of the cyclin-dependent kinases is subject to multiple levels of regulation (Grana and Reddy, 1995; Clarke, 1995; Nurse, 1997). They must be complexed with their appropriate cyclin partner and so are limited by the factors that regulate the temporal expression and binding propensity of the cyclins. The action of the activated complexes may be inhibited by a group of small proteins known as CDK-inhibitory proteins, or CDKIs. Additionally, CDK activity depends on activating and deactivating phosphorylations by a CDK-activating kinase (CAK) (Clarke, 1995), one of which has itself been shown to be a cyclin-dependent kinase, composed of cyclin H and CDK7 (Solomon and Kirschner, 1992; Fisher and Morgan, 1994). The mechanism of control of these regulatory influences involves the

induction of a conformational change at the catalytic cleft of the kinase, suggesting that an intrinsic flexibility exists within the kinase that allows it to respond to regulatory signals (Pavletich, 1999). When active, these cyclin/CDK complexes ensure orderly progression through the cell cycle by the appropriate temporal phosphorylation of specific substrate proteins that facilitate cell cycle progression (reviewed in Dynlacht, 1997; Reed, 1997). An additional level of regulation is exerted via controlled protein degradation. Because it is rapid and irreversible, it represents an efficient means of promoting unidirectional cell cycle progression. Ubiquitin-proteosome mediated proteolysis involves the ATP-dependent covalent attachment of ubiquitin chains to target substrates. Such proteins are subsequently recognized by the 26S proteosome which catalyses their degradation. This non-lysosomal mechanism of protein breakdown controls the temporal availability and therefore activity of various proteins central to the progression of the cell cycle, including several growth factor receptors, various cyclins, the p27 CDKI and members of the E2F family of transcription factors (Pagano, 1997; Elledge and Harper, 1998a).

Cyclins & their CDKs

The cyclins are a family of regulatory proteins that function in the cell cycle to activate specific cyclin-dependent kinases. Their expression and degradation are temporally regulated such that they exert their influence only at a precise time during the cell cycle. More than a dozen cyclins have been described to date, of which four

have been characterized as essential components of the core cell cycle machinery: cyclins D and E are fundamental G_1 phase cyclins, cyclin A functions to move cells through S phase and cyclin B is the key G_2M cyclin (Sherr, 1993, Grana and Reddy, 1995).

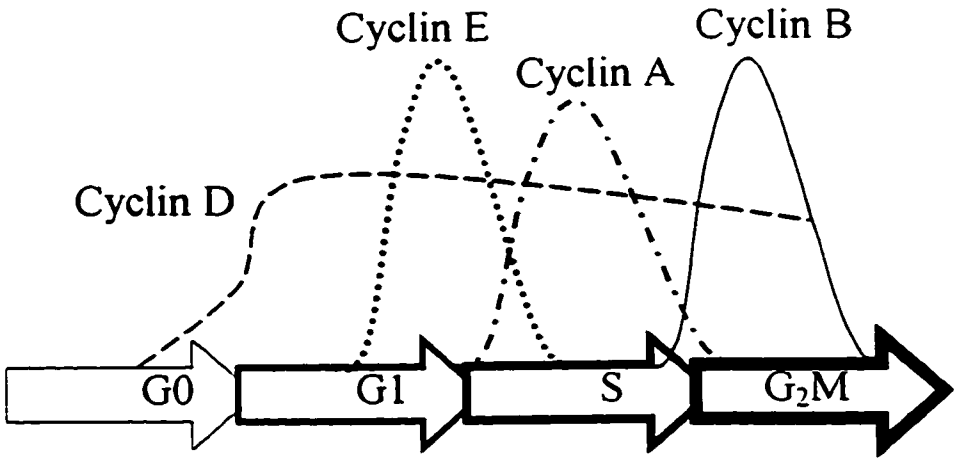


Figure 7 . Temporal expression of cyclin proteins during the cell cycle

Extracellular growth factors induce expression of the D-type cyclins (D1, D2 and D3) as part of an early response to favourable extracellular growth conditions. The levels of cyclin D are low in quiescent cells but, upon mitogenic stimulation, increase to a maximum in mid-G_1, decline slightly as cells enter S and then remain at persistently elevated levels as long as favourable conditions prevail (Matsushime et al., 1994). In fact, constitutive activation of cyclin D can overcome the requirement for extracellular growth factors and result in mitogen-independent cell proliferation

(Resnitzky et al., 1994). Thus, cyclin D plays an important role in the initiation of a new cell cycle, acting in the capacity of a growth factor sensor, ensuring that cells replicate only in response to appropriate regulatory signals (Sherr, 1994).

D-type cyclin proteins associate with their kinase partners (CDK4 or CDK6), are translocated to the nucleus and are activated by a cyclin dependent kinase-activating kinase (CAK) prior to acquiring kinase activity. These complexes serve to move the cells through G_1 toward S phase by phosphorylation of the Retinoblastoma protein (Rbp), which indirectly regulates transcription of genes associated with DNA replication (Sherr, 1993; Hamel and Hanley-Hyde, 1997; Nurse, 1997). Although the kinase activity of cyclin D associated CDKs is directly inhibited by the INK4 class of cyclin dependent kinase inhibitors (CDKIs), the cyclinD/CDK complexes are also responsible for sequestering other inhibitors, namely $p21^{cip1}$ and $p27^{kip1}$, which have a less powerful inhibitory effect on CDK4 and CDK6 than on CDK2. Binding of these Cip/Kip peptides by cyclinD/CDKs relieves their inhibitory influence on CDK2, which functions later in G_1 and S phase when coupled with cyclin E or A. Removal of growth factors from the extracellular environment results in a decline in cyclin D levels with a release of p21/p27 and subsequent inactivation of cyclin E/CDK2, and a return to the quiescent state. Degradation of cyclin D is mediated by phosphorylation-dependent ubiquitination (Brennan et al., 1981; Polyak et al., 1994b).

Expression of cyclin E occurs later than cyclin D and is limited to a brief interval at the G_1/S transition (Lew et al., 1991; Koff et al., 1992; Dulic et al., 1992).

It complexes exclusively with CDK2, activating this kinase immediately prior to S phase entry, coincident with passage of the cell through the Restriction point (Dou et al.,1993). CyclinE/CDK2 activity is required for phosphorylation of the Retinoblastoma protein, reportedly following initial phosphorylation of Rbp by cyclin D and its partner kinase (Lundberg and Weinberg, 1998). Unlike cyclinD/CDK complexes which demonstrate a high degree of substrate specificity for Rbp, cyclinE/CDK2 complexes show broad specificity, also phosphorylating p27 and histone H1 in *in vitro* kinase assays. Activity of the cyclin E/CDK2 complex is subject to positive regulation by CAK-mediated phosphorylation (Solomon and Kirschner, 1992; Fisher and Morgan, 1994) and inhibitory regulation through association with members of the Cip/Kip family of CDKIs, including p21 and p27. The rapid turnover of cyclin E is mediated by ubiquitination and proteosomal degradation secondary to autophosphorylation by CDK2. This auto-phosphorylation promotes dissociation of the active complex, rendering unbound cyclin E susceptible to ubiquitin-mediated proteolysis (Won and Reed, 1996; Clurman et al., 1996; Pagano, 1997).

Cyclins A and B

Cyclin A is expressed immediately following the onset of DNA replication, as cells enter S phase. It associates with CDK2, imparting an activity that is required for DNA synthesis, as inhibition of cyclinA/CDK2 activity arrests cells in the S phase of the cell cycle (Pagano et al., 1992). The activated cyclinA/CDK2 complex can be

localized to DNA replication foci and may have a role in ensuring that the DNA is copied only once per cycle. CyclinA/CDK2 phosphorylates and thereby induces the relocation of an inhibitory DNA binding protein (RPA p34) to the cytoplasm during S phase. Upon completion of DNA synthesis, cyclin A levels decline as the cell progresses into G_2, kinase activity is lost and the inhibitory binding protein is subjected predominantly to the action of phosphatases allowing its return to the nucleus to again bind DNA (Pan et al., 1994). Activated cyclinA/CDK2 complexes also phosphorylate Rbp to help maintain it in its inactive state for the duration of its kinase activity.

Cyclin B is the "mitotic" cyclin, an important member of the "M-phase promoting factor" that regulates the onset, sequence and completion of mitosis It associates with cdc2 (also known as CDK1) during late S and throughout G_2 and mitosis when levels are sufficiently high to bind and activate the kinase. This complex requires a sequence of activating phosphorylations and dephosphorylations to achieve activity, imposing an elaborate control system on mitotic regulation, ensuring that the cell does not initiate cell division prematurely (Elledge, 1996; Nurse, 1997). The cyclinB/cdc2 complex phosphorylates critical nuclear proteins, inducing cytoskeletal reorganization, nuclear lamin disassembly and cell shape changes characteristic of mitosis (Nurse, 1990; Nigg, 1993; Glotzer, 1995, Gorbsky, 1997).

CDK Inhibitors

In addition to the activating influences of cyclin association and CAK-mediated phosphorylation, there exists a means for modulation of cyclin/kinase activity through the action of cyclin-dependent kinase inhibitor proteins (CDKIs). These small molecular weight proteins are divided into 2 groups based on structural and functional similarities, those being the INK4 and Cip/Kip families of CDKIs. These inhibitors are able to effectively arrest cell cycle progression in response to such diverse stimuli as extracellular anti-mitogenic signals, cell-cell contact and DNA damage (reviewed in Hunter, 1993; Biggs and Kraft, 1995; MacLachlan et al., 1995).

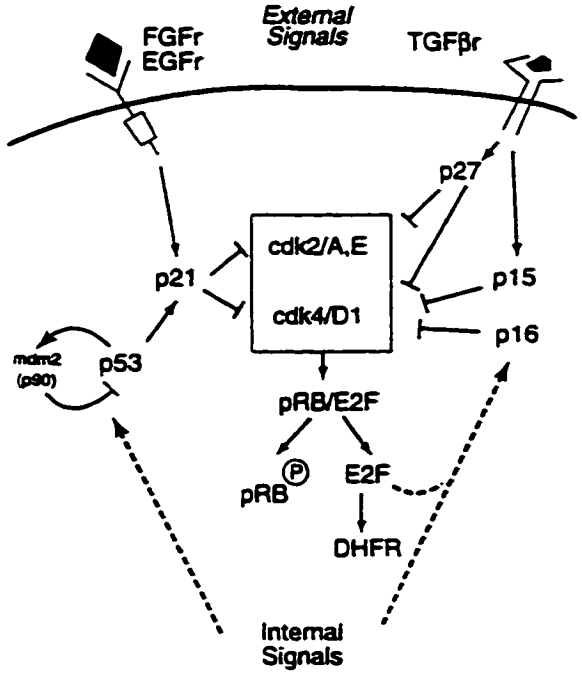


Figure 8. CDK regulation by CDKIs (reprinted from Cordon-Cardo, 1995). Diverse intracellular and extracellular signals regulate expression of the p16, p21 and p27 CDKIs which inhibit the kinase activity of the CDKs and ultimately disallow the deactivating phosphorylations of the Retinoblastoma protein.

INK4

The INK4 (inhibitors of CDK4) family of CDKIs includes p15, p16, p18 and p19, which share structural similarities and form complexes only with D-type cyclin-related CDKs (i.e. CDK4 and CDK6) (Serrano, 1997; Liggert and Sidransky, 1998). The prototype INK4 inhibitor, p16, ultimately exerts its effects by inhibiting phosphorylation of Rbp and therefore requires an intact Rb-pathway in order to induce cell cycle arrest (Medema et al., 1995). This inhibitory peptide can dissociate cyclinD/CDK4 complexes *in vitro*. It binds CDK4 at a site distinct from the putative cyclin D binding site and may block CDK4 activity by stabilizing it in an inactive conformation, thereby decreasing its affinity for cyclin D (Serrano, 1997, Liggert and Sidransky, 1998). Thus, p16 lessens the ability of cyclinD/CDK complexes to phosphorylate and inactivate Rbp and also frees the Cip/Kip proteins from these complexes, allowing their redistribution to CDK2-containing complexes (McConnell et al., 1999; Parry et al., 1999). Basal levels of p16 mRNA are very low in most tissues and cultured cells, but can usually be detected by RT-PCR (Stone et al., 1995, Schwaller et al., 1997). This inhibitor is frequently inactivated in human cancers, especially those of hematopoetic origin, in which *Rb* gene mutations are rare (Ragione and Iolascon, 1997; Drexler, 1998).

Cip/Kip Family

Both $p21^{cip1}$ and $p27^{kip1}$ are members of a family of inhibitors with the capacity to alter function of all G_1 cyclin/kinase complexes, with predominantly

inhibitory effects on cyclin/CDK2 complexes in late G_1 (reviewed in Sherr and Roberts, 1999). Although p21-mediated cell cycle arrest in response to DNA-damaging agents is known to be dependent on functional p53, it has been shown to be upregulated in a p53-*in*dependent manner by various extracellular signals mediated through growth factor receptors via different signal transduction pathways including the Ras/Raf/MAPK pathway for NGF and the JNK/STAT pathway for EGF and INF-γ (Sheikh et al., 1994; Gartel and Tyner, 1999). There is also evidence of a p53-independent upregulation of this protein upon myeloid cell differentiation, such as that induced by the phorbol ester 12-O-tetradecanoyl phorbol-13-acetate, TPA (Jiang et al., 1994; Zhang et al., 1995). Association of p21 with a cyclin/CDK2 complex is believed to block the activating phosphorylation of specific threonine residues by CAK (Kato et al., 1994; Firpo et al., 1994).

In contrast to p21, p27 is detectable at basal levels in unstimulated cells, beyond which its expression can be increased in response to inhibitory extracellular signals such as cell-to-cell contact, growth factor deprivation and anti-mitogenic factors like TGF-β (Polyak et al., 1994a). The observed increase in cellular levels of p27 following deprivation of serum mitogens is coincident with decreased activity of cyclinE/CDK2 complexes and a G_1 phase cell cycle arrest (Kato et al., 1994). The observation that anti-sense against p27 mRNA prevents the G_1 arrest in response to growth factor withdrawal suggests an important role for this peptide in linking extracellular conditions to the core proteins of the cell cycle that regulate transition from G_1 into S phase (Sherr, 1996; Coats et al., 1996).

The p27 CDKI can interact with cyclins and CDKs alone or in complex but binds the complex with much greater affinity (Russo et al., 1996). It appears that p27 binding induces a conformational change in the catalytic cleft of CDK2, dismantling the ATP binding site and thereby inhibiting its kinase activity (Russo et al., 1996, Pavletich, 1999). Like p21, p27 is also believed to exert an inhibitory effect by preventing the activating phosphorylation of the CDK by the CDK-activating kinase (CAK) (Aprelikova et al., 1995).

The inhibitory activity of p27 is regulated not only via increased expression (Hengst and Reed, 1996), but also post-translationally through phosphorylation that is required for its degradation. Because it is a substrate of activated cyclinE/CDK2, its phosphorylation and therefore, destruction, is cell cycle-dependent (Sheaff et al., 1997; Montagnoli et al., 1999). Ubiquitin-mediated proteolysis is the primary means of p27 degradation (Pagano et al., 1995; Alessandrini et al., 1997) and upregulation of this system may contribute to the decreased levels of p27 that accompany tumour development in certain tissue types (Lloyd et al., 1999).

The influence exerted by p21 and p27 is affected by their distribution between various cyclin/CDK complexes. Recently, it has been shown that both Cip/Kip proteins, when sequestered into complexes with excess cyclin D/CDK in early G_1 may in fact promote their association and activation when present in equimolar concentrations (LaBaer et al., 1997; Cheng et al., 1999) despite their previously demonstrated inhibitory effects on these complexes in *in vitro* systems in which they had been abundantly overexpressed (Kato et al., 1994; Toyoshima and Hunter, 1994).

With the Cip/Kip "inhibitors" participating in cyclinD/CDK complexes, cyclin E/CDK2 and cyclin A/CDK2 complexes are left uninhibited. Following an anti-mitogenic stimulus which may decrease availability of cyclin D or CDK4 or increase expression of CDKIs, p21 and p27 may then saturate the CDK2 complexes, thereby inhibiting their kinase activity toward Rbp (Sherr and Roberts, 1995, Sherr, 1996, Sherr and Roberts, 1999). Thus, it would appear that all the G_1 CDKIs co-operate to ultimately dampen the ability of CDK2 to phosphorylate and thereby inactivate Rbp

Retinoblastoma and E2F

A critical substrate for phosphorylation by the activated cyclin-dependent kinases is the protein product of the Retinoblastoma tumour suppressor gene (Rbp). When active, this protein binds and inhibits transcription factors required for progression from G_1 into S phase (reviewed in Weinberg, 1995; Sellers and Kaelin Jr., 1997; Grana et al., 1998). The activity of this protein is temporally regulated by cycles of inactivating phosphorylations and activating dephosphorylations on specific serine/threonine residues (Chen et al., 1989; Buchkovich et al., 1989; Knudsen and Wang, 1996). This protein has 15 highly conserved consensus phosphorylation sites, which are differentially regulated throughout G_1 and early S phase by the cyclin/CDK complexes active during those stages of the cell cycle (i.e. cyclin D/CDK4,6 during G_1, cyclinE/CDK2 at the G_1/S transition, and cyclin A/CDK2 throughout S phase) (Decaprio et al., 1992; Knudsen and Wang, 1996; Zarkowska and Mittnacht, 1997a). It is likely that the G_1 cyclin/CDK complexes co-operate in the regulation of Rbp

inactivation, as they are responsible for the phosphorylation of different residues (Connell-Crowley et al., 1997; Zarkowska et al., 1997b; Pan et al., 1998) and are not sufficient alone to induce the required degree of phosphorylation to inactivate the protein (Zarkowska and Mittnacht, 1997a; Lundberg and Weinberg, 1998) Because the cyclin/CDK complexes that mediate Rbp activity are themselves subject to different regulatory influences, inactivation of Rbp constitutes an integration point of various proliferative and anti-proliferative signals affecting the cycling cell.

The active, hypo-phosphorylated form of Rbp exerts its growth suppressive effect throughout G_1 by indirectly influencing transcription of cell cycle-related genes through sequestration of critical transcription factors, most prominently E2F (Weintraub et al., 1995; Knudsen and Wang, 1997; Dyson, 1998). The E2F family of transcription factors links the core cycle machinery to the expression of genes that are essential for replication of the genome. Functional E2F transcription factors, existing as heterodimers of an E2F polypeptide complexed with a DP polypeptide, alternate between transcriptional activators and repressors depending upon their association with members of the Rb family, including Rbp and the closely related pocket-proteins, p107 and p130 (Hiebert et al., 1992; Mayol and Grana, 1997; Nevins, 1998). Because of the regulatory influence of Rbp on E2F factors, E2F's transcriptional activity is dependent also on the regulators of Rbp activation. CDK-mediated hyperphosphorylation of Rbp in mid- and late G_1 reverses the association of Rbp with the E2F factor, allowing transcription of rate-limiting regulators of DNA synthesis including DNA polymerase α, dihydrofolate reductase, thymidine kinase

and thymidylate synthase (reviewed in Sladek, 1997). The DNA-binding site for E2F found in the promoters of these genes is also found within the promoter region of cyclin E, cyclin A and E2F itself, forming a positive feedback loop to maintain the mitogen-independent phosphorylated state of Rbp and hence, freedom of E2F (Ohtani et al., 1995; Johnson and Schneider-Broussard, 1998). Phosphatases of the PP1 family are responsible for the removal of phosphates from Rbp and reconstitution of its activity in late M phase (Nelson et al., 1997).

It is clear that loss of functional control over Rbp activity could result in a loss of control over the G_1/S transition, producing a population of cells that is unresponsive to the normal factors governing growth control. Because cyclinD/CDK complexes and the p16 CDKI exert their regulatory influence exclusively through Rbp, altered regulation of these factors would upset Rbp-mediated cell cycle control. In fact, disruptions of this "Rb-pathway" are among the most common genetic alterations in cancer cells (Sellers and Kaelin Jr., 1997) and together with inactivation of p53 account for the majority of neoplastic phenotypes (Tortora et al., 1999).

Differences in Cell cycle Control in Tumour cells: the Rb pathway and p53

In the fields of both cell cycle and cancer research, the importance of the competence of the cell cycle is continually stressed, wherein the slightest loss of integrity can result in the initiation of uncontrolled cell proliferation detrimental to the host.

Because of its key regulatory role at the G_1/S transition, the Rb-pathway is a potent oncogenic target. In fact, its deregulation may be mandatory for cell transformation, as mutations of its key components are among the most common aberrations in tumour cells (Weinberg, 1995; Sellers and Kaelin Jr., 1997; Bartkova et al., 1997). Overexpression of cyclin D1 was first described in parathyroid adenoma (and designated PRAD1), while amplification of *cdk4* has been noted in subsets of gliomas and sarcomas (reviewed in Hall and Peters, 1996). Intrinsic in their nature then, *cyclin D* and its partner *cdk4*, are proto-oncogenes. When cyclin D was found to be overexpressed secondary to gene amplification in human tumours (especially breast, esophageal and head and neck squamous cell carcinomas) (Hunter and Pines, 1994; Hall and Peters, 1996), or following induction of cyclins D and E in cell culture models (Resnitzky et al., 1994), cells were forced prematurely through the G_1 checkpoint coincident with hyperphosphorylation of Rbp. Conversely, loss or inactivation of the *INK4a* (encoding p16) or *Rb* tumour suppressor gene results in a similar dilemma of unrestrained cell division (Bartek et al., 1997; Liggert and Sidransky, 1998). In such a sequential pathway, multiple mutations would be redundant, as deregulation of a single component would be sufficient to abrogate the growth suppressive effect of Rbp. In fact, this ontogenetic conservatism is borne out in most tumours studied to date, as overexpression of cyclin D1 or loss of p16 activity is consistently observed in the context of wild-type *Rb*, while cells that lack functional Rbp express normal p16 and cyclin D1 (reviewed in Sherr, 1996; Sellers and Kaelin Jr., 1997). Genetic alterations of cyclin E or cyclin A in human cancers

are rare and have not yet proven to have a causative role in tumourigenesis (Hall and Peters, 1996).

