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# Tumour suppressor proteins in proliferating and differentiating cells

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# TUMOUR SUPPRESSOR PROTEINS IN PROLIFERATING AND DIFFERENTIATING CELLS

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A thesis submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in fulfillment of the requirements for the degree of Doctor of Philosophy.

Johannesburg 2013

# Declaration

I declare that this thesis is my own unaided work. It is being submitted for the degree of Doctor of Philosophy in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

Umesh Bodalina

# Abstract

Cells have evolved the ability to change continuously and adapt to their environment. An important way in which this dynamic modulation is achieved is by reversible phosphorylation, mediated by protein kinases and phosphatases. This thesis focuses on the temporal variations in expression of the proteins protein phosphatase 1 (PP1), protein phosphatase 2A (PP2A) and p53 tumour suppressor protein in proliferating and hexamethylene bisacetamide (HMBA) induced differentiating murine erythroleukaemic (MEL) cells. The study included analysis of variations of p53 mRNA in these cells. Protein variations were analysed in cell extracts using western immunoblotting. The p53 variations were evaluated further using enzyme-linked immunosorbent assay (ELISA); p53 mRNA was determined by reverse transcriptase polymerase chain reaction (RT-PCR).

Dynamic variations in protein expression and mRNA were detected in both the untreated and HMBA treated MEL cell preparations. For PP1, an immunospecific band of molecular mass 36 kDa, corresponding to the catalytic subunit, was detected, while for PP2A, two immunospecific bands of 32 and 36 kDa were observed. For the PP2A, the 36 kDa band corresponded to the catalytic subunit of this protein and the 32 kDa band was believed to be a proteolytically cleaved form of the catalytic subunit. The mean values of results showed little significant difference between proliferating and differentiating MEL cells, emphasising that single time-point studies give incomplete and probably misleading information. Multiple time analysis for expression clearly showed evidence of oscillatory behaviour and modulation by the differentiating agent. The influence of HMBA on PP1, PP2A and p53 expression was variable for the different experiments and affected both the frequency and phasing of rhythms. The results add support to the view that dynamic oscillatory control processes play an important role in regulating cellular behaviour. Modulation of the dynamics of key proteins in the

cell, such as PP1, PP2A and p53, may be an important mechanism of controlling cellular function and reversing neoplastic transformation.

# Dedication

I dedicate this work to my parents and family. Thank you for all your understanding, patience and support.

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# Abbreviations

| | |
|---|---|
| Abl | Abelson tyrosine-protein kinase |
| ADP | Adenosine diphosphate |
| AEA | Antigen extraction agent |
| AgT | SV40 large T antigen |
| AIP1 | Actin-interacting protein 1 |
| Akt | Ak thymoma viral proto-oncogene (PKB) |
| AP-1 | Activator protein 1 |
| ATCC | American Type Culture Collection |
| ATP | Adenosine triphosphate |
| ATRA | All-trans retinoic acid |
| BAX | Bcl-2-associated X protein |
| BALB/c | Bagg Albino inbred albino mice |
| BALB/3T3 | Mouse embryonic fibroblast cell line |
| bcl-2 | B-cell lymphoma 2 |
| bp | Base pair |
| BSA | Bovine serum albumin |
| C | Competimer |

| | |
|---|---|
| c-Fos | FBJ murine osteosarcoma viral oncogene cellular homologue |
| c-Jun | Jun oncogene |
| c-Myb | Avian myeloblastosis virus oncogene cellular homologue |
| c-Myc | Avian mycelocytomatosis virus oncogene cellular homologue |
| c-Raf-1 | Cellular Raf-1 oncogene |
| C1NH | Complement component 1 inhibitor |
| CaM | $Ca^{2+}$-calmodulin-dependent |
| CBP | Creb-binding protein |
| cDNA | Complementary DNA |
| cdc2 | CDK1, Cyclin-dependent kinase 1 |
| cdc25p | Cell division control protein 25 |
| cdc55 | Cell division control protein 55 or regulatory B subunit of protein phosphatase 2A |
| cdk | Cyclin-dependent protein kinase |
| cdk1 | Cyclin-dependent protein kinase 1 |
| cdk2 | Cyclin-dependent protein kinase 2 |
| cdk4 | Cyclin-dependent protein kinase 4 |
| cdk6 | Cyclin-dependent protein kinase 6 |

| | |
|---|---|
| cds | Coding sequence |
| Cip1 | Cyclin-dependent kinase inhibitory protein-1 |
| $CO_2$ | Carbon dioxide |
| Cop1 | Constitutively photomorphogenic 1 |
| cyl | Cyclin |
| DAB | Diaminobenzidene |
| DARPP-32 | Dopamine- and cAMP-regulated phosphoprotein of 32 kDa |
| DBA/2 | Dilute Brown Non-Agouti |
| DEAE | Diethylaminoethyl |
| DEPC | Diethyl pyrocarbonate |
| DMSO | Dimethyl sulphoxide |
| DNA | Deoxyribonucleic acid |
| dNTP | Deoxyribonucleoside triphosphate |
| DS19 | Mus musculus endogenous virus Friend spleen focus-forming virus cell-line (MEL) |
| dT | Deoxy-thymine |
| E2F | E2 transcription factor |
| E2F-DP | E2 transcription factor dimerisation partner |
| E4orf4 | Adenovirus E4 open reading frame 4 |

| | |
|---|---|
| E4F1 | Transcription factor E4F |
| E6AP | E6-associated protein |
| ECL | Enhanced chemiluminescence |
| EDTA | Ethylene-diamine-tetra-acetic acid |
| ELISA | Enzyme-linked immunosorbent assay |
| EMEM | Eagles Minimal Essential Medium |
| ERCC2 | Excision repair cross-complementing group 2 |
| ERK | Extracellular-signal-regulated kinase |
| FAK | Focal adhesion kinase |
| FCS | Foetal calf serum |
| FK506 | Tacrolimus or fujimycin |
| FKBP12 | FK506-binding protein 12 |
| Fos | FBJ osteosarcoma oncogene |
| GADD45 | Growth arrest and DNA-damage-inducible |
| GST3 | Glutathione-s-transferase 3 |
| HCl | Hydrochloric acid |
| HDAC | Histone deacetylase |
| HIV | Human Immunodeficiency Virus |
| HIPK2 | Homeodomain-interacting protein kinase 2 |

| | |
|---|---|
| HL60 | Human promyelocytic leukaemia cells |
| HMBA | Hexamethylene bisacetamide |
| hMOF | Human orthologue of males absent on the first |
| HOX11 | Homeo box 11 |
| HSP | Heat shock protein binding site |
| HPV | Human papilloma virus |
| HRX | Human trithorax gene |
| $I_1^{PP2A}$ | Protein inhibitor 1 of PP2A |
| $I_2^{PP2A}$ | Protein inhibitor 2 of PP2A |
| I-1 | Inhibitor-1 |
| I-2 | Inhibitor-2 |
| Ia8 | I-A subregion class II antigen 8 |
| IGF | Insulin-like growth factor |
| IGF-BP3 | Insulin-like growth factor binding protein3 |
| Ig | Immunoglobulin |
| IgG | Immunoglobulin G |
| IgG2b | Immunoglobulin G2b |
| JAK2 | Janus-activated kinase 2 |
| M-MLV | Moloney murine leukaemia virus |

| | |
|---|---|
| MAP | Mitogen-activated protein |
| MAPK | Mitogen-activated protein kinase |
| MCF7 | Human breast adenocarcinoma cell line |
| Mdm2 | Murine double minute 2 homologue |
| MEK | Mitogen-activated protein kinase kinase |
| MEL | Murine erythroleukaemic |
| mRNA | Messenger ribonucleic acid |
| *MYB* | Myeloblastosis oncogene |
| *MYC* | Myelocytomatosis oncogene cellular homologue |
| MYPT1 | Myosin phosphatase target subunit 1 |
| MYT1 | Myelin transcription factor 1 |
| NCBI | National centre for biotechnology information |
| NCp7 | Nucleocapsid protein p7 |
| NF-κB | Nuclear factor kappa beta |
| NIPP-1 | Nuclear inhibitor of PP1 |
| NLS | Nuclear localisation signal |
| NMR | Nuclear magnetic resonance |
| NP40 | Nonyl phenoxypolyethoxylethanol |
| OA | Okadaic acid |

| | |
|---|---|
| OLIGO | Oligomerisation domain |
| p107 | Retinoblastoma-like protein p107 |
| p130 | Retinoblastoma-like protein 2 |
| $p16^{Ink4}$ | Cyclin-dependent kinase 4 inhibitor |
| $p21^{WAF1\backslash Cip-1}$ | Cyclin-dependent kinase inhibitor 1 |
| p300 or EP300 | E1A binding protein p300 |
| p53BP2 | p53 Binding protein 2 |
| p53Con | p53 Consensus sequence |
| p70S6K | p70 ribosomal S6 kinase |
| pAb-122 | p53 Antibody clone 122 |
| PAK1 | p21 Activated kinase 1 |
| PBS | Phosphate buffered saline |
| PCAF | P300/CBP-associated factor |
| PCNA | Proliferating cell nuclear antigen |
| PCR | Polymerase chain reaction |
| PDB | Protein data bank |
| PI3K | Phosphoinositide 3-kinase |
| Pirh2 | p53-Induced RING-H2 domain protein |
| PKB | Protein kinase B |

| | |
|---|---|
| PKC | Protein kinase C |
| PMSF | Phenylmethanesulphonyl fluoride |
| PP | Primer pair |
| PP1 | Protein phosphatase 1 |
| PP2A | Protein phosphatase 2A |
| PP2B | Protein phosphatase 2B |
| PP2C | Protein phosphatase 2C |
| PP3 | Protein phosphatase 3 |
| PP4 | Protein phosphatase 4 |
| PP5 | Protein phosphatase 5 |
| PP6 | Protein phosphatase 6 |
| PP7 | Protein phosphatase 7 |
| PPM | Protein phosphatase $Mg^{2+}$ or $Mn^{2+}$ dependent |
| PPP | Phosphoprotein phosphatase |
| PPP1A | Protein phosphatase 1 alpha |
| PPP1CA | Protein phosphatase 1 alpha catalytic subunit |
| PPP2R4 | Protein phosphatase 2A activator, regulatory subunit 4 |
| PPT | Protein tyrosine phosphatase |
| PR55 | 55-kDa Regulatory subunit of protein phosphatase 2A |

| | |
|---|---|
| PR61 | 61-kDa Regulatory subunit of protein phosphatase 2A |
| PR65 | 65-kDa Regulatory subunit of protein phosphatase 2A |
| PR72 | 72-kDa Regulatory subunit of protein phosphatase 2A |
| PTP | Protein tyrosine phosphatase |
| PTPA | Protein tyrosine phosphatase activator |
| PUMA | p53 Up-regulated modulator of apoptosis |
| Raf-1 | V-Raf-1 murine leukaemia viral oncogene homologue 1 |
| Ras | Rat sarcoma oncogene |
| Rb | Retinoblastoma |
| RCSB | Research collaboratory for structural bioinformatics |
| RSA | Republic of South Africa |
| RGC | Ribosomal gene cluster |
| RIPP-1 | Ribosomal inhibitor of PP1 |
| Rm | Rainbow marker |
| RNA | Ribonucleic acid |
| RNases | Ribonucleases |
| rRNA | Ribosomal ribonucleic acid |
| RPMI | Roswell Park Memorial Institute medium |
| RT | Reverse transcription |

| | |
|---|---|
| RT-PCR | Reverse transcription polymerase chain reaction |
| SDS | Sodium dodecyl sulphate |
| sds22 | Suppressor 2 of dis2-11 mutation |
| SDS-PAGE | Sodium dodecyl sulphate poly-acrylamide gel electrophoresis |
| SI | International System of Units |
| sIg | Surface immunoglobulin |
| SV40 | Simian Vacuolating virus 40 |
| SV80 | Human fibroblasts transformed by SV40 |
| TAD | Transcriptional activation domain |
| TAE | Tris-acetate-EDTA |
| Taf31 | TATA-associated factor 31 |
| Taf70 | TATA-associated factor 70 |
| TAg | T antigen |
| Tap42 | Type 2A-associated protein of 42 kDa |
| Tau | Beta 2 transferrin |
| TBS | Tris buffered saline |
| TEMED | Tetramythylethylene-diamine |
| TFIIH | Transcription factor II H |

| | |
|---|---|
| Thr | Threonine |
| TIP60 | Tat-interacting protein 60 |
| TP53 | Tumour protein p53 |
| TSG | Tumour suppressor gene |
| Tyr | Tyrosine |
| UK | United Kingdom |
| USA | United States of America |
| UTR | Untranslated region |
| UV | Ultra violet |
| V | Volts |
| Vpr | Viral protein R |
| WAF1 | Wild-type activating fragment-1 |
| Wee1 | Small cell protein, a mitotic inhibitor kinase |
| WT1 | Wilms tumour 1 |
| Xpd | Xeroderma pigmentosum D |

# Conference Proceedings

This work was presented at the following congresses:

1. Expression of the p53 tumour suppressor protein in proliferating and differentiating cells. Proceedings of the 33rd Annual Congress of the Federation of S.A. Societies of Pathology, Sun City (July 1993).

2. The p53 tumour suppressor protein: Expression and phosphorylation during cell differentiation. Proceedings of the 12th Congress of the S.A. Biochemical Society, Stellenbosch (January 1994).

3. The p53 tumour suppressor protein: Phosphorylation during cell differentiation. Proceedings of the 34th Annual Congress of the Federation of S.A. Societies of Pathology, Cape Town (July 1994).

4. Chemiluminescent detection of the p53 protein in murine erythroleukaemic cells. Proceedings of the 35th Annual Congress of the Federation of S.A. Societies of Pathology, Bloemfontein (July 1995).

5. Expression of protein phosphatases in murine erythroleukaemic cells. Proceedings of the 36th Annual Congress of the Federation of S.A. Societies of Pathology, Kruger National Park (July 1996).

6. Protein phosphatases and reversal of transformation in murine erythroleukaemic cells. Cancer 97: Recent Advances in the Molecular and Cellular Aspects of Cancer, Langebaan, Cape (January 1997).

7. Expression of p53 and protein phosphatases in murine erythroleukaemic cells. Proceedings of the 37th Annual Congress of the Federation of Societies of Pathology, Cape Town (July 1997).

8. Expression of the p53 protein in proliferating and differentiating murine erythroleukaemic cells. Proceedings of the 2nd FASBMB Meeting: Biochemistry of Africa, Potchefstroom, South Africa (September 1998).

9. Expression of the p53 tumour suppressor protein in proliferating and differentiating murine erythroleukaemic cells. Miami Nature Biotechnology Winter Symposium, Miami, USA (February 1999). This was published in *Miami Nature Biotechnology Short Reports* volume 10 which is a supplement to *Nucleic Acids Symposium Series* volume 40.

# Publications

Hammond, K. D., Bhoola, R., Bodalina, U. and Gilbert, D. 1998. Dynamic cells: Temporal organisation and control of phosphorylation. *Trends in Comparative Biochemistry and Physiology*, 4, 75-88.

Bodalina, U. and Hammond, K. D. 1999. Expression of the p53 tumour suppressor protein in proliferating and differentiating murine erythroleukaemic cells. Published in *Miami Nature Biotechnology Short Reports* volume 10 which is a supplement to *Nucleic Acids Symposium Series* volume 40.

Bodalina, U., Hammond, K. D. and Gilbert, D. A. 2005. Temporal changes in the expression of protein phosphatase 1 and protein phosphatase 2A in proliferating and differentiating murine erythroleukaemic cells. *Cell Biol Int*, 29, 287-299.

Bodalina, U., Hammond, K. D. and Gilbert, D. A. 2007. Temporal variation in the expression of the p53 protein in proliferating and differentiating murine erythroleukaemia cells. *Mol Cell Biochem*, 294, 155-162.

# Chapter 1

# Introduction and literature review

## 1.1. Cancer, an evolutionary process; the dynamics of life

"Unless you have gone through the experience yourself, or watched a loved one's struggle, you really have no idea just how desperate cancer can make you. You pray, you rage, you bargain with God, but most of all you clutch at any hope, no matter how remote, of a second chance at life." (Gorman *et al.*, 1998)

Most of what we know about the origin of life and cells comes from modern research done on the kinds of chemical reactions and interactions that may have taken place on the earth 3.5 billion years ago (Ridley, 1993). Theories and speculation about these origins of life are put forward; as technology advances these theories are refined, reinforced or proved wrong and later modified. Nature has evolved such that organisms strive to sequester energy and use it to survive, grow and multiply. With the evolution of life, cells developed unique characteristics and adaptive methods in order to continue to live. This adaptive progress of life and evolution was intensely studied by many people with the foremost being Charles Darwin, who published some of these insights in his theory on natural selection (Darwin, 1859). Important in these unique survival mechanisms is the ability of genes to adapt to environmental changes. These alterations are brought about by inheritable transformation in the genes which leads to changes in encoded proteins (Merlo *et al.*, 2006). One such disease of genetic change is cancer.

Cancer is one of the most prominent diseases of the modern world. This disease arises from changes that take place at critical positions in the genetic material of a cell. The cell does have defence mechanisms to prevent cancer but often these protection systems are rendered inoperable by further genetic alterations thus leading to its cancerous behaviour. There are many forms of cancers all of which have one common characteristic; that of uncontrolled division. As yet there is no universal form of treatment that can heal all varieties of cancer. Thus this is one of the goals that drives the initiative to seek more knowledge on this disease with the intention of finding that cure.

Some of the questions researchers ask in their quest for this knowledge are "Why do these things behave the way that they do? What is driving the process?" Often, the answers to these questions are based on some kind of standard measurement; a simple example being the measurement of distance. Fundamentally, science is based on these standard measurements. This allows experiments to be replicated and their results compared. Another ubiquitous measure that is frequently used is the standard measure of time. This measure is used to track changes that take place in a system and to quantify these rates of alteration. In this way the comparison of different interrelated studies can be done.

Every facet of life uses time, it has been a major subject of religion, philosophy, and science. Throughout the ages, time has been applicable to all fields of study. Its definition has been the subject of a lot of controversy and it has been difficult to describe. Often it was reduced to a simple measure of change. From the *Collins English Dictionary* time is defined as "A nonspatial continuum in which events occur in apparently irreversible succession from the past through the present to the future." (Collins, 2003). The "International System of Units" (SI) defines time as one of the seven base units, the others being length, mass, electric current, thermodynamic temperature, amount of substance and luminous intensity

(Lehmann, 1979). These units independently or collectively are used as the bases of all measurement. In this study we relate time to the quantity of change of specific proteins and mRNA in a population of cancerous cells.

### 1.1.1. Dynamic processes

We live in a world that is highly complex and continuously changing; material is being formed and broken down together with the exchange of energy. From an evolutionary perspective nature has evolved efficient mechanisms for adapting to these environmental changes thus allowing living matter to survive and strive in it. Some of these adaptations are simple while others involve highly complex interactions. From a genetic perspective, if a particular process was efficient in respect to carrying out its function then that particular trait would be passed on to its offspring. Thus if this attribute were critical it would be ubiquitous in nature and its presence would be found in many different organisms. Consequently, to a certain extent it is the environment that dictates which traits are conserved. An example of this inheritance with respect to signal transduction, is the tumour suppressor gene *p53* which is found in most living organisms (Merlo *et al.,* 2006; Xue *et al.,* 2013). As Richard Dawkins mentions in his book *Climbing Mount Improbable* "Often living bodies have converged upon the same shape as each other, not because they are mimicking each other but because the shape that they share is separately useful to each of them" (Dawkins, 1996). Thus living organisms are highly dynamic with extremely efficient mechanisms of survival.

All of these adaptive mechanisms have an additional factor to contend with which is the synchronising of activities to timely cycles (daily, monthly, seasonal, yearly). Again this is driven by changes in energy and nutrient levels in the environment. An

example of this is the circadian rhythm which has developed as a result of recurring environmental events (changes of day and night) such as the 24 hour period of the earth's rotation on its axis. Similar to changes that synchronise to environmental time, an understudied aspect of biology is the study of changes and reactions that molecules within a cell undergo with respect to time. This dynamic aspect of the Universe has been intensely investigated in many other disciplines and only recently have studies from this perspective begun to grow in the biological field.

Cycles and rhythms are everywhere in nature and are central to life; they form the basis of adaptation and natural selection. Oscillatory systems occur at all levels of biological organisation, from unicellular to multicellular organisms, with periods ranging from fractions of a second to years (Goldbeter, 1996) (see table 1.1). Some of these rhythms, like the cardiac rhythm, are maintained throughout life, and even a brief interruption could lead to death. Other rhythms, some under conscious control and some not, make their appearance for various durations during an individual's life. These rhythms form a complex network of interactions with each other and with the external environment. Variation of rhythms outside of normal limits, or the appearance of new rhythms where none existed previously indicates change and in some cases could be associated with disease (Gilbert, 1995; Gilbert, 1968; Gilbert, 1982; Gilbert, D.A. and MacKinnon, H., 1992; Hammond *et al.*, 1998; Hammond *et al.*, 1989b).

Table 1.1 A list of time periods of important biological rhythms taken from *Biochemical oscillations and cellular rhythms* (Goldbeter, 1996).

| Rhythm | Period |
|---|---|
| Neural rhythms | 0.01 to 10 seconds (and more) |
| Cardiac rhythm | 1 second |
| Calcium oscillations | 1 second to several minutes |
| Biochemical oscillations | 1 minute to 20 minutes |
| Mitotic cycle | 10 minutes to 24 hours |
| Hormonal rhythms | 10 minutes to several hours |
| Circadian rhythms | 24 hours |
| Ovarian cycle | 28 days (humans) |
| Annual rhythms | 1 year |
| Epidemiology and ecological oscillations | years |

Due to the complex nature of these dynamic systems, new models of organisation have been generated involving advanced mathematics and the processing power of computers. These models make use of the branch of mathematics called nonlinear dynamics. Simply put, nonlinear dynamics is a language to talk about dynamic systems. It does not only focus on a system on its own but also takes into consideration the various other factors that influence the system. Nonlinear dynamics also focuses on the effects that small changes can have on the overall system.

Most of these observations involve a study of change with respect to time. Such time series have been categorised into four main mathematical ideas: steady states, oscillations, chaos, and noise (Glass and Mackey, 1988). A brief explanation of each of these terms follows:

A. Steady state - In mathematics, steady state refers to a constant solution of a mathematical equation (Glass and Mackey, 1988). This concept is similar to that of homeostasis in the biological field. Homeostasis refers to the relative constancy of the internal environment with respect to variables such as, blood sugar, osmolarity, electrolytes, waste products, blood gases and pH. The elucidation of the mechanisms that constrain variables to narrow limits constitutes a key area of biological research (Glass and Mackey, 1988).

B. Oscillations – In mathematics, oscillations refer to periodic solutions of mathematical equations (Glass and Mackey, 1988). Oscillations arise due to an action or behaviour that is regularly (or irregularly) repeated. Some examples of oscillations are the rhythms of heartbeat, respiration, the normal sleep-wake cycle, release of insulin and luteinising hormone, and cellular division (Goldbeter, 1996).

C. Chaos – Chaos refers to the randomness or irregularity that arises in a deterministic system (Glass and Mackey, 1988). In other words, chaos is

observed even in the complete absence of environmental noise. An important aspect of chaos is that there is a sensitive dependence of the dynamics to the initial conditions (Glass and Mackey, 1988). Thus, although in principle it should be possible to predict future dynamics as a function of time, in reality this is presently impossible as any error in specifying the initial conditions, no matter how small, leads to an erroneous prediction at some future time (Glass and Mackey, 1988). Do not misunderstand the definition; chaos is not random: it is apparently random behaviour resulting from specific set of laws. It is an enigmatic form of order.

D. Noise – Noise refers to chance fluctuations in a system. In most cases, noise is usually referred to a system in which the underlying mechanisms leading the irregularities are not understood at this current point in time (Glass and Mackey, 1988). Noise is characterised by total randomness of the oscillations and is not dependent on the initial conditions of the system.

Thus nonlinear dynamics would be an ideal tool to study nature. Its use in the study of biology is not a new concept but it is one that has not been used often. Goldbeter extensively reviews some of the most common studies done from the perspective of nonlinear dynamics (Goldbeter, 1996; Goldbeter *et al.*, 2012). Some of these studies included looking at glycolytic, cAMP, calcium, mitotic and circadian oscillations (Gerard and Goldbeter, 2012a; Gerard and Goldbeter, 2012b; Gerard *et al.*, 2012; Goldbeter, 1996; Goldbeter *et al.*, 2012). In order to make use of these tools efficiently, one would have to be proficient in both mathematics and biology. This study will not incorporate any mathematical explanation of nonlinear dynamics; instead it will focus on providing evidence on dynamics in the cell.

### 1.1.2. The dynamic cell

All cells have natural rhythmic patterns which are dependent on the dynamics of the macromolecules within each cell and the requirements of their environment (Glass and Mackey, 1988). This behaviour is also a reflection that all living cells are far from equilibrium due to the continuous creation and destruction of components within the cell and the interaction with its changing environment. The dynamics of most of these interactions are not fully understood. Part of this behaviour is driven by elements that make up the system when they respond automatically to feedback signals within the environment. A consequence of this is that environmental feedback can be seen as providing information about the system's efficiency and effectiveness.

There are a number of metabolic and genetic control systems in the cell, each having a different level of influence and interaction. The dynamic control mechanisms within each cell type have their own pattern of temporal organisation that reflects the cell's own unique pattern of metabolism under those particular conditions. This gives rise to cellular characteristics that are not constant (contributing to the dynamic nature) but operate within certain limits, an upper and lower limit which forms the boundary of operation. This profile, in turn, could be used to label and characterise that particular cell under those conditions, its limits dictating the health of the cell. Permanent changes that occur during differentiation and disease (e.g. cancer) involve changes in this dynamic state (Calvert-Evers and Hammond, 2002; Gilbert and Lloyd, 2000; Gilbert, 1968; Gilbert, 1984; Gilbert, D.A. and MacKinnon, H., 1992; Hammond *et al.*, 1998; Hammond *et al.*, 2000a; Hammond *et al.*, 1989b; Hammond *et al.*, 2000c). Hence studying these patterns of oscillation may help shed more light on predicting the survival or death of cells. Also, the subtle manipulation of these oscillations could help reverse certain disease states and return the cells to within their normal parameters.

To appreciate what gives rise to this type of behaviour, the fundamental operations of the key players involved need to be understood. Dynamic behaviour of individual molecules is influenced directly or indirectly by every other molecule in its environment. Thus small changes from a few molecules can cause further changes and adjustments to other elements which eventually lead to one of the mathematical states discussed in section 1.1.1. These alterations eventually contribute to the efficient working of the cell and its harmonisation with its environment. Almost everything is implicated in the dynamics of the cell with the key players being the interactions of cellular material e.g. DNA, RNA, proteins, macromolecules, and other cellular components. The reactions of these molecules are intricate and vast leading to some complex interactions that are continuously changing. The state of all these various processes is continuously being relayed back to the brain of the cell, the DNA. The DNA, in turn, reacts and brings about the necessary changes in protein activity. Hence the dynamics of the cell is greatly dependent on relaying of information; this includes information from the environment. The relaying processes have been labelled as signal transduction and are defined into various signal transduction pathways.

In order to survive, multicellular organisms must sense their internal and external environment which includes the detection of neighbouring cells and hormones. This sensing allows the cell to respond appropriately by growing, proliferating, moving or dying. These processes require the transfer of information from the detection system referred to as receptors (usually on the outside of cells) through a network of interacting molecules within the cell, leading to changes in the expression of genes on the DNA and the activity of proteins including enzymes. The DNA contains the code that controls and coordinates all these internal and external factors that ultimately influence cellular behaviour. This mechanism of biological information transfer has been previously described as signal transduction. Most signal

transduction pathways involve second messengers which activate enzymes via an ordered sequence of biological reactions inside the cell, leading to a cascade of signal or the transfer of information.

Signal transduction pathways are multifaceted, engaging a huge number of molecules at any one time and all having very complex interactions. These processes vary in time with some being rapid, lasting in the order of milliseconds in the case of ion flux (Dupont, 1984), or minutes for the activation of protein- and lipid-mediated kinase cascades (Felix *et al.*, 1989; Martiel and Goldbeter, 1981; Sun *et al.*, 2005; Tertyshnikova and Fein, 1998), or a period of several minutes in microtubule polymerisation (Carlier *et al.*, 1987) whilst some can take hours, and even days - as in the case with gene expression (Menegazzi *et al.*, 1994; Pigolotti *et al.*, 2007). Included in the complex matrix of interactions is the self regulation of most molecules by positive and negative feedback mechanisms involving the DNA. The DNA in turn reacts to these changes using its vast database of codes which are pre-programmed on how to respond appropriately for that situation to generate the required proteins. It is such feedback mechanisms that, to an extent, lead to the oscillatory behaviour of the molecules which ultimately adds to the dynamics of the cell. This is just one aspect of cellular activity (amongst many others) which contributes to the dynamic nature of cells.

There are many different types of signal transduction pathways. Some are involved in growth, division of the cell, energy conversion, metabolism, whilst others are involved in the protection of the cell by getting rid of waste products, and survival of the cell via apoptosis. All of these processes are directly or indirectly driven via signalling.

The whole process of signal transduction is a well orchestrated and harmonised machine. The proteins involved are often precisely controlled. The most widespread and simple type of control is the activation and deactivation of proteins. A frequent way of achieving this is by phosphorylation, a rapid and readily reversible mechanism.

Gene transcription can be thought of as the final step in the signal transduction process. The molecules directly involved in gene transcription are called transcription factors. Again, changes in gene transcription lead to dynamic behaviour, which in turn are manipulated by the activity of transcription factors. A major part of this regulation is influenced by the mechanism of phosphorylation. However, phosphorylation not only regulates transcription factors, but is also involved in energy production, and the control of various signal transduction molecules. A simplified illustration of signal transduction is shown in figure 1.1.

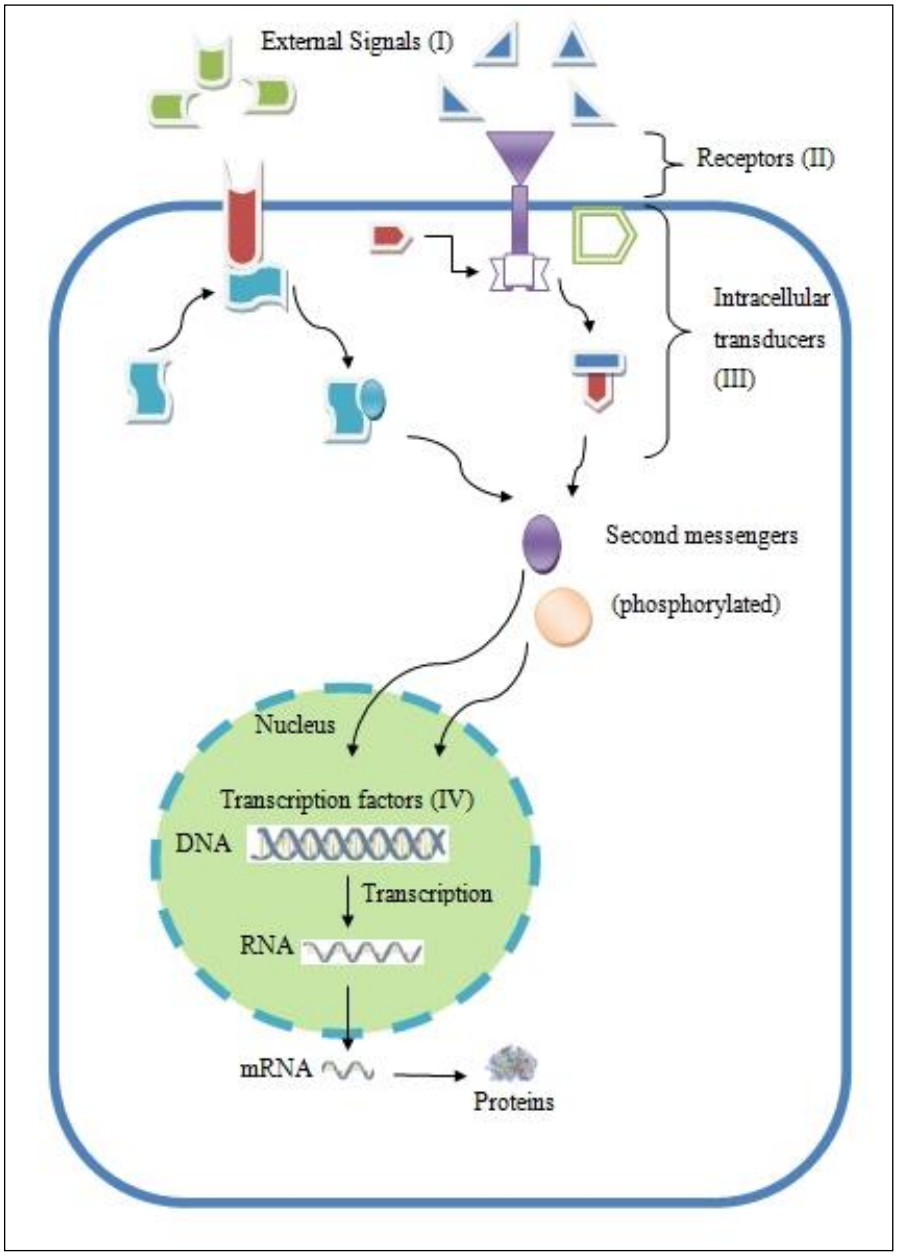



Figure 1.1 An illustration of the sequence of events in a signal transduction pathway adapted from *Cancer Explained* (Leong and Leong, 1989)

An example of the general steps involved in the signal transduction process. Components involved are extra cellular factors (I), their receptors (II), transducers (III), and intranuclear factors (IV) (Leong and Leong, 1989).

### 1.1.3. Phosphorylation and dephosphorylation of proteins

Protein phosphorylation involves the transfer of the terminal phosphate group from a nucleotide (usually ATP) to an amino acid in a protein. Phosphorylation and dephosphorylation is one of the most common types of reversible covalent modification mechanisms that is known to regulate biological activity (Krebs and Beavo, 1979). Phosphorylation and dephosphorylation reactions are catalysed by protein kinases and phosphoprotein phosphatases, respectively. They can be depicted as follows:

$$\text{ATP } Mg^{2+} + \text{protein} \longrightarrow \text{protein-P} + \text{ADP } Mg^{2+}$$

$$\text{protein-nP} + nH_2O \longrightarrow \text{protein} + nP$$

(P = phosphate group)

The phosphorylation and dephosphorylation of proteins was first described by Edmond H. Fischer and Edwin G. Krebs over four decades ago, and their determining contributions to research in this area was recognised in 1992 with the Nobel Prize (Raju, 2000). The process of reversible phosphorylation is now recognised as a universal dynamic mechanism for the posttranslational control of protein function. All manner of physiological and biochemical processes are subject to this type of regulation, including, transcription, translation, ion transport, cell structure and motility, mitosis, and cell cycle progression. Protein kinases and their complementary phosphoprotein phosphatases make ideal systems for the generation and transmission of signals in the cell (Jin and Pawson, 2012). In addition, protein kinases and phosphatases act catalytically, thus providing direct signal amplification. They are also pleiotropic, each type of kinase or phosphatase can act on several different protein substrates. It was initially thought that all the control in a phosphorylation-based system could simply be exerted at the level of protein

kinases, with the phosphatases acting at a constant rate. However, it has subsequently been shown that the activities of protein phosphatases are regulated as well (Hunter, 1987).

Reversible protein phosphorylation is a critical component of the signal transduction process where extracellular signals regulate homeostasis and cell growth (Barford, 1996). These effectors act by modulating protein kinases and phosphatases, with the addition or removal of a phosphate molecule that causes the conformational change in regulated proteins thus altering the protein's biological properties (Hunter, 1987). The level of phosphorylation at any instant as a result reflects the relative activities of the protein kinases and phosphatases that catalyse these interconversion processes. The phosphatidylinositol 3-kinase (PI3K) pathway is an important pathway that highlights the role of kinases and phosphatases. This pathway has a pivotal role in the maintenance of processes such as cell growth, proliferation, survival, and metabolism in all cells and tissues (Zhang and Claret, 2012). Often a dysregulation of this signalling pathway occurs in patients with many cancers and other disorders. The disruption of the pathway is primarily caused by loss of function of all negative controllers known as inositol polyphosphate phosphatases and phosphoprotein phosphatases (Zhang and Claret, 2012). The possibility that phosphatases may be tumour suppressors has attracted much interest in the regulation of these enzymes. A description of protein phosphatases is documented in section 1.2.

### 1.1.4. Disruption of dynamic systems

The ability to regulate gene expression in response to the environment is of vital importance during cell growth and differentiation. In many instances, alterations in

patterns of gene expression are usually the endpoint of a signal triggered by agents such as growth factors. Hence, signal pathways result in the activation or inactivation of key molecules that regulate transcription (Mitchell and Tjian, 1989) which results in specific changes in gene transcription. These changing patterns provide characteristic behaviour which can be studied and recorded. Often, a drastic change in these patterns reflects the appearance of certain diseases which in themselves have a characteristic pattern. By studying these different patterns and behaviours we could ultimately direct the type of activities we want and in the case of disease hope to cure it.

#### 1.1.4.1. Models of disease

The normal individual displays a complex mosaic of rhythms in the various body systems. These rhythms rarely display absolute periodicity. Indeed, quantitative measurements of such rhythms as the heart rate and respiration frequently reveal much greater fluctuations in these systems than might be naively expected. It is known that numerous pathologies are readily identifiable by abnormal rhythmicities.

The human body is a spatially and temporally complex organised unit. In many different diseases, the normal organisation breaks down and is replaced by some abnormal dynamics. With the current rate of improvement in technology, in the near future it will be possible to utilise principles and concepts from biological and mathematical models of dynamic studies for diagnosing disease with the rationale to design better therapies.

The signature of a dynamic disease is a marked change in the dynamics of some variable. Three types of qualitative changes in dynamics are possible and all have been observed: (1) variables that are constant or undergoing relatively small-amplitude "random" fluctuations can develop large-amplitude oscillations that may be more or less regular (Glass and Mackey, 1988). Thus, there may be the appearance of a regular oscillation in a physiological control system not normally characterised by rhythmic processes; (2) new periodicities can arise in an already periodic process; and (3) rhythmic processes can disappear and be replaced by relatively constant dynamics or by aperiodic dynamics (Glass and Mackey, 1988).

The notion that it is possible to formulate mathematical models that capture qualitative characteristics of human disease is certainly not new (Glass and Mackey, 1988). Studies in which mathematical models for biological systems have been proposed is steadily increasing (Bowsher *et al.*, 2013; Kirk *et al.*, 2013; Nomura and Okada-Hatakeyama, 2013; Sadot *et al.*, 2013). In the mathematical model often the identification of bifurcations reflects transformed dynamics (Bani-Yaghoub and Amundsen, 2008; Glass and Mackey, 1988; Mackey and Glass, 1977; Villasana and Radunskaya, 2003) for example, the fluctuation in peripheral white blood cell counts in chronic granulocytic leukaemia (Gatti *et al.*, 1973). The great advantage in working with a model for dynamic disease is that it is possible to make systematic manipulations on the model without affecting live subjects.

A potential pitfall in models for dynamic diseases is that the model prediction might not always reflect the true situation. For example, models showing altered dynamics in disease may mimic those observed clinically but in the case of the clinical condition this behaviour might not reflect disease. Glass and Mackey explain that a possible reason for this could be that the types of bifurcation from stable or oscillating states are often limited and it may be possible to establish qualitatively similar dynamics in many different ways (Glass and Mackey, 1988). Despite this,

combined systematic experimental and theoretical work on biological models of dynamical disease is essential. Even if the particular model is shown to be inappropriate as a model for a particular disease, the careful working-out of the theory for bifurcations in dynamics as a function of system parameters is bound to be of interest (Glass and Mackey, 1988). Unfortunately, the current structuring of most institutions of higher learning combined with the inadequate training of both theoretician and experimentalists and the current nature of research support conspire to make the requisite interdisciplinary studies logistically difficult to design, implement, and complete (Glass and Mackey, 1988).

A further target to aim for in the application of nonlinear dynamics to biochemistry is to extend these practical techniques for the development of rational therapeutic strategies in treating diagnosed diseases. Very few research initiatives have been made in this direction; one such study was done by Mackey in 1978 (Mackey, 1978). It is hoped that by extending these studies, using the tools of nonlinear dynamics, a better understanding of various diseases will be obtained, especially with the ubiquitous first world disease cancer.

#### 1.1.4.2. Cancer

Occasionally, the exquisite controls that regulate cell multiplication break down. The cell begins to grow and divide uncontrollably even though the body has no need for further cells of its type. When the descendants of such a cell inherit the propensity to grow without responding to regulation, the result is a clump of cells able to expand indefinitely. Ultimately, a mass called a tumour may be formed by this clone of unwanted cells.

Often, this process of genetic change arises due to mutations taking place in the DNA. Mutations arise as a result of a drastic change in the internal or external environment of the cell, an example being the accumulation of waste products like free radicals. The greater the exposure to these extreme stimuli, the greater the number of mutations. In most cases, the mutagen dictates the type of mutation that will arise. In the case of cancer, the mutation has to take place at a critical spot or at a few critical spots on a gene.

From the evolutionary perspective, mutations provide a mechanism for cells to adapt to their environment. The rate of mutation in humans is extremely low and the chance of a gene mutating is minute due to evolution developing excellent repair mechanisms; an adaptive strategy to improve the procreation process.

The majority of mutations occur at random sites on the DNA. Most of these arise at non-critical coding sites (i.e. an alteration in the gene at this site does not affect the survival and functionality of the cell in any significant way) and are ignored; often in these cases the cell dies off unnoticed. On the other hand, mutations might occur at extremely critical locations that result in adverse cellular changes which eventually hasten to cell death. In between these two extremes, there are mutations that cause altered cellular functioning with no cell death. The DNA in these cells, over a period, undergoes a series of minute changes. These cells continue to reproduce and become pernicious free agents that multiply out of control, spreading through the body, and ultimately killing their host. This phenomenon has been characterised as the disease called cancer.

The development of cancer is a multi-step process in which oncogenes are activated and tumour suppressor genes are inactivated (Chang *et al.*, 1995), and in which inhibition of apoptosis may occur. The development of a fully malignant tumour appears to involve multiple stages or steps; the activation or altered expression of proto-oncogenes and the loss or inactivation of tumour suppressor genes, which control normal cellular activity. Inactivation of a tumour suppressor gene appears to involve first a mutational event that inactivates one allele, followed by a deletion during mitosis that results in homozygosity. A normal cell has multiple independent mechanisms that regulate its growth and differentiation potential. Hence, in order to override these control mechanisms several separate events are needed as well as the induction of the other aspects of the transformed phenotype. The activation of some of these genes to express their oncogenic potential can occur in several ways: point mutation, chromosome rearrangement, gene amplification or viral insertion.

As human longevity continues to increase, diseases that were moderately unimportant are assuming an ever-greater importance (Basilico and Newport, 1988). Cancer is one of these diseases and thus impetus to develop successful strategies for tumour therapy is growing. One aspect of cancer research that is growing phenomenally is the study of tumour suppressor genes (TSGs).

TSGs are genes that function to prevent neoplastic transformation. These genes may counteract the function of oncogenes (Thompson, 1995) by slowing down cell division or by initiating apoptosis, thus killing the cell. Some of the main functions of TSG proteins can be categorised as follows:

1. They inhibit cell division by suppressing genes involved in the cell cycle (Sherr, 2004).

2. They monitor the cell cycle and any DNA damage that might occur. If there is DNA damage, the cell should not be allowed to divide. They stop the cell cycle and allow DNA repair to take place, once the DNA is repaired, the cell cycle can continue (Sherr, 2004).

3. Apoptosis is initiated if the damaged DNA cannot be repaired (Sherr, 2004).

4. They prevent metastasis by activating proteins involved in cell adhesion. These proteins are called metastasis suppressors (Hirohashi and Kanai, 2003; Yoshida *et al.*, 2000).

5. They are involved in DNA repair (Markowitz, 2000).

An important TSG is *TP53* which codes for the tumour-suppressor protein p53. This protein will be discussed in detail in section 1.3 and provide greater insight into the workings of TSGs.

Cancer can be described as a process of continuous cell division. In a normal cell, the cell cycle is generally divided into the following groups; growth in mass ($G_1$) is followed by DNA replication (S-phase), synthesis of proteins required for mitosis ($G_2$), mitosis and cell division (M-phase) and return to $G_1$. Interphase is composed of $G_1$, S and $G_2$ phases and comprises over 90% of the cell cycle time in proliferating mammalian cells. Mitosis itself usually lasts for 1 to 2 hours.

In certain circumstances, the circular description of the cell cycle is not always obeyed and some differentiated cells exit the cell cycle, in the early $G_1$ phase, into a quiescent phase known as $G_0$. During this phase, the cells usually become differentiated. In the late $G_1$ phase there is another pause called R-point which if

passed results in cellular commitment to DNA replication. In addition, after treatment of proliferating cells with infrared rays or chemotherapeutic drugs, further DNA synthesis or division is prevented by the execution of these 'cell cycle checkpoints'. Both normal and cancer cells possess checkpoint controls which prevent the initiation of key cell cycle events pending the completion of critical preceding events (Hartwell, 1992; Hartwell and Weinert, 1989; Murray, 1992; Verdugo *et al.*, 2013). At these checkpoints, cells may become committed to enter a quiescent state, differentiate, senesce, resume cycling, or undergo apoptosis depending on prevailing environmental conditions (Verdugo *et al.*, 2013).

As the molecular mechanisms that control these events are dissected, it is becoming clear that tumour cells are defective in many checkpoints, and in some circumstances, events are completed in the incorrect order, causing genomic instability or cell death (Hartwell, 1992). Therefore, these weak checkpoint controls in malignant cells may provide opportunities for new therapeutic strategies.

The finer details between signal transduction and the cell cycle are still uncertain, however, one thing is certain, a critical mechanism in the regulation of all phases of the cell cycle is protein phosphorylation/dephosphorylation. A variety of different kinases and phosphatases with different amino acid and protein specificities control the activation and inactivation of cell-cycle dependent genes. The principle mechanism relating signal transduction and the cell cycle is phosphorylation and dephosphorylation of various transcription factors like *p53*. The phosphorylation of cyclin (proteins that are critical in the passage of a cell through the cell cycle) by a variety of cyclin-dependent kinases (cdks) results in varying levels of phosphorylation during different stages of the cell cycle (Gerard and Goldbeter, 2012b). On the other side of the coin there is an opposed behaviour where the removal of phosphate groups from cyclins by the phosphatases (example PP1 and PP2A) results in cell cycle inhibition (Jin and Pawson, 2012; Mochida and Hunt,

2012). This behaviour coupled with the rapid synthesis and degradation of cyclins and other cell cycle-dependent proteins during the cycle, allows the cell cycle to take place in a reproducible manner. It has been shown by Hartwell and Kastan in 1994 that excessive production of any cyclin or cdk or an absence of one of the transcription factors leads to cancer (Hartwell and Kastan, 1994).

## 1.2. Protein phosphatases

### 1.2.1. Classification of protein phosphatases

Until 1983, the serine/threonine-specific protein phosphatases were not formally classified. At this time it was suggested that most, if not all, of the protein phosphatases in the literature could be divided into four classes according to their catalytic subunits which had broad and overlapping substrate specificities (Ingebritsen and Cohen, 1983a; Ingebritsen and Cohen, 1983b; Ingebritsen *et al.*, 1983). Various simple criteria were introduced to distinguish these proteins. The enzymes were subdivided into two groups (Type 1 and Type 2) depending on whether they dephosphorylated the β subunit of phosphorylase kinase specifically and were inhibited by nanomolar concentrations of two small heat- and acid-stable proteins, termed inhibitor-1 (I-1) and inhibitor-2 (I-2) (Type 1, protein phosphatase-1), or whether they dephosphorylated the α subunit of phosphorylase kinase preferentially and were insensitive to I-1 and I-2 (Type 2) (Cohen, 1989; Eto and Brautigan, 2012). Type 2 phosphatases were further subclassified into three distinct enzymes, 2A, 2B, and 2C (PP2A, PP2B, and PP2C) due to their dependence on divalent cations. PP2B and PP2C had absolute requirements for $Ca^{2+}$ and $Mg^{2+}$, respectively (Ingebritsen and Cohen, 1983a; Ingebritsen and Cohen, 1983b), while PP2A, like protein phosphatase 1 (PP1), was active toward most substrates in the

absence of divalent cations (Cohen, 1989). Since that time many other useful criteria were introduced for categorising protein phosphatases.

Eukaryotic protein phosphatases (PPs) are structurally and functionally diverse enzymes that are represented by three main classes based on their sequence, structure and catalytic function. One of these, the phosphoprotein phosphatase (PPP) and the protein phosphatase $Mg^{2+}$ or $Mn^{2+}$ dependent (PPM) families, dephosphorylate phosphoserine and phosphothreonine residues (Guan and Dixon, 1991; Schubert *et al.*, 1995) and form the most abundant serine/threonine phosphatases. These two families are unrelated in sequence, with the PPP family having PP1, PP2A, PP2B (or PP3), PP4, PP5, PP6 and PP7 and the PPM family, PP2C (Das *et al.*, 1996). The second group of PPs are the protein tyrosine phosphatases (PTPs) which dephosphorylate phosphotyrosine and have a catalytic signature of $CX_5R$. This group has a domain structure and substrate preference which is varied and some of its members can dephosphorylate complex carbohydrates, mRNA and phosphoinositides (Alonso *et al.*, 2004; Moorhead *et al.*, 2007; Tonks, 2006). The third group, classified as the aspartate-based phosphatases, consists of enzymes involving an aspartic acid signature for catalysis (Moorhead *et al.*, 2009). Within each family of phosphatase, the catalytic domains are highly conserved, with structural and functional diversity endowed by the regulatory domains and subunits.

### 1.2.2. Protein serine/threonine phosphatases: the PPP family

The PPP family of phosphatases includes PP1, PP2A and PP2B. These are metalloenzymes with a central β-sandwich of 11 β-strands surrounded by α-helices and a three-stranded β-sheet (Goldberg *et al.*, 1995). The two metal ions ($Fe^{2+}$ and

$Mn^{2+}$ in PP1, $Fe^{2+}$ and $Zn^{2+}$ in PP2B) form a binuclear metal centre, which is coordinated by invariant residues emanating from the carboxyl terminus of the central β-strands of the β-sandwich (Goldberg *et al.*, 1995). These metal-coordinating residues occur within five sequence motifs dispersed throughout the sequence, which are characteristic of a family of enzymes involved in phosphate ester hydrolysis. In the PPPs, the dephosphorylation reaction is catalysed by a metal-activated water molecule, which attacks the phosphorous atom of the substrate in a single-step nucleophilic mechanism.

### 1.2.3. Physiological functions of phosphoprotein phosphatases

Protein serine/threonine phosphatases of the PPP family have numerous roles in mediating intracellular signalling processes (Cohen, 1989; Wera and Hemmings, 1995). PP1 and PP2A are specifically and potently inhibited by a variety of naturally occurring toxins, such as okadaic acid (Hata *et al.*, 2013), a diarrhetic shellfish poison and strong tumour promoter, and microcystin, a liver toxin produced by blue-green algae (MacKintosh and MacKintosh, 1994). On the other hand PP2B is only poorly inhibited by these toxins. PP2B is the immunosuppressive target of FK506 and cyclosporin, which mainly associate with the cellular binding proteins, cis-trans peptidyl prolyl isomerases FKBP12 and cyclophilin, respectively (Liu *et al.*, 1991).

PP1 and PP2A are responsible for regulating many diverse cellular functions, including glycogen metabolism, muscle contraction, control of cell cycle and RNA splicing. Due to the structural complexity of the catalytic subunits of these proteins, a relatively small number is needed to carry out these functions. The specificity of these proteins *in vivo* is achieved by altering the selectivity of the enzyme towards a

particular substrate or by targeting the phosphatase to the subcellular location of its substrates (Barford, 1996). This is controlled by regulatory or targeting subunits that bind to the phosphatase catalytic subunits (Faux and Scott, 1996; Hubbard and Cohen, 1993). The activity of the PPPs is modulated by the regulatory subunits using reversible protein phosphorylation and second messengers (Faux and Scott, 1996; Hubbard and Cohen, 1993).

### 1.2.4. Protein phosphatase 1

PP1 was discovered in the early 1940s as the first interconverting enzyme that inactivates phosphorylase *a* (Cori and Cori, 1945; Cori and Green, 1943), although it took another two decades to realise that it was a phosphatase (Fischer and Brautigan, 1982). During the 1970s, much energy went into purifying phosphatases and in 1983; these mammalian phosphatases were classified according to their biochemical and enzymatic properties (Ingebritsen and Cohen, 1983b).

PP1 is one of the most highly conserved eukaryotic proteins and this reflects the fundamental role it plays in cellular processes. This protein is a key enzyme in the protein phosphorylation/dephosphorylation apparatus of the cell. PP1 initially was identified as a regulatory enzyme of glycogen metabolism and as the mediator of insulin-stimulated dephosphorylation and activation of glycogen synthase. PP1 has since been shown to be involved in the co-regulation of mitosis and chromosome segregation. It is also involved in muscle contraction, cell progression, neuronal activities, splicing of RNA, mitosis, cell division, apoptosis, protein synthesis and regulation of membrane receptor and channels (Fong *et al.*, 2000; Kurimchak and Grana, 2012b; Tournebize *et al.*, 1997; Wurzenberger and Gerlich, 2011).

PP1 has three genes coding for four distinct isoforms designated α, γ1, γ2, and δ (this has now been renamed 1β) (Berndt, 1999). Their primary structures are identical, except for a few N-terminal residues and the extreme C-terminal region (Sasaki *et al.*, 1990). All isoforms are ubiquitously expressed except for PP1γ2, an alternative-splicing product that is found only in testes (Shima *et al.*, 1993). While PP1β is part of the glycogen particle (Shima *et al.*, 1990), PP1α, along with PP1γ1, is over-expressed in hepatomas and other malignant tumours, suggesting that these two isozymes might contribute to tumourigenesis (Saadat *et al.*, 1994; Sasaki *et al.*, 1990; Sogawa *et al.*, 1994a; Sogawa *et al.*, 1994b; Sogawa *et al.*, 1995; Yamada *et al.*, 1994). The crystal structures of two isoforms of PP1 are shown in figure 1.2. These structures are complexed with microcystin LR which is a potent inhibitor of PP1.

Barker *et al*. (Barker *et al.*, 1990) isolated a cDNA encoding 1 isoform (PP1α) of the catalytic subunit of human protein phosphatase 1 and used it to map the gene (PPP1A) to 11q13 by Southern analysis of somatic cell hybrids and by *in situ* hybridisation. Because of evidence that PP1α is a tumour suppressor and because of the assignment to 11q13, PPP1A is a candidate gene for type I multiple endocrine neoplasia, which maps to the same area. Translocations involving breakpoints at 11q13 have been observed in lymphomas, chronic lymphocytic leukaemia of the B-cell type, and multiple myeloma. Richard *et al*. (Richard *et al.*, 1991) described a high resolution radiation hybrid map of 11q12-q13, which placed the PPP1A locus at the extreme end of a cluster of more than 12 genes with complement component 1 inhibitor (C1NH) at the other end and with glutathione-s-transferase 3 (GST3) as its closest neighbour in the linear array. Using fluorescence *in situ* hybridisation, Saadat *et al*. (Saadat *et al.*, 1995) mapped PPP1CA to human 11q13, rat 1q43, and mouse chromosome band 7E3-F2. These results indicated that PPP1CA is a member of a group of genes showing homology of synteny.

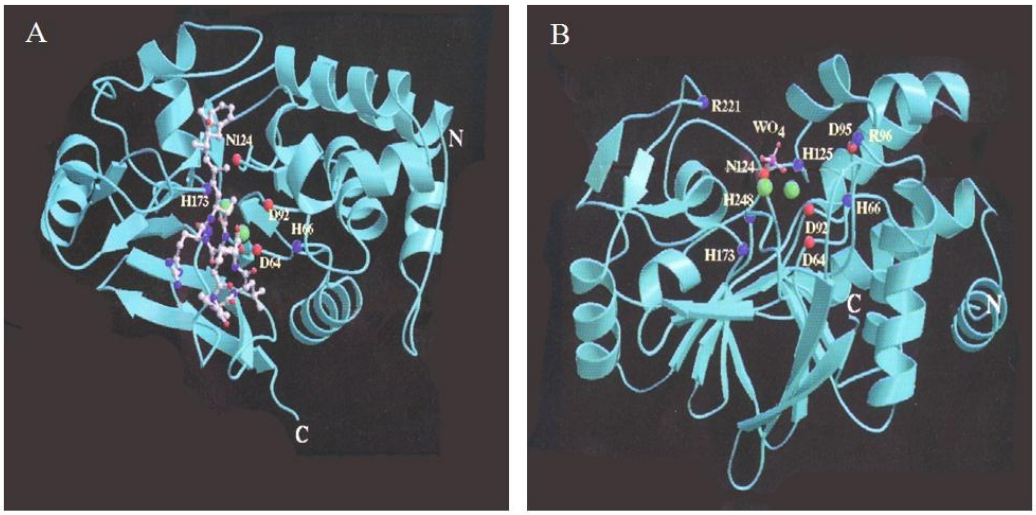



Figure 1.2 The crystal structure of two isoforms of protein phosphatase 1 obtained from the research collaboratory for structural bioinformatics protein data bank (Egloff *et al.*, 1995; Goldberg *et al.*, 1995)

The crystal structure of protein phosphatase 1α (A) complexed with microcystin LR. This view is perpendicular to that of B. Microcystin LR, a potent inhibitor of PP1, interacts with the hydrophobic groove, the metal sites and to Cys273 (Goldberg *et al.*, 1995). Figure 1.2B is a crystal structure of protein phosphatase 1γ, showing a perpendicular viewpoint to that of A. This structure shows two divalent metal ions (shown in green) within a binuclear metal site are coordinated by residues from the central β-strands of the β-sandwich whose Cα positions are shown as red and blue spheres. A tungsten ion (an analogue of phosphate) is bound to the catalytic site and directly coordinates the metal ions (Egloff *et al.*, 1995). Images were obtained from the research collaboratory for structural bioinformatics (RCSB) protein data bank (PDB) (www.pdb.org) (Berman *et al.*, 2000) with PDB ID 1FJM (Goldberg *et al.*, 1995).

PP1 is the target for a number of environmental toxins/tumour promoters such as okadaic acid and microcystin-LR, which inhibit its dephosphorylation activity. Okadaic acid produced by marine dinoflagellates is a major cause of diarrhetic shellfish poisoning, and microcystins from freshwater cyanobacteria are a worldwide threat to the health of animals and humans.

The free catalytic subunit of PP1 is a 37 kDa protein. This protein has a broad substrate specificity overlapping with that of other phosphatases *in vitro*. In the cell, PP1 (as well as PP2A) is often complexed to regulatory subunits that modify their activity or target them to particular locales (Berndt, 1999). The first regulatory subunits were discovered by conventional protein purification and became known as inhibitor-1 and inhibitor-2 (Huang and Glinsmann, 1976). Meanwhile, novel approaches including the yeast two-hybrid screen and affinity chromatography on immobilised microcystin (Campos *et al.*, 1996) have identified a growing number of proteins that appear to specifically bind PP1 (Lee *et al.*, 1999; Oliver and Shenolikar, 1998).