Surveillance proteins that monitor cell cycle progression, although not required for actual cell division, are indispensable for the maintenance of genomic stability. The p53 tumour suppressor gene, dubiously designated "Guardian of the Genome" (Lane, 1992), encodes the quintessential checkpoint regulator protein, whose loss allows a cell to sustain and propagate genetic alterations. Any such alteration that conferred a selective growth advantage would be preferentially selected, resulting in populations of cells with enhanced characteristics of tumourigenicity.

The p53 protein is a nuclear phosphoprotein that binds within the regulatory region of target genes functioning as a transcription factor, regulating cell cycle arrest in the face of DNA damage or nutrient deprivation (Vogelstein and Kinzler, 1992). If damage sustained by a cell is in excess of its repair capacity, p53 initiates a program of apoptotic cell death, in an attempt to eliminate pre-malignant cells. There is also evidence that p53 is involved in the induction of differentiation. The common theme underlying the diverse biological roles of this protein is that p53 responds to events that could result in uncontrolled cellular proliferation in an attempt to limit the tumourigenic potential of affected cells (reviewed in Gottlieb and Oren, 1996, Ko and Prives, 1996). The importance of this tumour suppressor gene is exemplified by the fact that it is the most frequently mutated gene in human cancers (Hollstein et al., 1991).

HL-60 cells

The HL-60 cell line was established in Dr. R.C. Gallo's laboratory from peripheral blood leukocytes taken from a patient with acute promyelocytic leukemia (Collins et al., 1977). These cells display unlimited proliferative potential in culture that is unusual for myeloid leukemias, which tend to undergo only a limited number of cell divisions prior to their arrest and subsequent death *in vitro* (Collins, 1987). HL-60 cells produce CSF-like autocrine growth factors, which have been isolated from conditioned media (Elledge and Harper, 1998b) and may help explain their tolerance for culture conditions and their density-dependent growth characteristics.

These cells exhibit continuous proliferation in suspension culture with a doubling time of approximately 24-36 hours (Collins et al., 1977). They have the potential to differentiate into a variety of cell types of the myelomonocyte lineage (i.e. granulocytes, monocytes or macrophages), depending on the selected inducing agent (reviewed in Collins, 1987). However, the resulting terminally differentiated cells are deficient in certain characteristics present in their normal counterparts.

HL-60 cells are known to harbour specific alterations in several cellular oncogenes (Collins, 1987). A 30-fold amplification of c-*myc* has been documented, which is the homologue of the transforming gene v-*myc*, of the avian myelocytoma virus. This oncogene, in co-operation with *ras*, is capable of transforming fibroblasts in culture. HL-60 cells also display a specific point mutation in the N-*ras* gene, which can induce the malignant transformation of NIH-3T3 cells.

The *p53* gene is deleted in HL-60 cells and thus, there is no expression of this tumour suppressor protein (Wolf and Rotter, 1985). Other cell cycle control abnormalities have been described in this cell line known to involve the Rb-pathway Although HL-60 cells are known to have wild-type *Rb*, they commonly have a heterozygous deletion of *p16* with a point mutation in the other allele (Drexler, 1998). This potential tumour suppressor CDKI is often inactivated in myelogenous cancer cell lines, which rarely harbour mutations in the *Rb* gene (Ragione and Iolascon, 1997; Drexler, 1998).

Chapter 2

Characterizing Mitochondrial Properties During the HL-60 Cell Cycle

To gain a more complete understanding of the energetic requirements of cell cycle progression, we investigated the cell cycle-specificity of several mitochondrial characteristics. Although commitment to and completion of the cell division cycle are known to be sensitive to losses of mitochondrial function (Kroll and Schneider, 1984; Van den Bogert *et al.*, 1986), the relationship between cell cycle and mitochondrial physiology is poorly understood. By defining specific mitochondrial characteristics, such as membrane potential and the magnitude of the ATP pool at different phases of the cell cycle, it was our goal to elucidate predictable relationships between this organelle's function and the requirements of passage through a given cell cycle phase. Using the technique of elutriation centrifugation to achieve relatively homogeneous cell cycle populations while minimizing the metabolic insult to individual cells, we have investigated the relationship between mitochondrial mass, mitochondrial membrane potential and cellular ATP content over the course of the HL-60 cell cycle. Employing vital, mitochondrial-specific fluorochromes to differentiate between mitochondrial mass and mitochondrial membrane potential, together with a quantification of total cellular ATP levels, it was possible to generate profiles of these particular characteristics in HL-60 cells at different stages of their cell cycle. Our data confirm the need for different indicators of mitochondrial quantification and activity, as a change in one parameter does not necessarily equate

with a proportional change in the other. The data also suggest that the availability of ATP changes in a cell cycle-specific manner that cannot be predicted by changes in mitochondrial mass or membrane potential. Furthermore, transition points in the cell cycle where ATP availability is low with respect to the amount of functional inner mitochondrial membrane have been observed, suggesting periods of increased demand and utilization. We propose that these cell cycle phase transitions demonstrate basal levels of available ATP that are nearer to a theoretical "minimal threshold", below which cell cycle progression may be inhibited, rendering these points potentially sensitive to inhibition of mitochondrial activity This further delineation of the nature of mitochondrial changes over the course of the cell cycle (i.e. the relationship between mass, membrane potential and ATP production) provides a basis for understanding the energetic requirements of cell cycle progression and suggests phase transitions where cell division may be most susceptible to manipulation through pharmacological inhibition of ATP synthesis.

MATERIALS and METHODS

Cell culture. The human leukemic cell line HL-60, isolated originally by Collins (Collins *et al.*, 1977) was obtained from the American Type Culture Collection (Rockville, Maryland) and maintained in suspension culture in RPMI supplemented with 10% FBS and 1% penicillin/streptomycin.

Cell Synchrony using Nocodazole. To synchronize cells in the G_2M phase of the cell cycle for subsequent release as a synchronous population, HL-60 cells were incubated with the reversible microtubule inhibitor, nocodazole, at 60 ng/ml for the duration of one cell cycle (24 hours) (Zieve *et al.*, 1980; Kung *et al.*, 1990). Cells were released into fresh media and DNA profiles were obtained during the subsequent cell cycle.

Cell Synchrony through Crowding. HL-60 cells were cultured in the same media without splitting for 6 days, reaching a density of 6.5 million cells per milliliter of culture media. On the seventh day, the culture was split into fresh media and cells were seeded at a density of 3.5 million/ml. Cell cycle profiles and viability assessments were taken each day at the same time.

Elutriation centrifugation. To separate cells according to their position in the cell cycle, a heterogeneous population of 10^7 cells was suspended in 10 ml of "supplemented PBS" (5 mM glucose, 10 mM sodium citrate and 5% w/v albumin)

and loaded into a Beckman J2-21 centrifuge equipped with a JE-6B elutriation system and rotor. Cells were loaded at an initial speed of 3500 RPM with a continuous flow rate of 17 ml/min. Samples of 100 ml were collected starting at 2900 RPM, with the rotor speed decreasing by 100 RPM for each consecutive sample. All steps were conducted at 4°C. A total of 10 eluted fractions were subject to DNA analysis and those containing cells of similar DNA content were pooled, resulting in six discreet fractions for further analysis. Due to the inexact nature of the separation (based on cell size, which correlates to position in the cell cycle), and the requirement for replication in triplicate, there is variation in the degree of sample homogeneity between experiments. Representative samples were named according to the most enriched cell cycle population (i.e. G_1: 95-96%, S: 67-68% and G_2M: 72-73%) Samples with a transitional amount of DNA were also included for continuity

Cell Cycle analysis. Cell cycle profiles were obtained using a variation of the procedure of Vindelov *et.al.* (Vindelov, 1977). A cell pellet of $\approx 10^6$ cells was suspended in Tris buffer (0.10 M Tris, 0.10 M NaCl in deionized H_2O, pH 7.6). Ice cold lysis solution was added (0.01 M glycine, 0.3 M NaCl, 0.10% v/v Triton-X, pH 10) followed by RNase A (100 µL of 1 mg/ml) and ethidium bromide (50 µL of 0.1mg/ml, 0.013 mM). The samples were incubated for at least 10 minutes at 4°C and warmed to room temperature prior to analysis. Cell cycle profiles of no less than 2×10^4 cells were generated on a Coulter EPICS IV Profile flow cytometer. Results were given as number of cells vs. amount of DNA indicated by fluorescence intensity of ethidium bromide.

Raw data were analysed on MCYCLE (Phoenix Flow Systems Inc., 1991) to achieve cell cycle distribution histograms.

Cell staining. Rhodamine 123 (Rh123) (Sigma Chemical Company, St Louis, Mo) was prepared fresh in PBS and used at an end concentration of 25 μM for 30 minutes at 37°C. Samples were washed and resuspended in normal media for an additional 30 minutes prior to flow cytometric analysis of total Rh123 fluorescence. Stock 10-N-nonyl acridine orange (NAO) (Molecular Probes, Inc., Eugene, Or.) was dissolved in ethanol, diluted with PBS and used at an end concentration of 5 μM for 30 minutes at 37°C. Final ethanol concentrations did not exceed 0.1% and had no effect on cell viability. Samples were washed once with PBS to remove unbound NAO, resuspended in media and read directly on the flow cytometer. Samples were also subjected to these same conditions for analysis by fluorescence microscopy, which confirmed the specificity and stability of the fluorochromes.

Dissipating the Membrane Potential. In order to confirm that Rh123 did, in fact, accumulate in response to the membrane potential while NAO staining was independent of that physiologic parameter, cells were incubated for 1 hour in either high potassium media (137mM to neutralize the effects of the plasma membrane) or 5 μM carbonyl cyanide m-chlorophenylhydrazone (CCCP) which is an ionophore that dissipates the mitochondrial membrane potential by allowing the free passage of H^+ ions through the inner mitochondrial membrane. Cells were then stained with either

25μM Rh123 or 5μM NAO for at least 15 minutes at 37°C. Samples were washed, resuspended in fresh media for 30 minutes, and their mean fluorescence was read by flow cytometry.

Ionophore Treatment and ATP. To investigate the relationship between membrane potential, Rh123 uptake and ATP, cells were incubated in 5μM trifluoromethoxy-phenylhydrazone (FCCP), 5μM CCCP or 4.5 μM valinomycin (a K^+ ionophore) for 2 hours after which time 25 μM Rh123 was added to the incubation medium. After 40 minutes, samples were washed once and resuspended in fresh media for at least 40 minutes, at which time their ATP content and total fluorescence were evaluated.

ATP assay. Whole cell ATP levels were measured using the luciferin/luciferase assay (Garewal *et al.*, 1986). Cells were permeabilized prior to the addition of luciferin substrate and luciferase enzyme (ATP Assay Mix, Sigma). Bioluminescence was assessed on an EG&G Berthold Lumat LB9507 luminometer and whole cell ATP content was determined using the equation of the line achieved by luminescence of standard dilutions of ATP. ATP content per sample (in picograms) was divided by cell number to determine average ATP content per cell.

RESULTS

Cell Cycle Synchrony using Nocodazole

Incubation of an HL-60 cell population in 60 ng/ml nocodazole for the duration of one cell cycle led to a cell cycle distribution in which over 90% of the population was in the G_2M phase of the cycle (Table 1). When this microtubule inhibitor was washed from the culture however, cells recovered in a non-synchronous manner displaying a cell cycle profile similar to control after 18 hours in fresh media. Nocodazole, therefore, did not prove useful for generating cell cycle-specific populations of HL-60 cells that would be appropriate for comparing mitochondrial attributes during the cell cycle.

Synchrony through Crowding

In an attempt to achieve a relatively synchronous cell population without the insult of drugs that disrupt the cell cycle, cells were allowed to grow in crowded culture conditions, a technique which had previously been reported in a culture of murine leukemic L1210 cells (LePrat *et al.*, 1990). This process ultimately resulted in an HL-60 population having 87% of cells in the G_1 phase with a cell density of more than 6.5 million/ml (Table 2). However, the population viability was less than 75% that of the control population and the viability was not restored when cells were passed into fresh media. Viability continued to drop to 50% that of control after 24 hours in fresh culture conditions and the cell cycle profile demonstrated that the

degree of synchrony induced by this method was less than optimal with 50% of the cells in G_1, 45% in S phase and 5% in G_2M after a full cell cycle

Cell Cycle Distribution of Elutriated Fractions

To obtain subpopulations of HL-60 cells representative of the various cell cycle phases while inflicting the least degree of metabolic insult, heterogeneous populations were separated by elutriation centrifugation. This technique proved to be ideal for generating cell cycle-specific populations and six distinct fractions were used for all analyses; their cell cycle distributions are summarized in Table 3 The fraction chosen to represent the G_1, S and G_2M compartments of the cell cycle are those most enriched with cells of that phase; absolute homogeneity was not achieved. Fractions with distributions transitional from G_1 to S were labeled T1 and T2. likewise, a fraction representing the transition from S into G_2 is denoted by T3. These samples were included to demonstrate continuity of the progressive changes in the parameters under study. Cell cycle histograms of representative fractions are shown in Figure 9. Representative fractions were divided for individual fluorochrome analysis or ATP assessment.

Effect of Dissipating the Membrane Potential on Rh123and NAO

Consistent with previous reports, Rh123 fluorescence was decreased in the presence of either high potassium media or media containing the ionophore CCCP, while NAO fluorescence was relatively preserved (Figure 10). This confirmed that

Rh123 is taken up into the cell and then into the mitochondria in response to the electrical potential maintained across both the plasma and mitochondrial membranes, confirming its usefulness as an indicator of membrane potential. Since NAO fluorescence was not diminished when the electrical potential was dissipated, it is most likely that this fluorochrome binds its target molecule cardiolipin, independent of the energetic state of mitochondria (Figure 10).

Effect of Ionophore Treatment on Rh123 Uptake and ATP Levels

Incubation of a heterogeneous sample of HL-60 cells with various ionophores induced a decrease in the mitochondrial membrane potential as monitored by uptake of Rh123. In these samples, the amount of detectable whole cell ATP level was similarly reduced, suggesting the dependence of mitochondrial ATP production on the maintenance of a transmembrane electrochemical gradient (Table 4).

NAO and Rh123 Uptake by Elutriated Cell Cycle Fractions

Mean fluorescence of both NAO (as an indicator of inner mitochondrial membrane mass) and Rh123 (reflecting mitochondrial membrane potential) was assessed by flow cytometric analysis of $>2x10^4$ cells from each sample and expressed as a percentage of the fluorescence representative of the G_1 population (Figures 11 and 12). From these data, it appears that neither mass nor membrane potential increases in a linear fashion during the course of the cell cycle. Instead, NAO uptake increases steadily until mid-S phase, after which point, total NAO fluorescence is not

exceeded as cells continue through to mitosis (Figure 11). This observation indicates that synthesis of inner mitochondrial membrane components is completed prior to entering G_2 phase (i.e. 95% of the total increase in NAO fluorescence occurs between G_1 and S phases). In contrast to this, Rh123 uptake shows a profile more representative of a biphasic pattern wherein there exists a significant increase in fluorescence as G_1 cells begin moving into S phase (as represented by the T1 fraction) and then again as cells exit S and traverse G_2, immediately prior to cell division (Figure 12). Changes in Rh123 uptake with respect to changes in NAO fluorescence are represented in Figure 13, where the "expected" line indicates the result if membrane potential increased in direct relation to increases in inner membrane mass It is evident from this representation of the data that fractions transitional into S phase show rapid synthesis of mitochondrial membrane components while, during the progression from S to G_2M, there is a noticeable increase in membrane potential, without a concomitant increase in inner membrane mass.

ATP Content of Elutriated Fractions

Whole cell ATP content was determined in the eluted fractions of cells in various phases of the cell cycle (Figure 14). Although mitochondrial membrane mass (NAO) and membrane potential (Rh123) have both increased significantly by the time cells leave G_1 (49% and 42%, respectively), total ATP content increases only marginally (10%). While cells are undergoing DNA synthesis, the pool of available ATP increases coincident with increases in NAO and Rh123. The amount of ATP

decreases again as cells prepare for mitosis and physical cell division. Cells of the G_1/S and G_2/M transitions display the biggest discrepancy between mitochondrial membrane potential and ATP level (Figure 15), suggesting periods of pronounced ATP demand.

Table 1. Cell cycle distribution following treatment and release from Nocodazole.

Treatment	%G_1	%S	%G_2M
control	49	39	12
24 hours in nocodazole	1	7	92
6 hours post-release	66	12	22
18 hours post-release	52	45	3

Data represent cell cycle distributions following treatment with 60 ng/ml nocodazole for 24 hours with subsequent release into fresh media.

Table 2. Synchrony of an HL-60 cell population through crowded culture conditions.

Time (days)	cell number / ml	$\%G_0G_1$	% control viability
4	4.4×10^6	55	92
5	5.1×10^6	80	88
6	6.5×10^6	87	74
7 (cells split)	3.5×10^6	70	52
8	3.0×10^6	50	50

Cells were cultured in the same media for 7 days then split into fresh culture media. DNA profiles were acquired at the same time each day

Table 3. Cell cycle distribution of elutriated fractions representing the different cell cycle phases

sample	% G_1 ± s.d.	% S ± s.d.	% G_2M ± s.d.
G_1	95.44 ± 2.71	4.55	----
T1	84.52 ± 8.42	15.32 ± 8.45	----
T2	36.32 ± 8.16	58.80 ± 3.14	5.5
S	14.04 ± 6.28	68.08 ± 5.89	18.87 ± 7.85
T3	2.25	38.55 ± 7.56	61.40 ± 4.32
G_2M	3.33	23.64 ± 8.81	72.95 ± 8.11

All data represent n≥3. Where standard deviations are not included, the percentage of cells in that cycle phase was too small to perform meaningful statistical analysis.

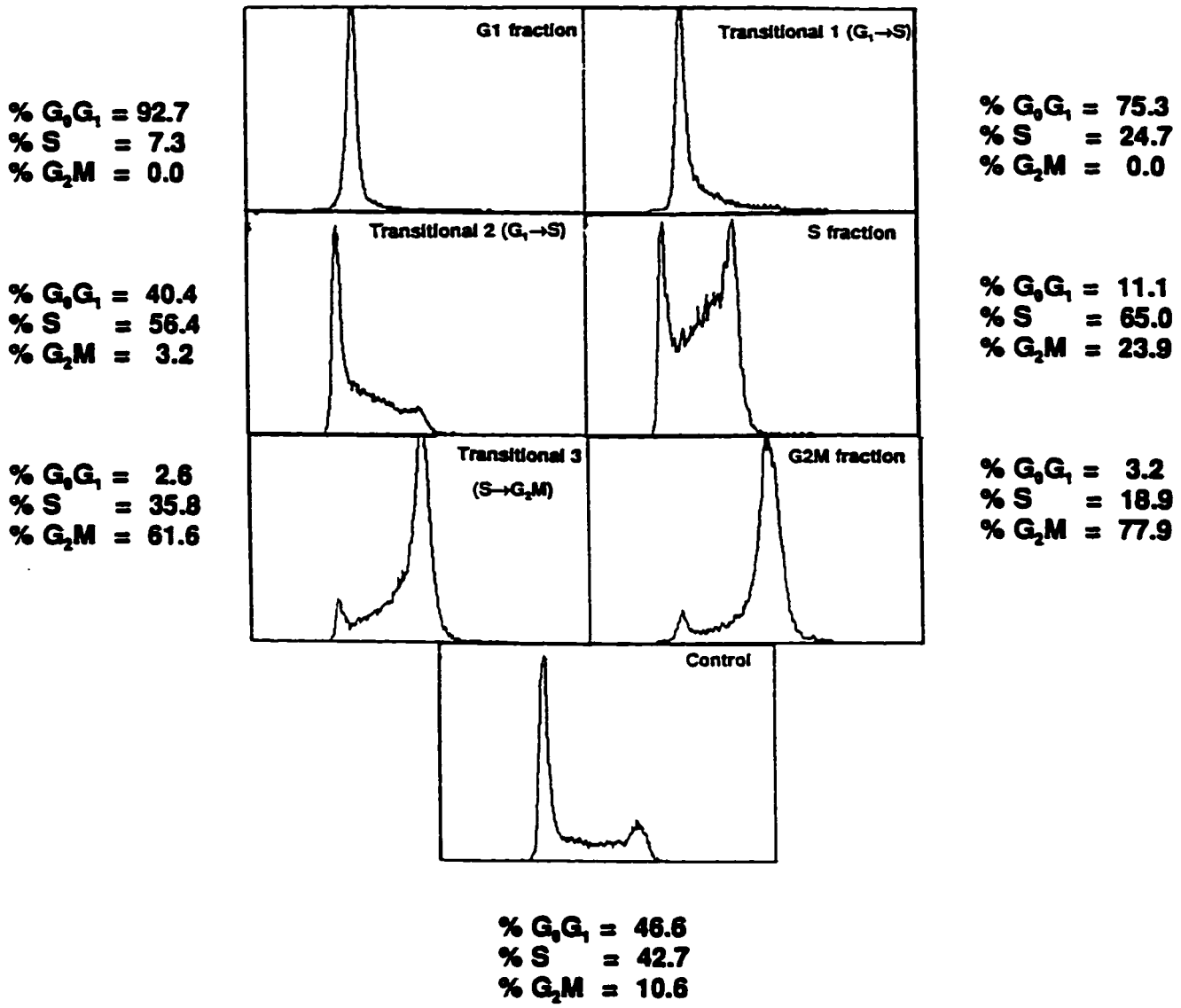


Figure 9. Representative cell cycle histograms of eluted fractions of HL-60 cells from a control heterogeneous population (n=1). Each histogram is expressed as cell number (y-axis) versus DNA content (x-axis) along with the corresponding cell cycle analysis. The control panel represents the HL-60 population immediately prior to elutriation.