The existence in most cells of three PP1 isozymes and close to twenty regulatory subunits means that there are - at least in theory - sixty or so distinct forms of PP1 alone (Cohen, 2002). The situation with PP2A is similar if not more complicated by the fact that this enzyme mostly occurs in heterotrimeric form. Even conceding that some cell types might not express all of the putative PP1 binding proteins, this still means that various PP1 heterodimers are probably involved in distinct cellular processes. The free PP1 in the cell has no specificity of substrates. Rather, its specificity is closely controlled by its interaction with more than 200 known targeting proteins (Bollen *et al.*, 2010; Watanabe *et al.*, 2001). These targeting proteins first localise PP1 to distinct regions of the cell and secondly, they modulate substrate specificity of PP1 (Choy *et al.*, 2012). This modulation can be achieved by PP1-specific inhibitor proteins, e.g. I-2 (Alessi *et al.*, 1993; Huang *et al.*, 1999) and

DARPP-32 (dopamine- and cAMP-regulated phosphoprotein of 32 kDa) (Greengard *et al.*, 1999; Hemmings *et al.*, 1984; Kwon *et al.*, 1997), which bind and block the PP1 active site thus inactivating PP1 to all substrates. On the other hand, regulatory proteins like spinophilin and MYPT1 (myosin phosphatase target subunit 1) bind to PP1 forming a highly stable holoenzyme which regulates PP1 specificity via a variety of ways (Choy *et al.*, 2012).

For the control of skeletal muscle glycogen metabolism and acto-myosin contraction, the catalytic subunit of PP1 binds to $G_m$ and $G_L$, and $M_{110}$ and $M_{21}$, respectively (Faux and Scott, 1996; Hubbard and Cohen, 1993). Other regulatory subunits are p53 binding protein 2 (p53BP2), a kinesin-related protein, ribosomal proteins L5 and RIPP-1, an RNA splicing factor, a nuclear inhibitor of PP1 (NIPP-1), the retinoblastoma (Rb) gene product, the two cytosolic proteins, inhibitor-1 and inhibitor-2, and the yeast sds22 gene product which regulates PP1 during mitosis.

The first set of evidence linking PP1 and the cell cycle came from work done in 1989. These studies revealed that in *Aspergillus nidulans* and *S. pombe*, proteins that are over 80% identical to mammalian PP1, are required for the separation of daughter nuclei and completion of anaphase or chromosome segregation (Booher *et al.*, 1989; Doonan and Morris, 1989; Ohkura *et al.*, 1989). As in fungi and fission yeast, PP1 was found to be essential for mitosis in *Drosophila* (Axton *et al.*, 1990). Supporting the notion that cell cycle-regulatory mechanisms are conserved across distant species was the finding that PP1α can rescue the *Aspergillus* mutant *bim*G (Doonan *et al.*, 1991). These data were confirmed biochemically in vertebrate cells: Microinjection of neutralising PP1 antibodies into early mitotic cells arrests them in metaphase (Fernandez *et al.*, 1992). The same study also showed that PP1 translocates from the cytoplasm to the nucleus during $G_2$ (Fernandez *et al.*, 1992). One crucial mitotic function of PP1 is to bring about the necessary inhibition of cdk1 by dephosphorylating threonine 161 in cdk1 (Lorca *et al.*, 1992). Another

important role of PP1 is to dephosphorylate the nuclear cdk1 substrate lamin (Enoch *et al.*, 1991), thereby permitting the repolymerisation that is necessary to rebuild the nuclear envelope at the onset of cytokinesis (Thompson *et al.*, 1997). An important substrate for PP1, at least in mammals, is the Rb tumour suppressor protein that is dephosphorylated by a type-1 phosphatase activity during mitosis (Ludlow *et al.*, 1993).

The full details of the role of PP1 in the cell cycle are still being decrypted. It is believed that PP1 promotes $G_1$ entry and thereby contributes to cellular integrity. These proteins are also involved in maintaining cells in $G_1$ unless the conditions for commitment to DNA replication are met. Research done on PP1 and its role in the cell cycle has been directed mainly from the viewpoint of the Rb protein because of the interactions between these two molecules.

Rb is a tumour suppressor protein, which is phosphorylated in a cell cycle-dependent manner by cdks. The active form of Rb (unphosphorylated form) normally causes inhibition of cell cycle progression. Rb is the key player in regulating passage through the R point, and there is accumulated evidence, suggesting that one or more members of the Rb-pathway ($p16^{Ink4}$, cdk4/cyclin D, Rb itself) are mutated in most human cancer (Ludlow *et al.*, 1993). PP1 is responsible for keeping the distinct phosphorylation pattern of Rb (active, unphosphorylated Rb during $G_1$, and hyper-phosphorylated Rb during late $G_1$ through M-phase) and thus plays an important role in $G_1$/S regulation (Levine, 1997). Rb binds and hinders transcription factors of the E2F family, which are dimers of E2F and DP protein. Normally the E2 promoter-binding-protein-dimerisation partners (E2F-DP) encourage a cell into the S phase (Kalendo and Kuzin, 1966; Kolupaeva and Janssens, 2013; Kurimchak and Grana, 2012b; Ranjan and Heintz, 2006). When Rb binds to this complex it acts as a growth suppressor and halts the cell cycle (Munger and Howley, 2002; Ranjan and Heintz, 2006). This

complex also attracts a histone deacetylase (HDAC) protein to the chromatin, inhibiting the transcription of S phase promoting factors and thus repressing DNA synthesis. A diagrammatic view of the action of PP1 on the cell cycle can be seen in figure 1.3.

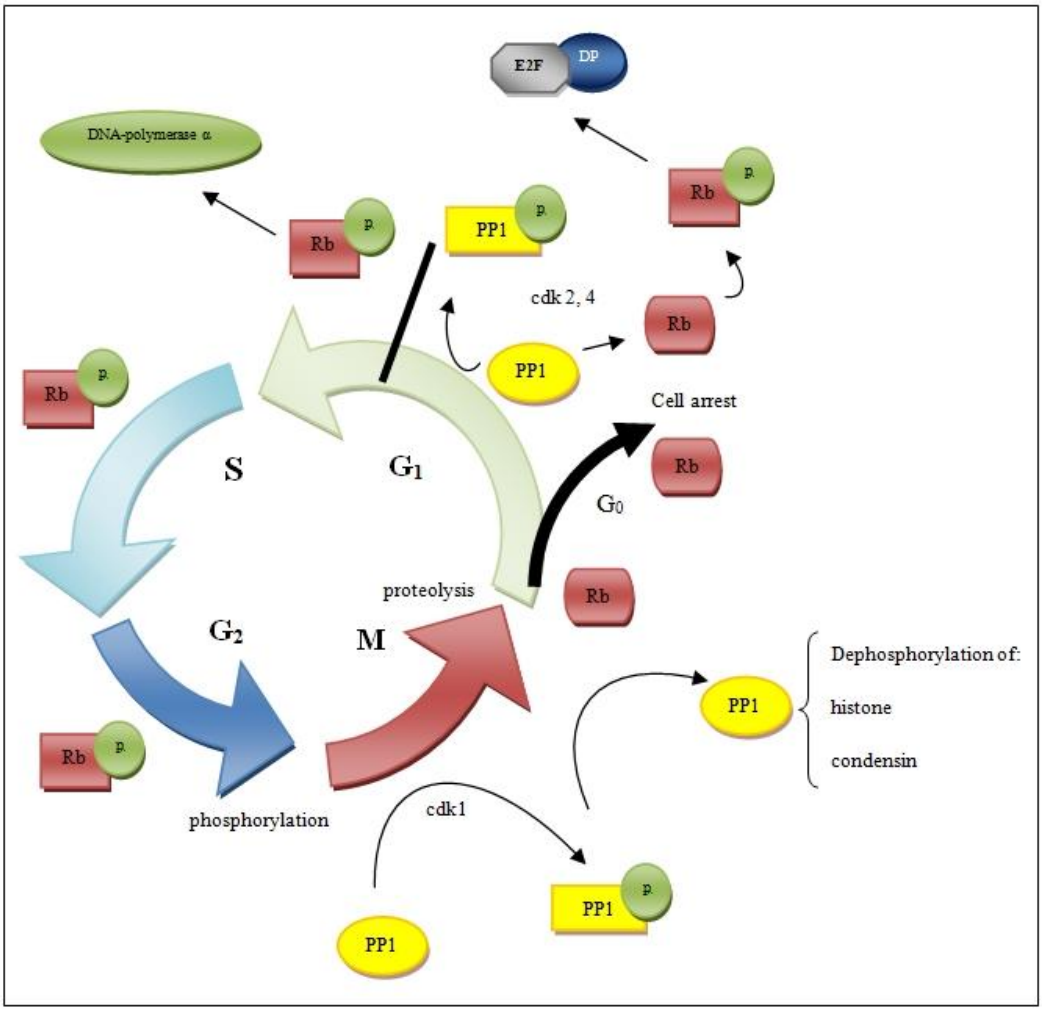



Figure 1.3 The influence of PP1 on the cell cycle adapted from (Berndt, 1999)

A simplified diagram of the cell cycle showing the sites of action of PP1 and Rb. Reversible phosphorylation and proteolysis cooperate to drive cell cycle progression. PP1 is needed to get into and remain in $G_1$. At $G_2$/M, PP1 undergoes inhibitory phosphorylation by cdk1; later PP1 is required to exit from mitosis and enter $G_1$. Dephosphorylation of several other proteins including Rb is also needed for this process.

### 1.2.5. Protein phosphatase 2A

As was the case with PP1, PP2A also plays an important role in the cell. Traditionally it was believed that this protein together with the other phosphatases was involved in reversing the effects of phosphorylation created by kinases. This discovery has now been altered in that the major substrates for the PP2A protein are the protein kinases themselves.

One of the outstanding features of PP2A is that its catalytic subunit can bind to a wide variety of regulatory and targeting subunits. An important molecule that this protein complexes with is a 65 kDa regulatory subunit called PR65 (Millward *et al.*, 1999). This subunit's primary function is to recruit further regulatory subunits (Millward *et al.*, 1999). There are three gene families, which encode subunits similar to PR65; these are PR55, PR61 and PR72 (Millward *et al.*, 1999). Each of these subunits has multiple isoforms and together with the catalytic subunit of PP2A, they form a range of trimeric holoenzymes that allow the enzyme to dephosphorylate an array of distinct substrates.

Several examples of what makes PP2A such an important protein in the cell have been put forward in the review written by Millward, *et al*. (Millward *et al.*, 1999). This importance is emphasised by the fact that PP2A forms a target for viruses and toxins. A listing of these interactions and toxins is depicted in table 1.2 and 1.3, respectively. In order for viruses to replicate and survive in a host cell, they must weaken the signal transduction machinery of the cell (Millward *et al.*, 1999). The most economical way for these viruses to achieve this is to target molecules that have central roles in cellular signal transduction and replication. This scenario is highlighted by the interaction of various virus particles with the PP2A protein (table

1.2). Regulatory B-subunits individually assemble with a core heterodimer consisting of PP2A catalytic subunit and a 65 kDa A-subunit (Groves *et al.*, 1999; Xu *et al.*, 2006). The core heterodimer of PP2A is also a target of tumour antigens simian virus 40 (SV40) and polyoma viruses (Ruediger *et al.*, 1992). *In vivo*, small T antigen inhibits PP2A dephosphorylation of mitogen-activated protein kinase kinase (MEK) and extracellular-signal-regulated kinase (ERK) resulting in receptor mediated activation of these kinases and cell proliferation (Kurimchak and Grana, 2012b; Sontag *et al.*, 1993; Wurzenberger and Gerlich, 2011).

*In vivo* PP2A exists as a holoenzyme in which a core heterodimer composed of the 35 kDa catalytic subunit is associated with a regulatory subunit of 65 kDa referred to as PR65 or the A-subunit. This core AC heterodimer associates with different B subunits to form the physiologically relevant ABC hetero-trimer. The B subunits are responsible for conferring the diverse properties upon the PP2A holoenzyme. The PR65 subunit appears to act as a scaffold by simultaneously interacting with the PP2A catalytic subunit and the regulatory B-subunit. The crystal structure of the PR65 regulatory subunit of PP2A is shown in figure 1.4.

The physiological functions of PP2A are diverse, ranging from regulation of signal transduction pathways including the MAP kinase pathway and the PKB pathway, to regulation of protein synthesis, the cell cycle (and substrates of the cdks) and transcription factors (Kolupaeva and Janssens, 2013; Kurimchak and Grana, 2012b; Okamoto *et al.*, 2002; Tournebize *et al.*, 1997; Virshup, 2000; Wurzenberger and Gerlich, 2011). These diverse functions are mediated by different forms of the protein in which the substrate specificity and intracellular location of the catalytic subunit is modulated by a wide variety of regulatory subunits.

Table 1.2 Virus particles that interact with PP2A taken from (Millward *et al.*, 1999)

| Virus | Type of action |
|---|---|
| SV40 small t antigen | Displaces some types of the B subunit from the PR65-C core dimer |
| Polyomavirus small t and middle T antigens | Bind to the catalytic subunit of PP2A |
| Adenovirus protein, E4orf4 | Binds to trimer PP2A through PR55α B subunit- down regulates AP-1 |
| HIV encoded proteins – NCp7 and Vpr | Binds to trimer PP2A with PR61 |

Table 1.3 A list of toxins and their source that inactivate PP2A taken from (Millward *et al.*, 1999)

| **Toxin** | **IC 50 (nM)** | **Source** |
|---|---|---|
| Okadaic acid | 0.20-2.00 | Marine sponges |
| Calyculin A | 0.30-7.30 | Marine sponges |
| Tautomycin | 10.00-23.10 | Streptomyces spiroverticillatus |
| Microcystin (hepatotoxin) | 0.04-2.00 | Cyanobacteria |
| Nodularin (hepatotoxin) | 0.03-1.00 | Cyanobacteria |
| Cantharidin | 160.00 | Blister beetles |

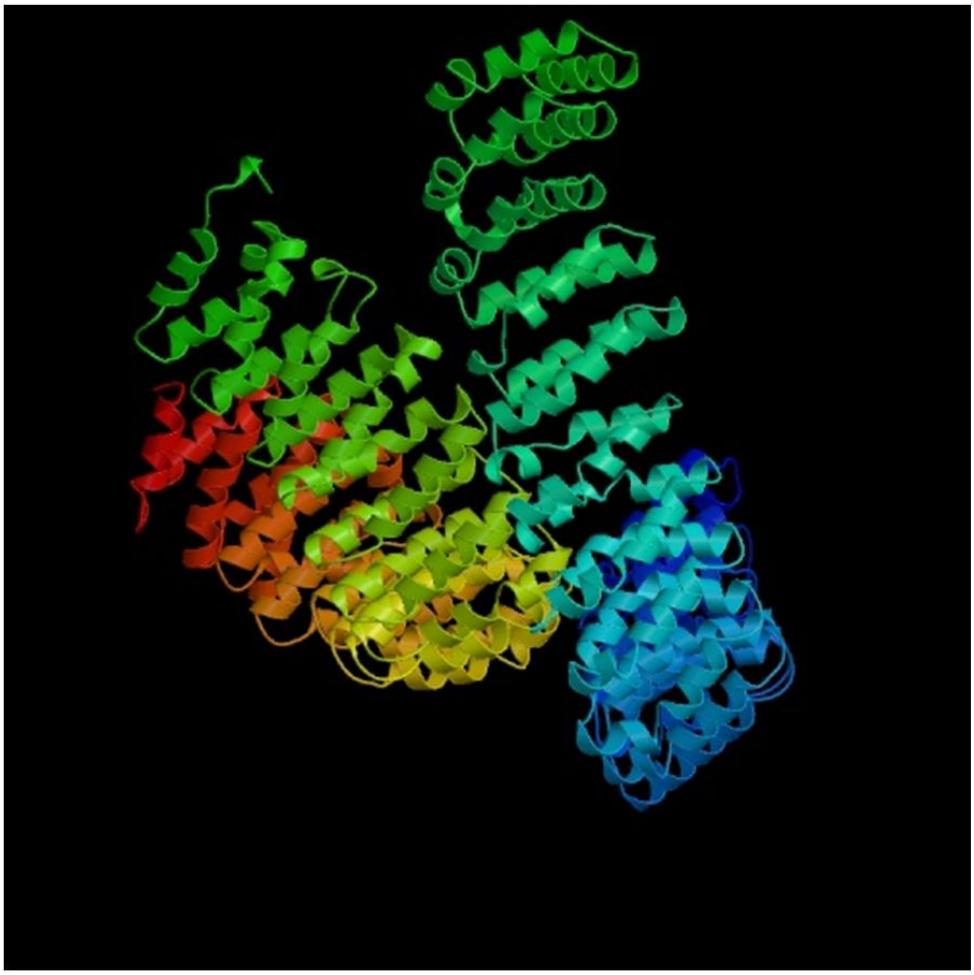


Figure 1.4 The crystal structure of the human PP2A regulatory subunit obtained from the research collaboratory for structural bioinformatics protein data bank (Groves *et al.*, 1999)

The crystal structure of the constant regulatory domain (PR65) of human PP2A, PR65Alpha, at 2.3 A resolution. The structure shows the conformation of its 15 tandemly repeated HEAT sequences, degenerate motifs of approximately 39 amino acids (Groves *et al.*, 1999). The PR65/A subunit of PP2A serves as a scaffolding molecule to coordinate the assembly of the catalytic subunit and a variable regulatory B subunit, generating functionally diverse heterotrimers (Groves *et al.*, 1999). The image was obtained from the RCSB PDB (www.pdb.org) (Berman *et al.*, 2000) of PDB ID 1B3U (Groves *et al.*, 1999).

The finding that okadaic acid is a tumour promoter, combined with the observation that this compound efficiently inhibits PP2A, has led to the suggestion that PP2A, and potentially some other phosphatases, may function as tumour suppressors (Berndt, 1999). It is thought that the tumour suppressing function could be accomplished by the enhanced dephosphorylation of activated kinase cascades, which would revert oncoprotein-activated signalling pathways back to their inactive state. As many oncogene products cause the sustained activation of growth-regulatory kinase cascades, it is a distinct possibility that increased serine/threonine protein phosphatase activity might counteract elevated levels of protein phosphorylation and block cellular transformation (Cohen and Cohen, 1989).

One of the functions of PP2A is to inhibit the entry of the cell into mitosis. This effect on mitotic entry may be explained by its regulation of the mitosis-specific form of cdk1 in mammalian cells (Fujiki and Suganuma, 1993; Yamashita *et al.*, 1990), *S. Pombe* (Kinoshita *et al.*, 1990) and *Xenopus* (Felix *et al.*, 1990). PP2A most likely inactivates cdc25p, which removes the inhibitory phosphate groups at $Thr^{14}/Tyr^{15}$ in cdk1, and activates the dual-specificity kinase Wee1/Myt1, which phosphorylates $Thr^{14}/Tyr^{15}$. The inhibition of mitotic entry by PP2A cannot be absolute otherwise cells would never be able to proliferate. In *S. cerevisiae*, the activity of PP2A towards the cdk1-regulating enzymes appears to be reduced by the PP2A-regulatory subunit Cdc55 (a homologue of the mammalian B subunit). In mammalian cells, the potentially oncogenic homeobox gene product of *HOX11* primarily inhibits PP2A, thereby eliminating a $G_2$/M checkpoint. PP2A's role in mitotic progression is more complicated (Kurimchak and Grana, 2012b), however. In addition to preventing cdk1 activation, PP2A may act downstream of cdk1(Wassarman *et al.*, 1996), *i.e.* reverse the phosphorylations catalysed by cdk1. Evidence for this scenario also comes from at least two angles: Loss of the B subunit in *Drosophila* results in abnormal anaphase (Wassarman *et al.*, 1996), and PP2A dephosphorylates physiological cdk substrates *in vitro* (Kurimchak and Grana, 2012a; Kurimchak and Grana, 2012b).

Numerous observations suggest that PP2A plays a major role in downregulation of the ERK MAP-kinase pathway and probably acts at multiple points in the cascade (Junttila *et al.*, 2008; Switzer *et al.*, 2011). PP2A is not the only phosphatase that is important for the inactivation of the ERKs (Junttila *et al.*, 2008; Switzer *et al.*, 2011). Another important product that PP2A targets are the $Ca^{2+}$-calmodulin-dependent (CaM) kinases (Dudits *et al.*, 2011; Kolupaeva and Janssens, 2013). The activation of these kinases takes place by CaM-kinase kinases and autophosphorylation. PP2A can dephosphorylate activated preparations of CaM kinase I, CaM kinase II and CaM kinase IV. PP2A can also dephosphorylate CaM itself, which affects several effector proteins. In the same way that PP1 interacts with the Rb protein, PP2A also influences this protein and negatively and positively regulates cell cycle progression (Kolupaeva and Janssens, 2013; Kurimchak and Grana, 2012a; Kurimchak and Grana, 2012b). Thus these are only some of the examples of PP2A's interaction with signal transduction pathway molecules. There are many others with which PP2A is involved (refer to table 1.4).

Table 1.4 Proteins that form stable complexes with protein phosphatase 2A taken from (Millward *et al.*, 1999)

| **Protein** | **Comments** |
|---|---|
| **Protein kinases** | |
| p70 S6 kinase | p70 S6 kinase is a PP2A substrate |
| CaM kinase IV | Binds toPR55-containing ABC complex; substrate for C subunit |
| Casein kinase IIα | Binds to AC dimer in quiescent cells; stimulates activity of C subunit |
| RAF-1 | RAF-1 can be dephosphorylated by PP2A |
| p21-activated kinase (PAK1) | PAK1 is a PP2A substrate |
| JAK2 | Transient association upon interleukin-11 stimulation of adipocytes |
| **Other cellular proteins** | |
| $I_1^{PP2A}$ (PHAPI, mapmodulin) | Endogenous, heat-stable inhibitor of PP2A |
| $I_2^{PP2A}$ (SET) | Endogenous, heat-stable inhibitor of PP2A |
| Tap42/$\alpha^4$ | Binds to C subunit; interaction dependent upon TOR1 (FRAP/RAFT) |
| Cyclin G1 | Binds to B subunits of the B' (PR61) family |
| p107 (pRB-related) | Binds to PR59-containing AB''C complex |
| HOX11 | Binds to C subunit; inhibits phosphatase activity |
| HRX | Binds PP2A through $I_2^{PP2A}$; commonly mutated in acute leukaemias |
| Caspase-3 | Activates PP2A during apoptosis by proteolysis of the PR65 subunit |
| Phosphotyrosyl-phosphatase activator (PTPA - PPP2R4) | Binds to AC dimer through A subunit; confers tyrosine phosphatase activity |
| TAU | PR55-containing trimer dephosphorylates TAU; promotes microtubule binding |
| Neurofilament proteins | AC dimer binds and dephosphorylates NF proteins; promotes assembly |
| Erf1 | Binds to AC dimer through C subunit; might target AC dimer to ribosomes |

| **Viral proteins** | |
|---|---|
| SV40 small t | Binds to and inhibits AC dimer; displaces PR55-type B subunits |
| Polyomavirus small t | Confers tyrosine phosphatase activity |
| Polyomavirus middle t | Confers tyrosine phosphatase activity |
| Adenovirus E4 or f4 | Binds to PR55-containing ABC complex; causes downregulation of AP-1 |
| HIV NCp7:Vpr | Binds to PR61-containing AB'C complex; activates C subunit |

## 1.3. The p53 tumour suppressor protein

### 1.3.1. Introduction to p53

The most frequently encountered genetic events in human malignancy are the alterations of the *p53* gene and its encoded protein (Malkin, 1994; Sun *et al.*, 1993; Symonds *et al.*, 1994; Van Meir *et al.*, 1995). The p53 protein is a surveyor of nuclear and cellular integrity. The loss of this role of p53 in most cases results in oncogenesis and tumour development.

Initial experimental work with p53 and viruses characterised the p53 protein as an anti-oncogene. This was the first host protein shown to bind specifically to the product of a DNA tumour virus oncogene (Lane and Crawford, 1979; Linzer and Levine, 1979). It was first discovered in 1979 with the culmination of two types of study involving a virological and a serological approach (Soussi, 1995). It had been shown in SV40 transformed cells that the SV40 large T antigen (AgT) is specifically complexed with a 53 kDa cellular protein (Lane and Crawford, 1979; Linzer and Levine, 1979; McCormick and Harlow, 1980). Viral AgT proteins are responsible for the loss of cell growth that is induced by the virus. Subsequently, this protein was found to complex with the adenovirus E1b 58 kDa protein of adenovirus type 5 and 2 (Sarnow *et al.*, 1982). It was initially suggested that *p53* was an oncogene, a hyperactive, altered, stimulatory gene which dominant retro-viruses act as vectors for (Malkin, 1994), as it was capable of immortalising cells by itself or capable of transforming them in conjunction with the *Ras* oncogene. It was later shown that the mutant forms of *p53* were responsible for the effects just described, while the wild type p53 protein actually suppressed transformation (Alberts *et al.*, 1994; Malkin, 1994). This and the observation that *p53* is frequently lost or mutated in human tumours combined with the fact that introduction of wild

type p53 protein blocks transformed cell growth, convinced researchers that the function of *p53* is actually a growth inhibitor, or suppressor (Malkin, 1994).

The importance of the *p53* gene mutation in carcinogenesis is reflected by *Science* naming it molecule of the year in 1993 (Koshland, 1993). Evidence which suggests that *p53* is an important tumour suppressor gene includes the observations that the wild type p53 protein suppresses or inhibits cell transformation mediated by either viral or cellular oncogenes; missense gene mutations occur frequently in diverse cancers (Chang *et al.*, 1993); germline mutations of the gene are associated with the Li-Fraumeni familial cancer syndrome (Frebourg and Friend, 1992); binding of the protein to murine double minute 2 homologue (MDM2) protein is associated with the development of malignancy (Wang and Hu, 2012; Wang and Jiang, 2012); restoration of wild type p53 into neoplastic cell lines with *p53* defects suppresses cellular growth and/or tumourigenicity *in vitro* and *in vivo*; and transgenic mice deficient for the *p53* gene, or carrying altered p53, are significantly more prone to the spontaneous development of a variety of neoplastic disorders (Alberts *et al.*, 1994; Chang *et al.*, 1995).

### 1.3.2. Structure of p53

The *p53* gene is located on the short arm of the human chromosome 17, band 13, and is approximately 20 kilobases (kb) in length (Isobe *et al.*, 1986; Matlashewski *et al.*, 1984). This gene yields a 2.8 kb mRNA transcript and encodes for a 53 kDa nuclear phosphoprotein (hence, p53). The open reading frame of *p53* is 393 amino acids long. The gene is composed of 11 exons with the first being non-coding. Nucleotide analysis and amino acid sequencing has revealed five evolutionarily conserved domains, from *Xenopus* to human, these are contained in exons 1, 4, 5, 7,

and 8 (Malkin, 1994; Xue *et al.*, 2013). These conserved domains are considered essential for normal function of the p53 protein (Malkin, 1994; Xue *et al.*, 2013). Properties of the *p53* gene include two DNA-binding domains in the central region (consisting of amino acids from about 100 to 300), two SV40 Ag-T binding sites, a nuclear localisation signal, an oligomerisation domain at the C terminus, a strong signal transcription activation signal at the amino-terminal end and several phosphorylation sites (refer to figure 1.5) (Malkin, 1994).

In 1994 Cho *et al*. succeeded in co-crystallising the core domain of p53 bound to DNA (refer to figure 1.6) (Cho *et al.*, 1994). The structure of p53 appears to be unique, consisting of a large beta-sandwich that acts as a scaffold for three loop-based elements. The sandwich is composed of two anti-parallel beta-sheets containing four and five beta-strands. The first loop binds to DNA within the major groove, the second loop binds to DNA within the minor groove, and the third loop packs against the second loop to stabilise it. This conformation correlates well with the mutation hot spots. Mutations of *p53* in most cancers are missense mutations and reside in the DNA binding domain. Most of these base substitution mutations are clustered between exons 5 and 8 and are localised in four evolutionarily conserved domains of *p53*: domain II (codons 117 to 142), domain III (codons 171 to 181), domain IV (codons 234 to 258) and domain V (codons 270 to 286) (Caron de Fromentel and Soussi, 1992; Hollstein *et al.*, 1991; Levine *et al.*, 1991).

In 1995 Jeffrey *et al*. confirmed that p53 formed a tetramer via an oligomerisation domain at the COOH-terminal portion of the protein and suggested that it binds DNA sites that typically contain four copies of a consensus sequence (refer to figure 1.7) (Jeffrey *et al.*, 1995).

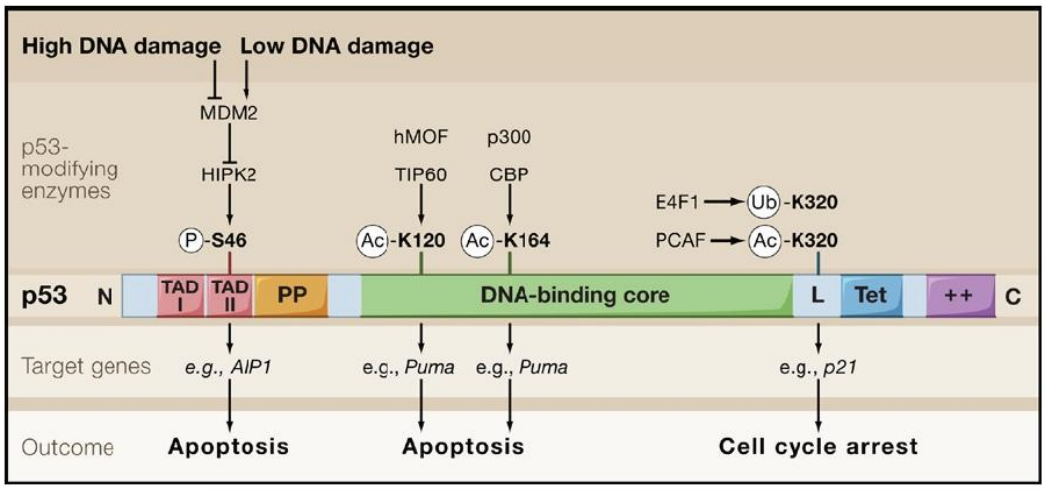



Figure 1.5 Structural features of the p53 protein and the impact of its modifications taken from (Vousden and Prives, 2009)

The following domains have been defined on the p53 protein; the transcriptional activation domain I (TAD I, residues 20-40), the transcriptional activation domain II (TAD II, residues 40-60), the proline domain (PP, residues 60-90), the sequence-specific core DNA-binding domain (DNA-binding core, residues 100-300), the linker region (L, residues 301-324), the tetramerisation domain (Tet, residues 325-356), and the lysine-rich basic C-terminal domain (++, residues 363-393) (Vousden and Prives, 2009).

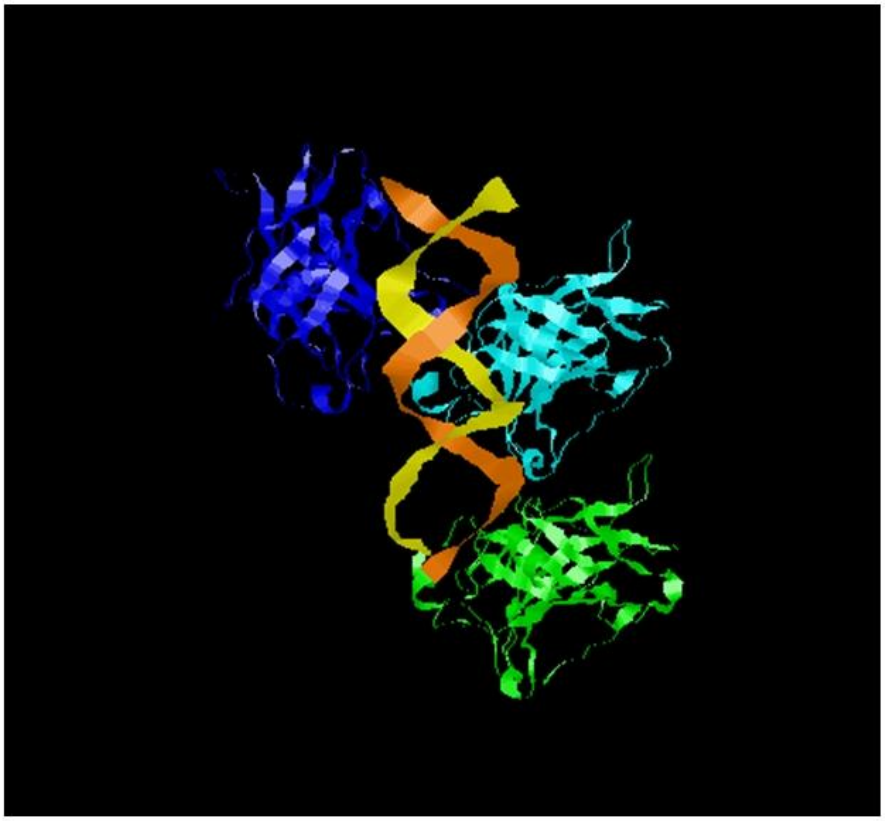


Figure 1.6 Three p53 molecules interacting with DNA obtained from the research collaboratory for structural bioinformatics protein data bank (Cho *et al.*, 1994)

A schematic ribbon drawing of the three p53 molecules and one DNA duplex (X-ray diffraction). The image was obtained from the RCSB PDB (www.pdb.org) (Berman *et al.*, 2000) of PDB ID 1TUP (Cho *et al.*, 1994).

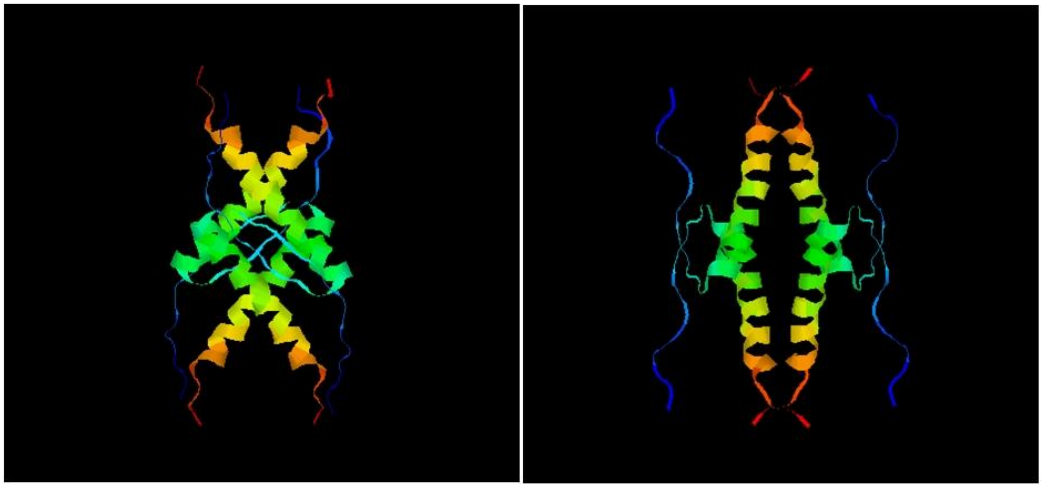


Figure 1.7 The p53 tetrameric oligomerisation domains obtained from the research collaboratory for structural bioinformatics protein data bank (Clore *et al.*, 1994)

Two different axis views of p53 tetrameric oligomerisation domains (NMR, minimised average structure). The two images were obtained from the RCSB PDB (www.pdb.org) (Berman *et al.*, 2000) of PDB ID 1OLG (Clore *et al.*, 1994).

### 1.3.3. The function of p53

To date, important functions of the p53 protein have been explained. However, its interactions with other molecules, and the underlying principles involved in its functioning are still being elucidated. The p53 protein has been implicated in the control of the cell cycle, DNA repair and synthesis, cell differentiation, genomic plasticity, inhibition of angiogenesis and programmed cell death (Carvajal and Manfredi, 2013; Hunten *et al.*, 2013). This protein has a half-life of about 20 minutes and is generally located in the cell nucleus (Oren *et al.*, 1981). The p53 tumour antigen is found in increased amounts in a wide variety of transformed cells. It is detectable in many actively proliferating, non-transformed cells, but is undetectable or present at low levels in resting cells. This low concentration in normal cells is a reflection of the potency of this protein. The synthesis of p53 increases in resting cells after stimulation with mitogen, and stimulated cells can be blocked from entering S phase by microinjections of p53-specific antibody. There is much evidence that depicts its involvement in cell cycle regulation (Carvajal and Manfredi, 2013; McCubrey and Demidenko, 2012). The *p53* gene has structural and functional characteristics to the *MYC* family of protooncogenes (Richon *et al.*, 1989). The Myc protein normally acts in the nucleus as a signal for cell proliferation; excess quantities of Myc cause the cell to enter the cell cycle in circumstances that a normal cell would halt (Richon *et al.*, 1989; Tan *et al.*, 2006).

In some cases *p53* can be thought of as an oncogene since it has been shown that alteration or inactivation of *p53* by mutation, or the interaction of p53 with oncogene products of DNA tumour viruses, can lead to cancer (Levine *et al.*, 1991). In this case p53 can complement activated *Ras* genes (genes that can cause the cell to proliferate or divide) in the transformation of rat fibroblasts and act as an oncogene (Harris and Hollstein, 1993; Levine *et al.*, 1991).

#### 1.3.3.1. p53 In DNA damage and apoptosis

It is now known that *p53* is intimately involved in the induction of apoptosis after DNA damage with infrared rays and certain drugs in a number of cell types. The *p53* gene product is activated by the presence of double strand DNA breaks, which may occur as a direct consequence of DNA injury (by infrared rays or cytotoxic drugs), or even by inappropriate initiation of DNA synthesis (Adams and Carpenter, 2006; Nelson and Kastan, 1994). In several cell types, *p53*-mediated apoptosis initiated by DNA damage occurs in the presence of actinomycin D, cyclohexamide (compounds that can block RNA or protein synthesis) (Caelles *et al.*, 1994), or by amino acids residues 1-214 of p53 that fail to bind to DNA. This suggests that transcriptional activation or the translation of p53-regulated gene products is not required for apoptosis. Other p53 regulated genes can influence p53-mediated apoptosis, these are *BAX* and IGF-BP3 (Adams and Carpenter, 2006; Buckbinder *et al.*, 1995; Miyashita and Reed, 1995).

The ability of p53 to pause the cell cycle allows the cell to repair damage and if the damage is too excessive, to undergo apoptosis. These two effects of p53 appear distinct in that bcl-2 is able to block the apoptosis effect of p53, but not the cell cycle block for DNA repair. If the *p53* gene is mutated or missing, the pause for repair does not occur and the cell cycle continues unchecked. These cells are consequently genetically unstable and will accumulate mutations and altered DNA at an increased rate, resulting in the rapid selection of a malignant clone (Prokocimer and Rotter, 1994). This would ultimately lead to the formation of cancer. Refer to figure 1.8 for an illustration of p53 protein accumulation and DNA damage.

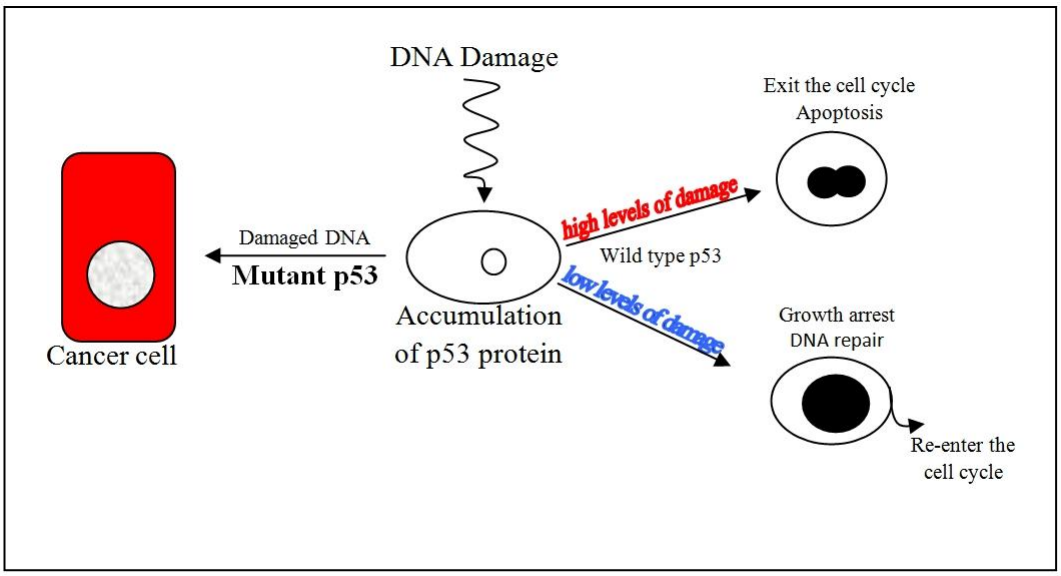



Figure 1.8 A model of p53 protein accumulation and DNA damage adapted from (Prokocimer and Rotter, 1994)

A model of the involvement of wild-type p53 protein in the DNA repair machinery and deregulation in activity mediated by mutant p53 protein (Prokocimer and Rotter, 1994).

### 1.3.3.2. p53 Stability and its various associations

Presently, some of the regulatory mechanisms of p53 are known with new information constantly being unravelled. The mechanism that controls p53 function is extremely intricate and its control is critical to the cell. The principal method by which this is achieved is by controlling the concentration of p53 protein in the cell and by manipulating its stability.

One of the most prominent biochemical properties of the p53 protein is its ability to interact with a variety of proteins of viral and cellular origin. The p53 protein forms complexes with DNA tumour virus oncoproteins such as SV40 Tag, adenovirus E1b 55 kDa protein, and E6 proteins of the human papillomaviruses HPV 16 and HPV 18 (Sato and Tsurumi, 2013). These proteins inactivate the p53 protein.

The p53 protein is usually present at low levels in normal cells, with only a few thousand molecules per nucleus. In contrast cells transformed by the SV40 and adenoviruses, contained elevated levels of p53 protein of up to 100 000 molecules per nucleus (Lane, 1989). The principle mechanism accounting for the high levels of p53 protein in these transformed cells was found to operate post-translationally, since mRNA levels are similar in non-transformed and SV40 transformed 3T3 cells (Oren *et al.*, 1981). In non-transformed cells and in normal tissues p53 protein characteristically has a very short half-life of approximately 10-20 minutes (Rogel *et al.*, 1985). In cells transformed by these viruses, the half-life of the p53 protein is extended to at least 20 hours (Oren *et al.*, 1981) due to the complex formation of p53 and virus protein. The SV40 Tag complex with p53 protein blocks the p53 DNA binding domain; the adenovirus E1b complex with p53 blocks the p53 transcriptional activation domain (Ko and Prives, 1996; Levine, 1997; Sato and

Tsurumi, 2013). Blockage of these domains results in the inactivation of p53 protein, which in turn affects the other molecules that p53 interacts with (Refer to table 1.5 and 1.6).

In contrast, to the SV40 and adenovirus proteins, the HPV E6 protein targets p53 degradation. This mechanism involves the E6 protein recruiting a cellular protein, E6AP, to cause the ubiquitination of p53 protein. It is this p53-ubiquitin conjugate that is targeted for degradation by a multi-protease complex called the proteasome (Kubbutat and Vousden, 1996). Normal degradation of p53 also depends on the ubiquitin dependent pathway, but this pathway does not recruit E6AP.

In non-virally induced tumours, the half-life of p53 is also extended (Reich *et al.*, 1983). This is indicative of mutation of the *p53* amino acid sequence. Naturally arising (Finlay *et al.*, 1988; Mowat *et al.*, 1985; Rovinski *et al.*, 1987) and artificially engineered (Jenkins *et al.*, 1985; Sturzbecher *et al.*, 1987) mutations of the *p53* sequence have the effect of extending the half-life of the protein, by making the protein a poor substrate for the ubiquitin dependent degradation pathway, which is responsible for the rapid turnover of wild-type p53 protein (Gronostajski *et al.*, 1984).

In addition to proteasomal degradation, p53 is also a substrate of the $Ca^{2+}$-dependent neutral protease calpain. Calpains are a family of cysteine proteases that cleave a variety of proteins (such as N-Myc, c-Jun, c-Fos and fodrin), and appear to play a role in platelet aggregation, neuronal long-term potentiation and apoptosis (Atencio *et al.*, 2000; Liang, 2010; Tan *et al.*, 2006). The tertiary structure of p53 is an important determinant of calpain cleavage.

Table 1.5 Proteins that have functional interactions with p53 adapted from (Brooks and Gu, 2006; Golubovskaya and Cance, 2011; Levine, 1997; Sato and Tsurumi, 2013)

| Viral proteins | |
|---|---|
| Protein | Interaction |
| SV40 T antigen | Blocks p53 DNA binding domain |
| Adenovirus E1b | Blocks p53 transcriptional activation domain |
| Human papilloma virus E6 | Promotes p53 degradation |
| Epstein–Barr virus | p53 Inhibition and degradation |
| Kaposi's sarcoma-associated herpesviru | p53 Inhibition and degradation |
| Influenza virus | p53 Protein activation |

| Oncogene products | |
|---|---|
| Protein | Interaction |
| Mdm2 | Blocks p53 transcriptional activation domain |
| c-Abl | p53 Mediated cell cycle arrest |
| Cop1 | Ubiquitin ligase for p53 |
| Pirh2 | Ubiquitination and degradation of p53 |
| FAK | p53 Inhibits FAK transcription |

| Transcriptional factors | |
|---|---|
| Protein | Interaction |
| TATA-binding protein | Binds amino and carboxyl termini of p53 |
| TAF 70 and TAF 31 | Binds to amino-terminal domain of p53 |
| TFIIH | Helicase modulated by wild-type p53, not mutant p53 |
| ERCC2 XPD | Helicase modulated by wild-type p53, not mutant p53 |
| Wilms tumour-1 (WT1) | Alters p53 activities |

Table 1.6 Products of genes transcriptionally activated by p53 taken from (Levine, 1997)

| Proteins | Function |
|---|---|
| p21, Waf1, Cip1 | Inhibits several cyclin-cyclin-dependent kinases; binds cdk's, cyclins and PCNA; arrests the cell cycle |
| Mdm2 | Product of an oncogene; inactivates p53-mediated transcription and so forms an autoregulatory loop with p53 activity |
| GADD45 | Induced upon DNA damage; binds to PCNA and can arrest the cell cycle; involved directly in DNA nucleotide excision repair |
| Cyclin G | A novel cyclin ( it does not cycle with cell division); fairly new |
| Bax | A member of the Bcl2 family that promotes apoptosis, not induced by p53 in all cells |
| IGF-BP3 | The insulin-like growth factor binding protein-3; blocks signalling of a mitogenic growth factor |

It has been reported that cleavage of p53 *in vitro* results in the generation of p53 fragments lacking either the N- or C-terminus, or both (Okorokov *et al.*, 1997). These autoproteolytic activities are activated by incubation with either double-stranded or single-stranded DNA, and the smallest fragment of p53 (DNA-binding core), was shown to possess proteolytic activity, with characteristics of an aminopeptidase (Okorokov *et al.*, 1997). Products of autoproteolytic cleavage were detected in cells following DNA damage.

Mdm2 promotes the destabilisation of p53, which depends on the interaction of both proteins, and is mediated by the proteasome (Pei *et al.*, 2012; Wade *et al.*, 2013; Wang and Hu, 2012; Wang and Jiang, 2012). In undamaged cells Mdm2 is necessary for keeping p53 levels low. A model of this role is summarised in figure 1.9.

In response to DNA damage, the half-life of the p53 protein is extended. This occurs in the absence of any change in the expression of p53 mRNA. It has been speculated that enhanced translation contributes to the rapid and dramatic rise of p53 protein levels. The translation of p53 mRNA is inhibited through an element in its 3' untranslated region (UTR), and this inhibition is alleviated in response to DNA damage (Galy *et al.*, 2001). It has been found that p53 binds to a region within the 5' UTR of its own mRNA which results in the inhibition of its own translation (Galy *et al.*, 2001). This observation suggests the existence of a negative autoregulatory feedback loop at the level of translation (Kartasheva *et al.*, 2002; Mosner *et al.*, 1995).

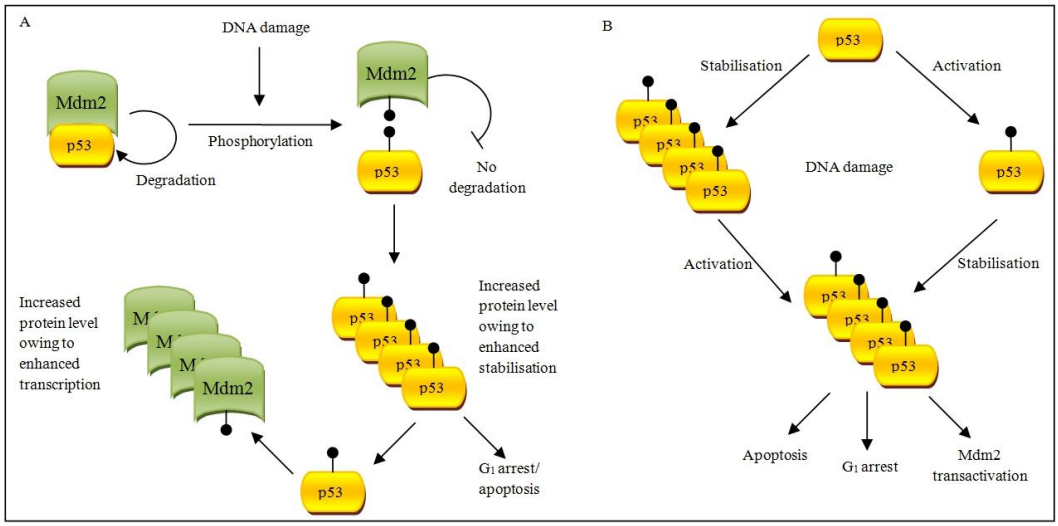



Figure 1.9 A model of the regulation of p53 stability by Mdm2 adapted from (Pei *et al.*, 2012; Wang and Jiang, 2012)

(a) In response to genotoxic stress such as DNA damage, interaction between p53 and Mdm2 can be disrupted by modification by either protein, such as phosphorylation, resulting in elevated p53 levels and induction of $G_1$ arrest or apoptosis. p53 Also transactivates Mdm2 but, as long as both proteins remain phosphorylated, degradation of p53 does not take place. Therefore, after DNA damage, high levels of p53 can co-exist with high levels of Mdm2. Establishing high levels of Mdm2 after DNA damage might be important for re-entry into the cell cycle once the damaged DNA has been repaired, because as soon as the signals that activate phosphorylation of p53 and Mdm2 are removed, the Mdm2-mediated degradation is reactivated and p53 is rapidly removed. (b) Although genotoxic stress such as DNA damage usually results in an increase in active p53 protein levels, there is evidence that stabilisation and activation of p53 can be regulated independently. In certain tumours, a high level of inactive wild-type p53 protein is synthesised and differential regulation of activation and stabilisation can occur under certain conditions in normal cells. A full p53 response would therefore require signals for both stabilisation and activation.

#### 1.3.3.3. The role of p53 in the cell cycle

The Rb protein, mentioned previously in section 1.2.4, alters the cell cycle at the level of cyclin E just before the important "checkpoint" at the $G_1$-S transition (Caillava and Baron-Van Evercooren, 2012; Hunter and Pines, 1994; Takahashi *et al.*, 2012). Phosphorylation of the Rb protein prevents its interaction with both cyclins and transcription factors, allowing the cycle to continue (Caillava and Baron-Van Evercooren, 2012; Draetta, 1994; Takahashi *et al.*, 2012). Phosphorylation of Rb by cyclin D1-cdk4 prevents its interaction with E2F-DP transcription factor complexes that are responsible for transcription of cyclin E, cyclin A, dihydrofolate reductase, and proliferating cell nuclear antigen (PCNA). These genes are important in propagating the S phase of the cell cycle. Therefore, Rb regulates the start signal of the cell cycle at the $G_1$-S transition and propagation through the S phase. The $p16^{Ink4}$ gene product is another protein that can regulate the cycle at the level of cyclin D1-cdk4 (Mirzayans *et al.*, 2012a). It is a negative regulator of cyclin D1-cdk4 and can be shut off in some cancers by methylation.

The *p53* gene acts as a transcription factor while preventing continuance of the cell cycle when there is DNA damage (Carvajal and Manfredi, 2013; Wu and Levine, 1994). In response to some forms of DNA damage, p53 accumulates and turns on the transcription of one of its downstream genes, p21 (WAF1, Cip1) (Carvajal and Manfredi, 2013; El-Deiry *et al.*, 1993; Mirzayans *et al.*, 2012b; Ye and Field, 2012). Increased p21 activity can inhibit PCNA and cyclin-kinase activities and block cell cycle progression (Mirzayans *et al.*, 2012b; Ye and Field, 2012). p21 May also be induced in response to mitogens.

p21 Binds to the cyclin dependent kinases (cdk2, cdk4 and cdk6) and inhibits their activity (Ye and Field, 2012). This results in cell cycle arrest prior to DNA synthesis, allowing repair of the damaged DNA. If repair is unsuccessful then the presence of p53 protein may alternatively induce apoptosis. Cell cycle arrest occurs at the $G_1$ checkpoint, just before the S phase. The action of this $G_1$ checkpoint is lost in the presence of the mutated p53 protein.

After genotoxic stress, p53 also induces the expression of GADD45, which binds to and inhibits PCNA. PCNA directly activates DNA polymerase δ, one of the primary replicative DNA polymerases (Smith and Seo, 2002; Taylor and Stark, 2001). GADD45 inhibits entry of the cell cycle into S phase and also stimulates DNA excision repair (Kastan *et al.*, 1992; Smith and Seo, 2002; Taylor and Stark, 2001). GADD45 is an established link between p53, cell cycle arrest and DNA repair. WAF1/Cip1 is also able to directly inhibit PCNA.

Thus if DNA is damaged before the S phase, p53 is able to prevent cells from starting their DNA replication by WAF1/Cip1 mediated inhibition of cyclin-cdk complexes, whereas if DNA is damaged during the S phase, the WAF1/Cip1 or GADD45 is able to halt replication by binding to PCNA. Figure 1.10 summarises the p53 pathway based on much of the evidence, however, there are great gaps in our understanding of the signal transduction processes that control and regulate cell cycle control.

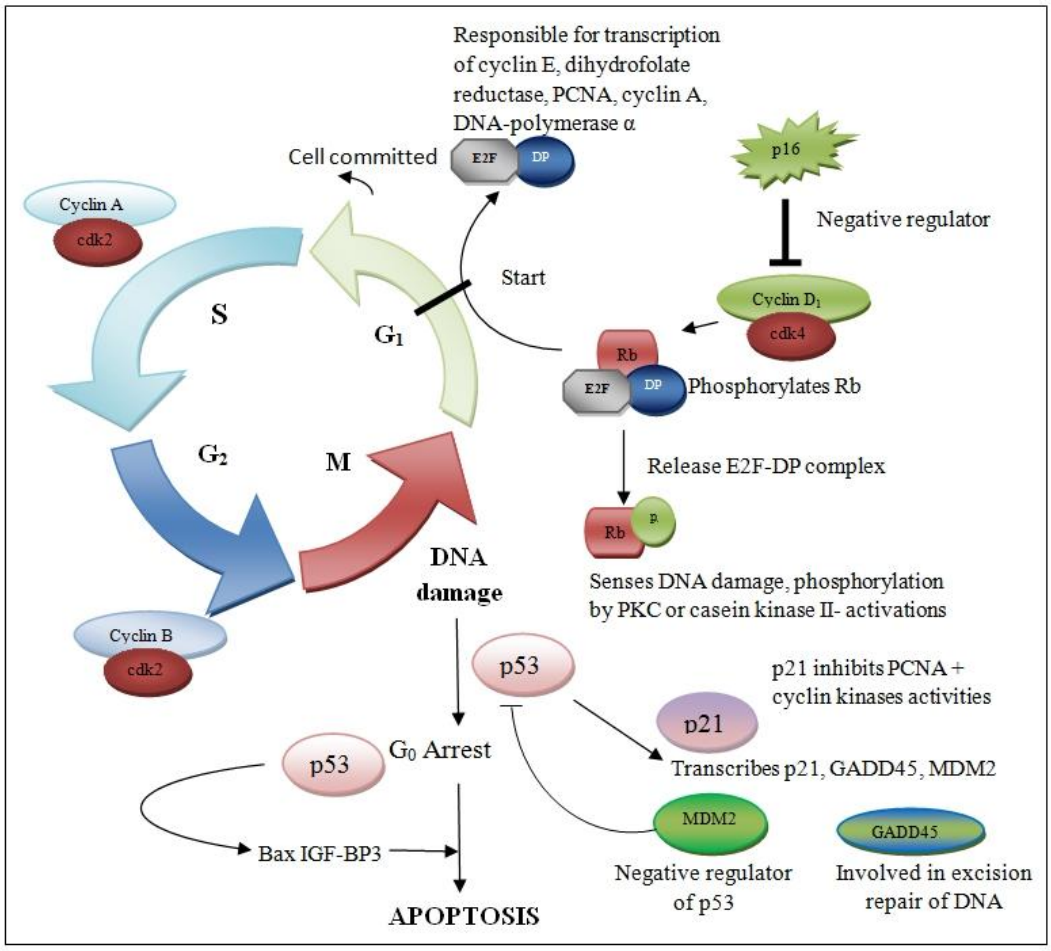



Figure 1.10 The influence of p53 on the cell cycle adapted from (Harris, 1996; Polager and Ginsberg, 2009; Sherr and McCormick, 2002)

This is a hypothesised pathway of p53's role in cell cycle regulation and apoptosis. This is meant to be a general overview of some of the literature on this subject and definitely doesn't represent the entire picture.

## 1.4. Murine erythroleukaemic cells

Murine erythroleukaemic (MEL) cells are erythroid precursor cells derived from mouse spleens and transformed by the Friend virus complex. They grow easily as stationary suspension cultures and can be adapted to stirred suspension conditions. These cells are ideal for laboratory study because they can be induced to undergo differentiation by a wide variety of agents and are excellent for the study of the reversal of malignant transformation.

In 1957, Charlotte Friend reported recovery of a virus from the spleen cells of Swiss mice inoculated 14 months previously with filtrate from disrupted Ehrlich ascites tumour cells (Friend, 1957). Initially these were considered a reactive reticulum cell sarcoma (Buffett and Furth, 1959; Friend and Haddad, 1960) associated with a reactive erythroblastosis (Metcalf *et al.*, 1959). In 1962, based on pathological studies and radio-iron uptake, Zajdela *et al*. (Lacassagne *et al.*, 1962) suggested that the primary disease initiated by Friend virus complex was a neoplastic proliferation of erythropoietic elements.

The primary erythropoietic nature of Friend disease was subsequently documented by histopathological studies, including electron microscopy (Ikawa and Sugano, 1967), demonstration of erythrocytic antigens on the surface of tumour cells (Ikawa *et al.*, 1972), and the isolation, by Mirand (Mirand, 1966) of a variant of Friend virus complex that induces not only the typical manifestations of Friend Disease, including splenomegaly and erythroblastosis, but a pronounced polythaemia as well (Marks and Rifkind, 1978).