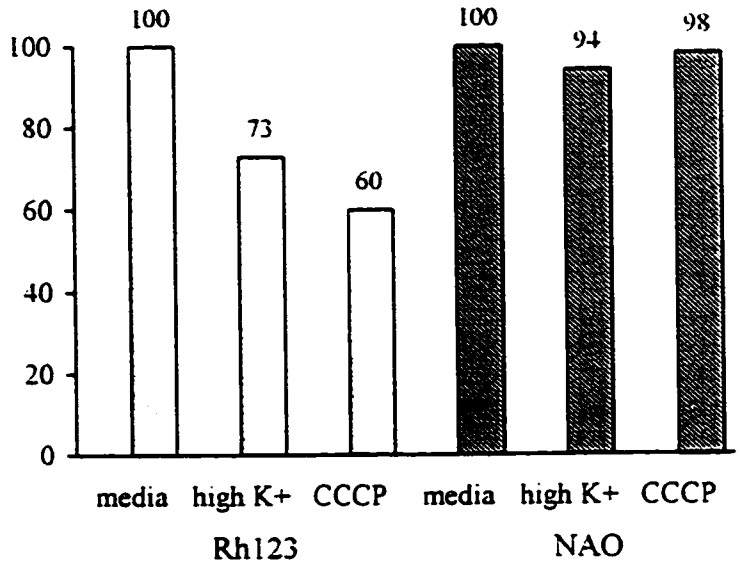


Figure 10. Changes in Rh123 and NAO fluorescence expressed as percent control following a 1 hour incubation in media supplemented with 137 mM potassium or 5μM CCCP.

Table 4. Changes in Rh123 Fluorescence and cellular ATP content following ionophore treatment.

Ionophore	Rh123 fluorescence (%control)	ATP content (%control)
FCCP	64.7 ± 7.5	67 8 ± 9 0
CCCP	71.2 ± 15 2	64 1 ± 8 5
valinomycin	52.1 ± 9.5	51 7 ± 10.0

Data are expressed as percent control from one representative experiment performed in triplicate.

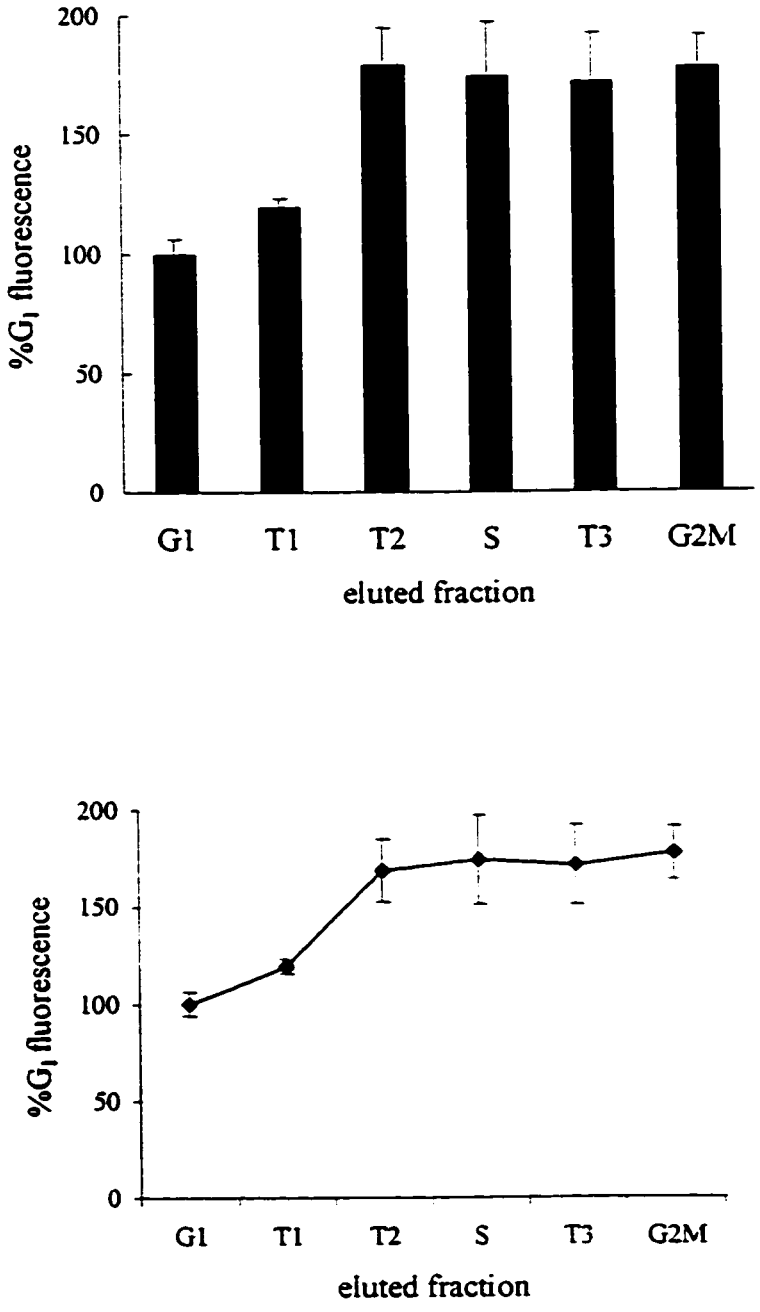


Figure 11. Mean NAO fluorescence of eluted fractions representing the various cell cycle phases. Fluorescence is expressed as a percentage of the fluorescence elicited by the G_1 population (set as 100%). Values represent the mean and standard deviation calculated from 3 independent experiments.

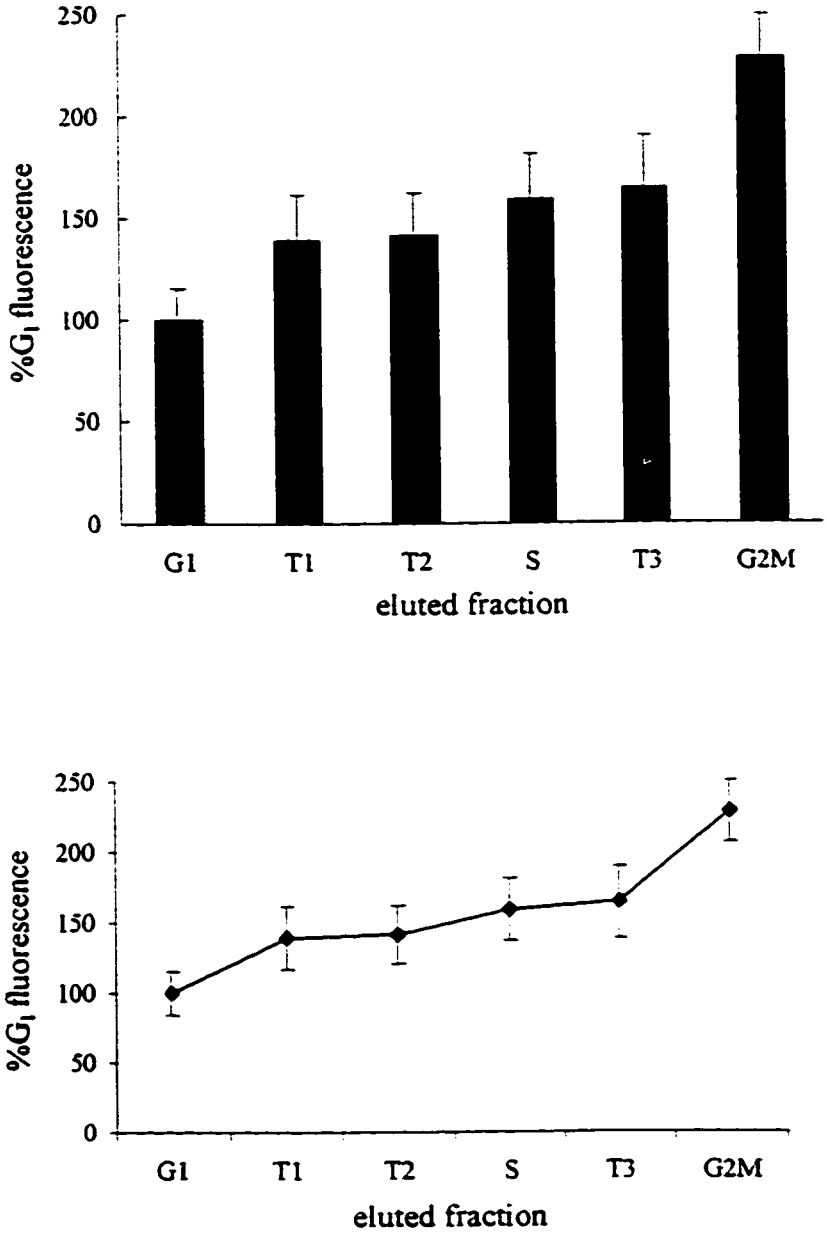


Figure 12. Mean Rh123 fluorescence of eluted fractions. Fluorescence is expressed as a percentage of that elicited by the G_1 population (set as 100%). Values represent the mean and standard deviation calculated from 3 independent experiments.

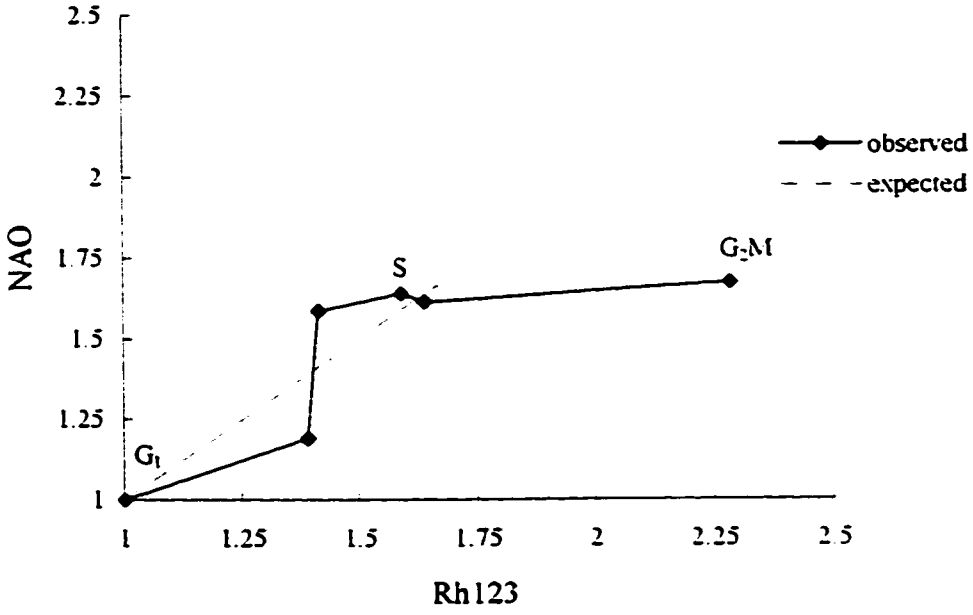


Figure 13. Changes in Rh123 fluorescence with respect to changes in NAO fluorescence as cells progress through the cell cycle. Note the rapid increase in NAO staining as cell progress from G_1 into S. The subsequent increase in Rh123 fluorescence as S cells enter G_2M is not accompanied by any substantial increase in NAO staining.

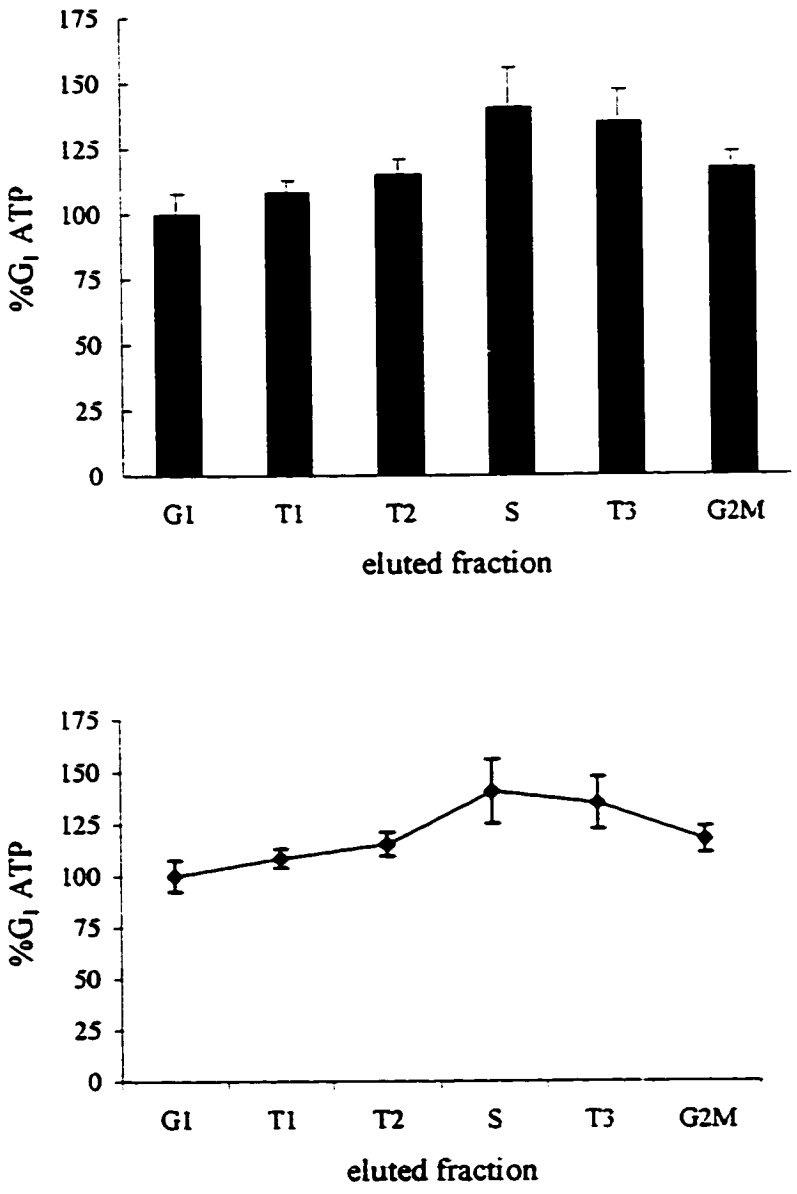


Figure 14. Mean cellular ATP content of eluted fractions. Whole cell ATP levels are expressed as a percentage of the ATP content of the G_1 population (set as 100%). Values represent the mean and standard deviation derived from 3 independent experiments.

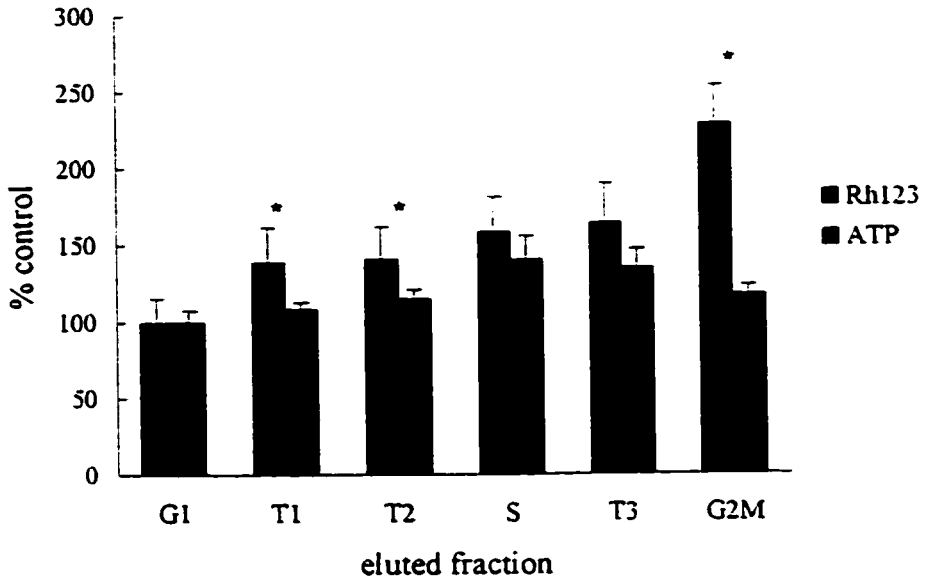


Figure 15. Changes in Rh123 fluorescence compared to total cellular ATP. *Note the discrepancy between increases in Rh123 uptake and total ATP in the samples that are transitional from G_1 into S (T1 and T2) and in the G_2M sample.

Chapter 3

Energetic Cell Cycle Checkpoints in HL-60 Cells

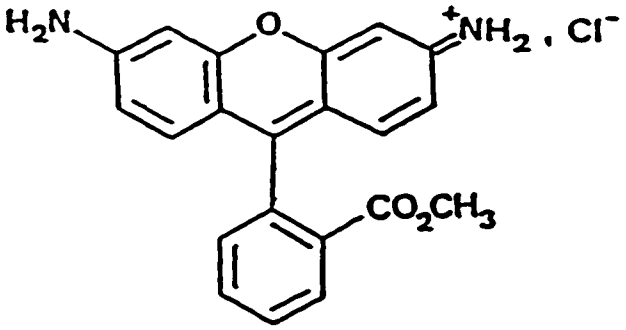
Investigations into the mechanisms of cell cycle control have provided insight into the means through which a cell transfers its genetic information to its progeny. In doing so, the importance of the competence of the cell cycle has been stressed, where the slightest loss of integrity can result in the initiation of a cell death program or uncontrolled cell proliferation detrimental to the host. The focus in cell cycle research has largely centred around elucidation of the molecular events that drive the cell cycle engine. The importance of extracellular influences (Pardee, 1989), the role of the cyclin proteins and the cyclin dependent kinases at various stages of the cell cycle (Sherr, 1993, Grana and Reddy, 1995; Roberts, 1999; Sherr and Roberts, 1999), the phosphorylation status of the Retinoblastoma tumour suppressor gene (Goodrich et al., 1991; Helin and Harlow, 1993; Weinberg, 1995; Sellers and Kaelin Jr., 1997), the influence of p53 (Levine et al., 1991; Vogelstein and Kinzler, 1992; Wang and Harris, 1997; Tortora et al., 1999) and the various molecular events that must occur to initiate and end the characteristic processes of mitosis (McIntosh and Koonce, 1989; Solomon, 1993, Glotzer, 1995) have constituted the major advances in this area of research. Although these molecular events and their regulatory influences are popular areas of research, there has been no consensus on the *energetic* dependence of the cell cycle machinery or any description of the link between decreased ATP availability and the resultant cell cycle effects.

As most of a cell's energy is derived from oxidative phosphorylation within the inner mitochondrial membrane, it is not surprising that alterations to mitochondrial function result in changes in the rate of cell division and ultimately cell death. The inhibition of mitochondrial protein synthesis is known to affect cell cycle distribution, resulting in G_1 accumulation similar to that induced by nutrient deprivation (Kroll and Schneider, 1984; Van den Bogert et al., 1986). We examined the effect on cell cycle progression when mitochondrial ATP production is directly inhibited. Oligomycin, a specific inhibitor of the F_0F_1 ATPase located in the inner mitochondrial membrane, was used as a positive control for reducing the availability of mitochondrially-derived ATP Rhodamine 123 (Rh123) and dequalinium chloride (deca) were subsequently chosen due to their lipophilic cationic properties that result in their accumulation within mitochondria (Figure 16)(Weiss et al., 1987; Chen, 1988). Because many tumour cells are known to maintain a higher mitochondrial membrane potential than their non-transformed counterparts (Chen, 1988), agents like Rh123 and deca will preferentially accumulate in cancer cells, making mitochondria an attractive target for induction of cytostasis or cytotoxicity.

The results of our investigation show that agents which result in decreased cellular ATP content induce a G_1 and G_2M phase accumulation, depending on the degree of ATP depletion. A small reduction (<15%) in the level of whole cell ATP will induce a significant increase in the G_1 portion of the population while further decreases (>20%) will elicit a G_2M accumulation and the onset of cytotoxicity. These data suggest that the checkpoints which regulate passage through those cell cycle positions are

sensitive to alterations in the ATP status of the cell. This study, the first to describe G_1 and G_2 energetic checkpoints, presents a novel means for both analysis and intervention of the cell cycle.

a).



b).

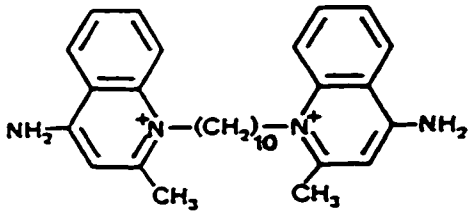


Figure 16. Chemical structures of a) Rhodamine 123 and b) dequalinium. Note the monovalent cationic property of Rh123 and the delocalised divalent nature of dequalinium, making these compounds ideal for accumulation within mitochondria.

MATERIALS and METHODS

Drugs, cell lines and cell culture. Oligomycin, Rhodamine 123 and dequalinium chloride were obtained from Sigma Chemical Company (St. Louis, Mo.). Oligomycin was dissolved in ethanol with final experimental ethanol concentrations not exceeding 0.1%. Rhodamine 123 was prepared fresh daily in PBS while dequalinium chloride was dissolved in DMSO prior to dilution in PBS. The HL-60 cell line was obtained from American Type Culture Collection (Rockville, Maryland) and maintained in RPMI supplemented with 10% FBS and 1% penicillin/streptomycin.

Counting and viability. Cells were cultured in media containing the appropriate concentration of drug for 24 or 48 hours. After the specified incubation time, cells were counted on a hemocytometer and viability was assessed using trypan blue exclusion.

ATP assay. Whole cell ATP levels were measured using the luciferin/luciferase assay (Garewal et al., 1986). Briefly, cells were rinsed with Hank's Balanced Salt Solution before being resuspended in deionized water and boiled for 5 minutes. Samples were cooled to room temperature prior to addition of luciferin substrate and luciferase enzyme. Bioluminescence was assessed on a BioOrbit 1253 Luminometer and whole cell ATP content (in picograms per cell) was determined using the equation of the line achieved by luminescence of standard dilutions of ATP.

Cell Cycle Analysis. Cell cycle profiles were obtained using a variation of the procedure of Vindelov *et.al.*(Vindelov, 1977). A cell pellet of $\approx 10^6$ cells was suspended in Tris buffer (0.10 M Tris, 0.10 M NaCl in deionized H2O, pH 7.6). Ice cold lysis solution was added (0.01 M glycine, 0.3 M NaCl, 0.10% v/v Triton-X, pH 10) followed by RNase A (100 µL of 1 mg/ml) and ethidium bromide (50 µL of 0.1mg/ml, 0.013 mM). The samples were incubated for 10 minutes at 4°C and warmed to room temperature prior to analysis. Cell cycle profiles of no less than 20000 cells were generated on a Coulter EPICS IV Profile flow cytometer. Results were given as number of cells vs. amount of DNA indicated by fluorescence intensity of ethidium bromide. Raw data were analysed on MCYCLE (Phoenix Flow Systems Inc., 1991) to achieve cell cycle distribution histograms.

DNA Synthesis using Bromodeoxyuridine incorporation. A two colour cell cycle analysis using anti-BrdU antibodies and propidium iodide was employed to determine whether S phase cells were actively synthesising DNA. Bromodeoxyuridine (BrdU) is a uridine analogue incorporated into DNA in place of thymidine and was detected using an anti-BrdU antibody and a FITC-labelled goat anti-mouse IgG (Becton-Dickinson, San Jose, CA). Samples of 10^6 cells were incubated in 10 µM BrdU at 37°C for 30 minutes, washed twice, resuspended in ice cold PBS and fixed in 70% ethanol. After 30 minutes, cells were pelleted, the supernatant was aspirated and cells were suspended in 2N HCl containing 0.5% TX-100 at room temperature for 30 minutes to partially denature the DNA into single stranded molecules. Again, cells were pelleted and

resuspended in a solution of 0.1M sodium tetraborate, pH 8.5 to neutralize the acid. Cells were centrifuged again and suspended in 0.5% Tween20 containing 1% BSA in PBS prior to the addition of 1 µg anti-BrdU antibodies. After 30 minutes, cells were pelleted and resuspended in the Tween/BSA/PBS solution prior to adding 1 µg of FITC-labelled goat-anti-mouse (GAM) IgG. Following another 30 minute incubation, cells were spun and resuspended in 1 ml of PBS containing 5 µg/ml propidium iodide for the detection of double stranded DNA. Cells were then analysed by dual-parameter flow cytometry.

Elutriation Protocol. No fewer than 10^7 cells were suspended in 10 ml of supplemented PBS (containing 5 mM glucose, 10 mM sodium citrate and 5% w/v albumin) and loaded into a Beckman J2-21 centrifuge equipped with a JE-6B elutriation system and rotor. The loading speed was 4000 rpm at a continuous flow rate of 17 ml/min. Samples of 100 ml were collected starting at 3000 rpm with the rotor speed decreasing by 100 rpm for each consecutive sample. A total of 15 samples were collected and their DNA profiles were analysed by flow cytometry.

RESULTS

Effect of Oligomycin on ATP Content of HL-60 Cells

Using doses of oligomycin ranging from 1.25 ng/ml (1.6 nM) up to 2.5μg/ml (3.2 μM), viability of the treated samples remained at or above 90% that of controls. Significant dose-dependent reductions in ATP were achieved with doses of oligomycin that did not significantly reduce population viability, ensuring that cell death was not responsible for the decrease in population ATP level (Figure 17). Cells that had been cultured from a frozen stock in liquid nitrogen and used in our laboratory for an extended period of time (denoted "culture A" in Figure 17a) displayed less sensitivity to oligomycin than a new culture received from ATCC (Figure 17b). Despite the age of the culture used however, the degree of ATP depletion, and not the dose of oligomycin, predictably correlated with subsequent cell cycle changes.

Effect of Oligomycin on the HL-60 Cell Cycle

From the DNA profile achieved directly from the flow cytometer, it is apparent that in the presence of oligomycin, fewer cells are leaving G_1 and entering S phase than in the control population (Figure 18a). This population characteristic is lost in the analysis of the cell cycle distribution by MCYCLE, as the program "smooths" the data to assess the mean number of cells in each cell cycle phase (Figure 18b). At the smallest dose of oligomycin that could elicit a detectable change in cellular ATP (i.e. when ATP is reduced by >12%), a significant increase in the proportion of cells in G_1 is observed

(Table 5, Figure 18). There are no further increases in the proportion of cells in G_1 with increasing doses of oligomycin. However, higher doses of oligomycin which correspond to decreasing levels of ATP, cause an accumulation of cells in the G_2M phase of the cycle (Table 5). The most substantial change in the G_2M phase occurs when ATP is reduced from 80% to 60% that of control, inducing an increase in the G_2M population of ≈60% although these doses were accompanied by losses in population viability. The effects of oligomycin were reversible at doses that reduced ATP by as much as 25%; the cell cycle profiles of these samples were similar to those of controls after 36 hours in fresh media. Furthermore, when an eluted "pure G1" population was exposed to doses of oligomycin ranging from 0.05-5.0 μg/ml, approximately 15% more cells were detected in the G_1 compartment after 24 hours (Table 6).

Effect of Oligomycin on BrdU Incorporation

Samples of HL-60 cells treated with 5 μg/ml oligomycin were subjected to dual parameter analysis to simultaneously assess position in the cell cycle and ability to incorporate BrdU, a uridine analogue, in place of thymidine. Figure 19 shows that treated S-phase cells continue to take up BrdU, suggesting that S phase cells are not metabolically compromised or arrested in this phase of the cell cycle The magnitude of BrdU fluorescence is decreased with respect to control cells owing to the fact that oligomycin has induced a redistribution of the cell cycle, reducing the S phase population from 35% to 15% so that there are fewer cells available to incorporate the BrdU.

Effect of Rhodamine 123 on ATP and Cell Cycle Progression

Incubation of HL-60 cell cultures in 2.5-5.0 μM Rh123 for 24 hours was sufficient to cause a significant redistribution of the cell cycle and a concurrent reduction in whole cell ATP (Figure 20, Table 7). The initial increase in the G_1 population is not surpassed at higher doses, although a longer incubation time does induce further accumulation at each dose. Increases in dose do however elicit an increase in the G_2M fraction of the cell cycle which also increases with time. Viability was not significantly affected up to and including 2.5 μM Rhodamine 123. The whole cell ATP levels showed a dose-dependent decrease with increasing Rhodamine 123 levels.

Effect of Dequalinium Chloride on Energetic Checkpoints

The cell cycle changes observed with dequalinium chloride were similar to those induced by both oligomycin and Rhodamine 123 (Figure 21, Table 8). The viability of the cells in doses up to 1 μM deca was not different from control. Whole cell ATP levels were observed to decrease with increasing doses of dequalinium chloride.

Generalized Effect of Altering ATP on Cell Cycle Progression

Regardless of the agent used to reduce the total amount of cellular ATP, cell cycle redistribution was predictable based on the degree of ATP depletion. A small decrease in ATP elicits a maximal accumulation of cells in G_1 that is not surpassed when ATP is further diminished (Figure 22), while higher doses of ATP-depleting agents show escalating proportions of cells in the G_2M phase of the cycle (Figure 23).

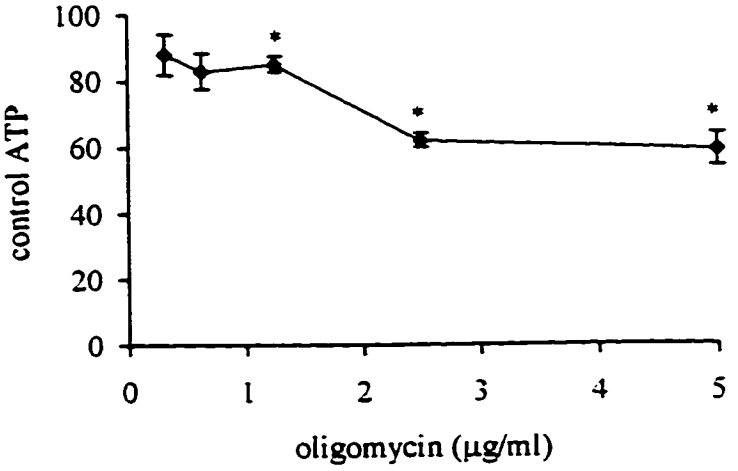


Figure 17a). ATP-response curve of HL-60 culture "A" cells to increasing doses of oligomycin.

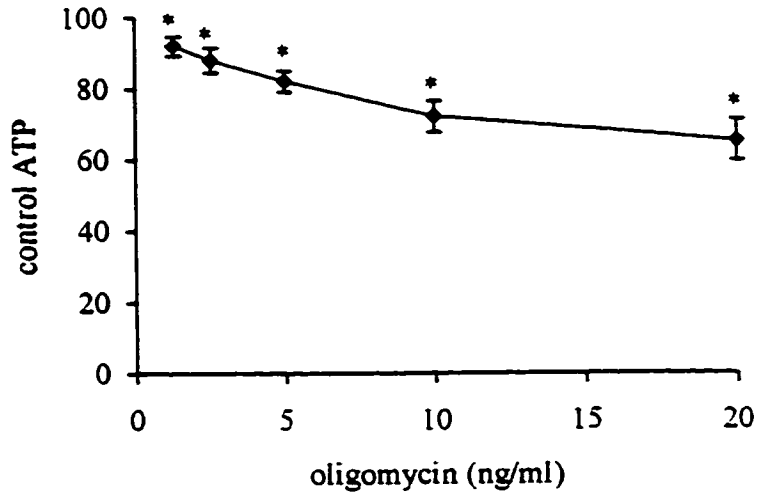


Figure 17b). ATP-response curve of HL-60 culture "B" cells to increasing doses of oligomycin.

Content of whole cell ATP in the presence of oligomycin in old stock culture "A" cells and in newer culture "B" cells. ATP content was determined using the luciferin/luciferase assay. The values are expressed as a percentage of control [ATP] (pg/cell). * $P<0.05$ for treated vs. control as determined by a Student's T-test (n=3).

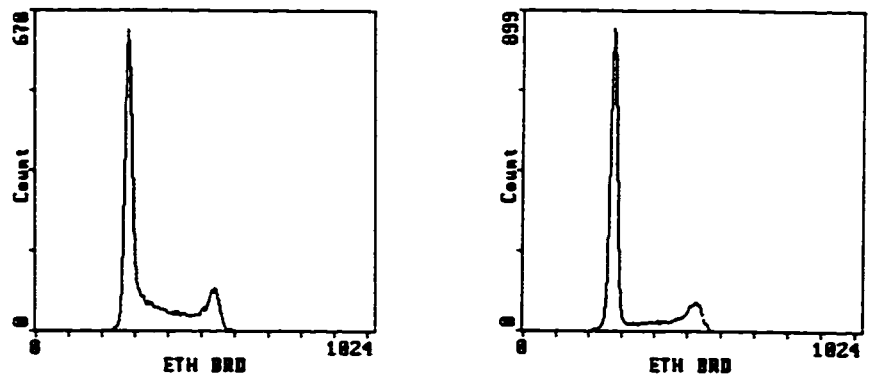


Figure 18a). Raw data showing representative cell cycle changes when HL-60 cells are exposed to 5 ng/ml oligomycin for 24 hours. The x-axis represents DNA content as measure by ethidium bromide fluorescence (FL3) and the y-axis represents cell number.
control: 62 % G_1, 22% S, 16% G_2M
treated: 71 % G_1, 10% S, 19% G_2M

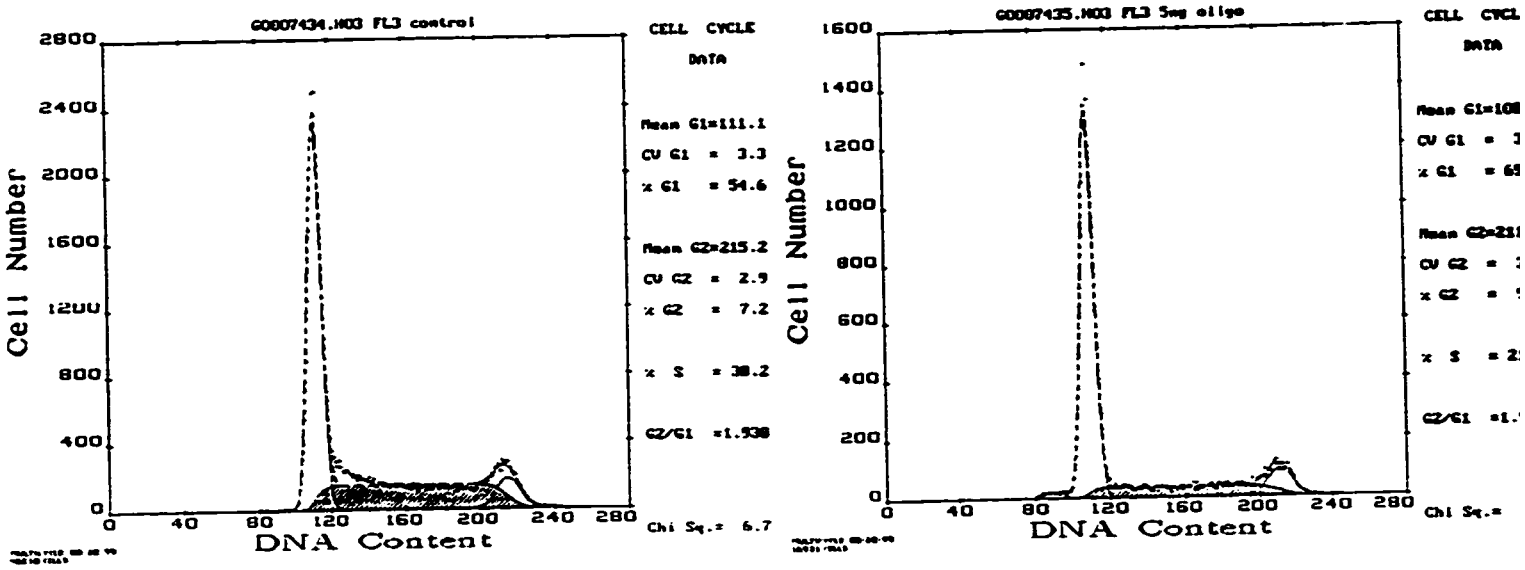


Figure 18b). Cell cycle profiles following MCYCLE analysis with 24 hour oligomycin treatment, as above.

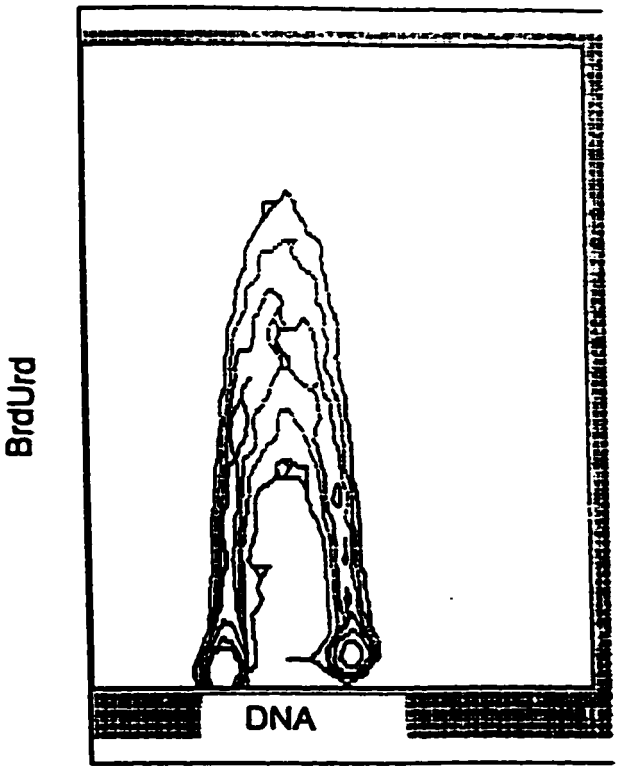


Figure 1 Control

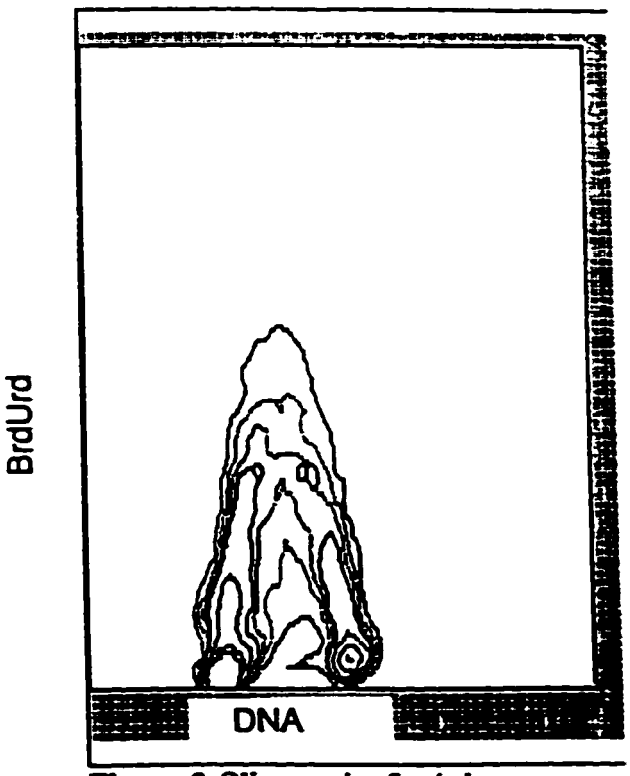


Figure 2 Oligomycin, 5ug/ml

Figure 19. Contour plots showing DNA content on the x-axis versus BrdU uptake on the y-axis. The oligomycin-treated sample continues to take up BrdU, although to a lesser degree owing to the fact that fewer cells are in S phase in this sample (15% vs. 35% control)

dose	%control		
(μg/ml)	ATP	G_1	G_2M
0.05	88	121 ± 5.9*	106 ± 5.8
0.625	83	124 ± 4.2*	128 ± 9.4
1.25	85	126 ± 2.5*	127 ± 10.3*
2.5	62	119 ± 3.9*	152 ± 9.2*
5.0	59	119 ± 2.8*	159 ± 10.1*

Table 5. Changes in G_1 and G_2M phase accumulation with corresponding changes in whole cell ATP in the presence of increasing doses of oligomycin. Cells were treated for 24 hours after which time whole cell ATP was measured using the luciferin/luciferase bioluminescence assay. Cell cycle distributions were achieved on a Coulter Epics Profile flow cytometer using standard DNA staining techniques. All values are expressed as % control; data represent n=3. ATP data shown above represents one typical experiment. (*significant at $p<0.05$)

	$\%G_0G_1$	%S	$\%G_2M$
control	37	44	19
0.05 μg/ml	50	50	--
0.5 μg/ml	52	46	2
5.0 μg/ml	52	47	1

Table 6. Cell cycle distribution of a pure, elutriated G_1 population 24 hours after release into fresh media with or without the indicated dose of oligomycin. Note that 63% of the control G_1 cells progressed into S with 19% reaching G_2M while fewer than half of the treated cells left G_1.