Unlike normal erythropoiesis, Friend Virus infected erythroid cells will express erythropoietin-independent erythroid cell differentiation when explanted under *in vitro* conditions (Hankins and Krantz, 1975; Liao and Axelrad, 1975; Marks *et al.*, 1977). Thomson and Axelrad in 1968 demonstrated the malignant features of MEL cells by transplantation to a murine host genetically resistant to infection by the Friend virus complex, and confirmed their unrestrained growth potential (Thomson and Axelrad, 1968). However, under these conditions, and using serial mouse passages, it has been shown that MEL cells do not have indefinite transplantability unlike typical virus transformed malignant cells (Steeves *et al.*, 1970; Thomson and Axelrad, 1968). Evidence suggests that host environmental factors may be critical in modulating the malignancy of MEL cells (Rossi and Friend, 1970).

The earliest cellular response of the Friend virus was proliferation in haemopoietic organs, principally the spleen, but also marrow and liver, of immature erythroid elements such as proerythroblasts and their progeny(Marks and Rifkind, 1978). The malignant features of the MEL cells were demonstrated by transplantation of the cells into a murine host, genetically resistant to the Friend virus complex and an unrestrained growth potential was observed (Marks and Rifkind, 1978).

### 1.4.1. Characteristics in culture

MEL cells in culture appear pleomorphic (cells of immature morphology and all stages of nucleated erythroblast maturation). Most strains of MEL cells grow with ease in standard tissue culture media supplemented with foetal calf serum (+/- 5%) (Friend *et al.*, 1966; Ikawa *et al.*, 1973; Singer *et al.*, 1974). These lines display a low level of spontaneous differentiation, ranging from 0.5-20%, as determined by the benzidine reaction for haemoglobin (Friend *et al.*, 1966; Ikawa *et al.*, 1976;

Ikawa *et al.*, 1975; Orkin *et al.*, 1975; Preisler *et al.*, 1976b; Rovera and Bonaiuto, 1976). This spontaneous differentiation depends on the culture conditions as well as cell strain. As *in vivo*, MEL cell cultures display neither requirement for nor response to erythropoietin (Ikawa *et al.*, 1973).

The addition of inducers to the culture medium initiates the process of differentiation. Initially these metabolic changes can be reversed. After continued incubation of cultured cells with the inducer, these cells become committed to irreversible differentiation. It has been shown that commitment to terminal differentiation is a multistep process (Ramsay *et al.*, 1986). With HMBA, commitment has been detected after 12 hours but only after 48 hours were 95% of the cells committed to differentiate (Ramsay *et al.*, 1986).

### 1.4.2. Murine erythroleukaemic cell proliferation and differentiation

As mentioned before, neoplastic transformation of precursor cells leads to uncontrolled cell division and failure of cells to differentiate normally. There is abundant evidence that transformation does not necessarily destroy the capacity of transformed cells to express their differentiated phenotype, including terminal cell division (Marks and Rifkind, 1984; Marks and Rifkind, 1978; Michaeli *et al.*, 1992b; Pierce and Wallace, 1971; Preisler *et al.*, 1976a; Reuben *et al.*, 1976; Sachs, 1987). Conceptually, using agents that could restore or force the cells to enter differentiation might be effective in treating or in preventing the progression of cancers (Marks and Rifkind, 1984). Induced terminal differentiation of transformed cells involves both the cessation of DNA synthesis and the expression of genes for the differentiated phenotype.

MEL cells can be stimulated to mature along the erythroid pathway when cultured in the presence of a variety of structurally unrelated chemical compounds, see table 1.7 (Gazitt and Friend, 1981). The onset of MEL cell differentiation and the rate of commitment are highly dependent on the type and concentration of inducer used.

From all the inducers, the planar-polar compounds are the most active as inducers. These compounds have relatively low molecular masses (< 300 Da), with a polar, hydrophilic group and a planar, hydrophobic portion (Preisler *et al.*, 1976a; Reuben *et al.*, 1976; Tanaka *et al.*, 1975). These are not the only types of inducers; other inducers include short chain fatty acids, purines and purine derivatives, haemin, and inhibitors of DNA or RNA synthesis. Some of these inducers, like diamines, certain purines, haemin, and fatty acids form part of the normal cellular components.

Evidence suggests that the pathways of induction of these inducers on MEL cells are not all the same. The main reason for this is the difference in structure and the site of action of these inducers (Marks and Rifkind, 1978). Some of these compounds may also induce apoptosis if exposed to the cells for too long.

Table 1.7 Active agents as inducers of MEL cell differentiation adapted from (Marks and Rifkind, 1978) and (Tsiftsoglou and Wong, 1985)

| **Generally strong inducers[a]** | **Generally weak inducers[a]** |
|---|---|
| Polar-planar compounds | Polar-planar compounds |
| Hexamethylene bisacetamide | 2-Pyrrolidinone |
| Dimethylsulphoxide | Propionamide |
| 1-Methyl-2-piperidone | Pyridine-N-oxide |
| N, N-Dimethylacetamide | Piperidone |
| N-Methylpyrriolidinone | Pyridazine |
| N-Methylacetamide | Dimethylurea |
| N, N-Dimethylformamide | |
| N-Methylformadlehyde | |
| Tetramethyl urea | |
| Triethylene glycol | |
| Polymethylene bisacetamides | |
| Hexamethylene bispropionamide | |
| Acetamide | |

| Antibiotics/Antitumour agents | Antibiotics/Antitumour agents |
|---|---|
| Bleomycin | Vinoristine |
| N-Dimethylrifampicin | 5-Fluorouracil |
| Actinomycin D | Methramycin |
| Actinomycin C | Cycloheximide |
| | X irradiation |
| | UV irradiation |
| | Adriamycin |
| | Cytosine arabinoside |
| | Mitomycin C |
| | Hydroxyurea |

| Purine/Purine derivatives | Diamines |
|---|---|
| Hypoxanthine<br>1-Methylhypoxanthine<br>2, 6 Diaminopurine<br>6-Mercaptopurine<br>6-Thioguanine<br>6-Amin-2-mercaptopurine<br>2-Acetylamino-6-mercaptopurine | Cadaverine |

| Fatty acids | Fatty acids |
|---|---|
| Butyrylcholine | Acetate<br>Proprionate<br>Butyrate<br>Isobutyrate |

| Other | Other |
|---|---|
| Ouabain | Hemin<br>Methylisobutylxanthine<br>Proteases<br>$Ca^{++}$ and $K^{+}$ metabolism<br>Nucleic acid methylation |

[a]An agent is generally listed as a strong inducer if it is reported to induce 50% or more of a population of MEL cells, at the optimal concentration of the inducer while the weak inducers induce 5-50% of MEL cells (Marks and Rifkind, 1978).

### 1.4.3. Characteristics of induced murine erythroleukaemic cells

Erythroid differentiation of MEL cells is characterised by the appearance of an erythrocyte membrane, increase in haem synthesis and iron uptake, together with induction of haemoglobin synthesis, and the loss of proliferation (Orkin *et al.*, 1975). These biochemical and morphological changes parallel most of the changes that occur during normal red blood cell formation with some of the regulatory mechanisms being different from normal erythropoiesis e.g. the ejection of the nucleus (Friend *et al.*, 1987).

### 1.4.4. Hexamethylene bisacetamide

The most extensively studied model of induced differentiation in MEL cells has been with the use of hybrid polar compounds, of which HMBA is the prototype. From the compounds listed in table 1.7, HMBA was found to be the most potent in causing MEL cell differentiation. Evidence by Marks and Rifkind showed that HMBA caused (a) the entire population of MEL cells to differentiate, (b) a higher proportion of the total protein synthesised was haemoglobin, and (c) relatively low concentrations were effective (Marks and Rifkind, 1978). The two and three dimensional structure of HMBA is shown in figure 1.11.

Evidence suggests that HMBA activates a signalling pathway by modulating the membrane surface potential of MEL cells (Arcangeli *et al.*, 1993). No evidence has been obtained suggesting a membrane receptor for HMBA or its related compounds on these cells (Reuben *et al.*, 1980). Experiments have shown that within minutes of initiating a culture of MEL cells with HMBA, there is a transient increase and then

a fall in diacylglycerol content (Michaeli *et al.*, 1992a), followed by an isozyme-specific translocation of protein kinases, PKC δ and PKC ε, from the cytosol to the membrane (Leng *et al.*, 1993). This suggests that the PKC signalling pathway maybe one of the pathways that HMBA uses for induction of MEL cells. Some of the characteristics of HMBA induced differentiation of MEL cells are listed in table 1.8.

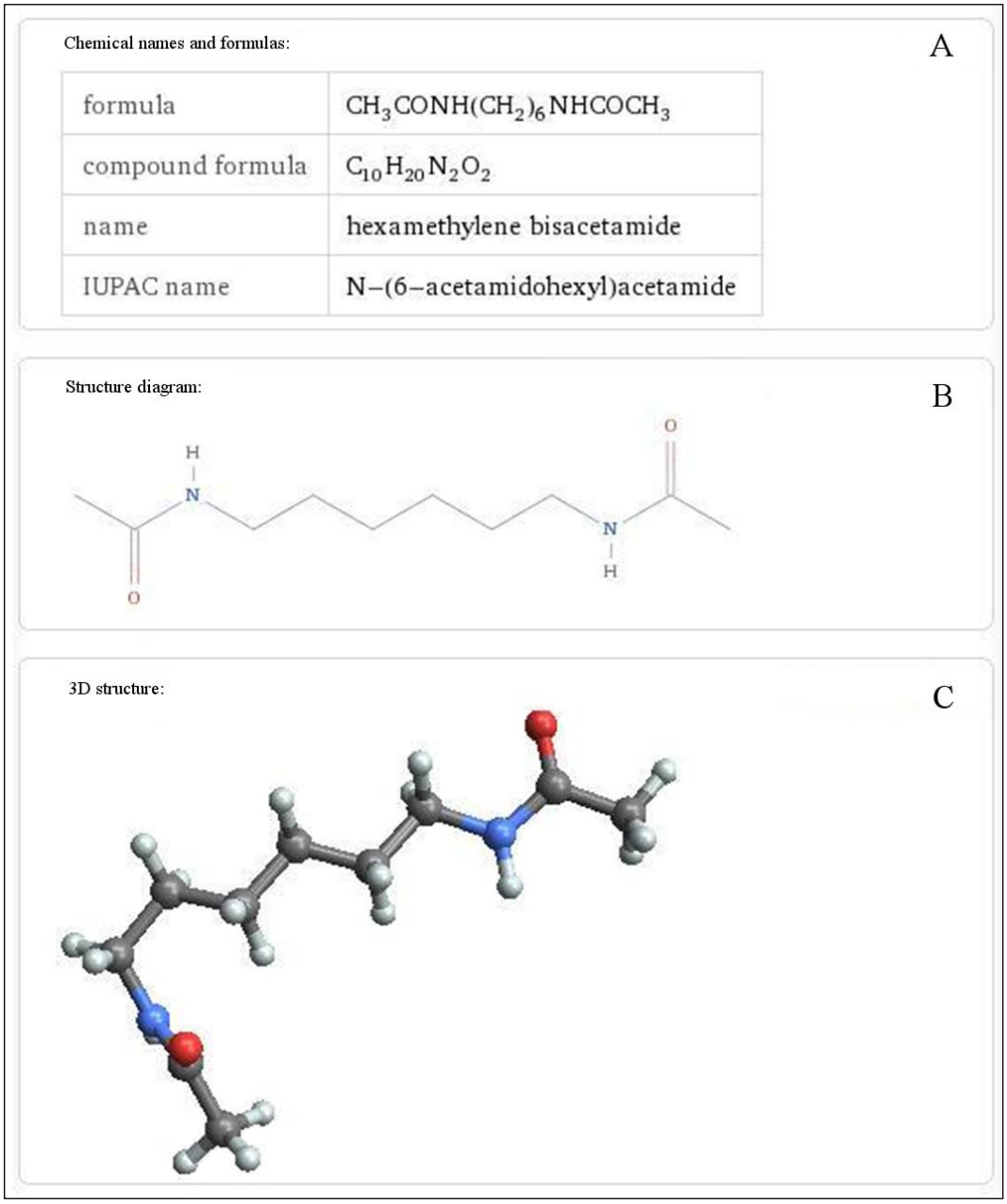



Figure 1.11 The structure and chemical formula of HMBA obtained from the national centre for biotechnology information (PubChem, 2013).

The molecular formula for HMBA is $C_{10}H_{20}N_2O_2$ and its molecular mass is 200.3 Da (A). The structure diagram and three dimensional layout of HMBA, images were obtained from the National Centre for Biotechnology Information (NCBI) (B and C).

Table 1.8 Characteristics of HMBA induced differentiation of MEL cells taken from (Marks *et al.*, 1994)

| **Morphological changes** |
|---|
| Chromatin condensation |
| Disappearance of nucleoli |
| Decrease in the nuclear/cytoplasmic ratio |
| **Early metabolic changes** |
| Decrease in membrane fluidity |
| Alteration of membrane potential |
| Alteration at $Na^+/K^+$ transport |
| Increase of $Ca^{2+}$ uptake |
| PKC activity translocated from cytosol to membrane |
| Transient increase, then decrease in diacylglycerol |
| Cessation of c-myb and c-myc gene transcription |
| Decrease in c-myb, c-myc, and p53 protein levels |
| Increase in c-fos mRNA level |
| **Later metabolic changes** |
| Cessation of DNA synthesis |
| Increase in globin gene transcription |
| Increase in globin synthesis |
| Sequential increase in enzyme activities involved in de novo heme synthesis: |
| δ-aminolevulinic acid synthetase, aminolevulinic acid dehydrogenase; uroporphyrinogen-I synthetase; ferrochelatase |
| Accumulation of haemoglobin |

### 1.4.5. Involvement of HMBA in the cell cycle

MEL cells cultured with HMBA have a prolongation in the first $G_1$ phase, resulting in an increase in cell cycle time from ~ 12 hours (for untreated cells) to ~ 16 hours (Gambari *et al.*, 1979; Gambari *et al.*, 1978). This prolongation in $G_1$ required exposure to HMBA during the preceding $G_1$ and early S phases (Gambari *et al.*, 1978). It is during the initial prolonged $G_1$ phase that commitment of a portion of the cells (~ 15%) to terminal cell division and gene expression were first noted (Fibach *et al.*, 1977; Gambari *et al.*, 1979); after an additional five to six divisions, > 95% of the cells were differentiated. These results reflect the involvement of HMBA with respect to the cell cycle, in particular at the $G_1$ and S phases.

It has been discussed that cdks, which are activated by cyclins, are involved as positive regulators of cell cycle progression. In contrast, Rb and its related proteins, like p107 and p130, act to suppress cell cycle progression, especially at the $G_1$ phase (Cobrinik *et al.*, 1992; Takahashi *et al.*, 2012). In turn, these proteins are regulated by the protein phosphatases, like PP1 and PP2A. These phosphatases activate Rb and p107 by dephosphorylation and thus blocking the $G_1$ to S phase transition (Ewen, 1994; Kolupaeva and Janssens, 2013; Kurimchak and Grana, 2012a; Kurimchak and Grana, 2012b; Ranjan and Heintz, 2006).

In untreated MEL cells, the protein levels of cyclins D3, E, and A fluctuate during the cell cycle, whereas the level of cyclin D2 is unchanged (Kiyokawa *et al.*, 1994) and cyclin D1 was not detected. Cyclin D3 increases as cells progress through $G_1$ phase to a maximum level in S phase (Marks *et al.*, 1994). Cyclin E peaks at the transition from $G_1$ to S phase (Marks *et al.*, 1994). Cyclin A increases later than cyclin E, peaking in $G_2$/M phase (Marks *et al.*, 1994). The level of cdk4 fluctuates

in a pattern similar to that of cyclin D3, peaking in S phase (Fisher *et al.*, 2012; Marks *et al.*, 1994; Yasutis and Kozminski, 2013). cdk2 Kinase activity is low in $G_1$ phase and increases during S phase, whereas the level of cdk2 protein is constant during the cell cycle (Fisher *et al.*, 2012; Marks *et al.*, 1994; Yasutis and Kozminski, 2013).

In HMBA treated MEL cells, there is a change in the pattern of expression of these cyclins and cdks. During the prolonged $G_1$ phase, the level of cyclin A and the activity of cdk2 remain low (Marks *et al.*, 1994). The levels of cyclin A protein and cdk2 kinase activity increase as cells enter S phase (Marks *et al.*, 1994). The pattern of fluctuation of cyclin E remains the same as that in the untreated cells. There is a 20-fold decrease in the expression of cdk4 protein, a 75% decrease in cdk4 dependent kinase activity, and a 3-fold increase in cyclin D3 protein level in the prolonged $G_1$ phase (Marks *et al.*, 1994). The decrease in cdk4 protein is due to its decrease in stability since the rate of synthesis of cdk4 protein and the level of mRNA remain the same (Marks *et al.*, 1994). The increase in cyclin D3 (because of an increase in expression and a decrease in binding to cdk4) results in some of them binding to the activated Rb protein (Fisher *et al.*, 2012; Marks *et al.*, 1994; Yasutis and Kozminski, 2013). Some of these cyclin D3s are also bound to the transcription factor E2F (Fisher *et al.*, 2012; Marks *et al.*, 1994; Yasutis and Kozminski, 2013). Genes with E2F sites include DNA polymerase` α, dihydrofolate reductase, thymidylate synthase, *Myc*, and *Myb*. Their proteins are all involved in cell cycle regulation.

The E2F factor also binds to the Rb and p107 proteins. During the mid to late $G_1$ phase, phosphorylation of Rb (by cyclin D-cdk4 complex) results in the release of E2F from the E2F-Rb complex (Cobrinik *et al.*, 1992; Ewen, 1994). This allows the E2F to bind to the genes mentioned earlier. In the treated MEL cells, the Rb protein

is hypophosphorylated. Other proteins that bind to E2F are cyclins A and E and the cdks. Figure 1.12 shows a diagrammatic form of some of the proteins involved in the MEL cell cycle.

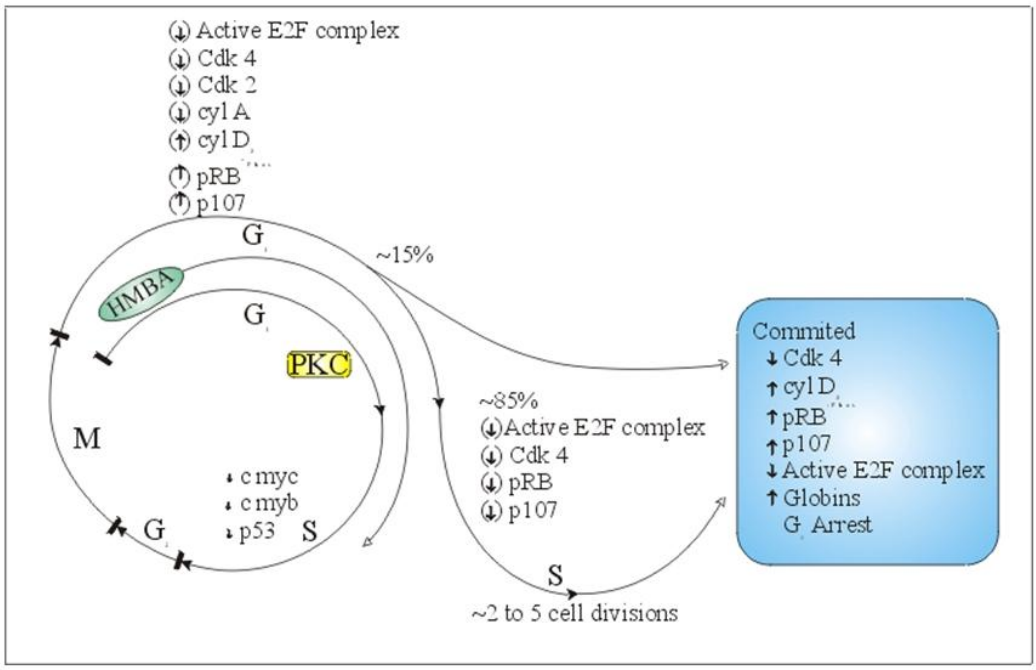



Figure 1.12 Control of the cell cycle in murine erythroleukaemic cells taken from (Marks *et al.*, 1994)

A Schematic representation of HMBA induced changes in MEL cell cycle progression, cyclins (cyl), cdks, pRb, p107, and globin gene expression. (↑), Increased; (↓), decreased. Phos, phosphorylated (Marks *et al.*, 1994).

## 1.5. Objectives of this study

Around 1860 a German microscopist, Johannes Mueller, showed that cancers were made up of cells, a discovery that began the search for changes that would help to pinpoint the specific differences between normal and cancer cells. Since then, cancer has been exhaustively studied in many different disciplines - chemistry, biochemistry, microbiology, molecular biology, genetics, pharmacy, medicine, and immunology. Now, with the rapid advances in computer and biological technology, one can further investigate and obtain a better understanding of the nature of these cells. On a daily basis new information on cancer is being unravelled at an exponential rate.

Cancer has no boundaries. It affects people of all races and ages, and it has been found in almost all types of cells and tissues. Historically, cancer has been difficult to understand for two main reasons. Firstly, the generic term 'cancer' involves dozens of different tumour types, which occur in different tissues, behave in slightly different ways as they grow, and respond differently to surgery, chemotherapy and radiotherapy. Secondly, these cancers seem to be provoked by a host of apparently unrelated causes. For years, this diversity in cause and effect led researchers down dozens of parallel paths and confounded their search for common origins and cures. However, with the revolution in molecular biology this has changed. All cancers can be traced back to damage or changes in DNA that causes cells to ignore signals that normally informs them how to function properly within the tissues of the body.

This research project involves a study of the dynamic behaviour of tumour suppressor proteins during proliferation and induced differentiation of murine erythroleukaemic cells. The study focuses on temporal changes in expression of the

protein phosphatases, protein phosphatase 1 and protein phosphatase 2A, and the p53 protein and it's mRNA; it aims to provide further understanding of the reversal of malignant transformation at the molecular level and the ways in which cellular behaviour can be influenced by modifying the dynamics.

The protein phosphatases are involved in the dephosphorylation of proteins which are phosphorylated by kinases. Phosphatases are believed to be tumour suppressor proteins because of their role in deactivation of certain key proteins (Berndt, 1999). The *p53* gene is an important gene in cancer, it is found mutated in more than 50% of all cancers (Hollstein *et al.*, 1994; Hollstein *et al.*, 1991; Vogelstein, 1990) and has been classified as a tumour suppressor gene (Hollstein *et al.*, 1991; Levine *et al.*, 1991; Oren, 1992; Rotter and Prokocimer, 1991) involved in the critical functioning of the cell. The protein phosphatases and *p53* have important roles in the cell cycle and are connected in a complex web of interaction.

Experimental evidence supports the view that temporal organisation determines the properties and behaviour of cells, and disturbances in these oscillatory patterns with regard to frequencies, amplitudes and phasings could reflect the alteration of cellular activity e.g. the development of differentiation or cancer (Gilbert, 1984; Gilbert, 1974b; Gilbert, D.A. and MacKinnon, H., 1992; Goldbeter, 1996; Goldbeter *et al.*, 2012; Hammond *et al.*, 1998; Hammond *et al.*, 1989b). Previously, studies from this laboratory have demonstrated oscillatory changes in the activities of protein kinase A and protein kinase C (Hammond *et al.*, 1987; Sprott *et al.*, 1991), in the expression of certain isoenzymic forms of protein kinase C (Hammond *et al.*, 2000b), and in the expression of the *Ras* protein and its messenger RNA and c-Raf-1 (Hammond *et al.*, 2000a; Hammond *et al.*, 2000c). Similar behaviour was also noted in the extractable protein content, phosphorylation potential, and the expression of protein kinases and phosphoamino acid phosphatases (Bhoola and Hammond, 2000; Calvert-Evers

and Hammond, 2002; Calvert-Evers and Hammond, 2003; Calvert-Evers and Hammond, 2000; Ferreira *et al.*, 1996c; Ferreira *et al.*, 1994a; Ferreira *et al.*, 1994b; Hammond *et al.*, 1998; Hammond *et al.*, 1989a; Hammond *et al.*, 1989b; Hammond *et al.*, 1985). This investigation extends these studies by examining the patterns of expression of PP1, PP2A, p53 protein and its mRNA under the action of the differentiating agent HMBA in murine erythroleukaemic cells.

In this analysis we look at changes that take place in the frequency, amplitude, difference or phasing of a particular set of rhythms, highlighting the vital role that these rhythmic qualities play in modulating cellular behaviour. The fact that PP1 and PP2A influence many other cellular proteins, makes them crucial in cellular functioning. p53 Which is involved in various cellular defence mechanisms is also critical to cell function. Hence by manipulating the way these proteins act and influence other molecules we could use them to control or adjust certain cellular functions and ultimately reverse diseases associated with cell transformation.

# Chapter 2

# Expression of protein phosphatases 1 and 2A, in proliferating and differentiating murine erythroleukaemic cells

## 2. Summary

This chapter describes the methods used and the results obtained during the investigation of the expression of the important regulatory serine/threonine phosphatases, PP1 and PP2A, in proliferating and differentiating MEL cells. Samples were isolated at different time periods from untreated and HMBA treated MEL cells. Total protein was extracted from the samples and the concentration was determined. Proteins were resolved on sodium dodecyl sulphate (SDS) polyacrylamide gels and then electroblotted onto nitrocellulose membranes. The membranes were blocked and an appropriate primary antibody was added to detect either PP1 or PP2A. The blots were then incubated with a secondary antibody, donkey anti-rabbit antibody, and visualised using diaminobenzidene (DAB). The detected bands were densitometrically scanned and analysed.

Evidence of oscillatory behaviour in the expression of PP1 and PP2A proteins from untreated and HMBA treated MEL cells is presented together with in-depth analysis of the time series experiments. Time dependent variations in expression of PP1 were observed using 15, 30 and 60 minute sampling times. Similar variations were observed for PP2A in which the sampling times of 15, 30, 60, 720 and 1 440 minutes were used. In the PP1 experiments a 33 kDa band was detected. For PP2A,

two bands of 32 and 36 kDa were observed. In general, the patterns of expression of the proteins were complex and there were differences between the effects of HMBA under various conditions.

## 2.1. Methods

### 2.1.1. Materials and equipment

The primary antibodies, anti phosphatase 1 or anti phosphatase 2A, used in this study were obtained from Upstate Biotechnology Incorporated in the USA (refer to Appendix E.1.1 and E.1.2 for more details on the antibodies). The secondary antibody (donkey anti-rabbit Ig secondary antibody IgG linked to horseradish peroxidase) was purchased from Amersham in the United Kingdom Amersham Bioscience. A detailed description of the antibodies can be found in Appendix E. Other reagents and equipment were obtained from standard commercial sources - see Appendix A.

### 2.1.2. Cell culture and the differentiation of murine erythroleukaemic cells

MEL cells purchased from the American Type Culture Collection (ATCC) were used in these experiments. For a full description of the characteristic profile and maintenance of these cells refer to Appendix B. MEL cells were cultured in 250 ml plastic tissue culture flasks in Eagles Minimal Essential Medium (EMEM) with Hanks salts. Heat inactivated foetal calf serum (FCS) 5% v/v and antibiotics (0.27 g/l penicillin and 0.45 g/l streptomycin) were added to the medium (refer to

Appendix B.2 and B.3). The medium was replaced every 2 to 3 days. The tissue culture flasks were replaced every 4 weeks. Cells were examined microscopically on a daily basis and mycoplasma detection were regularly performed (refer to Appendix B.4). Frozen stocks were maintained and re-established as necessary (refer to Appendix B.5). Cultures were maintained in a humidified 5% $CO_2$ atmosphere incubator at 37 °C.

### 2.1.3. Differentiation of murine erythroleukaemic cells

In each experiment, proliferating MEL cells were allowed to grow to a concentration of approximately 1 x $10^7$ cells/ml before they were split into two equal portions; one representing the untreated control (labelled "control") and the other treated with 5 mM HMBA to stimulate the cells to differentiate (labelled "HMBA") (refer to Appendix B.6). These stock cultures had cell concentrations of 1 x $10^5$ to 1 x $10^6$ cells/ml for the experiment. Cell differentiation was tested by the nitroblue tetrazolium technique described by Kamijo and co-workers (Kamijo *et al.*, 1990) (refer to Appendix B.7). Prior to analysis, cell viability was determined by exclusion of 0.2% trypan blue dye and the estimation of the cell population was performed by microscopic examination using a haemocytometer (refer to Appendix B.8 and B.9). The cell viability of the stock culture had to be above 95% before it was used in any of the experiments.

### 2.1.4. Preparation of cell extracts

Stock cultures of 700 ml of untreated and HMBA treated cells were prepared for each experiment. At time 0, HMBA was mixed into one of the stock cultures and the initial extraction of samples (50 ml) was performed. Aliquots of the cultures, of volume 50 ml, were removed from these stocks at various time intervals. Experiments were performed using extraction time intervals of 15, 30 or 60 minutes. The cells were washed 3 times (300 g for 10 minutes) with 0.9% NaCl solution. The pellets were resuspended in 0.1 ml of lysing buffer containing 50 mM Tris-HCl at pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% NP40 and 1 mM PMSF (refer to Appendix C.1). The suspension was left at 4 °C for 30 minutes, after which it was freeze-thawed 3 times and centrifuged at 12 000 g for 30 minutes. The supernatant was collected, and kept frozen for further analysis. This isolation process after the initial centrifugation was carried out on ice.

### 2.1.5. Determining the protein concentration

The protein concentration of samples was determined using the Lowry technique as described by Lowry *et al*. (Lowry *et al.*, 1951). Protein standards (duplicates) of 0.0, 25, 50, 75, 100 and 150 μg of bovine serum albumin (BSA) in 1 ml $H_2O$ were prepared using a 0.1% stock solution. Reagent C (5 ml) (see Appendix C.2) was then added to 1 ml of a 1:100 dilution of each sample in duplicate and to each standard tube. The samples were allowed to stand at room temperature for 10 minutes, after which 0.5 ml of Folin Reagent (50% Folin Ciocalteau in $H_2O$, see Appendix C.2) was added. These samples were left in a dark environment for 30 minutes. The absorbance of each was read at 750 nm using the Beckman DU69 spectrophotometer. This spectrophotometer was programmed to plot the standard curve and calculate the protein concentration of each of the samples.

### 2.1.6. Protein electrophoresis and immunoblot detection

#### 2.1.6.1. Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Protein samples, containing 40 to 80 µg of protein, were mixed with an equal volume of SDS sample loading buffer (0.0625 M Tris/HCl at pH 6.8, 2% SDS, 10% glycerol, 0.5% mercaptoethanol and 0.1% bromophenol blue; see Appendix D.1). These were boiled for 3 minutes. Standard rainbow markers (5 µl) were prepared in a similar manner (refer to appendix D.2 for a listing of components). The sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) procedure was based on the method developed by Laemmli *et al*. (Laemmli, 1970). Samples were loaded onto an SDS-PAGE gel consisting of a 4% stacking and a 10% resolving gel (see Appendix D.3 and D.4). Equal amounts of protein were loaded into each lane on the polyacrylamide gels. The gels were electrophoresed using the Hoefer SE 250 Mighty Small unit. An electrophoresis running buffer consisting of 25 mM Tris-HCl (at pH 8.3), 192 mM glycine and 0.1% w/v SDS was used (see Appendix D.4.3). The proteins were separated at a constant current of 10 mA per gel through the stacking gel and 20 mA per gel through the resolving gel. To evaluate the electrophoresis process initially the gels were stained with Coomassie blue stain (see Appendix D.5.1) for 2 hours on a shaker. Thereafter they were destained with a destain solution (see Appendix D.5.2) for 6 hours on the shaker. The effectiveness of the electrophoresis process was demonstrated by the separation of the proteins into clear bands.

#### 2.1.6.2. Transfer onto nitrocellulose paper

Proteins separated on an electrophoresis gel were transferred to nitrocellulose membrane of 0.22 µm pore size (Hybond-C). The transfer process was carried out in a Hoefer TE 22 Mini transfer tank. The buffer used in this process contained 250 mM Tris, 192 mM glycine in 20% methanol at pH 8.3 (see Appendix D.6). Transfer was at a constant current of 200 mA for 150 minutes, at 4 °C. Initially, the protein transfer process was evaluated by staining the blots overnight with India ink stain (see Appendix D.7). This would demonstrate the efficiency of the transfer process.

#### 2.1.6.3. Immunoblot with anti protein phosphatase 1 and anti protein phosphatase 2A

The following steps were carried out at room temperature using glass petri dishes. The nitrocellulose membranes containing the transferred proteins were blocked overnight in 10 ml of blocking buffer (3% BSA in Tris buffered saline (TBS; 20 mM Tris, 150 mM NaCl, pH 7.4)) with constant agitation, followed by three 5 minute washes in TBS with 0.1% Tween-20 (refer to Appendix E.2). Appropriate primary antibody solution containing PP1 or PP2A at a dilution of 1:100 in 7.5 ml antibody buffer (1% BSA in TBS, see Appendix E.2.7) was placed on the membrane. Both antibodies were rabbit polyclonal IgG antibodies. To test for non-specific antibody binding 7.5 ml of antibody buffer (without primary antibody) was added to a second nitrocellulose membrane that had protein transferred to it and was blocked overnight with blocking buffer. This test was performed initially as a separate experiment to detect any non-specific binding by the secondary antibody. These membranes were incubated overnight at room temperature with agitation. After three 5 minute washes in TBS with 0.1% Tween-20, 7.5 ml of a second antibody (donkey anti-rabbit IgG linked to horseradish peroxidase; see Appendix E.1.3) was added at a 1:3 000 dilution in antibody buffer (1% BSA in TBS). This was agitated for 60 minutes at room temperature. The immunoblots were washed

again as before and developed with a colour development reagent (25 µg diaminobenzidine (DAB) in 50 ml TBS with 50 µl $H_20_2$ (30%)). Once the colour intensity was adequate the reaction was stopped with the addition of distilled water. The blots were rinsed in water, dried and stored in a light free environment. These immunoblots were photographed and scanned using a camera and scanning software from Biomed Instruments Inc., California, USA.

### 2.1.7. Time studies and data analysis

Analyses of the data were done using computer programmes designed by D. A. Gilbert called DATANAL which included AUTOCORR and PERAMP (Bodalina *et al.*, 2005; Bodalina *et al.*, 2007; Calvert-Evers and Hammond, 2002; Calvert-Evers and Hammond, 2003; Tsilimigras and Gilbert, 1977). AUTOCORR was the programme that was run first to capture all the data values and generate the graphs of expression given in terms of the peak area (arbitrary units) of the scanned bands. These data points were plotted as a function of time using a fourth power polynomial fit through the data points. The difference plot for each of the curves was based on calculating the difference between successive points. Where there were sufficient time points the PERAMP programme was used which determined the timing of peaks and troughs in the data and thereby estimated the instantaneous period and amplitude of a rhythm at different times and plotted the data. The mean and range for these period and amplitude values were also calculated and summarised in table form.

It should be noted that as far as possible, experimental conditions were kept the same for each experiment and for the different time experiments i.e. the volume of cells extracted for each sample was 50 ml, the amount of lysing buffer used was

100 µl, the volume of sample loaded on the polyacrylamide gels was 30 µl when using a 10 well comb and 20 µl when using a 15 well comb.

## 2.2. Results for protein phosphatase 1 expression in murine erythroleukaemic cells

### 2.2.1. Immunoblots of protein phosphatase 1

Typical results showing the expression of PP1 are given in figures 2.1 and 2.2. A single band corresponding to a molecular mass of 33 kDa was detected. The position of this band in relation to the position of the rainbow marker bands 30 and 46 kDa is shown in figure 2.1. An example of typical results showing the expression of PP1 isolated from untreated and HMBA treated MEL cells is illustrated in figure 2.2. No bands were detected on the non-specific blot in which the PP1 primary antibody was excluded (results not shown). All the samples isolated at the different time intervals showed time dependent changes for the expression of PP1. Samples were isolated with time sequences of 15, 30, and 60 minutes.

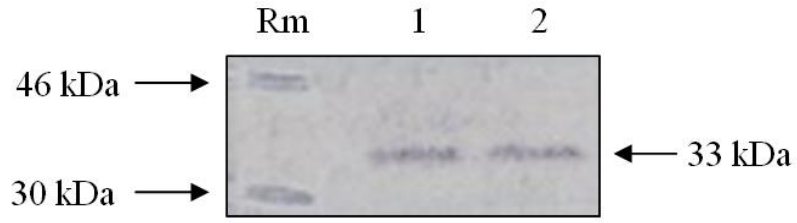



Figure 2.1 Western blot of PP1 in relation to markers

An example of a nitrocellulose blot showing the position of the PP1 band in relation to two marker bands ($M_r$ = 30 and 46 kDa, Rm lane). Equal amounts of protein (58 µg) were loaded in each well. Samples were isolated at 0 time (lane 1) and after 12 hours (lane 2). Rm is the rainbow marker lane.

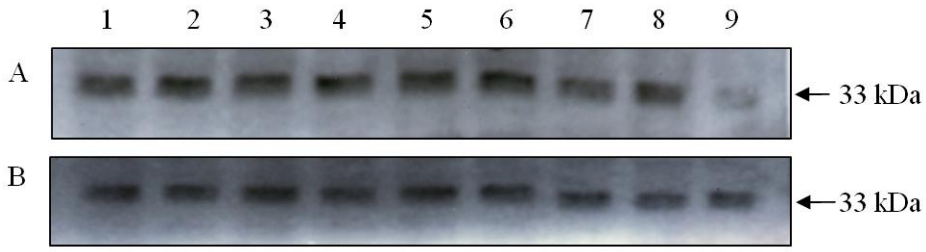



Figure 2.2 Western blot analysis of PP1 from untreated and HMBA treated MEL cells

An example of a typical nitrocellulose blot showing the expression of the PP1 band measured at 15 minute time intervals from untreated (A) and HMBA treated (B) MEL cells. Equal amounts of protein (92 µg) were loaded in each well. Lanes 1 to 9 had protein samples isolated from 0 to 105 minutes at 15 minute intervals.

### 2.2.2. Expression of protein phosphatase 1 at 15 minute time intervals

Three different experiments were prepared in which untreated and HMBA treated MEL cells were isolated at 15 minute time intervals and analysed for PP1 expression. The mean values of PP1 expression for each time point together with the standard deviation were calculated and plotted in graph form (figure 2.3). There was no significant difference in the means between the untreated and the HMBA treated samples. The fitted curves for these samples are shown in figure 2.4 A, B and C. Their corresponding difference curves are depicted on the right (figure 2.4 D, E, and F).

In the first experiment (figure 2.4 A) a dampening effect by HMBA on the expression of PP1 in MEL cells was observed. In contrast, HMBA had a stimulatory effect on the expression in the second experiment (figure 2.4 B). While in the third (figure 2.4 C) there was no marked influence by HMBA. In the first and second experiments, the fitted curves for the untreated and treated samples were out of phase most of the time while the fitted curves in the third experiment were almost in phase. The difference curve (figure 2.4 D, E, and F) of each experiment illustrates the phase relationship between the untreated and HMBA treated cells.

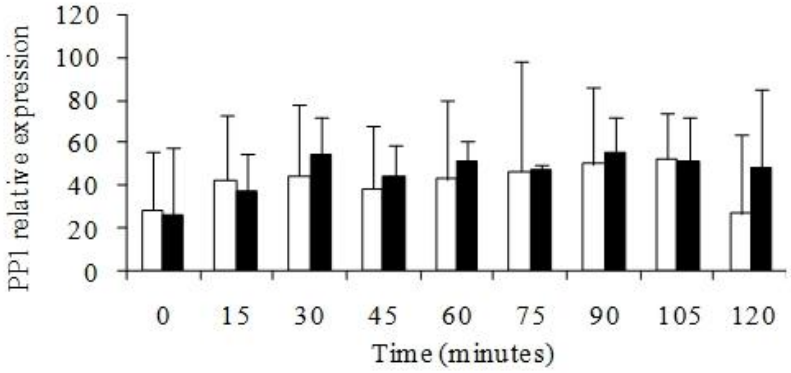



Figure 2.3 Mean values of the expression of PP1 at 15 minute time intervals

Means of the values obtained from densitometric scans of immunoblots of PP1 from three different experiments (refer to figure 2.4). Error bars represent the standard deviation of the mean values for each time interval. No significant differences between the mean values for the control and the HMBA treated cells were noted. □ - Untreated MEL cells. ■ - HMBA treated MEL cells.

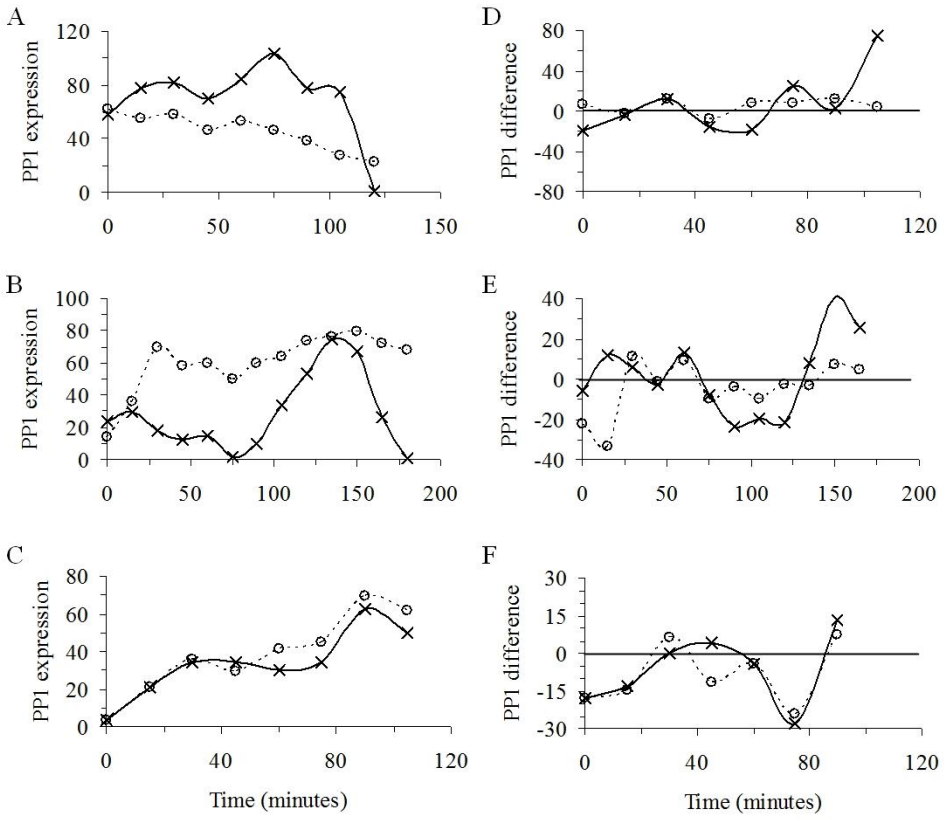



Figure 2.4 Expression of PP1 measured at 15 minute time intervals

Temporal variations in the expression of PP1 measured at 15 minute time intervals in different experiments (A, B, and C). Their respective difference curves (D, E, and F) are included. Proliferating control cells (x—x); cells treated with HMBA (o--o).

### 2.2.3. Expression of protein phosphatase 1 at 30 minute time intervals

Two experiments were performed at 30 minute time intervals to determine the expression of PP1 in MEL cells. The results are illustrated in figure 2.5. As was the case for the 15 minute time intervals, the fitted curves (figure 2.5 A and 2.5 C) varied considerably between the two experiments. In both experiments the period (figure 2.5 B and 2.5 D) and amplitude (figure 2.5 F and 2.5 H) graphs could be obtained. A summary of the range and mean values for the period and amplitude is given in table 2.1.

Some difference in expressional pattern involving a slight phase shift and amplitude change was noted between the untreated and the treated samples (figure 2.5 A). This phase shift was emphasised by the difference curve which confirms the oscillatory behaviour of PP1 in this experiment. The mean period for PP1 (figure 2.5 B) was greater for the untreated samples (82 minutes) than for the treated ones (78 minutes) (see table 2.1). Whereas the mean amplitude for the untreated samples (6.7 arbitrary units) was less than that of the treated samples (9.1 arbitrary units) (see table 2.1).

In the second experiment (figure 2.5 C) a dampening effect by HMBA was noted after 60 minutes indicating inhibition of PP1 expression. An overall increase in expression for PP1 was observed in untreated cells which was emphasised by the greater proportion of the curve below the zero axis in the difference plot (figure 2.5 G). The mean period for PP1 in this experiment was greater for the untreated samples (88 minutes) than for the treated (75 minutes) (see table 2.1). In contrast to the first experiment, the mean amplitude for the untreated samples (10.2 arbitrary units) was greater than that for the treated samples (7.0 arbitrary units) (see table

2.1). The variation of the mean period between the untreated and the treated samples was smaller in the first experiment (4 minutes) compared with the second (14 minutes), similarly the mean amplitude was smaller in the first experiment (2.4 arbitrary units) compared with the second one (3.2 arbitrary units).

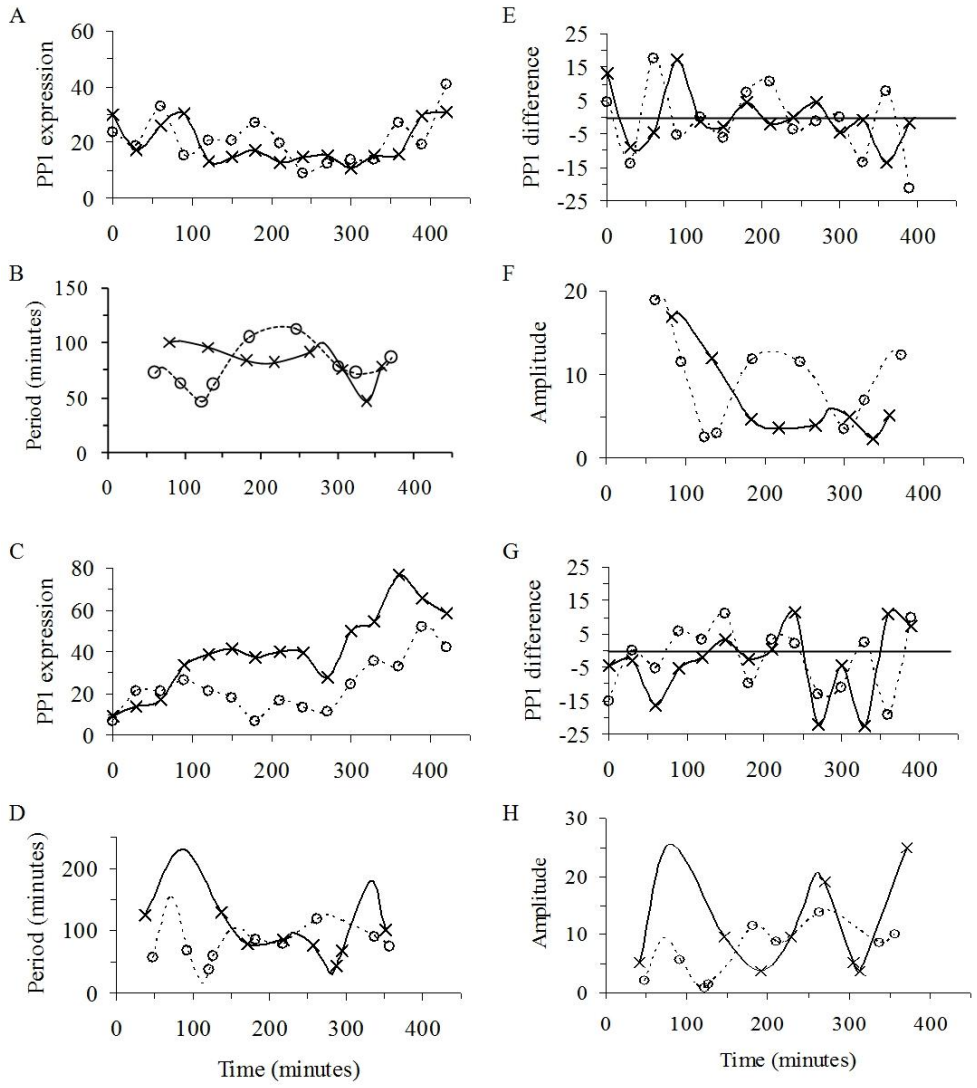



Figure 2.5 Expression of PP1 measured at 30 minute time intervals

Temporal variations in the expression of PP1 as measured at 30 minute time intervals in different experiments (A and C). The respective difference (E and G), period (B and D) and amplitude curves (F and H) are included. Proliferating control cells (x—x); cells treated with HMBA (o--o).

### 2.2.4. Expression of protein phosphatase 1 at 60 minute time intervals

Two experiments were carried out. Samples were isolated from untreated and HMBA treated MEL cells (figure 2.6 A and 2.6 B) at 60 minute intervals. In the first experiment (figure 2.6 A) the HMBA treated cells showed an increase in the expression of PP1. In the second experiment (figure 2.4 B) there was no stimulatory or inhibitory effect of HMBA on PP1 and the curves for the untreated and treated samples were almost in phase with each other. The period and amplitude (figure 2.6 C and 2.6 F) curves were obtained for the second experiment (the range and mean values are summarised in table 2.1). In this experiment the mean period and amplitude for PP1 was less for the untreated samples (132 minutes and 15.42 arbitrary units) than that of the treated samples (189 minutes and 16.8 arbitrary units) (see table 2.1). These period and amplitude values were greater than those obtained for samples isolated at 30 minute intervals.

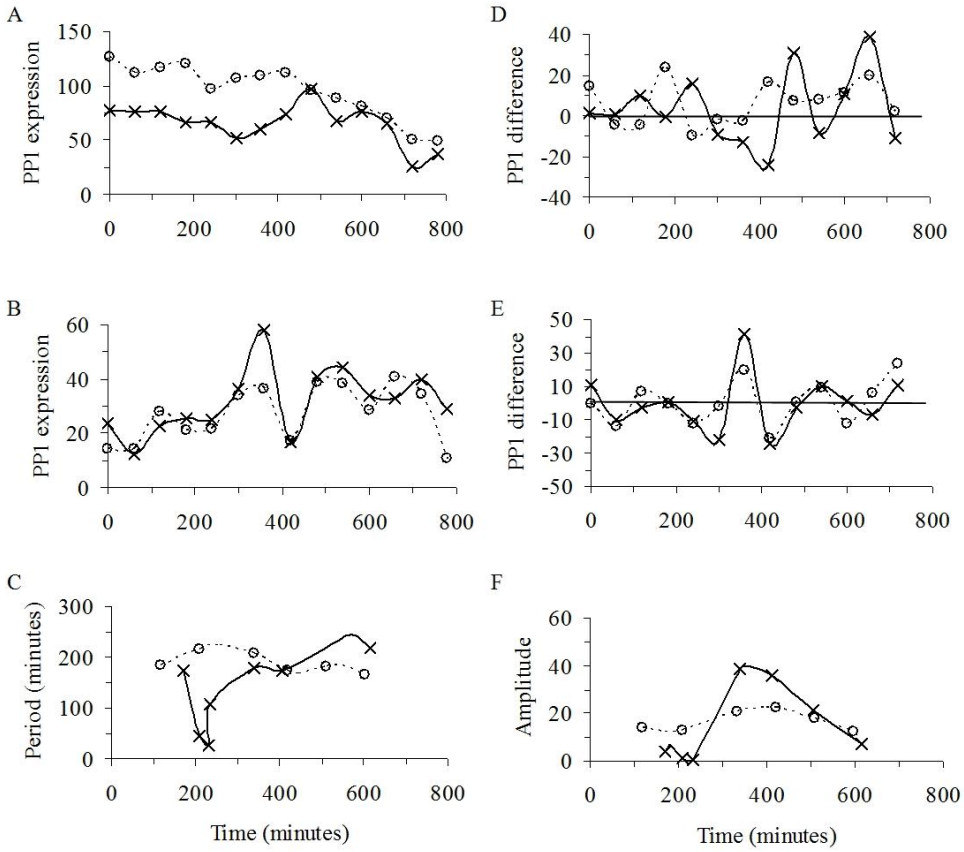



Figure 2.6 Expression of PP1 measured at 60 minute time intervals

Temporal variations in the expression of PP1 as measured at 60 minute time intervals in different experiments (A and B). The corresponding difference curves are included (D and E). Period (C) and amplitude (F) plots were obtained for experiment B. Proliferating control cells (x—x); cells treated with HMBA (o--o).

Table 2.1 Comparisons between the period and amplitude for PP1 expression at different time intervals from untreated and HMBA treated MEL cells

| Experiment | Period (minutes) | | | | Amplitude (arbitrary units) | | | |
|---|---|---|---|---|---|---|---|---|
| | Untreated cells | | HMBA treated cells | | Untreated cells | | HMBA treated cells | |
| | Range | Mean | Range | Mean | Range | Mean | Range | Mean |
| PP1 30 A | 46 - 101 | 82 ± 17 | 47 - 113 | 78 ± 20 | 2.3 - 17.0 | 6.7 ± 5.1 | 2.5 - 18.8 | 9.1 ± 5.5 |
| PP1 30 B | 44 - 129 | 88 ± 29 | 37 - 118 | 75 ± 23 | 3.9 - 25.0 | 10.2 ± 7.8 | 0.9 -13.8 | 7.0 ± 4.7 |
| PP1 60 B | 27 - 219 | 132 ± 74 | 167 - 217 | 189 ± 20 | 0.4 - 38.6 | 15.4 ± 16.5 | 12.6 - 22.4 | 16.8 ± 4.2 |
| | | | | | | | | |

## 2.3. Results for protein phosphatase 2A expression in murine erythroleukaemic cells

### 2.3.1. Protein phosphatase 2A expression using immunoblots

Typical results showing the expression of PP2A are given in figures 2.7, 2.8 and 2.9. Two protein bands were detected using the PP2A antibody, corresponding to molecular masses of 32 kDa and 36 kDa. The position of these bands in relation to the position of the rainbow marker bands 30 and 46 kDa is shown in figure 2.7. No bands were detected on the non-specific blot (results not shown). As was the case with PP1 all the samples isolated at the different time intervals showed time dependent changes for the expression of PP2A. This was emphasised in figure 2.8 where samples isolated from both untreated and HMBA treated MEL cells at 24 hour time intervals were analysed on the same blot. Another example of typical results showing the expression of PP2A isolated from untreated and HMBA treated MEL cells isolated at 30 minute intervals is illustrated in figure 2.9.

Time dependent variations in expression of both the 32 kDa and 36 kDa PP2A subunits were observed at the different time intervals of 15, 30 and 60 minutes including 12 and 24 hours. Representative results are shown in figures 2.11 to 2.24. In general, the patterns of expression for the two PP2A subunits were complex and there were differences between the effects of HMBA in different experiments.

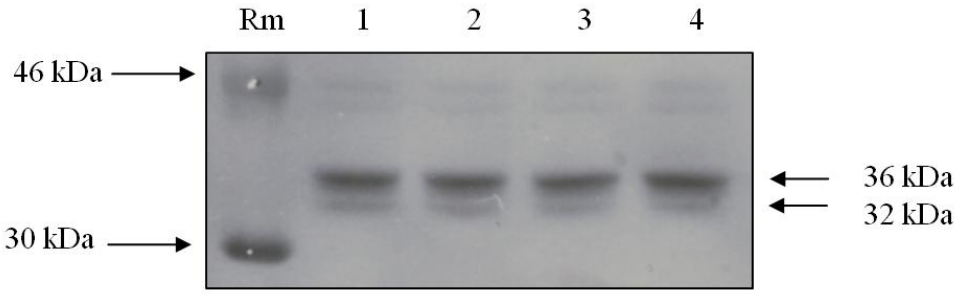



Figure 2.7 Western blot analysis of PP2A with marker bands

An example of a nitrocellulose blot showing the position of immunospecific PP2A bands. Two bands were detected, a 32 kDa and a 36 kDa band. The rainbow marker (Rm) bands were 30 kDa and 46 kDa. Equal amounts of protein (120 µg) were loaded in each well. Lanes 1 to 4, were samples isolated from untreated MEL cells at 30 minute time intervals, starting at 0 time (lane 1).

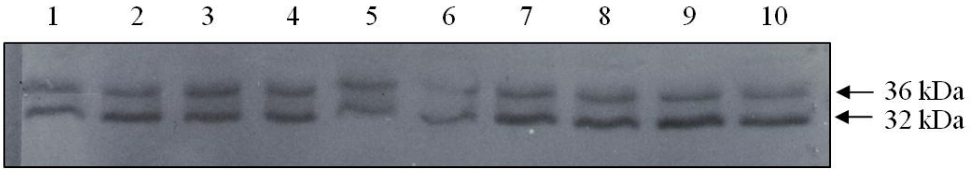



Figure 2.8 Western blot analysis of PP2A measured at 24 hour time intervals

A typical example of a nitrocellulose blot showing the expression of PP2A. Equal amounts of protein (80 µg) were loaded in each well. Samples were taken from untreated (lanes 1, 3, 5, 7 and 9) and HMBA treated (lanes 2, 4, 6, 8 and 10) MEL cells in periods of 24 hours; the lanes on the left represent zero time (lanes 1 and 2).

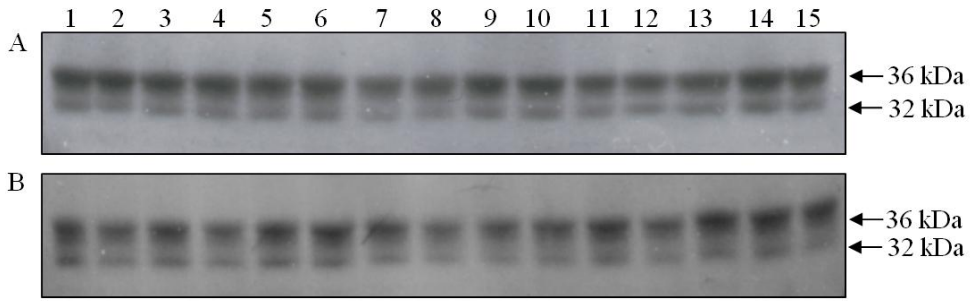



Figure 2.9 Western blot analysis of PP2A from untreated MEL cells

An example of a nitrocellulose blot showing the expression of PP2A measured at 30 minute time intervals from untreated (A) and HMBA treated (B) MEL cells. Equal amounts of protein (80 µg) were loaded in each well. Lanes 1 to 15 represents expression of PP2A from samples isolated at 30 minute intervals (0 to 450 minutes).

### 2.3.2. Expression of protein phosphatase 2A at 15 minute time intervals

The average values of expression of the two PP2A subunits are shown in figure 2.10. Results were obtained from five sets of experiments performed at 15 minute time intervals. As was the case with PP1, no significant difference between the values for the untreated and the treated samples were observed for either of the PP2A subunits. The fitted curves of the 32 kDa band are presented in figure 2.11 and those for the 36 kDa band in figure 2.12. The corresponding difference curves are shown on the right in these figures. For the last two experiments (figure 2.11 D and E for the 32 kDa subunit and figure 2.12 D and E for the 36 kDa subunit) the period and amplitude plots (figure 2.13 for the 32 kDa subunit and figure 2.14 for the 36 kDa subunit) were obtained. The total expression and the ratio of changes between the two subunits are shown in figure 2.15. The total PP2A expression plots (figure 2.15 A, B, C, D and E) were calculated by adding the expression of the 32 kDa band to the 36 kDa band for the 15 minute time interval experiments (figure 2.11 and figure 2.12). The correlation in expression between the two subunits was also determined (figure 2.16).