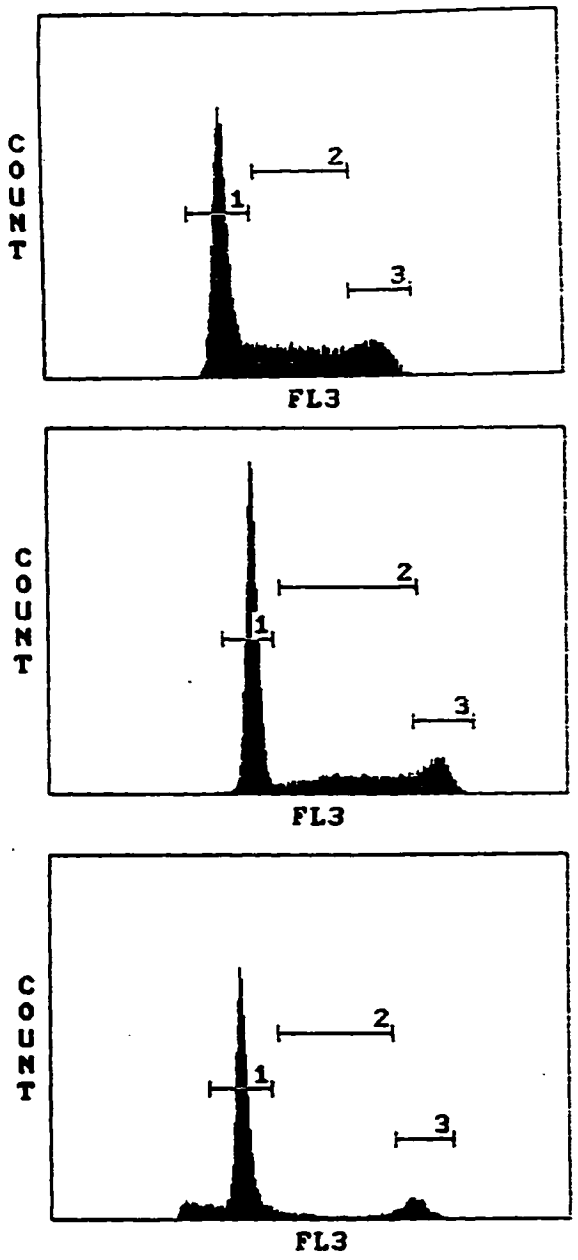


Figure 20. Representative cell cycle changes in the presence of 2.5 μM Rh123 for 24 and 48 hours.

control	52% G1, 29% S, 19% G_2M
24 hours	60% G1, 21% S, 19% G_2M
48 hours	72% G1, 7% S, 21% G_2M

dose	% control					
	ATP		G1		G2M	
	24hr	48hr	24hr	48 hr	24 hr	48 hr
2.5 μM	69	56	114±4.2*	150±13.4*	143±9.4*	169±27.5
5.0 μM	67	55	118±3.9*	143±9.0*	145±13.4*	175±14.1*
10 μM	56		117±4.1*		168±39	

Table 7. Changes in G_1 and G_2M phase accumulation with corresponding changes in whole cell ATP in the presence of increasing doses of Rhodamine 123. Cells were treated for either 24 or 48 hours prior to cell cycle and ATP analysis. At 48 hours, 10 μM Rhodamine123 induced significant cell death. (* significant at $p<0.05$)

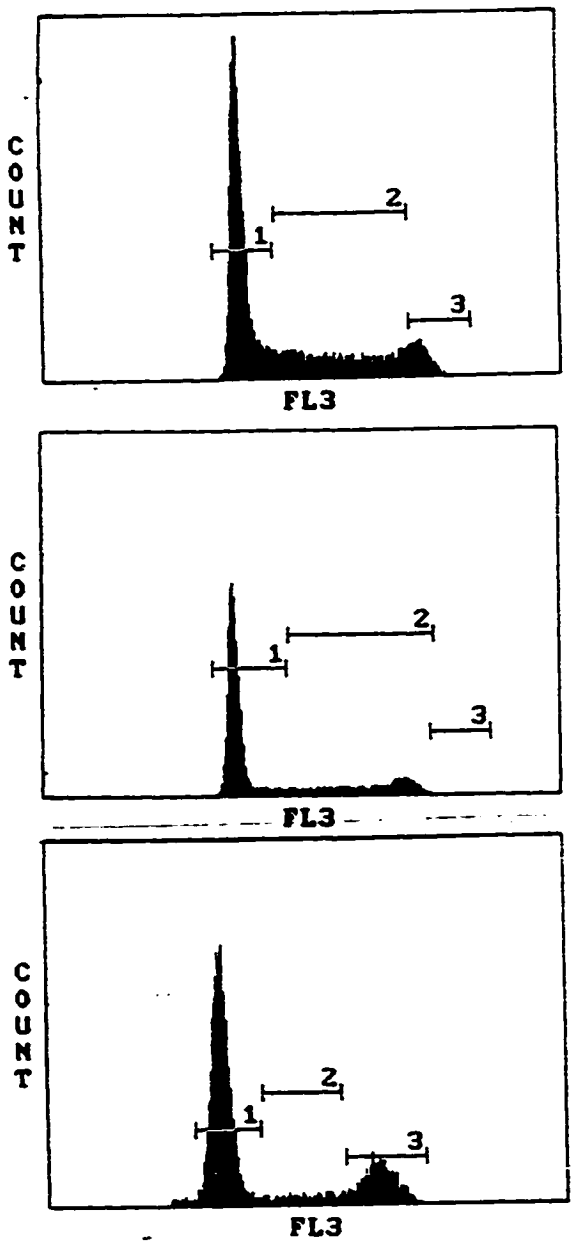


Figure 21. Representative cell cycle changes in the presence of 2 μM dequalinium chloride for 24 and 48 hours.

control	59% G1, 24% S, 17% G_2M
24 hours	67% G1, 15% S, 17% G_2M
48 hours	67% G1, 10% S, 23 % G_2M

dose	% control					
	ATP		G_1		G_2M	
	24hr	48hr	24 hr	48 hr	24 hr	48 hr
0.5 μM	83	81	111±0.6*	108±0.6*	110±3.6	114±5.9
1.0 μM	79	61	116±1.2*	124±4.2*	121±7.6*	148±7.9*
2.0 μM	78	58	118±3.2*	143±5.7*	123±6.7*	169±1.35*

Table 8. Changes in G_1 and G_2M phase accumulation with corresponding changes in whole cell ATP in the presence of increasing doses of dequalinium chloride. Cells were treated for either 24 or 48 hours prior to cell cycle and ATP analysis. At 48 hours, 2.0μM dequalinium chloride caused population viability to fall to ≈80% control. (*significant at $p<0.05$)

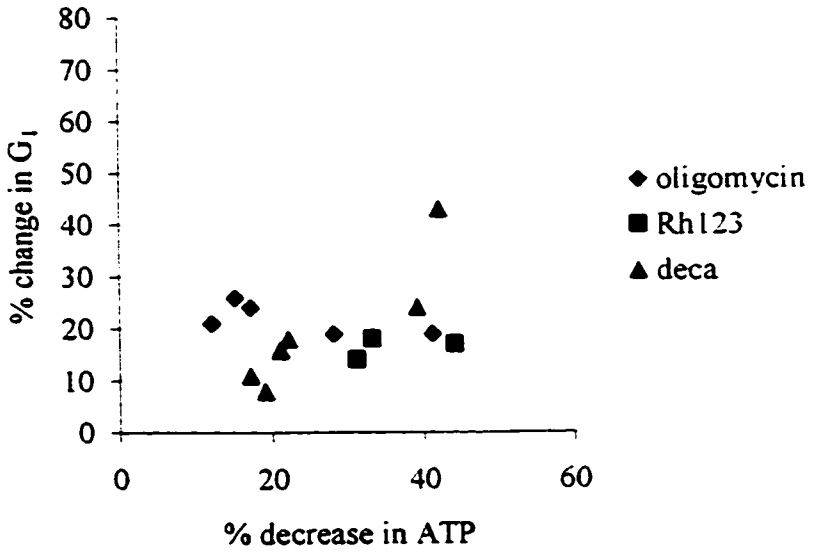


Figure 22. Changes in the proportion of cells in G_1 following incubation with various agents with respect to control G_1. Decreases in ATP of up to 45% (regardless of agent) induce moderate G_1 accumulation (When a regression line is fitted to these data, the slope of the resultant line is 0.240 with an R value of 0.103, indicating that the data do not fit a pattern wherein one property varies directly with the other).

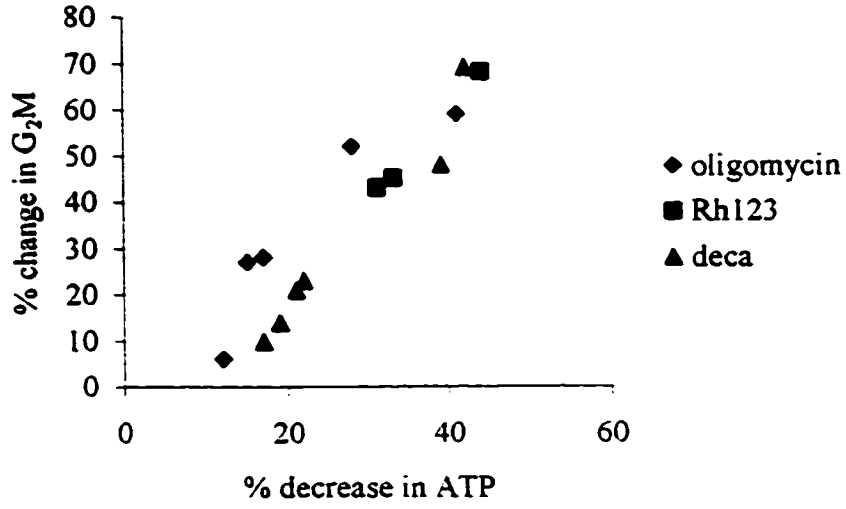


Figure 23. Changes in the proportion of cells in G_2M following incubation with various agents with respect to control G_2M. Decreases in ATP cause a dose-dependent increase in the proportion of cells in G_2M (The slope of the line best fitting these data is1.5 with an R value of 0.956).

Chapter 4

Regulation of the G_1 to S Phase Transition of the Cell Cycle in Response to Inhibition of Mitochondrial ATP Production

Although the energetic requirements for cell cycle progression have been largely overlooked in the literature, we believe that the mitochondrial generation of ATP adds an additional and important level of control that governs cell cycle passage. Numerous studies employing agents which selectively disrupt mitochondrial function have demonstrated that cell division is sensitive to alterations in the cellular energy pool (Van den Bogert et al., 1986; Hapala, 1989; Van den Bogert et al., 1992; Heerdt et al., 1997). We have shown that one such agent, oligomycin, which blocks the ATP synthase, reduces the amount of total cellular ATP and induces a substantial accumulation of cells at the G_1/S boundary. It would not be unlikely then that the regulatory events that normally drive cells through the G_1/S transition are affected by ATP depletion secondary to oligomycin treatment.

The core cell cycle machinery is composed of complexes of kinases and their regulatory cyclin partners. These complexes ensure orderly passage through the cell cycle by the appropriate temporal phosphorylation of specific substrate proteins that facilitate unidirectional cell cycle progression (reviewed in Dynlacht, 1997, Nurse, 1997; Gillet and Barnes, 1998). Of the 14 cyclins described to date, four have been characterized as essential components of the core cell cycle machinery: cyclins D and

E are fundamental G_1 phase cyclins, cyclin A functions to move cells through S phase and cyclin B is the critical G_2M cyclin (Sherr, 1993; Grana and Reddy, 1995)

Extracellular growth factors induce expression of the D-type cyclins as part of an early response to favourable extracellular growth conditions. Thus, cyclin D plays an important role in the initiation of a new cell cycle, acting in the capacity of a growth factor sensor (Sherr, 1994). D-type cyclins associate with CDK4 and CDK6 and serve to move the cells through G_1 toward S phase by phosphorylation of the Retinoblastoma protein (Rbp), which indirectly regulates transcription of genes associated with DNA replication (Sherr, 1993; Hamel and Hanley-Hyde, 1997; Nurse, 1997). The cyclinD/CDK4 complex is directly inhibited by the INK4 class of cyclin dependent kinase inhibitors. Removal of growth factors from the extracellular environment results in a decline in cyclin D, and a return to the quiescent state.

Expression of cyclin E occurs later than cyclin D, appearing only briefly at the G_1/S transition (Lew et al., 1991; Koff et al., 1992; Dulic et al., 1992). It complexes exclusively with CDK2, activating this kinase immediately prior to S phase entry, coincident with passage of the cell through the Restriction point (Dou et al., 1993). CyclinE/CDK2 activity is required for phosphorylation of the Retinoblastoma protein, subsequent to the initial phosphorylation of Rbp by cyclinD/CDK4,6 (Lundberg and Weinberg, 1998). Activity of the cyclin E/CDK2 complex is inhibited through association with members of the Cip/Kip family of CDKIs, including p21 and p27.

A critical substrate for phosphorylation by cyclin-dependent kinases is the protein product of the Retinoblastoma tumour suppressor gene, which is minimally

phosphorylated in early G_1 (Chen et al., 1989; Buchkovich et al., 1989; Knudsen and Wang, 1996; Zarkowska and Mittnacht, 1997; Lundberg and Weinberg, 1998). This active, hypo-phosphorylated form of Rbp exerts a growth suppressive effect by indirectly influencing transcription of cell cycle-related genes through sequestration of critical transcription factors, such as E2F (Weintraub et al., 1995; Knudsen and Wang, 1997; Dyson, 1998). Hyperphosphorylation of Rbp by activated cyclin/CDK complexes reverses its association with E2F, allowing transcription of proteins directly involved in DNA synthesis (reviewed in Nevins, 1992; Sladek, 1997).

Superimposed upon this level of cell cycle regulation is the potential for inhibition of cyclin/kinase activity through the action of cyclin-dependent kinase inhibitor proteins (CDKIs). Both p21 and p27 are members of the Cip/Kip family of inhibitors that interact with all G_1 cyclin/kinase complexes, preferentially inhibiting cyclin/CDK2 complexes in late G_1 (reviewed in (Sherr and Roberts, 1999)). The p21 CDKI is normally undetectable compared to p27 which is detectable at basal levels in unstimulated cells and increases as cells enter quiescence.

The p16 CDKI, which binds only D-type cyclin-dependent kinases, reportedly blocks activity by decreasing the affinity of CDK4/6 for cyclin D following a conformational change when p16 is bound to the kinase (Serrano et al., 1993; Serrano, 1997; Liggert and Sidransky, 1998). This inhibitor is frequently inactivated in cancers of hematopoetic origin. HL-60 cells commonly lack functional p16 protein secondary to a heterozygous deletion of p16 with a point mutation in the other allele (Drexler, 1998).

Our data show that although the levels of the CDKIs do not change, Rbp is hypo-phosphorylated in oligomycin-treated cells resulting in delayed entry into S phase. There is a significant decrease in the amount of cyclin D protein in response to depletion of ATP which may facilitate this alteration in cell cycle by disallowing activation of its partner kinase and thereby resulting in an underphosphorylated and persistently active form of Rbp.

MATERIALS AND METHODS

Drugs, cell lines and cell culture. The HL-60 acute myelogenous leukemic cell line was obtained from American Type Culture Collection (Rockville, Maryland) and maintained in RPMI media supplemented with 10% FBS and 1% penicillin/streptomycin. Oligomycin (Sigma Chemical Company, St. Louis, Mo.) was dissolved in ethanol with final experimental ethanol concentrations not exceeding 0.3% All additional reagents were obtained from Sigma unless otherwise stated.

Elutriation centrifugation. To separate cells according to their position in the cell cycle, a heterogeneous population of $\geq 10^7$ cells was suspended in 10 ml of "supplemented PBS" (5 mM glucose, 10 mM sodium citrate and 5% w/v albumin) and loaded into a Beckman J2-21 centrifuge equipped with a JE-6B elutriation system and rotor. Cells were loaded at an initial speed of 3500 rpm with a continuous flow rate of 17 ml/min. Samples of 100 ml were collected starting at 2500 rpm, with the rotor speed decreasing by 100 rpm for each consecutive sample. All steps were conducted at 4°C. A total of 15 eluted fractions were subject to DNA analysis and those containing cells of similar DNA content were pooled.

Oligomycin Treatment. Cells were treated with doses of oligomycin that were sufficient to alter the cell cycle profile without significantly affecting population viability. Cells were incubated continuously in the indicated dose of oligomycin for

at least one cell cycle, usually 24 hours, unless stated otherwise. At the end of the incubation period, cell viability was assessed, total population ATP was determined, and cell cycle profiles were obtained prior to protein lysate preparation.

DNA analysis. DNA profiles were obtained using a variation on the procedure of Vindelov (Vindelov, 1977), described previously. Briefly, cell pellets were lysed in ice cold Triton-X buffer and supplemented with RNase and ethidium bromide. Samples were left on ice for at least 10 minutes and warmed to room temperature prior to analysis. Cell cycle profiles of $\geq 10^4$ cells were obtained using a Coulter XL Pro flow cytometer. Data were subsequently analyzed using the MCYCLE Programme for cell cycle distribution histograms (Phoenix Flow Systems Inc., 1991)

Antibodies. Polyclonal anti-Rb, anti-CDK2, anti-p16, anti-p21 and anti-p27 antibodies were purchased from Santa Cruz Biotechnology Co. (Santa Cruz, CA.), as was the monoclonal anti-E2F antibody. Monoclonal antibodies against cyclins E and D1 were purchased from Oncogene Research Products (Cambridge, MA), while the anti-CDK4 antibodies were from Transduction Laboratories (Lexington, KY). All polyclonal primary antibodies (rabbit) were detected using HRP-conjugated Protein A (Bio-Rad), while murine monoclonal antibodies were detected using Bio-Rad goat-anti-mouse HRP secondary antibodies.

Immunoprecipitation and Western Blotting. Experimental cultures of HL-60 cells were collected by centrifugation following treatment with oligomycin and washed once with PBS prior to preparation of cell lysates. Cell pellets were resuspended in lysis buffer (containing 1% NP-40, 50 mM Tris and 150 mM NaCl with freshly added 10 mM DTT, 0.014 inhibitory units of aprotenin and 1 μg/ml leupeptin) and left on ice for at least 30 minutes. The lysates were clarified by centrifugation at 14 000 rpm for 15 minutes at 4°C and protein content was assessed using the Bio-Rad Protein Reagent. For immunoprecipitations, 100μg of total protein was added to 1 ml of Protein A-Sepharose beads (Pharmacia) which had been pre-associated with the appropriated antibody. Samples were rocked for ≥2 hours at 4°C. The immunoprecipitate was washed four times with lysis buffer and resuspended in SDS-loading buffer (containing glycerol, SDS, Tris pH 6.8, bromophenol blue and DTT) and boiled for 5 minutes. For Western blotting, 20μg of protein from whole cell lysates was similarly boiled in loading buffer prior to analysis by SDS-PAGE. Proteins were then transferred to nitrocellulose membranes and probed with the appropriate primary antibodies. Secondary reagents conjugated to HRP allowed detection of the protein following exposure to X-ray film using the enhanced chemiluminescence system (Amersham).

λ Protein Phosphatase Assay. Using 20 μg protein from whole cell lysates, each sample was incubated with 0.25 μg of λPPase in 30 μl of reaction mixture containing phosphatase buffer and $MnCl_2$, purchased from New England Biolabs, Inc. The

reaction mixture was incubated at 30°C for 30 minutes at which time sample buffer was added, samples were boiled for 5 minutes and loaded onto an SDS gel.

In Vitro Kinase Assay: Kinase activity of CDK2 was assessed following immunoprecipitation of this protein from 500 µg whole cell lysates as described above. The CDK2 immunoprecipitate was mixed with kinase buffer (containing HEPES/NaOH, 10 mM $MgCl_2$, 1mM DTT, 16µg histone H1 (Boehringer Mannheim), 50 µg ATP and 2.5 µCi [γ^{32}-P]ATP) for 15 minutes at 37°C prior to SDS-PAGE analysis.

RESULTS

Cell Cycle Redistribution with Oligomycin Treatment

The alteration in HL-60 cell cycle following oligomycin treatment was predictable and reproducible throughout all experiments. For example, a dose of 5 ng/ml of oligomycin was consistently sufficient to induce a 15% reduction in the total amount of ATP and a concomitant 20% increase in the proportion of cells in the G_1 compartment of the cell cycle (average increase from approximately 55-60% G_1 to 70-75% G_1 with treatment) with no significant effect on viability (Figure 24). These examples in Figure 24(b) demonstrate increases in the proportion of cells in G_1 from 50% (control) to 63% with 10 ng/ml oligomycin, which is consistent with our previous reports.

Retinoblastoma protein and E2F

Relatively homogeneous samples of HL-60 cells representing various phases of the cell cycle were achieved by elutriation centrifugation (Figure 25) and used to compared the phosphorylation status of the Retinoblastoma protein. The degree of Rbp phosphorylation increases as cells traverse the cell cycle (Chen et al., 1989; Buchkovich et al., 1989). Figure 26(a and b) demonstrates that this protein undergoes a shift in mobility from approximately 110 kDa to 118 kDa as HL-60 cells progress through their cycle. Figure 27 demonstrates that the apparent shift in mobility of this protein is in fact due to phosphorylation events, as immunoprecipitated samples

treated with λ protein phosphatase demonstrate no such shift. To determine whether the level of Rb protein was being affected by the oligomycin treatment, protein was extracted from treated and control cells at various times during a single cell cycle and compared by Western blot analysis (Figure 28). There is no apparent decrease in the amount of Rbp in response to oligomycin treatment. However, there appears to be an increase in the proportion of the hypo-phosphorylated species of the protein, as indicated by the lower border of the band in those samples treated with oligomycin (Figures 28, 29, 30).

As Rbp becomes increasingly phosphorylated as normal cells progress through G_1 towards S, the E2F transcription factor is released and thereby activated (reviewed in Mittnacht, 1998). Immunoprecipitations of Rb protein from eluted early phase cells subsequently probed for the E2F transcription factor demonstrate that oligomycin-treated G_1 cells pull down more E2F than controls (Figure 31), which is consistent with the observation that less Rb is phosphorylated and more cells are remaining in the G_1 phase. It would be interesting to have conclusive immunoprecipitates of Rbp in heterogeneous populations showing its degree of association with E2F to corroborate that the observed hypophosphorylation of Rbp seen in oligomycin treated cells prevented the release and subsequent activity of that transcription factor, which is being pursued by other members of our lab.

Cyclin-dependent kinase inhibitors

In order to determine whether the G_1 accumulation observed following oligomycin treatment was mediated by an upregulation of the CDKIs, Western blots were performed on whole cell lysates from both treated and control cell heterogeneous samples. Using 50 ng/ml TPA (12-O-tetradecanoyl phorbol-13-acetate) as a positive control for induction of p21 expression (Jiang et al., 1994; Zhang et al., 1995), we demonstrate that oligomycin treatment sufficient to induce G_1 accumulation did not stimulate an increase in the amount of detectable p21 protein (Figure 32). Note that this inhibitory protein is detectable in TPA-treated cells prior to any alteration in the cell cycle distribution pattern (Table 9).

When lysates were resolved and probed for induction of p27, it was apparent that the absolute amount of this protein did not increase above basal levels in response to oligomycin (Figures 33a and b). When similar investigations were carried out for detection of p16 using a control GST-fusion protein for p16 (running at 43kDa), no induction or even detection of this protein could be observed in HL-60 cells (Figure 34).

Cyclins and cyclin-dependent kinases

Because the cyclin dependent kinases must associate with an appropriate cyclin partner in order to have kinase activity, we first investigated the level of cyclin E protein in control cell populations following elutriation of a heterogeneous population into fractions enriched with cells of the various cell cycle phases to

confirm our ability to detect its temporal expression (Figure 35). Cyclin E was detected in late G_1 and early S populations in both control and oligomycin-treated elutriated samples (Figure 36). Western blots conducted on lysates from heterogeneous control and treated samples also show no change in the absolute amount of cyclin E suggesting that the amount of this protein is not decreased following treatment (Figure 37).

The total amount of CDK2 protein was also determined by Western blot analysis to ensure that expression of the protein itself is not being down-regulated in response to oligomycin. Figure 38 demonstrates that there is no change in the amount of this kinase as cells traverse the cell cycle, nor is there an apparent difference in the level of protein in treated vs. control cell populations. An immunoprecipitation of CDK2 with subsequent probing for cyclin E in heterogeneous control and treated samples shows no change in the degree of association of cyclin E with its kinase partner (Figure 39).