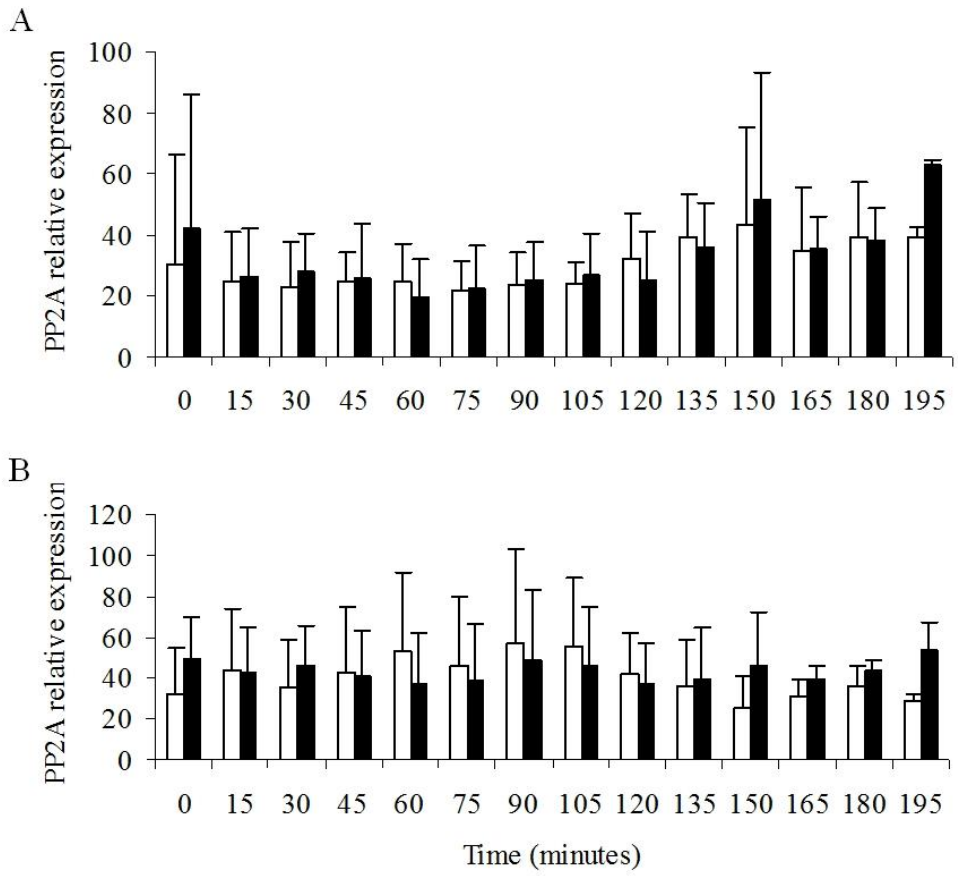



Figure 2.10 Mean values of expression of PP2A measured at 15 minute time intervals for the 32 and 36 kDa subunit

Means values of expression obtained from densitometric scans of immunoblots of PP2A for the 32 (A) and the 36 kDa (B) subunits from five different experiments (refer to figure 2.11). Error bars represent the standard deviation of the mean values for each time interval. No significant difference between the mean values for the control and HMBA treated cells were noted. □ - Untreated MEL cells. ■ – cells treated with HMBA.

In the first three experiments (figure 2.11 A, B and C), the expression of the 32 kDa subunit of PP2A showed oscillatory behaviour that was often out of phase between the untreated and HMBA treated samples. Also, HMBA influenced (inhibitory or stimulatory) the expression of this subunit. In experiments one, three, four and five (figure 2.11 A, C, D and E) the initial values between the untreated and treated samples at time zero were markedly different. In contrast experiment two (figure 2.11 B) had initial values that were similar. In experiment one, the expression of untreated samples generally increased during the experiment while that of the treated samples decreased for the first half of the experiment and then increased. The period of untreated samples was approximately 30 minutes and that of the treated around 120 minutes. The second experiment (figure 2.11 B) showed an initial partial phase shift which started synchronising into phase after 50 minutes. In the third experiment (figure 2.11 C) there was a dampening effect by HMBA after 50 minutes, this extended for the next 90 minutes. The fourth and fifth experiments (figure 2.11 D and 2.11 E) had fitted curves of the untreated and HMBA treated samples almost in phase; with the effect of HMBA on expression being minimal.

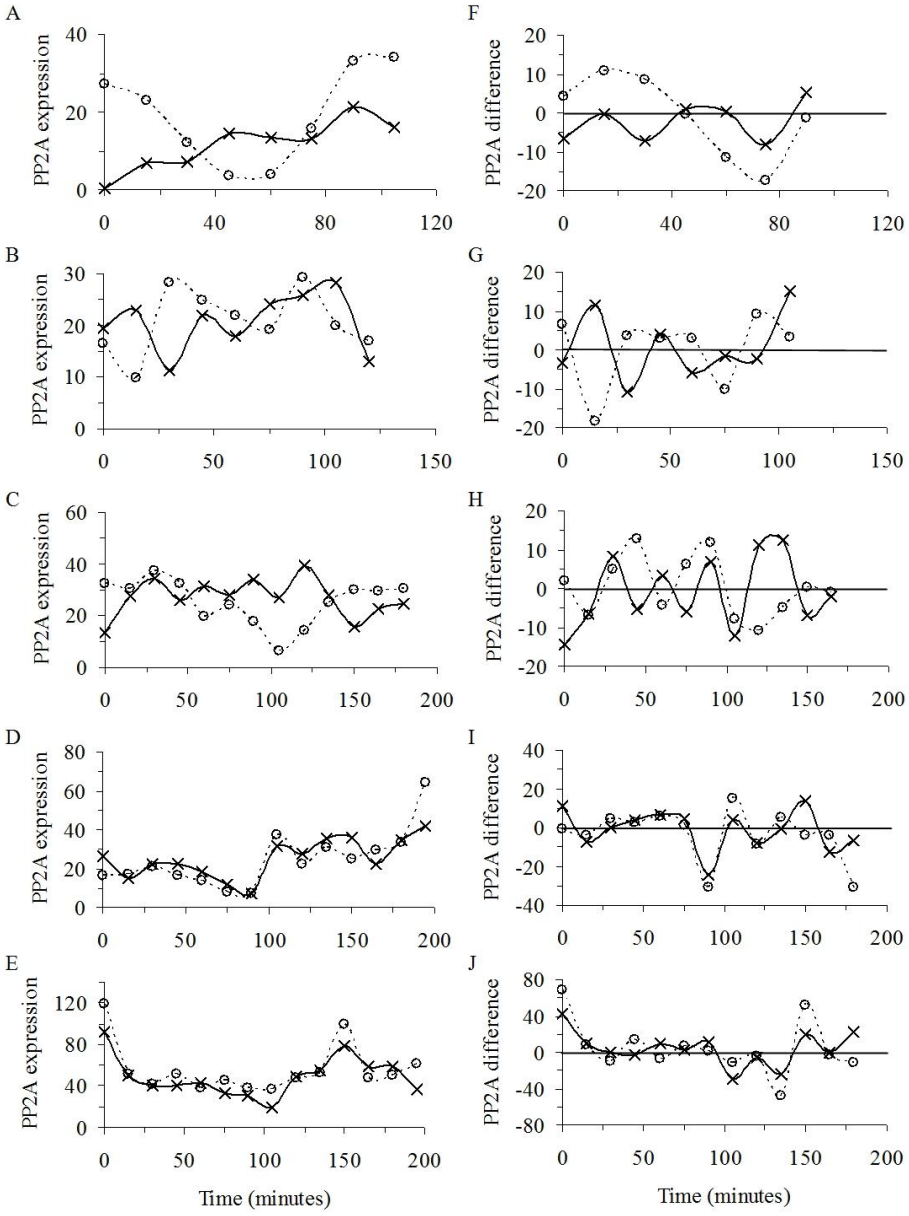



Figure 2.11 Expression of the 32 kDa band of PP2A measured at 15 minute time intervals

Temporal variations in the expression of PP2A 32 kDa subunit as measured at 15 minute time intervals in five different experiments (A, B, C, D and E). The corresponding difference curves are shown on the right (F, G, H, I and J). Proliferating control cells (x—x); cells treated with HMBA (o--o).

The fitted curves of the 32 kDa subunit were different to those of the fitted curves for the 36 kDa subunit. In experiment one (figure 2.12 A), after 30 minutes there was a dampening of expression of the 36 kDa subunit of PP2A by HMBA. In the next experiment (figure 2.12 B) a similar effect was noted after 15 minutes, extending for 1 hour and thereafter the expression synchronised into phase. Experiment three (figure 2.12 C) displayed random oscillatory behaviour with the initial values of the untreated and the treated samples being different by a magnitude of five times. The curves of the untreated and treated samples were almost in phase in the fourth experiment (figure 2.12 D), with HMBA having little influence on the expression compared with the untreated sample. In contrast to experiment one (figure 2.12 A), the fifth experiment (figure 2.12 E) showed that HMBA stimulated the production of the 36 kDa subunit. Experiments one, three and five had initial values of expression that were dissimilar between the untreated and HMBA treated samples. The other two experiments (experiment two and four) had similar starting values of expression.

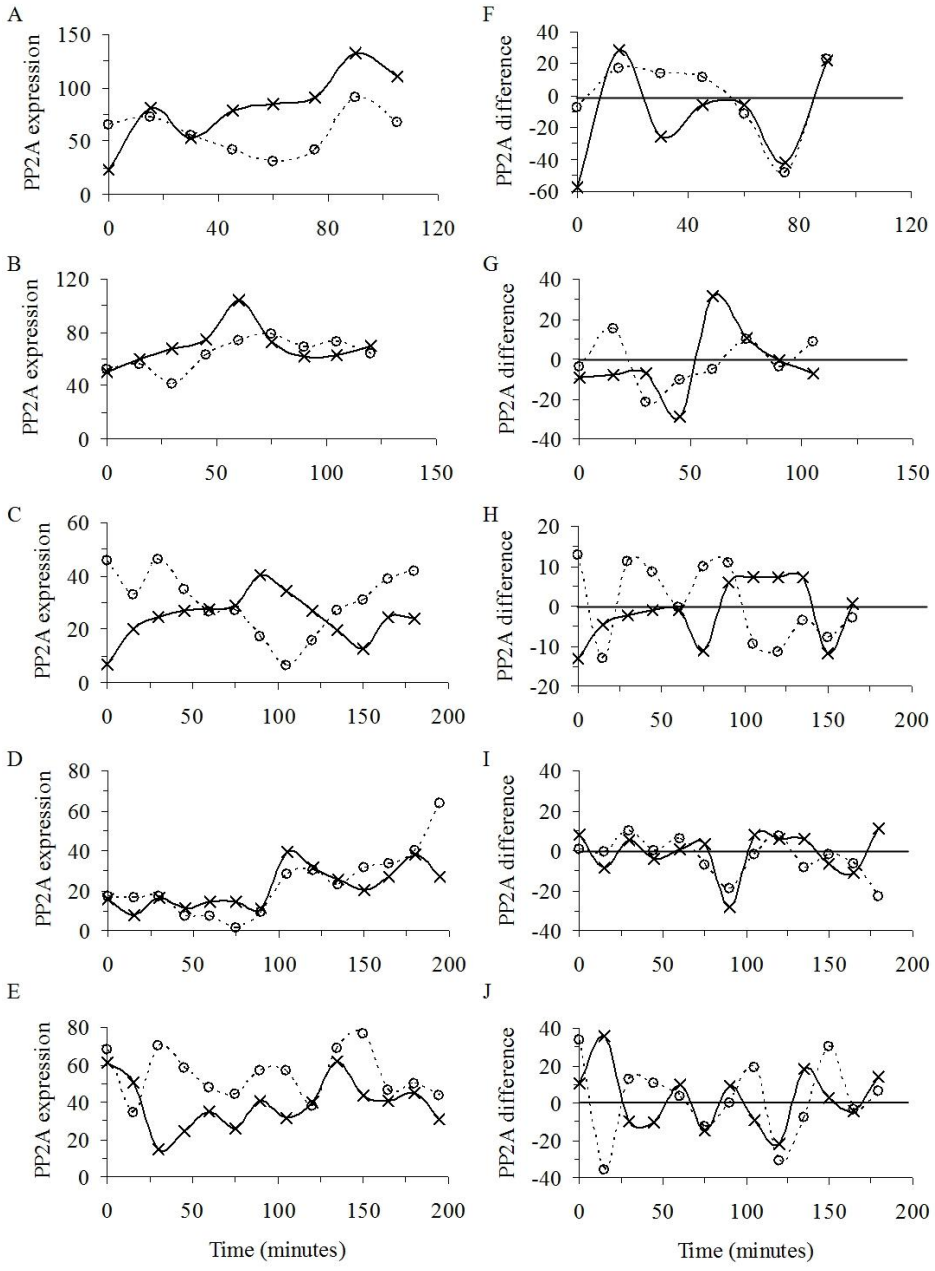



Figure 2.12 Expression of the 36 kDa band of PP2A measured at 15 minute time intervals

Temporal variations in the expression of PP2A 36 kDa subunit as measured at 15 minute time intervals in five different experiments (A, B, C, D and E). The corresponding difference curves are shown on the right (F, G, H, I and J). Proliferating control cells (x—x); cells treated with HMBA (o--o).

The period and amplitude curves for experiment four (figure 2.11 D and 2.12 D) and five (figure 2.11 E and 2.12 E) were obtained for each of the subunits. These are shown in the figure 2.13 for the 32 kDa subunit and figure 2.14 for the 36 kDa subunit. The values for the range and mean of the period and amplitudes are summarised in table 2.2.

For the 32 kDa subunit (experiment four - figure 2.11 D), the fitted curves for the untreated and treated samples were almost synchronised. This effect was further emphasised by the period (figure 2.13 A) and amplitude (figure 2.13 C) curves which were similar for the untreated and treated samples. The mean period for the untreated samples (52 minutes) was higher than that for the treated samples (49 minutes) (see table 2.2). In contrast, the mean amplitude for untreated samples (14.6 arbitrary units) was lower than that for the treated samples (16.3 arbitrary units) (see table 2.2).

Similarly to experiment four, experiment five (figure 2.11 E) showed the fitted curves for the untreated and treated samples to be almost synchronised, but their period (figure 2.13 B) and amplitude (figure 2.13 D) curves were different. The mean period for the untreated samples (42 minutes) was higher than that for the treated samples (37 minutes) (see table 2.2). The mean amplitude for untreated samples (14.6 arbitrary units) was lower than that for the treated samples (21.0 arbitrary units) (see table 2.2). Both these trends in period and amplitude were similar to those observed for experiment four.

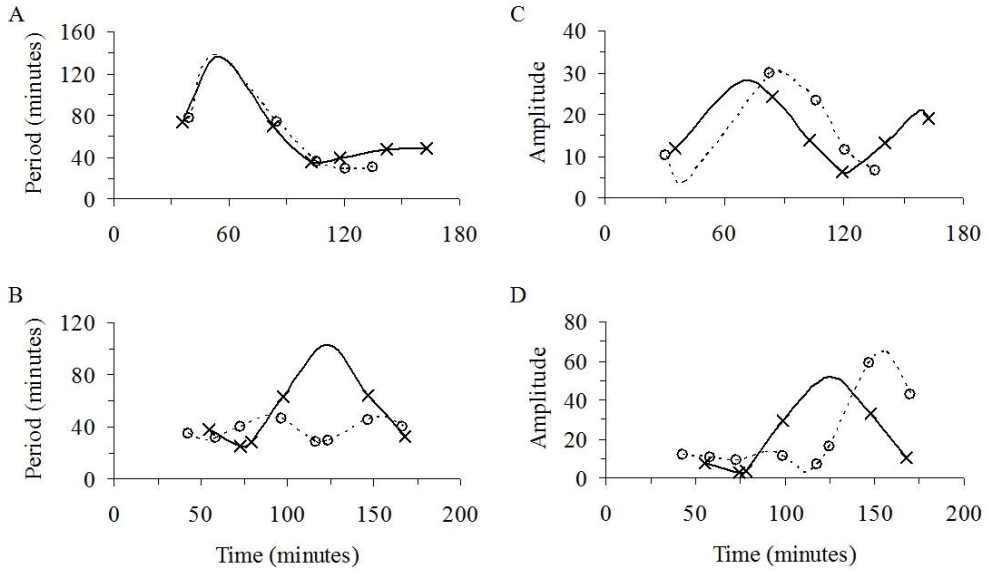



Figure 2.13 Period and amplitude plots for PP2A measured at 15 minute time intervals for the 32 kDa band

Period (A and B) and amplitude (C and D) plots of PP2A for the 32 kDa band. These plots were calculated using the values from figure 2.11 D and 2.11 E, respectively. Proliferating control cells (x—x); cells treated with HMBA (o--o).

The fitted curve patterns between the 32 and the 36 kDa subunits of PP2A were very dissimilar and showed no discernible relationship between each other. This was accentuated by the unrelated period and amplitude curves of experiments four and five. For the 36 kDa subunit (experiment four and five - figure 2.12 D and 2.12 E) the period (figure 2.14 A and 2.14 B) and amplitude (figure 2.14 C and 2.14 D) curves showed similar patterns for the untreated and treated samples, although in experiment five the treated samples had higher values.

For experiment four the mean period and amplitude for the untreated samples (50 minutes and 14.0 arbitrary units) were higher than of the treated samples (44 minutes and 8.9 arbitrary units) (see table 2.2). In experiment five the mean period and amplitude for the untreated samples (42 minutes and 18.7 arbitrary units) were lower than for the treated samples (56 minutes and 24.8 arbitrary units) (see table 2.2).

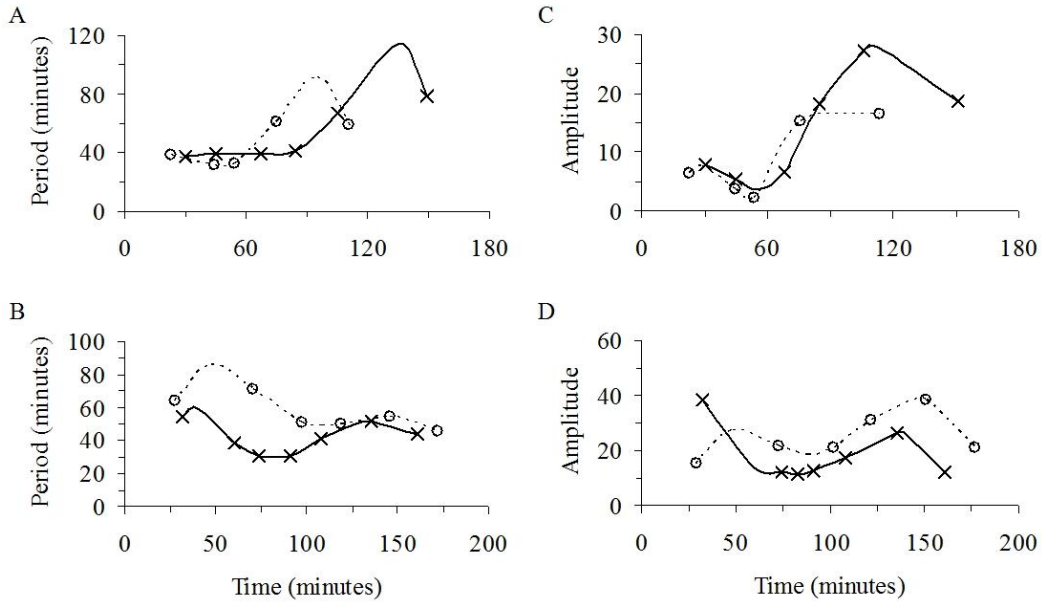



Figure 2.14 Period and amplitude plots for PP2A measured at 15 minute time intervals for the 36 kDa band

Period (A and B) and amplitude (C and D) plots of PP2A for the 36 kDa band. These plots were calculated using the values from figure 2.12 D and 2.12 E, respectively. Proliferating control cells (x—x); cells treated with HMBA (o--o).

Using the values of expression for the two PP2A subunits (figure 2.11 and 2.12), total expression, ratio and correlation curves were plotted. The total expression curves (figure 2.15) were obtained by adding the expression of the two subunits together. The first, third and fifth experiments (figure 2.15 A, C and E) showed oscillatory patterns that were out of phase while the other two (figure 2.15 B and D) exhibited fitted curves that were in phase. From all the experiments, only experiment five showed that HMBA treatment gave rise to increased production of PP2A during the time course (figure 2.15 E).

The ratios of expression of the 32 kDa subunit to the 36 kDa subunit for the 15 minute time study are plotted on the right of each of the total expression curves (figure 2.15 F, G, H, I and J). In the first four experiments (figure 2.15 F, G, H and I) the curves between the untreated and treated samples are relatively out of phase. While in the last experiment (figure 2.15 J) they are in phase for most of the time. These patterns were distinct from those observed for each of the subunits.

An assessment of the influence of the expression of the two subunits on each other was made (figure 2.16). There was no consistency in the relationship between the two subunits and the values ranged from 0.00 to 0.90. Experiment two (figure 2.16 B) showed very little correlation between the subunits from both the untreated and treated samples. Untreated samples from experiment one (figure 2.16 A) and treated samples from experiments three (figure 2.16 C) and four (figure 2.16 D) showed high correlation. There was no distinct overall relationship between the two subunits from these experiments at 15 minute time intervals in both untreated and treated samples.

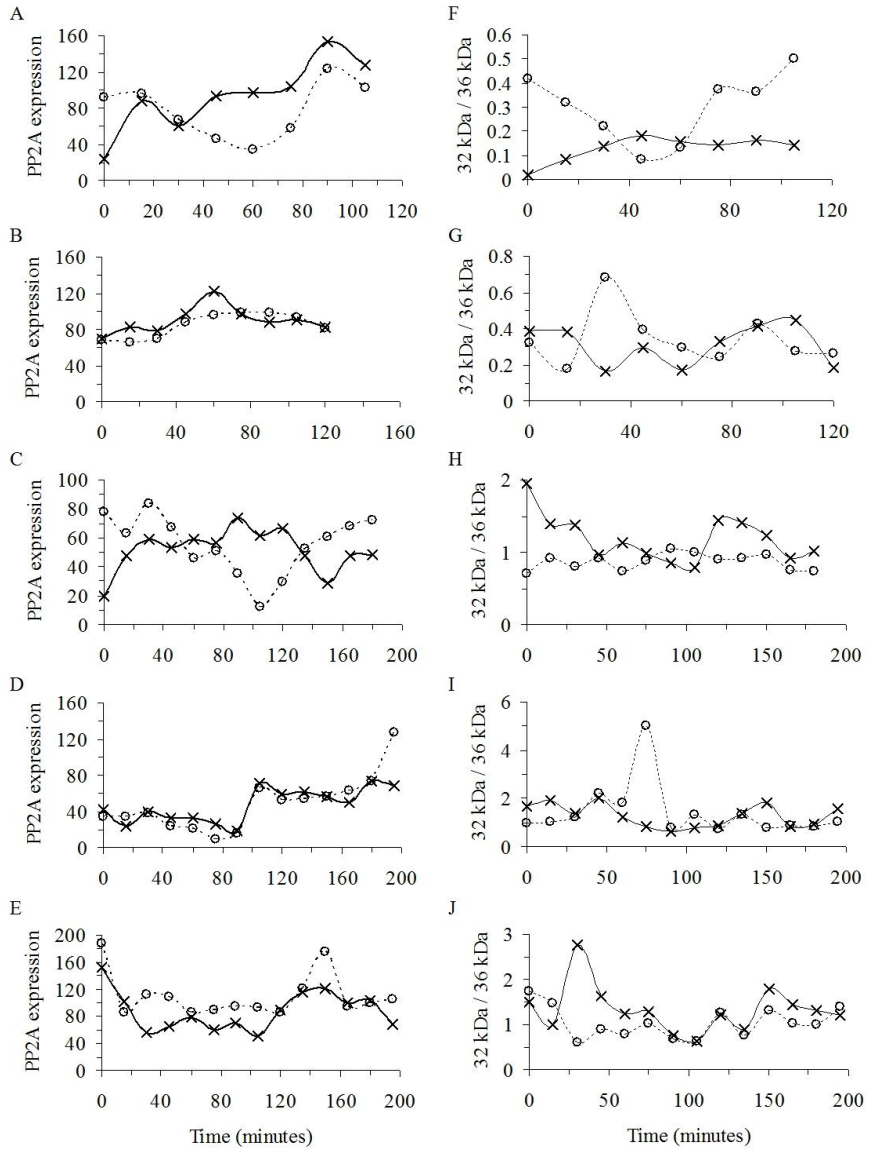



Figure 2.15 Total expression and ratio plots of PP2A for the 15 minute time interval experiments

Total PP2A expression plots (A, B, C, D and E), calculated by adding the expression of the 32 kDa band to the 36 kDa band for the 15 minute time interval experiments (figure 2.11 and figure 2.12). The ratio plots (F, G, H, I and J) of the 32 kDa band to the 36 kDa band for the 15 minute time interval experiments (figure 2.11 and figure 2.12). Proliferating control cells (x—x); cells treated with HMBA (o--o).

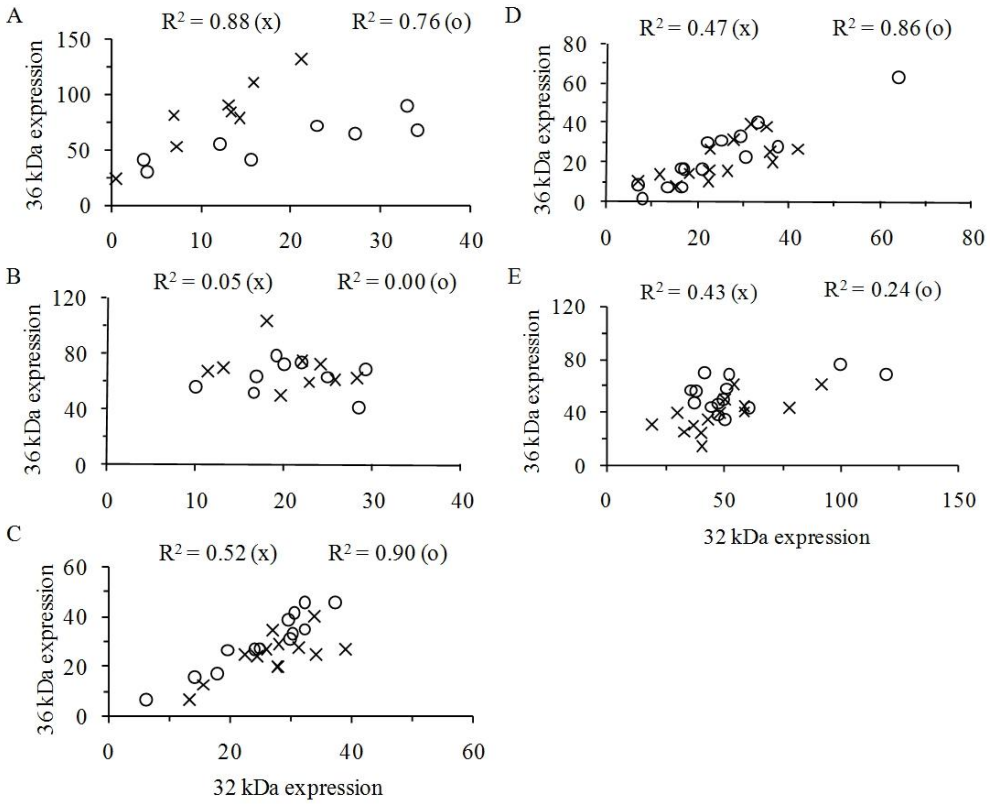



Figure 2.16 Correlation plots of PP2A for the 15 minute time interval experiments

Correlation graphs (A, B, C, D and E) comparing the expression of the 32 kDa band with the 36 kDa band of PP2A for the 15 minute time interval experiments (figure 2.11 and figure 2.12). Proliferating control cells (x—x); cells treated with HMBA (o--o).

### 2.3.3. Expression of protein phosphatase 2A at 30 minute time intervals

Expression of PP2A at 30 minute time intervals is presented in figures 2.17 and 2.18 corresponding to the 32 kDa and the 36 kDa subunits respectively. Two experiments were performed and the period and amplitude curves (figure 2.17 C, F and figure 2.18 C, F) were calculated for the second experiment.

In the first experiment (figure 2.17 A) for the 32 kDa subunit the expression between untreated and treated samples was initially in phase up to the first 100 minutes then out of phase for the next 100 minutes and remained in phase up to the end of the experiment. In the second experiment (figure 2.17 B) both curves seemed to be in phase with HMBA having a stimulatory effect on the subunit expression.

The period and amplitude curves for the untreated and the treated samples were in phase with the values for the treated samples being higher. The mean period for this subunit was greater in the untreated samples (79 minutes) than that for the treated samples (73 minutes) and the mean amplitude for the untreated samples (5.6 arbitrary units) was less than that for the treated samples (12.7 arbitrary units) (see table 2.2).

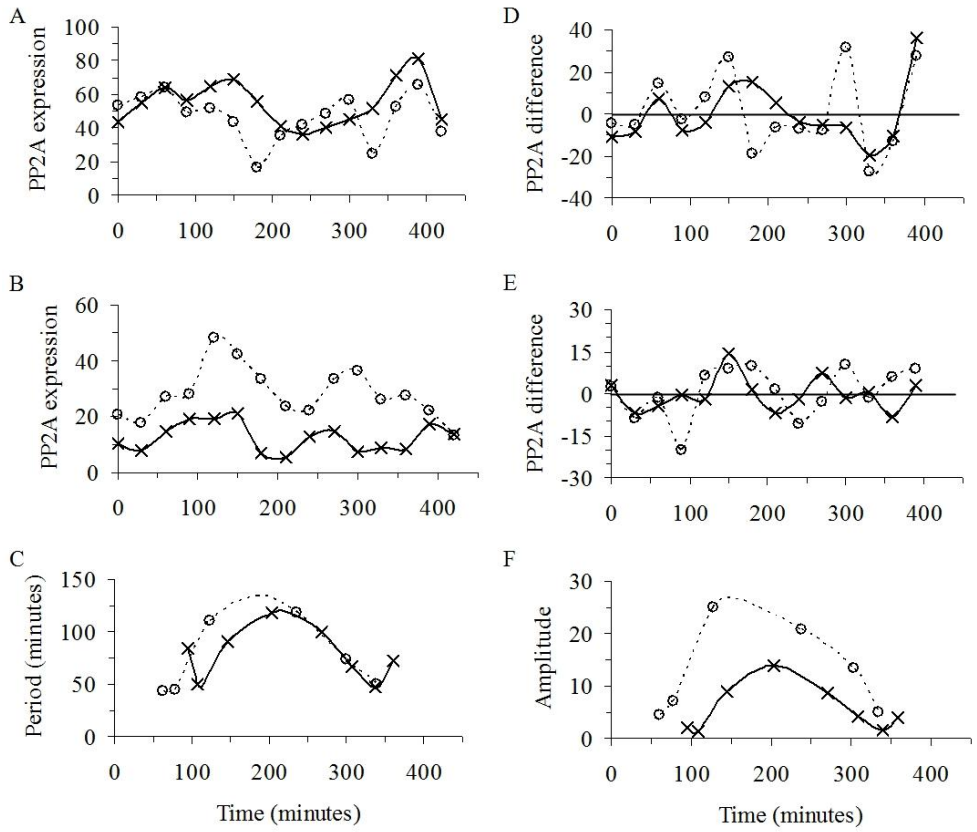



Figure 2.17 Expression of the 32 kDa band of PP2A measured at 30 minute time intervals

Temporal variations in the expression of PP2A 32 kDa subunit as measured at 30 minute time intervals in two different experiments (A and B). The corresponding difference curves are shown on the right (D and E). Period (C) and amplitude (F) plots were obtained for experiment B. Proliferating control cells (x—x); cells treated with HMBA (o--o).

As noted for the 15 minute time periods, the trends in expression for the 32 kDa subunit were different from those observed for the 36 kDa subunit. There was a stimulatory effect by HMBA on the treated cells in the first experiment (figure 2.18 A). Both experiments (figure 2.18 A and B) had oscillatory behaviour that was mostly out of phase with an occasional in phase section. The period (figure 2.18 C) and amplitude (figure 2.18 F) curves formed a smooth single wave shaped curve for the untreated samples and an oscillatory curve for the treated samples. As was the case with the 32 kDa subunit, the mean period for the 36 kDa subunit was greater in the untreated samples (97 minutes) than that for the treated samples (62 minutes) (see table 2.2). In contrast to the 32 kDa subunit the mean amplitude for the untreated samples (11.7 arbitrary units) was greater than that for the treated samples (9.8 arbitrary units) (see table 2.2). These values of mean period and amplitude for both the 32 kDa and the 36 kDa subunits were similar to those of PP1 isolated at 30 minute (section 2.2.3) time intervals but were less than those for the 60 minute PP1 (section 2.2.4) samples.

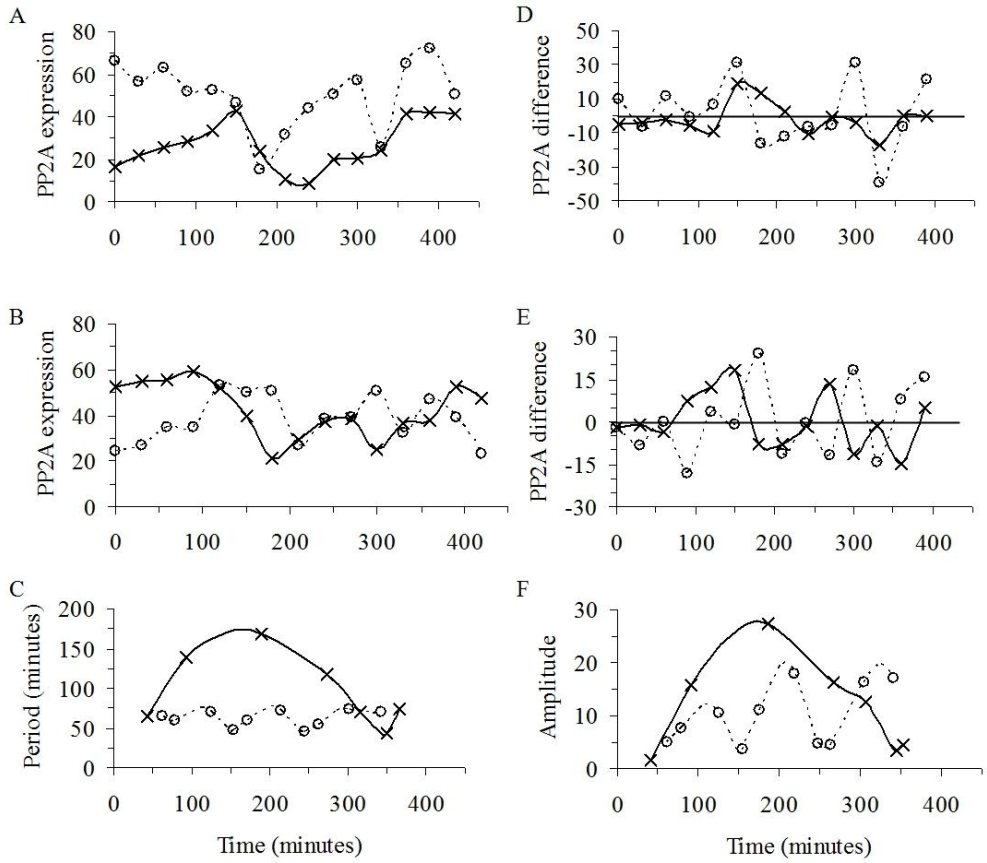



Figure 2.18 Expression of the 36 kDa band of PP2A measured at 30 minute time intervals

Temporal variations in the expression of PP2A 36 kDa subunit as measured at 30 minute time intervals in two different experiments (A and B). The corresponding difference curves are shown on the right (D and E). Period (C) and amplitude (F) plots were obtained for experiment B. Proliferating control cells (x—x); cells treated with HMBA (o--o).

For the 30 minute time period, the total expression, ratio plots together with the correlation curves are shown in figure 2.19. The first experiment (figure 2.19 A) of PP2A showed random behaviour while in the second (figure 2.19 B) the fitted curves were almost in phase with HMBA having a stimulatory effect. With regard to the ratio plots a clear overall influence was seen on comparing the untreated and treated samples. In the first experiment (figure 2.19 D) HMBA had an inhibitory influence over the ratio of expression. An opposite effect was noticed in the second experiment (figure 2.19 E) where the influence of HMBA was stimulatory. The correlation (figure 2.19 C and F) between the two subunits showed varying influence with the treated samples in experiment one having the highest correlation at 0.89.

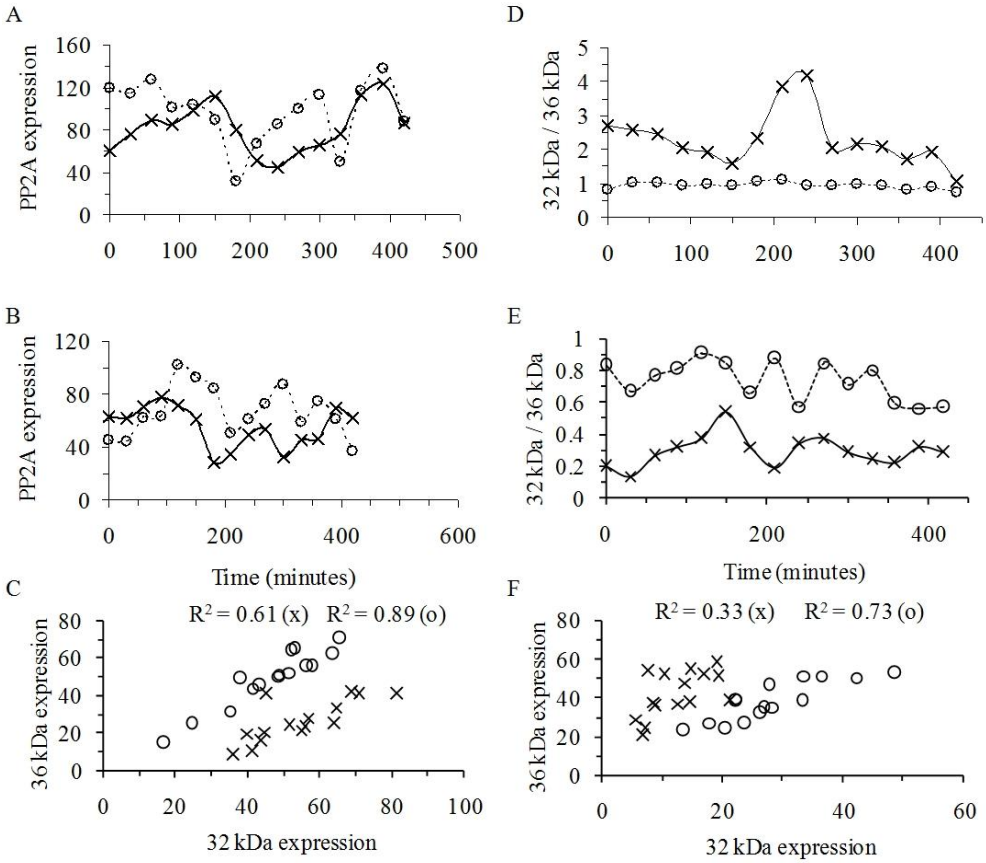



Figure 2.19 Total expression, ratio and correlation plots of PP2A for the 30 minute time interval experiments

Total expression plots (A and B) were calculated by adding the expression of the 32 kDa band to the 36 kDa band. The ratio plots (D and E) of the 32 kDa band to the 36 kDa band. Correlation graphs (C and F) comparing the expression of the 32 kDa band with the 36 kDa band of PP2A. Values were obtained from experiments for the 30 minute time intervals (figures 2.16 and 2.17). Proliferating control cells (x—x); cells treated with HMBA (o--o).

Table 2.2 Comparisons between the period and amplitude for PP2A expression at different time intervals from untreated and HMBA treated MEL cells

| Experiment | Period (minutes) | | | | Amplitude (arbitrary units) | | | |
|---|---|---|---|---|---|---|---|---|
| | Untreated cells | | HMBA treated cells | | Untreated cells | | HMBA treated cells | |
| | Range | Mean | Range | Mean | Range | Mean | Range | Mean |
| PP2A 15 D 32 | 35 - 74 | 52 ± 16 | 29 - 77 | 49 ± 22 | 6.3 - 24.1 | 14.6 ± 6.2 | 6.5 - 30.0 | 16.3 ± 8.9 |
| PP2A 15 D 36 | 37 - 79 | 50 ± 18 | 31 - 61 | 44 ± 15 | 5.4 - 27.3 | 14.0 ± 8.8 | 2.3 - 16.6 | 8.9 ± 6.6 |
| PP2A 15 E 32 | 25 - 64 | 42 ± 17 | 28 - 46 | 37 ± 7 | 2.9 - 32.9 | 14.6 ± 13.3 | 6.8 - 59.0 | 21.0 ± 19.1 |
| PP2A 15 E 36 | 31 - 54 | 42 ± 9 | 46 - 71 | 56 ± 10 | 11.5 - 38.3 | 18.7 ± 10.2 | 15.2 - 38.6 | 24.8 ± 8.5 |
| PP2A 30 B 32 | 47 - 118 | 79 ± 24 | 43 - 118 | 73 ± 34 | 1.3 - 14.0 | 5.6 ± 4.5 | 4.6 - 25.0 | 12.7 ± 8.7 |
| PP2A 30 B 36 | 44 - 168 | 97 ± 45 | 45 - 74 | 62 ± 10 | 1.7 - 27.3 | 11.7 ± 9.2 | 3.6 - 18.0 | 9.8 ± 5.7 |
| | | | | | | | | |

### 2.3.4. Expression of protein phosphatase 2A at 60 minute time intervals

Two experiments showing the expression of PP2A when analysed at 60 minute time intervals are presented in figures 2.20 and 2.21, corresponding to the 32 kDa and the 36 kDa subunits respectively. In the first experiment (figure 2.20 A), an inhibitory effect of HMBA on the expression of the 32 kDa subunit was noted. While in the second experiment (figure 2.20 B), a stimulatory effect by HMBA was observed with the oscillatory patterns being in phase. In the first experiment (figure 2.21 A) for the 36 kDa subunit, no difference between the patterns in the untreated and the HMBA treated samples could be perceived. In the second experiment (figure 2.21 B) the curves were in phase and no major influence was exhibited by HMBA.

Similarly to the fitted curves for the individual subunits, the fitted curves for total expression (figure 2.22 A and B) of PP2A were in phase in the untreated and the treated samples; HMBA had little effect. The ratio plot in the first experiment (figure 2.22 D) showed an inhibitory effect of HMBA whereas there was no distinguishable effect in the second experiment (figure 2.22 E). The correlation curve (figure 2.22 C) for both the treated and untreated samples showed high correlation in the first experiment and less in the second (figure 2.22 F).

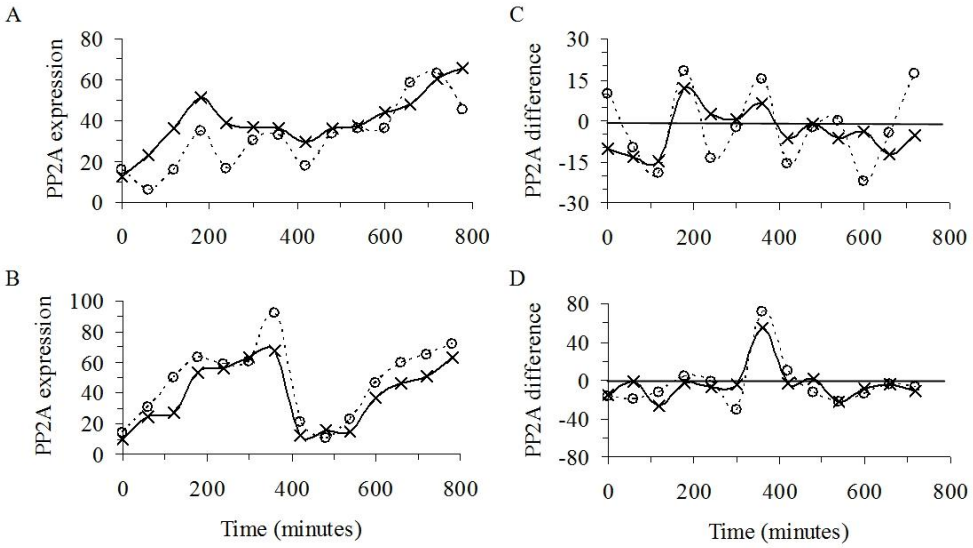



Figure 2.20 Expression of the 32 kDa band of PP2A measured at 60 minute time intervals

Temporal variations in the expression of PP2A 32 kDa subunit as measured at 60 minute time intervals in two different experiments (A and B). The corresponding difference curves are shown on the right (C and D). Proliferating control cells (x—x); cells treated with HMBA (o--o).

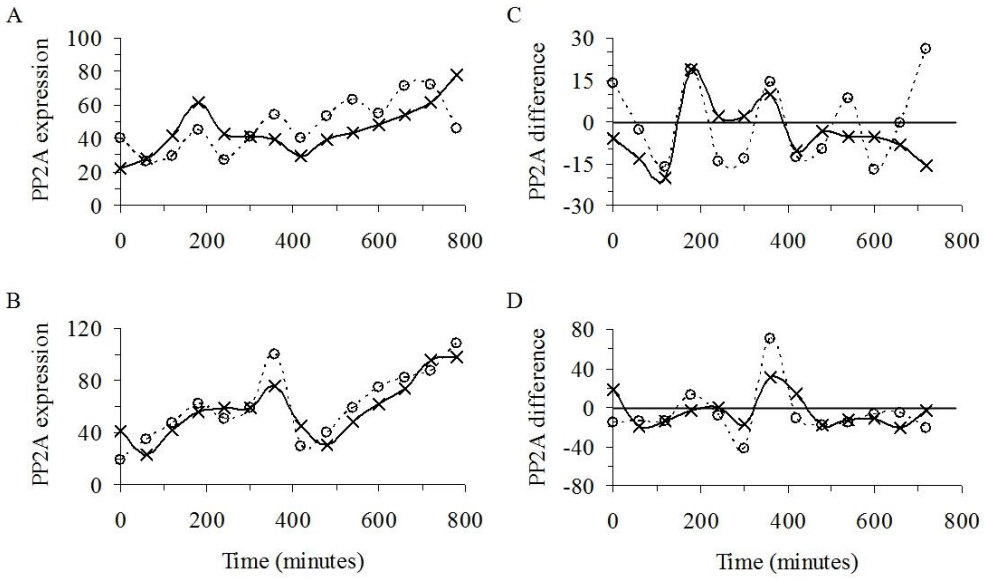



Figure 2.21 Expression of the 36 kDa band of PP2A measured at 60 minute time intervals

Temporal variations in the expression of PP2A 36 kDa subunit as measured at 60 minute time intervals in two different experiments (A and B). The corresponding difference curves are shown on the right (D and E). Proliferating control cells (x—x); cells treated with HMBA (o--o).

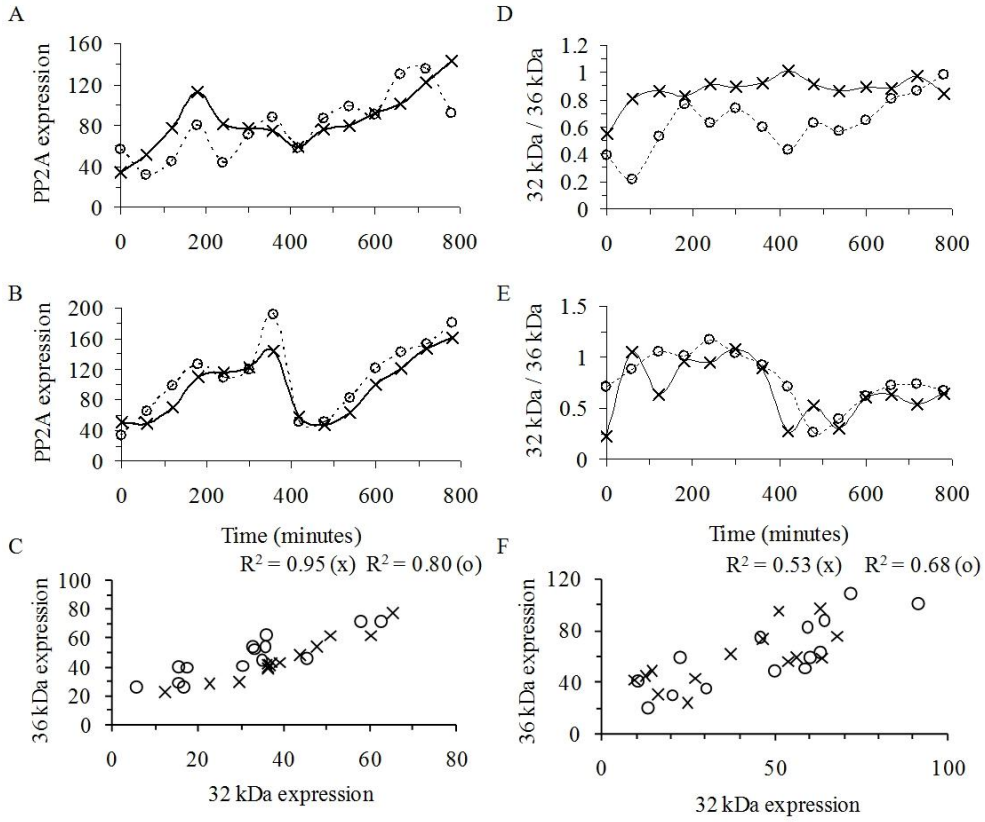



Figure 2.22 Total expression, ratio and correlation plots of PP2A for the 60 minute time interval experiments

Total expression plots (A and B) calculated by adding the expression of the 32 kDa band to the 36 kDa band. The ratio plots (D and E) of the 32 kDa band to the 36 kDa band. Correlation graphs (C and F) comparing the expression of the 32 kDa band with the 36 kDa band of PP2A. Values were obtained from experiments for the 60 minute time intervals (figures 2.20 and 2.21). Proliferating control cells (x—x); cells treated with HMBA (o--o).

### 2.3.5. Expression of protein phosphatase 2A at 12 and 24 hour time intervals

Two experiments measuring the expression of PP2A at time intervals of 12 hours (figure 2.23) and 24 hours (figure 2.24) were performed. For the 12 hour period, the fitted curves for the untreated and the treated samples for both the 32 kDa (figure 2.23 A) and the 36 kDa (figure 2.23 B) subunits were in phase, with HMBA having a small inhibitory influence on the subunits. The total expression (figure 2.23 C) and ratio plots (figure 2.23 G) emphasised what was noted with the fitted curves for each of the subunits. The correlation (figure 2.23 D) curve shows high correlation between the expression of the two subunits.

For the 24 hour experiment, the fitted curves for the untreated and the treated samples for both the 32 kDa (figure 2.24 A) and the 36 kDa (figure 2.24 B) subunits were partially out of phase, with HMBA having a small inhibitory influence on the subunits. A similar trend was noted for the total expression (figure 2.24 C) of PP2A. The ratio plot (figure 2.24 G) shows the curves as being totally out of phase. The correlation (figure 2.24 D) curve shows high correlation between the expression of the two subunits, with the treated samples being 0.99, the highest from all the experiments.

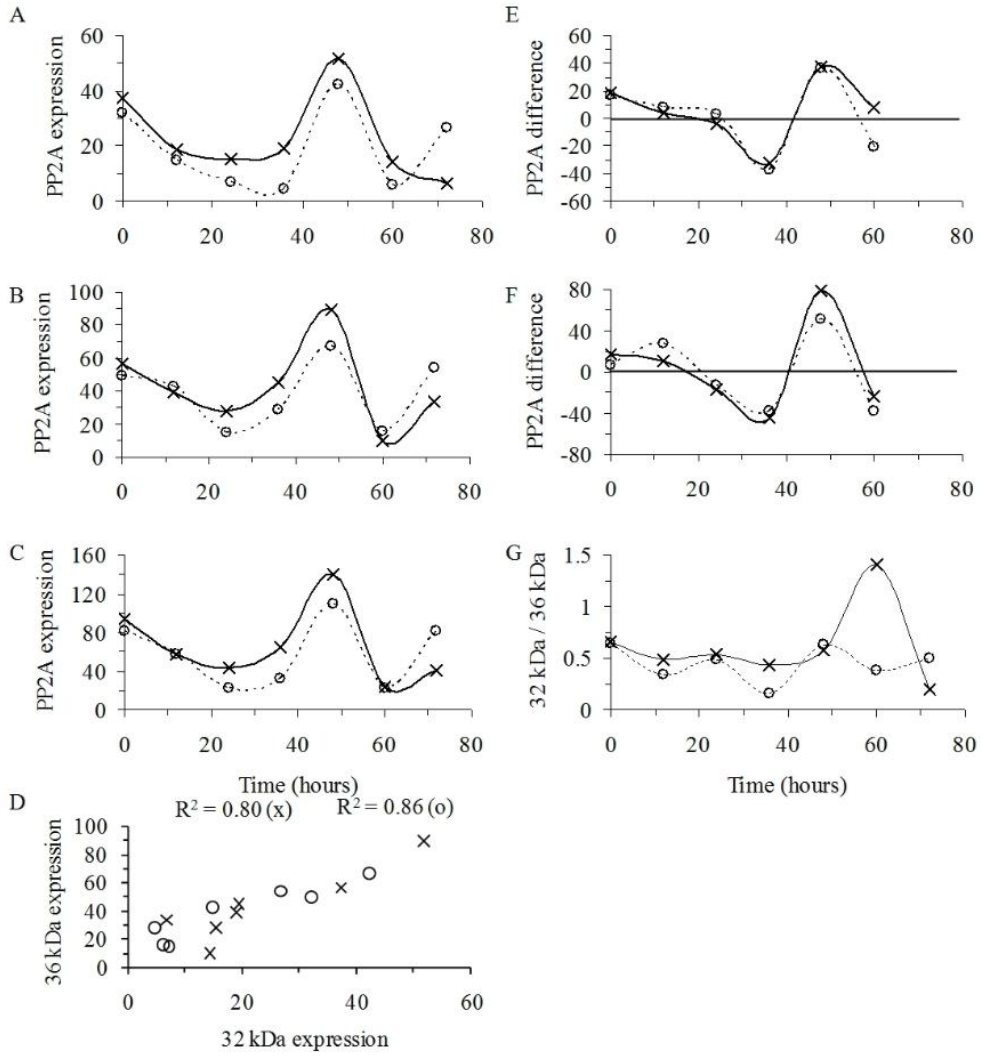



Figure 2.23 Expression, total expression, ratio and correlation of the 32 kDa and 36 kDa band of PP2A measured at 12 hour time intervals

Temporal variations in the expression of the 32 (A) and 36 kDa (B) subunits of PP2A as measured at 12 hour time intervals. The corresponding difference curves are shown on the right (E and F). Total expression plot (C) calculated by adding the expression of the 32 kDa band to the 36 kDa band. The ratio plot (G) of the 32 kDa band to the 36 kDa band of PP2A. The correlation graph (D) comparing the expression of the 32 kDa band with that of the 36 kDa band of PP2A. Proliferating control cells (x—x); cells treated with HMBA (o--o).

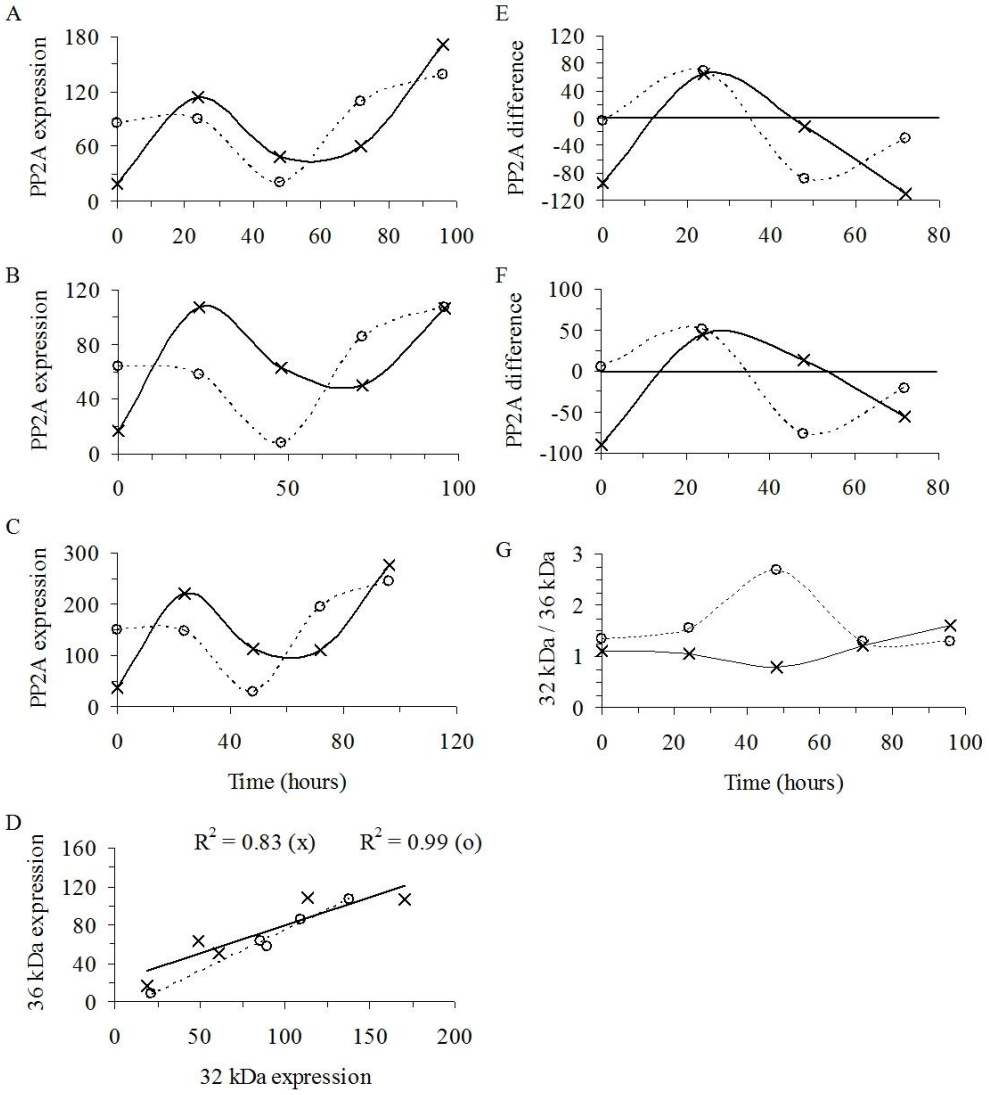



Figure 2.24 Expression, total expression, ratio and correlation of the 32 kDa and 36 kDa band of PP2A measured at 24 hour time intervals

Temporal variations in the expression of the 32 (A) and 36 kDa (B) subunits of PP2A as measured at 24 hour time intervals. The corresponding difference curves are shown on the right (E and F). Total expression plot (C) calculated by adding the expression of the 32 kDa band to the 36 kDa band. The ratio plot (G) of the 32 kDa band to the 36 kDa band for PP2A. The correlation graph (D) comparing the expression of the 32 kDa band with that of the 36 kDa band of PP2A. Proliferating control cells (x—x); cells treated with HMBA (o--o).