An *in vitro* kinase assay was performed using histone H1 as a substrate to determine if the activity of CDK2 changes in response to a decrease in availability of ATP (Aprelikova et al., 1995; Akiyama et al., 1997). Figure 40 shows that the oligomycin treatment has little effect on the ability of this kinase to phosphorylate histone H1 in vitro.

Although the amount of CDK4 protein did not change following a reduction of cellular ATP with oligomycin treatment (Figure 41), the level of cyclin D protein was shown to be dramatically reduced (Figure 42). This is especially noteworthy as

there are >25% *more* cells in the G_1 phase in these samples where we would normally expect to find even more of this crucial G_1 cyclin. Given this, it would not be surprising to find a decrease in the level of CDK4 activity secondary to a reduced availability of its activating cyclin partner. Histone H1 is not phosphorylated by cyclin D/CDK4 and so an assessment of kinase activity would require the use of Rbp as the phosphorylation substrate to accurately assess the ability of the complex to carry out its required role in the face of ATP depletion (Matsushime et al., 1992; Pan et al., 1998).

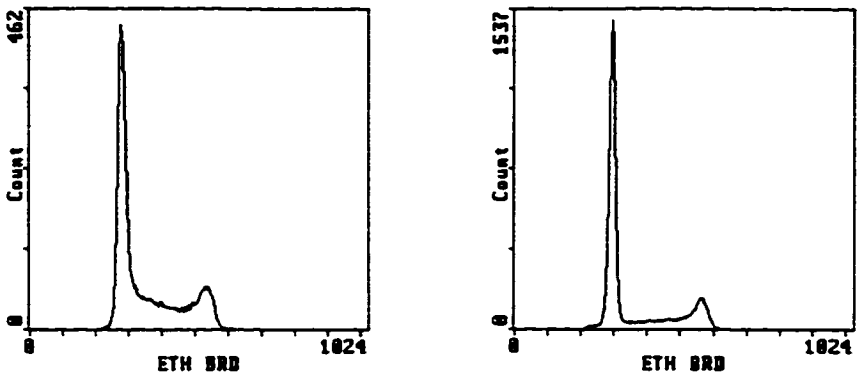


Figure 24a). Cell cycle distribution in control HL-60 cells and in cells treated with 5 ng/ml oligomycin. In this example, 24 hours of oligomycin treatment was sufficient to induce an increase in the proportion of cells in G_1 from 55% in the control sample to 68% (an increase of 24%) in the treated sample without affecting the viability of the population.

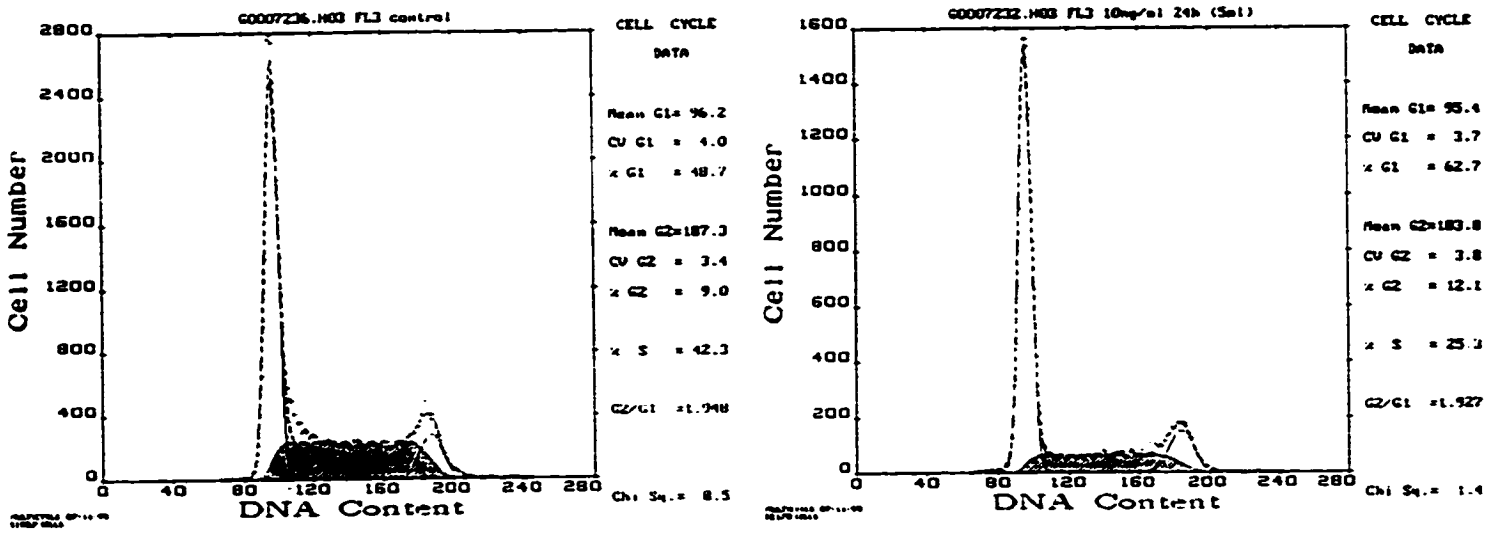


Figure 24b). Additional cell cycle analysis using MCYCLE which generates a curve of "best fit" across the data and gives proportions of cells in the various cell cycle phases, accounting for overlap at cell cycle boundaries.

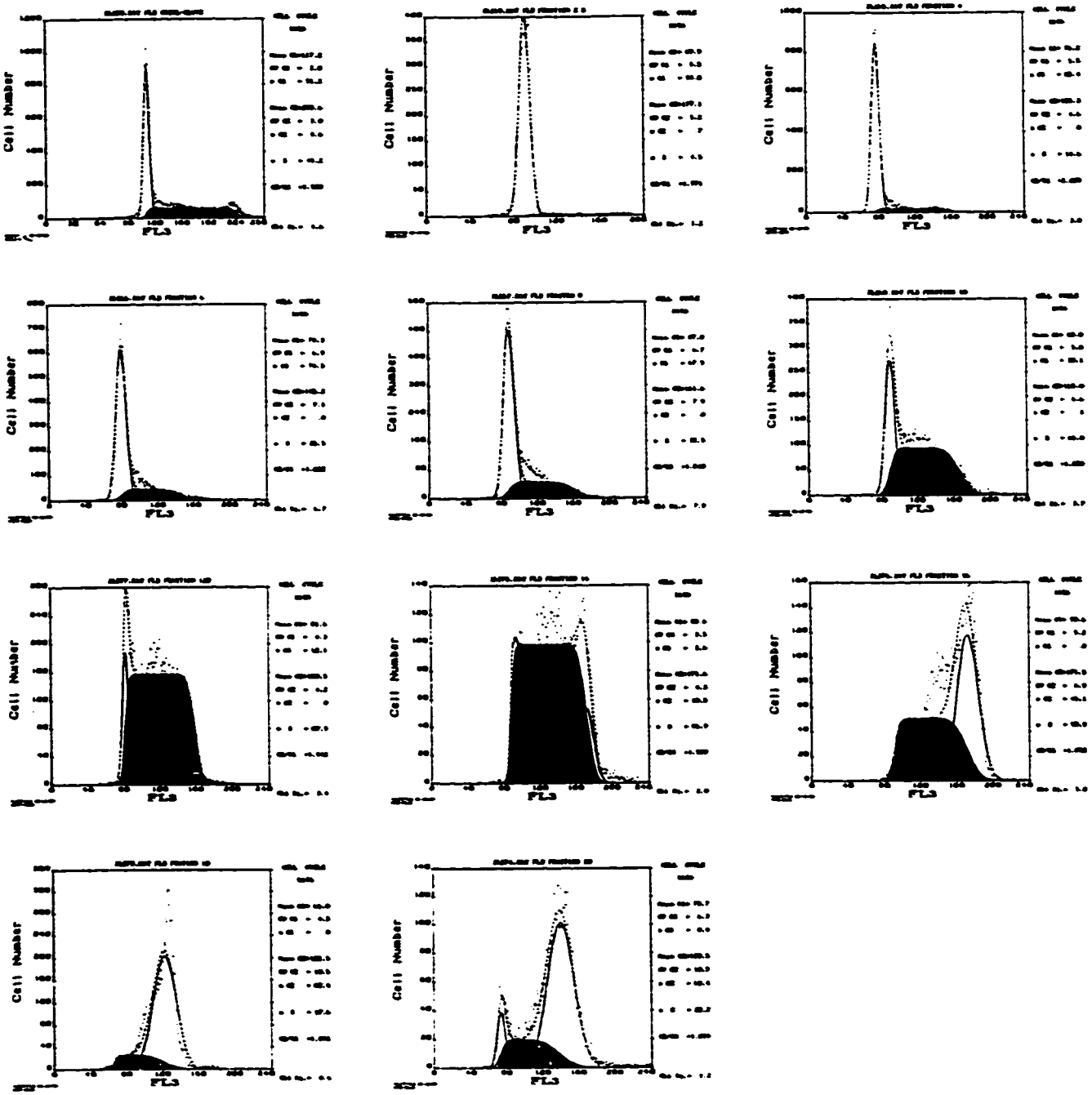


Figure 25. Eluted fractions of HL-60 cells showing the progressive elutriation of cells of increasing size corresponding to cells with a successively increasing amount of DNA, i.e. cells eluted in the final fractions correspond to those in the G_2M phase of the cell cycle.

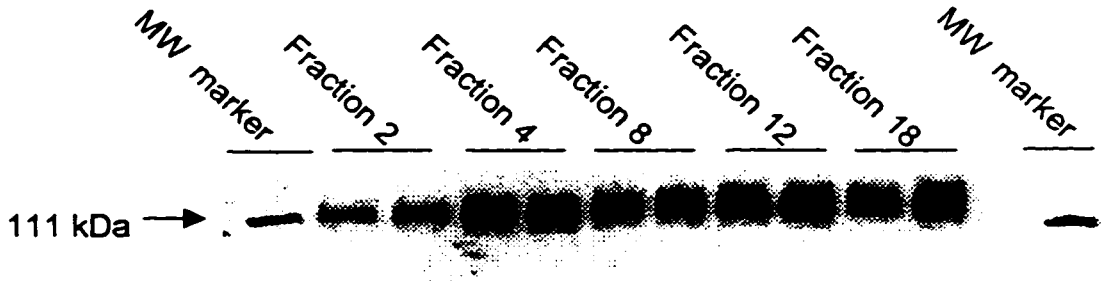


Figure 26a. Normal cell cycle changes in the degree of phosphorylation of the Retinoblastoma protein, showing an increasing trend as cells progress through the cell cycle

Fraction 2:	95% G_1		
Fraction 4:	85% G_1	15% S	
Fraction 8:	67% G_1	33% S	
Fraction 12:	12% G_1	88% S	
Fraction 18:		18% S	82% G_2M

Figure 26b. Increased degree of phosphorylation of cells in G_2M versus cells in G_1 is demonstrated by the more slowly migrating species of increased molecular weight in the G_2M sample.

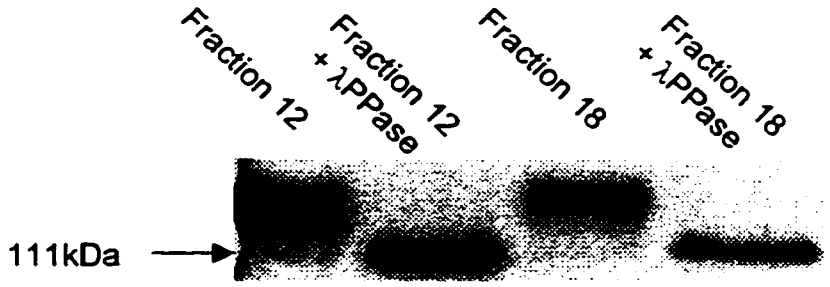


Figure 27. The decreased mobility of the Rb protein in late cycle cells is due to phosphorylation, as protein samples treated with a phosphatase that strips serine/threonine residues of their phosphate groups show increased mobility when resolved by SDS-PAGE.

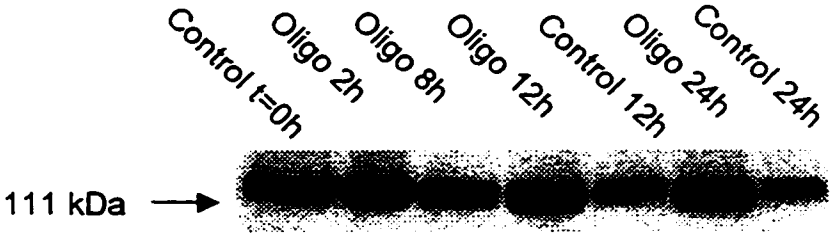


Figure 28. The total amount of Rb protein does not decrease in cells treated for up to 24 hours with 50 ng/ml oligomycin, although the amount of faster migrating, hypophosphorylated species begins to appear with increasing exposure in this heterogeneous population.

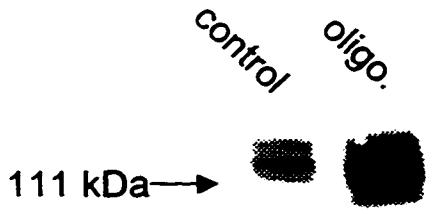


Figure 29. The degree of hypophosphorylated Rb protein increases with oligomycin treatment sufficient to cause and increase in the G_1 population from 52% in control cells to 69% in cells treated for 24 hours with 5 ng/ml oligomycin.

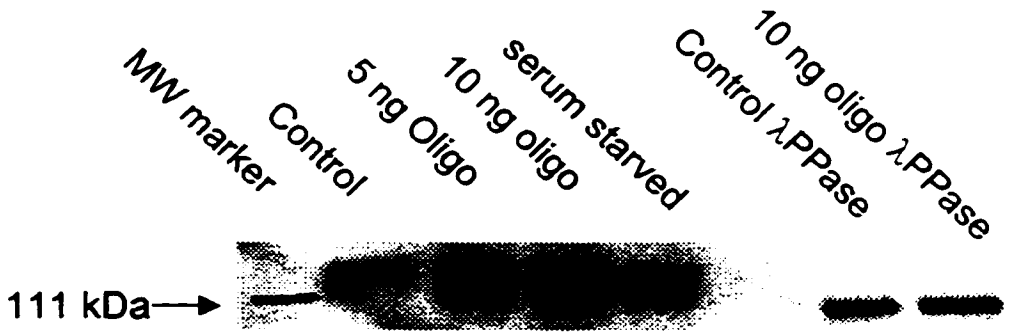


Figure 30. Rb protein phosphorylation in control, serum starved and oligomycin treated HL-60 cells. Both doses of oligomycin were sufficient to induce an increase in the G_1 population of more than 25%. These cells clearly show more of the hypophosphorylated species of Rb when compared to controls.

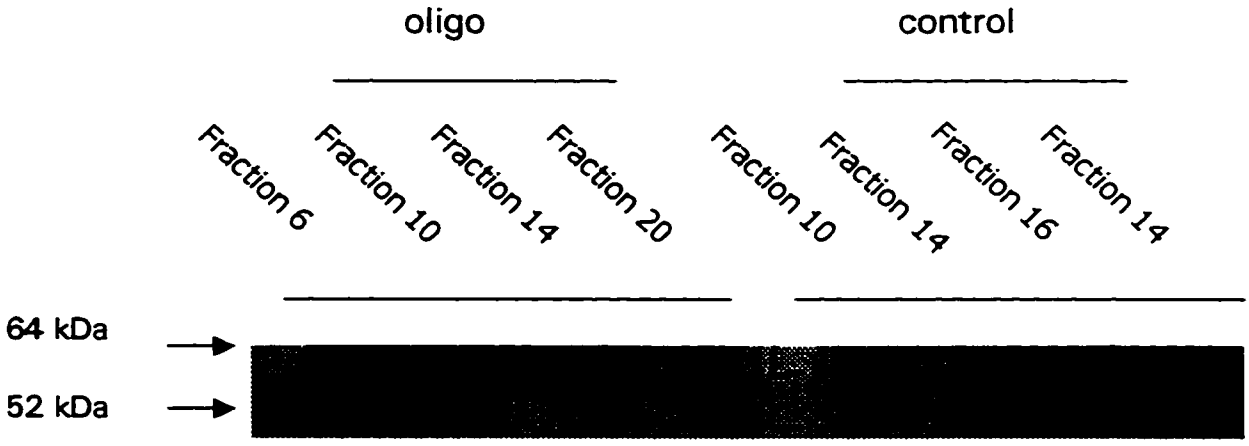


Figure 31. Immunoprecipitation of Rb protein from eluted early phase cells subsequently probed for E2F demonstrates that oligomycin-treated G_1 cells pull down more E2F than controls, which is consistent with the observation that more cells are remaining in the G_1 phase.

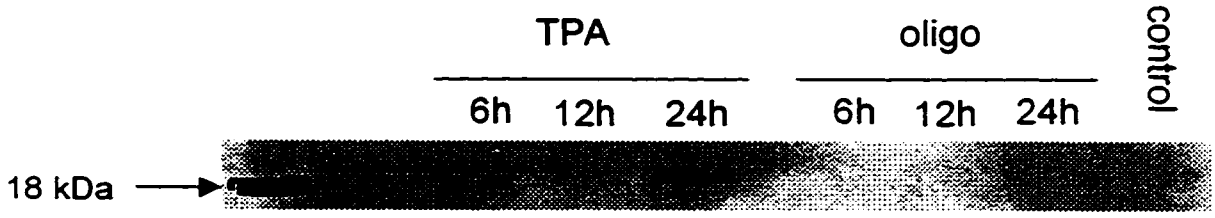


Figure 32. Expression of the CDKI $p21^{cip1}$ in response to continuous exposure to 50 ng/ml TPA or 50 ng/ml oligomycin. The cell cycle redistributions induced by these treatments are described in Table 9.

Table 9. Cell Cycle Accumulation using Oligomycin and TPA

time	condition	cell number ($x10^6$)	viability (%)	$\%G_0G_1$	%control
6 hours	control	4.28	95	53.6	100
	TPA	4.10	94	51.5	96
	oligomycin	4.3	94	60.2	112
12 hours	control	5.6	92	46.1	100
	TPA	5.2	95	46.2	100
	oligomycin	4.6	93	59.5	129
24 hours	control	6.6	93	53.0	100
	TPA	5.8	94	52.0	98
	oligomycin	5.7	89	62.0	117

note: data represent mean of 3 experiments; oligomycin and TPA used at 50 ng/ml continuous exposure

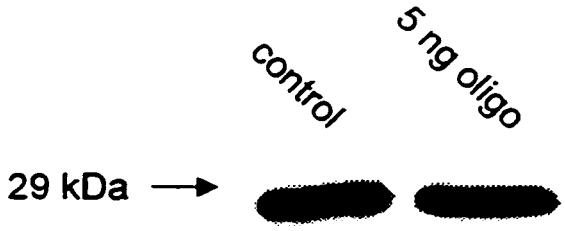


Figure 33a. $p27^{kip1}$ protein levels do not change in response to 5 ng/ml oligomycin, which caused an increase in the G_1 population from 50% in control cells to 70% in treated cells

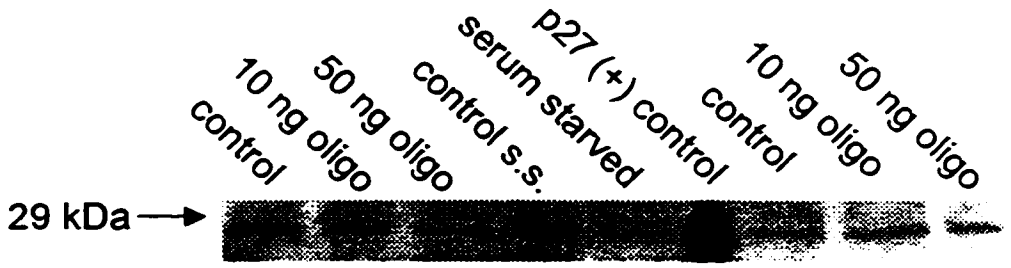


Figure 33b. Levels of $p27^{kip1}$ protein in two different experiments where oligomycin treatment was sufficient to increase the G_1 population by more than 25% show no significant change in the amount of this CDKI.

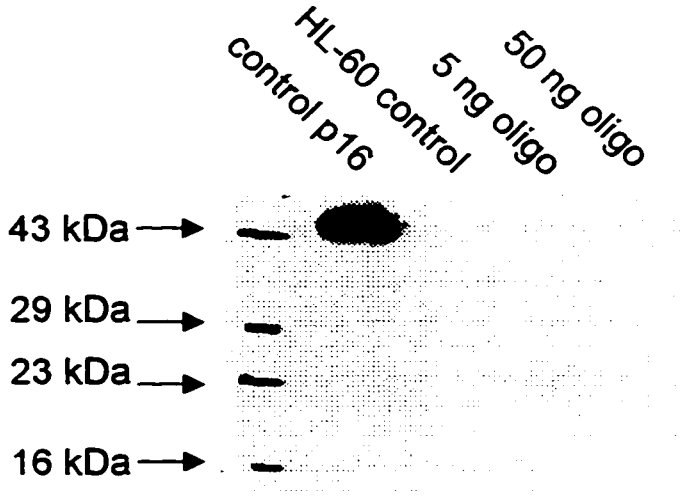


Figure 34. Western blot analysis for $p16^{INK4a}$ in HL-60 cells that show a 30% increase in the proportion of G_1 cells in response to treatment show no induction or even detection of this CDKI.

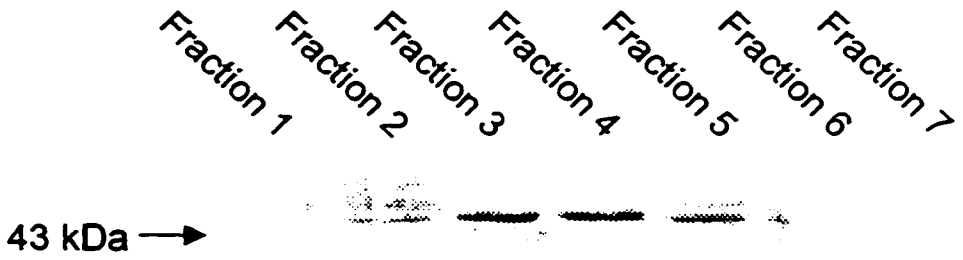


Figure 35. Cyclin E expression, as detected by Western blot analysis, is evident only in cells at the G_1-S boundary of the cell cycle in these eluted control cells.

Fraction 1:	99% G_1		
Fraction 2:	97% G_1		
Fraction 3:	89% G_1	11% S	
Fraction 4:	68% G_1	32% S	
Fraction 5:	63% G_1	33% S	4% G_2M
Fraction 6:	35% G_1	53% S	12% G_2M
Fraction 7:	4% G_1	43% S	53% G_2M

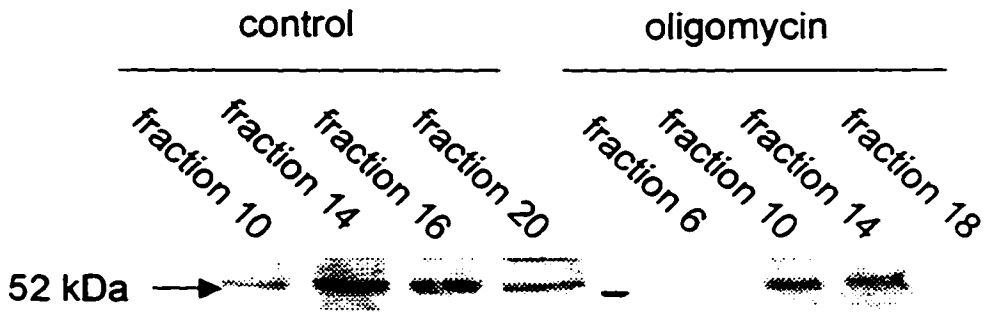


Figure 36. Western blot detection of cyclin E expression is most markedly apparent at the G_1-S boundary in both eluted control and treated cells.

Control:	Fraction 10:	96% G_1		
	Fraction 14:	89% G_1	10% S	
	Fraction 16:	64% G_1	28% S	8% G_2M
	Fraction 20:	15% G_1	30% S	55% G_2M
Treated:	Fraction 6:	96% G_1		
	Fraction 10:	91% G_1	7% S	2% G_2M
	Fraction 14:	61% G_1	26% S	13% G_2M
	Fraction 18:	18% G_1	42% S	40% G_2M

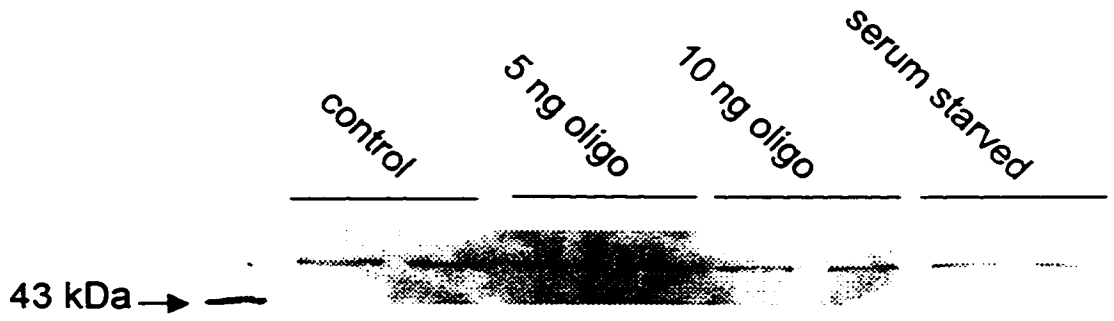


Figure 37. Cyclin E levels in control and treated heterogeneous populations shows no significant decline in protein level following treatment sufficient to cause an increase in the G_1 population from 53% in controls to 62% in both treatment samples.

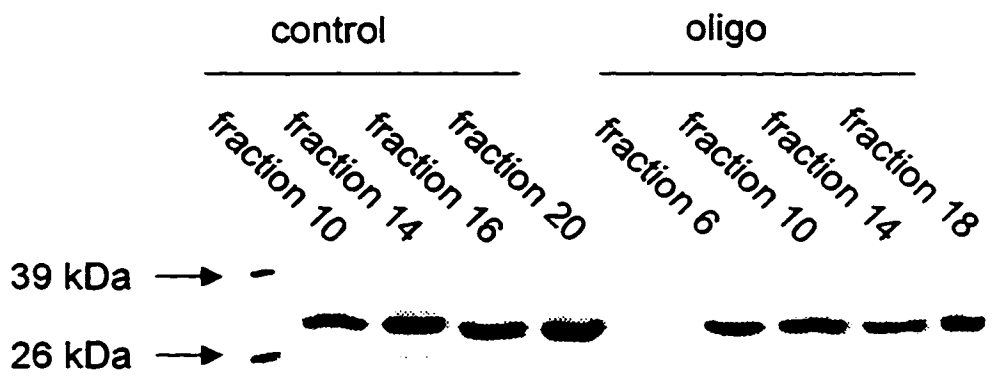


Figure 38. Using the same fractions described in Figure 36, CDK2 protein levels in eluted fractions of HL-60 cells show no change in amount throughout the cell cycle nor with oligomycin treatment. As can also be observed in Figure 36, there was proportionally less protein loaded into the treated lanes, therefore, the observed decrease represents less overall protein rather than reflecting its downregulation

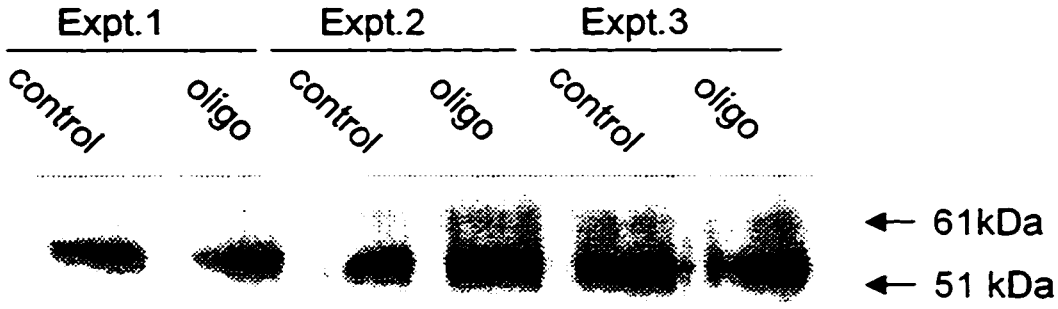


Figure 39. Immunoprecipitation of CDK2 with subsequent probing for cyclin E shows no change in the degree of association between CDK2 and cyclin E following oligomycin treatment in each of three different experiments. In each case, treatment evoked >20% increase in the proportion of cells in G_1 versus control.

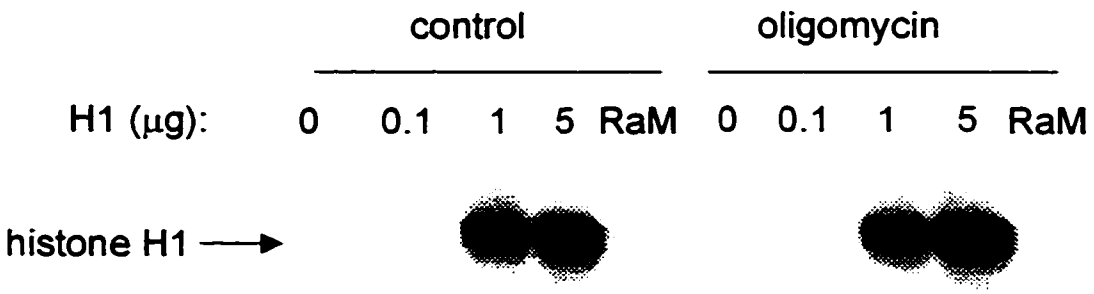


Figure 40. In vitro kinase assay of CDK2 on histone H1 shows no change in the ability of the activated kinase to phosphorylate substrate H1 in response to oligomycin treatment.

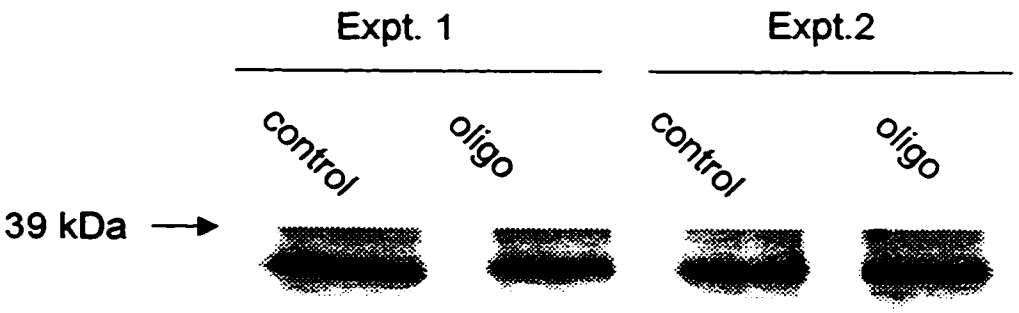


Figure 41. Total CDK4 protein levels assessed by Western blot from 2 different experiments show no significant alteration in the amount of this protein following treatment sufficient to increase the G_1 population from 49% to 70% in Experiment 1 and from 49% to 63% in Experiment 2.

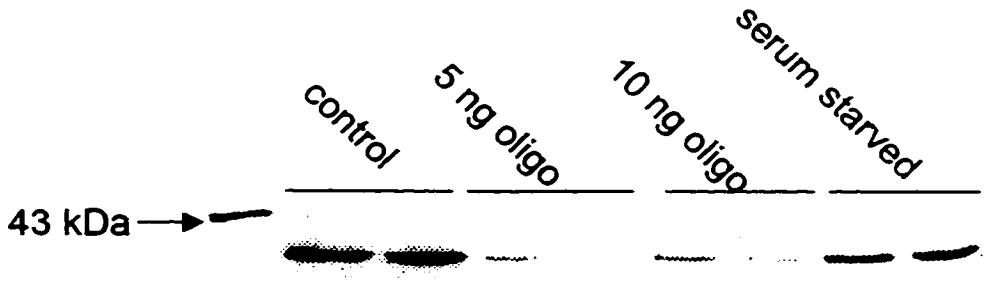


Figure 42. Cyclin D levels in control and treated cells show a marked decline in response oligomycin, concomitant with a shift in the G_1 population from 54% in controls to 70% in the treated cells.

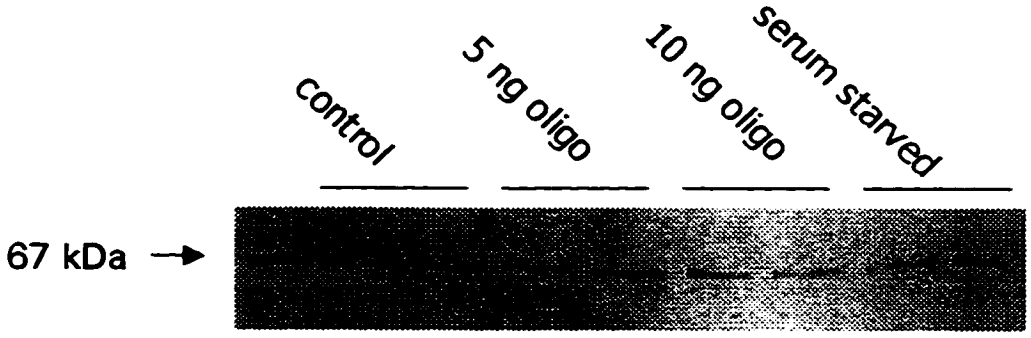


Figure 43. Protein loading control for the previous cyclin D western blot showing equivalent non-specific bands staining with similar intensity in each well

Chapter 5

DISCUSSION

Overview

This body of work encompasses several lines of investigations that aim to describe the contribution made by mitochondrial function to the mechanics of cell cycle progression. Initially, we defined two transition periods during the cell cycle where the availability of ATP is less than would be expected by the mitochondrial membrane potential and pursued these points as potentially sensitive to ATP depletion. Using agents that compromise mitochondrial function, we described two energy-sensitive checkpoints existing at precisely these transition phases, confirming that the energetic status of a cell is a potent influence on cell cycle control. Further investigations into the key regulators of the extremely sensitive G_1/S transition revealed that the Retinoblastoma protein was persistently underphosphorylated when mitochondrial function was inhibited. This prolonged activity of Rbp was not mediated through the CDK inhibitors, which normally initiate cell cycle arrest in the face of hostile conditions for replication. Rather, decreased availability of a key G_1 cyclin was demonstrated, suggesting that the cell is equipped with an intracellular sensing mechanism that is responsive to changes in the energy balance within the cell. This discussion will address the conclusions of each stage of these investigations corresponding to the order in which the data have been presented in the preceding Results sections.

Changes in Mitochondrial Properties During the Cell Cycle

Agents which affect normal mitochondrial function have been shown to reduce the level of total cellular ATP and induce a substantial change in cell viability and cell cycle dynamics (Kroll and Schneider, 1984; Van den Bogert et al., 1986, Van den Bogert et al., 1987; Muus et al., 1987; Giraud and Velours, 1997) To better understand why such changes arise, an investigation into the normal properties of mitochondria throughout the cell cycle was undertaken in an effort to define periods of cell cycle transition that might be more dependent upon and therefore susceptible to manipulation of mitochondrial function.

In order to characterize mitochondrial behaviour at different times during the cell cycle, it is essential to sample a relatively homogeneous population, enriched with cells of one particular phase. Reports employing various methods of population synchrony have previously described changes in mitochondria in relation to the cell's position in the cell cycle. Electron microscopy studies revealed that the process of drug-induced synchronization was itself sufficient to produce changes in mitochondrial morphology (Dewey and Fuhr, 1976), and that an initially synchronous population did not remain so as it progressed through the cycle, demanding further means for selection of desired cells (Posakony et al., 1977). Also, the limited sample number and the variable nature of mitochondrial form make a precise evaluation of mitochondrial mass challenging by electron microscopy alone. To avoid the complications of synchrony, other groups have related Rh123 uptake to cell size (inferring position in the cell cycle) (James and Bohman, 1981) or have semi-

permeabilized the plasma membrane to allow simultaneous dual labelling of DNA and mitochondria (Hammerle and Loffler, 1989); neither of which resulted in conclusive data in our lab. A comprehensive report by LePrat *et.al.* followed a crowded culture of murine leukemic cells through their first cell cycle after release into fresh medium, periodically recording changes in mitochondrial characteristics (LePrat et al., 1990). However, by the time the bulk of the population reached the G_2M phase, it was only approximately 55% synchronous, a disappointing observation encountered in our own attempts at synchrony. Our experiments using crowding as a method of synchrony, despite achieving approximately 87% of the sample in the G_1 phase at a cell density of more than 6.5 million/ml, showed a decrease in population viability by 25% which was not restored when cells were spilt and cultured into fresh media. After 24 hours in fresh culture conditions, viability had dropped to 50% that of control and the cell cycle profile demonstrated that the population was not progressing synchronously with 50% in G_1, 45% in S phase and 5% in G_2M (Table 2). Furthermore, synchronization through crowding of HL-60 cells has itself been shown as a metabolic insult sufficient to alter the parameters of mitochondrial physiology under investigation (Ashihara and Baserga, 1979; Grdina et al., 1987).

In an attempt to generate discreet samples of the various cell cycle phases without significant metabolic disturbance, a heterogeneous population was subjected to fractionation by elutriation centrifugation (Figure 9). Using this method, cells are maintained in a nutritive medium and subject only to low speed centrifugation prior

to analysis. Consistently high recovery of relatively homogenous populations (i.e. 96% G_1, 68% S, 73% G_2M) is possible with little effect on cell viability (Table 3)

Rh123 uptake studies indicate that the membrane potential increases throughout G_1 followed by a plateau phase as cells traverse S and then another significant increase as cells leave S phase and enter G_2 (Figure 12). Other studies describing mitochondrial activity in the context of cell cycle position in various cell types have reported Rh123 accumulation patterns similar to those presented here, despite differences in model and protocol (i.e. dual labeling of mitochondria and DNA in Ehrlich ascites tumor cells (Hammerle and Loffler, 1989); synchrony through crowding of murine leukemic cells (LePrat et al., 1990)). Thus, there appears to be a consensus regarding the pattern of change in mitochondrial membrane potential across the cell cycle in different cultured cell lines.

The introduction of the mitochondrial-specific vital dye NAO allows the discrimination of mitochondrial mass separately from membrane potential, and has overcome the limitations of earlier studies of mitochondrial biogenesis employing only Rh123 (James and Bohman, 1981; Collins and Foster, 1983). LePrat *et.al.* were first to employ both NAO and Rh123 in murine L1210 cells to follow biosynthesis and function of the inner mitochondrial membrane (IMM), reporting a biphasic increase in NAO uptake occurring mainly in G_1 but also again in late S through G_2, directly preceding a similar profile for Rh123 uptake (LePrat et al., 1990). The fact that we fail to observe the secondary increase in NAO fluorescence in G_2M cells may represent a cell-type specific phenomenon or, alternately, could result from the

influence of contaminating G_1 cells arising from the division of cells that were undergoing mitosis at the time of elutriation separation. The latter effect would likely be negligible in our samples however, as fewer than 8% of the G_2M cells had entered G_1 by the end of the analysis period. The reported second NAO increase may perhaps reflect a downstream effect of their method of synchrony by crowding for 8 cell-doubling times which, upon release into fresh media, may represent an artifact reflecting a compensatory increased biosynthesis resulting from the metabolic insult of starvation. Furthermore, it is unlikely that the time between peak NAO fluorescence and peak Rh123 fluorescence represents the time required for functional assembly of synthesized components of the IMM (as has been suggested by LePrat *et. al.*), as this 7 hour period represents almost one third of the total cell doubling time in HL-60 cells as compared to the significantly smaller 2 hour difference reported in the L1210 model.

Alternatively, we propose that HL-60 cells have nearly completed synthesis of IMM components (specifically cardiolipin) by mid-S phase. Because the period from S to G_2M shows significant increase in Rh123 accumulation with no concomitant increase in NAO (Figure 13), we suggest that a given mass of IMM does experience changes in membrane potential, presumably in accordance with the metabolic demands of the cell.

Cell populations with most cells in a phase transitional between G_1 and S (i.e. T1 and T2) have only marginally more ATP but significantly greater Rh123 and NAO fluorescence as compared to an early G_1 population (T3 shows >40% increase

in both Rh123 and NAO). Likewise, cells that are entering and traversing G_2 (prior to cell division) have significantly increased both membrane potential (by >200%) and inner membrane mass (by ≈178%) while the detectable levels of ATP has increased by less than 20% at this point in the cell cycle. Although this may represent a slower production rate of ATP during these cell cycle phases, it is also possible that it reflects periods of increased ATP utilization. The latter explanation is supported by both our observation that NAO and Rh123 have significantly increased and the generally held belief that the magnitude of the membrane potential is tightly coupled to the rate of ATP synthesis under normal physiological conditions as the proton gradient generated by the enzymes of the electron transport chain provides the energy for ATP synthesis (reviewed by Senior, 1988). In support of this idea, we were able to confirm that a decrease in mitochondrial membrane potential induced by incubation in any one of several ionophores (FCCP, CCCP, valinomycin) consistently resulted in a simultaneous decrease in whole cell ATP levels (Table 4). Therefore, if a given membrane potential translates into a predictable level of ATP production under normal physiological conditions, it is likely that points in the cell cycle where ATP is lower than would be expected by the increase in mitochondrial membrane potential represent transitions sensitive to ATP inhibition. At these points, the basal level of available ATP would already be close to a theoretical "minimum threshold" required for passage through that phase (Figure 15).

The development of increasingly photostable mitochondrial-specific fluorochromes which respond to changes in mitochondrial membrane potential but

are insensitive to fixation procedures such as CMXRos (Molecular Probes Inc., Eugene, Oregon) (Poot et al., 1996) will aid in the morphological analysis of discreet mitochondrial changes between cells of various stages of the cell cycle via quantitative confocal microscopy. Such experiments, which yielded inconclusive results in our lab due to photobleaching effects on Rh123, will provide representative, quantitative, visual results and complement the current data on cell cycle-specific mitochondrial characteristics.

Overall, these data emphasize the need for distinct markers of mitochondrial activity and mass, as a given mass has the capacity to alter its activity. Furthermore, this is the first report to directly relate mitochondrial characteristics to position in the cell cycle via direct mitochondrial labeling and ATP assessment of metabolically unperturbed elutriated populations, providing a foundation in basic science for investigating the energetic requirements of cell cycle progression. And finally, these data lead us to speculate that the points in the cycle where ATP utilization is greatest with respect to its production may be most susceptible to manipulation of mitochondrial activity as a reduction of ATP beyond the already low basal levels would quickly drop it below the "threshold" and the cell would be unable to fulfill the energetic requirements of transition through that cell cycle phase.

Influence of Mitochondrial Activity on Cell Cycle Distribution

The observation that there are two critical transition points in the HL-60 cell cycle where ATP availability is less than that predicted by the mitochondrial membrane potential led to the hypothesis that those transitions represent points of increased ATP requirement, and to an investigation of the specific effects of ATP inhibition on cell cycle dynamics.

Our data suggest that progress through G_1 into S is exquisitely sensitive to small decreases in whole cell ATP evidenced by the fact that the smallest detectable decrease in whole cell ATP was sufficient to cause a G_1 accumulation that was not surpassed at higher drug concentrations that resulted in lower ATP levels (Figure 22). Consistent with this idea is the observation that oligomycin-treatment of a "pure G_1" population (achieved by elutriation) causes substantially fewer cells to leave G_1 (i.e. 48% vs. 63% of controls) when assessed after 24 hours (Table 6). Progress through the G_2/M checkpoint also appears to be sensitive to changes in ATP although accumulation continues to increase as ATP levels decrease (Figure 23). Significant G_2M accumulation is not observed until ATP is reduced by approximately 20% whereas G_1 accumulation is evident earlier, by ≈12% reduction in whole cell ATP.