## 2.4. Discussion

This study of temporal variations in expression of two major serine/threonine dephosphorylating enzymes, PP1 and PP2A, during differentiation of MEL cells, is a continuation of earlier work on cell dynamics and the expression of phosphoprotein phosphatases (Bhoola and Hammond, 2000). In those experiments the expression of PP1, PP2A and protein tyrosine phosphatase-1B (PTP-1B) protein were shown to be dynamically regulated in proliferating human acute promyelocytic leukaemic (HL-60) cells and modulated after being induced to differentiate along the macrophage pathway using phorbol-12-myristate-13-acetate and along the granulocytic pathway using dimethyl sulphoxide, all-trans retinoic acid and 9-cis retinoic acid (Bhoola and Hammond, 2000). The study also reported similar behaviour with PTP-1B mRNA using northern blot analysis. In the present study, the expression of PP1 and PP2A was studied in MEL cells under the influence of HMBA; dynamic behaviour was demonstrated which was consistent with the varying activities of proteins in the cell and other similar studies (Bhoola and Hammond, 2000; Bodalina *et al.*, 2005; Bodalina *et al.*, 2007; Brodsky *et al.*, 2012a; Brodsky *et al.*, 2012b; Calvert-Evers and Hammond, 2002; Calvert-Evers and Hammond, 2003; Ferreira *et al.*, 1996b; Ferreira *et al.*, 1994a; Ferreira *et al.*, 1994b; Hammond *et al.*, 1998; Hammond *et al.*, 1989b; Hammond *et al.*, 1985).

Like many other proteins in the cell, the PP1 and PP2A enzymes both have multimeric structures consisting of a catalytic subunit interacting with an array of regulatory subunits (Cohen, 1989; Mumby and Walter, 1993). These regulatory subunits modulate the activity of the catalytic subunit so as to perform a specific function in a particular region of the cell (Sim and Ludowyke, 2002). It is this combination of a single catalytic unit binding with multiple regulatory subunits that make these proteins ubiquitous with regard to dephosphorylating target proteins.

In these experiments in which MEL cell preparations were probed for PP1, a 33 kDa immunoreactive protein was detected; this most likely correspond to a form of PP1c, a 37 kDa protein transformed during purification to a 33 kDa unit through loss of approximately 30 residues from the C-terminus (Cohen, 1989; Silberman *et al.*, 1984; Tung *et al.*, 1984). The core enzyme of PP2A exists as a heterotrimer consisting of a conserved catalytic subunit (C), a conserved scaffold subunit (A) and a variable B subunit (Virshup, 2000). The B subunit comprises three main families, each with multiple isoforms which bestow modulation and specificity towards PP2A (Mayer-Jaekel and Hemmings, 1994; Sim and Ludowyke, 2002). Extracts prepared from MEL cells, when probed for PP2A, showed two immunoreactive bands corresponding to molecular masses of 32 and 36 kDa. The 36 kDa band most probably represents the catalytic subunit; this mass corresponds to that reported for the catalytic subunit of PP2A from rabbit skeletal muscle (Tung *et al.*, 1984). A 33.5 kDa proteolytically nicked form was also isolated (Paris *et al.*, 1984; Silberman *et al.*, 1984; Tung *et al.*, 1985), and we suggest that our 32 kDa band might represent a similar cleaved species of the catalytic subunit.

The mean values of expression for PP1 and PP2A were calculated, there were no significant differences between time points or between untreated and HMBA-treated cells. Variations in expression were small and standard deviations were high. Calculation of the mean values in this way actually masks what is happening in the cell; in some cases it is not desirable and may be misleading (Hammond *et al.*, 2000a; Hammond *et al.*, 2000c). Also it needs to be noted that since a sample of cells was taken during each isolation, the results showing dynamic changes in expression of these proteins are the variations from the population of MEL cells extracted at that time. Thus these results are not the reflection of what is taking place in a single cell. Although most of the cells would be in the same phase of growth, the concentration of each type of protein in some of the cells could be

different from that of the population. Often the expression of proteins from the population would be an indication of what is being expressed in the majority of the individual cells. Ideally it would be good to study the changing patterns of individual proteins in a single cell as well but this is not possible with current technology. Thus the individual fitted curves for PP1 and PP2A showed clear evidence of oscillatory behaviour reflecting a dynamic environment and the changes taking place.

The period and amplitude curves for PP1 and PP2A expression varied with time. Variations were also noted between the untreated and the HMBA treated samples indicating that changes were taking place during cell differentiation. The mean period and amplitude values for PP1 and PP2A for the 30 minute time intervals were less than those obtained using 60 minute intervals. These observations again demonstrate that the sampling frequency is an important factor in determining period and the true values may be much lower, as noted previously (Calvert-Evers and Hammond, 2002; Hammond *et al.*, 1998). The relationships between the 32 and 36 kDa PP2A components showed some variability; clearly the two proteins are oscillating and there is evidence that in certain instances they do so in phase with one another; this suggests a common underlying process, but on the other hand there are differences which imply a degree of independence. The value of extending such studies is evident.

Expression of PP1 and PP2A in proliferating and HMBA treated MEL cells clearly showed temporal changes for the time intervals studied. The results provide further evidence for the existence of oscillations, consistent with our earlier studies relating to protein phosphorylation and the expression of kinases and phosphatases, and to the expression of other key proteins in these and HL60 (human promyelocytic leukaemia) cells (Bhoola and Hammond, 2000; Calvert-Evers and Hammond, 2002; Calvert-Evers and Hammond, 2003; Calvert-Evers and Hammond, 2000; Ferreira *et*

*al.*, 1996b; Hammond *et al.*, 1998; Hammond *et al.*, 2000a; Hammond *et al.*, 1989b; Hammond *et al.*, 2000c; Hammond *et al.*, 1985).

# Chapter 3

# Expression of p53 protein in proliferating and differentiating murine erythroleukaemic cells

## 3. Summary

This chapter describes an investigation of p53 protein expression in MEL cells. Samples were isolated at different time periods from proliferating and differentiating cells. The cells were incubated with or without HMBA and/or OA. Total protein was extracted from the samples and concentrations determined. Protein samples were analysed for p53 expression by either resolving them on SDS polyacrylamide gels followed by western blotting or using a quantitative ELISA method. For the western blot analysis, a specific p53 antibody was used. The results were analysed densitometrically. In the ELISA study, a specific antibody towards p53 produced a colour reaction proportional to the amount of p53 present.

Evidence of oscillatory behaviour in the expression of the p53 protein from untreated and HMBA treated MEL cells is presented together with detailed analysis of the time series experiments. Time dependent variations in expression of p53 were observed using 15, 30, and 60 minute sampling times. In general, the expression patterns for the protein were complex and there were differences between the effects of HMBA in different experiments. The expression of the p53 protein was inhibited in the presence of OA.

## 3.1. Methods

### 3.1.1. Materials and equipment

The primary antibody used in these experiments was obtained from Boehringer Mannheim in Germany. The secondary antibody (sheep anti-mouse IgG linked to horseradish peroxidase) was purchased from Amersham Bioscience in the United Kingdom. A detailed description of the antibodies can be found in Appendix E. Okadaic acid was obtained from Boehringer Mannheim in Germany and the p53 ELISA kit from Oncogene Research Products based in the USA in Massachusetts. Other reagents were obtained from standard commercial sources – for details see Appendix A.

### 3.1.2. Cell culture and the differentiation of murine erythroleukaemic cells

MEL cells were cultured, maintained and differentiated exactly as was described previously in Chapter 2 (see section 2.1.2 and 2.1.3; also refer to Appendix B on MEL cell culture).

### 3.1.3. Addition of okadaic acid to murine erythroleukaemic cells

This procedure was similar to that used for MEL cell differentiation, as described in Chapter 2. In each experiment proliferating MEL cells were grown to a

concentration of 1 x $10^7$ cells/ml then diluted to 1 x $10^6$ cells/ml for each experiment. In a study of the effects of OA concentration, 10 ml MEL cell suspensions were incubated with 0, 1, 10, 100 and 500 nM of OA (refer to Appendix B.10). The cells were incubated for 24 hours. In time series experiments, MEL cells were incubated with 100 nM OA and analysed at 12 hour time intervals. Cell viability of 95% (refer to Appendix B.8) and above was ensured before each experiment.

### 3.1.4. Preparation of cell extracts and the determination of their protein concentration

For each of the experiments, cell samples were prepared in the same way as described in Chapter 2 (see section 2.1.4 and Appendix C.1). Protein concentration was determined as described in Chapter 2 section 2.1.5 (also refer to Appendix C.2). Samples were isolated and analysed either using western blotting or ELISA techniques to determine the expression of the p53 protein.

### 3.1.5. Expression of the p53 protein

#### 3.1.5.1. Electrophoresis and immunoblot detection of the p53 protein

The electrophoresis and transblot methods used here were similar to those described in Chapter 2 section 2.1.6.1 and 2.1.6.2.

After protein transfer, nitrocellulose membranes were blocked overnight at 4 °C in 10 ml of blocking buffer (3% BSA in phosphate buffered saline (PBS; 80 mM $Na_2HPO_4$, 20 mM $NaH_2PO_4$, 100 mM NaCl, pH 7.4)) with constant agitation (see Appendix E.2). The blocking solution was poured off and primary anti-mouse p53 antibody was poured on the membrane at a dilution of 1:100 in 7.5 ml antibody buffer (1% BSA in PBS, see Appendix E.2.8). The primary antibody was a monoclonal IgG2b antibody from mouse-mouse hybrid cells (clone Pab-122) and was specific to an epitope on the p53 protein (see Appendix E.1.4). The membrane was incubated with the solution for 4 hours at room temperature with constant agitation. After four 5 minute washes with 0.1% Tween-20 in PBS (see Appendix E.2.6), 7.5 ml of a secondary antibody (sheep anti-mouse Ig linked to horseradish peroxidase, see Appendix E.1.5) was added at a 1:100 dilution with 0.1% Tween-20 in antibody buffer (1% BSA in PBS, see Appendix E.2.6). This was agitated for 60 minutes at room temperature. As before the immunoblot was washed four times with 0.1% Tween-20 in PBS (see Appendix E.2.6) and developed using 25 µg DAB in 50 ml PBS and 50 µl $H_20_2$ (30%). The blot was rinsed in water, dried and stored away from light. The immunoblots were photographed and densitometrically analysed.

#### 3.1.5.2. Enzyme-linked immunosorbent assay detection of p53 expression

Isolated MEL cell samples were initially diluted so as to have equal amounts of total protein. These samples were then analysed using a specific p53 ELISA "sandwich" enzyme immunoassay kit obtained from Oncogene Research Products in Massachusetts, USA (Appendix F). This kit has wells coated with mouse monoclonal antibody which are exposed to the sample and a biotinylated antibody (mouse monoclonal), the detector. Any p53 present in the sample binds to the

capture and detector antibody and a colour reaction is produced which is proportional to the amount of p53 protein in the sample.

Standard p53 protein (2 ng/ml) was serially diluted with sample diluents (see Appendix F). The following p53 standards were prepared in duplicate: 0.00, 0.05, 0.13, 0.32, 0.80, and 2.00 ng/ml. To each microtiter well, 100 µl of detector antibody (biotinylated monoclonal p53 antibody) was pipetted. Duplicates of each sample were prepared and pipetted (100 µl) into the wells. The wells were covered with a plate sealer and incubated at 37 °C for 4 hours in a humidified incubator. The wells were washed three times with a 1X wash buffer (a dilution of a 20X solution of PBS and surfactant with 2.0% chloroacetamide). Into each well, 100 µl of 1X streptavidin conjugate (peroxidase streptavidin conjugate) was pipetted. The plate was covered with a plate sealer and incubated at room temperature for 30 minutes. The wells were again washed three times with wash buffer. The entire plate was then filled with double distilled water; the water was removed by inverting the plate over the sink and tapping it on a paper towel. Each well then had 100 µl of substrate solution (chromogenic substrate containing tetra-methylbenzidine) added to it. The plate was incubated at room temperature in the dark for 30 minutes. Finally, 100 µl of stopping solution (2.5 N sulphuric acid) was added to each well. The absorbance was measured in each well using a spectrophotometric plate reader at 450 nm wavelength. The standard curve for the assay was drawn and the value for each sample calculated.

### 3.1.6. Time studies and data analysis

The analysis of data here was done using the programmes mentioned in Chapter 2 section 2.1.7 Time studies and data analysis. As was stated in that section all experimental conditions were kept the same for each experiment.

## 3.2. Results for p53 protein expression in murine erythroleukaemic cells

### 3.2.1. Time analysis of p53 protein using western blot

#### 3.2.1.1. Immunoblots of p53

Typical examples of results obtained for blots probed for p53 protein are shown in figures 3.1, and 3.2. The band detected had a molecular mass corresponding to 53 kDa (figure 3.1). No bands were detected on the non-specific blot in which the p53 primary antibody was excluded (results not shown).

Although experimental conditions, as far as possible, were kept constant, the expression of p53 protein always varied with time. This variation was seen in all the western blots but with differences between experiments. In figure 3.1, the position of the detected p53 protein is shown in relation to the rainbow marker masses. Time dependent changes in p53 expression can be seen in both the untreated (figure 3.2 A) and the corresponding HMBA treated (figure 3.2 B) samples.

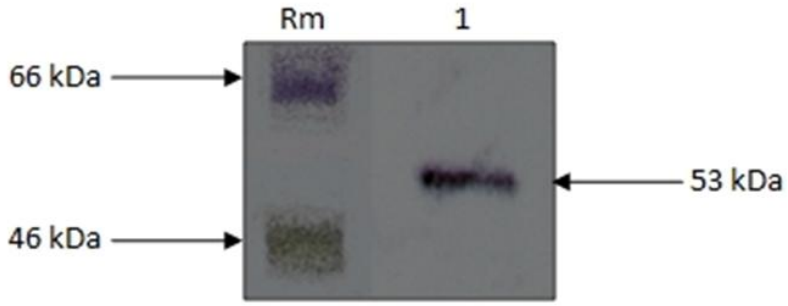



Figure 3.1 Western blot analysis of p53

An example of a nitrocellulose blot showing the position of the p53 band in relation to two rainbow marker bands (46 and 66 kDa, Rm lane). Total protein (200 µg) isolated from untreated MEL cells was loaded in lane 1 and probed for p53 protein.

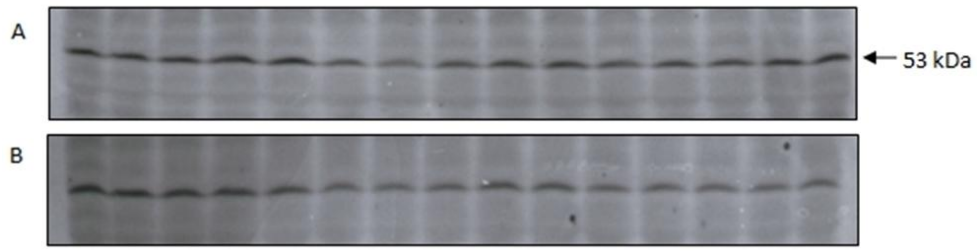



Figure 3.2 Western blot analysis of p53 at 30 minute time intervals

Typical western blots showing time dependent changes in expression of p53 protein from untreated (A) and HMBA treated (B) MEL cells. Samples were isolated at 30 minute time intervals with time zero beginning on the left. Each lane was loaded with 84 µg of total protein.

#### 3.2.1.2. Samples isolated at 15 and 30 minute time intervals

The densitometric interpretation of western blot results for samples isolated at 15 and 30 minute time intervals are illustrated in figure 3.3. Both the 15 minute and the 30 minute fitted curves show peaks and troughs depicting oscillatory behaviour. The corresponding difference curves are shown on their right and enhance the illustration of the oscillatory patterns.

Time dependent variations differed between the untreated and HMBA treated samples. For the 15 minute time interval experiment, the p53 protein expression plots (figure 3.3 A) for the untreated and HMBA treated samples were out of phase. In the 30 minute time interval experiment (figure 3.3 B) the curves were initially out of phase but after 150 minutes they become synchronised. For both time intervals HMBA slightly depressed the expression of the p53 protein.

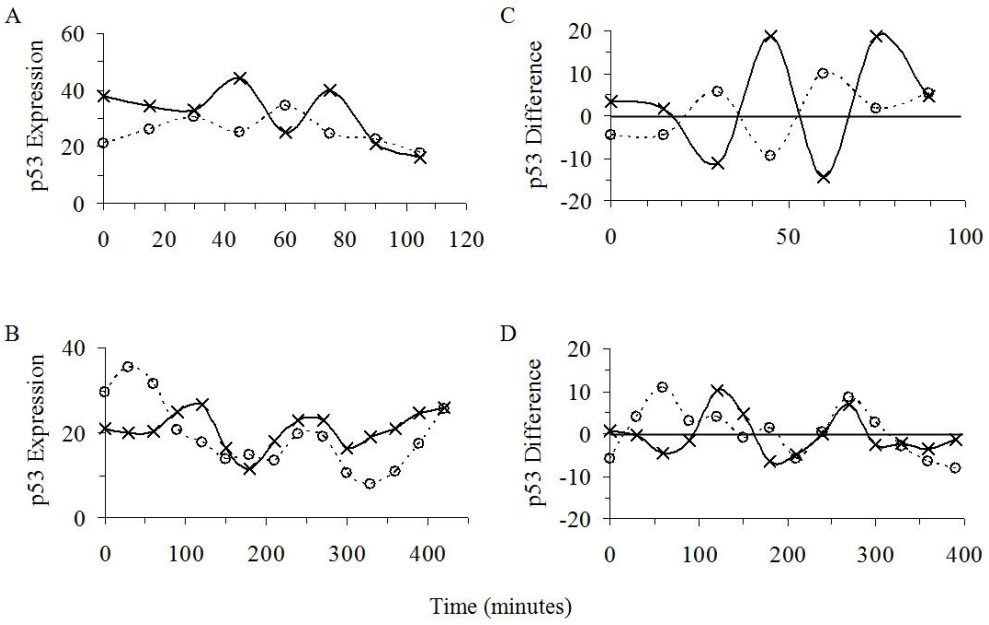



Figure 3.3 Western blot analysis of p53 at 15 and 30 minute time intervals

Temporal variations in the expression of p53 protein measured at 15 (A) and 30 (B) minute time intervals. The respective difference curves (C and D) are included. Proliferating control cells (x—x); cells treated with HMBA (o--o).

### 3.2.1.3. Samples isolated over periods of hours

An additional experiment was performed in which MEL cells were isolated at 0, 4, 15, 18, 25, 29, 41, 48 and 71 hours. Total protein was extracted from these samples and they were analysed at three different times for p53 protein expression using western blot analysis. The mean value of expressed p53 protein for each time point together with its standard deviation was calculated and plotted in graph form (figure 3.4). There were no significant differences between the means for the untreated and treated samples. The fitted curves for these samples are shown in figure 3.5 A, B and C. The corresponding difference curves are depicted on the right (figure 3.5 D, E and F). There were slight differences in p53 expression at certain times but the trends in the three fitted curves were very much the same. The untreated samples were in phase with the HMBA samples for most of the time periods. Again HMBA had a slight dampening effect on the expression of p53. All three expression graphs showed similar patterns of behaviour for p53 with some differences possibly being indicative of experimental error.

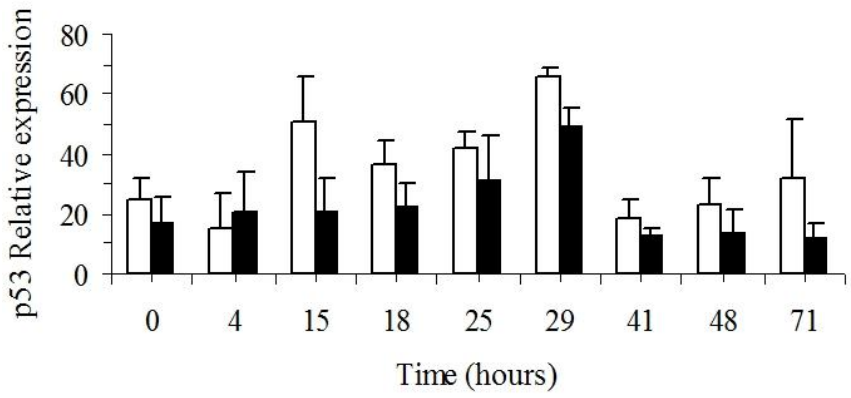



Figure 3.4 Mean values of the expression of p53 measured at various hours

Means of the values obtained from densitometric scans of immunoblots for p53 from the same initial samples analysed at three different times using western blot analyses. Samples were isolated at 0, 4, 15, 18, 25, 29, 41, 48 and 71 hours. Error bars represent the standard deviation of the mean values for each time interval. No significant differences between the mean values for the control and HMBA treated cells were noted. □ - Untreated MEL cells. ■ - cells treated with HMBA.

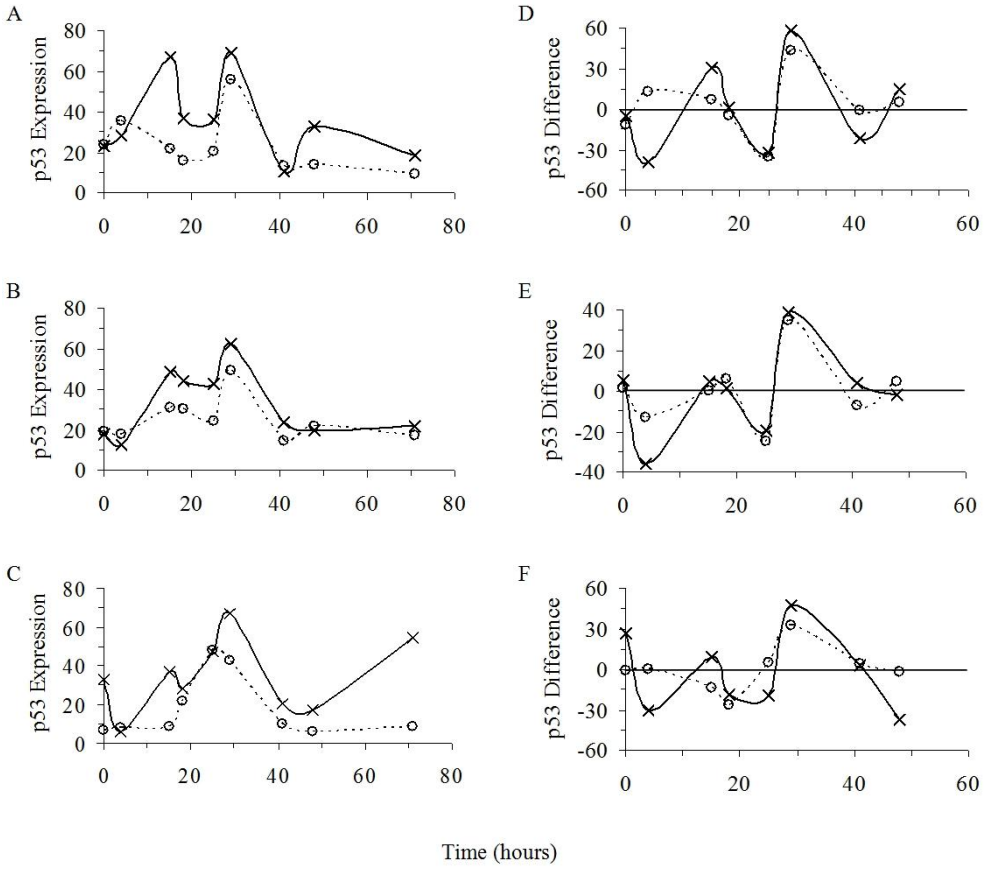



Figure 3.5 Western blot analysis of p53 at various periods of hours

Temporal variations in the expression of p53 protein isolated at 0, 4, 15, 18, 25, 29, 41, 48 and 71 hours. Triplicate analyses of p53 expression are shown (A, B and C). The respective difference curves (D, E and F) are included. Proliferating control cells (x—x); cells treated with HMBA (o--o).

### 3.2.2. Time analysis of p53 protein using an enzyme-linked immunosorbent assay kit

Further studies were carried out using the ELISA technique to monitor and quantitate the changes in expression of the p53 protein in MEL cells. The temporal variations of p53 concentration from samples isolated at 15, 30 and 60 minute time intervals are shown in figures 3.6, 3.8 and 3.10, respectively. In these experiments it was possible to calculate the period and amplitude curves (C and D in these figures). The mean values for the period and amplitude in the different experiments are depicted in table 3.1. For each time interval the variation of p53 protein concentration was compared with its respective cell number and total protein concentration (figures 3.7, 3.9 and 3.11).

#### 3.2.2.1. Samples isolated at 15 minute time intervals

Temporal changes of the fitted curve for p53 concentration from samples isolated at 15 minute time intervals (figure 3.6) were observed. Initially the changes in the untreated and HMBA treated samples were synchronised but after 75 minutes the concentration changed and HMBA inhibited p53 expression. Both the average period and amplitude values were higher in untreated than in HMBA treated samples (table 3.1 – 15 minutes). Changes in relation to the cell number and total protein concentration for these experiments were also observed (figure 3.7). The variations in the total protein concentration (figure 3.7 B) of samples were not as extensive as those observed in cell number (figure 3.7 A) and p53 concentration (figure 3.7 C). No correlation was noted between any of these components (figures 3.7 D, E and F) i.e. cell number, total protein concentration and p53 concentration.

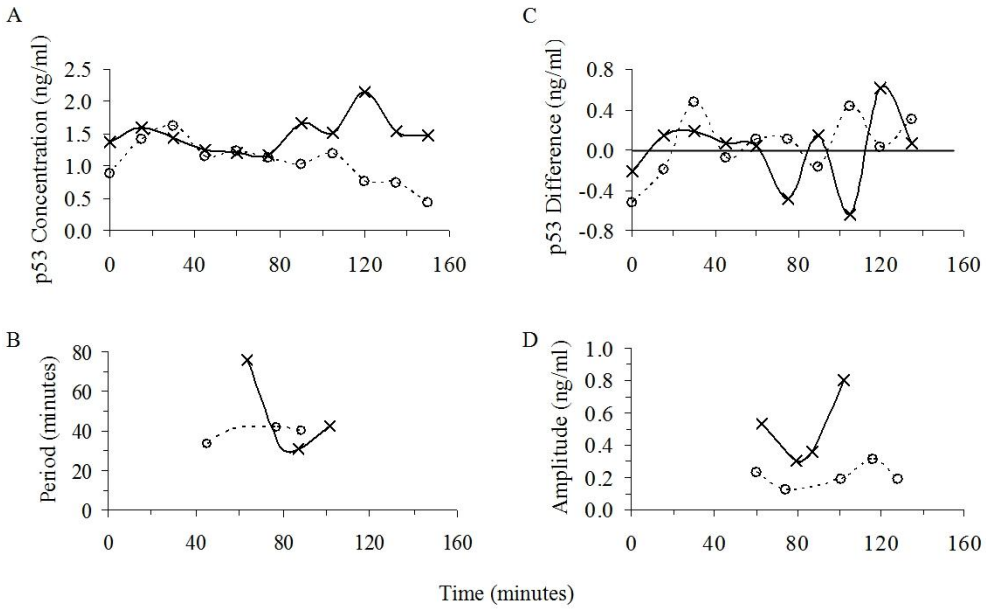



Figure 3.6 ELISA analysis of p53 at 15 minute time intervals

Temporal variations in the concentration of p53 protein measured at 15 minute (A) time intervals. The corresponding difference (C), period (B) and amplitude (D) plots are included. Proliferating control cells (x—x); cells treated with HMBA (o--o).

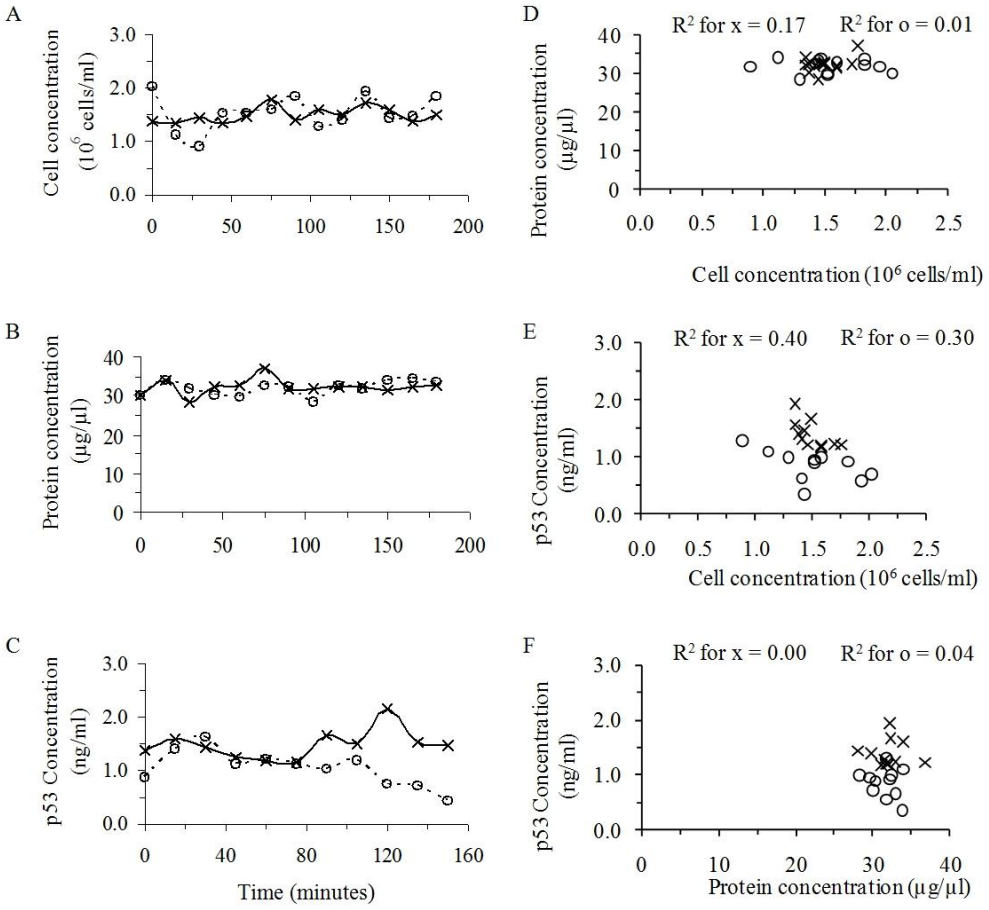



Figure 3.7 Comparison of p53 concentration in relation to the cell and total protein concentration; 15 minute time intervals

Changes in the concentration of cells (A), total protein (B) and p53 protein (C) measured at 15 minute time intervals. The correlation curves comparing each of these components are included on the right. Proliferating control cells (x—x); cells treated with HMBA (o--o).

#### 3.2.2.2. Samples isolated at 30 minute time intervals

As was the case for the 15 minute time intervals, temporal changes in p53 concentration from samples isolated at 30 minute time intervals (figure 3.8) were also noted. In this case, the p53 concentration drifts in and out of phase when comparing the untreated and HMBA treated samples. The average period and amplitude values were again higher in the untreated than HMBA treated samples (table 3.1 – 30 minutes). Cell number and total protein concentration for this experiment exhibited temporal variations (figure 3.9). The cell number for both the untreated and HMBA treated samples showed a steady increase in number, starting from about 250 000 cells/ml going to around 1 000 000 cells/ml. There was no comparable increase in total protein concentration and p53 concentration. The values for total protein extracted from untreated samples were higher than those from the HMBA treated samples at each time period. There was little correlation between cell number, total protein concentration and p53 concentration (figure 3.9 D, E, and F respectively).

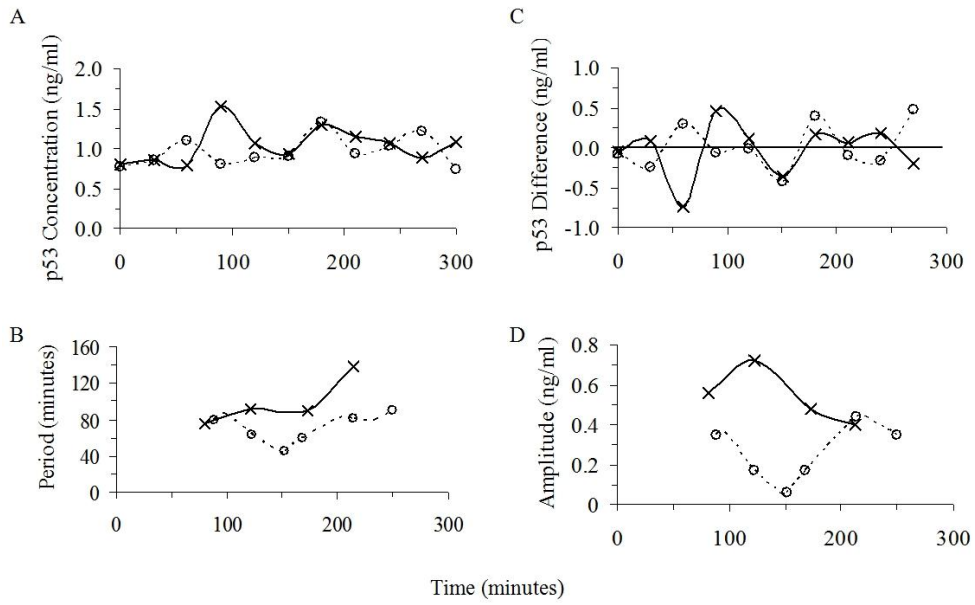



Figure 3.8 ELISA analysis of p53 at 30 minute time intervals

Temporal variations in the concentration of p53 protein measured at 30 minute (A) time intervals. The corresponding difference (C), period (B) and amplitude (D) plots are included. Proliferating control cells (x—x); cells treated with HMBA (o--o).

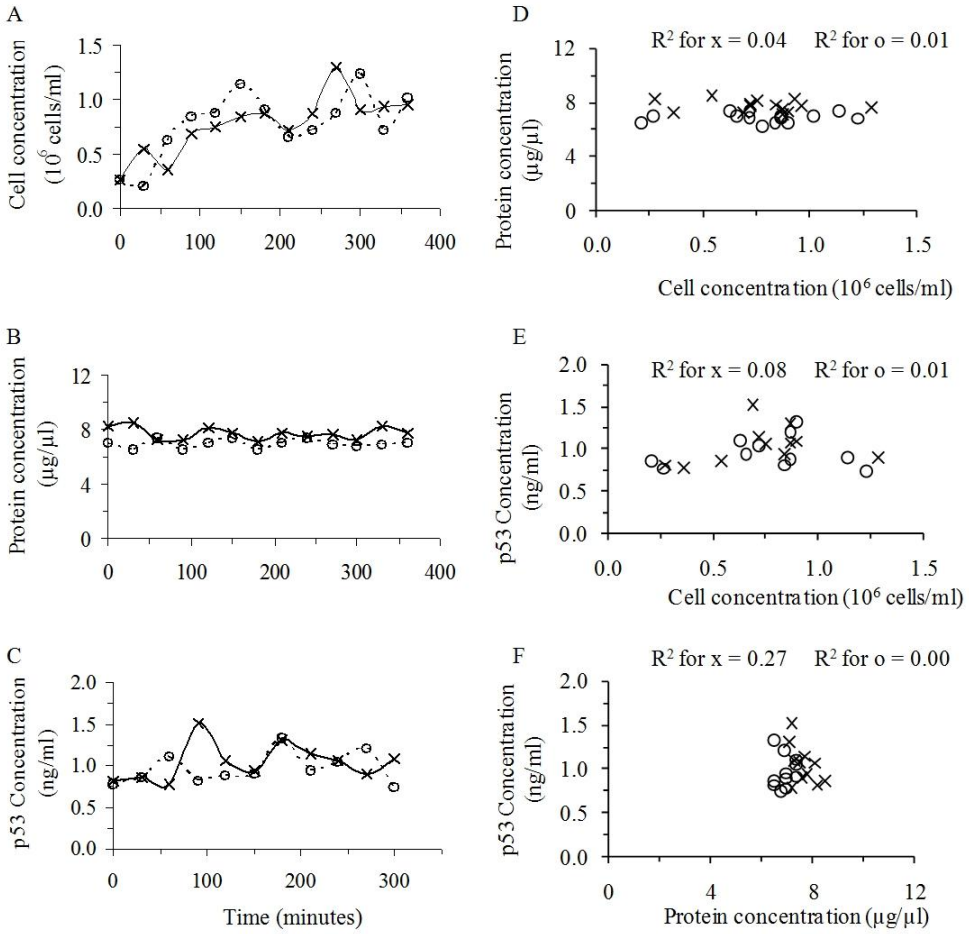



Figure 3.9 Comparison of p53 concentration in relation to the cell and total protein concentration; 30 minute intervals

Changes in the concentration of cells (A), total protein (B) and p53 protein (C) measured at 30 minute time intervals. The correlation curves comparing each of these components are included on the right. Proliferating control cells (x—x); cells treated with HMBA (o--o).

#### 3.2.2.3. Samples isolated at 60 minute time intervals

For the 60 minute time interval (figure 3.10) a dampening effect by HMBA was observed. The period and amplitude both showed changes with time and varied over a wide range. In contrast to the previous two experiments, the mean period (table 3.1 – 60 minutes) was lower in the untreated samples compared to the treated ones. The mean amplitude stayed consistent with trends and was higher to a lesser extent in the untreated samples than the treated. Cell concentration for this experiment showed pronounced oscillatory behaviour (figure 3.11 A). As before there was little correlation between the cell number, total protein concentration and p53 concentration of samples from this experiment (figure 3.11 D, E, and F respectively).

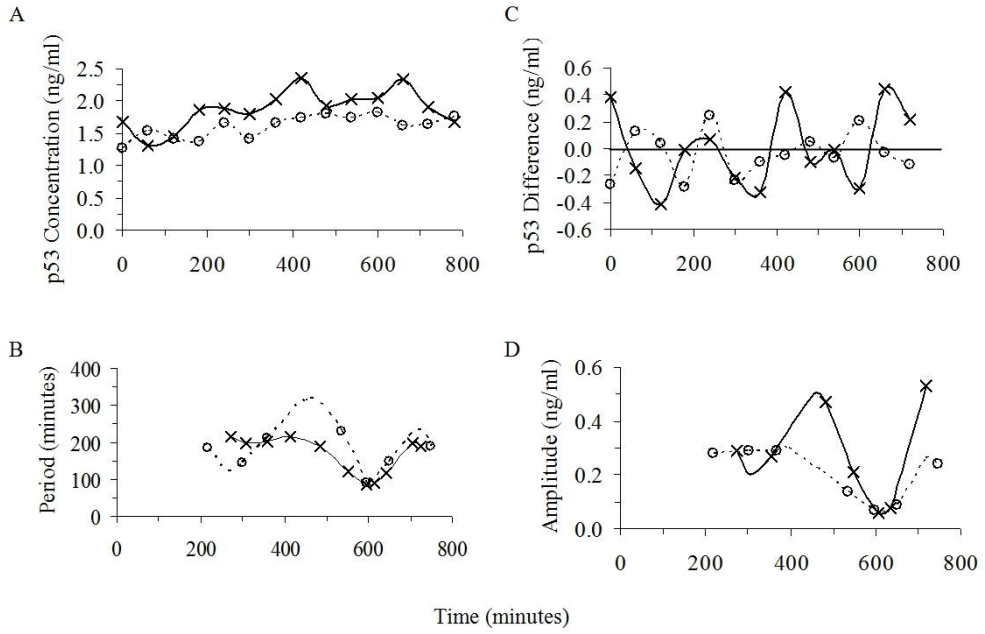



Figure 3.10 ELISA analysis of p53 at 60 minute time intervals

Temporal variations in the concentration of p53 protein measured at 60 minute (A) time intervals. The corresponding difference (C), period (B) and amplitude (D) plots are included. Proliferating control cells (x—x); cells treated with HMBA (o--o).

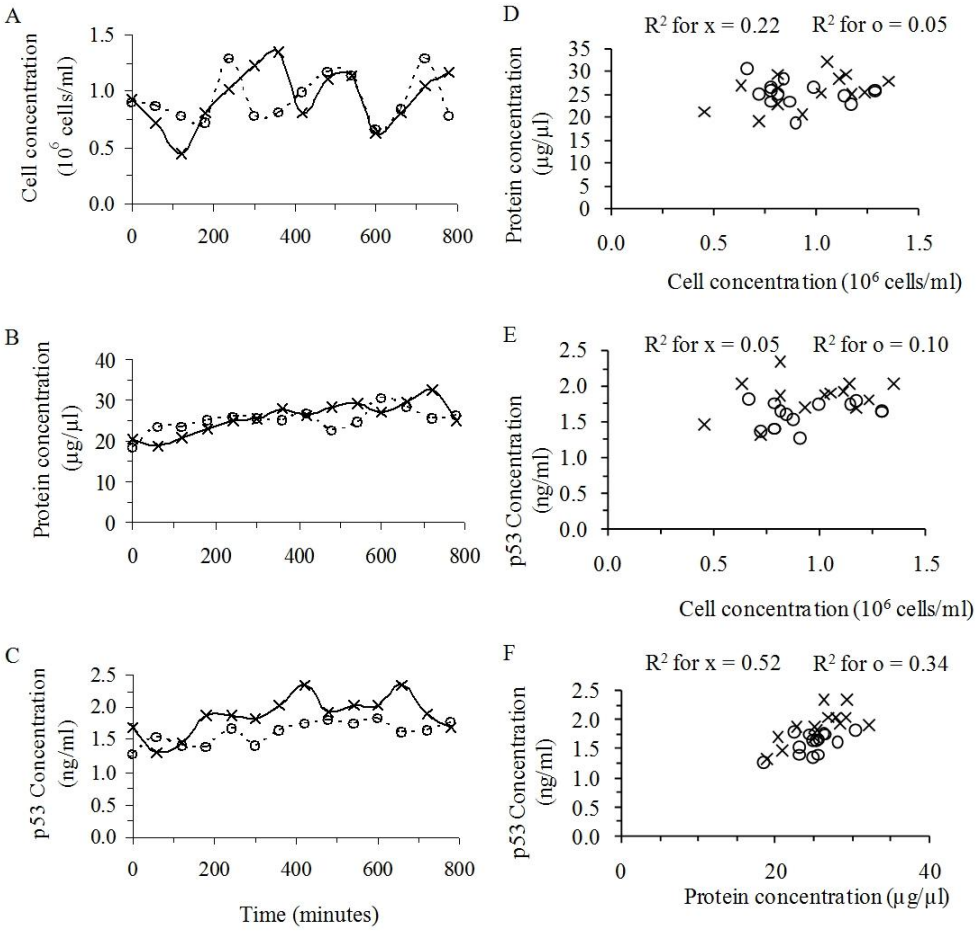



Figure 3.11 Comparison of p53 concentration in relation to the cell and total protein concentration; 60 minute intervals

Changes in the concentration of cells (A), total protein (B) and p53 protein (C) measured at 60 minute time intervals. The correlation curves comparing each of these components are included on the right. Proliferating control cells (x—x); cells treated with HMBA (o--o).

Table 3.1 Comparisons between the period and amplitude for p53 protein concentration at different time intervals from untreated and HMBA treated MEL cells analysed via the ELISA method

| p53 Experiment | Mean period (minutes) | | Range of period (minutes) | | Mean amplitude (ng/ml) | | Range of amplitude (ng/ml) | |
|---|---|---|---|---|---|---|---|---|
| | Untreated | HMBA Treated | Untreated | HMBA Treated | Untreated | HMBA Treated | Untreated | HMBA Treated |
| p53 at 15 min | 45 | 38 | 31 – 76 | 33 - 42 | 0.50 | 0.21 | 0.30 - 0.80 | 0.12 - 0.31 |
| p53 at 30 min | 99 | 71 | 76 – 138 | 43 - 90 | 0.54 | 0.26 | 0.40 - 0.72 | 0.05 - 0.44 |
| p53 at 60 min | 165 | 178 | 87 – 215 | 90 - 321 | 0.29 | 0.22 | 0.06 - 0.53 | 0.07 - 0.30 |

### 3.2.3. Effect of okadaic acid on p53 expression

#### 3.2.3.1. Immunoblot analysis of the effect of okadaic acid on p53 protein expression

MEL cells were incubated with OA, to investigate its effect on the expression of the p53 protein. The effect of different concentrations of OA on p53 expression was tested in MEL cells after 2 hours (figure 3.12). Maximum p53 was expressed in the cells incubated with 100 nM OA. Cells with no OA present showed a greater expression of p53 than cells with 1 nM OA, indicating that 1 nM of OA had negligible influence on p53 expression. On the other hand, very little expression was noted in cells that were incubated with 500 nM of OA, showing that this concentration of OA highly suppressed p53 expression. Using this concentration of OA would also cause massive cell death after two hours and would not be practical for our studies. These results reflect the influence of OA concentration on p53 expression with the greatest effect being obtained with 100 nM of OA.

Temporal changes on p53 expression were demonstrated in the presence of 100 nM of OA at 30 minute time intervals (figure 3.13). These temporal changes again reflect the oscillatory nature of the expression of p53 even under the influence of a different agent. The effect of OA on the expression of p53 in untreated and HMBA treated MEL cells after 24 hours of incubation was observed (figure 3.14). The lowest amount of expression of p53 was noted from cells which were incubated with HMBA and OA, reflecting the collective effect of the two agents on p53 expression.

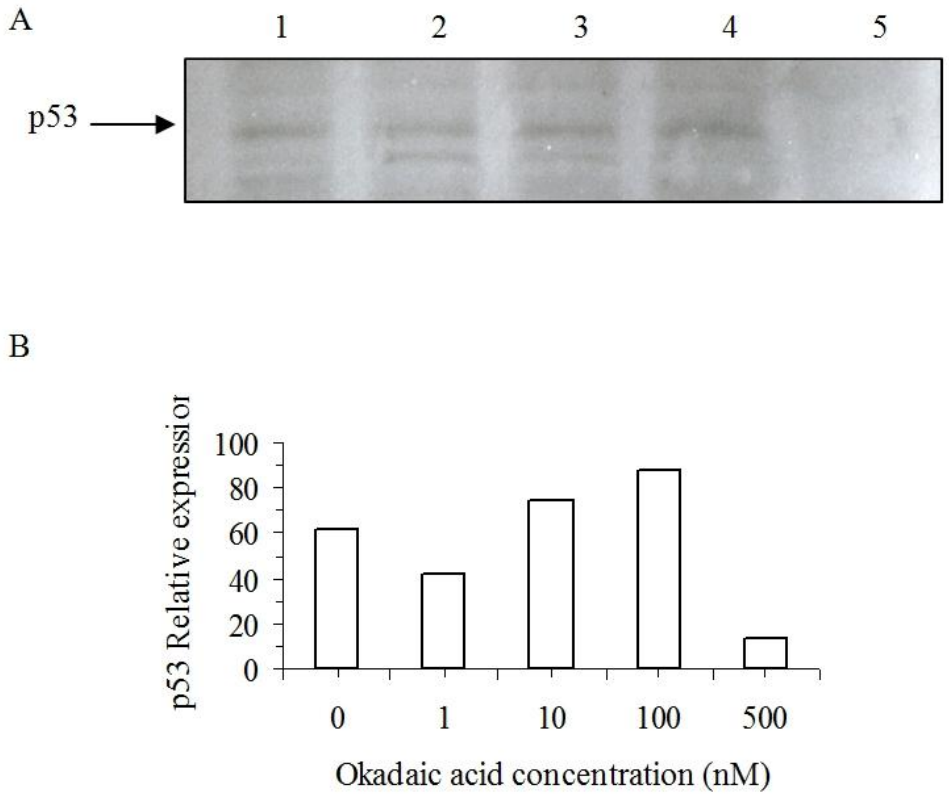



Figure 3.12 Analysis of p53 expression from MEL cells incubated with different concentrations of okadaic acid

A western blot (A) of p53 in MEL cells incubated with different concentrations of OA for 2 hours. Equal amounts of protein (70.5 µg) were loaded in each well. A corresponding graph (B) of the densitometric scan of the blot is shown. Lane 1 – 0 nM OA, lane 2 – 1 nM OA, lane 3 – 10 nM OA, lane 4 – 100 nM OA and lane 5 – 500 nM OA.

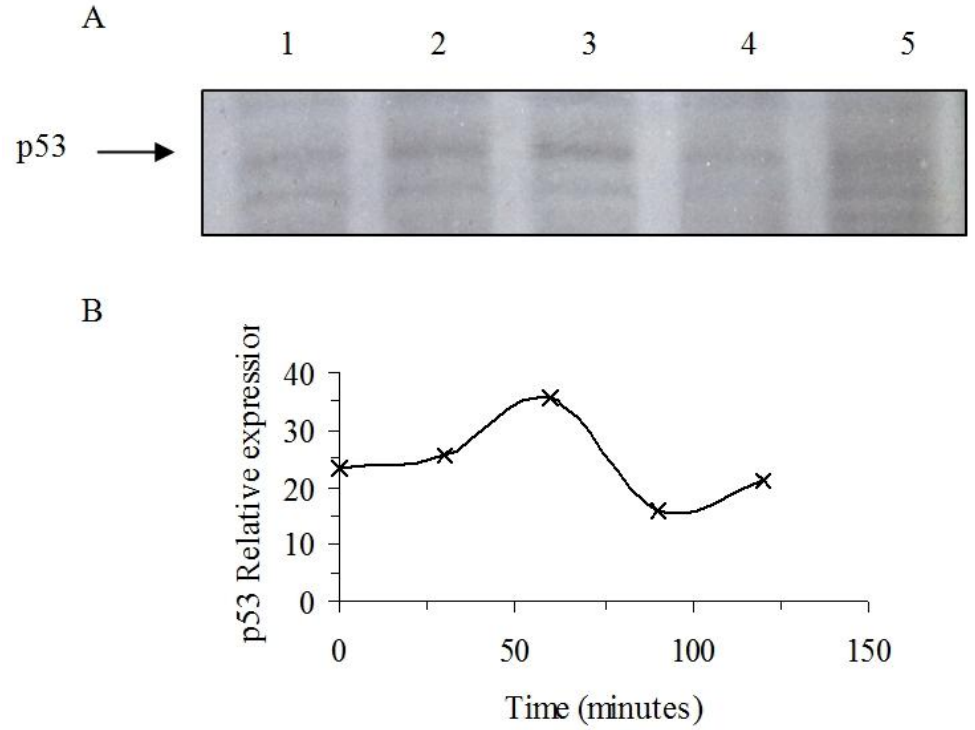



Figure 3.13 Temporal changes of p53 measured at 30 minute time intervals from MEL cells incubated with okadaic acid

A western blot (A) of p53 measured at 30 minute time intervals from MEL cells incubated with 100 nM of OA. Equal amounts of protein (70.5 µg) were loaded in each well. The corresponding relative expression graph (B) of p53 determined densitometrically is shown. Lanes 1 to 5 had samples isolated at 30 minute intervals with lane 1 containing the 0 minute sample.

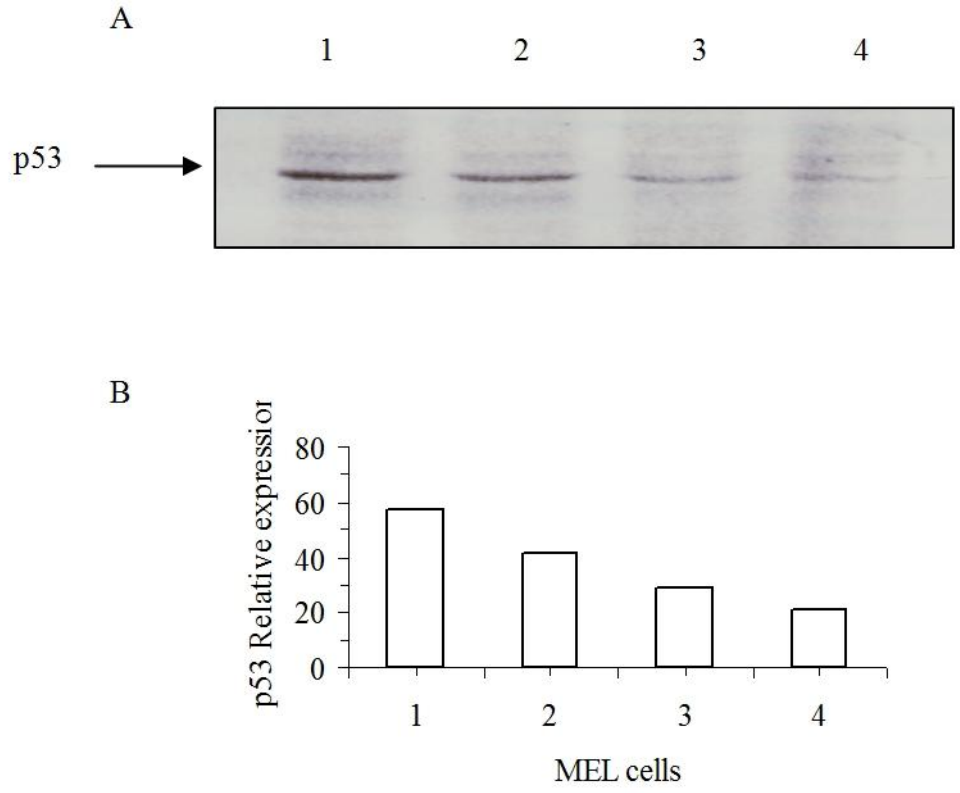



Figure 3.14 Effect of okadaic acid on p53 protein expression after 24 hours

A western blot (A) showing the effect of OA (100 nM) after 24 hours on the expression of p53 measured in MEL cells. Equal amounts of protein (100 µg) were loaded in each well. The corresponding relative expression graph (B) of p53 determined densitometrically is shown. Four cultures of MEL cells were used; one representing the untreated control (lane 1), the other treated with 5 mM HMBA (lane 2), another incubated with 100 nM OA (lane 3) and the final one treated with both 5 mM HMBA and 100 nM OA (lane 4).

### 3.2.3.2. Enzyme-linked immunosorbent assay analysis of the effect of okadaic acid on p53 protein expression

The ELISA analysis was again used to quantitate the influence of OA on p53 protein expression in MEL cells. The effect of OA on the expression of p53 protein after 24 hours was quantitated and displayed together with the cell and total protein concentration values (figure 3.15 and table 3.2). OA with HMBA caused the maximum reduction in MEL cell number (figure 3.15 A) with some difference in the total protein concentration (figure 3.15 B). Before the ELISA analysis no attempt was made to equalise the protein concentration. After 24 hours of incubation with OA there was a decrease in expression of p53 protein (figure 3.15 C). But when this value was adjusted for cell concentration (figure 3.15 D), most expression of p53 protein was noted in cells incubated with OA with the maximum amount being produced with OA and HMBA. While if the p53 concentrations were adjusted relative to the protein concentrations (figure 3.15 E), less p53 was produced under the influence of OA and the least with OA and HMBA.

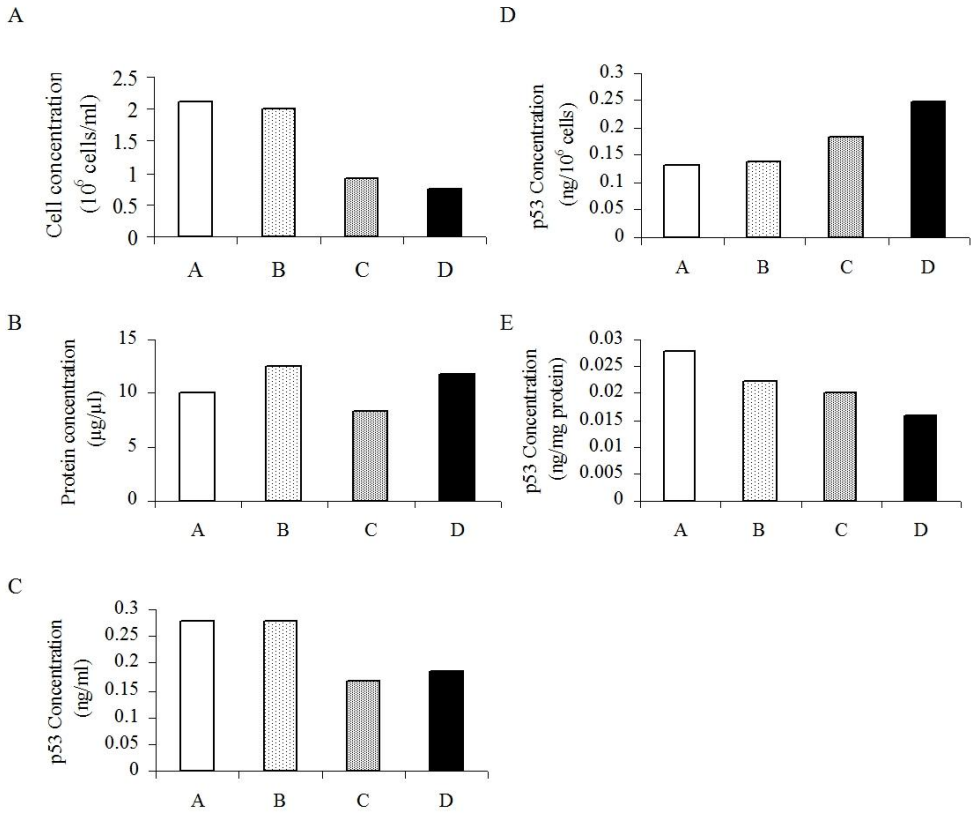



Figure 3.15 Concentration of p53 in MEL cells treated with okadaic acid

Graphs showing the effect of OA (100 nM) on the concentration of MEL cells (A), total protein (B) and p53 (C) protein concentration measured after 24 hours. Equal volumes of protein sample (5 µl) were loaded in each well. The concentration of p53 expression in relation to the cell number (D) and protein concentration (E) are presented. □ - untreated MEL cells, ▒ - HMBA treated MEL cells, ▓ - untreated MEL cells with OA and ■ - HMBA treated MEL cells with OA.

Table 3.2 - Analysis of the effect of OA on the concentration of p53 in MEL cells

| Agent | Cell concentration ($10^6$ cells/ml) | Protein concentration (mg/ml) | p53 concentration (ng/ml) | p53 concentration (ng/$10^6$ cells) | p53 concentration (ng/mg protein) |
|---|---|---|---|---|---|
| Non | 2.1 | 9.98 | 0.279 | 0.132 | 0.028 |
| HMBA | 2.0 | 12.6 | 0.279 | 0.140 | 0.022 |
| OA | 0.9 | 8.3 | 0.166 | 0.184 | 0.020 |
| HMBA + OA | 0.75 | 11.7 | 0.186 | 0.248 | 0.016 |

### 3.2.3.3. Effect of okadaic acid on murine erythroleukaemic cell and protein concentration over a 24 hour period

The influence of OA on MEL cells was studied at 0, 12 and 24 hours (figure 3.16 A). Total protein was extracted from these cells and their concentration determined (figure 3.16 B). During this time period a steady decrease in cell number was noted. After 24 hours there were fewer cells in samples incubated with OA than those without. This again confirms the poisonous effect of OA on MEL cell viability. A similar trend was noted for the total protein concentration. There was a steady decrease in total protein concentration from the 0 time to the 24 hour period. At the 24 hour time period, the samples incubated with OA had less total protein than those without, probably due to decrease in protein production.

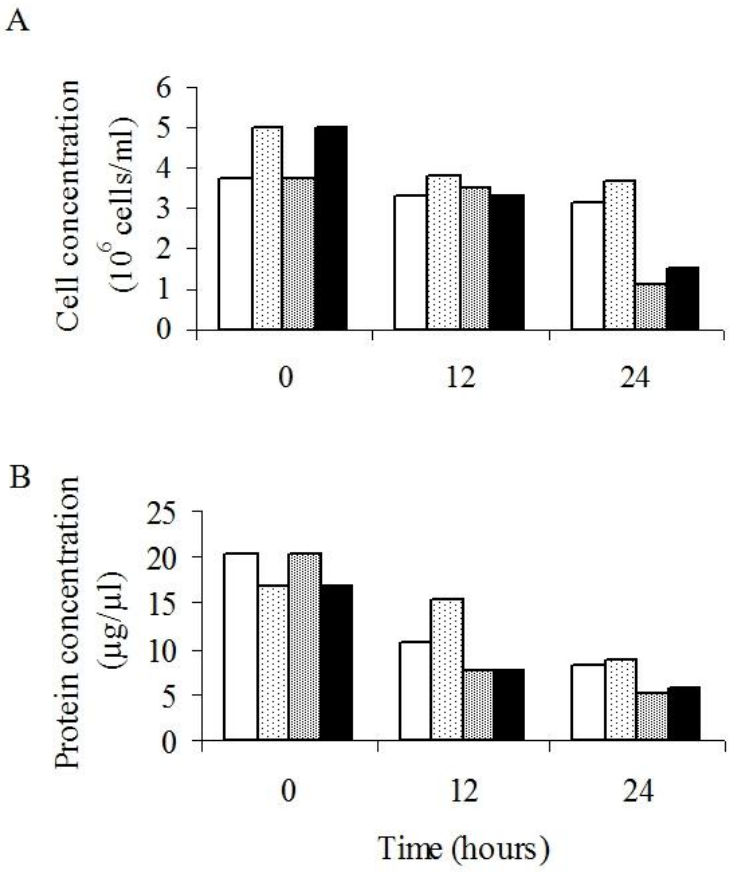



Figure 3.16 Cell and protein concentration of MEL cells treated with okadaic acid

A graph showing the effect of OA on MEL cell (A) and protein (B) concentrations isolated at 12 hour time intervals. □ - untreated MEL cells, ▒ - HMBA treated MEL cells, ▓ - untreated MEL cells with OA and ■ - HMBA treated MEL cells with OA.

### 3.3. Discussion

Currently studies on p53 are extensive and continually expanding. However, relatively little attention has been paid to its temporal aspect. It has been shown that in cultured mammalian cell lines the p53 protein, which forms part of a negative feedback loop with its transcriptional target Mdm2, shows dynamic oscillatory patterns in its expression (Lev Bar-Or *et al.*, 2000). In this study, observations were made at frequent time intervals monitoring the chronological changes of p53 expression in MEL cells and assessing how HMBA affected these changes. It is apparent that the regularity and magnitude of these periodic modifications cannot be dismissed as random experimental errors.

Samples from MEL cells which were probed for p53 showed a single band corresponding to its theoretical molecular mass of 53 kDa. Time dependent variations in expression were presented as fitted curves and difference plots. The difference plot gives additional visual information of trend changes and variations between successive data points seen on the fitted curves. The pattern of p53 expression was different in each of the experiments, even though attempts were made to keep experimental conditions the same. Because of the complex nature of the interactions that are influencing this protein it would be unrealistic to expect consistent and well defined behaviour. A corollary to this is the set of results shown in figure 3.5 which possibly indicates the impact of experimental error, specifically the accuracy of western blot analysis. In this experiment sample was isolated at various hour intervals and the total protein extracted. These were subsequently analysed in triplicate via the western blot method. Results showed that some of the data points differed slightly from each other but the general trends of the three graphs were very similar. Thus implying that the expression values obtained for p53 would be closely representative of those in the sample of cells. In this experiment

the sampling interval was irregular (times of 0, 4, 15, 18, 25, 29, 41, 48 and 71 hours) and still the graphs show oscillatory changes. Studies involving the western blot analysis of p53 and Mdm2 oscillations in MCF7 cell populations done by Lahav *et al*. (Lahav *et al.*, 2004), showed similar changing oscillatory patterns of p53. Lahav *et al*. extended their study by monitoring p53 in individual cells and concluded that in response to ionising radiation, cells emit p53 in pulses of fixed height and duration (Lahav *et al.*, 2004).

Although the effects were different in different experiments, HMBA often modified the dynamics of p53 expression; differences in the frequency and phasing of rhythms were seen in the treated cells compared with the controls. For the shorter time intervals of 15 and 30 minutes (figure 3.3, 3.6 and 3.8) there were no obvious effects on p53 expression by HMBA. But for the longer time intervals of 60 minutes (figure 3.5 and 3.10), HMBA had a propensity for dampening p53 expression. This trend corresponds with previous experiments in which induced differentiation of MEL cells via HMBA caused decreased expression of p53 (Marks and Rifkind, 1984). If this phenomenon was monitored for a longer time period, its outcome would have been more pronounced since p53 is involved in the $G_1/G_2$ phase prolongation and $G_1/G_2$ arrest (Aguda, 1999; Shen *et al.*, 1983). Other investigations done by Richon *et al* showed that the p53 protein decreased to very low levels between 4 and 8 hours after being induced to differentiate with HMBA and remained low in MEL cells (Richon *et al.*, 1989). A similar trend was observed in our experiments. Richon *et al* also state that the mRNA of p53 does increase after 24 and 48 hours (Richon *et al.*, 1989) which would signify that their levels of p53 protein should also increase.

The period and amplitude curves for p53 expression varied with time and complicated the estimation of the mean period and amplitude values. Periodic and amplitude variations were noted between the untreated and the HMBA treated

samples indicating that changes were taking place during cell differentiation. The mean period and amplitude values for p53 for the 15 minute time intervals were lower than those obtained for the 30 minute intervals (table 3.1). These observations further demonstrate that the sampling frequency is an important factor in determining period and the true values may be much lower, as noted previously (Calvert-Evers and Hammond, 2002; Calvert-Evers and Hammond, 2003; Hammond *et al.*, 1998).

Experiments analysed using the sensitive ELISA method similarly showed variations in p53 expression adding further imputes to their temporal organisation. For each of the ELISA experiments the concentration of p53 is presented together with its data on cell and total protein concentration. Variations can be seen in both the cell and protein concentrations. The trends of each of these three elements were different from each other with no direct correlations. As was the case with the western blot results, HMBA showed a dampening effect on the cells for the longer time periods, especially the 1 hour time interval.

It is known that OA directly influences phosphatases and their expression (Fernandez *et al.*, 2002). It was hoped that by studying its influence on p53 expression in MEL cells a connection could be established between p53 and the phosphatases in these cells. After 24 hours of incubation with OA a decrease in cell number was noted (figure 3.16). This is consistent with the role of OA which is a potent inhibitor of types 1, 2A and 3 serine/threonine protein phosphatases reviewed by Fujiki *et al*. (Fujiki *et al.*, 1997). It was interesting to note that when p53 expression was adjusted via the cell concentration, after 24 hours there was an increase in p53 expression in MEL cells incubated with OA (figure 3.15). When its concentration was adjusted against total protein, the opposite effect was noted in which a decreasing effect occurred. It is known that OA influences p53 and this has been shown here (figure 3.15) in MEL cells but the actual mechanism of operation

is not known. Many experiments on other cell lines have shown OA to have differing biochemical and functional effects on p53. For example, at the level of transcription of p53, OA treatment appeared to decrease p53 mRNA levels (Skouv *et al.*, 1994). In other cases, p53 protein has been shown to be stabilised by OA treatment in some cell types (Zhang *et al.*, 1994) but unaffected in others (Lohrum and Scheidtmann, 1996). In terms of DNA binding, p53 hyperphosphorylation mediated by OA treatment shows increased affinity for both the ribosomal gene cluster (RGC) and p53 Consensus sequence (p53Con) oligonucleotides (Fuchs *et al.*, 1995; Hecker *et al.*, 1996; Zhang *et al.*, 1994). Yet p53 transcriptional transactivation, as measured through the activities of RGC and p53Con luciferase constructs, is decreased by exposure to OA (Zhang *et al.*, 1994). However, in other cell types, OA treatment can lead to increased transactivation of Mdm2, $p21^{waf1}$ and BAX promoter constructs (Lohrum and Scheidtmann, 1996). Interestingly, in terms of programmed cell death, it has been shown that OA-induced, p53 dependent apoptosis correlated with increased overall levels of p53 phosphorylation, but did not require p53-mediated transactivation (Yan *et al.*, 1997).

The role played by p53 is critical with regard to the survival of the cell. It is influenced by abnormal changes which in turn manipulate appropriate modifications that could allow the cell to survive or die. Thus the expression of p53 is a highly dynamic process, varying constantly according to the needs of the cell. These dynamic changes and complex interactions are reflected in the oscillatory patterns of cellular components and may be used as a means of monitoring transformation, possibly modulating the process.

# Chapter 4

# Expression of p53 mRNA in proliferating and differentiating murine erythroleukaemic cells

## 4. Summary

This chapter describes an investigation of p53 mRNA expression in MEL cells. Samples were isolated, at time intervals of 15, 30, and 60 minutes, from untreated and HMBA treated MEL cells. Total RNA was extracted from the samples and analysed spectrophotometrically at 260 and 280 nm. The RNA integrity was confirmed on agarose gels. Identical amounts of RNA were reverse transcribed using the Ambion RETROscript kit for reverse transcriptase (RT). Samples were then subjected to polymerase chain reaction (PCR) using the Ambion Gene Specific RT-PCR kit for p53. This kit allows the semi-quantification of p53 mRNA using 18S RNA as the control. The PCR product was resolved on agarose gels. The bands were densitometrically scanned and analysed, as described in Chapter 2 and 3.

Evidence of oscillatory behaviour in the expression of p53 mRNA from untreated and HMBA treated MEL cells is presented together with in-depth analysis of the time series experiments. Time dependent variations in expression of p53 mRNA were observed using 15, 30 and 60 minute sampling times. In each of the time series the period and amplitude values were calculated. The p53 mRNA expression was also correlated with the corresponding cell population and total RNA concentrations. As with the protein expression, patterns for p53 mRNA

were complex and there were differences between the effects of HMBA in different experiments and at different time intervals.

## 4.1. Methods

### 4.1.1. Materials and equipment

The Gene Specific Relative Quantitative RT-PCR kit used in these experiments was obtained from Ambion in Texas in the USA (see Appendix I). Other reagents were obtained from standard commercial sources - see Appendix A.

### 4.1.2. Cell culture and the differentiation of murine erythroleukaemic cells

MEL cells were cultured and differentiated in the way described previously in sections 2.1.2 and 2.1.3. The cells were allowed to grow to a concentration of approximately 1 x $10^7$ cells/ml. Before each experiment, cells were centrifuged at 300 g for 5 minutes. The cells were reconstituted in 60 ml of EMEM. The viability and concentration of the cell population was determined (refer to Appendix B.8). As before (section 2.1.4), the cells were split into two equal portions of approximately 30 ml; one untreated and the other treated with 5 mM HMBA. Each flask contained approximately 2 x $10^6$ cells/ml.

### 4.1.3. Cell sample isolation

Samples were isolated at regular intervals of 15, 30 or 60 minutes in the different time series experiments. Approximately 1.5 ml of cells was removed from the untreated and treated preparations; these aliquots were placed in Eppendorf tubes. The cell concentration and viability of each sample was determined (refer to Appendix B.8). The samples were centrifuged in a microfuge at 300 g for 5 minutes in a cold room (~10 °C). The pellets were washed twice with saline at 300 g for 5 minutes. Samples were kept on ice between washes. After washing, 125 µl of TRI Reagent was added to each pellet and the samples stored in the freezer (~ -20 °C). Samples could be kept for about 1 to 3 months at -70 °C.

### 4.1.4. Total RNA extraction

RNAase free equipment and procedures were used for this and the following procedures detailed in sections 4.1.4 to 4.1.7 (refer to Appendix G). All samples were removed from the freezer and allowed to thaw for 10 to 15 minutes at room temperature. They were vortexed for 15 seconds then left at room temperature for 10 minutes. Samples were centrifuged at 12 000 g for 15 minutes in a microfuge at 10 ºC. The upper aqueous phase was carefully removed into new Eppendorf tubes and 0.075 ml of chloroform was added. Each sample was vortexed for 15 seconds and left at room temperature for 15 minutes. Samples were then centrifuged at 12 000 g for 15 minutes at 10 ºC. The upper aqueous phase of each sample was removed (approximately 0.06 ml) and transferred to a new Eppendorf tube. To each of these samples, 0.125 ml of isopropanol was added. Samples were vortexed for 15 seconds and left to stand for 10 minutes at room temperature. They were centrifuged at 10 ºC at 12 000 g for 10 minutes. The supernatant was carefully

removed from each sample. The pellets were washed with 0.25 ml of 75% ethanol, vortexed for 15 seconds and centrifuged at 12 500 g for 15 minutes at 10 ºC. The supernatant was removed. The pellets, containing pure RNA, were allowed to air dry for 20 minutes to permit the ethanol to evaporate. They were reconstituted with 0.02 ml RNAase free $H_20$ or DEPC $H_2O$. These samples were either stored at -70 ºC or used immediately for further analysis. Refer to figure G.1 in Appendix G for a visual diagram of this RNA extraction process.

### 4.1.5. Determination of RNA concentration

If samples were frozen, they were removed from the -70 °C freezer and allowed to thaw for 10 minutes at room temperature (approximately 24 °C). These were kept on ice for another 30 minutes before quantitative analysis. The RNA isolates were vortexed for 15 seconds, depending on the concentration of RNA, between 5 and 20 µl of sample was diluted in DEPC $H_2O$ at a dilution of between 100 to 200 times. The samples were vortexed for 60 seconds and quantitated by UV absorption spectrophotometry. A quartz cuvette was used for the readings which were performed at 260 and 280 nm. The concentration of RNA was determined using the following equation:

$$[RNA] = 40\ \mu g/ml \times absorbance_{260} \times dilution\ factor$$

This gave a concentration of RNA in µg per ml in each of the samples. The value of the ratio of the 260 nm reading over the 280 nm reading was used to assess the purity of samples. A ratio close to two indicated that the sample was mostly RNA and of pure quality. A lower ratio (below 1.5) signified the presence of other UV absorbers such as protein and DNA.

### 4.1.6. Confirmation of RNA integrity

After determining the concentration of total RNA, and calculating the 260 and 280 nm ratio, a visual check was performed to confirm RNA integrity. RNA samples were separated on an agarose gel (Appendix H) and their integrity was determined under UV light. For each RNA sample a volume of 1.0 µg/µl of RNA was mixed with 4 µl of loading buffer. The samples were loaded onto the gel and separated at a constant voltage of 75 V. The gel was viewed under UV light and photographed. Clear bands indicate intact RNA while a smearing of any of the lanes indicates that the RNA was probably degraded. A ratio of approximately two to one in the intensity of the 28S to the 18S rRNA band also indicated intact RNA.

### 4.1.7. Details of the p53-reverse transcriptase polymerase chain reaction kit

After confirming the integrity of the extracted RNA, samples were prepared for RT-PCR. For the analysis of p53 mRNA, the Gene Specific Relative RT-PCR kit for p53 (catalogue #'s 5300 and 5450), from Ambion, was used. This is a semi-quantitative analysis RT-PCR kit for RT-PCR of p53 with 18S rRNA as the control. This kit can modulate the amplification efficiency of a PCR template without affecting the performance of other targets in a multiplex PCR (refer to instruction manual of the product, also see Appendix I). The kit has 18S competimers which are modified at their 3' ends to block extension by DNA polymerase, thus allowing the decrease of the PCR amplification efficiency of 18S cDNA without the 18S primers becoming limiting and also preventing the loss of relative quantitation. Equal amounts of starting RNA were analysed and subjected to the same RT-PCR conditions which allowed the amplification of p53 mRNA in each sample. The products were analysed, the amount of product from each reaction being

proportional to the amount of p53 mRNA. Multiplex PCR was performed using two primer sets in a single PCR; one to amplify p53 and the other to amplify an endogenous control which in this case was 18S mRNA. The level of product of p53 was normalised against the 18S control. Before the samples were processed, the whole RT-PCR process had to be optimised (for more details refer to Appendix I).

The recommended annealing temperature for p53 mRNA was 61 ºC (as per the kit manual). The sequence of the p53 gene used in the kit had the Genbank ID/version number of AF051368 (Genbank, 2013). This represented the *Mus musculus* tumour suppressor p53 mRNA, complete coding sequence (cds).

The sequence of p53 mRNA:

1 ggtgtcacgc ttctccgaag actggatgac tgccatggag gagtcacagt

51 cggatatcag cctcgagctc cctctgagcc aggagacatt ttcaggctta

101 tggaaactac ttcctccaga agatatcctg ccatcacctc actgcatgga

151 cgatctgttg ctgccccagg atgttgagga gtttttttgaa ggcccaagtg

201 aagccctccg agtgtcagga gctcctgcag cacaggaccc tgtcaccgag

251 acccctgggc cagcggcccc tgccccagcc actccatggc ccctgtcatc

301 ttttgtccct tctcaaaaaa cttaccaggg caactatggc ttccacctgg

351 gcttcctgca gtctgggaca gccaagtctg ttatgtgcac gtactctcct

401 cccctcaata agctattctg ccagctggcg aagacgtgcc ctgtgcagtt

451 gtgggtcagc gccacacctc cagctgggag ccgtgtccgc gccatggcca

501 tctacaagaa gtcacagcac atgacggagg tcgtgagacg ctgcccccac

551 catgagcgct gctccgatgg tgatggcctg gctcctcccc agcatcttat

601 ccgggtggaa ggaaatttgt atcccgagta tctggaagac aggcagactt

651 ttcgccacag cgtggtggta ccttatgagc cacccgaggc cggctctgag

701 tataccacca tccactacaa gtacatgtgt aatagctcct gcatgggggg

751 catgaaccgc cgacctatcc ttaccatcat cacactggaa gactccagtg

801 ggaaccttct gggacgggac agctttgagg ttcgtgtttg tgcctgccct

851 gggagagacc gccgtacaga agaagaaaat ttccgcaaaa aggaagtcct

901 ttgccctgaa ctgcccccag ggagcgcaaa gagagcgctg cccacctgca

951 caagcgcctc tcccccgcaa aagaaaaaac cacttgatgg agagtatttc

1001 accctcaaga tccgcgggcg taaacgcttc gagatgttcc gggagctgaa

1051 tgaggcctta gagttaaagg atgcccatgc tacagaggag tctggagaca

1101 gcagggctca ctccagctac ctgaagacca agaagggcca gtctacttcc

1151 cgccataaaa aaacaatggt caagaaagtg gggcctgact cagactgact

1201 tctgc

The kit provides enough reagents for 100 reactions. However, it was found that the RT-PCR process could be modified and optimised to analyse 200 reactions. This was done by halving all the volumes of solutions used, including the amount of sample to be analysed.

### 4.1.8. Experimental method for reverse transcriptase polymerase chain reaction

An RT master mix (RTMM), containing 10x RT buffer, dNTP, oligo dT, RNasin, RT enzyme and nuclease free $H_2O$, was prepared. The volumes for each component are listed in Table 4.1. The RTMM was prepared such that there was an adequate amount to analyse the required number of samples. A volume of 10 µl of RTMM was pipetted into each of 30 mini Eppendorf tubes. To each tube, 1 µl of isolated RNA sample was added. The mixture was vortexed for 15 seconds.

Table 4.1 The volume of components used in the RT master mix

| Component | 1 sample (µl) | RTMM (µl) |
|---|---|---|
| 10x RT buffer | 1.00 | 31.0 |
| dNTP | 0.50 | 15.5 |
| Oligo dT | 0.25 | 7.75 |
| RNasin | 0.10 | 3.1 |
| RT enzyme | 0.05 | 1.55 |
| Nuclease free $H_2O$ | 7.10 | 220.1 |
| RNA | 1.00 | 31 |

The tubes were placed in the thermocycler and the following programme was used for RT:

| | |
|---|---|
| RT cycle: | 44 ºC for 1 hour |
| Inactivation cycle: | 92 ºC for 10 minutes |

After completion of the thermocycler programme the samples were removed and put onto ice. The tubes were centrifuged briefly at 200 g for 15 seconds. For PCR, 5 µl of RT sample was used from each tube.

A volume of 20 µl of PCR master mix (PCRMM) (Table 4.2) was pipetted into each of 30 Eppendorf tubes and 5 µl of RT sample was added to each one. The mixture was vortexed for 15 seconds.

Table 4.2 The volume of components used for the PCR master mix

| Component | 1 sample (µl) | PCRMM (µl) |
|---|---|---|
| p53 | 0.80 | 24.80 |
| 18SPP | 0.625 | 19.40 |
| 18SC | 0.625 | 19.40 |
| MM | 12.50 | 387.50 |
| $H_2O$ | 5.45 | 168.95 |
| cDNA | 5.00 | 155.00 |

The tubes were placed in the thermocycler and the following programme was used for PCR:

| | |
|---|---|
| Denature: | 94 ºC for 4 minutes |
| 30 cycles: | 94 ºC for 30 seconds |
| | 61 ºC for 30 seconds |
| | 72 ºC for 59 seconds |
| Final extension: | 72 ºC for 5 minutes |

Once the programme was completed (about 3 hours), the tubes were removed and placed on ice. They were centrifuged at 200 g for 15 seconds to coalesce the solution to the bottom of the tube. These samples were then prepared for separation on an agarose gel.

### 4.1.9. Preparation and gel electrophoresis of the polymerase chain reaction product

The PCR products were separated on an agarose gel as described in Appendix H but with modifications as these were DNA samples. DNA 6X loading dye (1 µl) was mixed with 5 µl of each sample. The gel was prepared as per Appendix H. Samples were loaded into each well on the gel. The gel was subjected to a constant voltage of 75 volts. Once the dye front was close to the end of the gel the power was switched off; the bands were viewed using a UV light source and photographed. The photographs were later scanned and the data analysed.

### 4.1.10. Time studies and data analysis

The photographs of the separated PCR bands were analysed densitometrically by SYNGENE GeneTools (version 3.06) analysis software. The intensity of the bands (arbitrary units) for 18S and p53 were obtained. A ratio of the p53 value to that of 18S was calculated; the result corresponded to the relative expression of p53 mRNA in the sample. The analysis of the data here was done using the programmes mentioned in Chapter 2 section 2.1.7 Time studies and data analysis. As was stated in that section all experimental conditions were kept the same for each experiment.

## 4.2. Results for reverse transcriptase polymerase chain reaction of p53 in murine erythroleukaemic cells

### 4.2.1. Agarose gels of p53 polymerase chain reaction product

A typical example of a gel with intact RNA is shown in figure 4.1. From the gel there are two indicators that reflect RNA integrity; the first is the clear and distinct bands (no smearing) and the second is the ratio of the 28S band to the 18S band which should be approximately double.

Representative results showing PCR product of p53 and 18S are shown in figure 4.2. These samples were electrophoresed together with a standard DNA marker ladder and an RNA sample for comparison. Some of the bands on the DNA marker are labelled and represent: 10 000 bp, 1 031 bp, 500 bp and 300 bp. The 18S PCR product forms a band close to the 500 bp marker while the p53 band is located close to the 300 bp marker. According to the kit the p53 product should be 292 bp. The volumes of reagents used for the experiment in figure 4.2 are listed in table 4.3.

A typical example of results showing the expression of p53 mRNA compared with 18S is shown in figure 4.3. These samples were isolated at regular time intervals of 15 minutes. The results showed time dependent changes in the expression of p53 mRNA. All lanes were densitometrically scanned and a ratio of the p53 value over that of the 18S was calculated and plotted against time. In each of the time series (15, 30, and 60 minutes) two sets of experiments were carried out. Period and amplitude graphs could be calculated for all the experiments. Values for cell and total RNA concentrations were also recorded and correlation graphs were plotted.

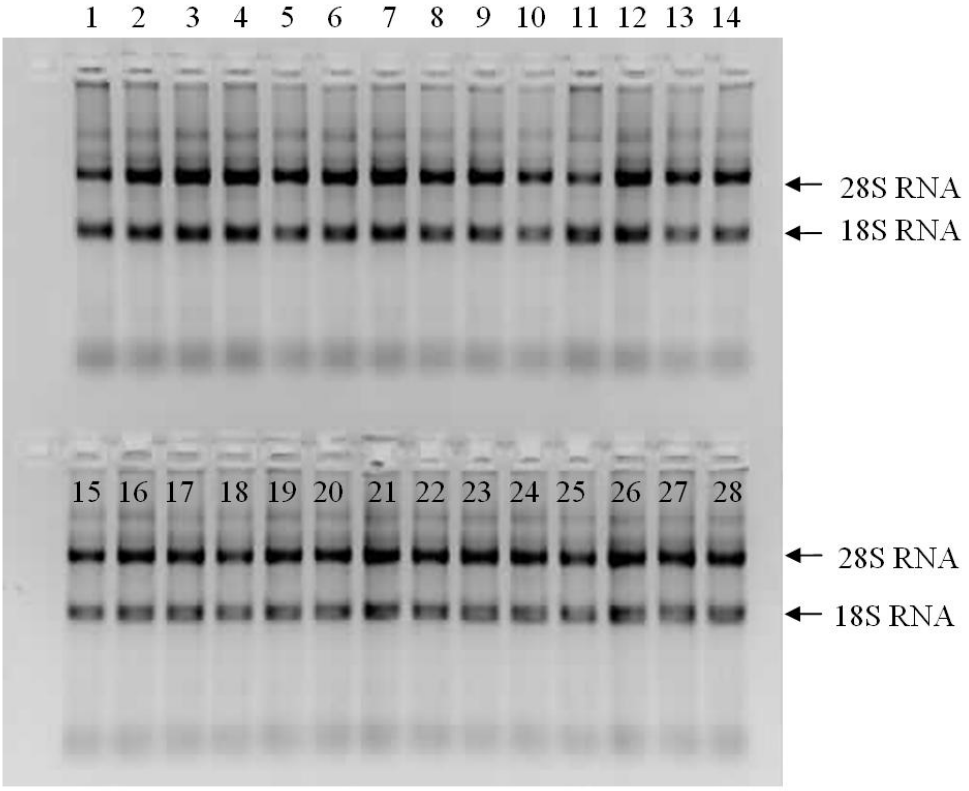



Figure 4.1 A typical example of an agarose gel (2%) showing intact RNA isolated from untreated and HMBA treated cells

Proliferating and differentiating MEL cells were isolated at 30 minute time intervals. Their RNA was extracted and separated on a 2% agarose gel. Clear bands representing 28S and 18S RNA is visible, reflecting the presence of intact RNA. Lanes 1 to 28 had 2 µl of isolated RNA mixed with 8 µl of DEPC $H_2O$ and 2 µl of 6x loading dye solution. Lanes 1, 3, 5, 7, 9, 11, 13, 15,17, 19, 21, 23, 25, and 27 were from untreated MEL cells isolated from 0 to 390 minutes at 30 minute intervals. Lanes 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, and 28 were from HMBA treated MEL cells isolated from 0 to 390 minutes at 30 minute intervals. These samples were electrophoresed on an 8 cm length 2% agarose gel, 1x TAE buffer, 10 V/cm, for 60 minutes.

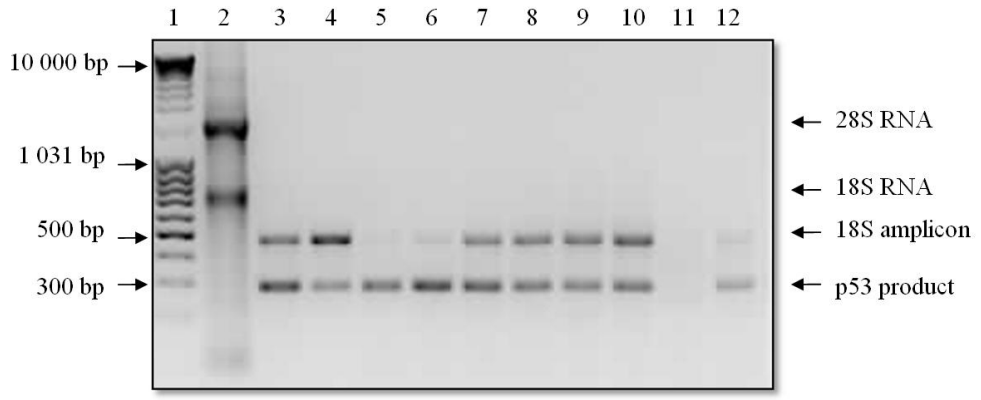



Figure 4.2 Sample of intact total RNA and RT-PCR product on a 2% agarose gel

An example of a 2% agarose gel showing the position of the 28S and 18S RNA (lane 2) in relation to the MassRuler DNA ladders (10 kbp to 80 bp, lane 1). Lane 1 had 10 µl of MassRuler DNA ladders loaded with 6X loading dye solution. Lane 2 had 2 µl of RNA with 2 µl DNA loading buffer. Different concentrations of RT-PCR product are apparent in lanes 3 to 12, dependent on the initial concentrations of cDNA, p53 primers and the ratio of 18S PCR primer to 18S PCR competimer (See table 4.3 for the volumes used). These samples were run on an 8cm length 2% agarose gel, 1x TAE buffer, 10 V/cm, for 60 minutes.

Table 4.3 The volume (µl) of components used in the PCR of samples

| | Lane number | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| p53 | 1.00 | 1.000 | 0.800 | 0.800 | 0.800 | 0.800 | 0.800 | 0.800 | 0.800 | 0.800 |
| 18S PP | 0.62 | 0.625 | 0.125 | 0.250 | 0.375 | 0.500 | 0.625 | 0.750 | 0.500 | 0.500 |
| 18S PC | 0.62 | 0.625 | 1.125 | 1.000 | 0.875 | 0.750 | 0.625 | 0.500 | 0.750 | 0.750 |
| MM | 12.5 | 12.50 | 12.50 | 12.50 | 12.50 | 12.50 | 12.50 | 12.50 | 12.50 | 12.50 |
| $H_2O$ | 5.25 | 7.75 | 5.45 | 5.45 | 5.45 | 5.45 | 5.45 | 5.45 | 5.45 | 5.45 |
| cDNA | 5.00 | 2.50 | 5.00 | 5.00 | 5.00 | 5.00 | 5.00 | 5.00 | 5.00 | 5.00 |
| | | | | | | | | | | |

PP = PCR primer Pair, PC = PCR Competimers, MM = Master Mix

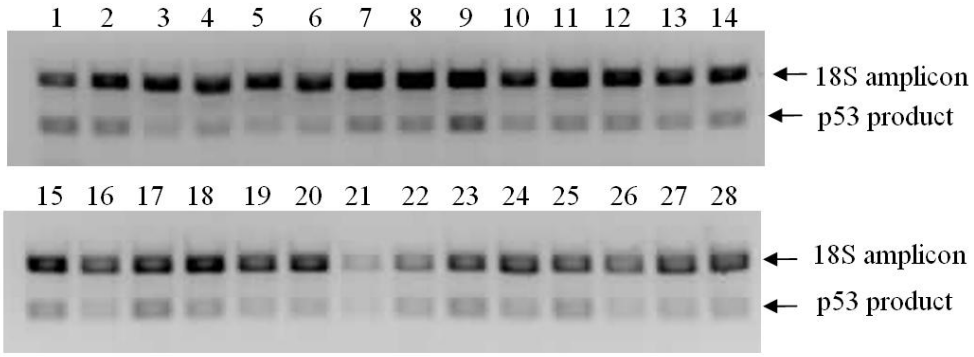



Figure 4.3 A typical example of an agarose gel of RT-PCR product of p53 mRNA and 18S from untreated and HMBA treated MEL cells

An example of a typical agarose gel reflecting the expression of p53 mRNA from untreated and HMBA treated MEL cells together with the 18S RNA. Equal amounts of isolated RNA were subjected to RT-PCR. Each lane had 10 µl of product mixed with 2 µl of 6x loading dye. Lanes 1, 3, 5, 7, 9, 11, 13, 15,17, 19, 21, 23, 25, and 27 were from untreated MEL cells isolated from 0 to 195 minutes at 15 minute intervals. Lanes 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, and 28 were from the HMBA treated MEL cells isolated from 0 to 195 minutes at 15 minute intervals. These samples were electrophoresed on an 8 cm length 2% agarose gel, 1x TAE buffer, 10 V/cm, for about 60 minutes.

### 4.2.2. Expression of p53 mRNA at 15 minute time intervals

Samples were isolated, in two different experiments, at 15 minute time intervals, from untreated and HMBA induced cells. The total RNA was extracted from the samples and analysed for p53 mRNA. The fitted curves for these samples are shown in figure 4.4 A, and C. Their corresponding difference curves are depicted on the right (figure 4.4 E, and G). The respective period and amplitude curves were calculated and are represented in figure 4.4 B, F, D, and H. A summary of the range and mean values for these curves is presented in table 4.4.

In the first experiment (figure 4.4 A) p53 mRNA expression from the untreated and the treated samples was initially in synchrony; HMBA had only a small effect at 60, 120, 165, and 180 minutes, when expression of p53 mRNA was inhibited as compared with the untreated samples, the only exception being at 90 minutes. In contrast, HMBA had a stimulatory effect on the cells in the second experiment during most of the first 105 minutes (figure 4.4 C). Thereafter, it inhibited expression. Initially, the expression was out of phase between the treated and untreated samples but was mostly synchronised again after 75 minutes until the end. In both experiments the difference curves exhibited values that fluctuated around the zero point mark (figure 4.4 E and G).

Period and amplitude curves could be calculated for both experiments, confirming that the p53 mRNA expression was changing in an oscillatory manner. In the first experiment, the period and the amplitude (figure 4.4 B, and F) values for the untreated samples were generally larger than those of the treated samples. In contrast, in the second experiment, this behaviour was only reflected in the period graph and not in the amplitude graph (figure 4.4 D, and H). In both experiments, the

period and amplitude graphs from the treated samples were noticeably oscillatory than those from the untreated samples.

The cell and total RNA concentrations for both experiments were plotted against time. These are shown in figure 4.5 A and B for experiment one, and figure 4.6 A and B for experiment two. The corresponding correlation graphs are given in figure 4.5 D, E, and F and figure 4.6 D, E, and F. In experiment one, the cell concentrations of untreated and HMBA treated samples were initially out of phase, up to 50 minutes, and thereafter they became synchronised, with the HMBA treated cells showing a higher cell count after 105 minutes. In experiment two, the cell concentrations were in phase with the untreated cells being higher in number. In contrast, the RNA concentrations for both the experiments showed variations, being in and out of phase around similar averages.

In neither of the two experiments were there any correlation between cell concentration, RNA concentration and p53 mRNA expression. The highest correlation was seen between p53 mRNA expression and RNA concentration in HMBA treated cells which was 0.43 (figure 4.6 F).

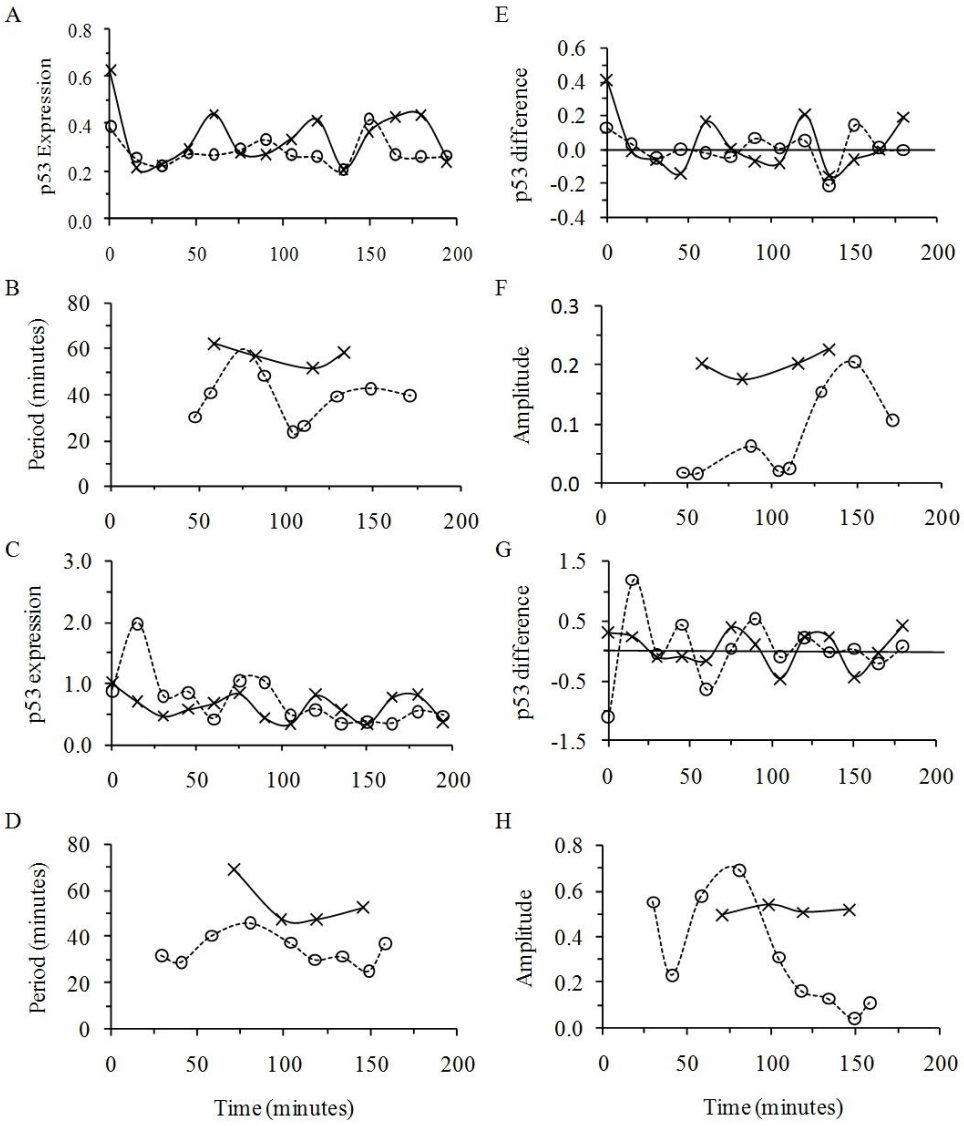



Figure 4.4 Expression of p53 mRNA

Temporal variations in the expression of p53 mRNA as measured at 15 minute time intervals in two different experiments (A and C). The respective difference (E and G), period (B and D) and amplitude (F and H) curves are included. Proliferating control cells (x—x); cells treated with HMBA (o--o).

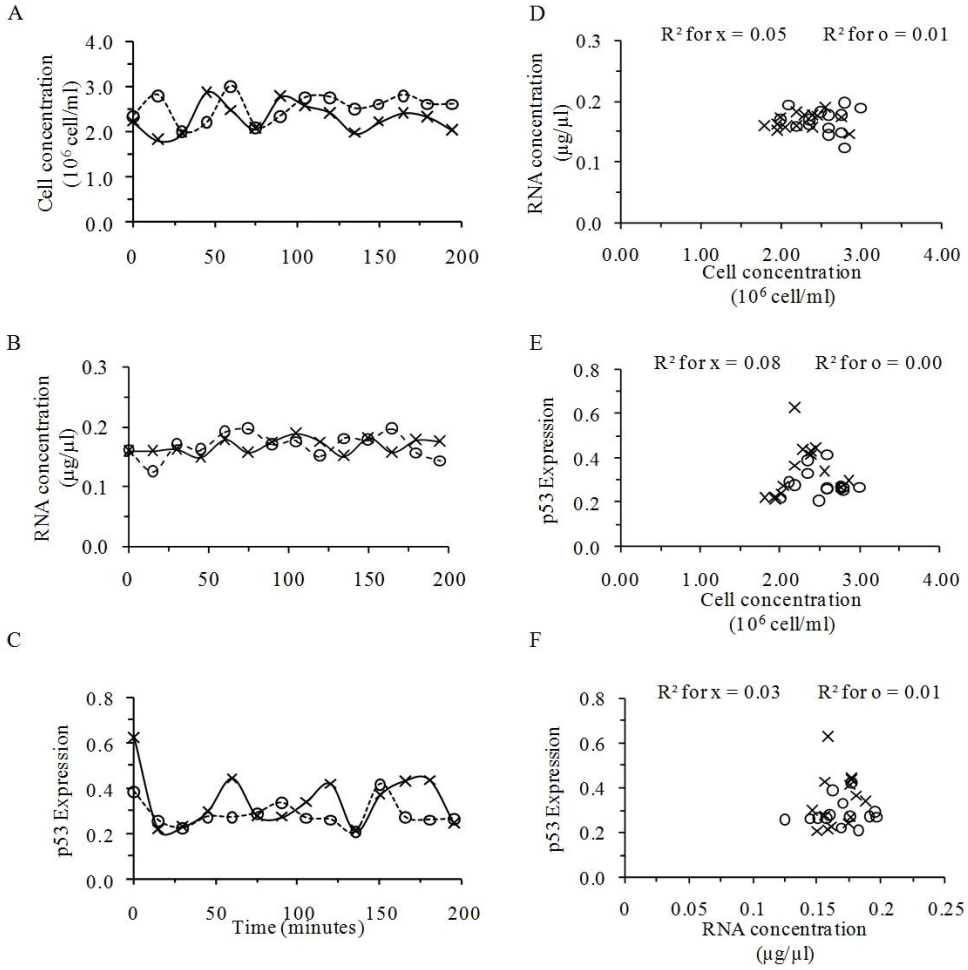



Figure 4.5 Comparison of p53 mRNA expression, cell concentration and total RNA concentration

Changes in the concentration of cells (A), total RNA (B) and relative p53 mRNA (C) expression measured at 15 minute time intervals. These values correspond to graph 4.4 A. The correlation curves comparing each of these components are included on the right (D, E, and F). Proliferating control cells (x—x); cells treated with HMBA (o--o).

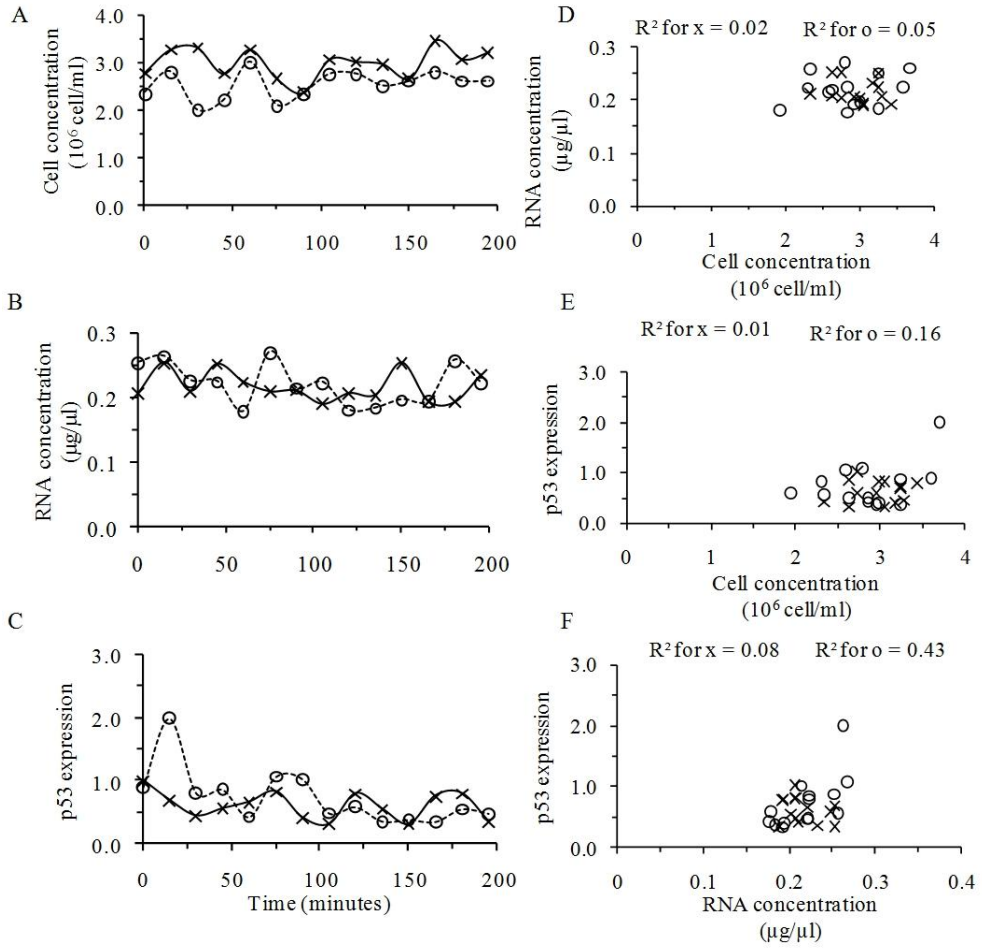



Figure 4.6 Comparison of p53 mRNA expression, cell concentration and total RNA concentration

Changes in the concentration of cells (A), total RNA (B) and relative p53 mRNA (C) expression measured at 15 minute time intervals. These values correspond to graph 4.4 C. The correlation curves comparing each of these components are included on the right (D, E, and F). Proliferating control cells (x—x); cells treated with HMBA (o--o).

### 4.2.3. Expression of p53 mRNA at 30 minute time intervals

Two experiments were performed at 30 minute time intervals. The results are illustrated in figure 4.7. As was the case for the 15 minute time intervals, the fitted curves showed oscillatory changes in the expression of p53 mRNA (figure 4.7 A, and C). The difference curves were calculated from the fitted curves (figure 4.7 E, and G). The period (figure 4.7 B, and D) and amplitude (figure 4.7 F, and H) graphs are shown as well. The cell and total RNA concentrations are shown in figure 4.8 A, and B for experiment one and figure 4.9 A, and B for the second experiment. The corresponding correlation graphs are given in figure 4.8 D, E, and F and figure 4.9 D, E, and F. A summary of the range and mean values for these curves is presented in table 4.4.

The p53 mRNA expression patterns in the two 30 minute experiments were very different. In the first experiment the expression of p53 mRNA in the untreated and HMBA treated samples showed oscillatory behaviour that was out of phase for most of the experiment (figure 4.7 A). In the second experiment they were in phase for the first half of the experiment (figure 4.7 C), with the untreated cells showing an unusual peak at around 240 minutes. The difference curves in both experiments were predominantly negative (figure 4.7 E, and G) with most of the values being below the zero point mark.

The period graphs (figure 4.7 B, and D) for both experiments reflected the oscillatory behaviour, with no major displacement between the HMBA treated and untreated cells. In both experiments the changing period of the untreated and HMBA treated samples reflected what was shown in the expressional patterns of p53 mRNA, the patterns were out of phase for most of the experiment. The

amplitude curves (figure 4.7 F, and H) also demonstrated wave patterns but these were not as clear and regular as for the period graphs. The amplitude curve in the first experiment (figure 4.7 F) was predominantly higher for the HMBA treated cells then the untreated ones. In the second experiment (figure 4.7 H) the untreated cells exhibited a peak rise at 240 minutes. In both experiments, both the period and amplitude graphs showed predominantly out of phase patterns of expression of p53 mRNA in the untreated and HMBA treated samples.

For the cell concentration, the untreated and HMBA treated samples in experiment one (figure 4.8 A) showed out of phase patterns of concentration while in experiment two (figure 4.9 A) the concentrations showed synchronised behaviour which was slightly dampened by HMBA until 220 minutes after which they moved out of phase. For total RNA in experiment one (figure 4.8 B), concentrations were initially synchronised (up to 60 minutes), with HMBA dampening and causing an out of phase shift for RNA expression for the next 120 minutes after which there was synchrony again for 80 minutes. For the second experiment (figure 4.9 B) the RNA concentration started out of phase, altering into phase at 60 minutes with synchrony for the next 90 minutes, and again changing - out of phase for 120 minutes. A spike in p53 mRNA expression in untreated cells was noted at around 240 minutes and this corresponded to a similar slight increase in the total RNA concentration.

As was the case with the experiments in the 15 minute time interval, there was no correlation between any of the data values collected for the 30 minute experiments (figure 4.8 D, E, and F for experiment one and figure 4.9 D, E, and F for experiment two). The highest correlation observed was between p53 mRNA expression and total RNA concentration in untreated cells with a value of 0.46 (figure 4.9 F).

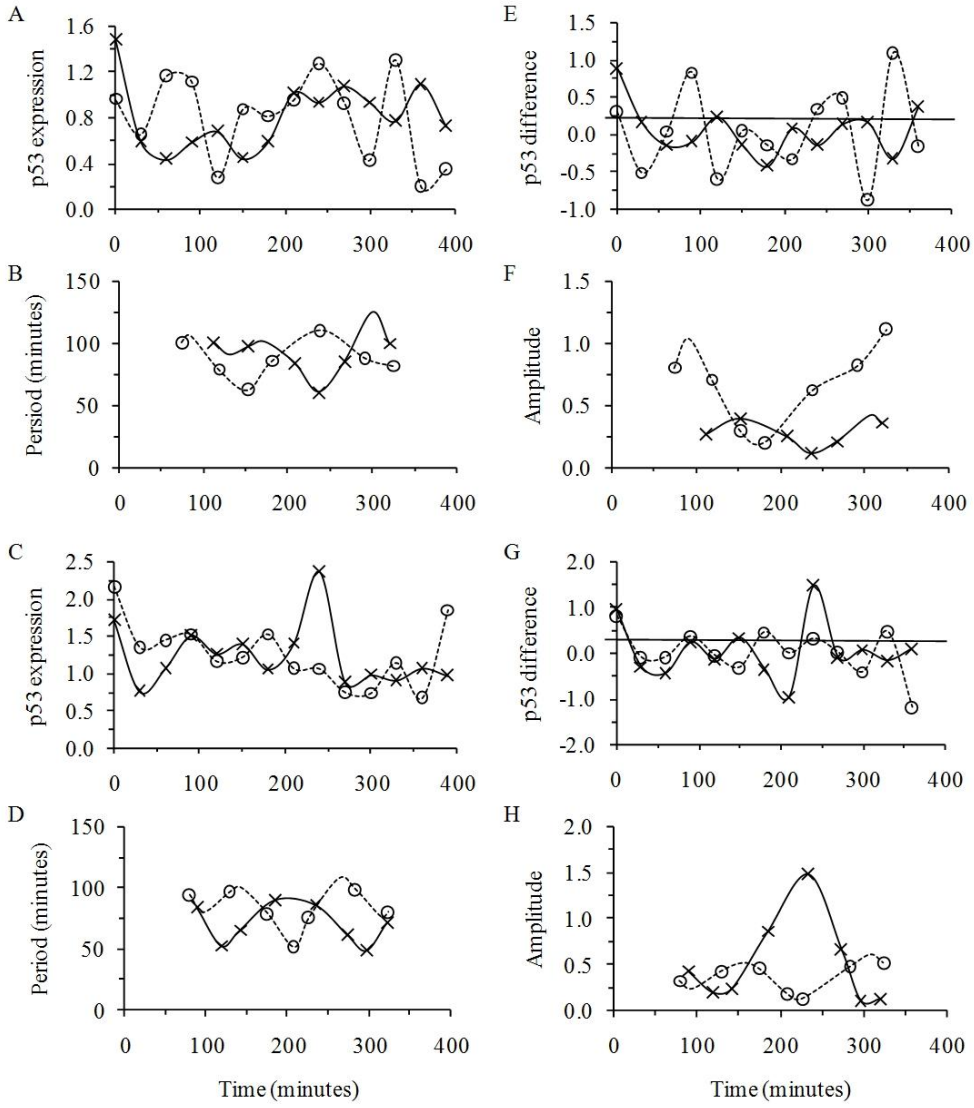



Figure 4.7 Expression of p53 mRNA

Temporal variations in the expression of p53 mRNA as measured at 30 minute time intervals in different experiments (A and C). The respective difference (E and G), period (B and D) and amplitude curves (F and H) are included. Proliferating control cells (x—x); cells treated with HMBA (o--o).

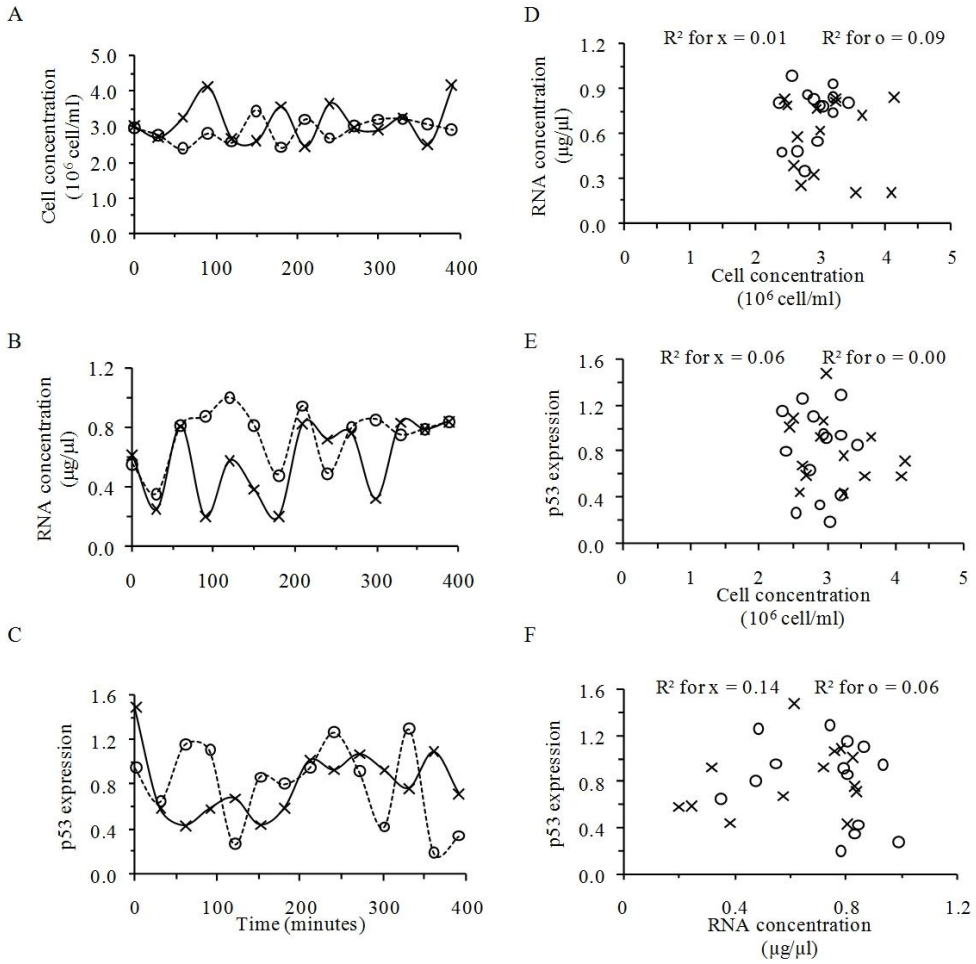



Figure 4.8 Comparison of p53 mRNA expression, cell concentration and total RNA concentration

Changes in the concentration of cells (A), total RNA (B) and relative p53 mRNA (C) expression measured at 30 minute time intervals. These values correspond to graph 4.7 A. The correlation curves comparing each of these components are included on the right (D, E, and F). Proliferating control cells (x—x); cells treated with HMBA (o--o).

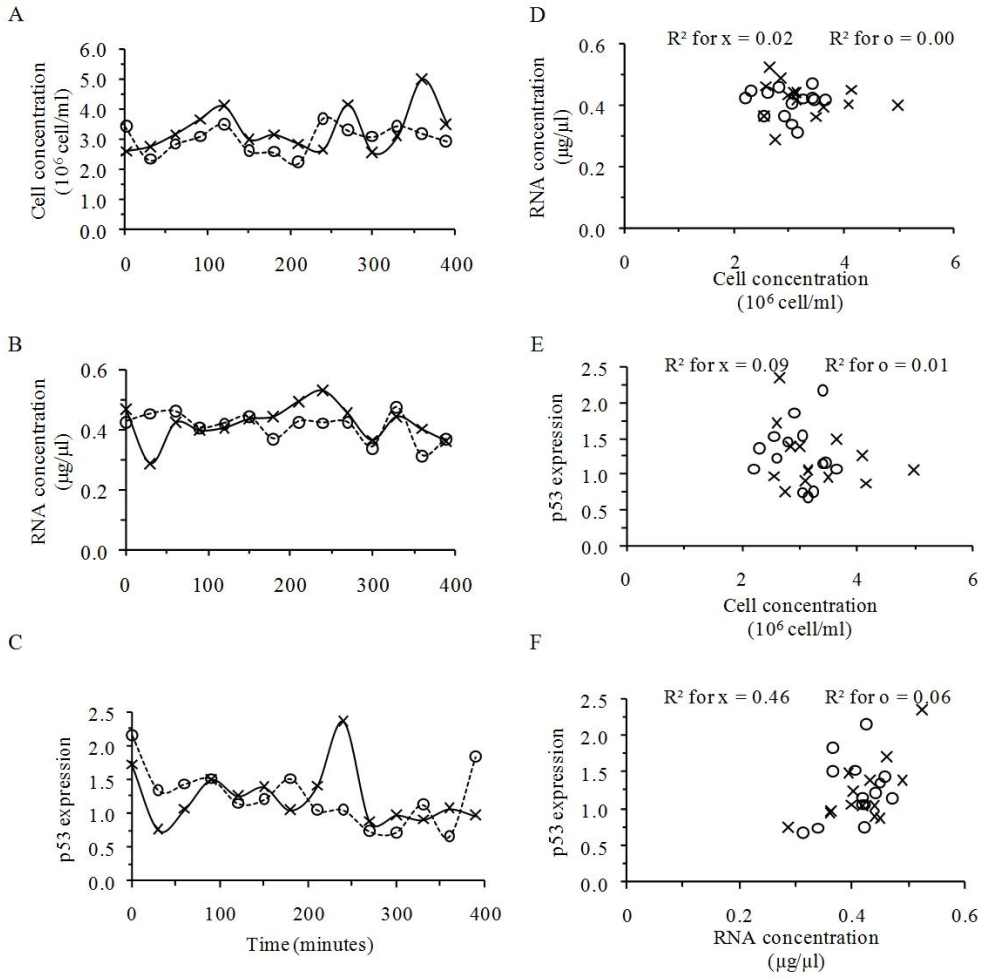



Figure 4.9 Comparison of p53 mRNA expression, cell concentration and total RNA concentration

Changes in the concentration of cells (A), total RNA (B) and relative p53 mRNA (C) expression measured at 30 minute time intervals. These values correspond to graph 4.7 C. The correlation curves comparing each of these components are included on the right (D, E, and F). Proliferating control cells (x—x); cells treated with HMBA (o--o).