The phenomenon of cell cycle redistribution observed in this study lends itself to several possible, but unlikely, lines of interpretation: a) only a selective portion of cells at a vulnerable stage in their cycle are susceptible to death induced by these agents and their loss is the source of both the change in the cell cycle proportions and the fall in ATP; b) the agent used to reduce ATP content is interfering directly with one or several

of the molecular cell cycle events and the decrease in ATP is merely a reflection of the arrested population; or c) the agent is directly interfering with some other physiologic function within the cell (such as the plasma membrane pump or DNA/protein synthesis) and this signals the cell cycle controls to initiate arrest due to an environment of suboptimal conditions for proliferation. The results presented here however, do not conform to the above explanations for a variety of reasons. Firstly, in any study of cell cycle redistribution, it is important that a significant proportion of the population is not lost to toxicity. If cells are dying from the test agent, both the cell cycle distribution and the total population ATP levels would be affected, particularly if cells of a given phase were selectively sensitive. For this reason, doses that did not induce substantial cell loss were deliberately chosen.

The possibility that the agents used are inducing a "generalized" arrest of cells in all phases is likewise a doubtful scenario. Not only has such a "master controller" of cell cycle progress affecting all phases never been described (short of irreversible, external influences such as flash freezing or fixation wherein viability is invariably lost), but also review of the raw data clearly demonstrates that with increasing time and dose, cells continue to pile up in G_1 and G_2M eventually depleting the S phase compartment of the cell cycle (Figures 18, 20, 21). Furthermore, if it were a generalized arrest, the cell cycle profile would resemble that of the untreated population and cells would not be actively synthesizing DNA and therefore, BrdU incorporation would be not be maintained, as it is with oligomycin treatment (Figure 19).

To confirm that the decrease in ATP was not solely a reflection of a redistribution of the population, the ATP content of homogeneous G_1, S and G_2M populations achieved through elutriation centrifugation were measured. By calculating the average ATP content of cells in the different cell cycle phases, we were able to show that cells in G_2M had more ATP than G_1 and S phase cells, which is not surprising given that G_2 cells have double the mass and contain more mitochondria. As the resultant cell cycle distribution following agents that affect mitochondrial ATP production consistently shows an accumulation of cells in G_2M, one would expect a higher ATP reading from that population when, in fact, it is predictably lower in these samples and correlates closely with an increasing proportion of cells in G_2M. Therefore, the redistribution alone is not sufficient to account for the observed decreases in whole cell ATP.

Similar cell cycle effects were observed regardless of the agent used or the duration of incubation. Despite any other potential effect each agent may have had within the cell, each drug independently induced a decrease in ATP content that resulted in a predictable alteration in the cell cycle profile of these cells. If one or more of these agents were affecting some other physiologic system, it would seem unlikely that their resultant effects on both ATP and cell cycle distribution would be as consistent as those observed (Figures 22 and 23).

Our interpretation of these results includes the existence of an energy-sensitive cell cycle checkpoint at both the G_1/S and G_2/M transitions in HL-60 cells. This checkpoint would, in theory, assess the cell's availability of ATP and its capacity to

generate ATP throughout the cycle. For example, following a moderate decrease in ATP (≈10%), the ATP status of a cell that had passed the G_1/S transition when the ATP pool was compromised, would again be assessed at the G_2/M border. As this checkpoint appears to have a lower ATP threshold (i.e. more tolerant to depletion), these cells would complete the cycle and return to G_0G_1 where they would be prevented from initiating another cell cycle by the high ATP threshold that exists at that checkpoint. As ATP levels are further decreased, the cells no longer meet the energetic requirement to pass the G_2 checkpoint and so the population exhibits G_2 accumulation at higher doses of ATP inhibitors when the minimum requirement of the checkpoint cannot be met.

It seems likely that the ATP pool itself is the key influence on cell cycle progression and not the changes to mitochondrial physiology, as glycolysis can compensate for decreased mitochondrial function and restore both viability and cell cycle progress. For example, it has been reported that depletion of ATP, independent of mitochondrial membrane depolarization, is sufficient to alter the cell cycle profile and induce lethal cell injury (Nieminen et al., 1994). Using oligomycin and cyanide on rat hepatocytes, it was shown that ATP levels must fall by more than 15% before viability is affected; an influence that could be completely reversed if glycolysis was stimulated with fructose, replenishing the ATP pool. Our data on ATP reduction and viability are consistent with these findings and the idea that non-essential cell functions are first to be affected while persistent insults to ATP generating capacity will eventually influence functions essential for survival. Furthermore, it had been shown in our lab that direct inhibition of glycolysis using inhibitors of hexokinase results in similar cell cycle effects

reported here with comparable degrees of ATP depletion (T. Shaw, Master's Thesis, 1996).

It is known that nutrient deprivation will induce cell cycle arrest at the G_1/S border due to decreased macromolecular synthesis necessary for the remaining cell cycle phases (Hartwell *et al.*, 1974). Consistent with our results, Van den Bogert *et.al* (Van den Bogert *et al.*, 1986; Van den Bogert *et al.*, 1988) used doxycycline to selectively block mitochondrial protein synthesis and showed proliferation arrest in the G_1 phase of the cell cycle which is accompanied by an increasing shortage of ATP. The same group also demonstrated that following doxycycline treatment, mammalian cells were no longer able to proliferate if the activity of enzymes involved in oxidative phosphorylation was significantly reduced and suggested that the lack of ATP generating capacity was the primary cause of arrest (Van den Bogert et al., 1992). Proliferation continued only if culture conditions allowed for an enhanced rate of glycolysis, which could compensate for the loss of mitochondrial ATP production. These studies however did not report a G_2 accumulation. The absence of the G_2 phenomenon may be attributable to the different cellular characteristics of the cell lines involved, or result from the fact that the ATP levels were not reduced sufficiently to induce G_2 arrest. Nonetheless, our data are consistent with these previously reported results suggesting that energetic checkpoints exist which must be satisfied if a cell is to continue through its proliferative cycle.

Because similar cell cycle effects were seen with reduced ATP regardless of the agent used to achieve the reduction (Figures 22 and 23), it is likely that the observed cell

cycle phenomena are related to the change in the energy status of the cell. The practical implications of these findings involve the potential use of mitochondrially-directed agents in cancer therapy, where the elevated membrane potential of tumour cells renders them particularly sensitive to cytostasis and cytotoxicity secondary to cation accumulation and subsequent compromise of ATP generating capacity. At the basic cell biology level, the existence of energetic checkpoints at the G_1/S and the G_2/M borders presents a field of inquiry which has experienced only limited investigation. It will now be possible to complement the existing information on the machinery of the cell cycle engine with a consideration of the fuel required to make the engine run. These data also reinforce the idea that mitochondria represent sensitive targets for cancer chemotherapy, as may all the processes of energy production, including glycolysis, the creatine kinase pathway as well as oxidative phosphorylation.

In conclusion, it is evident that progression through the cell cycle of HL-60 cells is sensitive to changes in mitochondrially-derived ATP. Energetic checkpoints exist at both the G_1/S and the G_2/M borders that prevent the cell from continuing through its proliferation cycle when the energetic status of the cell is not sufficient to allow competent cell division.

Mitochondrial Function Influences the Core Cell Cycle Machinery

Oligomycin-induced ATP-depletion results in significant G_1 phase accumulation. It was our intention to investigate which aspects of the cell cycle machinery are responsive to alterations in the energetic status of the cell. The key regulators of the G_1-S phase transition are the cyclin-dependent kinases and their cyclin partners. Their job of phosphorylating and inactivating Retinoblastoma protein is modulated in several ways. The levels of cyclin available for CDK binding, the phosphorylation status of the CDKs themselves and the association of activated cyclin/CDK complexes with small inhibitory peptides all govern kinase activity. The first cyclin to be expressed in response to favourable growth conditions is cyclin D. In conjunction with CDK4/6, cyclin D mediates the initial phosphorylation of Rbp, followed sequentially by kinase activity of the cyclin E/CDK2 and cyclin A/CDK2 complexes, to maintain Rbp in the inactive hyperphosphorylated state (Lundberg and Weinberg, 1998). Any factor affecting the activity of these kinases could abrogate the normal inactivation of Rbp and cause an accumulation of cells in G_1.

Our data demonstrate that HL-60 cells treated with oligomycin show more of the hypo-phosphorylated species of Rbp than untreated controls, represented by the lowest band of faster-migrating protein (Figures 29 and 30). This observation supports the idea that the status of the Rb protein acts as an integration point for various types of growth regulatory signals, as it is already known to reflect changes in growth factor availability and cell density. This, however, is the first demonstration of a response to internal energetic parameters, suggesting that some mechanism exists

within the cell that is sensitive to changes in the availability of ATP. Following this observation, we attempted to demonstrate which of the known regulators of Rbp phosphorylation were altered in the face of decreased ATP availability.

Using Western blotting techniques on whole cell lysates of control and oligomycin-treated HL-60 cells, we investigated whether the key G_1 cyclin-dependent kinase inhibitors were being induced and downregulating the activity of the cyclin/CDK complexes.

The INK4 CDKI p16 is often mutated in cells with wild-type Rbp. We were unable to detect any p16 protein in either treated or control samples by probing whole cell lysates (Figure 34). Studies addressing the p16 status in HL-60 cells report a heterozygous deletion of one allele with a point mutation in the other (reviewed in (Drexler, 1998)). Others report that while basal levels of this inhibitor are very low, stable transcripts are detectable using RT-PCR (Stone *et al.*, 1995). One group employing this technique in HL-60 cells found that p16 expression was less than 0.001% that of β-actin in both control and PMA-stimulated cells, as compared to p27 levels, which were approximately 5% in controls and rose to 40% in stimulated cells (Schwaller *et al.*, 1997). Taken together, it does not seem unreasonable to conclude that the observed G_1 accumulation following oligomycin treatment of HL-60 cells is not mediated by an increase in the p16 cyclin-dependent kinase inhibitor.

Linking extracellular growth signals to the machinery of the cell cycle, the p27 CDKI is known to respond to changes in growth factor availability, increases in cell density and inhibitory cytokines (reviewed in (Coats *et al.*, 1996; Lloyd *et al.*,

1999)). Although the p27 mRNA transcript is relatively stable and changes little over the course of the cell cycle, the level of p27 protein shows cell cycle related fluctuations peaking in G_1, reflecting its post-transcriptional control (Hengst and Reed, 1996).

Because of its central role in monitoring conditions permissive or restrictive of proliferation, and its capacity to associate with all G_1 cyclin/CDK complexes, p27 has been described as the CDKI most directly involved in passage through the G_1-S transition (Sherr, 1996). Although we were able to detect p27 protein in both untreated cells and those cells treated sufficiently to induce significant G_1 accumulation, the overall amount of this protein did not change significantly when assayed by Western Blot and controlled for equivalent protein loading (Figure 33) Thus, upregulation of the p27 CDKI does not appear to mediate the observed G_1 accumulation. The possibility that p27 is exerting some influence on the cell cycle distribution in the presence of oligomycin through a temporal shift in its normal binding partners, although unlikely, cannot be ruled out and will be discussed in the context of the observed changes in the cyclins and CDKs that follows.

The last CDKI that was considered as a candidate for mediating the G_1 phase accumulation was p21. Although HL-60 cells express no p53, they have been shown to exhibit p21 induction in response to various differentiation signals, such as DMSO and TPA (Jiang et al., 1994; Kuliczkowski et al., 1995). Using doses of oligomycin sufficient to induce a 29% increase in the proportion of cells in G_1 (see Table 9), no detectable induction of p21 was evident. In samples treated with TPA as a positive

control, upregulation of p21 was evident at 12 hours and maximal at 24 hours, prior to the onset of any G_1 accumulation (Figure 32). Therefore, it is our belief that p21 is not involved in mediating the oligomycin-induced G_1 accumulation in HL-60 cells, as increased levels of this CDKI were not detected prior to or coincident with the cell cycle change.

Another obvious candidate for control of Rbp phosphorylation is the cyclin E/CDK2 complex. If either protein showed decreased levels or if there was a decrease in the degree of association between these partners, then a decrease in the degree of Rbp phosphorylation would be expected. Our results demonstrate no obvious changes in the absolute amounts of these proteins following an oligomycin-induced G_1 accumulation (Figures 36, 37, 38). There does not appear to be a diminution in their degree of association (Figure 39) nor is there any difference in the ability of the complex to phosphorylate histone H1 in an *in vitro* kinase assay (Figure 40). Therefore, it is unlikely that a decrease in the kinase activity of the cyclin E/CDK2 complex is solely responsible for holding HL-60 cells in G_1 in response to decreased levels of cellular ATP.

Cyclin D is known to play at least two important roles in facilitating the transition from G_1 phase into S. Firstly, it regulates the kinase activity of CDK4/6, allowing the important initial phosphorylation of Rbp in mid-G_1 (Connell-Crowley et al., 1997; Lundberg and Weinberg, 1998). Secondly, it acts in complex with its partner kinase to sequester the Cip/Kip kinase inhibitors (Polyak et al., 1994; Nourse et al., 1994), relieving their inhibitory effect on cyclin E/CDK2 which is then free to

further phosphorylate Rbp and also to enhance its own activity via the targeting of p27 for degradation (Sheaff et al., 1997). Given these significant roles for cyclin D, it would not be surprising that any impediment to this cyclin's normal function would affect cell transit through G_1. Our data demonstrate that in response to ATP depletion sufficient to induce >20% increase in the proportion of cells in G_1, the total amount of cyclin D protein is significantly reduced when compared to that of untreated controls (Figure 42). The effects of this reduction in cyclin D availability could be reasonably linked to the mechanics of the observed cell cycle arrest both through the resulting inability of unbound CDK4 to phosphorylate Rbp and through the potential decrease in the association of cyclin D/CDK4 complexes with p21 and/or p27. With limited sequestration of the Cip/Kip proteins, these inhibitors would be free to associate with, and hence decrease the ability of, cyclin E/CDK2 complexes to phosphorylate Rbp. The observed increase in the degree of Rbp hypophosphorylation with oligomycin treatment supports this scenario as a possible contributor to the observed cell cycle redistribution in the face of ATP depletion. A CDK4 kinase assay using Rbp as a substrate would support this hypothesis and will be pursued by my colleagues in the lab.

Although plausible, a shift in p21/p27 affiliation to cyclin E/CDK2 following a decrease in cyclin D seems insufficient alone to explain the observed cell cycle changes as the ability of CDK2 to phosphorylate histone H1 *in vitro* was maintained. This may be accomplished through the ability of activated cyclin E/CDK2 complexes to phosphorylate and, in so doing, target p27 for destruction. Although no significant

change in the amount of p27 protein was detectable by Western blot analysis in treated cells, it is possible that a potential change was beyond the sensitivity of the assay and may be enhanced by its analysis following immunoprecipitation with both CDK4 and CDK2 following elutriation of G_1 cells rather than in a heterogeneous population. Furthermore, despite the observation that CDK2 activity is maintained on histone H1, it is possible that the activated cyclin E/CDK2 complex is less efficient at phosphorylation of Rbp as a substrate without Rbp's prior modification by cyclinD/CDK4 (Lundberg and Weinberg, 1998). This may contribute to the observed hypophosphorylation of Rbp despite CDK2 *in vitro* kinase activity.

With respect to the regulation of cyclin D levels, the observed decrease in this critical G_1 protein in response to lowered ATP availability may be related to a decrease in the stimulus for cyclin D production and/or an increase in the rate of its degradation. Expression of cyclin D1 is known to be an early event in commitment to the cell cycle. Its expression is highly growth factor dependent and its short half-life ensure that levels drop precipitously following mitogen withdrawal (Sherr, 1993). In fact, cyclin D has been described as a critical link coupling extracellular growth factors to the nuclear cell cycle machinery (Peeper and Bernards, 1997). External mitogenic stimuli work through receptor tyrosine kinases at the cell membrane to activate members of the Ras/Raf-1/MAPK signaling pathway which, in turn, increase expression of cyclin D, thus facilitating phosphorylation of Rbp (reviewed in Lavoie et al., 1996; Peeper and Bernards, 1997; Downward, 1997). Despite the availability of extracellular mitogens, cyclin D levels are low in the context of decreased cellular

ATP (Figure 42). Therefore, it is possible that some steps between activation of the growth factor receptor and cyclin D expression is/are sensitive to changes in ATP availability. This is not an unreasonable scenario as these are kinase reactions requiring the donation of a phosphate from the intracellular pool, which may be sufficiently depleted so as to direct the limited remaining high energy phosphates to processes critical for cell survival (such as maintenance of membrane potential), while cell division is relegated to the "non-essential" backbench of cellular priorities.

Another possible contributing factor to the observed decrease in cyclin D protein levels may be enhanced degradation of this protein. Cyclin D is ubiquitinated and degraded via the 26S proteosome pathway secondary to its phosphorylation by a glycogen synthase kinase (GSK-3β) and subsequent nuclear export (Diehl et al., 1998). This pathway is normally held in check by another Ras signaling pathway working through phosphatidylinositol-3-kinase (PI3K) and protein kinase B (Akt) (Diehl et al., 1998). Perhaps our data represent a scenario where these opposing regulatory enzymes are competing for a limited pool of intracellular phosphates wherein GSK-3β is preferentially successful or one in which the stability of cyclin D is significantly reduced, either due to decreased assembly with CDK4 or secondary to decreased activity of the Ras pathway which loosens control over the activity of GSK-3β. It is conceivable then, that decreased Ras activation may exert a two-fold influence over cyclin D levels; firstly, playing a role in the decreased expression of this protein via decreased activity of the Raf-1/MAPK pathway and, secondly via the increased proteosomal degradation of cyclin D. Assessing the activity of these

regulators of cyclin D expression in situations of compromised ATP generating capacity will help define the cellular mechanisms of the subsequent cell cycle arrest.

Although our data open up the field of cell cycle energetics and the dependence of signaling pathways on adequate intracellular high energy phosphate pools (i.e. is there a rate limiting step in the Ras pathway that is most sensitive to ATP depletion?), it also serves as the first demonstration of an *intra*cellular signal associated with cyclin D regulation and subsequent Rbp hypophosphorylation despite an adequate extracellular environment. These data further support our previous investigations suggesting that there exists within the cell some bioenergetic "sensor" that will affect cell cycle progress independent of external growth factor provision. Although the final details of such a mechanism remain to be described, it will likely involve the known regulators of cyclin D including members of the Ras signaling pathways and the ubiquitin-proteosome degradation system. Defining the participants in this unexplored "ATP-sensitive" pathway will not only contribute to our understanding of the energetics of cell cycle progression but will also further our ability to define and exploit potential targets for cancer chemotherapy, as members of the cyclin D/Rb pathway are often dysregulated in tumour cells.

Future Directions and Overall Conclusions

The data presented here could be complemented in future in a variety of ways. Regarding quantification of mitochondrial parameters during the course of the cell cycle, better stains which are resistant to photobleaching and fixation procedures are becoming available which will facilitate analysis by confocal microscopy. This technique creates a three-dimensional image of a cell based on its staining characteristics which will allow direct comparison of cells in the various phases of the cell cycle. It will also be important to broaden these observations to include other cancer cell types as well as non-transformed cell lines.

To further strengthen the idea of ATP-dependent checkpoints at the G_1/S and G_2/M boundaries, homogeneous populations of cells could be subjected to various levels of ATP depletion and used to clearly define the energetic thresholds in terms of picograms of ATP per cell below which cells would stop cycling. It would also be valuable to attempt rescue experiments with the provision of exogenous ATP to solidify the claim that this is in fact the limiting factor to cell cycle progression.

With respect to changes at the level of the cell cycle proteins, it will be imperative to assess the degree of association between cyclin D and its partner CDK4, as well as the kinase activity of these complexes on Rbp to confirm that the decrease in the level of cyclin D protein is responsible for the persistently underphosphorylated state of Rbp in these conditions. Further studies could include a dissection of the signal transduction pathways that regulate cyclin D expression and the influences

upon the ubiquitin system that orchestrates its destruction to explain why levels of this protein are affected by compromised mitochondrial function.

In an attempt to broaden the understanding of the interaction between mitochondrial function and the cell's capacity for division, we have described several important aspects of this inseparable relationship, highlighting the extent to which these two physiologic parameters are dependent upon one another. The discovery that the availability of ATP cannot be predicted by the absolute mass of mitochondria and that ATP availability changes in a cell cycle-dependent manner provides a basis for understanding the energetic requirements of cell cycle progression and suggests transition periods which may be susceptible to inhibition of ATP synthesis for manipulation of cell division. This discovery fits nicely with our observations that there do indeed exist two distinct transition points in the division cycle where progress is most sensitive to pharmacological inhibition of ATP synthesis. These two transitional phases, which exist at the G_1/S and the G_2/M boundaries, coincide precisely with points in the cell cycle where the mitochondrial membrane potential is high but whole cell ATP levels are low, suggesting a period during which the available pool of ATP may already be close to a critical threshold below which cell cycle progress would be inhibited. This study was the first to describe these "energetic checkpoints" thus strengthening the idea of mitochondrial-directed interventions as a novel means of cell cycle manipulation. Finally, the investigation into the mechanisms through which decreased mitochondrial function elicits an

altered cell cycle profile reveals the first account of an intracellular sensor arising in response to conditions within the cytoplasm, and not the DNA damage-sensing mechanism of the nucleus. This sensor, which is sensitive to disturbances in the energetic balance within the cell, ultimately results in prolonged inhibitory activity of the Retinoblastoma protein, signalling a change in cell cycle progression resulting from a change in the core proteins of the cell cycle engine that drive a cell from G_1 into S phase.

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