### 4.2.4. Expression of p53 mRNA at 60 minute time intervals

Results for the two experiments for the longer time period of 60 minutes are shown in figure 4.10 A, and C. The expression of p53 mRNA in the first experiment (figure 4.10 A) showed a small variation between the untreated and HMBA treated cells which was mostly out of phase and there was a dampening effect from HMBA in the middle and latter stages of the experiment. The untreated cells exhibited a spike at the end of the experiment starting at 600 minutes rising to a value of 1.69. In the second experiment (figure 4.10 C), both untreated and treated cells showed oscillatory behaviour of p53 mRNA expression, which was for the most part out of phase. The expression became out of phase at 60 minutes, then after 400 minutes HMBA dampened the expression until 650 minutes when the samples synchronised. These changes are again emphasised by the difference curves for the two experiments (figure 4.10 E, and G). As was previously noted these graphs have values predominantly below the zero value mark.

The period and amplitude (figure 4.10 B, D, F, and H) curves were calculated for both experiments. In the first experiment, these curves were oscillatory in form and pronounced in the untreated cells. In the second experiment, only the amplitude curve for untreated cells was oscillatory with treated cells having fairly constant amplitude which increased at the end. A summary of the range and mean values for these curves is presented in table 4.4.

The cell concentration in the first experiment (figure 4.11 A) displayed a very regular oscillating pattern for both the untreated and HMBA treated cells. The cells varied around the 2.4 million cells per ml concentration. In contrast, the total RNA concentration (figure 4.11 B) was fairly constant for untreated cells while HMBA

treated cells showed variation after 360 minutes. In the second experiment (figure 4.12 A) the cells initial concentrations are different and variation throughout the experiment is mostly out of phase. There is some synchrony between the 180 and 240 minute time period. The total RNA concentration (figure 4.12 B) in this experiment is in phase for 420 minutes at which time the treated cells exhibit a lower production of RNA. This behaviour corresponds with lowered p53 mRNA production (also after 420 minutes) in the treated cells.

In neither of the 60 minute time period experiments was there any correlation between cell concentration, RNA concentration, and p53 mRNA expression (figure 4.11 D, E, and F for experiment one and figure 4.12 D, E, and F for experiment two). The highest correlation observed was between total RNA concentration and cell concentration in HMBA treated cells with a value of 0.34 (figure 4.11 D).

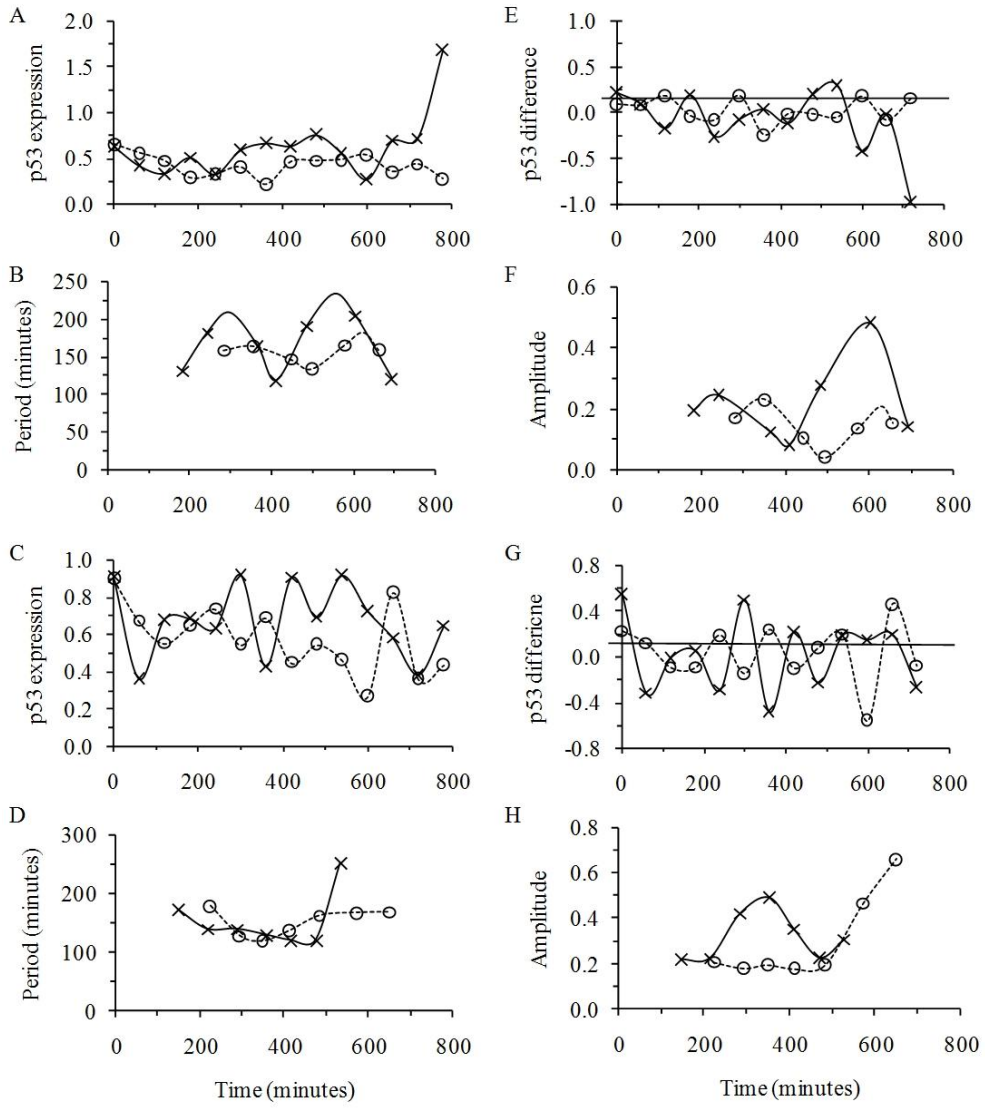



Figure 4.10 Expression of p53 mRNA

Temporal variations in the expression of p53 mRNA as measured at 60 minute time intervals in different experiments (A and C). The corresponding difference curves are included (E and G), together with period (B and D), and amplitude (F and H) plots. Proliferating control cells (x—x); cells treated with HMBA (o--o).

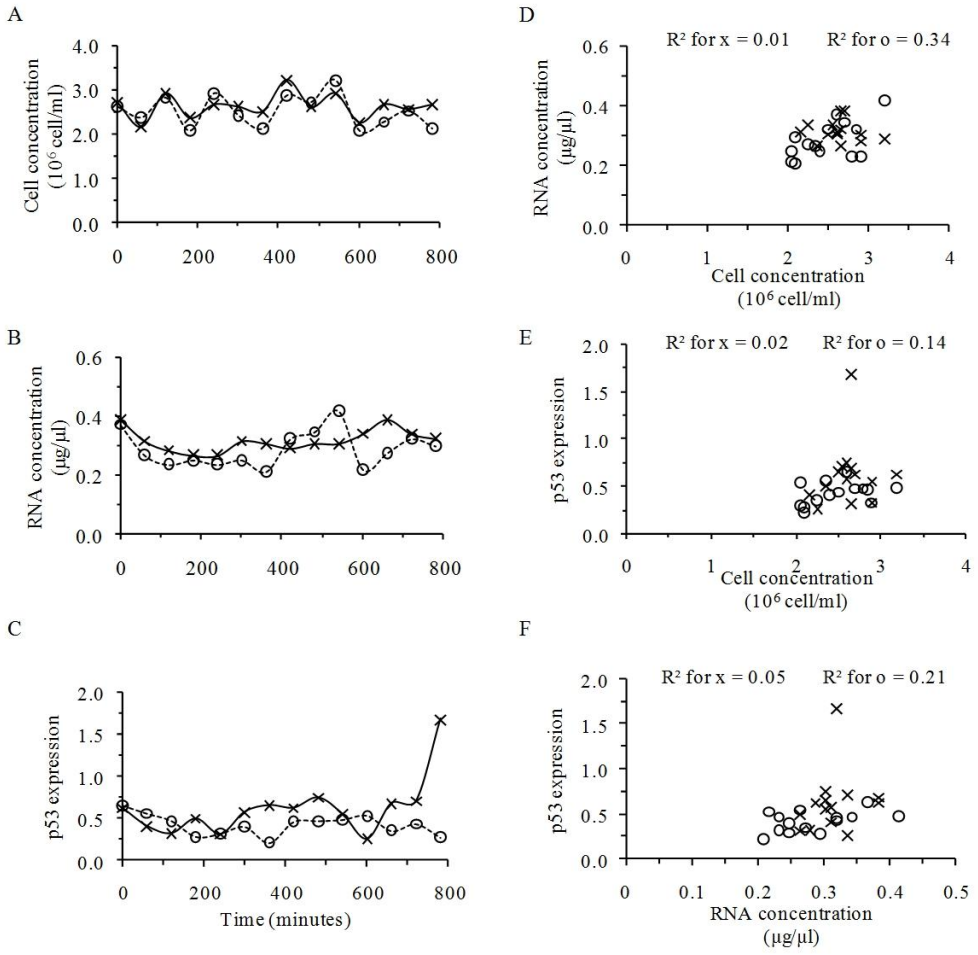



Figure 4.11 Comparison of p53 mRNA expression, cell concentration and total RNA concentration

Changes in the concentration of cells (A), total RNA (B) and relative p53 mRNA (C) expression measured at 60 minute time intervals. These values correspond to graph 4.10 A. The correlation curves comparing each of these components are included on the right (D, E, and F). Proliferating control cells (x—x); cells treated with HMBA (o--o).

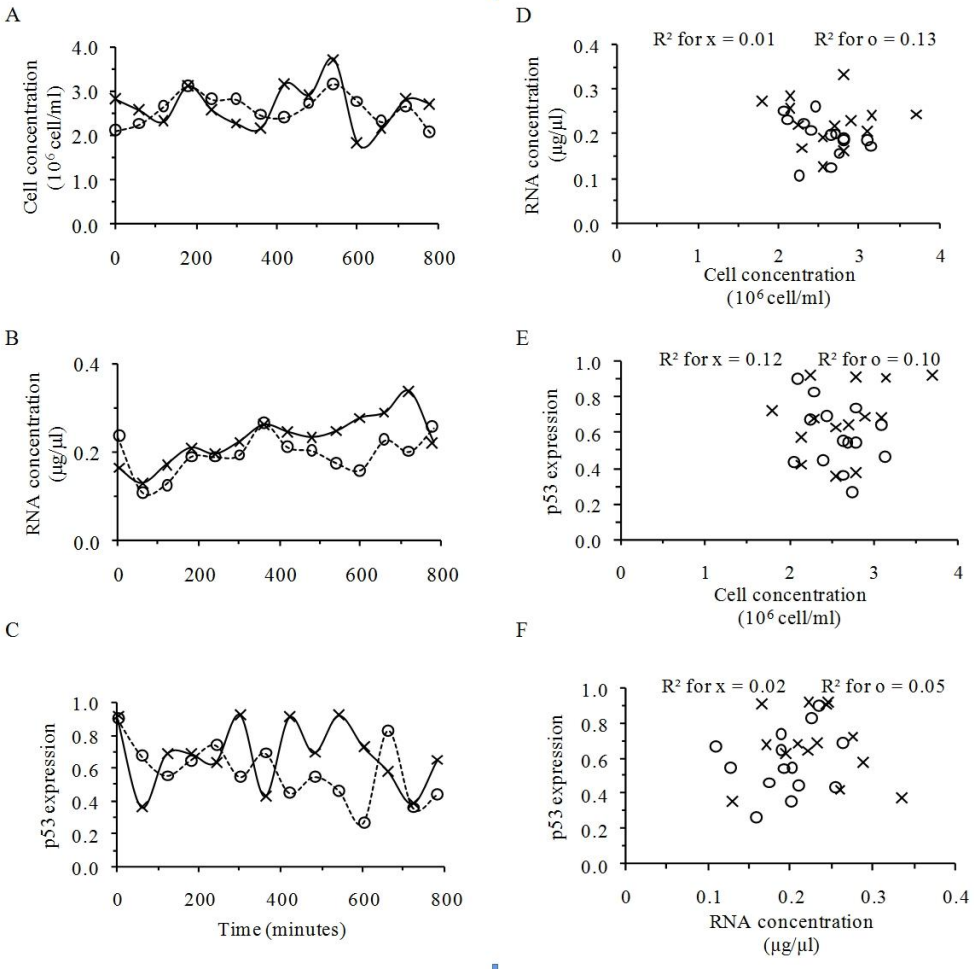



Figure 4.12 Comparison of p53 mRNA expression, cell concentration and total RNA concentration

Changes in the concentration of cells (A), total RNA (B) and relative p53 mRNA (C) expression measured at 60 minute time intervals. These values correspond to graph 4.10 C. The correlation curves comparing each of these components are included on the right (D, E, and F). Proliferating control cells (x—x); cells treated with HMA (o--o).

### 4.2.5. The range and mean of the period and amplitude curves for p53 mRNA expression at different time intervals

A summary of the range and mean values for each of the period and amplitude curves is given in table 4.4. Both the range and the mean values got larger for the period curves in each of the experiments (from 15 minutes to 60 minutes). The range and mean values for the 15 minute time interval experiments showed a dampening effect by HMBA on p53 mRNA expression for the period and amplitude curves. This behaviour was not as obvious in the amplitude graphs of the second experiment (figure 4.4 H). The range varied between 48 and 69 minutes for untreated cells and 23 and 48 minutes for HMBA treated cells (in both experiments).

For the 30 minute time period experiments the range was between 48 and 101 minutes for untreated cells and 52 and 111 minutes for the HMBA treated cells. Their mean value increased to 70 and 87 minutes for untreated cells and 87 and 83 minutes for treated cells. In the longer 60 minute time period experiments the range was 118 and 253 minutes for the untreated cells and 120 to 180 minutes for the HMBA treated cells. Again the mean values increased to 159, 154 (untreated) and 155, 152 (treated) minutes when compared to the 15 and 30 minute experiments.

The amplitude values for all the experiments varied from a minimum of 0.04 to a maximum of 0.66 (refer to Table 4.4). Unlike the trend with the periods of each experiment, no discernible pattern was reflected in the amplitudes.

Table 4.4 Comparisons between the range and mean values of the period and amplitude for p53 RNA expression at different time intervals from untreated and HMBA treated MEL cells

| Experiment | Period (minutes) | | | | Amplitude (arbitrary units) | | | |
|---|---|---|---|---|---|---|---|---|
| | Untreated cells | | HMBA treated cells | | Untreated cells | | HMBA treated cells | |
| | Range | Mean | Range | Mean | Range | Mean | Range | Mean |
| 15 minute p53 RNA sample 1 | 51 – 63 | 57 ± 5 | 23 - 48 | 36 ± 9 | 0.18 – 0.23 | 0.20 ±0.02 | 0.02 – 0.21 | 0.08 ± 0.07 |
| 15 minute p53 RNA sample 2 | 48 – 69 | 55 ± 10 | 25 – 46 | 34 ± 7 | 0.50 - 0.54 | 0.52 ± 0.02 | 0.04 – 0.70 | 0.31 ± 0.24 |
| 30 minute p53 RNA sample 1 | 61 – 101 | 87 ± 15 | 62 – 111 | 87 ± 16 | 0.11 – 0.40 | 0.26 ± 0.10 | 0.20 – 1.12 | 0.65 ± 0.31 |
| 30 minute p53 RNA sample 2 | 48 – 91 | 70 ± 16 | 52 – 99 | 83 ± 16 | 0.10 – 1.49 | 0.51 ± 0.48 | 0.13 – 0.52 | 0.36 ± 0.15 |
| 60 minute p53 RNA sample 1 | 118 – 205 | 159 ±36 | 133 – 165 | 155 ± 12 | 0.08 – 0.48 | 0.22 ± 0.14 | 0.04 – 0.23 | 0.14 ± 0.07 |
| 60 minute p53 RNA sample 2 | 120 – 253 | 154 ± 47 | 120 – 180 | 152 ± 23 | 0.22 – 0.50 | 0.32 ± 0.11 | 0.18 – 0.66 | 0.30 ± 0.19 |
| | | | | | | | | |

## 4.3. Discussion

The p53 protein plays a crucial role in determining a cell's destiny after DNA damage which could either lead to temporary cell growth arrest (Lacroix *et al.*, 2006) or activation of programmed cell death (El-Deiry, 2003; Jackson *et al.*, 2011; Prives and Hall, 1999; Riley *et al.*, 2008; Sionov and Haupt, 1999; Vousden and Lu, 2002; Zamzami and Kroemer, 2005). The pathways involved in these processes are highly interconnected and eventually will affect the expression of p53 via various positive and negative feedback mechanisms (Geva-Zatorsky *et al.*, 2010; Hat *et al.*, 2009; Toettcher *et al.*, 2010; Toettcher *et al.*, 2011; Zhang *et al.*, 2011).

Previously it has been observed that the total expression of different proteins in the cell varies with time (Ferreira *et al.*, 1994b). This variation is dependent on the levels and changes of the individual proteins in the cell. These concentrations are highly regulated and are dependent on the rate of production minus the rate of removal of each of the proteins which in turn are dependent on the rate of mRNA expression (Rosenfeld, 2011a). All of these are ultimately dependent and controlled by the signals that direct these processes. Thus a measure of mRNA levels of a particular protein should give an indication of the production rate of that protein in the cell. But this is not the only factor, there are many other processes in the cell that will affect the rate of production, one such influence is post-translational modification. In this Chapter we measured the change of p53 mRNA expression at regular intervals, comparing them in proliferating and differentiating cells.

As was the case in previous experiments for protein expression of PP1, PP2A, phosphatases, PTP-1B, (Bhoola and Hammond, 2000; Bodalina *et al.*, 2005; Hammond *et al.*, 1989a) and p53 (Bodalina *et al.*, 2007), the expression of p53 mRNA was dynamic. Each of the experiments showed that this expression oscillated with a variable period, both in the untreated and HMBA treated MEL cells. No discernible or significant pattern could be detected. For the shorter time periods of 15 minutes a more gradual change in oscillation was perceived as compared with the other intervals. Thus for the shorter time periods more details were observed which may be a reflection of the true period. An ideal situation would be to detect changes for periods shorter than 15 minutes, practically this was difficult. For the longer time periods of 60 minutes more oscillations were observed and HMBA had a dampening effect on p53 mRNA expression. A similar trend was noticed in Chapter 3 on the expression of the p53 protein (Bodalina *et al.*, 2007). As was the case with the p53 protein, the effects were diverse in each of the experiments with HMBA altering the dynamics.

These results correspond with the observations made by Khochbin and Lawrence in which they found that in the nucleus of MEL cells the steady-state level of mature p53 mRNA decreased after induction of differentiation (Khochbin and Lawrence, 1989). Richon and colleagues showed similar behaviour to that seen here; they reported that p53 mRNA and protein behaved in a discordant fashion (Richon *et al.*, 1989) in MEL cells induced with HMBA. Samples were collected at various time intervals. Their observations showed that p53 mRNA initially decreased and remained low but by 24 and 48 hours it increased again (Richon *et al.*, 1989) thus reflecting the dynamic nature of p53.

In this study of p53 mRNA, the period and amplitude values could be calculated for all the experiments, whereas for PP1, PP2A and p53 protein expression they were obtained only for some of the experiments. This is mainly due to the higher

number of samples collected here and that the values of expression between subsequent points were changing. The mean period for the untreated cells at 15 minute time intervals was higher than that of the HMBA treated cells. This behaviour was not reflected for the 30 and 60 minutes time interval experiments. In some of the experiments the period and amplitude curves exhibited peaks and troughs with the 30 minute experiment showing oscillations of the period curve (figure 4.7 D).

The mean period values increased steadily as the time intervals of the experiments increased (refer to Table 4.4). This behaviour was also seen in Chapter 2 and 3 and other investigations (Bodalina *et al.*, 2005; Bodalina *et al.*, 2007; Calvert-Evers and Hammond, 2002; Calvert-Evers and Hammond, 2003). This behaviour was not exhibited in the mean amplitudes. These changes in the period and amplitude values reflect the changing expression of p53 mRNA.

In each of the experiments the cell numbers and total RNA concentrations were measured. The population of cells in all of the experiments exhibited oscillatory behaviour with time. This is probably in line with changes taking place in their environment. In some cases the cell numbers in untreated and HMBA treated populations were similar and in others they varied. This again shows that even though a huge effort was made to keep all the conditions the same, differences still exist and maybe the micro environments are influencing behaviour and need to be looked at (Gilbert and Ferreira, 2000). The same was true for the total RNA concentrations.

There was no correlation in any of the experiments between any of the measured components (i.e. cell number, total RNA concentration, and p53 mRNA

expression), indicating that there was little dependence between any of the components.

Results of the previous chapter showed that the expression of the p53 protein was changing. This chapter focused on the expression of its mRNA which was also highly dynamic. Data for cell concentration and total RNA were collected showing varying concentrations. All of these alterations were intricate and there was no consistent pattern to any of them as would be expected in a complex system. Transcription of mRNA is the first stage of gene expression and changes on this level often reveal what is happening on the gene level (DNA), ultimately highlighting what is happening in the cell.

# Chapter 5

# Discussion and conclusion

## 5. General

The study of dynamics in molecular biology is rare with only a few papers being published each year. Much more information is needed and there is opportunity for new research incentives. This discussion is logically divided into three sections: the first explaining the theoretical nature of differentiation and cancer; the second explaining the experimental aspects of cellular processes and our results; and lastly our findings and their significance in relation to cancer.

## 5.1. Theoretical aspects

Theoretical studies on the nature of the cell division cycle, which differs from the mitotic cycle, showed that all the major facets can be understood if the induction of replication is due to the initiation of oscillatory behaviour in a control system governing division [see (Gilbert, 1974a) and (Gilbert, D.A. and MacKinnon, H., 1992) for a summary]. Cancer is not a matter of cells dividing too frequently but of them proliferating when they should not do so. Further considerations, supported by some experimental evidence, argue that pertinent regulators must exceed a threshold before they initiate division. This value can be seen as the sensitivity of the cell cycle switch and cancer is considered to result from a decrease in the threshold that is an increased sensitivity of the relevant control system determining

the onset of oscillatory behaviour that is proliferation. The theory enables one to understand how a wide variety of carcinogenic agents can produce the effect, particularly if the control system involves the interconversion of coenzyme forms. The arguments also show how the effect can result from changes in the autodynamic state, that is, in the pattern of temporal organisation of the cells concerned.

Living cells have evolved very fine tuned control mechanisms that result in highly dynamic entities which are able to effectively adapt and synchronise to their environment. This dynamic behaviour is considered to be the prime attribute of life [see (Gilbert and Lloyd, 2000; Gilbert, 1968; Gilbert, 1974a; Gilbert, 1984)] and it is one of the basic qualities by which we judge the existence of life, on the physiological level. Taking this further, maintained autodynamic behaviour is seen to be driven by control reactions that are operating in oscillatory mode. This results in cells that are multi-oscillators and which, under defined conditions, have characteristic patterns of temporal organisation reflecting distinct patterns of metabolism (Gilbert and Lloyd, 2000; Gilbert, 1968; Gilbert, 1984). These oscillations are further influenced by the reaction rates and kinetics of the various components involved (Gilbert and Lloyd, 2000). It follows that the modification of a pre-existing set of autodynamic reactions, that is the set of oscillations, will alter the characteristics of the cells concerned and that such effects can explain differentiation and cancer (Gilbert, 1968; Gilbert, 1974a; Gilbert, D.A. and MacKinnon, H., 1992). Considerable evidence now exists to support the basis for these views, as referenced at various places in the text; it includes the existence of distinct phase relationships between certain rhythms in untransformed and virally transformed cells and the effect of a variety of agents on the organisation of rhythms in time.

Current concepts of differentiation are based on genetic switching but such views do not account for the triggering processes, and especially the temporal aspects, not least the long time delays despite high reaction rate. A specific switch would seem to require a specific triggering factor and specific initiating reactions for each gene concerned. The same is true for each metabolic change in state (Gilbert, 1984) which may also be involved. Views founded on sequences of such effects yield even more complex considerations. In a multioscillator system, it must be asked if the dynamics associated with differentiation can be unaffected by fluctuations arising in systems not directly participating. In view of the highly integrated nature of cellular processes and their interdependence, lack of interaction appears inconceivable and it seems necessary to consider the overall dynamics of the cells. How then can a very particular time course of such specific events be determined? That problem is compounded when it is realised that most, if not all, switch systems must be individually influenced not by single components but several, each of which is capable of triggering the system by altering its kinetics. As suggested long ago (Gilbert, 1968), it is necessary to incorporate cellular dynamics into the considerations rather than attempt to circumvent them. In other words, any concept of differentiation should be based on the fundamental principles and the periodic operation of cellular control systems and hence the patterns of cellular rhythms. In this respect, it should be noted that distinct dynamics (in the form of phase differences, even between untransformed and transformed cells), were reported at the time such considerations were first proposed (Gilbert, 1968; Gilbert, 1984; Gilbert, 1974b). Our data can thus be viewed in relation to the concept of 'dynamical disease' expressed therein. It is therefore essential to consider the general properties of switch systems when the state is highly dynamic and that includes their reversibility and repetitive possibilities.

As a corollary, one can expect changes in cellular characteristics associated with differentiation and cancer to reflect alterations in the pattern of temporal organisation of the rhythms – that is, modification of the set of control systems

dynamics by agents (signals) (Gilbert, 1974b) and that has proven to be the case. For example, insulin has been found to disturb cellular dynamics, including those affecting cell morphology (Visser *et al.*, 1990), thereby explaining puzzling features of its action (Gilbert and Visser, 1993). Brodsky *et al*. showed similar behaviour in which cell to cell communications was disrupted by the addition of dopamine to hepatocyte cultures (Brodsky *et al.*, 2012a). In these experiments they used ultradian protein synthesis rhythm as the marker for cell to cell communication (Brodsky *et al.*, 2012a). In another experiment Brodsky *et al*. reported a novel cell type, the mesenchymal stromal cells, which exhibited ultradian protein synthesis rhythm after 2 nM melatonin was added to the medium for several minutes (Brodsky *et al.*, 2012b).

It is stressed that both the occurrence and exact timing of hysteretic switch systems depend not simply on an increase in the level of some ’signal’ but also on its duration of action and on its rate of change (Gilbert, 1973b). Where the ‘signal’ is periodic the last factor should be interpreted in terms of its frequency. Furthermore, when multiple oscillations can influence the system, the triggering effect and its absolute timing depend on the phasing between them. In short, in a highly dynamic system the critical conditions required for transition are complex (involving both synergistic and antagonistic effects) and depend on a coordinated set of reactions, not just a solitary, simple process. For these various reasons it is now proposed that switching associated with differentiation (and also cancer) (Gilbert, 1968; Gilbert, D.A. and MacKinnon, H., 1992) occurs through what may be termed ‘deterministic coincidence’; only when a number of factors are simultaneously suitable will state transitions take place. When they do, they will modify the dynamics further and hence effect other subsequent switch reactions, inhibiting some precipitating others. In that way, sequences of dependent reactions can occur with long time delays; thus even with just three rhythms of slightly different frequency, the repeat time (when all peak together) can be extremely long. These considerations form the basis of the original concept that differentiation involves changes in the set of control systems

dynamics, that is, in the patterns of temporal organisation, modification of the frequencies, amplitudes, means and phasings of cellular rhythms. As indicated earlier, oncogenesis can also be viewed in the same light (Gilbert, 1968; Gilbert, 1984; Gilbert, D.A. and MacKinnon, H., 1992). Other facets of development can find explanations in terms of oscillating cells (Gilbert, 1973a), positional information in developing tissues was considered from this point of view and the ideas can be extended to include contributions from several rhythms and not just one or two.

Science has always expected good reproducibility but such science was unfamiliar with cell biochemistry. The reasons why this topic is an exception can only be appreciated with someone experienced in theoretical knowledge of the behaviour of oscillator systems and, in particular, multioscillator systems. Even where a lone rhythm is present, good comparisons are possible only if the sampling is frequent and evenly spaced with respect to its period and the timing is the same. Contrary to common belief the characteristics of cellular rhythms are not fixed and can be critically dependent on minor distinctions in conditions, for example, the age of the culture. When, as is generally the case, the frequency of the periodicity is very high the requisite circumstances cannot be met especially when the state of the system is not known before the results are plotted (Gilbert, 1984; Gilbert, 1974b). Moreover, in general, the exact response to a disturbance depends on its timing relative to the phase of the cycle concerned and that is rarely known; and it can be distinct for different rhythms of a given parameter (the relevant set of rates and kinetics) see, for example, (Gilbert, 1974b). Where more than one periodicity is affected, conditions which are suitable for one may well be unsuitable for others. Of particular concern is the problem of aliasing (Gilbert, 1974b; Gilbert and Ferreira, 2000) in which the true behaviour (continuous expression) of a sample is masked (or becomes indistinguishable) due to the sampling times. Also, the results presented here are themselves enough to show that cells are multioscillators. Add to such problems the fact that various threshold conditions can exist within the cell

(While it is now widely appreciated that chaotic systems can exhibit great sensitivity toward disturbances, the same is true with respect to rhythmic systems in general but to a lower degree). One should also be aware that the cells concerned can be in a disturbed state without the fact being appreciated; even manipulation of a culture can give rise to non-steady state situations due, for example, to changes in the oxygen level. It should be evident that it is no simple matter to reproduce the exact circumstances required to obtain good reproducibility.

Oscillatory behaviour was detected in this and other studies despite there being no deliberate attempt to produce synchrony (Bhoola and Hammond, 2000; Calvert-Evers and Hammond, 2002; Calvert-Evers and Hammond, 2003; Calvert-Evers and Hammond, 2000; Ferreira *et al.*, 1996c; Ferreira *et al.*, 1994a; Ferreira *et al.*, 1994b; Hammond *et al.*, 1998; Hammond *et al.*, 1989a; Hammond *et al.*, 1989b; Hammond *et al.*, 1985). Owing to the nature of tissue culture techniques it is believed that the cells may have adapted to each other on a temporal basis forming a common periodicity. Normal changes in the environment such as feeding of cells, increased waste production or cell-cell signalling, may cause partial entrainment of cellular oscillations and produce a certain level of metabolic synchrony (Hammond *et al.*, 1998). With this degree of synchronisation, a periodic rhythmic behaviour is achieved where the metabolic switches are constantly changing in a steady state fashion. This state continues until dramatic changes are experienced in the environment which may then lead to irreversible changes, for instance those associated with cancer development, occurring in the cells.

Threshold values for the concentrations of oscillating components are of paramount importance; under suitable conditions, regulators of replication may initiate or inhibit oscillatory behaviour (Gilbert, 1974b). The cell is continually subjected to both random and deterministic disturbances of low magnitude and in order to induce differentiation or transformation it is necessary to disturb the pre-existing

dynamic state (Gilbert, 1995; Gilbert, 1974b). Endogenous or exogenous factors produce their effect on the concentrations of cellular constituents by inducing a change in the frequencies, amplitude and phasings of a particular set of rhythms (Gilbert, D. and MacKinnon, H., 1992; Gilbert, 1984; Gilbert, 1974b; Goldbeter, 1996; Goldbeter *et al.*, 2012; Hammond *et al.*, 1998; Hammond *et al.*, 1989b). The fascinating concept is that modulation of oscillatory characteristics is a feature of regulation of the differentiation process and the possible reversal of transformation.

### 5.2. Experimental aspects

Cellular processes interact with each other like a finely tuned orchestra producing harmonised music. As the concentration of particular components increase the concentrations of others decrease; some are activated while others are deactivated. The activation and deactivation of proteins accomplished by phosphorylation and dephosphorylation, via kinases and phosphatases, is the most common regulatory mechanism in eukaryotic cells (Zolnierowicz and Bollen, 2000). Its importance in the control of cell proliferation, differentiation and the development of cancer is well known (see, for example, (Feuerstein and Cooper, 1984; Hammond *et al.*, 1998; Hammond *et al.*, 1989b; Scheele, 1998; Watanabe *et al.*, 1992)). Reversible phosphorylation is a highly dynamic process which undergoes continual adjustment according to the needs of the cell. A disruption of this finely tuned balance may lead to an altered state of the cell, such as differentiation or cancer. These dynamic changes and complex interactions appear to be reflected in the oscillatory patterns of cellular components. Studying these patterns and collecting data with which to make comparisons, may be used as a means of monitoring transformation and possibly modulating the process.

The present study provides further evidence confirming our earlier views with regard to cellular oscillations and rhythmic behaviour. It is hoped that it will shed more light and influence the current views on the underlying principles governing cell proliferation. In this study, the p53 protein, its mRNA and two phosphatases, PP1 and PP2A were profiled in proliferating and differentiating MEL cells. The MEL cell line provides a convenient platform for examining these concepts since it has proved to be a useful model in studying the malignant proliferative properties of cells. In this cell line these properties can be reversed by the addition of certain polar chemical agents such as HMBA (Marks *et al.*, 1994; Marks and Rifkind, 1988). Moreover, we have previously reported on a wide range of oscillations in the cells as mentioned below. These studies were aimed at exploring the contribution of these proteins to the autodynamic state of cells in the absence and presence of HMBA.

As additional pathways are being elucidated in the cell, the link between the phosphatases and p53 grows. Phosphatases are linked with the p53 protein during its dephosphorylation process. It is known that PP1 directly dephosphorylates Rb to modulate its function (Ludlow *et al.*, 1993) thus in this way indirectly affecting p53. PP2A has been linked to the p53 pathway via various other pathways some being IGF/PI3K/Akt and MAPK. Both PP1 (Haneda *et al.*, 2004; Lee *et al.*, 2007; Li *et al.*, 2006; Llanos *et al.*, 2011; Long *et al.*, 2002) and PP2A (Okamoto *et al.*, 2002; Shouse *et al.*, 2008; Shouse *et al.*, 2010) are directly linked to p53's regulation and function. In this way our results reflect the effect of OA on p53 expression. OA which is a potent inhibitor of types 1, 2A and 3 serine/threonine protein phosphatases also has a dramatic effect on p53 expression as reflected by our results. This influence was enhanced with the effect of HMBA. The results obtained by Milczarek *et al*. (Milczarek *et al.*, 1999) might explain a possible mechanism for our results on OA. They suggest that wild-type p53 blocks the proliferative effects of OA through $p21^{waf1}$ –mediated growth arrest while in cells with disabled p53, the

arrest cannot take place, leading to elevated OA-mediated mitoses (Milczarek *et al.*, 1999).

Reversible phosphorylation during MEL cell differentiation was demonstrated earlier by (Watanabe *et al.*, 1992) and (Hammond *et al.*, 1998; Hammond *et al.*, 1989b). Investigations done by Morita *et al*. (Morita *et al.*, 1992) and Tawara *et al*. (Tawara *et al.*, 1993) in HL60 cells showed that PP1 and PP2A were involved during granulocytic differentiation by all-trans retinoic acid (ATRA). Tawara's group showed a decrease in phosphatase activity, as measured using myosin light chain substrate, over a period of 4 days after exposure of cells to ATRA (Tawara *et al.*, 1993). They showed that there was little change in the expression of a 33 kDa PP1 immunoreactive protein while the PP2A catalytic subunit, measured over several days, decreased during differentiation (Tawara *et al.*, 1993). These studies however were only done on treated cells; there were no controls and thus no possibility of temporal changes in expression, as was observed in our studies. Our results showed variations of expression for PP1 and PP2A 32 and 36 kDa bands, with differences being observed between control and treated cells. Similar trends were observed by Bhoola *et al*. (Bhoola and Hammond, 2000) in which expression studies of PP1 and PP2A were done in HL60 cells. Our results for PP2A expression for the longer time periods of 12 hours showed a decrease in expression during differentiation which is consistent with those of Bhoola *et al*. (Bhoola and Hammond, 2000) and Tawara *et al*. (Tawara *et al.*, 1993), although Tawara's group observed the effect after 5 hours. This dampening effect could possibly have regulatory significance. Our findings for PP1 were consistent with Bhoola's in that the expression was changing with time. This was contrasting to Tawara's results where the expression of PP1 did not change after induced differentiation with phorbol ester (Tawara *et al.*, 1993).

Studies on p53 have been extensive; they are continually growing and many new regulatory mechanisms are being elucidated. The protein serine/threonine phosphatases PP1 and PP2A catalyse 90% of serine/threonine dephosphorylation reactions in eukaryotic cells in highly controlled and selective manners (Moorhead *et al.*, 2007). Up to now still relatively little attention has been paid to the temporal aspects of these proteins. In the present study, the p53 protein, PP1 and PP2A were analysed by western immunoblotting. The p53 protein was also probed using the more sensitive ELISA procedure and its mRNA was investigated using a semi quantitative RT-PCR kit. The concentrations of these molecules in MEL cells were profiled with time; time-dependent changes were apparent and could not be dismissed as random experimental errors. As in earlier investigations of protein phosphorylation, kinase and phosphatase expression and the expression of other key proteins in these cells and in human promyelocytic leukaemia (HL-60) cells (Bhoola and Hammond, 2000; Bodalina *et al.*, 2005; Bodalina *et al.*, 2007; Calvert-Evers and Hammond, 2002; Calvert-Evers and Hammond, 2003; Calvert-Evers and Hammond, 2000; Ferreira *et al.*, 1996c; Ferreira *et al.*, 1994a; Ferreira *et al.*, 1994b; Hammond *et al.*, 1998; Hammond *et al.*, 1989b; Hammond *et al.*, 1985), the variations were complex. Indeed, it would be unrealistic to expect to find well-defined behaviour in the cellular environment where there are numerous interactions influencing protein expression. The characteristics of cellular rhythms are not fixed and can be critically dependent on just minor changes in conditions.

Various models of p53 dynamics and oscillations have been proposed, see (Abou-Jaoude *et al.*, 2009; Ciliberto *et al.*, 2005; Hat *et al.*, 2009; Mengel *et al.*, 2010; Ouattara *et al.*, 2010). Abou-Jaoude *et al.* (Abou-Jaoude *et al.*, 2009) have studied the p53, Mdm2 and DNA damage interaction model. This system consisted of an antagonist relation between p53 and nuclear Mdm2 set in a 3-element negative circuit with p53, and Mdm2 from the cytoplasm and nucleus. They explained that the qualitative dynamical properties of their network can be reassessed by a small number of bifurcation states explained in terms of the balance between the positive

and negative circuits of the core network (Abou-Jaoude *et al.*, 2009). They used their model to try and elucidate the qualitative nature seen in experimental observations, such as p53 pulses after irradiation, failure to respond to oscillation in a cell population, high and low frequency oscillations and they suggest unpredictability of behaviour due to cell to cell interactions and between different cell-types on the basis of post-translational modifications and transactivation properties of p53 (Abou-Jaoude *et al.*, 2009). In our studies some of our results correlated with these observations and could possibly be explained by them especially noting the cell to cell interaction.

In another finding Ouattara *et al*. (Ouattara *et al.*, 2010) found that the amplitude of the p53 and Mdm2 oscillations were highly variable and dependent on the bifurcation properties of the system. In contrast, the peak width and timing remain regular while noise can induce repeated pulses of p53 and Mdm2 that, at low damage, resemble the slow irregular fluctuations (Ouattara *et al.*, 2010). By adding the stochastic dimension in their modelling system they could account for the increase in part of cells oscillating with a higher frequency when the irradiation dose was increased. Their findings substantiate observations made by Geva-Zatorsky *et al*. (Geva-Zatorsky *et al.*, 2006). These findings substantiate our observations in that the pattern of oscillations, periods and amplitudes were complex and continuously changing.

Western blot analysis of p53 and Mdm2 in MCF7 cell populations by Lahav *et al*. (Lahav *et al.*, 2004) also showed oscillatory changes; in individual cells; however, in response to ionising radiation, p53 was emitted in pulses of fixed height and duration. In the studies of Lev Bar-Or *et al*. (Lev Bar-Or *et al.*, 2000), harmonic oscillatory patterns were observed, but it should be noted that these workers considered only a simplified interaction in the negative feedback loop of p53 and Mdm2. Consistent behaviour is possible only in simple systems, that is in

experiments where only a few isolated components are reacted together, as was the case in the studies of Lev Bar-Or *et al*. (Lev Bar-Or *et al.,* 2000), or in studies involving individual cells as carried out by Lahav *et al*. (Lahav *et al.*, 2004).

Prolonged oscillations were prominent in the research done by Geva-Zatorsky *et al*. (Geva-Zatorsky *et al.*, 2006) in which p53 and Mdm2 were fluorescently tagged in individual MCF7 (U280) cells following gamma irradiation. Their time studies were done over 3 days. They found that some of the cells showed undamped oscillations for at least 3 days with more than ten peaks; the amplitudes were unpredictable compared to the period; sister cells became unsynchronised after about 11 hours; and some cells showed low-frequency fluctuations that were uncharacteristic of oscillations (Geva-Zatorsky *et al.*, 2006). These observations are comparable to some of our findings but it should be noted that our studies were done on populations of MEL cells. Recently Toettcher *et al*. (Toettcher *et al.*, 2010) have been studying the dynamics of p53 pathway models in which they constructed a "tunable" oscillator made of natural and synthetic components in mammalian cells (clonal MCF7 cell line). They ascertained that this simplified p53 circuit with a single feedback loop maintained some features of the full network's dynamics, showing pulses of p53 with controlled timing (Toettcher *et al.*, 2010). This was dissimilar to the full natural p53 network in which the pulses were damped in individual cells, with amplitudes reliant on the input strength (Toettcher *et al.*, 2010). Supporting some of our findings, Toettcher *et al*. demonstrated that the three important features of oscillator dynamics, amplitude, period and the rate of damping can be controlled by influencing the stimulus level, interaction strength, and feedback topology (Toettcher *et al.*, 2010).

The difference plot gives a visual indication of trend changes and variations between successive data points, substantiating the effects seen with the fitted curves. They also highlight in/out of phase trends. The patterns of expression

studied here showed marked differences in different experiments even though the conditions were apparently the same. Although the effects were different in different experiments, HMBA often modified the dynamics. In fact it would be unrealistic to expect consistent and well defined behaviour because of the complex nature of the interactions that are influencing these proteins. Irregularities in periodic oscillations have been simulated in theoretical studies involving the combined effect of two or more modulating periodicities (Gilbert and Ferreira, 2000), and may arise as a result of superimposition of multiple rhythms, heterogeneity of cell populations, aliasing or even chaotic behaviour.

The results presented are consistent with the view that HMBA influences the dynamics; the patterns of expression of the molecules and the period and amplitude curves differed in proliferating and differentiating cells. However, another problem involved here is that the full effects of HMBA are not achieved instantaneously; the results for the different sampling times cover distinct time intervals and so diverse results can be expected even if the system were not subjected to aliasing. Furthermore, the changes in cell morphology, observed previously in these cells (Visser *et al.*, 1990) may be expected to affect the variation in expression. As was previously noted by Marks *et al.* (Marks and Rifkind, 1988) MEL cells (DS19) treated with HMBA showed an appreciable decrease of p53 protein by 8 hours but not its mRNA. Similar studies done by Ramsay *et al.* (Ramsay *et al.*, 1986) in which HMBA was used to treat MEL cells (DS19-sc9) for 240 minutes and p53 mRNA was probed at 0, 15, 30, 60, 120 and 240 minutes, showed that the level of p53 mRNA increased about two-fold at 15 minutes and returned to similar levels as those of the uninduced cells after 120 minutes. In our studies with p53 protein and mRNA expression we noticed that for the shorter time periods (15 minutes) HMBA treated cells had a lower mean period of oscillation than the untreated cells. This trend was not noticed in the longer time period studies and neither in the PP1 and PP2A experiments.

Support for the existence of numerous cellular periodicities is now considerable and includes rhythmic variations in the activities of a number of enzymes, in their isoenzyme patterns (effective and actual), coenzyme levels and redox state, gross and surface morphology (Visser *et al.*, 1990). Of especial interest are periodic variations in the total amount of cellular protein, in the amount of extractable protein, and in the extractable amount of particular proteins (for example isoenzymic forms of lactic dehydrogenase). Phase and frequency differences indicate the existence of essentially independent systems – that is the absence of a single rhythmic driving force. Particularly in view of the roles of phosphorylation reactions in cell replication, suggested in theoretical studies of the cell division cycle (see summary in MacKinnon and Gilbert) (Gilbert, D. and MacKinnon, H., 1992), we initiated investigations in that area. Despite problems associated with the study of cellular rhythms (Gilbert and Ferreira, 2000), the results presented here confirm the existence of oscillations affecting dephosphorylation processes and the ability of HMBA to influence the dynamics. However, in view of the phenomenon of aliasing (Gilbert and Ferreira, 2000), it is probable that the true frequencies involved are actually much higher than we report here.

The data confirm that all of these molecules are oscillating but the fact that the apparent estimated periods are sample time dependent indicates that the actual rhythm has a true period which is shorter than the lowest value estimated. It would be of interest to carry out analyses with different time intervals and to obtain information relating to the periods of the rhythms. This could be the case for the expression of the PP1, PP2A and p53 proteins, although the dynamics may differ from one protein to another and there may be differences between expression and activity. In the case of the PP1, PP2A and p53 proteins this was less than 31 minutes and for the mRNA of p53 it was less than 23 minutes while for protein tyrosine kinase and protein tyrosine phosphatase in HL-60 cells the estimated

period was lower and of the order of 10 minutes (Calvert-Evers and Hammond, 2002; Ferreira *et al.*, 1996a; Ferreira *et al.*, 1996b; Ferreira *et al.*, 1996c). In the case for lactate dehydrogenase activity and isoenzymes it was suggested that the period of activity oscillations may also be less than 10 minutes (Ferreira *et al.*, 1996a; Ferreira *et al.*, 1996b).

In these experiments, data from individual experiments were presented rather than statistical means (Hammond *et al.*, 1998). Although trends can be identified, variations in temporal behaviour are generally seen from one experiment to another. This is to be expected, since changes in metabolism arise from instability in and around the cells, a factor which is not always accepted (Ferreira *et al.*, 1994b). Even if oscillations are not evident, a variety of outcomes may be possible due to periodic behaviour. In experiments where only single time-points are used, standard deviations are often high, as would be expected in an oscillating system, and the information obtained may be incomplete and possibly misleading (Hammond *et al.*, 1998). As was stressed previously (Hammond *et al.*, 2000a; Hammond *et al.*, 2000c), it is essential that experimental design takes account of time-dependent behaviour so as to provide complete information without masking the dynamics. Often this aspect of a study is compromised as a result of extensive statistical evaluation, which leads to misleading or erroneous conclusions. Thus the complexity of such organisations has been highlighted and the need to investigate these systems from a time analyses perspective is needed.

## 5.3. Dynamic behaviour and cancer

Oscillations and complex adaptive behaviour are not just observed in cells or in the behaviour of particular proteins they are a fundamental concept of nature. Nature is nothing if not rhythmic, and its rhythms are many and varied. In biology, there are various levels of organisations, starting from the biggest, the Earth or biosphere, ecosystems, communities, populations, organisms, organ systems, tissues, cells and eventually to molecules. These systems can be profiled and characterised on each level. The dynamic behaviour as was presented in this study is exhibited on each level. Interactions between the various levels also takes place and further feeds back to the dynamics. By investigating these phenomena we could gain a better understanding of the workings of the process and it could lead to its better management. An ideal possible outcome of this would be the continuous monitoring of changes in the system so as to allow the manipulation of transformation in the desired direction.

Why do systems oscillate at all? According to Ian Stewart Professor of Mathematics at the University of Warwick "The answer is that this is the simplest thing you can do if you don't want, or are not allowed, to remain still" (Stewart, 1996). Many oscillations arise out of steady states, as conditions change, a system that has a steady state may lose it and begin to wobble periodically; this scenario is known as Hopf bifurcation (Stewart, 1996). A similar depiction involving the p53 and NF-κB system has been described by Hat *et al*. (Hat *et al.*, 2009). These are some of the fundamental aspects of nonlinear dynamics and chaos study all arising from the stochastic behaviour within the cell (Rosenfeld, 2011b). Together with systems biology these two fields could greatly enhance the study of the complex pathways in cells and their break down into disease (Rosenfeld, 2011b). In systems biology there are two major aspects that are considered; they are the better understanding of

complex natural networks and the *de novo* design of simple networks. Studying simple core network motifs in isolation offers the best potential for manipulating their behaviour, but not offering any insight into the complex natural context (Toettcher *et al.*, 2010). On the other extreme, natural biological systems can weave many layers of regulation on top of the core signalling pathway in its native context using nonnatural inputs and feedback loops, can help to quantitatively understand and perturb the full system (Toettcher *et al.*, 2010). These particular traits of the various systems could be a fundamental characteristic of molecules and nature and probably making dynamic behaviour a "tip of the iceberg". Studying these trends will lead to a deeper understanding of the nature of life and its diseases like cancer and eventually to their improved management.

These studies directly relate to the workings of molecules in the cell and possibly to various drug therapies. Another inference of these studies could be extended to the medical treatment of various diseases. At present doctors diagnose a patient and treat him/her with drugs and therapy depending on the diagnoses of the ailment. Usually these treatments are very none specific and targeted specifically towards the disease. An enhancement to this process would be the collation of additional data on the patient and not just on the particular diagnosis. The data that should be collected would be the patients rate of metabolism, his/her physical health, the ability to breakdown drugs, the mental state, various stress factors (the levels that can be tolerated), the sleep and wake cycle, whether the patient is refreshed or tired and the energy level. Additionally the type of nutrients that the body gets and over time what it has adapted to. These are only some of the factors needed. The body needs to be considered as a complete whole with its different interacting systems and their various influences. Environment plays a big factor in directing changes within a cell thus monitoring the internal and external factors would allow better control in manipulating further alterations.

The body is a finely tuned harmonious unit, when some of its systems are out of synchrony, others will try to compensate. Over time the body adapts to its environment. When exposed to bad influences it will try to adjust and minimise the effect. If the presence of the influence continues over time, some parts of the body will eventually succumb and lower their barriers. Depending on the time and the level of exposure, these will eventually deteriorate and degrade, ultimately leading to an altered state such as cancer. Going back to the evolutionary nature of cancer it was stated that it is a natural part of life allowing us to cope with the environment (Breivik, 2001; Kitano, 2007; Merlo *et al.*, 2006; Rosenfeld, 2011b). We should not look at this from a negative perspective instead we should acknowledge it and try to further understand its nature in order to guide it into a positive outcome. By considering the body in a holistic manner one could enhance the treatment process towards this disease. This is often a downfall of modern medicine as it fails to consider this. With the help of sophisticated technology such as computers this outlook is changing. Thus with the additional information on the patient together with the continuous monitoring of certain molecules in the disease doctors could create customised and targeted treatments for the patient. In this way the specific treatment could suitably be adjusted depending on the result of the patient's health. Appropriately, from this standpoint, researches would also have to alter their investigations. They would not just have to note the maximum and minimum effects of certain drugs but also monitor the changing consequences under different conditions so as to maximise the outcome. In other words they would have to do their research from the perspective of dynamics. Thus by considering the ups and downs (oscillation) of medication and its treatment we could further enhance the medical field with regard to diseases like cancer.

Moving back to the cellular level; changes at the cellular level are usually directed in a highly controlled fashion from the genetic hub of the cell. But when all of the interactions and regulatory mechanisms are considered simultaneously, they display very stochastic and nondeterministic characteristics. If each cell under defined

conditions displays its own pattern of periodicities, it can be inferred that modifications in cellular characteristics during differentiation and oncogenesis, for example, could result from adjustments in the pattern of rhythms (Gilbert, 1968). In this way both normal and abnormal (including malignant) modifications could result from differential alterations to the parameters of frequency, phasing, amplitude and/or mean levels of rhythmic processes exhibiting new patterns of periodicities (Gilbert, 1968). These changes in the pattern of rhythms would result from marked alterations to the size of the time delays proffered by any one or more of the segments in the relevant looped pathways (Gilbert, 1968). It is thus possible to interpret the action of changes (chemical, viral, oncogenic or environment) in terms of direct or indirect temporal disturbances – each specific one would have its own attribute action under given conditions. The temporal effects produced by such disturbances would depend on the position of action of the agent in relation to the state of the rhythmic processes that are disturbed. The system as a whole may be expected to exhibit a degree of resilience towards a tendency for alterations in the pattern of periodicities, dependent on the strength of the perturbation and its duration of action in relation to the period of the systems directly involved (Gilbert, 1968). If sufficiently marked or prolonged (i.e. if the agent has sufficient impulse), however, a rate difference at any locus is almost certain to overcome such inertia and hence lead to permanent change in the rhythmic characteristics and mean levels of cellular constituents (allowing for evolutionary adaptation). As a result of gradually changing degrees of interaction between the various feedback pathways, the frequencies of various rhythms would be likely to change until, eventually, the overall pattern of periodicities could itself again become periodic (Gilbert, 1968). Thus by gaining a better understanding of the timing, duration and characteristics of these pulsed perturbations we could expand our knowledge of how transformation takes place and possibly reverse these effects.

## 5.4. Conclusion

The results show that the expression of PP1, PP2A and the p53 protein including its mRNA is undoubtedly dynamic and modulated by the addition of HMBA. Our findings here contribute further evidence that modulation of the dynamics of certain key cellular components, as in the case of PP1, PP2A and p53, may change cellular functions; modulation may be achieved by altering the characteristics of frequency, phase and amplitude and wave shape of the rhythms of the enzymes and regulatory molecules involved in the system. It is hoped that an understanding of the oscillatory phenomena of expression of these proteins will provide a better understanding of the origin, as well as the physiological function, of these rhythms, and the circumstances under which malfunctioning regulation may give rise to malignant transformations. These studies of cellular periodicity contribute to understanding the changes that take place during cell replication, differentiation and neoplastic transformation.

Our results illustrate the importance of carrying out biochemical investigations on a temporal basis and emphasise the need to take multiple samples over as long a time interval as possible. They demonstrate that biochemical processes underlying cell function and behaviour are time dependent and should be regarded as such when designing experiments and interpreting results. Temporal aspects relating to PP1, PP2A, p53 and p53 mRNA have been considered in MEL cells. It is possible that modulation of the oscillatory characteristics of key proteins such as these by potential therapeutic agents may be crucial to the control of cell differentiation and transformation and treatment of diseases. Various strategies have been aimed at inhibiting the process of cancer. An alternative therapeutic approach may be to modulate the activities and/or expression of key regulatory proteins such as those studied here, the protein phosphatases and the p53 tumour suppressor protein.

# Appendix A

### 1. List of chemicals used

| | |
|---|---|
| Acetic acid | Saarchem-Holpro, RSA |
| Acrylamide | BDH Laboratory supplies, RSA |
| Agarose | Sigma-Aldrich, St Louis, USA |
| Ammonium persulphate | BDH Laboratory suppliers, RSA |
| Anti-p53-protein antibody | Boehringer Mannheim, Germany |
| Anti-mouse IgG secondary antibody | Amersham Life Science, UK |
| Anti-rabbit IgG secondary antibody | Amersham Life Science, UK |
| Anti-Protein phosphatase 1 antibody | Upstate Biotechnology Inc., USA |
| Anti-Protein phosphatase 2A antibody | Upstate Biotechnology Inc., USA |
| Baxter water | Sabax, RSA |
| Bisacrylamide | BDH Laboratory supplies, RSA |
| Bromophenol blue | Merck, Germany |
| Bovine serum albumin | Boehringer-Mannheim, Germany |
| 4-chloro-1-naphthol | Sigma-Aldrich, St Louis, USA |
| Chloroform | Saarchem-Holpro, RSA |

| | |
|---|---|
| Copper sulphate (Cu2SO4) | Saarchem-Holpro, RSA |
| Coomassie brilliant blue R-250 | Merck, Germany |
| Diaminobenzidine | Aldrich chemical co, UK |
| Diethyl pyrocarbonate | Sigma-Aldrich, St Louis, USA |
| Disodium hydrogen orthophosphate | Saarchem-Holpro, RSA |
| 6X DNA loading dye | Fermentas, Thermo Fisher Scientific, USA |
| 100 bp DNA ladder | Promega Corporation, USA |
| EDTA | Boehringer Mannheim, Germany |
| Ethanol | BDH Laboratory supplies, RSA |
| Ethidium bromide | Sigma-Aldrich, St Louis, USA |
| EMEM with Hanks salts | Highveld Biologicals, RSA |
| Formaldehyde | Merck, Germany |
| Formamide | Saarchem-Holpro, RSA |
| Foetal calf serum | Highveld Biologicals, RSA |
| Folin Ciocalteu reagent | Merck, Germany |
| Glycerol | BDH Laboratory supplies, RSA |
| Glycine | BDH Laboratory supplies, RSA |
| HCl | Saarchem-Holpro, RSA |

| | |
|---|---|
| Hydrogen peroxide | Merck, Germany |
| Isopropanol | BDH Laboratories Supplies, RSA |
| HMBA | Sigma-Aldrich, St Louis, USA |
| Leupeptin | Sigma-Aldrich, St Louis, USA |
| MEL cell line | ATCC, Maryland, USA |
| Millipore water | Millipore Corporation, USA |
| Methanol | Merck, Germany |
| β-Mercaptoethanol | Merck, Germany |
| M-MLV reverse transcriptase | Promega Corporation, USA |
| 3-N-Morpholino-propanesulphonic acid | Sigma-Aldrich, St Louis, USA |
| Mouse p53 relative RT-PCR kit | Ambion, Texas, USA |
| Non-fat milk powder | Clover, RSA |
| NP40 | Saarchem-Holpro, RSA |
| Okadaic acid | Boehringer Mannheim, Germany |
| Oligo (dT) 15 primer | Promega Corporation, USA |
| PCR master mix | Promega Corporation, USA |
| PCR nucleotide mix | Promega Corporation, USA |
| Penicillin | Sigma-Aldrich, St Louis, USA |
| Pepstatin | Sigma-Aldrich, St Louis, USA |

| | |
|---|---|
| PMSF | Boehringer Mannheim, Germany |
| 2x RNA loading dye | Fermentas life sciences, USA |
| RNA molecular mass markers | Inqaba Biotechnical, RSA |
| RNasin ribonuclease inhibitor | Promega Corporation, USA |
| RNase free water | Sabax, RSA |
| Sodium bicarbonate | Saarchem-Holpro, RSA |
| Sodium carbonate (Na2CO3) | Saarchem-Holpro, RSA |
| Sodium chloride (NaCl) | Saarchem-Holpro, RSA |
| Sodium dihydrogen phosphate | Saarchem-Holpro, RSA |
| Sodium dodecyl sulphate | BDH Laboratory supplies, RSA |
| Sodium hydroxide (NaOH) | Saarchem-Holpro, RSA |
| Sodium potassium tartrate | ACE, RSA |
| Standard rainbow markers | Amersham International, UK |
| Streptomycin sulphate | Sigma-Aldrich, St Louis, USA |
| Tetramethylethylenediamine | Merck, Germany |
| Tri-Reagent™ | Sigma-Aldrich, St Louise, USA |
| Tris | Boehringer Mannheim, Germany |
| Trypan blue dye | Merck, Germany |
| Tween 20 | Merck, Germany |

| | |
|---|---|
| Glycerol | BDH Laboratories Supplies, RSA |
| RNA molecular mass markers | Boehringer Mannheim, Germany |
| RNase-A | Boehringer Mannheim, Germany |
| RNase free water | Sabax, RSA |
| Sodium acetate | Saarchem-Holpro, RSA |

**2. List of equipment used**

| | |
|---|---|
| 4-Place castor for SE250 gels | Hoefer Inc, USA |
| 96 Microplate reader | Thermo Star, ADP, RSA |
| Cameo acetate filters | Millipore Corporation, USA |
| Confocal microscope | Zeiss, West Germany |
| Desktop centrifuge | Beckman Coulter, RSA |
| DNA/RNA gel chamber | Hoefer Inc, USA |
| DNA/RNA gel running chamber | Hoefer Inc, USA |
| DNA/RNA viewing apparatus | Syngene gel viewing system, USA |
| DU-65 spectrophotometer | Beckman Coulter, RSA |
| Eppendorf centrifuge | Beckman Coulter, RSA |
| Eppendorfs | BDH Laboratories supplies, RSA |

| | |
|---|---|
| Elmer flasks | BDH Laboratories supplies, RSA |
| Filtration unit | Millipore Corporation, USA |
| Filter paper (0.22 μm) | Whatmann, RSA |
| Gel chamber | Hoefer Inc, USA |
| Glass cuvette | Beckman Coulter, RSA |
| GS Millipore filters | Millipore Corporation, USA |
| Haemocytometer | Zeiss, Germany |
| Humidified incubator | Heraeus, USA |
| Hybond C nitrocellulose paper | Osmonics, Johannesburg, RSA |
| Light microscope | Zeiss, Germany |
| Magnetic stirrer | BDH Laboratories supplies, RSA |
| Medium filtration unit | Millipore Corporation, USA |
| Medium filtration unit pump | Millipore Corporation, USA |
| Mini gel system | Hoefer Inc, USA |
| Multichannel pipette | Socarex, England |
| p53 ELISA kit | Oncogene Research Products, USA |
| p53 RT-PCR kit | Ambion, USA |
| Pipetters | Socarex, England |
| Power supply | Hoefer Inc, USA |

| | |
|---|---|
| Prefilter filter paper | Whatmann, RSA |
| RNA viewing apparatus | Syngene gel viewing system, USA |
| Quartz cuvette | Beckman Coulter, RSA |
| Soft Pac Module Quant II linear for spectrophotometer DU-65 | Beckman Coulter, RSA |
| Sterile cryotubes | Nunc, Roskilde, Denmark |
| Sterile culture flasks | Nunc, Roskilde, Denmark |
| Sterile 50 ml nunc tubes | Nunc, Roskilde, Denmark |
| Sterile petri dishes | Nunc, Roskilde, Denmark |
| Water cooling system | Hoefer Inc, USA |
| Whatman no.1 filter paper | Whatmann, RSA |
| Vortex machine | Chilt Scientific, USA |

# Appendix B

### 1. Description of murine erythroleukaemic cells

The description of MEL cells as per the American Type Culture Collection catalogue. This is the description of the repository reference seed stock

| | |
|---|---|
| Certified cell line: | (TIB-56$^{TM}$) D1B (erythroblast, mouse) leukaemia |
| ATCC number: | TIB-56$^{TM}$ |
| Designations: | D1B |
| Depositors: | B. Chesebro |
| Biosafety level: | One |
| Growth properties: | Suspension |
| Organism: | *Mus musculus* (mouse) |
| Morphology: | Lymphoblast |
| Source organ: | Spleen |
| Source strain: | DBA/2 |
| Source disease: | Leukaemia |
| Source cell type: | Erythroblast |

| | |
|---|---|
| Cellular products: | Globin mRNA; haemoglobin |
| Freeze medium: | Complete growth medium supplemented with 5% (v/v) Dimethyl sulphoxide (DMSO) |
| Storage temperature: | Liquid nitrogen vapour phase |
| Subculturing protocol: | Cultures can be maintained by addition or replacement of fresh medium. Start cultures at 2 X $10^5$ cells/ml and maintain between 1 X $10^5$ and 1 X $10^6$ cells/ml. The cells may also be grown as ascites in syngeneic mice. Inject 2 X $10^5$ cells per mouse and harvest as soon as abdominal enlargement is observed (about 10 days). |
| Medium renewal: | Every 2 to 3 days |
| Tumourigenic: | Yes |
| Comments: | This line was derived from a leukaemia induced by B-tropic Friend murine leukaemia virus. The cells are negative for H-2d (H-2d-), surface immunoglobulin (sIg-), Ia8 (Ia8-) and Thy-1.2 (Thy-1.2-). Erythroid differentiation and haemoglobin synthesis are inducible by DMSO. Tested and found negative for ectromelia virus (mousepox). |
| References: | (Chesebro *et al.*, 1978)<br><br>(Chesebro *et al.*, 1976) |
| Prepared/characterised by: | ATCC, Rockville, Maryland, USA. |

## 2. Preparation of medium

According to ATCC recommendations, MEL cells should be grown in RPMI 1640 medium with 2 mM L-glutamine adjusted to contain 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 10 mM HEPES, and 1.0 mM sodium pyruvate and supplemented with 0.01 mM 2-mercaptoethanol, 90%; foetal calf serum, 10%. However it was noted that these cells also thrived in an environment consisting of EMEM with Hanks salts. Since this medium was readily available in the laboratory it was used for growing the cells. EMEM with Hanks salt was prepared by dissolving 13.53 g of EMEM, 3.7 g of sodium bicarbonate, 0.27 g of penicillin G and 0.45 g of streptomycin sulphate in 900 ml of Millipore water. These were mixed using a magnetic stirrer and the volume was adjusted to one litre with additional Millipore water. Once all the components were dissolved, the mixture was filtered using a filtration unit with a prefilter and filter paper of 0.22 μm. The medium was filtered into sterile one litre Elmer flasks. The filtration process was carried out using sterile conditions in a laminar flow hood. All components used such as the filtration unit, prefilter and filter paper and flasks were autoclaved prior to use. After the medium was filtered, it was incubated overnight at 37 °C. This was done to check for contamination which was identified from the change in colour of the medium due to an altered pH and turbidity. The medium was stored at 4 °C for a maximum period of 6 months. Heat inactivated FCS at 5% v/v, containing growth/stimulatory hormones, was added to the medium prior to use. This medium with FCS had to be used within a month before the stimulatory hormones decomposed. Prior to use, the FCS was inactivated by incubating it in a water bath at 56 °C for 30 minutes. The inactivated FCS was then filtered through a sterile 0.22 μm acetate filter into the EMEM medium.

## 3. Maintenance of murine erythroleukaemic cells

MEL cells were grown in EMEM with Hanks salts supplemented with 5% heat inactivated FCS, penicillin and streptomycin. The supplemented medium was prepared in Millipore water and sterilised by filtration through GS Millipore filters (0.22 μm) then stored at 4 °C. Inactivated FCS was added to the medium prior to use. Cells were kept in 250 ml tissue culture flasks which were incubated at 37 °C in a humidified 5% $CO_2$ environment incubator. The cells were regularly examined (daily during the week) under a microscope for infection and viability. The medium in the flasks were replaced every 2 to 3 days or when the bottom layer of the flask was confluent with cells or if the colour of the medium changed to orange. Prior to feeding, the tissue culture flasks were allowed to stand vertically in the incubator for 10 minutes. The top layer of medium was carefully poured off (approximately 80%) under a laminar flow hood, using sterile techniques. Fresh medium containing FCS was added. The tissue culture flasks were replaced every month. Changes in cellular morphology characteristics are the first indication that cell culture conditions are deteriorating. Thus routine microscopic examination of MEL cells was carried out. This was done to detect abnormalities in morphology such as nuclear granularity, cytoplasmic vacuolation and cellular clumping. These changes indicated either that the culture medium needed to be changed or some kind of infection due to bacteria or yeast might have taken place.

## 4. Contamination to cultures

Maintaining of cells in culture requires adherence of strict aseptic techniques. Often cultures can become contaminated with fungi or various types of microbes. Thus the sterility of medium and cultures was routinely monitored. Once every week day,

cultures were inspected visually and microscopically for signs of microbial and fungal contamination. Cells were also tested for mycoplasma infection if the cells showed changed morphology. Antibiotics such as penicillin and streptomycin were used in the preparation of the medium to prevent bacterial infection. The tissue culture room was kept sterile by switching on the UV light when not in use. The laminar flow hood was always kept clean and 95% alcohol was used to wipe the hood surface before and after use.

## 5. Cryopreservation of cells

### 5.1. Freezing of murine erythroleukaemic cells

If the cell culture became infected (bacterial or fungal), new MEL cells had to be grown from cryopreserved backup stocks. For cryopreservation, cultures were grown to a concentration of 5 x $10^6$ cells/ml and cryopreserved in liquid nitrogen to yield best results. A cell count was done and the viability of the cells checked. It had to be above 95%. MEL cells (100 ml) were centrifuged at 1 500 g for 10 minutes at 4 °C. The supernatant was discarded and the pellet resuspended in 10 ml of cryopreservation buffer (20% v/v fresh EMEM medium, 30% v/v glycerol and 50% v/v heat inactivated FCS). The suspension was dispersed in 1 ml cryopreseservation tubes and stored in liquid nitrogen until required. Some of these cells were also stored in the -70 °C freezer.

### 5.2. Reconstitution of cryopreserved murine erythroleukaemic cells

Cryopreserved MEL cells were thawed in a 37 $^{o}$C water bath over 1 minute. These cells were centrifuged at 1 500 g for 5 minutes at 4 °C. The supernatant was discarded and the pellet resuspended in freshly prepared EMEM medium containing 10% heat inactivated FCS. The cells were transferred to a culture flask and incubated at 37 °C in a humidified 5% $CO_2$ atmosphere.

**6. Murine erythroleukaemic cell differentiation**

For the differentiation of MEL cells, 5 mM HMBA solution was added to the cells. In the experiments the MEL cells ranged from 1 x $10^5$ to 1 x $10^6$ cells/ml, in concentration. For the preparation of 0.5 M HMBA, 0.1 g of HMBA was dissolved in 1 ml of sterile water. This solution was filtered through a 0.22 μm filter. Thus for experiments that had 700 ml of stock cells, 10 ml of 0.5 M HMBA was prepared and 7 ml was used. For experiments that had 30 ml or 50 ml of cells, 1 ml of 0.5 M HMBA was prepared and 0.3 ml or 0.5 ml of HMBA was used, respectively.

**7. Checking the differentiation of cells**

Cellular differentiation was monitored by using the nitroblue tetrazolium technique described by Kamijo and co-workers in 1990 (Kamijo *et al.*, 1990). Cells were incubated 1:1 (v/v) with 1.25 mg/ml nitroblue tetrazolium made up with EMEM for 30 minutes at 37 °C and then counted using a microscope and a haemocytometer. The percentage of cells containing intracellular formazan deposits was an indirect reflection of cellular differentiation.

## 8. Determination of cell concentration and viability

Cell concentration and viability assays were carried out at regular intervals using the trypan blue cell viability assay. Only cultures with viability greater than 90% and concentrations greater than 1 x $10^6$ cells/ml were used in experiments. A 1:1 (v/v) mixture of 1% trypan-blue dye and cell suspension was incubated at 37 $^oC$ for 15 minutes and then transferred to a haemocytometer chamber. The percentage of blue stained (non-viable) cells was used to calculate cell viability.

## 9. Trypan blue exclusion method of cell count

### 9.1. Trypan blue solution

A = 0.2% (w/v) of trypan blue dye

B = 5X saline solution (4.25% NaCl)

For the preparation of trypan blue solution four parts of A was added to one part of B.

### 9.2. Solution of 0.9% saline

A 0.9% saline solution was prepared by dissolving 9 g of NaCl in 1 000 ml of sterile water. The solution was autoclaved and stored at 4 °C.

### 9.3. Cell count using the haemocytometer

An appropriate volume of cell sample was extracted (~50 µl); if the cell concentration was too high, the sample was diluted with saline. An equal volume of trypan blue solution was added to the cell sample. The haemocytometer chamber was loaded with this mixture. The cells in the chamber were viewed under a microscope and the viable cells counted. Viable cells appeared colourless while non-viable ones were seen as dark blue dots. The concentration of the cells was determined by counting the viable cells in the four large grids (each having a volume of $1 \times 10^{-4}$ ml). The cell concentration was calculated using the following formula:

= (mean number of cells in the four blocks x dilution factor) ÷ ($1 \times 10^{-4}$ ml)

## 10. Addition of okadaic acid to murine erythroleukaemic cells

Okadaic acid was added to MEL cells to test the effect on p53 expression. The following concentrations of okadaic acid were used in the experiments with 10 ml of cells at $1 \times 10^{6}$ cells/ml: 1 nM, 10 nM, 100 nM and 500 nM. The initial stock solution had a concentration of 25 µg in 1 ml of sterile water. Thus for each concentration of okadaic acid the following volumes of the stock were used:

1 nM - 0.32 µl of the stock solution

10 nM - 3.2 µl of the stock solution

100 nM - 32 µl of the stock solution

500 nM - 160 µl of the stock solution

For 50 ml of cells, using a 100 nM concentration of okadaic acid, 160 µl of stock okadaic acid was used.

# Appendix C

## 1. Preparation of lysing buffer

The lysing buffer consisted of the following chemicals:

50 mM Tris-HCl at pH 8.0

150 mM NaCl, 5 mM EDTA

1% NP40

1 mM PMSF

These chemicals were prepared by adding them to 250 ml of sterile water: 1.51 g Tris-HCl, 2.19 g of NaCl, 0.465 g of EDTA, 2.5 ml of NP40 and 0.044 g of PMSF. The pH of the solution was adjusted to 8.0 with HCl and the volume was made up to 250 ml. Note that the PMSF was added just before the solution was used. The solution was stored at 4 °C.

## 2. Determination of protein concentration – Lowry method

### 2.1. Bovine serum albumin

A stock solution of 0.1% bovine serum albumin (BSA) was prepared by mixing 10 mg BSA with 10 ml of sterile water. Serial dilutions of the stock solution, ranging from 0 to 150 μg/ml were prepared as follows:

Table C.1 Concentration of BSA

| Stock BSA (µl) | $H_2O$ (ml) | Concentration (µg/ml) |
|---|---|---|
| 0 | 1 | 0 |
| 25 | 0.975 | 25 |
| 50 | 0.950 | 50 |
| 75 | 0.925 | 75 |
| 100 | 0.900 | 100 |
| 150 | 0.850 | 150 |
| | | |

## 2.2. Reagent A composed of 2% w/v sodium carbonate ($Na_2CO_3$) in 0.1 M sodium hydroxide (NaOH)

A 0.1 M solution of NaOH was prepared by adding 1 g of NaOH to 250 ml of sterile water. For a 2% $Na_2CO_3$ solution 5 g of $Na_2CO_3$ was added to 250 ml of 0.1 M NaOH. This solution was stored at room temperature.

## 2.3. Reagent B1 composed of 1% w/v copper sulphate ($CuSO_4.5H_2O$)

A 1% w/v solution was prepared by dissolving 0.1 g of $CuSO_4.5H_2O$ in 10 ml of sterile water. The solution was stored at room temperature.

## 2.4. Reagent B2 composed of 2% w/v sodium potassium tartrate (KNaC4H406.4H20)

For a 2% w/v solution of sodium potassium tartrate, 0.2 g was dissolved in 10 ml of sterile water and stored at room temperature.

### 2.5. Reagent C composed of 100 parts reagent A to 1 part reagent B1 to 1 part reagent B2

For 250 ml of reagent A, 2.5 ml of reagent B1 was added together with 2.5 ml of reagent B2. This solution was always prepared fresh just before use.

### 2.6. Reagent E composed of 50% Folin reagent

This solution was prepared by mixing 5 ml of Folin reagent with 5 ml water.

# Appendix D

### 1. Sodium dodecyl sulphate sample loading buffer

The SDS sample loading buffer was made up of:

0.0625 M Tris/HCl at pH 6.8, 2% SDS, 10% glycerol, 0.5% mercaptoethanol and 0.1% bromophenol blue

To prepare 25 ml of SDS sample loading buffer, 189 mg of Tris, 500 mg of SDS, 2.5 ml of glycerol, 0.126 ml of mercaptoethanol and 25 mg of bromophenol blue was dissolved in 10 ml of water. The pH was adjusted to 6.8 with HCl and the volume made up to 25 ml. The solution was stored at 4 °C and had to be used up within a month.

### 2. Composition of standard rainbow markers

Description: A mixture of individually coloured and purified proteins, combined to give bands of approximately equal intensity when electrophoresed on a polyacrylamide gel.

Form: Supplied in 50% glycerol containing the proteins listed in Table D.1.

Concentration: Approximately 1 mg/ml of each protein.

Table D.1 Colour and molecular mass of the standard rainbow marker

| Protein | Molecular Weight (Dalton) | Colour |
|---|---|---|
| Myosin | 220 000 | Blue |
| Phosphorylase b | 97 400 | Brown |
| Bovine serum albumin | 66 000 | Red |
| Ovalbumin | 46 000 | Yellow |
| Carbonic anhydrase | 30 000 | Orange |
| Trypsin inhibitor | 21 500 | Blue |
| Lysozyme | 14 300 | Magenta |

## 3. Polyacrylamide gel solutions

### 3.1. Stacking gel stock acrylamide solution

For the stacking gel a stock solution, containing 16% w/v acrylamide and 0.5% w/v bisacrylamide, was prepared. To prepare 250 ml of stock stacking solution, 40 g of acrylamide and 1.28 g of bisacrylamide, was dissolved in 200 ml of sterile water. Since acrylamide and bisacrylamide are both neurotoxic the necessary universal precautions for handling neurotoxic chemicals were followed. The pH of the solution was adjusted to 7.0 and the volume made up to 250 ml with sterile water. Since acrylamide is light sensitive the solution was stored in a dark bottle at room temperature.

### 3.2. Stacking gel buffer

The stacking gel buffer contained 0.5 M Tris-HCl, at a pH of 6.8 and 0.4% w/v SDS. For 250 ml of stacking buffer, 15.14 g of Tris-HCl and 1 g of SDS were dissolved in 200 ml of sterile water. The pH was adjusted with HCl and the volume made up to 250 ml. The solution was stored in a dark bottle at 4 °C.

### 3.3. Resolving gel stock acrylamide solution

The resolving gel stock solution contained 30% w/v acrylamide and 0.8% w/v bisacrylamide. A stock solution was prepared by dissolving 75 g of acrylamide and

2.5 g of bisacrylamide in 250 ml of sterile water. The solution was heated to 37 °C and the volume taken up to 250 ml with sterile water. This solution was stored in a dark bottle at room temperature.

### 3.4. Resolving gel buffer

The resolving gel buffer contained 1.5 M Tris-HCl at pH 8.8 and 0.4% w/v SDS. To prepare 250 ml of resolving gel buffer, 45.41 g of Tris and 1 g of SDS were dissolved in 200 ml of sterile water. The pH was adjusted to 8.8 with HCl and the volume made up to 250 ml with sterile water. The buffer was stored at 4 °C in a dark bottle.

## 4. Polyacrylamide gel preparation

### 4.1. Preparation of 10% resolving gel

The following components were put into a 100 ml degassing flask: 10 ml of resolving gel stock solution; 7.5 ml of resolving gel buffer and 12.5 ml of sterile water. The solution was degassed for a minimum of 10 minutes, 300 µl of a 10% w/v ammonium persulphate solution and 7.5 µl of TEMED were added to the mixture. This preparation was sufficient for two gels which were prepared in a 4-Place Castor for SE250 gels from Hoefer. The dimensions of the plates were 101.5 mm width by 82.5 mm in height with a thickness of 1.5 mm. The resolving gel preparation was poured into the gel setting sandwich and 2 ml of isopropanol was carefully layered above the gel to allow the gel to set evenly. The resolving gel was

allowed to set for about 30 minutes. Thereafter, the isopropanol was poured off and the gel air dried for 10 minutes. The stacking gel together with the lane producing comb was layered on top of the resolving gel. The stacking gel was allowed to set for 1.5 hours.

### 4.2. Preparation of 4% stacking gel

The following components were put into a 100 ml degassing flask: 5 ml of stacking gel stock solution, 5 ml stacking gel buffer and 10 ml of sterile water. After degassing for a minimum of 10 minutes, 100 µl of 10% w/v ammonium persulphate solution and 10 µl of TEMED were added to the solution (this was sufficient for two gels). Two 1.5 mm thick ten well combs or fifteen well combs were placed in the stacking gels.

### 4.3. Electrophoresis running buffer

The electrophoresis running buffer consisted of 25 mM Tris-HCl, 192 mM glycine and 0.1% w/v SDS. A 5X stock solution was prepared by dissolving 15.1 g of Tris, 72.0 g of glycine and 5 g of SDS in 900 ml of sterile water. The pH was checked (8.3) and the volume adjusted to 1 000 ml with sterile water. To prepare a 1X running buffer solution, 100 ml of 5X stock solution was added to 400 ml of sterile water. The stock solution and running buffer were stored at room temperature.

## 5. Coomassie brilliant blue stain and destain

### 5.1. Coomassie brilliant blue (0.1% w/v) stain

For 500 ml of Coomassie blue stain 0.5 g of Coomassie brilliant blue R-250 was added to 150 ml of methanol, 50 ml acetic acid and the volume was made up to 500 ml with sterile water. The stain was filtered through a Whatman No.1 filter paper and stored at room temperature.

### 5.2. Coomassie brilliant blue destaining solution

The destain solution contained 45% v/v methanol with 10% v/v acetic acid. A 250 ml solution was prepared by mixing 112.5 ml of methanol with 25 ml of acetic acid. This solution was made up to 250 ml with sterile water. The solution was stored at room temperature.

## 6. Protein blotting

### 6.1. Transblotting buffer

The transblotting buffer was made up of 250 mM Tris, 192 mM glycine in 20% methanol (pH of 8.3). To make up 2 000 ml of the transblotting buffer 60.6 g of Tris was mixed with 28.83 g glycine, 400 ml of methanol, made up to 1 900 ml with sterile water. The pH was adjusted to 8.3 and the volume was made up to 2 000 ml.

### 6.2. Assembling the transfer cassette

The nitrocellulose, blotting paper, sponges and cassette unit were all presoaked in transblotting buffer for 2 to 5 minutes. The cassette unit was assembled in a tray containing transblotting buffer. The unit was assembled as follows; a sponge was placed on the bottom cassette (grey colour), followed by the blotting paper then the nitrocellulose paper. All of which were covered by transblotting buffer. The electrophoresis gel was carefully removed from the glass and aluminium plates and placed onto the nitrocellulose paper. Care was taken to prevent any air bubbles from being trapped between the gel and nitrocellulose paper. The gel was covered with blotting paper followed by the sponge and the top part (black colour) of the transfer cassette. The transfer stack was assembled in this way so as to allow the proteins to migrate onto the nitrocellulose paper. Proteins would move towards the positive terminal since they are mainly negatively charged. The cassette was placed in the transfer unit with the grey side positioned towards the anode or the red lead (positive terminal). Transfer buffer was added to the unit until the cassette was completely covered.

**Note:** Gloves were used at all times to prevent contaminating the material. Air bubbles that were trapped or formed during the assembly process would have been carefully removed since they affect the transfer process.

## 7. India ink

### 7.1. Phosphate buffered saline solution with Tween-20

The phosphate buffered saline solution with Tween-20 contained 80 mM $Na_2HPO_4$, 20 mM $NaH_2PO_4$, 100 mM NaCl and 0.3% Tween-20 at pH 7.0. For 100 ml of solution, 1.14 g of $Na_2HPO_4$ with 0.24 g of $NaH_2PO_4$ and 0.58 g NaCl was mixed in 90 ml of water and 0.3 ml of Tween-20. The pH of the solution was adjusted to 7.0 and the solution was made up to 100 ml with distilled water.

### 7.2. India ink stain

The India ink stain contained 0.1% India ink v/v in PBS Tween-20 solution. For 100 ml of solution 0.1 ml of India ink was made up to 100 ml with PBS-Tween-20 solution.

### 7.3. India ink staining of nitrocellulose

The nitrocellulose membrane was washed four times in PBS-Tween-20 for 10 minutes. The membrane was rinsed in distilled water after each wash. The blot was soaked in 100 ml of India ink stain overnight on a slow shaker. The surface of the nitrocellulose membrane that was in contact with the electrophoresis gel was placed facing upwards so that it was exposed to the India ink stain. After being stained overnight, the blot was rinsed in distilled water for 5 minutes and allowed to dry.

# APPENDIX E

## 1. Anitibodies used in the western blots

### 1.1. Monoclonal antibody to protein phosphatase 1

The following description has been extracted from the PP1 package insert (Upstate Biotechnology Incorporated).

Description of Anti-Protein Phosphatase 1, rabbit (polyclonal IgG)

| | |
|---|---|
| Catalogue #: | 06-221. |
| Immunogen: | Synthetic peptide corresponding to the C-terminal amino acid residues 316-330 of human protein phosphatase 1 alpha conjugated to keyhole limpet haemocyanin. |
| Purification: | Protein A-Sepharose chromatography. |
| Immunoglobin type: | IgG. |
| Formulation: | Frozen liquid. 200 µg IgG in 33 µl of PBS. |
| Specificity: | Recognises and is monospecific for PP1 alpha. Does not react with PP2A or other protein phosphatases. |
| Molecular weight: | 34 kDa. |
| Species cross-reactivity: | Human, mouse, rat and rabbit. |

| | |
|---|---|
| Handling and use: | Centrifuge the original vial prior to removing the cap for maximum recovery of the product. |
| Storage and shelf life: | Two years at -20 ºC. Avoid repeated freezing and thawing. |
| Applications: | |
| Western Blotting: | At 1-5 µg/ml, this antibody preparation will detect 100 ng of enzyme using ECL or alkaline phosphatase detection systems. |
| Immunoprecipitation: | Use 2 µg/sample. |
| Neutralisation: | No test. |
| Immunochemistry: | Not tested. |

**1.2. Monoclonal antibody to protein phosphatase 2A**

The following description has been extracted from the PP2A package insert (Upstate Biotechnology Incorporated)

Description of Anti-Protein Phosphatase 2A, rabbit (polyclonal IgG)

| | |
|---|---|
| Catalogue #: | 06-222. |
| Immunogen: | Synthetic peptide corresponding to the C-terminal amino acid residues 296-309 of human protein phosphatase 2A conjugated to keyhole limpet haemocyanin. |

| | |
|---|---|
| Purification: | Protein A-Sepharose chromatography. |
| Immunoglobin type: | IgG. |
| Formulation: | Frozen liquid. 200 µg IgG in 33 µl of PBS. |
| Specificity: | Recognises and is monospecific for PP2A. Does not react with PP1 or other protein phosphatases. |
| Molecular weight: | 34 kDa. |
| Species cross-reactivity: | Human, mouse, rat and rabbit. |
| Handling and use: | Centrifuge the original vial prior to removing the cap for maximum recovery of the product. |
| Storage and shelf life: | Two years at -20 ºC. Avoid repeated freezing and thawing. |
| Applications: | |
| Western Blotting: | At 1-5 µg/ml, this antibody preparation will detect 10 ng of enzyme using ECL or alkaline phosphatase detection systems. |
| Immunoprecipitation: | Use 2 µg/sample. |
| Neutralisation: | Not tested. |
| Immunochemistry: | Not tested. |

**1.3. Anti-rabbit secondary antibody**

Anti-rabbit Ig, peroxidase-linked species-specific whole antibody

Description – obtained from the package insert

The antibody is produced by hyper-immunising donkeys with purified immunoglobulin fractions from normal rabbit serum to produce high affinity antibodies. The pooled antiserum is used to produce an immunoglobulin preparation which is affinity-absorbed to remove cross-reacting antibodies towards rat, human and mouse immunoglobulins. Finally, to select for specific binding to rabbit Ig, the antibodies are purified using an affinity column of rabbit Ig. After washing to remove non-specific serum components and low affinity antibodies, the species-specific antibodies are eluted using carefully selected, mild conditions which minimise aggregation and preserve immunological activity, yet which will elute high affinity antibodies. The enzyme horseradish peroxidase is attached to the immunoglobulin molecules using an adaptation of the periodate oxidation technique. This method has been found not to affect the effective binding of the antibody to the antigen or the activity of the enzyme. Finally the performance of the peroxidase-linked antibody is evaluated using an ELISA, and the concentration is adjusted to ensure that batch to batch variation is minimised and that the product will work in a consistent and reproducible manner.

Formulation

Horseradish peroxidase conjugated antibody is supplied in phosphate-buffered saline (0.1 M sodium phosphate, 0.1 M NaCl, pH 7.5, containing w/v bovine serum albumin) and an anti-microbial agent.

Application

Species-specific antibodies are designed to detect only immunoglobulins from the species against which they are raised. This is desirable for any application where Ig of a second species is present.

### 1.4. Monoclonal antibody to p53

The following description has been extracted from the p53 package insert

(Boehringer Mannheim)

Anti-p53-protein pan (clone PAb 122), Monoclonal antibody to a pan specific epitope on the p53 protein from mouse-mouse hybrid cells (clone PAb-122) lyophilised immunoglobulin, stabilised.

Catalogue # 1 413 147

Product description

Preparation:

To obtain monoclonal antibodies Balb/ mice received two injections of 1 x $10^6$ and one booster injection of 5 x $10^7$ B4 cells, a SV40-transformed Balb/c mouse cell line. The spleen cells were fused with NS1 myeloma cells. Hybridoma supernatant was screened on methanol fixed SV80 cells. The antibody was purified by ammonium sulphate precipitation followed by DEAE column chromatography. The antibody was diluted in potassium phosphate buffer, 10 mmol/l; pH 7.4; NaCl, 150 mmol/l; sodium azide, 0.09% (w/v); bovine serum albumin, 5 mg/ml; and lyophilised.

Dissolving the lyophilisate in 1 ml redistilled water results in a concentration of 50 µg antibody/ml.

The antibody belongs to the mouse IgG2b subclass.

Recommended work concentration:

For immunocytochemistry: 0.5-2.5 µg/ml.

Specificity: The antibody recognises a conserved, denaturation stable antigenic determinant of the p53 protein. The antibody shows strong nuclear staining on SV40- or BK virus-transformed cell lines and different tumour cell lines. The antibody precipitates a 53 kDa protein from extracts of SV40-transformed human, mouse, rat, and hamster cells and of SV40-infected late lytic monkey cells.

Stability: The lyophilised antibody is stable at + 4 ºC. The reconstituted antibody solution is stable for 6 months at + 4 ºC. Alternatively, it can be stored in aliquots at - 20 ºC, repeated freezing and thawing should be avoided.

**1.5. Anti-mouse secondary antibody**

Anti-mouse Ig, peroxidase-linked species-specific whole antibody

Description – obtained from the package insert

The antibody is produced by hyper-immunising sheep with purified immunoglobulin fractions from normal mouse serum to produce high affinity antibodies. The pooled antiserum is used to produce an immunoglobulin preparation which is affinity-absorbed to remove cross-reacting antibodies towards rat, human and mouse immunoglobulins. Finally, to select for specific binding to mouse Ig, the antibodies are purified using an affinity column of mouse Ig. After washing to remove non-specific serum components and low affinity antibodies, the species-specific antibodies are eluted using carefully selected, mild conditions which minimise aggregation and preserve immunological activity, yet which will elute high affinity antibodies. The enzyme horseradish peroxidase is attached to the immunoglobulin molecules using an adaptation of the periodate oxidation technique. This method has been found not to affect the effective binding of the antibody to the antigen or the activity of the enzyme. Finally the performance of the peroxidase-linked antibody is evaluated using an ELISA, and the concentration is adjusted to ensure that batch to batch variation is minimised and that the product will work in a consistent and reproducible manner.

## Formulation

Horseradish peroxidase conjugated antibody is supplied in phosphate buffered saline (0.1 M sodium phosphate, 0.1 M NaCl, pH 7.5, containing w/v bovine serum albumin) and an anti-microbial agent.

## Application

Species-specific antibodies are designed to detect only immunoglobulins from the species against which they are raised. This is desirable for any application where Ig of a second species is present.

## 2. Buffers used in immunoblotting

### 2.1. Tris buffered saline

The TBS solution contained 20 mM Tris with 150 mM NaCl at pH 7.4. For 100 ml of TBS 0.24 g of Tris together with 0.88 g of NaCl was dissolved in 90 ml of sterile water. The pH was adjusted to 7.4 and the total volume made up to 100 ml with sterile water.

### 2.2. Phosphate buffered saline

The PBS solution contained 80 mM $Na_2PO_4$, 20 mM $NaH_2PO_4$ and 100 mM NaCl at a pH of 7.4. PBS was prepared by dissolving 11.36 g of $Na_2PO_4$, 2.4 g of $NaH_2PO_4$ and 5.8 of NaCl in 900 ml of sterile water. Once dissolved, the pH was adjusted to 7.4. The solution was made up to 1 000 ml with sterile water. The buffer was stored at 4 °C.

### 2.3. Blocking buffer in Tris buffered saline

The blocking buffer consisted of 3% BSA in TBS at pH 7.4. To prepare 100 ml of blocking buffer, 3 g of BSA was dissolved in 90 ml of TBS. The volume was made

up to 100 ml with TBS. The blocking solution was freshly prepared before the immunoblot process.

### 2.4. Blocking buffer in phosphate buffered saline

The blocking buffer consisted of 3% BSA in PBS at pH 7.4. To prepare 100 ml of blocking buffer, 3 g of BSA was dissolved in 90 ml of PBS. The volume was made up to 100 ml with PBS. The blocking solution was freshly prepared before the immunoblot process.

### 2.5. Wash buffer containing Tris buffered saline

The wash buffer contained 0.1% v/v of Tween-20 in TBS at a pH of 7.4. To prepare 100 ml, 100 µl of Tween-20 was added to 90 ml of TBS and the volume made up to 100 ml with TBS. The solution was stored at 4 °C.

### 2.6. Wash buffer containing phosphate buffered saline

The wash buffer contained 0.1% v/v of Tween-20 in PBS at a pH of 7.4. To prepare 100 ml, 100 µl of Tween-20 was added to 90 ml of PBS and the volume made up to 100 ml with PBS. The solution was stored at 4 °C.

### 2.7. Antibody buffer with Tris buffered saline

The antibody buffer had 1% BSA in TBS at a pH of 7.4. To prepare 100 ml of the antibody buffer, 1 g of BSA was dissolved in 90 ml of TBS and the volume made up to 100 ml. The solution was freshly prepared.

### 2.8. Antibody buffer with phosphate buffered saline

The antibody buffer had 1% BSA in PBS at a pH of 7.4. To prepare 100 ml of the antibody buffer, 1 g of BSA was dissolved in 90 ml of PBS and the volume made up to 100 ml. The solution was freshly prepared.

# Appendix F

## 1. Reagents and buffers for the p53 enzyme-linked immunosorbent assay kit

### 1.1. Details of the p53 assay kit

The following sections 1.2 to 1.6 were taken from the package insert.

Pantropic p53 rapid format ELISA

Catalogue number: QIA26

### 1.2. Principle of the assay

The p53 ELISA is a "sandwich" enzyme immunoassay employing mouse antibody antibodies. A mouse monoclonal antibody, specific for the human p53 protein, has been immobilised onto the surface of the plastic wells provided in the kit. The sample to be assayed and biotinylated detector antibody (mouse monoclonal) is pipetted into the wells and allowed to incubate simultaneously for 4 hours at 37 ºC, during which any p53 present binds to the capture and detecting antibodies. Unbound material is washed away and horseradish peroxidase conjugated streptavidin is added, which binds to the detector antibody.

The horseradish peroxidase catalyses the conversion of the chromogenic substrate tetra-methylbenzidine from a colourless solution to a blue solution (or yellow after

the addition of stopping reagent), the intensity of which is proportional to the amount of p53 protein in the sample. The coloured reaction product is quantified using a spectrophotometer.

Quantitation is achieved by the construction of a standard curve using known concentrations of p53 (provided lyophilised). By comparing the absorbance obtained from a sample containing an unknown amount of p53 with that obtained from the standards, the concentration of p53 in the sample can be determined.

**1.3. Materials provided**

Standards were assayed in duplicate. Results for the standard curve were obtained from the same plate and during the same time as the samples were analysed. The p53 ELISA provided sufficient reagents to run two sets of standard curves, and 42 samples (if assayed all at once using one standard curve), or 36 samples (if assayed on two separate occasions using two standard curves).

The following materials were provided with the kit:

| | |
|---|---|
| Coated microtiter plate: | Removable wells (96) coated with p53 monoclonal antibody. |
| p53 Standard: | Two vials containing lyophilised p53. Reconstituted standards were discarded after one use. |

| | |
|---|---|
| Detector antibody: | A 12 ml solution of biotinylated monoclonal p53 antibody. |
| Conjugate 400X: | Peroxidase streptavidin conjugate: (0.2 ml) 400-fold concentrated solution. |
| AEA: | Antigen Extraction Agent (6 ml). Its use is described in the sample preparation section for the extraction of p53 from cell and tissue preparations. |
| Substrate: | 12 ml of the chromogenic substrate. |
| Sample diluent: | 25 ml of buffer used to dilute standards and samples. |
| Plate wash 20X concentration: | 100 ml 20-fold concentrated solution of PBS and surfactant. Contains 2.0% chloroacetamide. |
| Stop solution: | 2.5 N sulphuric acid (12 ml). |
| Plate sealers: | Used to cover plates during incubations. |

**1.4. Materials required for the kit**

The following precision pipettors with disposable tips were used 2-20 µl, 20-200 µl and 200-1000 µl.

Wash bottle or multichannel dispenser for washing.

A graduated cylinder, 500 ml.

Deionised or distilled water.

Spectrophotometer capable of measuring absorbance in 96-well plates using dual wavelength of 450/540 nm.

Cell resuspension buffer: 50 mM Tris, containing 5 mM EDTA, 0.2 mM PMSF, 1 µg/ml pepstatin, and 0.5 µg/ml leupeptin adjusted to pH 7.4.

A humidified incubator at 37 ºC.

## 1.5. Sample preparation for the p53 enzyme-linked immunosorbent assay kit

### 1.5.1. Preparation of 1X wash buffer

The 1X wash buffer was prepared by adding 50 ml wash buffer to 450 ml distilled water.

### 1.5.2. Standard curve for p53

Each time the assay was performed, lyophilised p53 standard was reconstituted by carefully and accurately pipetting the indicated amount of sample diluent, as indicated on the vial label, which gave a concentration of 1 500 pg/ml. This was gently mixed. The reconstituted standard was allowed to stand on ice for 15 minute with occasional swirling. Excessive agitation of the standards was avoided. The reconstituted p53 standard was diluted with sample diluent. Six tubes were labelled

as follows 1 500, 750, 375, 187.5, 93.75 and 0 pg/ml. Sample diluent of 300 µl was added to each tube except the first tube (1 500 pg/ml) which was the "undiluted" reconstituted standard. Into the first tube, 600 µl of the original lyophilised material was pipetted. From this tube 300 µl (1 500 pg/ml) of liquid was removed and added to the second tube (750 pg/ml). This was mixed gently. The procedure was repeated until the fifth tube (93.75 pg/ml). The last tube (0 pg/ml) contained only sample diluent. These reconstituted standards were discarded after one use.

### 1.5.3. Reporter antibody 1X preparation

Prior to use, a sufficient volume of p53 reporter antibody was diluted (1:20) with assay buffer to provide 100 µl of solution for each well. For a 1 ml 1X reporter antibody preparation, 50 µl of p53 reporter antibody was added to 950 µl assay buffer; this volume was sufficient for ten wells. The solution was mixed gently. An additional 5 to 10% of working solution was prepared to compensate for any loss that might occur during pipetting. Unused 1X reporter antibody was discarded.

### 1.5.4. Peroxidase conjugate 1X preparation

A sufficient volume of peroxidase conjugate was diluted (1:200) with assay buffer to provide 100 µl of 1X peroxidase conjugate for each well. For a preparation of 2 ml 1X peroxidase conjugate, 10 µl of peroxidase conjugate was added to 1 990 µl assay buffer. Any unused 1X peroxidase conjugate was discarded.

### 1.5.5. Peroxidase substrate solution preparation

Prior to use, enough substrate solution was prepared so as to provide 100 µl of solution for each well. For this a single peroxidase substrate tablet was dissolved in 4 ml of substrate diluent. The peroxidase substrate solution was used within 30 minute of preparation. Exposure to light was avoided**.**

## 1.6. The p53 enzyme-linked immunosorbent assay

The pantropic p53 ELISA was provided with removable strips of wells which allowed the assay to be performed on three separate occasions. A standard curve was determined each time a new assay was performed, this allowed for varying conditions. The standards and unknown samples were assayed in duplicate. In order to avoid cross-contamination of reagents or samples, disposable pipette tips and reagent troughs were used for all transfers.

Assay procedure:

All samples, controls, standards and reagents were prepared as described in the sample preparation section. Appropriate number of wells were removed from the foil pouch and placed into the empty well holder. Unused wells were returned to the foil pouch which was sealed with tape and stored at 4 °C. Samples were prepared and diluted with sample diluent. The wells were washed once by adding 300 µl 1X wash buffer to each well. The 1X wash buffer was removed by inverting the plate into a sink and allowing the buffer to pour off. The inverted plate was then gently tapped on a paper towel to remove excess buffer. Into each of the designated wells,

100 µl of each sample and p53 standard was pipetted. These were done in duplicates. Clean pipette tips were used for each sample. The wells were covered with plastic wrap and incubated at 4°C overnight. The next day all the contents of each well were removed by shaking the inverted plate over the sink. Excess liquid was removed by gently tapping the inverted plate on a paper towel. The wells were washed four times using wash buffer as was outlined previously. Thereafter, 100 µl of 1X reporter antibody was pipetted into each well. The wells were covered with plastic wrap and incubated at room temperature for 2 hours. The wells were again washed four times as outlined previously. The 1X peroxidase conjugate (100 µl) was added to each well; the plate was again covered with plastic wrap and incubated at room temperature for 1 hour. The content of the wells was removed by shaking the inverted plate over the sink. Excess liquid was removed by gently tapping the inverted plate on a paper towel. The wells were washed another four times as outlined before. The peroxidase substrate solution (100 µl) was pipetted into each well and incubated in the dark at room temperature for 30 minutes. Finally, 100 µl stop solution was added into each well in the same order as the previously added peroxidase substrate solution. The absorbance of the solution in each well was measured using a spectrophotometric plate reader set at dual wavelengths of 490/630 nm. The wells were read within 30 minute of adding the stop solution.

### 1.7. Calculation of the standard curve

A standard curve was prepared by plotting the average absorbance (A490/630) versus the concentration (pg/ml) of each of the p53 standard solutions. The best line was fitted to the points. For greater precision the data was analysed using linear regression. The concentration of p53 in each of the samples was determined by first calculating the average absorbance value for each of the duplicates. This value was

then entered into the linear regression equation to yield the p53 concentration of the sample. An alternative method was to locate the point at which the average absorbance value intersects the standard curve and follow a vertical line down to the X-axis. The p53 concentration was read directly from the standard values on the X-axis (i.e. if the vertical line fell midway between the 300 pg/ml and the 600 pg/ml standards, then the sample contained 450 pg/ml of p53). If the samples were diluted, the p53 concentration was multiplied by the dilution factor (i.e. if the sample was diluted fivefold, then the p53 value obtained from the standard curve was multiplied by five).

# APPENDIX G

## 1. Extraction of total RNA

### 1.1. DEPC-treated water, 0.1% v/v

DEPC water which is free of RNAase was prepared in a laminar flow chamber. For the preparation, 1 ml of DEPC was added to 999 ml of sterile water. The solution was allowed to stand overnight at room temperature. This was done to allow the DEPC to evaporate out. After 24 hours the solution was autoclaved for 20 minutes thus removing any trace of DEPC.

### 1.2. Maintaining RNAase free conditions

The following steps were maintained for RNAase free conditions:

Samples were kept on ice at all times.

The RNA running chamber was washed at least three times with DEPC $H_2O$ before use.

All equipment (epperndorfs, beakers, Erlenmeyer flasks, pipette tips, etc) that could be autoclaved were autoclaved before use.

All prepared solutions were prepared with DEPC $H_2O$.

All surfaces were washed/wiped with 100% ethanol.

Latex gloves were worn at all times when handling RNA. Care was taken to prevent these gloves from making contact with surfaces that might have been contaminated with RNAases.

An RNAase free hood was used when working with RNA.

### 1.3. TRI Reagent™

The following information was obtained from the package insert:

TRI Reagent™ is a reagent which allows for the rapid and convenient isolation of RNA, DNA and protein simultaneously (Chomczynski, 1993). Successful isolations have been performed from various samples including human, animal, plant, yeast, bacterial and viral. This is an improvement on the method previously reported by Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). This solution can effectively dissolve DNA, RNA and protein on homogenisation of tissue samples since it contains a mixture of guanidine thiocyanate and phenol in a mono-phase solution. This mixture separates into three phases after chloroform is added and it is centrifuged. There is an aqueous phase which contains RNA while the interphase contains DNA and the organic phase contains protein. After separating each of these phases the components can then be isolated.

This is one of the most effective methods for isolating total RNA and can be completed in only 1 hour starting with fresh tissue or cells. This procedure is effective for isolating RNA molecules of all types from 0.1 to 15 kb in length. The resulting RNA is intact with little or no contamination from DNA and protein. This RNA can be used for northern blots, mRNA isolation, *in vitro* translation, RNAase protection assay, cloning and polymerase chain reaction.

### 1.4. RNA extraction using TRI Reagent™

Different samples of MEL cells (1.5 ml ~ 2 x $10^6$ cells/ml), in suspension culture, were isolated by centrifugation. The pellet was lysed in 0.125 ml of TRI Reagent™ with repeated pipetting and vortexing for 30 seconds. These samples in TRI Reagent™ could be stored at -70 °C for up to 1 month. The samples were allowed to stand at room temperature for 10 minutes. The Eppendorf tubes were centrifuged at 12 000 g for 15 minutes. The upper aqueous phase was removed to new Eppendorfs and 0.075 ml of chloroform was added. This mixture was vortexed for 30 seconds and allowed to stand for 15 minutes at room temperature. The resulting mixture was centrifuged at 12 000 g for 15 minutes at 4 °C. Centrifugation separated the mixture into three phases: a red organic phase (containing protein), an interphase (containing DNA) and a colourless upper aqueous phase (containing RNA). The upper clear aqueous phase (~ 0.075 ml) was removed into new Eppendorfs. Isopropanol (0.125 ml) was mixed with the RNA solution and vortexed for 30 seconds at room temperature. This mixture was allowed to stand at room temperature for 10 minutes. The solution was centrifuged at 12 000 g for 10 minutes at 4 °C. The supernatant was discarded. The RNA pellet was mixed with 0.25 ml of 75% ethanol. This was vortexed for 30 seconds and the mixture centrifuged at 13 000 g for 15 minutes at 4 °C. The supernatant was removed and the RNA pellet was briefly allowed to air dry for 10 minutes. The RNA pellet was dissolved in 25 µl of DEPC water. The final preparation of RNA would have been free of DNA and protein and would have a 260/280 wavelength ratio greater than 1.7. For a graphical description of this process refer to figure G.1.

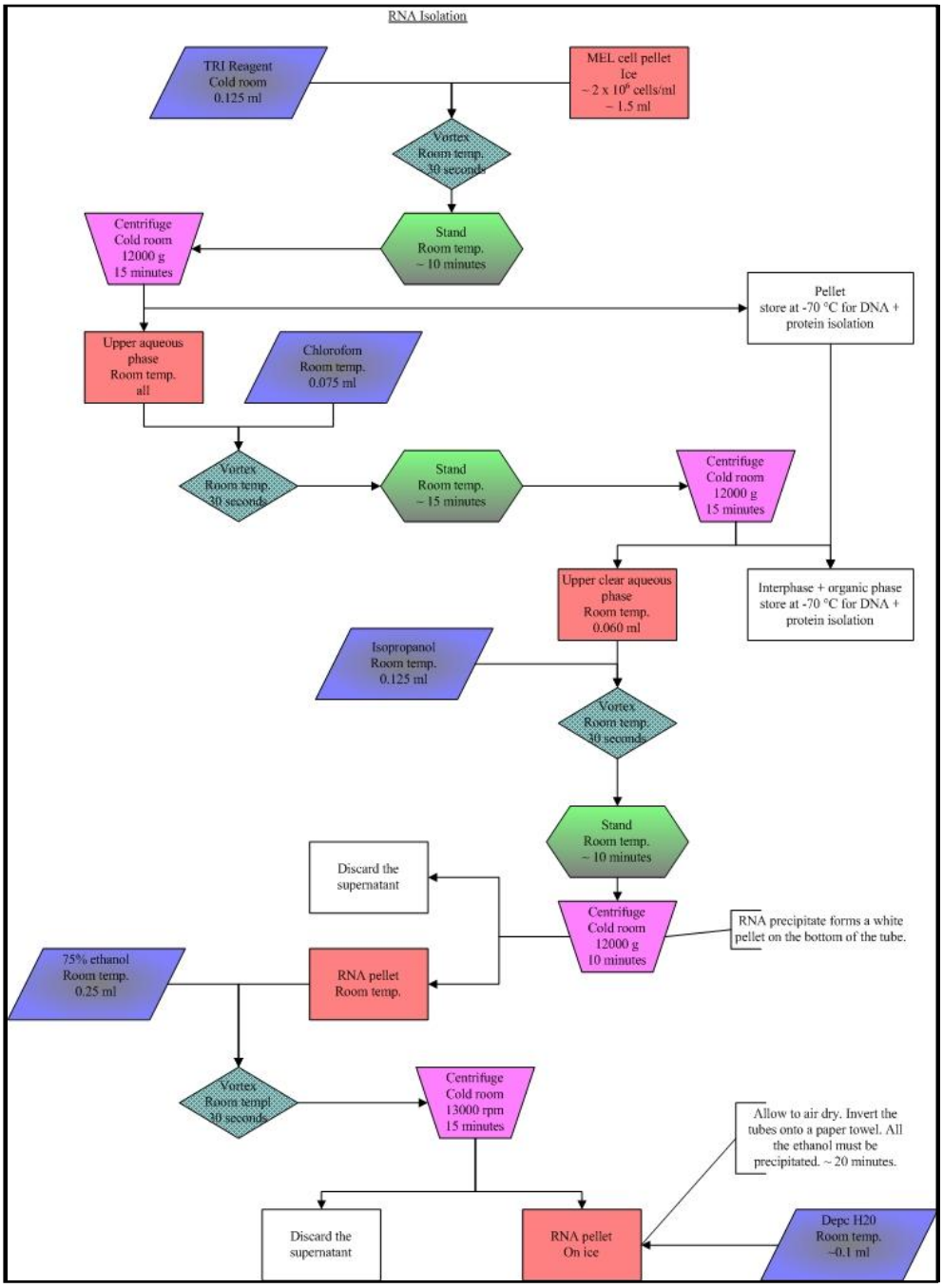



Figure G. 1 A visual representation of the process of RNA extraction

A visual diagram showing how RNA was extracted using TRI Reagent™.

### 1.5. RNA integrity and quantitation

A small aliquot (5 µl) of each RNA sample was pipetted into new Eppendorf tubes containing 0.995 ml of DEPC water. The solution was vortexed for 30 seconds and absorbance read using the Beckman spectrophotometer at 260/280 nm. Quartz cuvettes (1 ml) were used for the readings.

The concentration of RNA was determined using the following equation:

$[RNA] = 40\ \mu g/ml \times abs_{260} \times$ dilution factor

# APPENDIX H

## 1. Solutions for RNA and DNA electrophoresis

### 1.1. Solution of 0.5 M EDTA at pH 8.0

For a 100 ml of EDTA solution, 18.61 g EDTA was mixed with 80 ml of DEPC water. The pH was adjusted to 8.0 with NaOH pellets which would augment the dissolution of EDTA in the DEPC water. Additional DEPC water was added to make a final volume of 100 ml. This solution was stored at room temperature.

### 1.2. Solution of 50X Tris-acetate-EDTA buffer

For a solution of 100 ml, 24.2 g of Tris was added to 80 ml of DEPC water. To this solution 5.71 ml of glacial acetic acid was added together with 10 ml of 0.5 M of EDTA (pH 8.0). The final volume was adjusted to 100 ml with DEPC water and the pH was adjusted to 8.5 with glacial acetic acid. This solution was stored at room temperature. For a 1X solution 2 ml of the 50X TAE solution was dissolved in 48 ml DEPC water.

### 1.3. RNA loading dye (2X) from Fermentas

The 2X RNA loading dye contained the following:

95% formamide

0.025% SDS

0.025% bromophenol blue

0.025% xylene cyanol FF

0.025% ethidium bromide

0.5 mM EDTA

**1.4. DNA loading dye (6X)**

The 6X DNA loading dye contained the following:

0.09% bromophenol blue

0.09% xylene cyanol FF

60% glycerol

60 mM EDTA

**2. Preparation of agarose gel**

The gel casting chamber was prepared by taping the open ends with masking tape. Before being used the gel chamber, comb and running units were washed with DEPC water. The agarose gel was prepared by mixing 1 g of agarose in 50 ml of 1X TAE buffer. The solution was boiled in a microwave oven. The solution was removed from the microwave oven and allowed to cool to a temp of approximately 65 ºC. Ethidium bromide of volume 0.003 ml was added to the cooled solution. The solution was mixed and carefully poured into the gel casting chamber. Care was taken to prevent any bubbles from forming. The comb was carefully added to the gel and allowed to set for 1 hour. Once the gel was ready, the tape was removed and the gel was placed in the running chamber. The comb was carefully removed. The chamber was filled with 1X TAE buffer until it covered the gel.

**3. Loading of RNA samples**

Depending on the number of samples needed 4 µl of loading dye per sample was placed on RNAase free wax paper. These were spread out approximately 1 cm from each other. RNA sample of 2 µl was removed and mixed with one of the drops of loading dye. The solution was mixed by pipetting it up and down. Each sample was carefully loaded into a well on the gel.

**4. Loading of DNA samples**

Depending on the number of samples needed 1 µl of loading dye per sample was placed on cling wrap. These were spread out approximately 1 cm from each other. The PCR product (DNA) of 5 µl was removed and mixed with one of the drops of

loading dye. The solution was mixed by repeated pipetting. Each sample was carefully loaded into a well on the gel.

**5. Running of RNA or DNA gels**

Once all the samples were loaded onto the gel, the chamber was covered with the lid and the electrodes were connected to the power supply. Samples were separated by setting the voltage between 70 and 85 volts. They were allowed to run for 3 hours until the dye front was almost at the end of the gel. The power was switched off. The gel chamber with the gel was removed and the gel was placed in a UV light chamber. If the separation of the RNA or DNA was suitable, a photograph of the gel was taken.

# APPENDIX I

**1. Details from the reverse transcription polymerase chain reaction p53 kit package insert**

| | |
|---|---|
| Catalogue#: | 5431 |
| Reactions: | 100 |
| Storage conditions: | Store the kit at -20 ºC. |
| Concentration: | Primers and competimers are provided as a 5 µM mix of forward and reverse primers. Storage buffer for primers, competimers and DNA control is 1 mM Tris-HCL pH 7.5, 0.1 mM EDTA. |
| Quality control: | Primers are tested to yield a product of the correct size in a PCR with the positive control DNA template. All components are certified as nuclease free. |
| Primer design: | Primers are confirmed in multiplex RT-PCR on mouse and human RNA samples. Primers yield a gene-specific product of the correct size and multiplex efficiently with the supplied 18S primers. Where possible, primers are designed to flank intron sequences. |

| | |
|---|---|
| Species: | Primers designated mouse may also be used with rat RNA samples. |
| Amplicon size: | 292 bp. |
| Annealing temp: | 61 ºC. |
| 18S primer set: | The ‘classic’ 18S primer set produces a product of 494 bp. The set included in this kit produces a product sufficiently different in size from the gene specific product to be resolved on a 2% agarose gel. |
| Genebank #: | AF051368. |

Genebank sequences and literature references may be found at www.ncbi.nlm.nih.gov/Entrez/nucleotide.html (Benson *et al.*, 2010; Genbank, 2013; Sayers *et al.*, 2010).

**2. Reverse transcription of RNA**

The following concentrations were optimised to produce 10 µl of RT product.

RT mix for one sample:

| | | |
|---|---|---|
| RNA | = | 1.00 µl (at ~1 µg/µl initial concentration) |
| 10X RT buffer | = | 1.00 µl |
| Oligo dT primer | = | 0.25 µl |
| dNTP mix | = | 0.50 µl |
| RNase inhibitor | = | 0.10 µl |
| Reverse transcriptase | = | 0.05 µl |
| $H_20$ | = | 7.10 µl |

The samples were RTed at:

| | |
|---|---|
| RT cycle: | 44 ºC for 1 hour |
| Inactivation cycle: | 92 ºC for 10 minutes |

Samples could be stored at -20 ºC or used directly for PCR.

### 3. Determining the optimal ratio of 18S primer pair to 18S competimer

Since the primers and competimers compete for resources, it was critical that the control agent (18S) be amplified from the RT-PCR at a level roughly similar to the p53 amplicon. The following concentrations shown in Table I.1 were used in order to determine the optimal ratio of the 18S primer to the 18S competimer.

Table I.1 The volumes of 18S PCR primer pair to 18S PCR competimer used to determine the optimal ratio of the 18S primer to the 18S competimer

| | 1 (µl) | 2 (µl) | 3 (µl) | 4 (µl) | 5 (µl) |
|---|---|---|---|---|---|
| 18S PP | 0.5 | 1.0 | 1.5 | 2.0 | 2.5 |
| 18S C | 4.5 | 4.0 | 3.5 | 3.0 | 2.5 |

PP=primer pair (5 µM), C=competimer

A PCR mix for five samples was prepared (20 µl per tube):

| | | |
|---|---|---|
| Template (RT reaction) | = | 5 µl |
| PCR Master mix | = | 62.5 µl |
| Oligo (dT) primer | = | 25 µl |
| $H_20$ | = | 7.5 µl |

The samples were subjected to PCR with the following cycles:

| | |
|---|---|
| Denature: | 94 ºC for 4 minutes |
| 30 cycles: | 94 ºC for 30 seconds |
| | 61 ºC for 30 seconds |
| | 72 ºC for 59 seconds |
| Final extension: | 72 ºC for 5 minutes |

After the PCR process, the samples were electrophoresed on agarose gels for separation and subsequent visualisation.

**4. Determining the optimal number of polymerase chain reaction cycles**

A PCR mix for seven samples was prepared:

| | | |
|---|---|---|
| Template (RT reaction) | = | 7 µl |
| PCR master mix | = | 87.5 µl |
| Oligo (dT) primer | = | 35 µl |
| 18S PP | = | 3.5 µl |
| 18S C | = | 3.5 µl |
| $H_20$ | = | 38.5 µl |

Seven tubes were labelled from 1 to 7. Tube one was removed after 10 cycles with subsequent tubes being taken out at 5 cycle intervals.

Each tube was removed after the following cycles:

9, 14, 19, 24, 29, 34 and 39.

Once the PCR was complete the samples were separated on agarose gels.

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